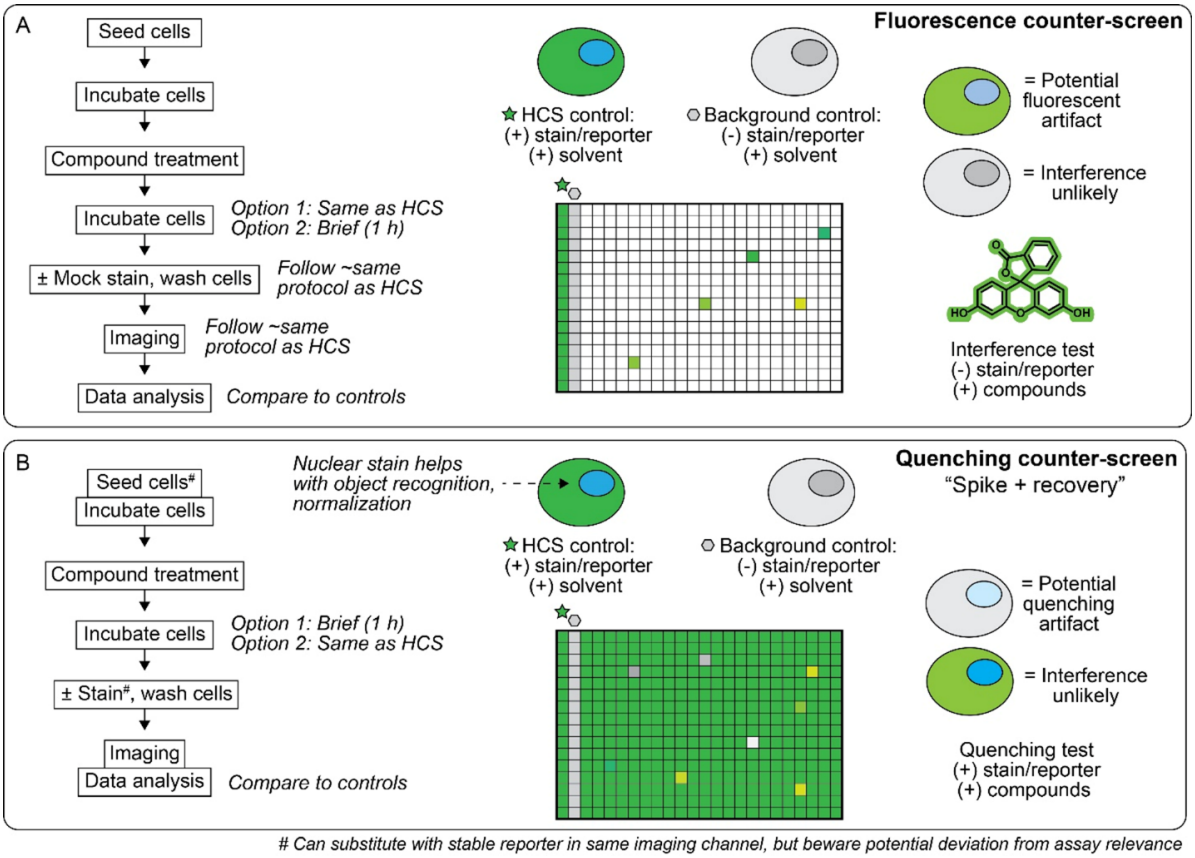


Figure 12. HCS assay counter-screens for compound fluorescence and quenching interferences. (A) Example workflow for fluorescence compound interference. Hits from the primary HCS are carried through the primary HCS procedure, except that the stains/reporter reagents are omitted from compound-treated wells. To quantify the magnitude of fluorescence, readouts are compared to control wells that are either stained per the primary HCS procedure or contain the original reporter. (B) Example workflow for quenching compound interference. Hits from the primary HCS are carried through the primary HCS procedure, except that the compounds are only briefly incubated with cells. To quantify the magnitude of quenching, readouts are compared to control wells. Quenchers will reduce the readout intensity in each imaging channel (e.g., GFP/FITC, etc.). Reduction in nuclear stain intensity further supports a broad-wavelength quenching mechanism.

Table 1. Generic protocol for fluorescence compound interference counter-screen in high-content screening assays

Step	Technical comments
1. Seed cells	Use identical conditions as primary assay (microplate, seeding density, etc.) to minimize bias
2. Incubate cells	Incubate under identical conditions as primary assay (temperature, humidity, incubator, time, media, etc.) to minimize bias
3. Add compounds to wells	Use identical conditions as primary assay (compound concentration, solvent, dispensing method, etc.) to minimize bias. Recommended control columns*: Solvent control (usually DMSO), (+) staining[#] Solvent control (usually DMSO), (-) staining Optional: positive control (bioactive compound), (+) staining Optional: positive control (bioactive compound), (-) staining Recommended: fluorescent compound control(s) **Can use multiple wells instead of entire columns, though this may require more manual manipulations* [#] *Useful for comparing magnitude of compound autofluorescence relative to bioactive readout*

Table 1. continued from previous page.

Step	Technical comments
4. Incubate cells	Option 1. Incubate under identical conditions as primary assay (temperature, humidity, incubator, time, media) Option 2. Incubate under identical conditions as primary assay (temperature, humidity, incubator, media) except use shortened incubation time* (1 h) **Shorter incubation times reduce likelihood of phenotypic changes*
5. (± Mock) stain and wash cells	Prepare staining solution(s), wash solutions, and imaging solution identical to primary assay (reagent(s), concentration, etc.) Prepare mock staining solution(s), wash solution(s), and imaging solution (contains no stains or reporter) Treat one or more solvent control columns with staining solution and washing* protocols. In parallel, (1) treat one or more solvent control columns and (2) compound-treated columns with mock staining and washing protocols* **Removes extracellular compounds, background media interferants; may not necessarily remove intracellular compounds*
6. Image acquisition of cells	Use identical imaging as primary assay (imager, filters, objective lenses, channel settings with exposure times, well location, etc.) to minimize bias
7. Data analysis	Calculate readouts and determine imprecision/cut-offs for: Positive staining/reagent controls Negative staining/reagent controls Compound treatments (- staining) Identify compound treatments with significant signs of autofluorescence: Usually, images with 3 SD above background (use negative staining controls) Assess significance of autofluorescence: Compare magnitude of suspected compound autofluorescence to activity of interest (use positive staining controls)

Additional notes:
To prevent carryover, use separate consumables (such as pipette tips, MultiDrop cartridges, and bottles) for staining and mock staining protocols. Certain reagents, like Hoechst or acridine orange, can carry over with polypropylene despite thorough washing.
Choosing the right autofluorescence counter-screen largely depends on the primary assay. Here are some factors to consider, though this list is not exhaustive:
Counter-screens with single bioluminescent reporter (e.g., GFP-labelled protein of interest): can test cell lines with transfected reporter and compare to cells transfected with non-luminescent reporter.
Counter-screens with dual bioluminescent reporters (e.g., GFP- and mCherry-labelled proteins of interest) or dual staining (Hoechst, AlexaFluor-X, and other visible fluorescent probes): can compare reporter/stained signal to signal from system with dual non-luminescent reporters/no staining.
Counter-screens with morphological profiling (e.g., cell painting): testing with pulsed compound treatment times (e.g., 1 h) can assess for simple autofluorescence, but will likely not produce changes in morphology; testing with identical compound treatment times (e.g., 24, 48 h) will control for morphological changes

Orthogonal assays

Compounds active in an HCS assay (especially a primary screen) should have their bioactivity confirmed in at least one orthogonal assay. The purpose of orthogonal assays is two-fold: to de-risk for technology-based interferences and to enhance overall confidence in the original bioactivity. An orthogonal assay should involve a different technology that is not susceptible to the same technology interferences and is usually lower throughput but more sensitive and specific. For example, simply swapping a GFP reporter with a mCherry reporter would not constitute a high-quality orthogonal assay, since both could be susceptible to fluorescent interferences. For HCS assays, potential orthogonal methods include more direct measurement of specific pathway components (e.g., immunoassays, mass spectrometry) or gene expression profiling of specific pathway components (e.g., qPCR).

Can fluorescent compounds still be viable HCS hits/leads or why do technology interferences persist despite washing steps? These and other frequently asked questions are summarized in Table 2.

Table 2. Frequently asked questions about HCS artifacts and interferences

Question	Answer
1. Can a fluorescent compound still represent a viable HCS hit/lead?	Compounds that interfere with an assay technology such as fluorescent compounds may represent bioactive compounds and viable hit/leads. In these cases, an orthogonal assay is crucial to confidently establish desirable bioactivity and to de-risk following up on artifacts. Assays with minimal technology interference should preferably drive SAR and lead optimization studies to minimize inadvertent optimization towards interference (structure-interference relationships, 'SIR').
2. If washing steps are including in an HCS assay, then why are technology interferences still present?	In the same way that washing steps do not remove intracellular stains, washing steps do not necessarily remove intracellular compound. Scientists should not assume that washing steps will completely remove unwanted compound from within cells.
3. Can technology-related compound interferences like fluorescence and quenching be predicted by chemical structure?	In general, compounds with conjugated electron systems ('aromatic') have higher likelihood of absorbing light and also emitting light by fluorescence. Predicting compound fluorescence can be performed with QM calculations, but more user-friendly calculations/tools are less common. For practical reasons, empirical methods using the HCS assay conditions are recommended. Some exceptions should also be considered: Sample impurities or compound degradation products can fluoresce. Non-optically active compounds may form fluorescent species in the cellular context based on metabolism, molecular binding, and local biochemical environments (pH, ions).
4. If a compound interferes in one HCS assay, how likely is it to interfere in another HCS assay?	Whether an interference compound interferes in another HCS assay depends on multiple factors: The type of interference (technology or non-technology) Specific experimental variables (compound concentration, treatment time, washing steps, fluorophores, imaging settings, etc.) The similarity in assayed biology (cell lines, target, etc.) The type of vessel or microplate fabrication materials used that can potentially bind compounds. Assays with similar readouts (e.g., GFP reporters) may show similar susceptibilities to green-fluorescent compounds. Interference compounds in one HCS assay may still represent starting points in another system if desirable bioactivity can be confirmed by an orthogonal assay and other experiments.
5. What should be done if an orthogonal assay is not available?	In the absence of an orthogonal assay, interference-specific counter-screens should be performed. Selectivity assays can help assess whether a compound effect occurs in related and unrelated biological systems. Modifications of the primary HCS method can be performed, such as genetic perturbations (e.g., KO or overexpression) of the putative compound target. It is highly recommended to develop an orthogonal method whenever possible. Without confirmation of activity by an orthogonal assay, there is risk for technology-based interference. While counter-screens may de-risk interferences, it is risky to rely on a single assay method.
6. Should antibiotics be routinely used in HCS media?	There are pros and cons to using antibiotics in HCS media. See microorganism contaminants section for additional information.

Conclusions

Interference and artifacts can be a significant burden in the application of HCS for chemical probe and drug discovery. Endogenous autofluorescent substances found in cells, tissues, and culture media may prevent the development and optimization of an effective HCS assay, especially when the specific signal of interest is not robust. Consistent with other cell based HTS assay formats, contaminants such as microbes and airborne particles can also represent a substantial interference problem for HCS assays. Autofluorescent compounds confound HCS assays by interfering with both the detection and analysis components of the technology (usually optical based), while other compounds compromise the assay by altering cell morphology, reducing cell

adherence, or inducing cytotoxicity. Several best practices that can mitigate interference in HCS assays include the use of sound tissue culture practices, careful technical decisions, and including the measurement of cell number into the primary HCS experiment. Recommended practices for identifying interference in HCS assays are conceptually like best practices in biochemical or other cell based HTS assay formats and include the use of statistical tools such as Z-scores, counter-screens for optical interference (quenching, autofluorescence), informer sets that incorporate known interferants, and orthogonal assays. The use of these best practices should lead to more efficient identification of higher-quality chemical matter from HCS assays.

Appendix 1: Derivation of the Z-score

A relatively straightforward and simple way to identify compound autofluorescence is to apply a simple Z-score statistical data processing analysis to the fluorescence intensity data collected for each of the channels acquired (13-17, 19, 20, 59-71). The Z-score (Equation 1) is a plate-based statistical score that is calculated only from the sample values (no controls) on an assay plate (16, 17, 66).

$$Z - score = \frac{X_i - \bar{X}}{s_x} \quad \text{Equation 1}$$

Where X_i is the raw measurement on the ith compound, $\bar{X}$ and s_x are the mean and standard deviation (SD) of all the sample measurements on a plate, respectively. A deviation of ±3 s_x from the sample average $\bar{X}$ is frequently utilized as a statistical threshold or cut-off for active compounds. The method assumes that in a randomly distributed diverse compound library, fluorescent compounds are relatively rare, and that most of the compounds on a plate are non-fluorescent and serve as controls. Compound measurements are rescaled relative to within-plate variation by subtracting the average of the plate compound well values and dividing the difference by the SD estimated from all sample measurements of the plate. Z-score analysis of multiparameter HCS data provides a statistical method to identify compounds that are auto-fluorescent in any of the acquired channels or that are cytotoxic or disrupt cell adhesion to substantially reduce the numbers of cells that are acquired and are available for analysis. (13-15, 19, 20, 59-66).

Appendix 2: Analysis of cytotoxic and autofluorescent compounds in the ToxCast compound collection

It is possible to identify compounds that are likely to be fluorescent artifacts using standard HTS screening data processing methods, including the Z-score analysis (see appendix 1, AGM chapter or primary ref (16).

Background

To illustrate the application of the Z-score analysis in HCS data to identify compounds that are autofluorescent or that are cytotoxic or disrupt cell adhesion we present data from a qHTS campaign of 2,000 compounds in the EPA ToxCast library screened in an Androgen Receptor (AR) Transcription Intermediary Factor 2 (TIF2) protein-protein interaction biosensor (AR-TIF2 PPIB) HCS assay (13,14,19,57,68).

Biological model

The protein-protein interaction biosensor HCS assay uses an AR “prey” protein interaction partner that can shuttle between the cytoplasm and nucleus in a ligand dependent manner and is composed of AR residues 662-919 expressed as a chimeric fusion protein with red fluorescent protein (RFP) and contains both a nuclear localization (NLS) and nuclear export (NES) sequence. The TIF2 “bait” protein interaction partner is targeted to and anchored in the nucleoli within the cell nucleus and expresses TIF2 residues 725-840 as a chimeric fusion protein with green fluorescent protein (GFP) and a high affinity nuclear/nucleolar localization (NLS/NoLS) sequence derived from HIV Rev protein.

Experimental Design

In unstimulated cells co-infected with the AR-RFP and TIF2-GFP adenovirus biosensors, AR-RFP expression is localized predominantly to the cytoplasm and TIF2-GFP expression is localized only to nucleoli within nuclei. Exposure to AR agonists such as dihydrotestosterone (DHT) induces the AR-RFP biosensor to traffic into the nucleus where it co-localizes with the TIF2-GFP biosensor within nucleoli (13,14,19, 57). Exposure to AR agonists alters the AR-RFP sub-cellular distribution phenotype in the Texas Red channel images from predominantly cytoplasmic to almost exclusively nucleolar.

To determine if compounds could block DHT-induced AR-TIF2 PPI formation, assay plates were pre-incubated with compounds for 3 h prior to the addition of DHT for 90 minutes. Fluorescent images of three fields of view in each of the DAPI (Hoechst-stained nuclei), FITC (TIF2-GFP) and Texas Red (AR-RFP) channels were acquired on the ImageXpress Micro (IXM) or Ultra (IXU) automated HCS platforms (Molecular Devices LLC, Sunnyvale, CA) using a 10X Plan Fluor 0.3 NA objective (13,14,19, 57).

Images were then analyzed using either the Translocation Enhanced (TE) or Multiwavelength Cell Scoring (MWCS) image analysis module of the MetaXpress software (Molecular Devices LLC, Sunnyvale, CA). The mean average inner fluorescence intensity (MAIFI) of AR-RFP within TIF2-GFP positive nucleolar masks output of the TE image analysis module was used to quantify AR-TIF2 PPIs (13,14,19, 57). The2,000 compound EPA ToxCast library was screened through plate 10-point 3-fold serial dilution qHTS format (67).

Data Analysis, Observations, and Outcomes

For each compound library plate, we calculated Z-scores based on the sample wells distributed across 10 assay plates for a total of 20,000 sample wells. Z-scores were also calculated for maximum (MAX - DHT + DMSO, n=32) and minimum (MIN - DMSO, n =32) plate controls on basis of sample well means. The sorted ascending order Z-score plots (low to high) for the mean average fluorescence intensity (MAFI) values obtained in the EPA ToxCast library compound wells in the DAPI (Hoechst-stained nuclei), FITC (TIF2-GFP) and Texas red (AR-RFP) channels of the AR-TIF2 PPIB HCS assay are presented in Appendix Figure A1, A2 and A4, respectively.

For comparison, the corresponding Z-scores for the MAX and MIN plate controls are also presented. In all 3 fluorescent channels > 98.4% of test compound and plate control well MAFI Z-scores fell within 3 standard deviations of the plate sample well means (Z-score > -3 and < 3), Appendix Figure A1, A2, A4, and Appendix Table A1. As anticipated, the exception was the MIN DMSO plate control wells in the Texas red channel where the AR-RFP MAFI values in TIF2-GFP positive nucleoli were more than 3 standard deviations below the plate sample well means and the MAX controls which were all exposed to DHT. In all three fluorescent channels there were compound wells with MAFI values that were more than 3 standard deviations higher (Z-scores >3) or lower (Z-scores < -3) than the plate sample well means (Appendix Table A1). Appendix Figure A1, A2, and A4, also show representative images of auto fluorescent compound treated wells in the DAPI (Hoechst), FITC and Texas red channels with MAFI Z-scores >3 compared to images from MAX and MIN control wells (Z-score > -3 and < 3).

In the DAPI (Hoechst) channel, images of some of the compounds with MAFI Z-scores >3 revealed that they had much lower numbers of Hoechst-stained nuclei, which may represent compounds that induced apoptosis and nuclear condensation. Hoechst (DAPI channel) images of some compounds with MAFI Z-scores < -3 revealed normal numbers of Hoechst-stained nuclei after the image stretch was adjusted, suggesting that these compounds either quenched Hoechst fluorescence or interfered with the staining of the DNA. Appendix Figure A3 and A5, show pseudo-colored images to illustrate the compound concentration dependence of auto fluorescence in the FITC (green) and Texas red (Red) channels respectively.

Across the 20,000-compound test wells and depending upon whether we used the mean integrated (MIFI) or average (MAFI) fluorescence intensity values, between 0.82% to 1.49% of wells were flagged as potential auto

fluorescent compounds (Z-scores > 3) in the Hoechst (DAPI channel), 0.12% to 0.46% in the FITC channel, and 0.19% to 0.87% in the Texas red channel (Appendix Table A1). If we had restricted our Z-score analysis to only the top concentrations of the EPA ToxCast library tested, then the % of auto fluorescent compounds would be higher. In an HCS campaign of three distinct compound libraries totaling 143,000 compounds conducted in the AR-TIF2 PPIB assay, 0.49% and 0.4% of compounds exhibited Z-scores >3 in the FITC and Texas Red channels respectively and were flagged as potentially auto fluorescent (14).

The value of this simple Z-score analysis of compound autofluorescence is that it flags potential artifacts or interference in the actives and hits identified in the HCS assay that can be readily confirmed or excluded by a direct inspection of the images. Several studies have taken advantage of compound autofluorescence to compare the uptake, accumulation, and distribution of fluorescent drugs in 2D monolayer and 3D multicellular tumor spheroid (MCTS) cell culture models (73-78).

For example, the dimethyl-pyrido-carbazole plant alkaloid ellipticine, and the anthracycline chemotherapeutics idarubicin, daunorubicin and doxorubicin exhibit autofluorescence in cells and have been used to study drug accumulation and distribution in 2D monolayer and 3D MCTS head and neck cancer cell cultures (77). A limitation of the Z-score analysis is that it fails to identify auto-fluorescent compounds when their fluorescence intensity values fall within the normal ranges of the sample wells.

For example, some of the lower concentrations of the auto-fluorescent compounds presented in Appendix Figure A3 and A5 would not be flagged because their MAFI and/or MIFI Z-scores were within 3 standard deviations of the plate sample well means (Z-score >-3 and <3).

Glossary

artifacts — Compounds that modulate an assay readout by interfering with some component of the assay technology, as opposed to modulating the intended target.

autofluorescence (compound) — Emission of light from compounds when they have absorbed light; this is unrelated to modulation of the assayed biology.

compound interference — Compounds that confound the interpretation of a bioassay readout by interfering with the assay technology or perturbing the assayed biology by undesirable mechanism(s) of action, ultimately impeding the identification of quality chemical matter.

cytotoxicity — The property of a compound to cause injury to cells; can include a variety of cellular fates including necrosis, decreased proliferation, or apoptosis.

high-content screening — Cell- or organism-based imaging assays that measure multiple parameters from the same set of images; often performed with multiple fluorophore channels.

nuisance compounds — Compounds with apparent bioactivity that is often due to interference with the assay technology and/or modulation of the assayed biology by undesirable mechanisms of action.

phenotype — The composite observable characteristics of a cell or organism; can include morphology, biochemical composition, and/or physiological properties.

promiscuous compounds — Compounds statistically-enriched in bioactivity across a variety of unrelated biological assays.

quenchers (fluorescence) — Compounds that decrease the observed fluorescence intensity that is unrelated to modulation of the assayed biology; can occur by different chemical mechanisms such as FRET, collision, and static processes.

Suggested readings (alphabetical order)

1. JL Dahlin, DS Auld, I Rothenaigner, S Haney, JZ Sexton, JWM Nissink, J Walsh, JA Lee, JM Strelow, FS Willard, L Ferrins, JB Baell, MA Walters, BK Hua, K Hadian, BK Wagner. Nuisance compounds in cellular assays. *Cell Chem. Biol.* 2021, 28 (3), 356-370. PMID 33592188.

A summary of nuisance compounds in cellular assays from academia, industry, and government experts. Includes recommended practices for mitigating and identifying cellular nuisance compounds, including those found in HCS assays.

2. G Ibáñez, PA Calder, C Radu, B Bhinder, D Shum, C Antczak, H Djaballah. Evaluation of compound optical interference in high-content screening. *SLAS Discov.* **2018**, 23 (4), 321-329. PMID 28467117.

An informative case report demonstrating the high hit rate of artifactual autofluorescent compounds in a GFP reporter readout in a phenotypic HTS.

Suggested resources

1. InterPred: Prediction of Chemical-Assay Interference.

An open-source cheminformatics tool for predicting compound fluorescence in blue, green, and red fluorescent channels. QSAR models are based on several compound fluorescence counter-screens with and without cells using the Tox21 library. Users can submit compound queries by SMILES (56).

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Conflicting interests

All authors hereby declare no conflicting interests pertaining to the material in this manuscript.

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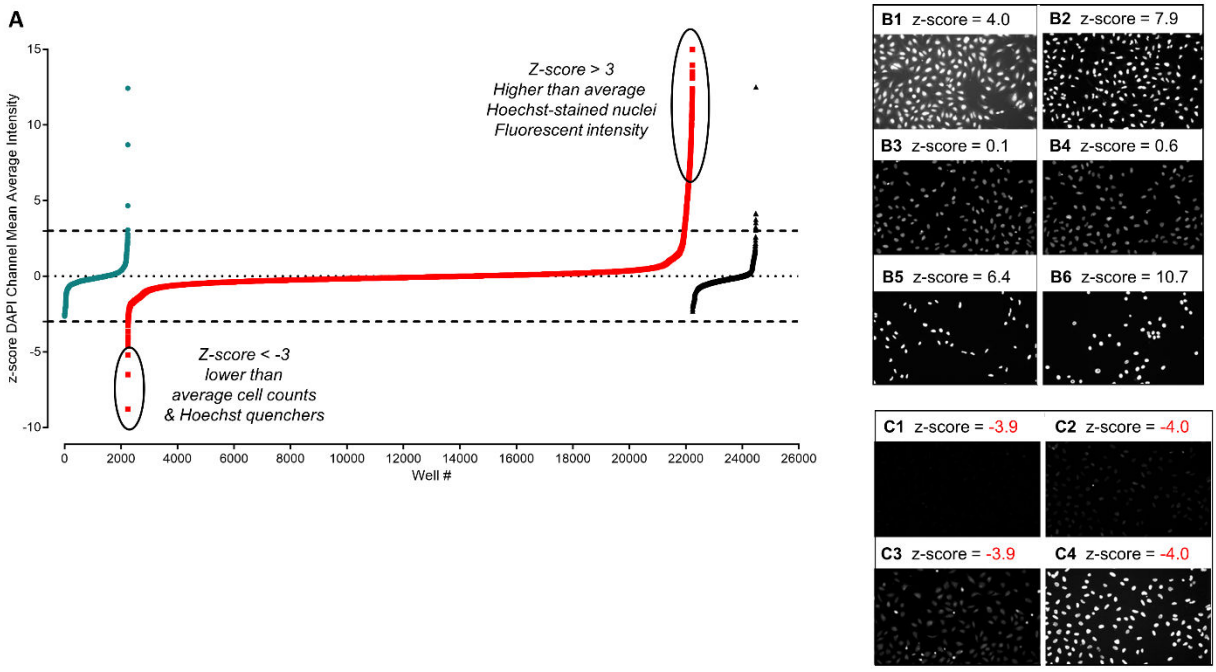
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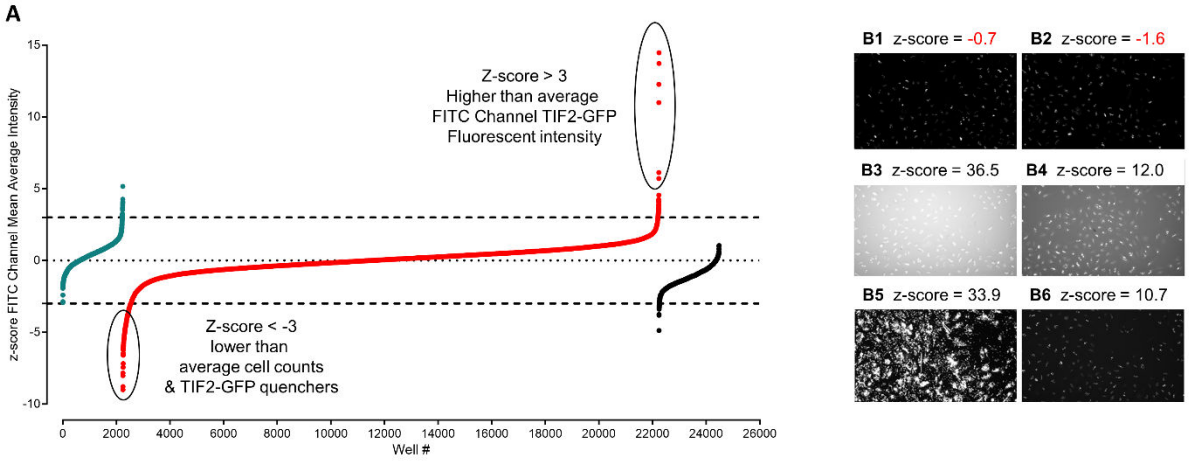
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Appendix Figures and Table



Appendix Figure A1. Compound autofluorescence as indicated by the nuclei Z-scores in the Hoechst (DAPI channel, ~450 nm).

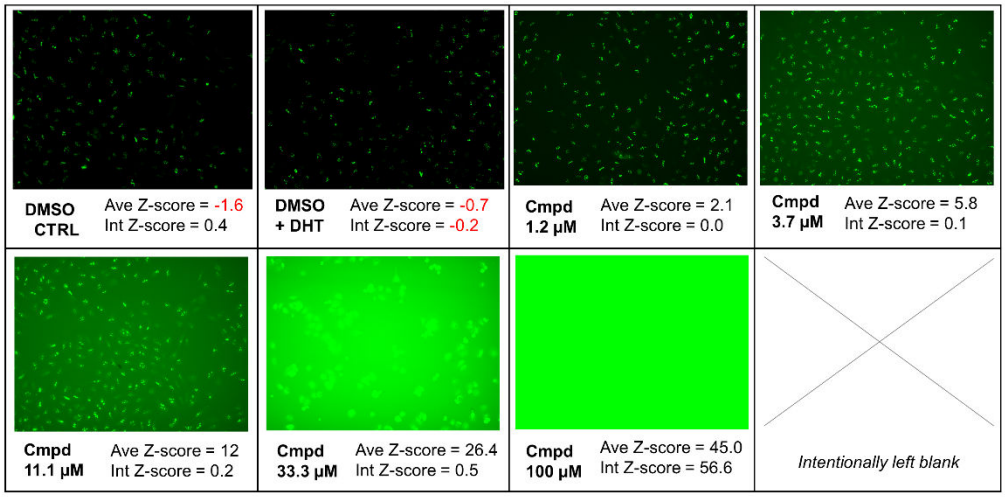
A. Max + DHT (green dots); Compounds (red dots); Min - DMSO (black dots). B: Hoechst-stained nuclei average intensity with cell counts Z-scores > -3 but < 3; 12-bit images. B1 = Compound; B2= Compound: B3 = Max + DHT control; B4 = Min – DMSO control; B5 & B6 = apoptosis inducer compounds. C: Hoechst-stained nuclei Quenchers with average intensity Z-scores < -3. C1 and C2 represent 12-bit image display range of 96-767; C3 & C4 represent a rescaled contrast stretch 8-bit image display range of 97-136 of the same fields of view for visualization.



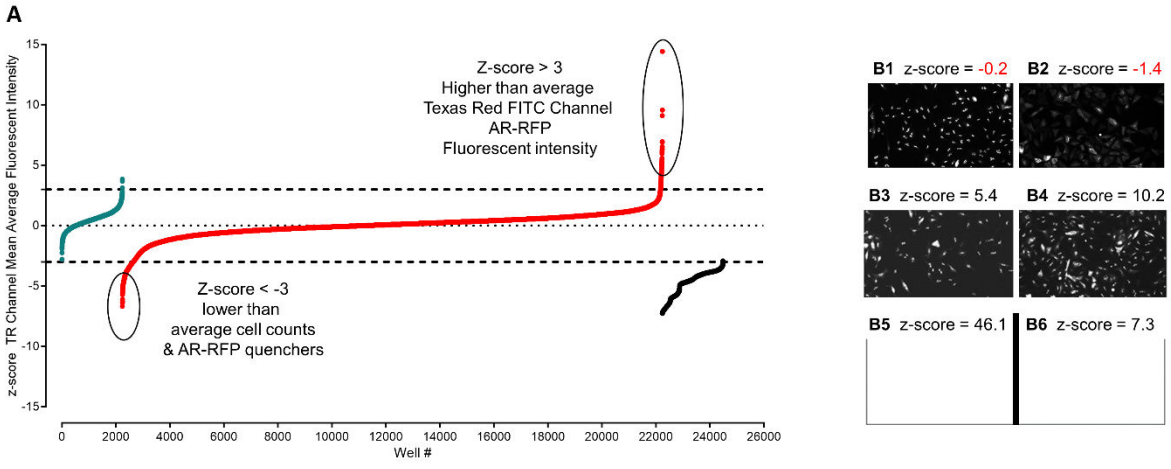
Appendix Figure A2. Compound autofluorescence as indicated in the TIF2-GFP positive nucleoli Z-scores (FITC channel, ~525 nm).

A. Max – DHT (green dots); Compounds (red dots); Min – DMSO Z-scores (black dots)

B: TIF2-GFP biosensor positive nucleoli average intensity Z-scores. B1 = Max + DHT control; B2 = Min – DMSO control; B3, B4, B5, B6 = compounds.



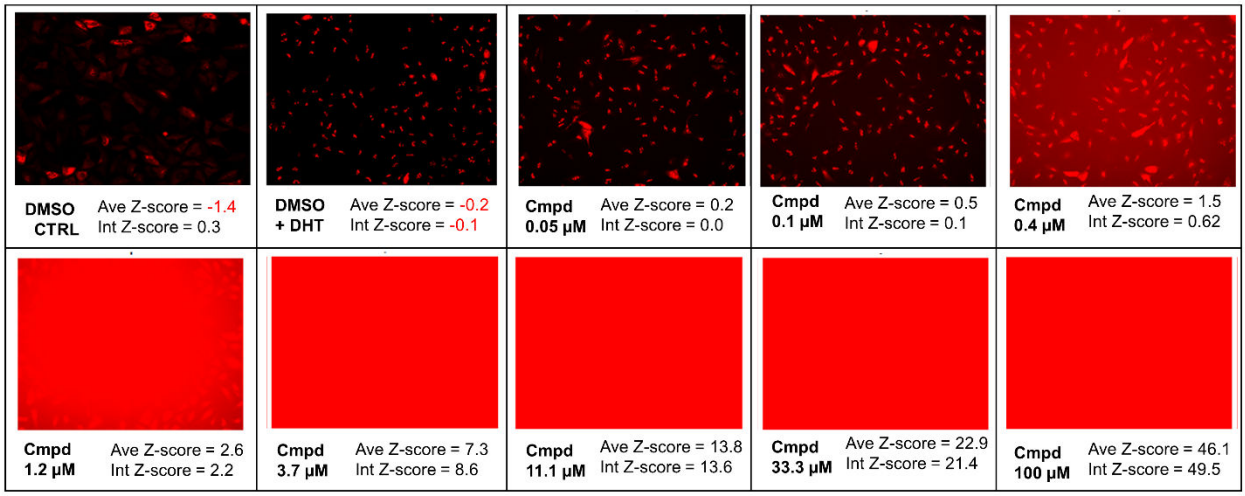
Appendix Figure A3. Images of TIF2-GFP positive nucleoli (pseudocolored green) from concentration response showing compound autofluorescence as compared to DMSO and DHT controls with increasing Z-scores expressed as the average (Ave) or integrated (Int) intensities in the FITC channel.



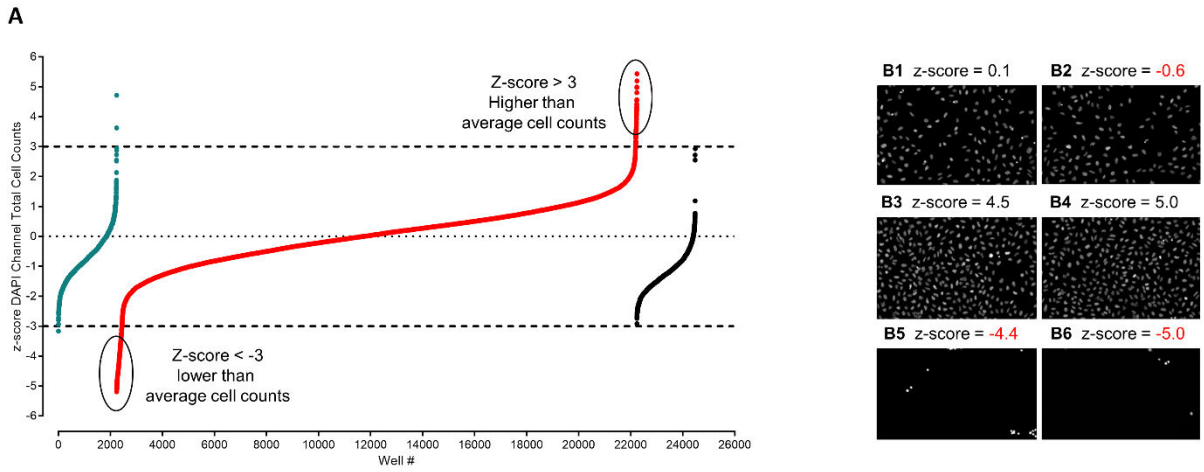
Appendix Figure A4. Compound autofluorescence as indicated by the average intensity Z-scores of the AR-RFP biosensor in cells (Texas Red channel, ~600 nm).

A: Z-scores: Max + DHT (green dots); Compounds (red dots); Min – DMSO (black dots)

B: Fluorescent images (10x) of AR-RFP (Texas Red) biosensor average intensity Z-scores. B1 = Max + DHT control (nucleoli); B2 = Min – DMSO control (cytoplasm); B3 and B4 = increased autofluorescence inside cells from compounds; B5 and B6 compound autofluorescence in solution showing saturation (white) that appears blank.



Appendix Figure A5. Images of AR-RFP nucleoli (pseudocolored red) from concentration response showing compound autofluorescence as compared to DMSO (cytoplasm) & DHT (nucleoli) controls with increasing Z-scores expressed as average (Ave) or integrated (Int) intensity values in the Texas Red channel.



Appendix Figure A6. Compound-mediated cytotoxicity and/or cell loss and higher-than-average cell counts.

A. Max + DHT (green dots); Compounds (red dots); Min – DMSO Z-scores (black dots)

B: Fluorescent images (10x) of total cell counts from Hoechst-stained nuclei (DAPI channel) Z-scores.

B1 = Max + DHT control; B2 = Min – DMSO control; B3 and B4 compounds with higher-than-average cell counts (Z-scores >3), B5 and B6 compounds with lower-than-average cell counts (Z-scores <-3).

Appendix Table A1. Z-score analysis of 2,000 compound EPA ToxCast library arrayed in 20,000 wells: Cell counts, Hoechst (DAPI channel), FITC channel, & Texas Red channel. MAFI = Mean average fluorescence intensity values from the three channels. MIFI = Mean integrated fluorescence intensity values from the three channels.

20,000 Compound wells	Cell Counts	%	DAPI MAFI	%	FITC MAFI	%	Texas Red MAFI	%
Z-score <-3	125	0.63	24	0.12	55	0.28	4	0.02
Z-score >-3 & <3	19869	99.35	19678	98.39	19853	99.27	19822	99.11
Z-score >3	6	0.03	298	1.49	92	0.46	174	0.87
20,000 Compound wells	**Cell Counts**	**%**	**DAPI MIFI**	**%**	**FITC MIFI**	**%**	**Texas Red MIFI**	**%**
Z-score <-3	125	0.63	92	0.46	136	0.68	88	0.44
Z-score >-3 & <3	19869	99.35	19745	98.73	19840	99.2	19874	99.37
Z-score >3	6	0.03	163	0.82	24	0.12	38	0.19

Compound-Mediated Assay Interferences in Homogeneous Proximity Assays

Nathan P. Coussens, Ph.D.,[1] Douglas Auld, Ph.D.,[2] Philippe Roby, Ph.D.,[3] Jarrod Walsh, Ph.D.,[4] Jonathan B. Baell, Ph.D.,[5] Stephen Kales, Ph.D.,[1] Kamyar Hadian, Ph.D.,[6] and Jayme L. Dahlin, M.D., Ph.D.[7,8]

Created: February 1, 2020.

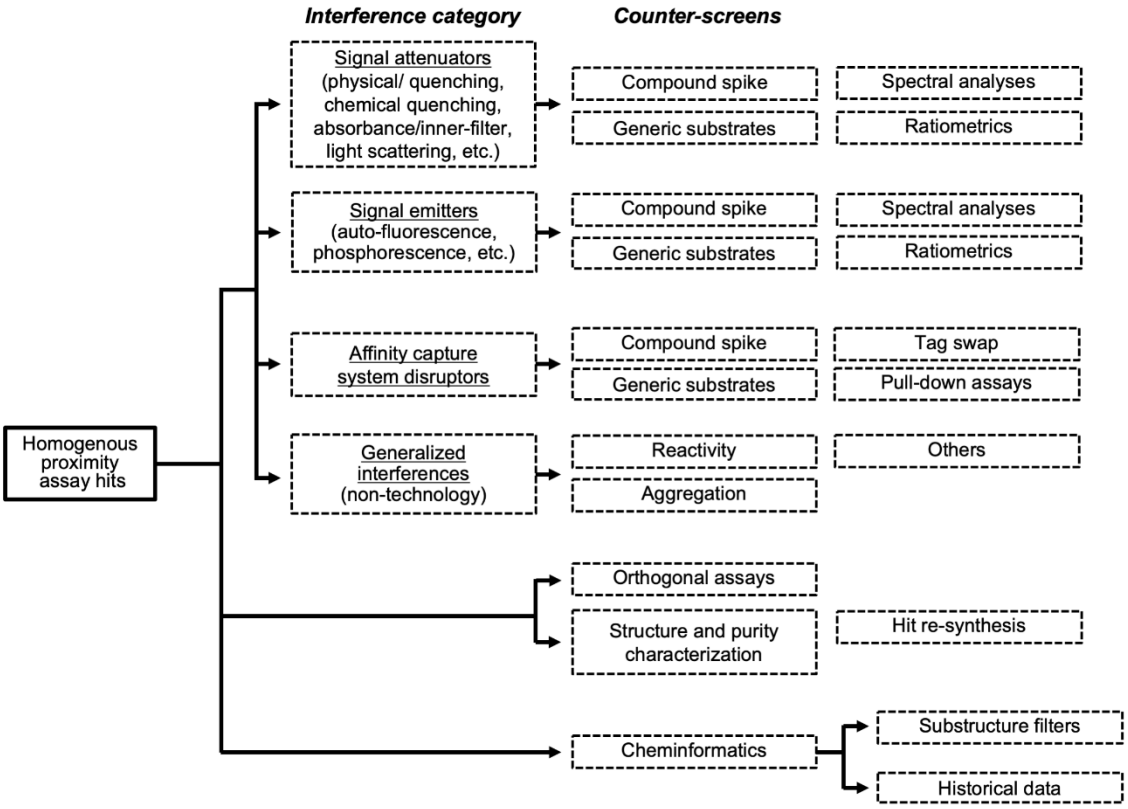
Abstract

Homogeneous proximity assays are widely implemented in high-throughput screening (HTS) of small-molecule libraries for drug and probe discovery. Representative technologies include amplified luminescent proximity homogeneous assays (ALPHA, which is trademarked by PerkinElmer; also informally referred to as Alpha), Förster/fluorescence resonance energy transfer (FRET), time-resolved FRET (TR-FRET) and homogeneous time-resolved fluorescence (HTRF, which is trademarked by CisBio), bioluminescence resonance energy transfer (BRET), and scintillation proximity assays (SPA). While highly useful, these assay technologies are susceptible to a variety of technology-related compound-mediated interferences, most notably signal attenuation (e.g., through quenching, inner-filter effects, light scattering), signal emission (e.g., auto-fluorescence), and disruption of affinity capture components such as affinity tags and antibodies. These assays are also susceptible to more generalized compound-mediated interferences such as nonspecific reactivity and aggregation. This chapter describes (a) the basic principles of proximity assays, (b) common sources of compound-mediated assay interference in homogeneous proximity assays, and (c) counter-screens and other strategies to classify compound-mediated assay interferences in homogeneous proximity assays. This information should be useful for prioritizing bioactive compounds in homogeneous proximity assays for drug and chemical probe discovery.

Author Affiliations: 1 National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD, USA. 2 Novartis Institutes for Biomedical Research, Cambridge, MA, USA. 3 PerkinElmer. 4 Oncology IMED, AstraZeneca, Cambridge, United Kingdom. 5 Medicinal Chemistry Theme, Monash Institute of Pharmaceutical Sciences, Monash University, Melbourne, Australia. 6 Helmholtz Zentrum Muenchen fuer Gesundeit und Umwelt (GmbH), Assay Development and Screening Platform, Institute of Molecular Toxicology and Pharmacology, Neuherberg, Germany. 7 Department of Pathology, Brigham and Women's Hospital, Boston, MA, USA; Email: jdahlin@bwh.harvard.edu. 8 Broad Institute, Cambridge, MA, USA; Email: jdahlin@bwh.harvard.edu.

Corresponding author.

Flowchart



Abbreviations

ALPHA (Alpha)	amplified luminescent proximity homogeneous assay
BRET	bioluminescence resonance energy transfer
CAC	critical aggregation concentration
DELFIA	dissociation-enhanced lanthanide fluorescence immunoassay
ELISA	enzyme-linked immunosorbent assay
FRET	fluorescence/Förster resonance energy transfer
GSH	glutathione
GST	glutathione *S*-transferase
His	histidine
HTS	high-throughput screening
IC_{50}	half-maximal inhibitory value
SIR	structure-interference relationships
TR-FRET	time-resolved FRET

Introduction to Proximity Assays

Proximity assays are widely utilized for the discovery and optimization of small-molecule therapeutics and chemical probes (1). This broad range of assay technologies relies on close proximity between detection components for optimal signal production. These assays are highly amenable to HTS and quantitative HTS

(qHTS) because they do not require separation steps and many key reagents are available commercially and/or amenable to customization. Many proximity assays may be classified as homogeneous, where all assay components are combined without any further separation steps ("mix and read"), or heterogeneous as with enzyme-linked immunosorbent assays (ELISA) or dissociation-enhanced lanthanide fluorescence immunoassays (DELFIA) that require wash steps to separate unbound components. Advantages of wash steps include higher signal-to-background that can be achieved and reduced spectroscopic interferences from test compounds due to the removal of these compounds prior to the signal acquisition. However, in general wash steps increase the time it takes to perform and automate an assay, increase waste generated, and increase imprecision.

The main advantage of homogeneous proximity assays from an HTS perspective is that they do not require washing or separation steps. However, a key disadvantage of the mix-and-read approach is that potential interference compounds are not removed prior to signal acquisition. When not diagnosed, compounds that interfere with homogeneous proximity assay readouts can waste scientific resources and even lead to flawed scientific conclusions (2,3).

In order to better understand the potential sources of compound-mediated interference, this section describes the general principles of several common homogeneous proximity assays including amplified luminescent proximity homogeneous assays (ALPHA, Alpha) technology, fluorescence/Förster resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET), time-resolved FRET (TR-FRET), and scintillation proximity assay (SPA). Also discussed are common affinity capture systems such as affinity tags and antibody-analyte combinations that are often used in these proximity assays. Understanding the underlying chemical and physical principles of each technology, including commonly used affinity capture components, allows one to better predict the potential sources of compound-mediated interferences and to design effective counter-screens and orthogonal assays for de-risking such interferences.

Basic Principles of Homogeneous Proximity Assays

Proximity assays are highly useful for measuring interactions between biomolecules, including protein-protein interactions, protein-nucleic acid interactions, post-translational modifications (e.g., protein phosphorylation), protein-ligand interactions, and specific analytes (e.g., cytokines, hormones). Such assays utilize two general components – a donor and an acceptor – to indicate proximity by a specific signal (Figure 1A). When the donor and acceptor components are sufficiently proximal, a signal from the donor is transmitted to the acceptor for emission of a signal indicative of proximity. Biomolecules amenable to study by homogeneous proximity assays include macromolecules, such as proteins or nucleic acids, as well as smaller ligands. Target biomolecules are often coupled to affinity capture systems, such as antibodies or affinity tags, that can be introduced chemically or by recombinant engineering. Alternatively, target biomolecules could be amenable to radiolabeling for SPA. Measurements can be based on direct (e.g., a discreet protein-protein interaction) or indirect (e.g., displacement of a labelled ligand by a related ligand) interactions. These proximity assays can be developed for the detection of an increased signal (such as assays for activators or inhibitors of analyte modification) or a decreased signal (such as inhibitors of protein-protein interactions). Common types of homogeneous proximity assays for HTS include: FRET, BRET, TR-FRET, Alpha, and SPA. Each technology has advantages and disadvantages (Table 1), which are described in more detail below.

FRET. Förster or fluorescence resonance energy transfer (FRET) is initiated by a donor fluorophore (also referred to as chromophore) that is excited to a higher electronic state by a lower-wavelength laser (approximately 350-550 nm). When there is significant overlap between the emission spectrum of the donor and the excitation spectrum of a proximal acceptor fluorophore (i.e., the initial excitation for the donor fluorophore does not excite the acceptor fluorophore), the acceptor can be excited by non-radiative, non-contact dipole–dipole coupling (Figure 1B). The dipole-dipole interaction gives a distance-dependence of $1/r^6$ where r = radius of the interaction, and this distance is approximately 1-10 nm. This excited acceptor fluorophore can then emit

longer-wavelength photons (approximately 500-650 nm) as it returns to its ground electronic state (4). For many biological assays, FRET donor and acceptor fluorophores are protein-based (e.g., CFP → YFP). There are a variety of donor and acceptor fluorophores amenable for FRET (Table 2).

TR-FRET. Time-resolved FRET (TR-FRET) combines the proximity features of the aforementioned FRET assays with time-resolved fluorometry (Figure 1B) (46,47). This variant of FRET assays utilizes donor fluorophores with longer emission times (1-2 ms), such as lanthanide ion complexes, to reduce potential interferences caused by the excitation energy. The optimal distances between donor and acceptor fluorophores are similar to FRET pairs (Tables 2 and 3); however, TR-FRET assays utilize a time-delay between fluorophore excitation and signal acquisition (approximately 50-150 µs in duration). This acquisition delay is sufficient to avoid interference by the usual short-lived fluorescence from test compounds, contaminants, matrix proteins, and aromatic biomolecules. After the delay, the fluorophore:background signal is typically enhanced relative to conventional FRET assays. Many TR-FRET reagents are commercially available (e.g., LANCE, Lanthanide chelate excite, and LANCE *Ultra* TR-FRET from PerkinElmer; HTRF, Homogeneous Time-Resolved Fluorescence from Cisbio). Advantages of TR-FRET are that it is not light sensitive, allows repeated measurements, and does not require radioactive isotopes like SPA.

BRET. A related assay technology is bioluminescence resonance energy transfer (BRET), which typically utilizes bioluminescent proteins and their substrates (e.g., luciferases and luciferin) to generate an initial excitation event. Analogous to FRET, a proximal acceptor fluorophore (e.g., YFP) can be excited through resonance energy transfer to produce the final emission readout (Figure 1C) (48,49). Bioluminescent proteins are typically introduced as fusion proteins. Common acceptor/donor pairs are RLuc8/GFP2 (BRET2) and NanoLuc and HaloTag (NanoBRET) (50-52). An advantage of BRET is that there is no need for bulk illumination of the sample with an excitation light source. This results in lower background signal through reduction of autofluorescent events, which can improve the sensitivity and ultimate performance of the assay.

ALPHA. AlphaScreen (amplified luminescent proximity homogeneous assay) is a proprietary proximity assay technology that utilizes singlet oxygen ($^1O_2$) transfer from an excited donor bead to an acceptor bead within a range of approximately 200 nm (Figure 1D) (53). Singlet oxygen is a short-lived, higher-energy state of molecular oxygen whereby the two normally unpaired HOMO electrons with the same spin state occupy the same degenerate π^* molecular orbital with opposite spin states. Donor and acceptor latex beads containing special chemical reagents are directed to biomolecules of interest through conjugation to capture reagents such as antibodies or capture tags. Phthalocyanine is a photosensitizer within the donor bead that excites ambient oxygen into a singlet state when irradiated by a high-wavelength laser (680 nm). This process produces singlet oxygen ($^1O_2$). Excitation of the standard donor beads generates about 60,000 oxygen singlets per second that can diffuse to a maximum distance of about 200 nm, due to the low microsecond half-life of singlet oxygen in solution and its diffusion rate (54). If the excited donor bead is in close enough proximity to the acceptor bead, a chemiluminescent signal is generated. The emission wavelength of this chemiluminescent signal depends on the composition of the acceptor bead.

Within AlphaScreen acceptor beads, thioxene forms an endoperoxide (ROOR) upon reaction with singlet oxygen. The endoperoxide which then decays and generates light that is transferred to anthracene and then to rubrene which results in chemiluminescence at a *lower* wavelength (520-620 nm; Figure 1D). Within AlphaLISA and AlphaPlex acceptor beads, anthracene and rubrene are replaced by either europium (λ_{Em} = 615 nm), terbium (λ_{Em} = 545 nm), or samarium (λ_{Em} = 645 nm) chelates that are directly excited upon fragmentation of the dioxetane intermediate formed by the reaction of thioxene with singlet oxygen (Table 4). This results in a more intense, narrower emission window optimized for biological matrices such as serum or plasma. In this chapter, discussion of AlphaScreen should be applicable to AlphaLISA and AlphaPlex. In addition to providing a large dynamic range and high sensitivity, Alpha technology has the advantage of being compatible with greater

distances than any other proximity approach, which enables larger molecular complexes to be studied. It also operates over a wider range of affinities of binding partners (K_D's or K_A's).

SPA. Scintillation proximity assay (SPA) is a solid-phase technique that links a scintillation matrix (beads or coated plates, FlashPlate) to a capture system (e.g., antibody, affinity tag) for measurement of a radioactive analyte by scintillation, a light-based readout (Figure 1E) (55,56). When a radiolabeled product (usually containing ^{3}H, ^{125}I, ^{33}P, or ^{14}C) is in close proximity to the scintillation matrix, the signal from radioactive decay of the radioisotope is amplified. In FlashPlate formats, radiolabeled analytes can be localized to the scintillation matrix by plate-bound capture reagents such as streptavidin or antibodies, or under certain circumstances nonspecific analyte absorption (57). The use of radiolabels enables a large dynamic range and increased sensitivity, and compared to more traditional radiolabeled filter-binding assays, SPA does not require a separation step. Important considerations for SPA include obtaining a radiolabeled reagent, obtaining institutional/regulatory approval for radioisotope work, specialized instrumentation (e.g., scintillation counters), disposal costs, and safety protocols.

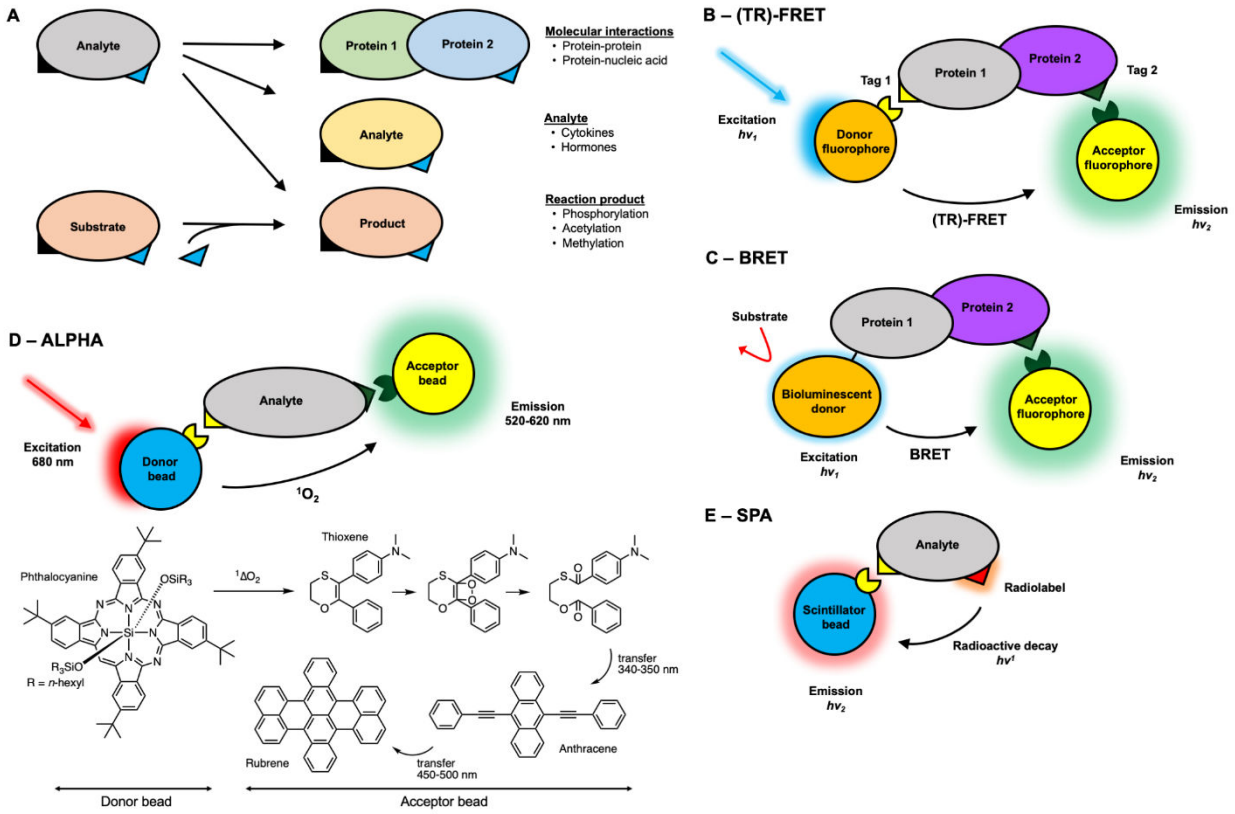


Figure 1 Schematics of common proximity assays. (A) The analyte promoting a signal from a proximity assay can be of many types, including a biomolecular interaction, a single molecule, or a reaction product, such as a protein post-translational modification. (B) FRET and TR-FRET. (C) BRET. (D) AlphaScreen, AlphaLISA, AlphaPlex. Additional details of the underlying Alpha chemistry are provided. (E) SPA. With proximity assays, proximal donor and acceptor components comprise a signal chain (*via* FRET/BRET, singlet oxygen transfer, or radioactive decay, respectively) for the assay readout. The donor and acceptor components can connect with analytes through affinity tags, antibodies, chemical modification, or fusion proteins, depending on assay requirements.

Table 1 Summary of common homogeneous proximity assays. Listed are general advantages, disadvantages, and potential technology-related sources of compound-mediated interference.

Assay type	Advantages	Disadvantages	Potential technology-related compound-mediated interferences
FRET	Commercial reagents available Compatible with intracellular readouts Relatively inexpensive Compatible with standard instrumentation	Lower dynamic range compared to Alpha or SPA Very short distance resulting in limited use	Excitation attenuation (quenching, inner-filter) FRET quenching Emission attenuation (quenching, inner-filter) Compound emission (auto-fluorescence) Affinity capture system disruption
TR-FRET	Commercial reagents available Generally larger dynamic range compared to FRET Reduced interference from excitation (due to Ratiometric readout reduces artifacts	Cost of reagents	Excitation attenuation (quenching, inner-filter) FRET quenching Emission attenuation (quenching, inner-filter); less likely given larger Stokes shift Compound emission (auto-fluorescence); less likely given time delay Affinity capture system disruption

Table 1 continued from previous page.

Assay type	Advantages	Disadvantages	Potential technology-related compound-mediated interferences
BRET	Commercial reagents available Compatible with intracellular readouts Donor is protein-encoded No issues with photobleaching Background fluorescence does not affect donor Spectral separation between donor and acceptor not needed	BRET signal can be weaker that FRET Donor is protein-encoded Expression levels of donor must be matched to acceptor.	Bioluminescence attenuation (quenching, inner-filter, luciferase inhibition) Luciferase stabilizers and inhibitors BRET quenching Emission attenuation (quenching, inner-filter) Compound emission (auto-fluorescence)
ALPHA (AlphaScreen, AlphaLISA, AlphaPlex)	Large dynamic range Commercial reagents available Highly amenable to miniaturization (1536-well plates) Compatible with distances up to 200 nm Works with wide range of affinities, even weaker ones	Cost of reagents Specialized plate reader required	Excitation attenuation (light quenching, light scattering, inner-filter) Singlet oxygen quenching (physical, chemical) Emission attenuation (light quenching, light scattering, inner-filter) Compound emission (auto-fluorescence) Affinity capture system disruption
SPA	Large dynamic range Commercial reagents available	Radioactive (additional safety, waste disposal) Specialized instrumentation required (e.g., CCD or microplate scintillation counter) Requires available radiolabeled reagent	Scintillation quenching Affinity capture system disruption

Table 2 Properties of select FRET pairs. Several donor and acceptor fluorophores are listed for each FRET pair with corresponding excitation and emission wavelengths and the R0 distance for FRET.

Donor fluorophore	λ_{Ex} (nm)	λ_{Em} (nm)	Acceptor fluorophore	λ_{Ex} (nm)	λ_{Em} (nm)	R_0 (nm)	Ref
EDANS	335	493	Dabcyl	453		3.3	(5)
IAEDANS	337	490	Fluorescein	490	525	4.6	(5)
Sirius	355	424	mseCFP	434	474	3.7	(6)
mTagBFP	402	457	sfGFP	485	510	4.6	(7-10)
mAmetrine	406	526	tdTomato	554	581	6.6	(7,11-14)
ECFP	433	475	EYFP	513	527	4.9	(13,15-17)
ECFP	433	475	mVenus	515	528	5.0	(13,16-20)
ECFP	439	476	YPet	517	530	N/A	(13,16, 21)
ECFP	433	475	mCherry	587	610	3.5	(13,16,22-24)
mTurquoise2	434	474	mVenus	515	528	5.8	(17,19,20,22,25,26)
mTurquoise2	434	474	sEYFP	515	528	5.9	(26-28)
CyPet	435	477	YPet	517	530	N/A	(13,29,30)
LSSmOrange	437	572	mKate2	588	633	7.0	(7,12,31)
mTFP1	462	492	mCitrine	516	529	5.7	(11,32,33)
EGFP	488	507	ShadowG	486	510	4.7	(13,34)

Table 2 continued from previous page.

Donor fluorophore	λ_{Ex} (nm)	λ_{Em} (nm)	Acceptor fluorophore	λ_{Ex} (nm)	λ_{Em} (nm)	R_0 (nm)	Ref
EGFP	488	507	PA-GFP	504	517	4.4	(7,13,35)
EGFP	488	507	Phanta	506	516	5.8	(7,13,36)
EGFP	488	507	sREACh	517	531	5.8	(13,34)
EGFP	488	507	mCherry	587	610	5.4	(13,24)
Fluorescein	490	525	Tetramethylrhodamine	557	576	5.5	(5)
Fluorescein	490	525	QSY 7 & QSY 9	560		6.1	(5)
Alexa Fluor 488	490	525	Alexa Fluor 555	555	580	7.0	(37)
CyOFP1	497	589	mCardinal	604	659	6.9	(7,31,38)
BODIPY FL	503	512	BODIPY FL	503	512	5.7	(5)
Clover	505	515	mRuby2	559	600	6.3	(13,27)
mClover3	506	518	mRuby3	558	592	6.5	(39)
mNeonGreen	506	517	mRuby3	558	592	6.5	(10)
EYFP	513	527	mCherry	587	610	5.7	(13,17,22,24)
mVenus	515	528	mKOκ	551	563	6.3	(7,17,19,20,40,41)
mOrange	548	562	mCherry	587	610	6.3	(21,42,43)
Alexa Fluor 594	590	617	Alexa Fluor 647	650	665	8.5	(37)
eqFP650	592	650	iRFP	690	713	5.8	(7,44,45)

Table 3 Properties of select TR-FRET reagents. Donors containing europium or terbium cryptates are paired with different acceptors for detection at 665 nm or 520 nm. Data adapted from www.cisbio.com.

Donor	λ_{Ex} (nm)	λ_{Em} (nm)	Acceptor	λ_{Ex} (nm)	λ_{Em} (nm)	R_0 (nm)
Europium (III) cryptate	320 - 340	615	XL665		665	9
Europium (III) cryptate	320 - 340	615	Allophycocyanine		665	9
Europium (III) cryptate	320 - 340		d2	620	665	9
Lumi4-Tb	320 - 340	490, 545, 620	d2	620	665	5.8
Lumi4-Tb	320 - 340	490, 545, 620	Fluorescein	490	520	4.6
Terbium (III) cryptate	320 - 340	490	GFP	488	520	7

Table 4 Properties of select Alpha reagents. Alpha proximity assays all utilize the same donor beads that are excited with a 680 nm laser. Signal emission depends upon the acceptor bead type and requires proximity within approximately 200 nm.

Donor	λ_{Ex} (nm)	Acceptor	λ_{Em} (nm)	Distance (nm)
Donor bead	680	AlphaScreen	520 - 620	200
Donor bead	680	AlphaPlex (Terbium)	545	200
Donor bead	680	AlphaLISA (Europium)	615	200
Donor bead	680	AlphaLISA (Samarium)	645	200

Affinity Capture Systems and Chemical Modifications

Proximity assays can utilize several different capture approaches to couple the donor and acceptor assay components to the analyte. Popular examples include biotin/streptavidin, glutathione (GSH)/glutathione-*S*-transferase (GST), nickel-NTA/polyhistidine (Ni-NTA/polyHis), FLAG/anti-FLAG, HA/anti-HA, Myc/anti-Myc, and antibodies/analytes. Each capture system has distinct advantages, disadvantages, and other nuances that must be considered (Table 5) (58). Many of these systems utilize tags that can be genetically engineered into recombinant proteins (e.g., 6xHis, GST, FLAG, Myc, or biotinylation in *Escherichia coli*), eliminating the need for any post-translational chemical modifications (59). Most commercial proximity assay technologies incorporate reagents specifically developed for common capture systems, such as donor and/or acceptor beads conjugated to streptavidin, nickel chelate, or antibodies to FLAG, GST, HA, or species-specific secondary immunoglobulins (e.g., anti-mouse IgG, anti-rabbit IgG). Post-translational modifications (e.g., histone methylations, phosphorylated proteins) or other analytes can also be targeted in proximity assays using custom antibodies; however, such antibodies must be carefully validated for specificity (60,61). For example, polyclonal or monoclonal antibodies can be conjugated to donor or acceptor beads with conventional techniques such as reductive amination. Proteins or peptides can also be directly conjugated to donor or acceptor beads for certain applications. The optimum affinity capture system and parameters for a particular proximity assay depends on multiple factors including the molecular target(s) and is beyond the scope of this chapter.

While highly useful, affinity capture components introduce several potential sources for compound-mediated assay interference, notably compounds that disrupt tag-capture system interactions. However, the variety of affinity capture systems can be exploited to develop certain interference counter-screens, discussed in subsequent sections.

Table 5 Summary of common affinity capture systems for homogeneous proximity assays. Advantages, disadvantages, and miscellaneous comments pertinent to homogeneous proximity assays utilizing test compounds are listed.

Capture reagent	Advantages	Disadvantages	Other comments
Biotin-strepavidin	Streptavidin or biotinylated tags can be genetically encoded Biotin amenable to chemical conjugation to various biomolecules Many commercial kits available Strong affinities (K_d ~ 10^{-15} M)	Steric bulk streptavidin tags Biotin-streptavidin interaction susceptible to biotin and biotin mimetics Biotin-streptavidin interaction can be sensitive to protein denaturants	Certain tissues contain biotin and avidins Certain bacteria produce avidins Streptavidin has increased specificity, lower nonspecific binding than avidin
GSH-GST	GST tag genetically encoded Tagged proteins very easy to purify Many commercial kits available	Steric bulk GST tags GSH/GST interaction susceptible to GSH mimetics GSH/GST interaction can be sensitive to protein denaturants GST can dimerize Potential for increased protein aggregation	Potentially redox sensitive
Anti-GST-GST	GST tag genetically encoded Tagged proteins very easy to purify Many commercial kits available	Steric bulk GST tags Anti-GST/GST interaction susceptible to mimetics Sensitive to protein denaturants GST can dimerize Potential for increased protein aggregation	

Table 5 continued from previous page.

Capture reagent	Advantages	Disadvantages	Other comments
Antibodies-analyte	Quality antibodies can possess high specify and sensitivity Many antibodies commercially available	Polyclonal and monoclonal antibodies can show lot-dependent variations Antibody-analyte interactions susceptible to analyte mimetics Sensitive to protein denaturants	Need to verify specificity of antibody
Polyhistidine-tag	His tag genetically encoded Many commercial kits available Tagged proteins are very easy to purify	Metal ion/His tag interactions are susceptible to chelators	Zinc and cobalt can be used in place of nickel ions
FLAG tag	FLAG tag genetically encoded Many commercial kits available	FLAG-Anti-FLAG interactions susceptible to mimetics	Needs other tags for protein purification
Myc tag	Myc tag genetically encoded	Myc-Anti-Myc interactions susceptible to mimetics	

Section Summary

Homogeneous proximity assays are widely used in HTS and follow-up assays for testing hits and compounds with bioactivity. Common technologies include Alpha, FRET, BRET, TR-FRET, and SPA, each of which exploits the proximity of a donor and acceptor source for optimal signal production. However, each technology, including commonly employed affinity capture systems, is susceptible to a variety of compound-mediated assay interferences that are discussed in detail in the subsequent section.

Chemical Mechanisms of Assay Interference in Homogeneous Proximity Assays

Homogeneous proximity assay technologies are susceptible to a range of compound-mediated assay interferences. This section describes the chemical mechanisms that can alter proximity assay readouts. Technology-related interferences can be attributed to different mechanisms related to the properties of compounds in solution and their impact on the assay technology. Such interferences can ultimately manifest as reductions of the assay signal due to mechanisms such as absorbance, quenching, light scattering, reporter enzyme modulation, and disruption of affinity capture components. For example, compounds that interfere with excitation of the donor are encountered in FRET- and Alpha-based proximity assays, as compared to SPA and BRET where initial excitation is caused by radioactive decay of a radioisotope or luciferase activity, respectively. Alternatively, depending on the assay technology employed, assay signals can be increased by interference mechanisms including auto-fluorescence, phosphorescence, and singlet oxygen generation. Sources of non-technology-related interferences that can constitute poorly tractable sources of bioactivity, such as nonspecific compound reactivity and aggregation, are also discussed. Each mechanism is discussed below, as understanding fundamental chemical mechanisms of assay interference allows the design of more effective counter-screens and the subsequent identification of likely interference compounds from homogeneous proximity assays utilized in HTS and follow-up campaigns (62).

Mechanisms of Interference

Fluorescence quenching. Quenching is a phenomenon by which fluorescence is decreased in intensity due to another chemical species. *Dynamic quenching* can occur through (1) FRET, whereby energy is transferred from excited donor to a proximal quencher with significant spectral overlap through nonradiative dipole–dipole interactions; (2) Dexter electron transfer, a short-range process dependent on molecular orbital overlap between the excited donor and a proximal quencher, and (3) exciplex formation. *Static* or *contact quenching* is a process

where the ground state donor comes into physical contact with the quencher to form a complex with altered spectral properties. Each of these quenching processes can induce a quencher to an excited state. As the excited quencher returns to the ground state, this can result in light emission through emissive decay, albeit at lowered intensities relative to quencher-free conditions. Other quenchers act through *dark quenching*, whereby the energy released from the excited to ground state transition is dissipated through molecular vibrations (heat). The end result can be a perturbation in the assay signal independent of the desired bioactivity. Signal attenuators can interfere with several stages in most proximity assays: (1) donor signal excitation/transmission; (2) donor to acceptor signal transmission; and (3) acceptor emission (Figure 2A).

The efficiency of such quenchers is directly related to their extinction coefficient (ε), a factor that represents the capacity of a compound to absorb light at a specific wavelength under a specific set of experimental conditions. The higher ε is, the more efficient the compound at absorbing light. This absorption capacity is also directly related to compound concentration as expressed by the classic Beers-Lambert law (Equation 1):

$$\text{Absorbance} = \text{Concentration} \times \text{Extinction coefficient } (\varepsilon) \times \text{path length} \qquad \text{Equation 1}$$

Chemical quenching. For Alpha-based proximity assays, compounds can interfere with the readout by quenching singlet oxygen, which defined broadly can include (1) energy and charge transfer mechanisms between singlet oxygen and the compound quencher that returns singlet oxygen to the ground state without oxygen consumption (physical quenching); and (2) direct chemical reaction of singlet oxygen with the quenching compound to consume oxygen (chemical quenching) (Figure 2B) (63,64). In organic synthesis, singlet oxygen classically reacts with cunsaturated chemotypes such as alkenes (ene reactions) and also certain heteroatoms to form various oxidation products such as peroxides (65). Singlet oxygen is also capable of oxidizing various phenols, sulfides, and amines. Some examples of singlet oxygen quenchers include carotenes, tocopherols, and tertiary amines like 1,4-diazabicyclo[2.2.2]octane (DABCO; Figure 2B) (66-68). There are also lingering questions about the quenching potential of certain chemotypes prevalent in screening collections including unsaturated carbonyls, and thiones (69-72). As much of the singlet oxygen chemistry has been performed in the context of organic synthesis under non-assay-like conditions (i.e., organic solvents, non-physiological temperatures), the chemical mechanisms, prevalence, and overall significance of singlet oxygen quenching by test compounds in Alpha-based assays are relatively uncharacterized and should be confirmed in conditions mimicking the parent assay.

Certain assay components or contaminants may also interfere with Alpha-based technologies. For example, azide (N_3^-), a common antimicrobial additive in biological reagents, may interfere with singlet oxygen transmission at sufficiently high concentrations. Certain metals (Al^{3+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, Ni^{2+}, Zn^{2+}) may also quench singlet oxygen, albeit at low millimolar concentrations (73). Notably, metals can be present as contaminates within test compound solutions (74).

Scintillation can be susceptible to chemical quenching. Compound disruption of the scintillation process causes non-radiative dissipation of energy manifesting as an apparent reduction of the energy of the decay event. This translates to a decrease in the number of photons produced (75). Some liquid scintillation counters have built-in functions for quenchers, often prepared by obtaining standard curves of known quenchers (75-78). However, it should not be assumed that such strategies completely correct for scintillation interference.

Absorbance. Test compounds can interfere with proximity assay readouts by absorbing light. Compounds that interfere with this assay step by inner-filter effects will absorb light in the excitation wavelength. For Alpha-based assays, these are usually blue-colored compounds that strongly absorb in the near-infrared region (Table 6 and Figure 3A). By contrast, the yellow and orange compounds more prevalent in screening libraries do not usually absorb in the light regions relevant to Alpha technology. For FRET-based assays, these types of interfering compounds usually absorb at shorter wavelengths corresponding to overlap with the FRET donor fluorophore. With SPA, some compounds, usually colored, can interfere with scintillation (also referred to as

'color quenching') (79). As with quencher, the ability of compounds to interfere with light can be expressed by the Beers-Lambert law (Equation 1), the consequence being that absorbance is concentration-dependent and dependent on the extinction coefficient for a particular compound at a given wavelength and under defined experimental conditions. For additional details on compound interference *via* light absorbance, refer to Interference with Fluorescence and Absorbance.

Light scattering. Another related mechanism of signal quenching is light scattering, whereby insoluble materials such as screening compounds effectively diffract light (Figure 2C) (80,81). Many of these compounds have high cLog*P* values and other poor physicochemical properties related to solubility in assay conditions. A characteristic of light-scattering compounds is a relatively continuous absorption spectrum (i.e., no apparent λ_{max}). Light scattering is most efficient at shorter wavelengths (decreases by factor of n^4), and scattered light is relatively polarized (81). This phenomenon can interfere with the transmission of the initial excitation light source (e.g., laser) and the final light excitation. Such compounds can occasionally be identified by simple visual inspection for cloudy suspensions or by centrifuging a sample and looking for gross precipitate. More quantitatively, this can be accomplished by nephelometry, turbidimetry, or dynamic light scattering. In certain cases, their apparent activity can be attenuated by the inclusion of detergents such as 0.1% Tween-20 (v/v) (Figure 3B; see also subsequent section Strategies to Mitigate Interference by Light-based Interferences).

Case example: Interpreting light-based interferences can be complex. For example, Figure 4 shows test compound activities in a TR-FRET assay for bromodomain inhibitors where binding of a His-tagged bromodomain to a biotinylated peptide is coupled to a Eu-labeled anti-His antibody (donor) and APC-labeled streptavidin (acceptor) for detection. Examining the acceptor and donor signals separately shows tight distributions for the active (AC) and neutral (NC) controls, whereas many test compounds interfere with the fluorescence signal (Figure 4A) and some would be classified as hits due to a reduction in the TR-FRET normalized ratio (Figure 4B). For this example, taking compounds that show a signal reduction greater than three standard deviations from the donor channel suggests that 30% of the putative hits are likely fluorescent quenchers. However, understanding the many interference mechanisms involved is complicated and building evidence for genuine biological activities of individual test compounds would benefit from orthogonal assay technologies and interference counter-screens.

Luciferase modulators. Certain proximity assays use luminescence reporters such as either BRET (Renilla luciferase and GFP) or NanoBRET (Nanoluciferase and Halotag enzymes) or split reporter enzymes as employed in NanoBiT technology that uses split forms of Nanoluciferase. Compounds that attenuate the luciferases signal either through inhibition of the enzyme or through light absorbance will interfere with these assay formats. For a review of luciferase inhibitors refer to Interferences with Luciferase Reporter Enzymes. For ratiometric assays such as BRET (or TR-FRET as mentioned above), it is important to examine both donor and acceptor channels in addition to the ratio data which is discussed in more detail in this chapter (see subsequent section Ratiometric Data Analysis).

Affinity capture system disruption. The next major category of compound-mediated proximity assay interference is disruptors of affinity capture components. These compounds interfere with affinity capture systems, leading to a disruption of the assay signal. Depending on the assay configuration, such interference could be misinterpreted as bioactivity. The chemical mechanisms of affinity capture interference depend on the specific donor and acceptor components.

One of the more popular affinity capture systems for proximity assays is the His-Ni^{2+} system. The popularity of this tag is given by its dual use for protein purification and affinity capture for proximity assays. While highly useful, several chemotypes have been shown to interfere with the His-Ni^{2+} interaction, including in AlphaScreen assays (Figure 5A and Table 4) (82). The most common motif in one explicit analysis of His-Ni^{2+} disruptors was the NCCN chemotype (Table 7). It is hypothesized that many of these chemotypes form bifurcated hydrogen bonds with NH-donating histidine side chain residues (present in His-tag) and/or chelate metal ions (present on

beads) (Figure 5B) (83-85). If bound to either the histidine tag side chains or nickel, proper tag function could be disrupted and the assay signal could be attenuated. A second prevalent category of His-Ni^{2+} frequent fitters are chelating agents. Examples for these chemotypes are picolylamines or pyridines (Table 4). Certain hydrazones may also interfere by a chelation mechanism (68).

The streptavidin-biotin interaction is another popular choice for coupling an analyte to a detection technology. Compounds with significant affinities (including fast kinetics) for avidins such as biotin analogs or isosteres may in principle compete with biotin to disrupt the intended streptavidin-biotin interaction (Figure 5C) (87). Biotin-avidin interactions are well characterized, and while biotin analogs have been described, the likelihood of such compounds actually interfering through this mechanism is probably low due to the remarkably strong affinity of the streptavidin-biotin interaction ($K_d \sim 10^{-15}$ M) (88-90). In one report, 2 of 24,779 (0.008%) compounds interfered by this mechanism in a TR-FRET HTS versus ROCK-II inhibitors (87). As another example, several compounds were found to interfere with the streptavidin-biotin interaction among 1,199 hits evaluated from a primary screen with an AlphaLISA assay (Figure 6) (91). In proximity assays utilizing streptavidin-biotin, the buffer should be otherwise free of biotin (as a contaminant, as an intended ingredient in the media such as RPMI, or as an endogenous component of biological samples such as serum). This biotin will compete with the affinity capture system and disrupt the intended streptavidin-biotin interaction. Furthermore, test compounds with structural similarity to biotin including the imidazoline-2-one motif can disrupt the affinity capture system (Figure 6) (92,93).

The GSH-GST interaction has also been successfully utilized to couple an analyte to detection reagents. There are several potential chemical mechanisms for compound-mediated interference of GSH-GST interactions: (a) binding of a compound with similar molecular features as GSH to the GSH-binding site on GST, (b) covalent modification of GST or GSH by electrophilic compounds, and (c) reversible binding to non-active sites on GST (Figure 4D) (94). Note that most reagents utilizing GSH will immobilize GSH at its *S*-position, so compound reactivity with GSH would presumably occur at its terminal *N*- or *C*- positions.

Several chemotypes have been shown to interfere with the GSH-GST interaction in the context of AlphaScreen technology (Table 8) (94). Note a similar affinity capture system utilizes an anti-GST antibody and a complimentary GST tag. In theory, compounds that bind GST or the anti-GST antibody could disrupt the intended antibody (anti-GST)-analyte (GST) interaction.

A subset of affinity capture systems susceptible to compound-mediated interference is antibody-analyte systems. Compounds with chemotypes similar to the antibody antigen may compete with the desired antigen, effectively preventing the antibody-antigen interaction. A notable example of this interference are acetamide-containing compounds that were enriched in an AlphaScreen HTS which utilized an acetylated lysine/anti-acetylated lysine antibody system (Figure 5E) (86). In another example, following a screen of ~350,000 with an AlphaLISA primary assay, 1,199 hits were further evaluated by a battery of counter-screens (95). By comparing results from TruHit counter-screen configurations with a counter-screen utilizing the primary assay and purified analyte, it was possible to rule out any interferences by antibody-mediated capture of the analyte (N. P. Coussens, unpublished data).

Auto-fluorescence. Auto-fluorescence can be induced by: (1) absorption of the donor excitation light, (2) absorption of the energy transferred from the donor to acceptor, and (3) compounds that absorb the signal from the acceptor (Figure 7A). Such compounds can interfere with the final assay readout by producing measurable signal even in the absence of the intended analyte.

Compared to other interferences, most compounds that emit light in the assay readout can be rather easily identified by signal intensities that are outside the range of the controls. This is especially apparent in assays where desired bioactivity produces an attenuated signal. With such an assay, a compound that emits light, but also has genuine bioactivity might not be identified and would thus be a false negative. By contrast, in assays

where desired bioactivity produces an augmented signal, emitters have the potential to function as false positives. Finally, it should be noted that compounds with genuine on-target activity but that also fluoresce can display a range of patterns, depending on the assay conditions and the compound properties (Figure 7B).

Singlet oxygen generators. In Alpha-based assays, aside from fluorescent compounds, certain compounds may produce singlet oxygen *in situ* (Figure 7C) (96,97). These photosensitizing agents characteristically possess high absorption coefficients in the excitation light spectral region, contain a triplet state allowing for efficient energy transformation to ground state oxygen, have high quantum yields for the triplet state, and have high photostabilities (64). Prototypic agents include organic dyes such as rose bengal, eosin derivatives, and methylene blue, as well as porphyrin derivatives (Figure 7C). These compounds are relatively uncommon in screening collections as they are non-lead-like by conventional metrics. Naphthalenes, anthracenes, quinones, and anthraquinones are aromatic hydrocarbons chemotypes that may be screening collections, and notably these and related derivatives have been shown to produce singlet oxygen in certain aqueous conditions (98,99). The significance of photosensitizing compounds in the context of biological screening conditions and Alpha assay interference is relatively unexplored, however. Additionally, note that some compounds that produce singlet oxygen *in situ* have been shown to damage SPA reagents (100).

Phosphorescence. During an evaluation of hit compounds, Erythrosin B was identified as an outlier by the EPIgeneous HTRF Methyltransferase assay (Cisbio), with a signal 10-fold beyond the 100% inhibition control (N. P. Coussens, unpublished observation). The assay measured SAH, which competitively displaces d2-labeled SAH that is pre-bound to anti-SAH labeled with Lumi4-Tb, resulting in a loss of FRET signal. In the absence of SAH (100% inhibition control), excitation of the terbium donor results in a long emission at 620 nm capable of exciting the d2 acceptor which emits at 665 nm. In the case of Erythrosin B, the donor emission (normalization control) was consistent with the other controls; however, the signal at 665 nm was 10-fold higher. It has been reported that excitation of Erythrosin B at 490-560 nm results in an emission at 665 nm that can persist for ~700 μs (101). The major emission peak for terbium is centered at 540 nm, which is in the excitation range of Erythrosin B.

Other mechanisms of assay interference in proximity assays. Recently it was reported that salicylic acids can interfere with TR-FRET assays by causing a large signal enhancement attributed to the interaction between the salicylate and the cryptand-ligated europium (102). In SPA, compounds that emit radiation may interfere via signal emission, though such compounds are not routine components of screening libraries and would be extremely rare. However, such compounds could occur as contaminants in screening centers making use of radioisotope-based assays and reagents.

Proximity assays are susceptible to a variety of generalized compound-mediated assay interferences, including nonspecific reactivity and aggregation (103). Reactive test compounds, including redox-active compounds that produce H_2O_2 *in situ*, can interfere with biological assays by reacting nonspecifically with assay reagent(s), substrate(s), target(s), and/or unrelated target(s) (104,105). Aggregating test compounds can interfere with biological assays by forming compound aggregates that nonspecifically interact with assay proteins, leading to altered protein structure and dynamics (106-109).

A common pitfall is to assume bioactivity confirmed by an orthogonal assay represents tractable bioactivity and excludes the possibility of false positives. In many cases, apparent bioactivity from aggregating or nonspecific reactive compounds is *highly reproducible* and *can be confirmed by orthogonal assays*. This is because reactive or aggregating compounds disrupt protein structure and dynamics nonspecifically in susceptible systems. However, unlike more desirable mechanisms of bioactivity, this reactivity and general protein perturbation is generally poorly tractable and difficult to optimize for specificity. Depending on the biological system and assay, aggregating compounds can display bell-shaped curves and some compounds can fail to reach maximal (100%) inhibition regardless of compound concentration (J. Walsh, unpublished observations) (110,111). Therefore, it is

crucial to perform appropriate orthogonal assays *and* counter-screens to rule-out generalized compound-mediated assay interference (Table 9).

For additional details on assay interference by aggregators in HTS, refer to Assay Interference by Aggregation. For additional details on assay interference by reactivity in HTS, refer to Assay Interference by Chemical Reactivity.

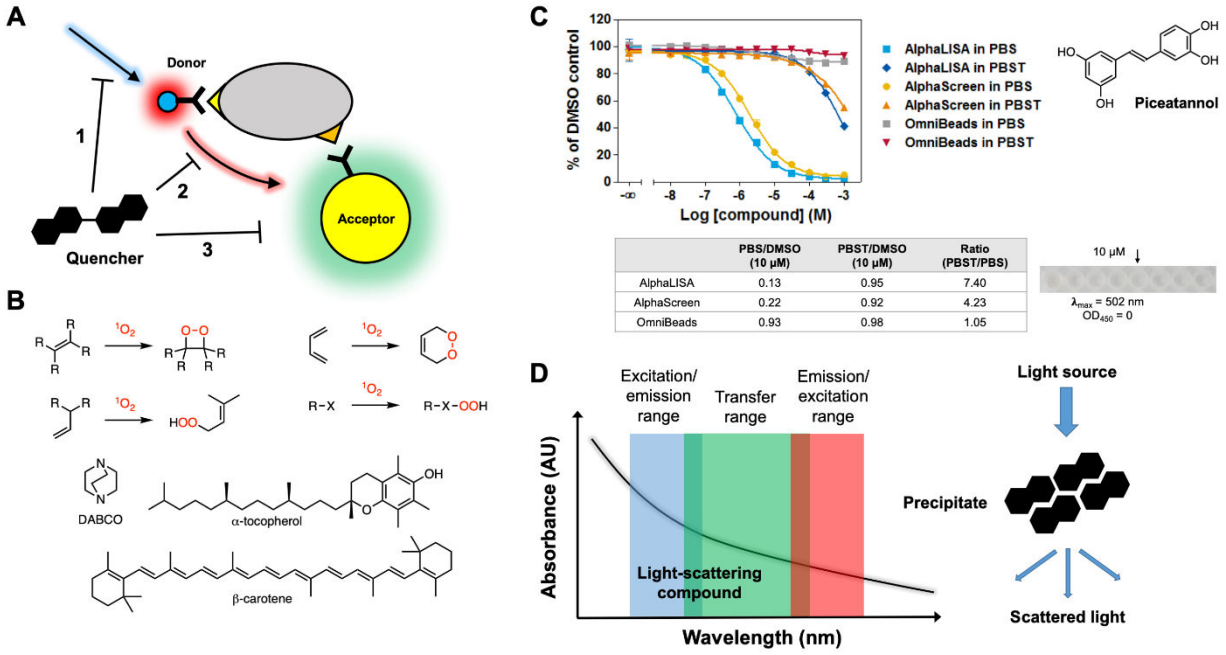


Figure 2 General schematic of compound-mediated interference in homogeneous proximity assays by signal-attenuating compounds. (A) Compounds can attenuate the signal of proximity assay readouts at several points: (1) excitation of donor (Note: for BRET this could be a luciferase inhibitor), (2) signal transmission from the donor to the acceptor, and (3) final emission event. Note: for SPA, interference can be observed at points (2) and (3). (B) Singlet oxygen quenching by test compounds. (left) Prototypical reactions of singlet oxygen. (right) Prototypical singlet oxygen quenchers. (C) Example of likely singlet oxygen quenching in Alpha technology by piceatannol. Compounds were tested in concentration-response format using AlphaScreen, AlphaLISA, and Omnibead formats in the presence and absence of Tween-20 detergent. The compound does not interfere significantly in the Omnibead format, as the singlet oxygen transmission occurs within the beads. Again note the interference presumably by light scattering is attenuated by the addition of 0.1% Tween-20 (v/v). Data courtesy P. Roby (PerkinElmer). (D) Light-scattering compounds can also attenuate assay light transmission through diffraction. Such compounds are often insoluble in assay conditions and have absorbance spectra with characteristically continuous light absorption.

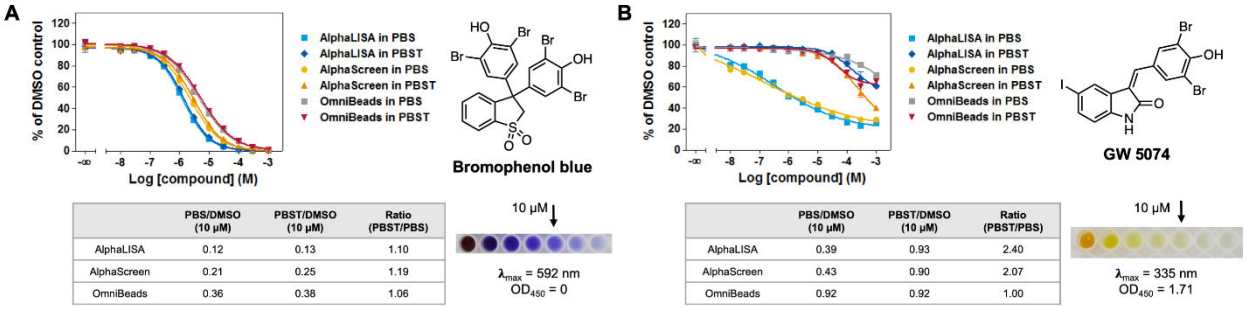


Figure 3 Examples of colored and scattering interferences in AlphaScreen. Compounds were tested in concentration-response format using AlphaScreen, AlphaLISA, and Omnibead (beads containing all necessary Alpha chemistry reagents) formats in the presence and absence of Tween-20 detergent. (A) Example of a colored compound, bromophenol blue, interfering with Alpha technology. The compound interferes similarly across all assay formats, as each format requires light transmission that overlaps the compound absorption. (B) Example of a poorly soluble compound, GW 5074, interfering with Alpha technology readouts. Note the interference presumably by light scattering can be attenuated by the addition of 0.1% Tween-20 (v/v). The compound does not interfere significantly in the Omnibead format, as the required light transmission occurs within the beads. Data courtesy P. Roby (PerkinElmer).

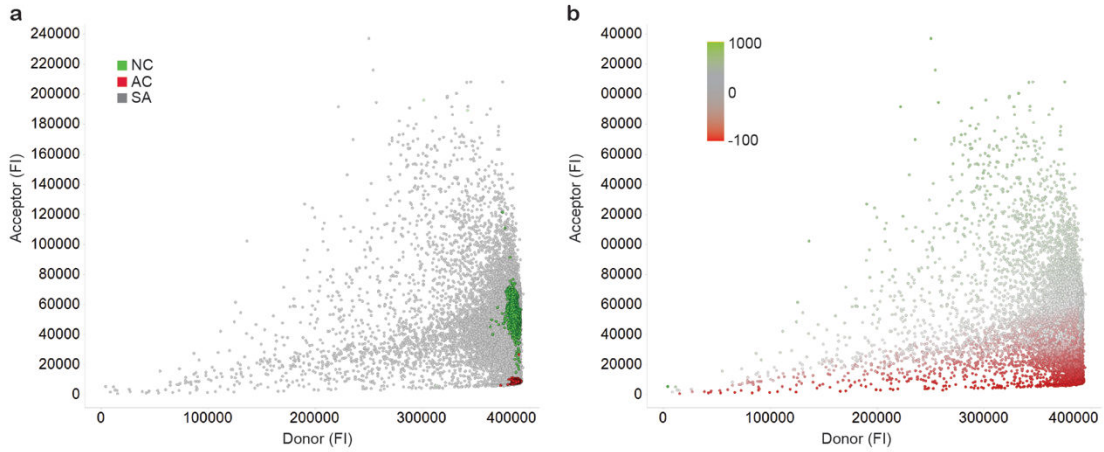


Figure 4 Example compound interference in a TR-FRET assay. (A) Neutral controls (DMSO treated, NC; green) and active controls (unlabeled peptide; red) show a tight distribution in both donor and acceptor fluorescent signals, while compound samples (grey data) significantly skew these signals with both quenching effects on the donor as well as increases in the acceptor signal, likely due to light scattering effects. (B) The same data colored by the normalized ratio (A/D) percent activity (-100% = 100% inhibition, red). Many of the quenchers are scored as hits. Data courtesy D. Auld (Novartis Institutes of Biomedical Research).

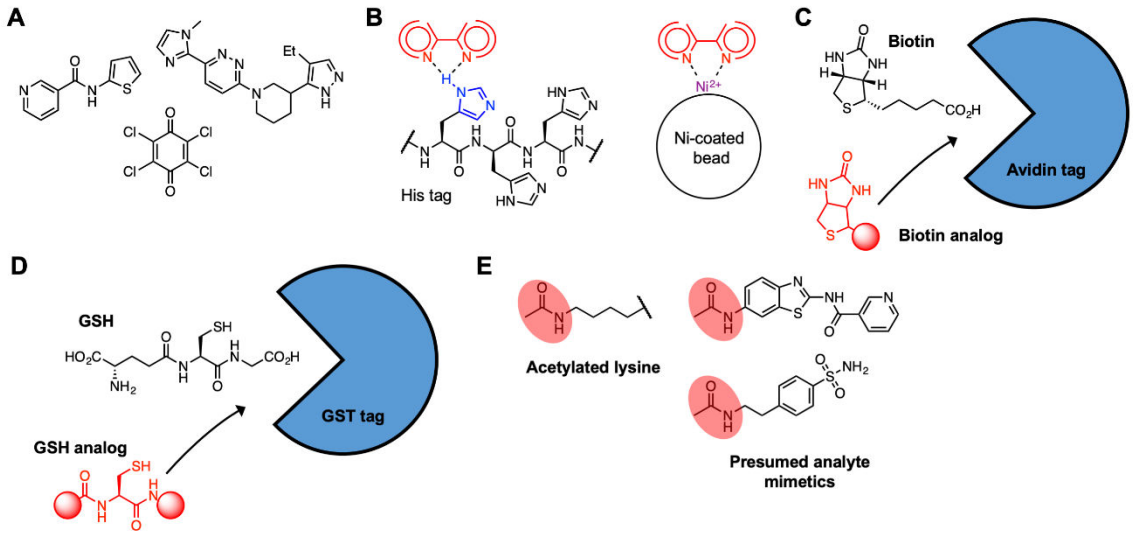


Figure 5 Compound-mediated interference of affinity capture systems in proximity assays. (A) Examples of reported His- Ni^{2+} interaction disrupters (82). (B) Potential chemical mechanisms of His-Ni^{2+} interference include interfering compounds (red) that are proposed to form hydrogen bonds with NH-donating histidine side chains (blue, left schematic) or coordinate with nickel ions (purple, right schematic). (C) Potential chemical mechanisms of biotin-streptavidin capture interference include compounds such as biotin analogs (red) that compete with biotin for binding. (D) Potential chemical mechanisms of interference of GSH-GST-mediated capture interference include compounds such as GSH analogs (red) that compete with GSH for the GSH-binding site on GST. (E) Potential chemical mechanisms of interference of antibody-analyte capture interference include analyte mimicry. In this example from an AlphaScreen for inhibitors of the MOZ histone acetyltransferase, the assay quantified the amount of acetylated lysine residues using an anti-acetyl lysine antibody conjugated to an acceptor bead (86). Compounds with an acetamide motif were enriched among the HTS primary actives and were triaged using a combination of counter-screens (product-spiking, antibody swap).

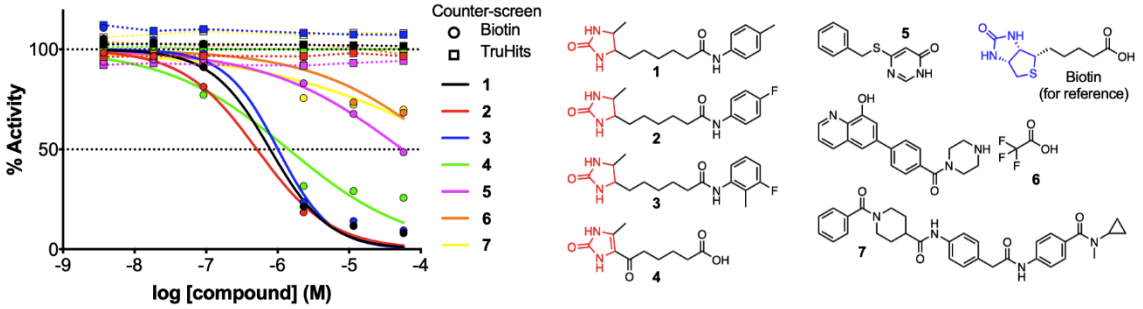


Figure 6 TruHit counter-screen flags compounds that disrupt the streptavidin-biotin interaction. Compounds **1**-**7** disrupt the streptavidin-biotin interaction were identified after performing both configurations of the TruHit counter-screen. Compounds **1**-**7** only showed activity when pre-incubated with streptavidin donor beads prior to the addition of biotinylated acceptor beads (circles), whereas no activity was observed when the beads were premixed before compound addition (squares). Note **1**-**4** contain the imidazoline-2-one motif (red) common to biotin (blue).

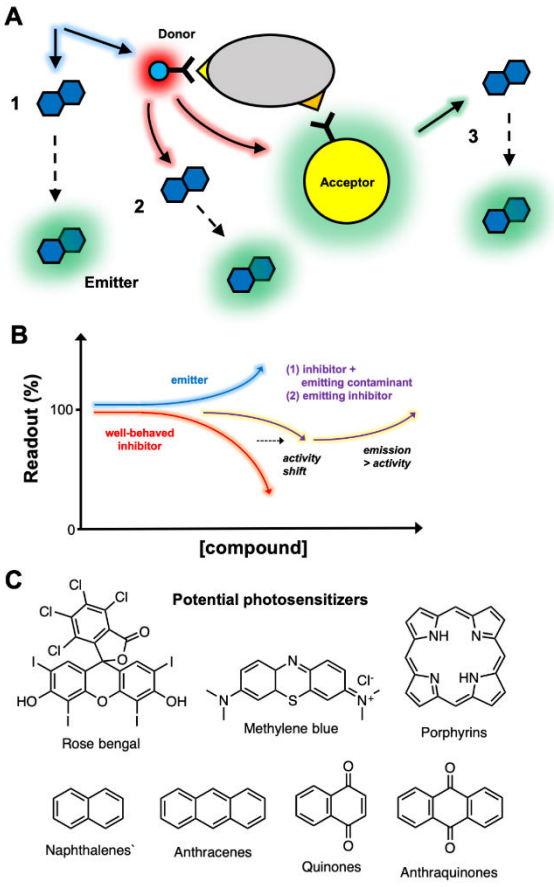


Figure 7 Compound-mediated interference in proximity assays by compound emission. (A) Compounds (blue hexagons) can interfere with proximity assay readouts through compound emission at several points: (1) compound excitation by initial excitation event, (2) compound excitation by signal transmission from donor to acceptor, and (3) compound excitation by final emission event. Interference can result when the test compound emits signal (usually light) in the same wavelength as the final assay readout. (B) Compounds that emit light can be easier to identify from assays with negative readouts (compare red and blue lines); however, emission from a compound with on-target activity could display a variety of patterns and result in a false-negative result (orange lines). (C) With Alpha-based assays, compound-mediated emission could result from photosensitizing agents that produce singlet oxygen under aqueous conditions causing emission from the acceptor bead. Structures of representative photosensitizing agents are shown.

Table 6 Examples of reported AlphaScreen quenchers via light absorbance. Colored compounds have the potential to interfere with AlphaScreen and FRET readouts due to their light absorbance properties.

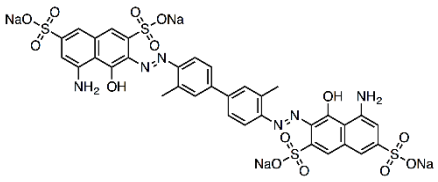
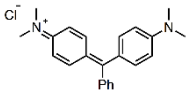
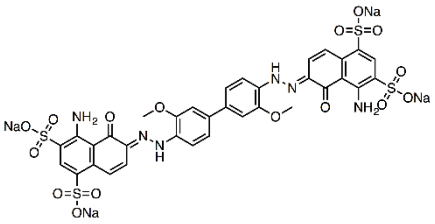
Compound name	Chemical structure	Concentration	% interference	λ_{max} (nm)	Reference
Trypan blue		1 μM	~90	607	(73)
Malachite green		1 μM	~20	615	(73)
Chicago sky blue		1 μM	~40	618	(73)
Sodium azide		0.01% (w/v)	~60		(73)

Table 7 Reported AlphaScreen His-Ni2+ interaction disrupting chemotypes. Chemotypes were derived from an AlphaScreen-based counter-screen (82). Many of the His-tag disruptors are thought to interfere by a chelation mechanism. See also https://ochem.eu (AlphaScreen-HIS-FHs) for SMARTS strings and online substructure screening tool.

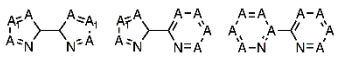
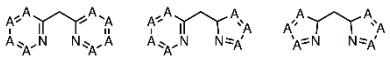
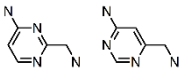
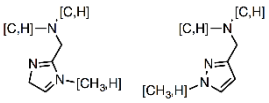
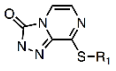
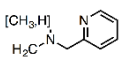
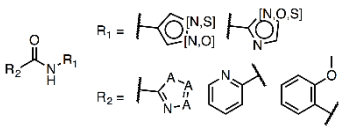
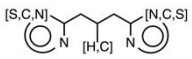
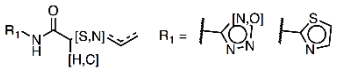
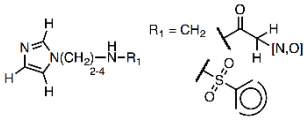
Name	Chemotype	Name	Chemotype
NCCN	 A = aromatic C or N; A1 = aromatic C, N or S	Diarylmethanes	 A = aromatic C or N
Picolylamines_A		Aminals	 Dashed line = any type of covalent bond
Picolylamines_B		Heterylsulfides	 R_1 = any atom, group
Picolylamines_C		Heterylamides_A	 A = any aromatic atom
Bis-picolylamines		Heterylamides_B	
Alkylimidazoles_A		Pyrimidinetriones	

Table 7 continued from previous page.

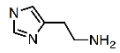
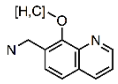
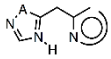
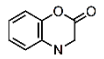
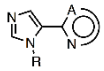
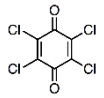
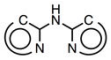
Name	Chemotype	Name	Chemotype
Alkylimidazoles_B		8-Quinolinols	
Alkylimidazole_C	 A = any aromatic atom	Benzoxozinones	
Heterylimidazoles	 R = any atom, group; A = any aromatic atom	Perchloroquinone	
Diarylamines			

Table 8 Reported AlphaScreen GST-GSH interaction disrupting chemotypes (94). Chemotypes were derived from an AlphaScreen-based counter-screen. See also https://ochem.eu (AlphaScreen-GST-FHs) for SMARTS strings and online substructure screening tool.

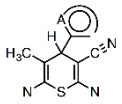
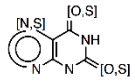
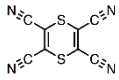
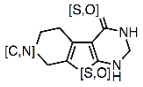
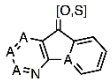
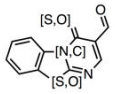
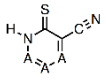
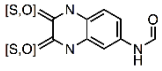
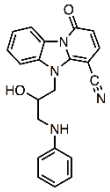
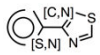
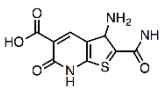
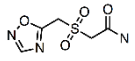
Name	Chemotype	Name	Chemotype
Cyanothiopyrans	 A = any aromatic atom	Pyrimidinodiones	
Cyanodithiine	 Singleton	Thienopyrimidinone_A	
Azafluorenones	 A = aromatic C or N	Thienopyrimidinone_B	
Cyanothioazinones	 A = aromatic C or N	Piperazinediones	
Cyanopyridinones		4-Heteryl-thiazoles	
Thienopyridinones		Oxadiazoles	

Table 8 continued from previous page.

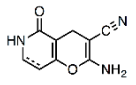
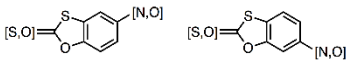
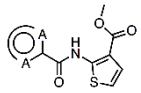
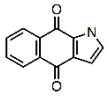
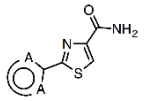
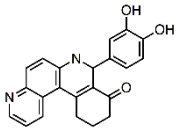
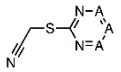
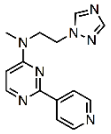
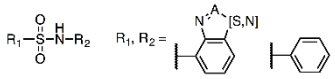
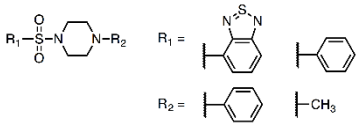
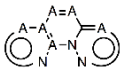
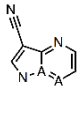
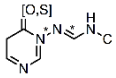
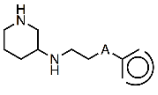
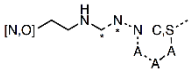
Name	Chemotype	Name	Chemotype
Pyranopyridinones	 Dashed line = double or aromatic bond	Cycloheptenones	
Benzoxathiolones		Thiophenes	 A = any aromatic atom
Indoloquinones		Thiazoles	
Catechols		Cyanothioethers	 A = aromatic C or N
Pyrazolones		Aminopyrimidines_A	
Sulfonylamide_A	 A = any atom	Aminopyrimidines_B	
Sulfonylamide_B		Fused azacycles_A	 A = aromatic C or N
Thiopyrylium		Fused azacycles_B	 A = aromatic C or N
Pyrimidinones_A	 * = bond also can be aromatic	Ethylenediamines	 A = any aliphatic atom
Pyrimidinones_B		Arylethanolamines	 Dashed line = aromatic bond; * = atoms that must belong to 6-membered ring
Pyrimidinones_C		Azaspironones	

Table 9 Interpretation of technology-independent compound-mediated assay interference in proximity assays. Well-behaved active compounds will show activity in both the proximity assay and the orthogonal assay. Technology-related artifacts will typically be inactive in truly orthogonal assays. Note compounds active through nonspecific mechanisms of action such as reactivity and aggregation of target assay components may show activity in both the proximity and orthogonal assays. Active compounds should be assessed for common sources for nonspecific bioactivity by additional counter-screens.

Mode of action	Proximity assay	Orthogonal assay	Interpretation
Well-behaved active compound	Active	Active	Rule-out nonspecific activity with aggregation, reactivity counter-screens
Quencher, capture system disruptor	Active	Inactive	Proximity assay-specific artifact
Nonspecific reactivity	Active	Active	Rule-out nonspecific activity with aggregation, reactivity counter-screens
Aggregation	Active	Active	Rule-out nonspecific activity with aggregation, reactivity counter-screens

Prevalence of Interferences in Homogeneous Proximity Assays

There are multiple factors that can influence the prevalence of interferences in a given homogeneous proximity assay, including:

- Compound library composition and concentration of test compounds (112)
- Assay technology
- Biological target (e.g., is it intrinsically sensitive to thiol-reactive compounds or aggregators?)
- Buffer/media components (detergent, scavenging reagents, chelating agents, salts, metal ions)
- Enzyme, substrate, and co-factor concentrations
- Reagent concentrations
- Spectrophotometric settings (wavelengths, filter widths, acquisition delay)
- Temperature, pH, oxygen saturation, ambient room fluorescent lights (for certain fluorophores and microplates)
- Assay procedure (incubation times, order of compound and reagent addition)

Section Summary

Based on the assay technology, proximity assays are susceptible to several sources of compound-mediated assay interferences. The main categories of technology-related compound-mediated assay interference include compounds that (1) attenuate signal transmission, (2) disrupt capture systems, (3) emit signal. Proximity assays are also susceptible to non-technology-related sources of assay interference, notably aggregation and nonspecific reactivity including thiol reactivity and redox activity. A sound knowledge of these chemical mechanisms enables the design of useful counter-screens and orthogonal assays for assessing compound-mediated assay interference, as discussed in the next section.

Strategies to Identify Compound-Mediated Interference in Homogeneous Proximity Assays

This section describes a number of counter-screens that can be devised for the identification of compound-mediated interference mechanisms associated with homogeneous proximity assays. Example counter-screen strategies include target-independent configurations, bypassing a reaction or process to only evaluate analyte detection, switching affinity capture components, and profiling studies, such as absorbance and emission tests. Data from these counter-screens can help to link technology-related interference mechanisms to test compounds; however, it is important to keep in mind that such compounds might also have on-target activity,

which should be evaluated by an orthogonal assay. The implementation of counter-screens will help to triage assay-specific artifacts, confirm truly bioactive compounds, and identify potentially useful bioactive technology-related interference compounds. Suggestions are given on the workflow design for both technology-related and generalized counter-screens, as well as orthogonal assays. Data analysis strategies are also discussed, including cheminformatics analyses to flag compounds associated with promiscuity and/or assay interference.

Employment of Counter-Screens in Workflows

An important question is, "How much effort should be devoted to investigating compound-mediated interferences?" We recommend performing at least one orthogonal assay to confirm bioactivity. In addition, we also recommend performing specific counter-screens for the primary biological assay interferences. The rationale for performing interference counter-screens even if the intended bioactivity is confirmed by orthogonal assays is to determine how much of the readout is due to true bioactivity versus interference. Note that certain counter-screens such as product spiking can efficiently identify a variety of interference mechanisms. More detailed counter-screens for specific interference mechanisms (e.g., pull-downs, emission spectral analyses) may be warranted under certain circumstances including further de-risking prior to more involved studies or compound progression.

Post-HTS screening triage should include counter-screens for technology-related assay interference and also orthogonal assays (62). Counter-screens and orthogonal assays can either be performed serially or in parallel. The exact order usually depends on the number of hits marked for follow-up, the throughput of each follow-up assay, the number of replicates and doses to be tested, and the pre-test probability of each interference type (the latter can be estimated by examining the structure of actives, and the behavior of the primary assay in response to known interference compounds). In practice, with sufficient planning, including pre-HTS optimization, technology-related counter-screens can be performed in a matter of weeks.

Compounds with confirmed bioactivity in orthogonal assay(s) and minimal interference in assay-related counter-screens should be considered for progression in a project. Compounds without confirmed bioactivity in orthogonal assay(s) that demonstrate interference in assay-specific counter-screens should generally be triaged. Compounds with confirmed bioactivity in orthogonal assay(s) and confirmed interference in counter-screens can be progressed, with the caveat that subsequent experiments should continuously monitor assay interference in addition to bioactivity. For target-based assays, biophysical measurements such as surface-plasma resonance should be performed to confirm compound binding and stoichiometry to target molecule and target-engagement in cell-based assay can be confirmed using techniques such as cellular thermal shift assays (CETSA). Additional details on CETSA can be found at the related Assay Guidance Manual chapter Screening for Target Engagement using the Cellular Thermal Shift Assay.

Target-Independent Configurations as Counter-Screens

Counter-screens can be performed to determine whether test compounds modulate the signal independent of the target. One approach is to utilize linker substrates to induce proximity between the donor and acceptor components. For example, a linker substrate might be a biotinylated peptide containing a polyhistidine tag (Figure 8A). Ideally the linker incorporates the same affinity capture components that are utilized in the primary assay. This enables the identification of compounds that disrupt the interactions between the affinity capture components in addition to other technology-related interference mechanisms. If strong-affinity capture components (e.g., streptavidin-biotin) are involved, two counter-screens can be performed to specifically identify disruptors of this interaction. For the first counter-screen, the test compounds should be added *before* the complementary capture component is added (see sample protocol below). For the second counter-screen, compounds are added after the capture components have bound the analyte. Greater activity in the first counter-screen would indicate interference with the affinity capture components, whereas similar activities in both counter-screens would be indicative of another technology-related interference mechanism. The necessary

reagents for target-independent counter-screen can sometimes be prepared in-house through standard recombinant and protein chemistry techniques; however, in many cases they are commercially-available or can be produced by vendors.

For Alpha-based assays, there are several commercially-available reagents that can be used to identify and distinguish technology-related interference compounds. The TruHit reagent includes streptavidin donor beads and biotinylated acceptor beads (Figure 8B). The TruHit beads can be used to develop counter-screen to identify different types of interference compounds, including biotin mimetics, light scattering compounds, singlet oxygen quenchers, light quenchers, and emitters. Omnibeads contain everything required to generate an AlphaScreen signal within each bead, and thus are not susceptible to interference by affinity capture disruption or singlet oxygen quenching (Figure 8B). Therefore, also including a counter-screen with Omnibeads allows one to distinguish light-based interference mechanisms (light absorbance, light scattering, quenchers, and emitters) from others that might be identified with the TruHit counter-screens.

Sample steps are provided below to illustrate how these counter-screens are performed and some points to consider. While counter-screens take time to develop and optimize, target-independent counter-screens can be applied to triage hits from many different screens performed with the same primary assay technology. Therefore, this strategy is likely to be well worth the initial investment.

Sample steps | Alpha Counter-Screens.

Using TruHit streptavidin-donor and biotin-acceptor beads

Configuration 1: Screening for singlet oxygen quenchers, color quenchers and, light scatters

Incubate donor (streptavidin) and acceptor (biotin) beads in assay buffer for 30 min.

Add test compounds, control compound (Malachite green), and vehicle control to corresponding reaction vessels from Step 1 using acoustic dispenser (e.g., Echo) or pintool transfer. Alternatively, transfer solutions from Step 1 to reaction vessels containing pre-dispensed compounds and vehicle controls.

Incubate beads and compounds from Step 2 for approximately 30 min.

Record assay readout (λ_{Ex} 680 nm/ λ_{Em} 615 nm).

Compare signals from test compounds to those of the controls.

Configuration 2: Screening for biotin mimetics (*Note that biotin mimetics are only identified after comparing the results of Configuration 2 against those of Configuration 1, because both configurations are susceptible to interference by singlet oxygen quenchers, color quenchers and light scatters*)

Incubate donor beads with test compounds, control compound (biotin), and vehicle control in assay buffer for 30 min.

Add acceptor (biotin) beads to corresponding reaction vessels from Step 1.

Incubate beads and compounds from Step 2 for 30 min.

Record assay readout (λ_{Ex} 680 nm/ λ_{Em} 615 nm).

Compare signals from test compounds to those of the controls.

Researchers should consider the following technical points:

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Beads can usually be prepared as working solutions in 1X assay buffer.

Titrate the beads to match the TruHit signal with the primary Alpha assay.

Incubation times should be altered to approximate the conditions of the parent assay

The concentration of test compounds should be the same as in the primary assay.

Ensure compound and bead mixtures are equilibrated.

Consider testing compounds in multiple concentrations.

In Configuration 1, the pre-formed biotin-streptavidin complex is difficult to disrupt. To investigate capture component interferences, use Configuration 2.

Compounds that decrease the assay readout in Configuration 2, but not in Configuration 1, are likely biotin mimetics.

Using Omnibeads

Incubate Omnibeads in assay buffer for 15 min.

Add test compounds, control compound (Malachite green), and vehicle control to corresponding reaction vessels from Step 1 using acoustic dispenser (e.g., Echo) or pintool transfer. Alternatively, transfer solutions from Step 1 to reaction vessels containing pre-dispensed compounds and vehicle controls.

Incubate beads and compounds from Step 2 for approx. 30 min.

Record assay readout (λ_{Ex} 680 nm/ λ_{Em} 615 nm).

Compare the signals from test compounds to those of the controls. Significant changes in signal likely result from light-based interference mechanisms.

Researchers should consider the following technical points:

Include positive and negative controls for relevant interferences (Table 10).

Beads can usually be prepared as working solutions in 1X assay buffer.

Incubation times should be altered to approximate the testing conditions of the parent assay.

Ensure compound and bead mixtures are equilibrated.

Titrate the Omnibeads to match the signal with the primary Alpha assay.

Consider testing compounds in multiple concentrations.

Compounds that decrease the assay readout are likely signal quenchers, while compounds that increase the assay readout are likely emitters.

A comparison between the results of the TruHit and the Omnibead counter-screens would distinguish compounds that interfere by quenching singlet oxygen.

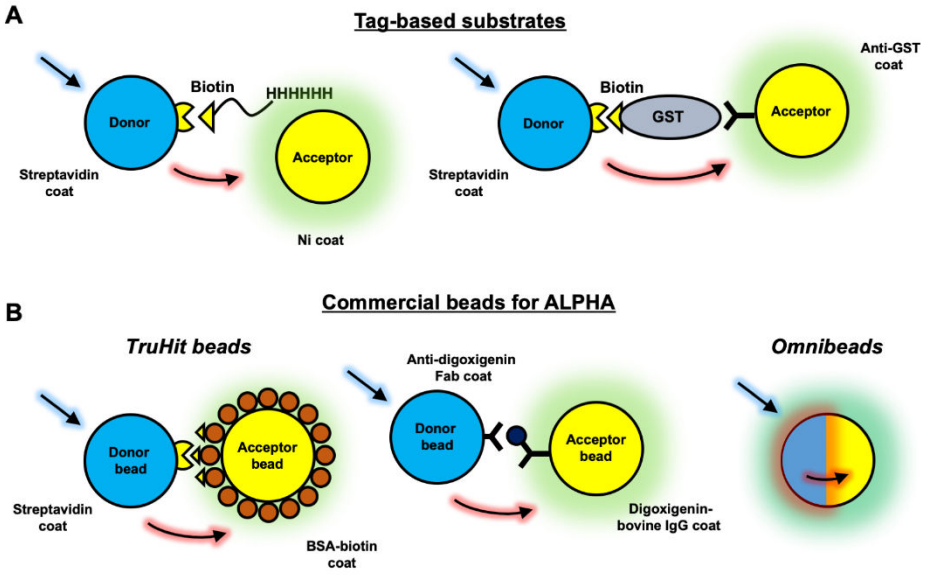


Figure 8 Schematic of reporter-based and tag substrate counter-screens for compound-mediated interference in proximity assays. (A) Reagents containing capture components such as a biotinylated peptide with a His tag (left) or biotinylated GST (right) can be constructed with standard recombinant and protein chemistry technologies or obtained commercially. This approach is applicable to most proximity assays. As with the TruHit beads, the addition of these substrates to complementary capture components will generate a stable assay signal in the absence of interfering compounds (see also **Figure 9**). To identify interference with strong affinity interactions, such as the biotin-streptavidin interaction, compounds can be added before or after reporter/analyte addition and equilibration (with strong-affinity tag interactions, compounds should be added *before* the complementary capture component is added). (B) For Alpha-based assays, TruHit and Omnibeads are commercially-available control beads to assess for target-independent changes in assay readout. TruHit beads contain streptavidin donor and biotinylated acceptor beads that generate a stable assay signal in the absence of interfering compounds. Omnibeads contain all the necessary reagents for the Alpha signal and thus can identify compounds that reduce the excitation light or emission signal.

Table 10 Suggested interference controls for homogeneous proximity assays.

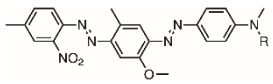
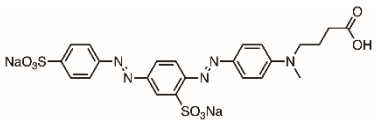
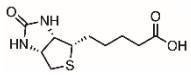
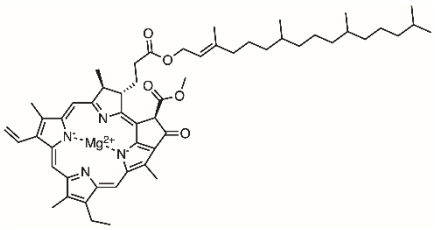
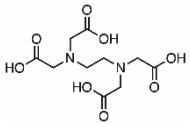
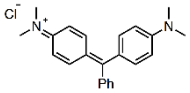
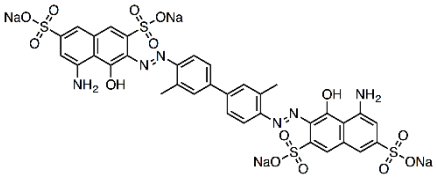
Compound name	Chemical structure	Mechanism	Notes
BHQ-1		Light absorbance	Absorbs light between 480-580 nm; amine/ carboxylic acid versions (at R position) commercially available Contains azo moiety – may be susceptible to singlet oxygen interference
BHQ-10		Light absorbance	Better water solubility than BHQ-1 (due to carboxylic acid) Contains azo moiety – may be susceptible to singlet oxygen interference
Biotin		Affinity reagent disruptor	Use for biotin-avidin systems
Chlorophyll A		Light absorbance	Absorbs at Alpha emission wavelength

Table 10 continued from previous page.

Compound name	Chemical structure	Mechanism	Notes
EDTA		Chelator	Water-soluble (salt poorly soluble in DMSO)
Malachite green		Light absorbance	Absorbs at Alpha emission wavelength
Trypan blue		Light absorbance	Absorbs at Alpha emission wavelength Contains azo moiety – may be susceptible to singlet oxygen interference
Creamer	-	Light scattering	Contains hydrogenated vegetable oils and sodium casein Prepare as 10-20X stock solutions in water Suggested starting concentration: 1 mg/mL

Counter-Screens to Bypass a Reaction or Process

Another strategy for counter-screens involves the addition of test compounds to a solution of pre-formed or purified analyte (Figure 9). This approach should not be taken if the analyte of interest is an equilibrium species, such as a protein-protein interaction, which could instead be countered by a target-independent assay (see above). However, this strategy is well-suited to assays that detect a product of some process, such as an enzymatic reaction or a cellular response. This type of counter-screen can be carried out in two ways: (1) the test compound is added after the reaction or process is completed and the detection reagents have equilibrated, or (2) the test compound is added after a purified version of the analyte has equilibrated with detection reagents (without the other components involved in the process, such as an enzyme or cells). Either variation can be useful for identifying multiple types of compound-mediated assay interferences, including light absorbance, quenching, light emitters, and capture system disruption.

Compared to the first variation utilizing products formed *in situ*, assays using a purified analyte might have better reproducibility due to less confounding factors, such as incomplete reactions or the presence of cells. For instance, an AlphaLISA primary assay was performed to measure IL-1β secreted from stimulated cells (95). A counter-screen was subsequently conducted with the same primary assay and purified IL-1β in the absence of cells to identify technology related interferences. Depending on the assay, a similar approach could be taken with purified cytokines, hormones, or even peptides containing relevant post-translational modifications.

There are some limitations and important considerations for counter-screens designed to bypass a reaction or process. When conducting the first variation that follows a completed process, it is important to ensure that the assay readout is stable prior to spiking in test compounds. Changing readouts are typically due to incomplete/non-quenched reactions (for enzymatic systems), non-equilibrated binding (protein-protein, analyte-reagent complexes), and/or analyte instability. For the second variation using a purified analyte, one should note that certain analytes might be difficult to purify in sufficient quantities and/or have stability limitations.

Sample steps | Counter-Screens to Bypass a Reaction or Process.

In situ format

Perform the complete proximity assay using standard procedures, but omit vehicle control, and test compounds. Include negative control to determine background (e.g., no-enzyme control for enzymatic reaction).

Add test compounds and vehicle controls to microplate wells containing reactions from Step 1.

Allow system to equilibrate.

Obtain counter-screen readout.

Compare the signals from samples treated with test compounds with those of the controls.

Purified analyte format

Perform proximity assay using a modified procedure: mix purified reaction product (analyte) and donor/acceptor reagents, omit vehicle controls and test compounds. Include negative control to determine background (e.g., no-analyte control).

Add test compounds and vehicle controls to microplate wells containing reactions from Step 1.

Allow system to equilibrate.

Obtain counter-screen readout.

Compare the signals from samples treated with test compounds with those of the controls.

Researchers should consider the following technical points:

Include positive and negative controls for relevant interferences (Table 10).

While the sample steps are suggestive of an enzymatic assay, the same approach can be applied to cell-based assays, where the proximity assay detects an analyte produced by the cell following stimulation.

Ensure that the counter-screen signal is comparable to that of the primary assay. Note that the absence of vehicle control (e.g., DMSO) may alter the initial readout before compound/control spiking.

Verify readout stability by obtaining serial readouts (typically 30 to 60 min) prior to performing this counter-screen.

When spiking in a product, the concentration of the purified product should be comparable to what it would be under primary assay conditions.

Depending on the kinetics, this counter-screen format may not be appropriate for certain protein-protein interaction studies. True actives may disrupt the interaction of interest after spiking detection reagents.

Counter-Screens with Different Affinity Capture Components

Developing a counter-screen that incorporates orthogonal affinity capture components can help to identify test compounds that interfere with the coupling of the analyte to the detection system. Antibodies represent a notable subset of capture components in proximity assays. Antibodies are often used to target specific analytes including post-translational modifications (e.g., phosphorylation, acetylation, methylation) and can be monoclonal or polyclonal. An ideal antibody is highly sensitive (i.e., strong affinity) and most importantly specific for its target epitope. However, certain test compounds may interfere with the antibody-analyte interaction by mimicking the analyte pharmacophore. Depending on the similarities of biomolecular

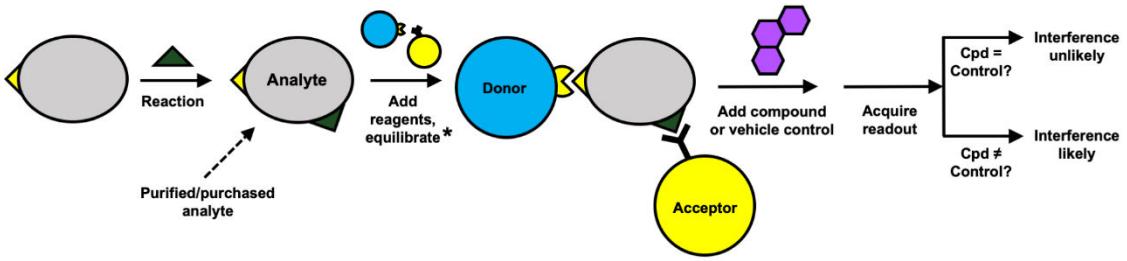


Figure 9 Schematic of product-spiking counter-screens for compound-mediated interference in homogeneous proximity assays. Compounds can be added to a proximity assay system before affinity capture of the analyte. Readouts can be compared to vehicle controls, with significant changes in readout likely attributable to compound-mediated assay interference. Note: with strong-affinity capture components such as streptavidin-biotin, compounds can be added *before* streptavidin reagents are added (*).

recognition, these analyte analogs can prevent the intended antibody-analyte interaction. For proximity assays utilizing an antibody for a highly specific analyte, a potential counter-screen is to substitute the antibody used in the primary assay with an independently-derived antibody (Figure 10). Depending on the assay technology, the suitability of an antibody for a proximity assay might vary. For example, FRET pairs are much more sensitive to the distance and orientation of the donor and acceptor, than Alpha pairs. In principle, if two antibodies are suitable for an assay, then the observed activities for a given test compound should be independent of the antibody choice. However, differences in activities might be attributable to antibody-specific compound interference due to different target epitopes (and thus different affinities to the test compounds).

Similarly, substitution capture components are available for some tags. For example, Ni^{2+}-NTA beads or anti-6xHis-antibody beads are commercially available to capture 6x-His-tags for Alpha-based assays. Likewise, GSH beads or anti-GST-antibody beads will bind GST tags. Compounds that only inhibit reactions using Ni^{2+}-NTA beads, but not anti-6xHis-antibody beads are likely to be chelators and not genuine hits. In general, a genuine hit should affect the signal output independent of the capture component (i.e. for example Ni^{2+}-NTA vs. anti-6xHis-antibody) used.

Absorbance Counter-Screens

Counter-screens can be performed to measure the absorbance spectrum of test compounds in assay-like conditions. Compounds with the potential to interfere by light absorbance will absorb at the excitation and/or emission wavelengths (Figure 11). Such compounds might lead to false-negative interpretations, false-positive interpretations, or indeterminate interpretations. For example, upon evaluating active compounds identified from an AlphaScreen proximity assay, absorbance at 680 nm (excitation) and/or 520-620 nm (emission) would indicate the potential for interference via light absorbance. A similar counter-screen for (TR)-FRET would focus on the excitation and emission wavelengths of the donor and acceptor fluorophores, respectively. Recall that the degree of interference is related to the concentration of compound and its extinction coefficient (Beers Law). Whenever possible, measure compound absorption in assay-like conditions. Light properties such as fluorescence can depend on experimental conditions such as ionic strength, temperature, buffer composition, and buffer pH.

Emission Counter-Screens

Counter-screens can also be conducted to measure the emission properties of test compounds in assay-like conditions. Compounds that emit light at assay-relevant wavelengths might cause false-negative interpretations, false-positive interpretations, or indeterminate interpretations. *A caveat is that such compounds are rare in practice and specific counter-screens for emitters may not be the highest yield unless such a mechanism is suspected or must be de-risked.*

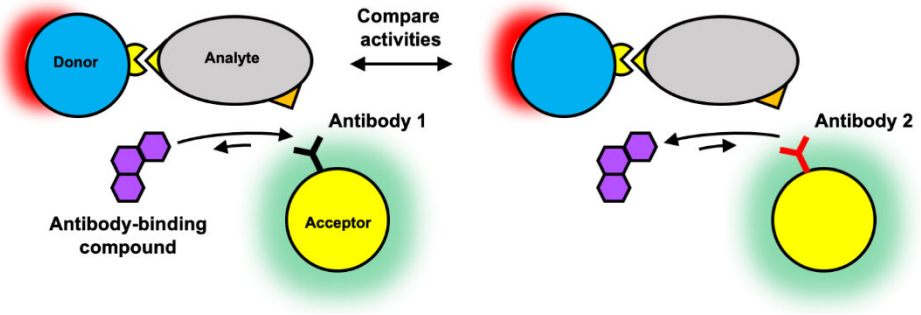


Figure 10 Schematic of an antibody-based counter-screen for compound-mediated interference in proximity assays. Certain test compounds may show antibody-dependent activity, which can be evaluated by comparing the activity of a suspected compound in otherwise identical assays constructed with independent antibodies to the target analyte.

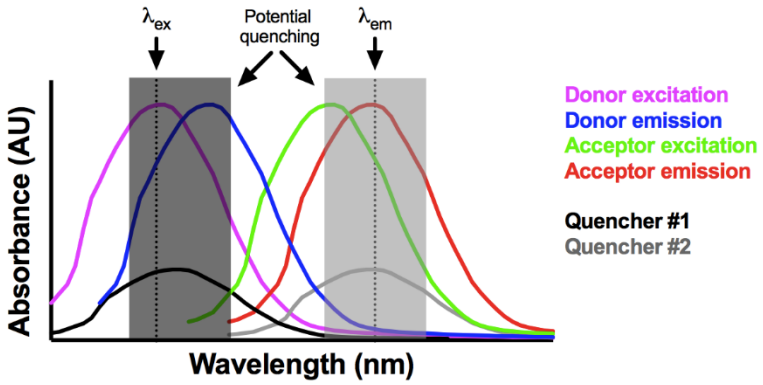


Figure 11 Schematic of absorbance counter-screen for compound-mediated interference in proximity assays. Shown in a hypothetical FRET assay with a donor fluorophore (purple, excitation spectra; blue, emission spectra) and acceptor fluorophore (green, excitation spectra; red, emission spectra). Compounds that absorb near the excitation (Quencher #1, dark grey) or emission wavelengths (Quencher #2, light grey) have the potential to quench the assay readout through inner-filter effects by effectively reducing the intensity of excitation and emission light, respectively.

A straightforward approach for flagging compound emission in homogeneous proximity assays is to measure the emission of a test compound under assay-like conditions using a fixed excitation wavelength. The most relevant, high-yield experiment would be to excite a compound at the donor excitation wavelength and then monitor for light emission in the acceptor emission channel. The magnitude of light emission can be expressed relative to a positive and negative controls for the assay readout and/or a pure fluorescent standard (mean fluorescent concentration (112)). Note that other combinations of excitation and emissions can be tested (e.g., excite at the donor emission wavelength, then monitor emission in acceptor emission channel).

Other pragmatic counter-assays capable of identifying emitting compounds include compound spiking, the use of target-independent linker substrates, and bead-based reagents for Alpha assays (i.e., Omnibeads, TruHit beads). As above, the magnitude of light emission can be quantified relative to positive and negative assay controls. For fluorescent compounds, absorbance spectra (see above) can be correlated with emission (i.e., compounds absorb light at assay-relevant wavelengths).

In cellular fluorescence-based assays where the desirable activity is associated with an increased fluorescence signal (e.g., enhanced promotor activity), hits should be tested on parental cells without the incorporated reporter. If the parental cells also show increased fluorescence upon compound treatment, the hits are likely to be flagged as fluorescence emitters.

There are several considerations when performing emission counter-screens:

- Emission spectra can be normalized to a known quantity of standard (positive control) to control for fluctuations in instrumentation and/or reporter systems (112).
- Whenever possible, obtain emission spectra in assay-like conditions. Light properties such as fluorescence can vary depending on experimental conditions such as ionic strength, temperature, buffer composition, and buffer pH.
- Obtain emission at several compound concentrations, including above and below the activity concentration. For a compound with an AC_{50} of 10 μM, this might include taking emission spectra at 1, 10, and 100 μM compound concentrations.

Ratiometric Data Analysis

A high-yield experimental design for certain proximity assays is to measure the readout (emission) in the donor fluorophore *and* the acceptor fluorophore channels. Readouts can then be analyzed in the donor fluorophore emission channel, the acceptor fluorophore emission channel, and as a ratio of these donor:acceptor channels (Equation 2) (113). This type of analysis is applicable to (TR)-FRET, BRET, and Alpha.

$$\text{Ratio} = \frac{\text{Acceptor}}{\text{Donor}} \qquad \text{Equation 2}$$

Analyzing activity in terms of each channel separately, the ratio can correct for some well-to-well effects and also identify certain light-interference compounds (Figure 12). Plotting the control well data for both donor and acceptor channels to determine the variation without compound addition can be used to judge if decreases/increases in the ratio data are due to either genuine complex disruption/formation or aberrant signal modulation of the donor or acceptor channels.

Well-behaved (i.e., non-fluorescent, non-quenching) bioactive compounds should perturb the readout in the acceptor channel and not the donor channel. Compounds that quench light in the donor and acceptor channels show decreases in signal intensities in both channels. Typically, this ratio is similar to the unmodulated (vehicle control) ratio as it affects both the donor and acceptor channels equally. Compounds that fluoresce in both channels will show increased intensities in both the donor and acceptor channels, and the donor:acceptor ratios are again unchanged from vehicle controls. Compounds that selectively attenuate readout in either the donor or acceptor channels may appear as bioactive compounds depending on the assay setup. Similarly, compounds that selectively fluoresce in either the donor or acceptor channels may also appear as bioactive compounds depending on the assay setup.

When analyzed as concentration response curves, light-interfering compounds may show variable patterns that will depend on the assay setup (up- or down-readout), fluorescence behavior, quenching behavior, and on-target bioactivity. Therefore, it is highly recommended to analyze the single channel and ratiometric readouts. Such a protocol can be implemented as a counter-screen or as a built-in data acquisition step in the routine performance of a given assay. In cases where there may be appreciable compound light interference, orthogonal assays and counter-screens should be performed to further examine technology-based interferences and on-target bioactivity.

Pull-Down Assays

Compounds that interfere with proximity assay capture components can also be flagged by conventional pull-down assays (94). In this counter-screen, test compounds are incubated with a target-independent substrate containing one of the capture components (e.g., GST-conjugated protein) in assay-like conditions, followed by the addition of the complementary solid-phase capture component (e.g., GSH-coated beads). Beads are washed to remove unbound compound and proteins, followed by protein elution from the washed beads, and the formerly bound proteins are quantified after electrophoresis separation by staining (e.g., Coomassie) or

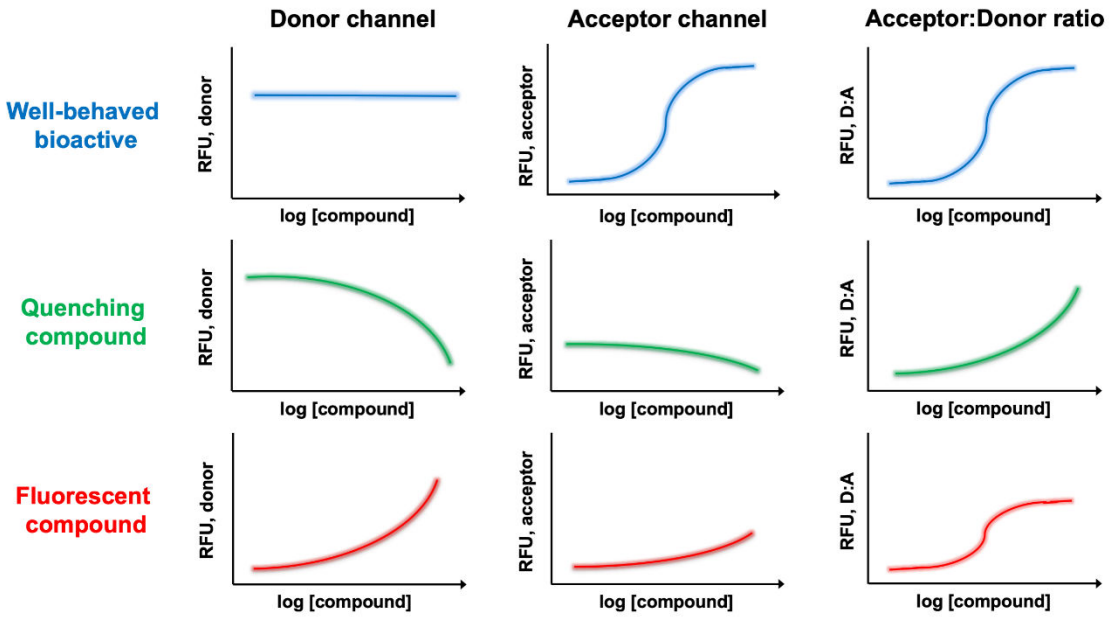


Figure 12 Ratiometric analysis of proximity assays to identify compound-mediated interferences. In this example, on-target bioactivity results in an increase in TR-FRET signal. Since light-based interferences by compounds are concentration-dependent, such compounds may be flagged by plotting the ratio of the acceptor:donor fluorescence as a function of compound concentration (see right-most panels). Depending on the magnitude of compound quenching and fluorescence in each fluorophore channel, quenching compounds (green) and fluorescent compounds (red) may show concentration-dependent changes in the acceptor:donor ratio. In such cases, orthogonal assays and counter-screens should be performed to further examine technology-based interferences and on-target bioactivity. Adapted from Imbert *et al* (113).

immunostaining (e.g., western blot). Compounds that disrupt the binding of the complementary binding reagents show decreased amounts of pulled-down product compared to vehicle controls and well-behaved compounds. Incubation of the test compound with capture component including stoichiometry and buffer conditions should attempt to mimic the parent assay. Unlike other counter-screens discussed in this chapter, these assays can be lower-throughput, semi-quantitative, and may not necessarily replicate assay-like conditions. Therefore, we recommend reserving for cases where previous counter-screens are inconclusive or other higher-throughput counter-screens cannot be performed for technical reasons.

Sample steps | Pull-Down Counter-Screen.

Prepare 100X compound stock solutions, (e.g., 1 mM in DMSO to test at 10 µM compound concentrations).

Prepare serial dilutions in stock solution solvent (e.g., DMSO).

Dilute non-target protein stock containing capture component acceptor (e.g., GST, His tag, antibody epitope) with 1X assay buffer.

Add 3 µL of 100X compound solution to reaction vessel such as standard Eppendorf tube with pipette.

Add 297 µL solution from Step 3 to reaction vessel from Step 4. Mix gently with inversion or brief vortex.

Incubate solution from Step 5 for 60 min at same temperature as parent assay.

Add beads containing immobilized complementary capture component to solution from Step 6 (e.g., agarose-GSH, agarose- Ni^{2+}, agarose-antibody).

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Incubate solution from Step 7 for additional 1-2 h at room temperature.

Centrifuge solutions from Step 8 for 5 min at 1000 *g* at room temperature. Remove supernatant. Replace supernatant with fresh 1X assay buffer.

Repeat Step 9 for 5 cycles.

Elute bound proteins with SDS-containing buffer (alternatively, low pH or high salt buffers).

Analyze eluates by standard SDS-PAGE followed by protein staining or Western blot.

Compare quantity of eluate proteins by treatment with test compounds versus vehicle control as well as relevant positive controls. Eluates with decreased amounts of protein are presumed capture tag disruptors.

Researchers should consider the following technical points:

Include positive controls that disrupt the interaction of interest (e.g., purified GSH, GST, anti-His, chelators).

Utilize assay buffer from proximity assay of interest, as compound behavior can depend on experimental conditions such as ionic strength, temperature, buffer composition, and buffer pH.

Keep in mind that the amount of protein used in pull down experiments will be much higher than in micro-well proximity assays.

For each system, experimental parameters such as the number of washes, wash buffer composition, and incubation should be optimized.

Consider testing compounds at multiple concentrations.

Include positive and negative controls. For example, nickel-chelating agents such as EDTA could be used as positive controls for Ni^{2+}-His pull-downs.

Orthogonal Assays

The purpose of performing orthogonal assays is to rule out technology-dependent activities that are usually hallmarks of compound-mediated assay interference, as useful bioactivity should ideally be independent of assay technology (Table 6). A typical high-throughput approach is to employ an independent proximity assay technology as the orthogonal assay (e.g., if Alpha was used for a primary screen, use SPA or TR-FRET as the orthogonal screen, as well as a different set of tags if feasible). However, efforts should also be placed in identifying non-homogeneous proximity secondary assays to avoid the possibility of technology-related interference, such as light-based interference or tag disruption. Non-proximity formats could include mass spectrometry assays or biophysical target engagement assays such as surface plasmon resonance (note: while showing direct binding, such assays do not necessarily indicate functional readouts such as enzymatic modulation or tractable/optimizable bioactivity). Otherwise, orthogonal proximity-based assays could include separation steps at the expense of throughput (e.g., filter-binding, ELISA).

Generalized Interference Counter-Screens

Counter-screens for non-technology-related interferences such as nonspecific reactivity and aggregation should also be performed. For brevity, extensive details on counter-screens for these interferences can be found at the related Assay Guidance Manual chapters on Assay Interference by Aggregation and Assay Interference by Chemical Reactivity.

Time-Course Measurements

Another strategy to identify light-based interferences is to obtain multiple readout measurements over a range of compound concentrations to obtain a set of reaction rates (Figure 13) (113,114). Light-based interferences are relatively static and are generally not time-dependent (e.g., unless compound instability is an issue). Therefore, in an unquenched reaction (e.g., enzymatic assay), compounds that perturb the readout at the same rate, independent of compound concentration, are potential interference compounds. Bioactive compounds should induce concentration-dependent changes in reaction rates. Bioactive compounds that also interfere with the readout may show a mixed pattern at a particular time point, but when readouts are plotted as reaction rates, show more well-behaved bioactivity. This strategy is most appropriate for assays that exhibit readout stability, activity not disrupted by detection reagents, and show steady-state-like, time-dependent activity.

Note this assay format is usually most relevant to certain enzymatic proximity assays used to detect a product (e.g., a kinase assay with AlphaScreen to measure a phosphopeptide). Most binding assays occur too rapidly to measure kinetically. Additionally, technical factors such as instrumentation may prevent rapid measurements, or system instability may hinder delayed measurements.

Cheminformatics

Cheminformatics can be useful tools for flagging potential compound interferences from proximity assay data sets. One common cheminformatics approach for identifying interference compounds involves substructure filters derived from larger data sets or historical records of distinct compounds. The former enables flagging compounds that may not have been tested previously, but share a chemotype associated with a particular assay misbehavior (e.g., Tables 6-8), but this strategy runs the risk of overgeneralizing. The latter approach can be especially useful if the same compounds are routinely assayed in multiple unrelated proximity assays or if the primary hit list is very large and a chemoinformatics filtering helps to reduce the number of compounds to be tested in counter- as well as orthogonal assays. An alternative cheminformatics approach is to calculate frequent hitting behavior for each compound in a screening collection (many of which may owe their bioassay promiscuity to various interference mechanisms) using their historical performance, without explicitly considering compound structure (115).

There are several substructure filter sets for AlphaScreen-based interferences, including general interference (Table 11) as well as interferences with His-Ni^{2+} and GSH-GST interactions (Tables 7 and 8) (94,116). These filters may be highly useful to flag actives for either triage or additional counter-screening.

There are several considerations for the use of these chemoinformatics filters, however: (1) substructure filters have the potential to be specific for a given assay type or capture technology depending on the training set, whereas generalized filters (e.g., REOS, Lilly) may flag more generally promiscuous compounds; (2) computational methods provide a hypothesis about the promiscuity of compounds and thus need confirmation through appropriate assays; (3) for many specific interferences or assay technologies, these filters do not exist and the generation of these filters needs a large dataset of several independent targets employing the same detection and capture technology.

Cheminformatics has distinct advantages and disadvantages. Most obvious is the speed of these approaches and the reduction in required experiments. However, compound interference can be highly assay-dependent. For example, the observed spectroscopic properties of compounds can be dependent on experimental parameters such as ionic strength, pH, temperature, and other factors such as compound solubility. As with experimental approaches, cheminformatics approaches are also susceptible to potential false positives and false negatives. An overly strict method may triage potentially useful non-interfering compounds. By comparison, reliance on a less strict method may provide a false sense of confidence in filtered compounds. Therefore, we recommend using

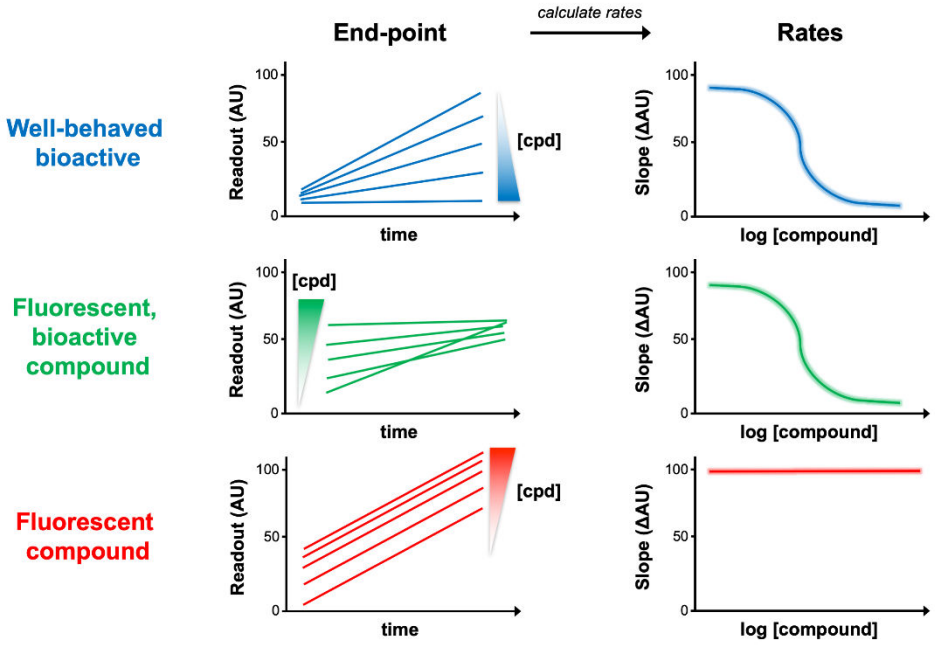
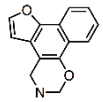
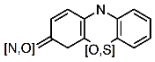
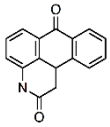
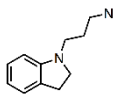


Figure 13 Time-course analysis of proximity assays to identify compound-mediated interferences. For certain proximity assays (usually enzymatic), readouts can be obtained at multiple time points to derive rates. Because light-based interferences are time-independent and concentration-dependent, some light-based interference compounds (red, bottom panel) can be identified by reaction rates independent of compound concentration. By contrast, well-behaved, bioactive compounds (blue, top panel) would show concentration-dependent changes in reaction rates. Certain bioactive light-based interference compounds (green) may also show concentration-dependent changes in reaction rates. Adapted from Imbert *et al* (113).

cheminformatics as a flagging tool as opposed to a triage tool to help prioritize compounds for interference counter-screens and orthogonal assays.

Table 11 Reported AlphaScreen frequent hitters. Chemotypes were derived from an AlphaScreen-based counter-screen (82). See also https://ochem.eu (AlphaScreen-FHs) for SMARTS strings and online substructure screening tool.

Name	Chemotype	Name	Chemotype
Naphthafurans		Imidazotriazoles	
Phenoxazines		Indenones	
Anthrones		Indolines	

Section Summary

Compound-mediated interferences in proximity assays can be identified through well-designed counter-screens. Users should also perform at least one orthogonal assay to confirm desired bioactivity, and counter-screens to identify compounds that interfere with capture components (affinity tag systems, antibody-analyte interactions)

and signal transmission (quenchers, emitters). Compounds should be triaged, progressed, or flagged for additional studies based on the combination of orthogonal and assay-specific interference counter-screens.

Strategies to Mitigate Compound-Mediated Interferences in Proximity Assays

This section describes several approaches to reduce the incidence of compound-mediated assay interference in proximity assays. Strategies to mitigate interference by light-based interferences such as the use of red-shifted fluorophores and ratiometric data analyses are highlighted. Steps to mitigate interference by aggregators and nonspecific reactive compounds are briefly discussed. The use of known interference compounds to guide the optimization of assay experimental conditions and to characterize the effects of various interference modalities is also discussed.

Strategies to Mitigate Interference by Light-Based Interferences

Many screening compound collections are enriched in sp^2-hybdridized atoms (117), and consequently absorb light in the UV light range. Such compounds can interfere with proximity assay readouts by auto-fluorescence and/or quenching. However, the use of red-shifted fluorophores can significantly reduce the burden of compound-mediated light interferences (81,103,112,118,119). This is because in general, the ability of a compound to absorb or be excited by a higher wavelength (lower energy) photon requires more extensive electronic delocalization. Such extensively conjugated compounds are usually blue by visible inspection and are relatively rare in conventional HTS collections.

Another strategy is to acquire multiple channel (donor, acceptor) signals to analyze individually and as ratios (113). Depending on factors such as the assay stability, available instrumentation, and overall throughput of data acquisition, generating data for multiple channels may be relatively straightforward (see above section, Ratiometric Data Analysis).

Finally, including detergents in assay buffer may attenuate the effects of light-scattering compounds. Detergents can effectively increase the solubility of certain compounds, attenuating the amount of suspended light-scattering particles. Care should be taken to remain below the critical micelle concentration (CMC) for the given detergent, however.

Special cases: TR-FRET time delay. One approach to reduce compound interference in TR-FRET assays is to measure apparent bioactivity as a function of the time delay of the TR-FRET measurement (113). Relatively short time delays (e.g., 25 µs) may still detect significant fluorescence from short-lived compound fluorescence. Increasing the time delay (e.g., 50 or 100 µs) typically reduces this background fluorescence (Figure 14). Compounds that show significant reductions in apparent bioactivity with increasing time delays are suspicious for fluorescence interference. While useful, certain long-lived fluorescent compounds may continue to show apparent bioactivity even with long time delays. However, as noted in the previous sections, TR-FRET will still be susceptible to compounds which absorb the donor fluorescence as well as light scattering effects.

Special cases: fluorescent lifetime (FLT) detection. Another method to mitigate against fluorescent compound interference is to perform rapid kinetic measurement of the fluorescence over time to characterize the fluorescent lifetime of a probe fluorophore (Figure 15A). Microtiter-plate readers are available which can perform these measurements in micro-well densities as high as 1536-well plates (TTP Lab Tech Ameon reader). Fluorescent probes such as FLEX17 (Figure 15B, 9-aminoacridine, from Almac; PT14, PT22, from GE Healthcare) with long lifetimes (τ = 14 to 22 ns) have been developed which show a change in fluorescent lifetime when the proximal environment changes due to events that occur in a variety of biochemical assays (Figure 15B). The long lifetime is rather easily distinguished from typical fluorophores and fluorescent compounds because such compounds show much faster fluorescent decay rates (τ = 1 to 5 ns). Therefore, the use

of longer-decaying fluorophores and/or analysis of fluorescent decay curves can be used to flag and even mitigate the burden of fluorescent compounds (120). Assays resulting in either longer or shorter (quenched) lifetimes can be configured for a variety of enzyme assays and have been developed for both serine/threonine and tyrosine kinases (Figure 15C), HATs, HMTs, HDACs, DUBs, and arginine-modifying enzymes.

Strategies to Mitigate Interference by Reactive Compounds

A popular and straightforward strategy to mitigate nonspecific compound reactivity in biochemical assays is to include scavenging reagents such as DTT (121). Often these assays use low millimolar concentrations (usually 0.5 to 1 mM). The principle of this strategy is that excess scavenging reagents can react with nonspecific reactive compounds, leaving the biomolecule(s) of interest unperturbed (104,122). Other scavenging agents can include β-mercaptoethanol (BME), L-cysteine, *tris*(2-carboxyethyl)phosphine (TCEP), and decoy proteins containing free cysteines such as BSA. However, addition of reducing agents can promote unwanted activity by redox cycling compounds (123). Therefore, compatibility of scavenging reagents with a given proximity assay and optimal concentrations should be verified experimentally. Ideally, the susceptibility of the protein target and assay system should be profiled against the suite of scavenging agent options in the presence of suitable control compounds for these mechanisms.

For additional details on mitigating interference by reactive compounds, refer to Assay Interference by Chemical Reactivity.

Strategies to Mitigate Interference by Aggregators

Several relatively facile strategies exist for mitigating the incidence of aggregators in biochemical assays, including proximity assays. Conveniently, utilizing detergents in assay buffer can reduce light-scattering compounds and aggregate formation. The inclusion of nonionic detergents such as Triton X-100 and decoy proteins such as BSA can usually increase the critical aggregation concentration (CAC) and saturate compound aggregates, respectively (106,107). As an example, Triton X-100 or Tween-20 is often used at 0.01% (v/v) concentrations in assay buffers to mitigate aggregation. As with scavenging reagents, the compatibility of detergent and/or decoy proteins with the assay technology and biological system should be verified, along with the optimal concentration. More specifically, detergents should usually be used below their corresponding critical micelle concentrations (CMCs), as detergent micelles themselves can perturb the assay system. Product literature indicates Alpha technology can typically tolerate the following detergents (73):

- Tween-20: up to 1% (v/v)
- Triton X-100: up to 1% (v/v)
- CHAPS: less than 0.1% (v/v)
- SDS: avoid (interference at 0.05% v/v)

Based on the stoichiometric model of enzyme inhibition for aggregators, increasing the concentration of enzymes may also mitigate the effects of aggregation (110,124). Finally, decreasing the concentration of test compounds such that more compound concentrations are below the CAC under testing conditions may also lower the incidence of aggregation. As an alternative to decreasing single-point screening concentrations, compounds can be tested in dose response by qHTS to sample testing concentrations below CACs (125). The final concentrations of enzymes (or other biomolecules of interest) and test compounds usually balance multiple competing factors including assay timing, readout intensity, and reagent costs.

For additional details on mitigating interference by aggregators, refer to Assay Interference by Aggregation.

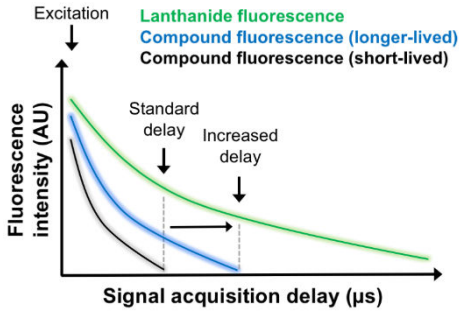


Figure 14 Effect of signal acquisition time delay on TR-FRET bioactivity. Many screening compounds can exhibit short-lived fluorescence (black). Increasing the signal acquisition delay can reduce interference from longer-lived fluorescent compounds (blue) provided the dynamic range is sufficient.

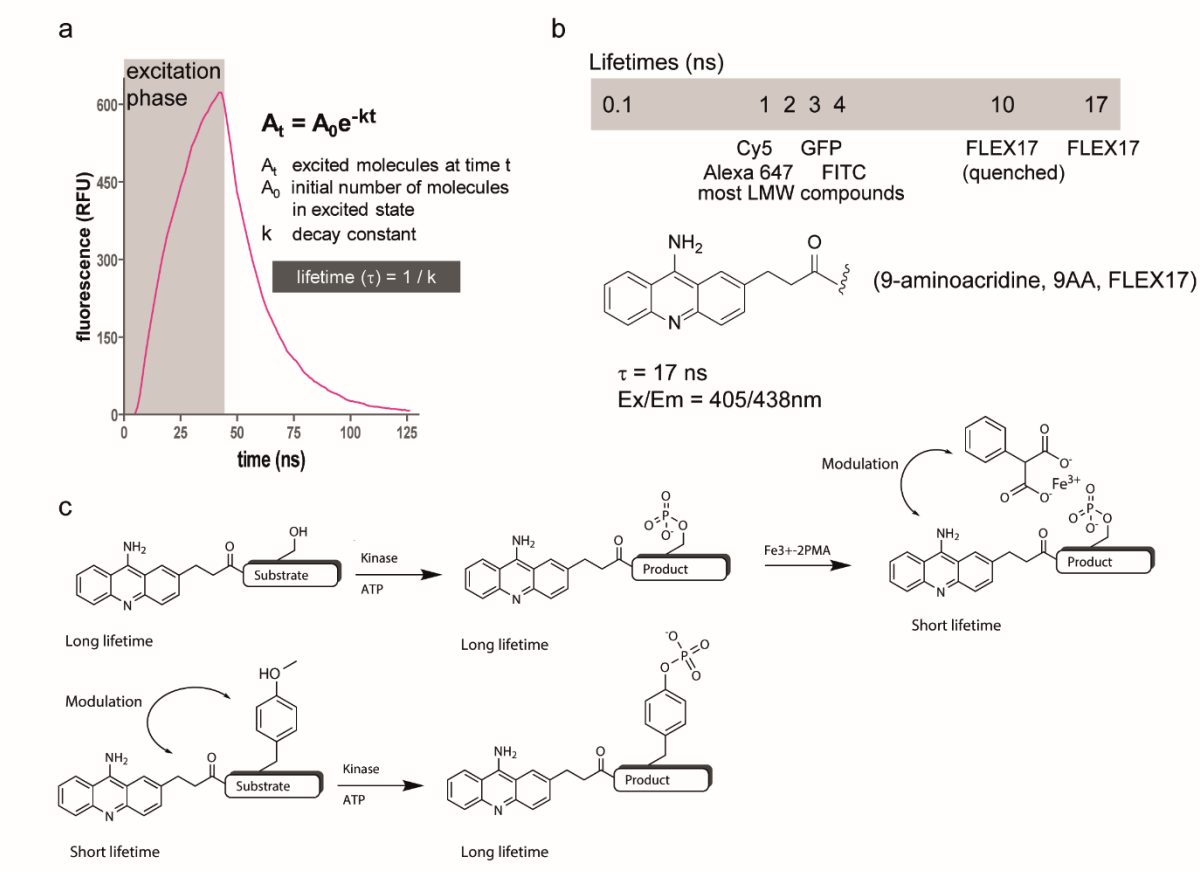


Figure 15 Principle of FLT and example assays. (A) Rapid detection of the fluorescent signal after excitation affords determination of the fluorescent lifetime through examining the slope of the fluorescent decay curve (fitting to the first-order equation to the data). (B) Standard fluorophores and typical fluorescent compounds show much shorter fluorescent lifetimes compared to FLT probes such as 9AA. (C) Assays for protein kinases can be developed where the phosphorylation event is monitored through the lifetime probe. Product formation can be followed when the by either a decrease or increase in fluorescent lifetime. Adapted from TTP Lab Tech material.

Interference Control Challenge to Mitigate Compound-Mediated Interference

During the assay design and validation phases, it is recommended to determine the effect(s) of known interference compounds on the assay readout (122,126). During the assay optimization process, experimental conditions can potentially be modified to reduce the effects of certain sources of compound-mediated assay interference (e.g., increasing detergent concentration in an assay prone to aggregator interference; increasing signal acquisition delay in a TR-FRET assay prone to auto-fluorescence compound interference). Challenging proximity assays with known interference compounds and key reagents permits the characterization of specific interference mechanisms on the assay readout (Table 10). For example, Ni^{2+}-His tag assays can be challenged with chelating agents (e.g., EDTA) while biotin-streptavidin assays can be challenged with biotin to gauge the interference phenotype and magnitude by capture component disruptors. Light-based interferences can be assessed using various colored, auto-fluorescent, and insoluble compounds. Several suggested electrophiles and aggregators can be obtained commercially (121,122,127). This information can facilitate compound triage, especially in complex assay systems, where the effects of interference compounds may not be immediately intuitive. Finally, the relative sensitivity of a given proximity assay to known interference compounds combined with preliminary estimates of specific interference incidences can help guide the design of post-HTS workflows (such as the selection and order of counter and orthogonal assays).

Section Summary

When combined with preliminary interference characterization on the assay readout, carefully designing certain assay features such as reagent selection, reagent concentration, and instrumentation settings can potentially reduce the burden of compound-mediated interference in proximity assays due to light-based interference, aggregation, and nonspecific reactivity. Some high-yield recommendations include (1) the use of red-shifted fluorophores, (2) the addition of detergents, decoy proteins, electrophilic scavenging reagents, and (3) the thorough characterization of known interference compounds on proximity assay readouts to inform on the appropriate counter-screens and orthogonal assays that will be required to characterize compound activity.

Conclusions

Homogeneous proximity assays – including Alpha, FRET, BRET, TR-FRET, FLT, and SPA – are powerful tools for lead and chemical probe discovery and development. These technologies obviate the need for analyte separation steps and are amenable to customization with off-the-shelf commercial reagents. While highly useful, these assays are susceptible to compound-mediated interferences. Sources of technology-related compound-mediated interferences include signal quenchers, signal emitters, and capture system disruptors. Additionally, homogeneous proximity assays are also susceptible to generalized (non-technology-related) compound-mediated interferences, including nonspecific reactivity and aggregation. Compound-mediated interferences in homogeneous proximity assays are best approached by a combination of orthogonal assays to confirm activities and counter-screens to rule-out or distinguish any technology-related interferences. The establishment of a primary assay along with orthogonal and counter-screens should be the task of every initial assay development phase. Each counter-screen strategy has advantages and disadvantages (Table 12). A combination of orthogonal assays and counter-screens helps to (1) triage assay artifacts, (2) to identify desired bioactivity among active compounds, and (3) to characterize potentially tractable compounds with interference properties.

Table 12 Summary of counter-screens for compound-mediated assay interference in proximity assays. Certain counter-screens are appropriate for specific proximity assay technologies. For each counter-screen, susceptible interferences, and notable advantages and disadvantages are listed. Mechanisms of interference should be confirmed or ruled-out with multiple counter-screens.

Counter-screen	Homogeneous proximity assay	Light / Fluorescence absorption	Quench	Singlet oxygen	Tag disruption[a]	Light/ Fluorescence emission	General[b]	Advantages, disadvantages
		Key interferences flagged						
TruHit beads	Alpha	+	+	+	± (usually Strep/ biotin)	+	±	Commercially available Straightforward protocol Expensive Limited tags available
Omni-beads	Alpha	+	+	?	-	+	-	Commercially available Straightforward protocol Expensive
Tag substrates	Alpha (TR)-FRET BRET SPA	+	+	+ (only Alpha)	+	+	±	Commercially available Synthesizable Straightforward protocol Can be used to identify specific tag interferences
Product spiking (*in situ*)	Alpha (TR)-FRET BRET, FLT SPA	+	+	+	+	+	±	Straightforward protocol May flag unstable and slow acting covalent compounds in addition to readout artifacts Requires stable product Excess product may place assay out of linear range Ensure system stability, equilibration

Table 12 continued from previous page.

Counter-screen	Homogeneous proximity assay	Light / Fluorescence absorption	Quench	Singlet oxygen	Tag disruption[a]	Light/ Fluorescence emission	General[b]	Advantages, disadvantages
		Key interferences flagged						
Product spiking (purified product)	Alpha (TR)-FRET BRET, FLT SPA	+	+	+	+	+	±	Straightforward protocol May flag unstable and slow acting covalent compounds in addition to readout artifacts Requires stable product Excess product may place assay out of linear range Ensure system stability, equilibration
Antibody swap	Alpha (TR)-FRET SPA				+			Depends on antibody
Absorbance spectra	Alpha (TR)-FRET BRET, FLT SPA	+	+					Straightforward protocol
Emission spectra	Alpha (TR)-FRET BRET, FLT SPA					+		Straightforward protocol
Cheminformatics	Alpha (TR)-FRET BRET, FLT SPA	±	±	±	±	±	±	Inexpensive Rapid False positives False negatives Needs to be confirmed with experiments
Ratiometrics	Alpha (TR)-FRET BRET SPA	+	+			+		
Kinetics	Alpha (TR)-FRET BRET, FLT SPA	+	+			+		Useful for non-quenched/ continuous assays
Pull-down	Alpha (TR)-FRET BRET SPA				+			Low throughput High concentration of protein needed

[a] Depends on tags used in beads
[b] Generalized compound-mediated interference including nonspecific reactivity, aggregation, chelation

Suggested Readings (alphabetical order)

Baell J.B., Holloway G.A. J Med Chem. 2010;53:2719. PubMed PMID: 20131845.

Describes several hundred chemical substructures exhibiting enriched bioactivity across six independent AlphaScreen HTS assays. See Supplementary Information for in-depth discussions of likely AlphaScreen and generalized interference chemotypes.

Brenke J.K., Salmina E.S., Ringelstetter L., Dornauer S., Kuzikov M., Rothenaigner I., Schorpp K., Giehler F., Gopalakrishnan J., Kieser A., Gul S., Tetko I.V., Hadian K. J Biomol Screen. 2016;21:596. PubMed PMID: 27044684.

An excellent analysis of compound-mediated GSH-GST tag interference including several counter-screens.

Falk H., Connor T., Yang H., Loft K. J., Alcindor J. L., Nikolakopoulos G., Surjadi R. N., Bentley J. D., Hattarki M. K., Dolezal O., Murphy J. M., Monahan B. J., Peat T. S., Thomas T., Baell J. B., Parisot J. P., Street I.F. J Biomol Screen. 2011;16:1196. PubMed PMID: 22086725.

Describes AlphaScreen post-HTS triage, including the use of TruHit beads and orthogonal assays. Also reports several compounds that appear to disrupt an antibody-analyte interaction.

Imbert P.E., Unterreiner V., Siebert D., Gubler H., Parker C., Gabriel D. Assay Drug Dev Technol. 2007;5:363. PubMed PMID: 17638536.

Details multiple counter-screens and data analysis strategies to mitigate and identify compound-mediated assay interference in TR-FRET assays. Many of the strategies are applicable to other proximity assay technologies.

Schorpp K., Rothenaigner I., Salmina E., Reinshagen J., Low T., Brenke J.K., Gopalakrishnan J., Tetko I.V., Gul S., Hadian K. J Biomol Screen. 2014;19:715. PubMed PMID: 24371213.

An analysis of compound-mediated assay interference encountered during a series of AlphaScreen HTS campaigns including several counter-screens.

Watson V.G., Drake K.M., Peng Y., Napper A.D. Assay Drug Dev Technol. 2013;11:253. PubMed PMID: 23679849.

Illustrative example of an AlphaScreen HTS assay development and interference triage. Describes an original counter-screen for AlphaScreen artifacts using a donor-acceptor peptide. Also reports several examples of presumed AlphaScreen artifacts.

Suggested Web Resources

Online Chemical Modeling Environment (OCHEM). (Available at: http://ochem.eu/alerts).

An open-access, user-friendly cheminformatics resource for identifying potential proximity assay artifacts, based on Suggested Readings #2 and #5. Test compounds can be provided as lists (SMILES), files (SDF, MOL2), or drawn. Submitted compounds can be filtered according to one of three relevant filters: AlphaScreen-HIS-FHs (His-tag disruptors), AlphaScreen-GST-FHs (GST-tag disruptors), and AlphaScreen-FHs (generalized AlphaScreen™ artifacts).

Glossary

Artifact – A compound whose apparent bioactivity is due to compound interference with a particular assay technology

Counter-screen – An assay that is designed to flag compounds for specific technology or general interference

False-positive – Compounds, including artifacts, whose bioactivity cannot be reconfirmed in subsequent testing; not to be confused with poorly tractable compounds whose bioactivity is reproducible but *via* undesirable mechanisms

Generalized interference – Modulation of an assay readout by compound-target engagement via poorly tractable or undesirable mechanisms; usually independent of assay technology

Homogeneous proximity assay – An assay that produces a detectable readout specifically when donor and acceptor components are in proximity, all without separation or filtration steps

Interference – Assay readout modulation by a compound due to mechanisms not related to the desired biological activity; due to technology and/or generalized interferences

Orthogonal assay – An assay that is designed to confirm compound bioactivity of interest compounds using an independent assay technology relative to the primary assay

Quenching, light – Attenuation of *fluorescence intensity* by a test compound; can be due to FRET, excitplex, or static (contact) mechanisms; distinct from light-based readout attenuation by absorption or light scattering

Quenching, singlet oxygen – Attenuation of singlet oxygen by a test compound; can be due to physical or chemical mechanisms

Technology-related interference – Modulation of an assay readout by compound that interferes with a specific assay technology

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Conflict of Interest Statement

The authors declare no conflicts of interest.

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Assay Interference by Chemical Reactivity

Jayme L. Dahlin, MD, PhD,[1] Jonathan Baell, PhD,[2] and Michael A. Walters, PhD[3]

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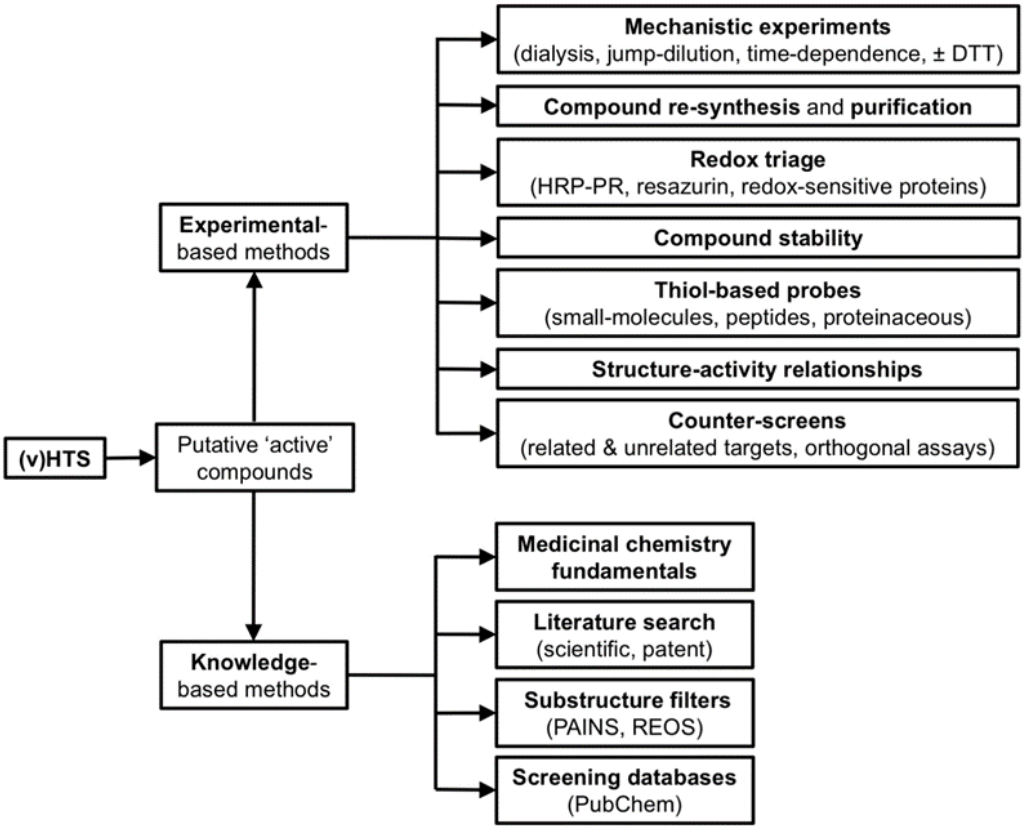
Abstract

Real and virtual high-throughput screens (HTS) are crucial components of the modern drug discovery process. Assays that determine the biological activity of compounds found by both of these approaches may be subject to interference by a variety of processes, one of which is non-specific chemical reactivity. Within this mode of assay interference, test compounds can chemically react with assay reagent(s) or biological molecule(s), confounding the assay readout by producing apparent biological activity. Failure to identify and triage (remove from consideration) these compounds can result in wasted resources and project dead-ends. This chapter contains practical strategies to mitigate the impact of assay interference by compound chemical reactivity. Two general approaches are described to identify and triage reactive compounds in the context of drug discovery and development: knowledge-based and experimental-based methods. Knowledge-based strategies covered in this chapter include substructure filters, literature and database searches, and the consulting of experienced medicinal chemists. Experimental-based strategies covered in this chapter include mechanistic experiments, thiol-based probes and multiple types of counter-screens. Employing a well-designed and comprehensive screening tree that incorporates these strategies should reduce the likelihood of pursuing reactive assay artifacts and intractable hits from HTS campaigns.

Author Affiliations: 1 Mayo Clinic College of Medicine, Rochester, MN. 2 Monash Institute of Pharmaceutical Sciences, Monash University, Melbourne, Australia. 3 Institute for Therapeutics Discovery and Development, University of Minnesota, Minneapolis, MN.

Corresponding author.

Flow Chart



Abbreviations

ALARM NMR, a La assay to detect reactive molecules by nuclear magnetic resonance; BME, β-mercaptoethanol; BSA, bovine serum albumin; CoA, coenzyme A; CPM, *N*-[4-(7-diethylamino-4-methylcoumarin-3-yl)phenyl]maleimide; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; ELSD, evaporative light scattering detection; GSH, glutathione; HMQC, heteronuclear multiple quantum coherence; HPLC, high-performance liquid chromatography; HRP-PR, horseradish peroxidase-phenol red; HTS, high-throughput screen or high-throughput screening; IC_{50}, half maximal inhibitory concentration; *m/z*, mass-to-charge ratio; MS, mass spectrometry; NMR, nuclear magnetic resonance; PAINS, pan assay interference compounds; PDA, photo diode array; REOS, Rapid Elimination Of Swill; SAR, structure-activity relationship; SIR, structure-interference relationship; UPLC, ultra-performance liquid chromatography.

Introduction and Background

Section Introduction

Some classes of compounds display activity that is associated with non-specific or generalized chemical reactivity rather than target binding. Many useful therapeutics that bind and react with their target are known. However, such reactivity was not rationally sought but generally unearthed after therapeutic relevance was demonstrated. Other, rationally designed, covalent-irreversible and covalent-reversible inhibitors are typically fit-for-purpose. That is, testing is performed with the full knowledge of potential target reactivity, or compound reactivity with the target is purposefully designed into the chemical leads to promote greater affinity (1-4). Additionally, throughout the lead optimization process, the contributions of covalent and non-covalent

interactions to target binding are characterized. For example, the FDA approved drugs afatinib (EGFR; conjugate addition of protein cysteine to α,β-unsaturated amide) (5), captopril (ACE; thiol binding of active-site zinc) (6, 7), carfilzomib (proteasome; double nucleophilic addition of N-terminal threonine to epoxyketone) (8), and telaprevir (hepatitis NS3/4A serine protease; serine addition to α-ketoamide) (9), all function by well-designed, targeted reactivity (target; mechanism of action). While these mechanisms can clearly prove efficacious when well-characterized, they are not necessarily appropriate for upstream discovery: when searching for a new starting point for a given biological target, non-covalent inhibition is desirable since a reactivity-driven screening hit has an extremely low chance of yielding to successful optimization. Undesired compound-target reactions have become recognized as a significant and costly source of assay artifacts (10-13). This section will discuss artifacts that may be caused by undesired and surreptitious compound reactivity*. Herein we will use the term 'active' to describe compounds that display activity in an assay. The term 'hit' will be reserved for compounds that have activity at a well-defined target.

*In this chapter, many of the examples and nomenclature will refer to target-based assays, specifically for enzymatic inhibition. This is meant to aid in readability. However, many of the general principles will hold true for non-enzymatic targets, such as transcription factors, protein-protein interactions, surface receptors and ion channels, as well as cell-based and organism-based ('*in vivo*') systems.

Chemical Reactivity Interference in Target-Based Assays

Chemical reactivity interference typically involves chemical modification of reactive protein residues or, less frequently, modification of nucleophilic assay reagents. Typical protein-modifying reactions include (**A**) oxidation of the cysteine sulfur (see Redox section, *vide infra*), (**B**) nucleophilic addition to activated unsaturation (referred to as Michael addition), (**C**) nucleophilic aromatic substitution, and (**D**) disulfide formation by reaction with thiol-containing compounds (Figure 1). Reaction of protein residues with highly electrophilic functional groups (such as acid halides, epoxides, α-halo carbonyls, and aldehydes) is also common.

While reactions of cysteine are highlighted in Figure 1, electrophilic reactions have also been observed at amino acid side chains such as Asp, Glu, Lys, Ser, and Tyr (15). In simple peptide models (6-12 mer), reactivity was shown to be Cys >> Lys >> Ser, Tyr with a small selection of electrophilic compounds (16). On the other hand, some probes that can undergo nucleophilic aromatic substitution have been observed to selectively react with Lys over Cys in proteomic experiments (15). Of relevance here is work that suggests that vinyl ketones readily react with protein cysteines, whereas tosylate esters readily react with a variety of residue types, including Asp, Glu, and Tyr (17). Protein microenvironment can greatly influence side-chain reactivity by altering the pK_a of amino acids (18). Therefore, while simplified models of amino acid reactivity may be helpful, they may not provide true evidence of non-interference of compounds in the context of a target protein.

Reactive moieties such as acid halides and aldehydes are easily recognized as covalent modifiers. Typically these can be discarded from screening 'actives' without further consideration. Many of these substructures can also be recognized by filters such as REOS (Rapid Elimination of Swill) (19). It is important to recognize that chemical reactivity is context specific. The reactivity of biological nucleophiles is affected by protein structure, electrophile structure, and reaction (assay) conditions. For example, not all epoxides (or other functional groups) in all molecules will be reactive under all assay conditions. The same holds true for other electrophilic or oxidizing species. For example, gradations of nitrile reactivity are well-documented (20). However, as a general rule compounds containing known reactive moieties should always be considered guilty (liable to cause false activity) until proven innocent by orthogonal assays.

Recently, several further classes of compounds, beyond those that are easy to spot and discard, have been added to the list of compound classes that can cause apparent bioactivity by reactive mechanisms. These classes of compounds, called PAINS (pan assay interference compounds), contain defined substructures that are more difficult to recognize as being potentially reactive. The most common of these structures are shown in Table 1.

These classes have been fully described (21-23). While not every PAINS substructure has a defined mechanism of assay interference, most of the substructures are presumed to be reactive. Certain PAINS classes can also interfere via metal chelation, and aggregation, but these chemical mechanisms of interference will not be discussed in this section.

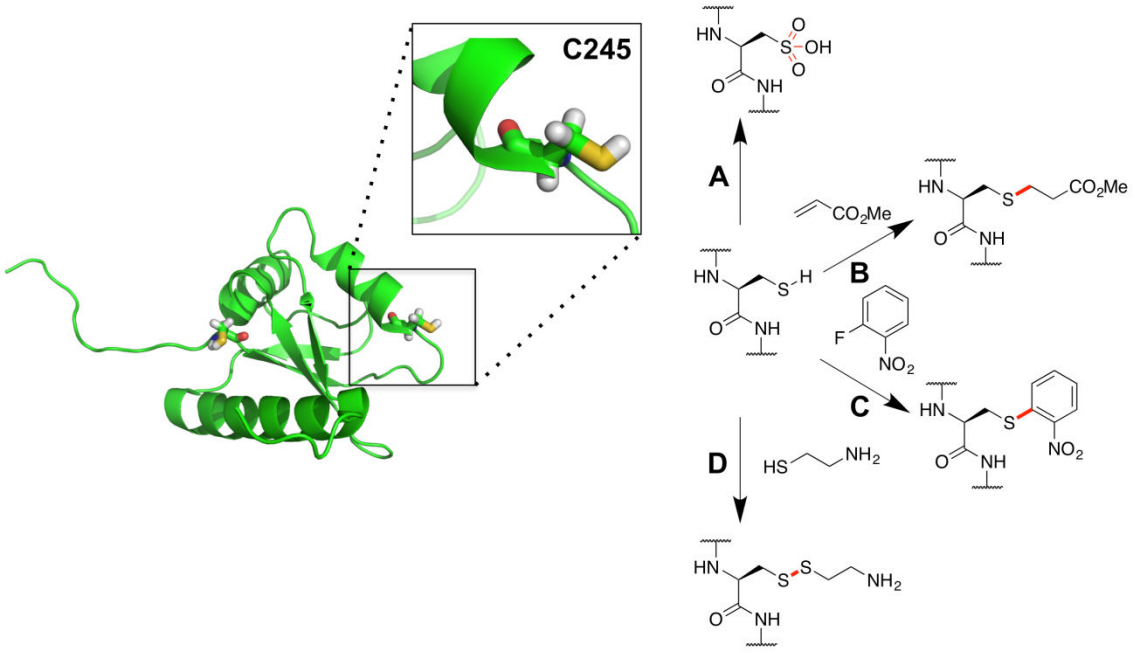


Figure 1: Typical reactions that are responsible for reaction-mediated assay interference involving cysteine reacting as a nucleophile with various electrophiles. (A) cysteine oxidation, (B) Michael-addition of the cysteine-thiol to an activated, unsaturated group, (C) nucleophilic-aromatic substitution, and (D) disulfide bond formation. The human La protein PDB ID 1OWX is used here for illustrative purposes (14). The bonds formed in these reactions are indicated in red.

Table 1: Examples of non-selective covalent modifiers commonly encountered in HTS triage. Shown are particularly notorious substructures linked to non-selective reactivity (in alphabetical order) and representative examples found in the scientific literature. This list is by no means comprehensive. Also included are noteworthy references describing the chemical basis of non-selective covalent reactivity for each class shown.

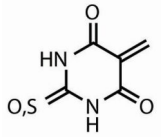
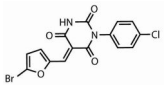
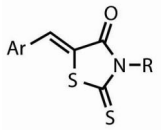
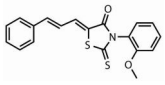
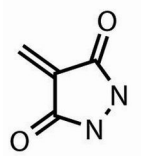
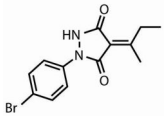
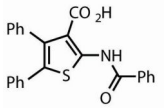
Substructure	Structure (example)	Class name	MOA comment[a]
		Alkylidene barbituates and thiobarbituates	Reactive
		Alkylidene rhodanines	Reactive, photo-reactive, chelation
		Alkylidene pyrazolidinediones	Reactive
		2-Amino-3-carbonylthiophenes	Redox-active

Table 1 continued from previous page.

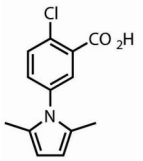
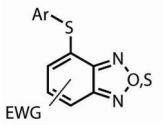
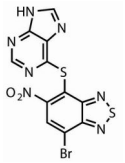
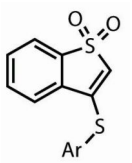
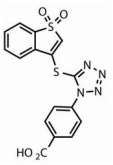
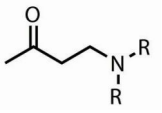
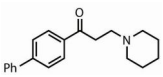
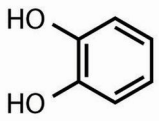
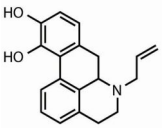
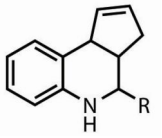
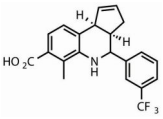
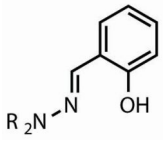
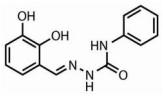
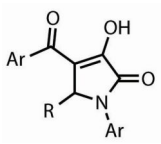
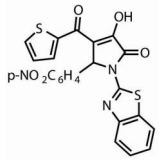
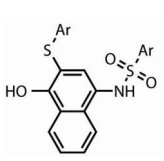
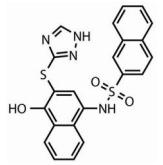
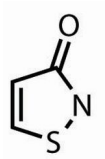
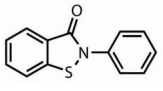
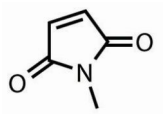
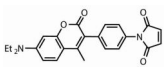
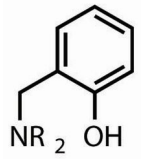
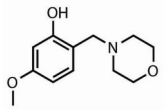
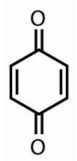
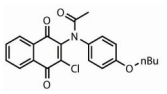
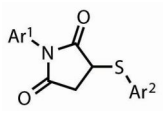
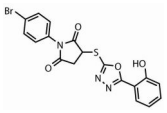
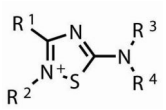
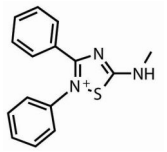
Substructure	Structure (example)	Class name	MOA comment[a]
		Aralkyl pyrroles	Reactive, can form bioactive polymers (24)
		Benzofurazans, Benzothiadiazoles (*S*-linked aromatic LGs)	Reactive (nucleophilic aromatic substitution) (25)
		Benzo[*b*]thiophene 1,1-dioxides (*S*-linked aromatic LGs)	Reactive (addition-elimination) (25)
		Beta-amino ketones	Elimination to form reactive species
		Catechols	Chelator; redox-active, oxidizes to quinones
		Cyclopentene-fused tetrahydroquinolines	Reaction catalyst impurities and/or formation of an electrophilic by-product in vitro
		Hydroxyphenylhydrazones	Chelation
		3-Hydroxy-pyrrolidin-2-ones	Reactivity, protein disruptors
		*N*-(4-Hydroxynaphthalen-1-yl)sulfonamides	Redox-active; reactive; buffer instability to form reactive quinones (25)
		Isothiazolones	Reactive

Table 1 continued from previous page.

Substructure	Structure (example)	Class name	MOA comment[a]
		Maleimides	Reactive (25)
		Phenolic Mannich bases (Betti bases)	Reactive
		Quinones	Redox-active; reactive
		Succinimides (*S*-linked aromatics)	Buffer instability to form reactive maleimide (25)
		1,2,4-Thiadiazole salts	Reactive (reversible, ring-opening disulfide bond formation) (25)

[a] Commonly encountered reactive mechanisms of action (MOA) for this series.

Chemical Reactivity Interference in Cell-Based and Phenotypic Assays

While this chapter focuses on target-based and cell-free assays, a critical point is that cell-based or phenotypic assays are also subject to interference by non-specific chemical reactivity. A prime example is the case of PTC124, a small-molecule that was discovered in a firefly luciferase-based assay targeting nonsense codon suppression (26). Several studies have provided evidence that this compound reacts with ATP to produce an adduct (PTC124-AMP) capable of stabilizing the firefly reporter enzyme, which ultimately confounds the cell-based assay readout by yielding apparent activation (27, 28). It is important to note that amphiphilic compounds, in particular those that are cationic in nature, can interfere with membranes and hence give artifactual results, not just in cell-based assays but also in membrane-associated target-based assays (29).

If a test compound has non-specific target activity, then it is highly unlikely that any observed effect could be specifically attributed to a well-defined compound-target interaction in a complex biological system such as a cell. Researchers performing cell-based screens should therefore be aware of the potential for off-target effects due to compound reactivity.

Prevalence in HTS

Most screening libraries contain reactive compounds and this must be considered in HTS triage. Natural products, which can be part of screening libraries, can also be electrophilic and highly reactive (30). Table 2 shows a comparison of the number of compounds found by HTS, REOS (19), and PAINS filters (*vide infra*) in a few general screening libraries (MLSMR: the Molecular Library Small Molecule Repository), Academic A and B (two representative academic screening libraries), and two recent (2014-2015) versions of eMolecules; a collection of commercially-available compounds). It has been suggested that PAINS compounds are often those

that are easily prepared by high-throughput synthesis (formerly referred to as combinatorial synthesis) and, thus, would most likely be highly represented in large, unfiltered collections of compounds. The HTS filter used here is the one implemented in Pipeline Pilot, and recognizes reactive compounds, metals, etc. REOS filters were designed to remove compounds likely to cause assay interference but also those that might be anticipated to cause late stage project failures because of toxicity (so-called 'toxicophores'). PAINS filters were carefully developed based on the actives in a select group of assays performed on a specific compound library. They recognize compounds that would likely interfere with assays. PAINS filters do not recognize the most reactive structural moieties because those functional groups were not included in the library upon which the filters were based. Other useful filtering strategies for removing potential interference compounds from data sets have been published (31, 32). The seminal point here is that the apparent hit rate due to PAINS compounds exceeds the typical 0.5 – 2% hit rates from screening of broad small molecule chemical libraries, so left unfiltered, authentic hits might not be identified from primary screening.

Table 2: Almost all libraries contain potential interference compounds. MLSMR: Molecular Library Small Molecule Repository (2014); Academic A and B: representative academic screening libraries; eMolecules: two versions of a curated library of commercially-available compounds (2014-2015); HTS: high-throughput screening filter in Pipeline Pilot (Accelrys); REOS: Rapid Elimination of Swill; PAINS: Pan assay interference compounds. REOS and PAINS filters as implemented in Schrödinger.

Collection	Compounds (n)	HTS	REOS	PAINS	PAINS (%)
MLSMR	314,651	7160	79,695	18,654	6
Academic A	208,887	5082	59,041	10,098	5
Academic B	234,304	3287	41,134	9831	4
eMolecules	6,580,176	200,805	NC	366,939	6
	5,498,347		982,150 (~20%)		

Section Summary

Compounds that are known to be reactive with proteins are likely to be found as "actives" in any primary HTS screen. The next sections will offer strategies to help triage these compounds so that resources can be directed to what might be better compound classes.

Knowledge-Based Strategies to Minimize Impact of Interference Compounds

Section Introduction

All libraries will most likely contain compounds capable of causing assay interference. Therefore, it is essential that a combination of knowledge and assay-based strategies be used to separate the true from false actives. There are several other knowledge-based strategies that will help the project team focus on the best chemical matter.

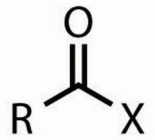
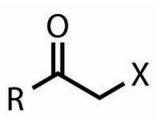
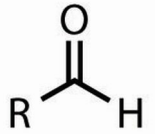
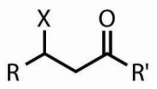
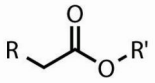
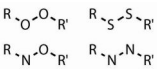
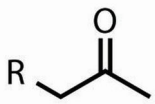
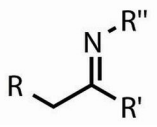
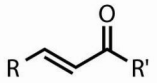
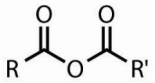
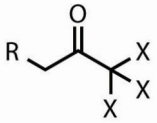
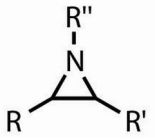
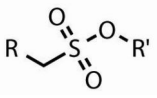
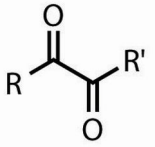
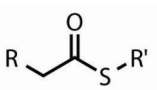
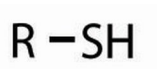
Understand Medicinal Chemistry Concepts and Reactive Substructures

Screening collections will inevitably contain reactive molecules, despite their limited potential utility as chemical leads. Many of these compounds represent synthetic intermediates submitted to corporate and commercial screening collections, or were developed for antineoplastic or antimicrobial projects. Many of these reactive functional groups have been characterized (Table 3). These can include acyl chlorides or aldehydes, for example. Medicinal chemists can usually identify these more obvious reactive groups.

Many of these functional groups are relatively straightforward to identify, and many cheminformatics programs have filters to rapidly identify the more conspicuous 'reactophores'. For example, the cheminformatics platform

in Pipeline Pilot has an 'HTS filter' that efficiently removes compounds with many of these problematic substructures. The NIH Molecular Libraries Small Molecule Repository has a similar set of functionality filters available. Another free and useful site for compound filtering currently available is FAF-Drugs3 (33). Compounds bearing these reactive groups should generally be avoided. However, not every reactive substructure filter can be all-inclusive, and in practice it is prudent to have trained medicinal chemists examine the results of a filtering process (34).

Table 3: Examples of notoriously reactive substructures. X, halogen; R/R'/R", alkyl.

Functional group	Structure	Functional group	Structure
Acyl halides		α-Halocarbonyls	
Aldehydes		β-Heterosubstituted carbonyls	
Aliphatic esters		Heteroatom-heteroatom single bonds	
Aliphatic ketones		Imines	
Alkyl halides		Michael acceptors	
Anhydrides		Perihalo ketones	
Aziridines		Sulfonate esters	
α,β-dicarbonyls		Sulfonyl halides	
Epoxides		Thioesters	
		Thiols	

Computational Methods

Computational strategies have been described to help identify reactive compounds and frequent hitters. One of the most straightforward methods available to academic researchers to flag problematic compounds is the use of substructure filters. In addition to filtering out compounds with the well-characterized reactive functional groups described above, screening actives should be flagged for PAINS substructures. In practice, this can be accomplished by several means. The computational chemistry suites from Tripos and Schrödinger have built-in, user-friendly PAINS filters. PAINS filtering can also be accomplished with open-source software, such as KNIME, or using the FAF-Drugs3 server (33, 35). Compounds that are not flagged as PAINS by substructure filters can still represent problematic compounds. This may be partly explained by the fact that the original PAINS substructures were identified by six HTS campaigns using AlphaScreen, and specific substructures were designed with specific cut-off criteria. Compounds closely resembling PAINS substructures may therefore not be flagged.

The potential for chemical reactivity can also be modeled and predicted using computational methods (36). These models can provide clues about the potential for reactivity, but they are not a substitute for experimental evidence.

Search Scientific Literature and Databases

In evidence-based medicine, systematic reviews and meta-analyses are routinely conducted to answer clinical questions. This consists of a thorough literature search, and expert synthesis of the available data to formulate a clinical guideline. Similarly, promising compounds should be put in the context of previous studies to formulate recommendations about future studies (34). This includes assessing the 'natural history' of a compound and related analogs. There are several resources that can be used for this purpose (Table 4).

A straightforward practice is to first search for potential lead compounds by their structure to examine what data, if any, are available for the parent compound. For example, one can search PubChem to see how a particular compound behaved in any NIH-funded HTS. Often, a compound will have been screened in tens to hundreds of assays with different targets and assay formats. This can provide valuable clues about bioassay promiscuity including potential reactivity (37-39). A useful web application that offers a convenient window into standardized data from PubChem assays is BADAPPLE (bioactivity data associative promiscuity pattern learning engine) (40). BADAPPLE serves as a plug-in for BARD, the standardized BioAssay Research Database of the MLP (NIH Molecular Libraries Project). The algorithm embedded in BADAPPLE accepts query structures and generates a pscore (promiscuity score) based on a statistical analysis of the bioactivity data in BARD associated with scaffolds present in the query molecule (41).

There is also important information that can be gained from examining related chemical analogs in the databases listed in Table 4. We have found it useful to examine structurally similar compounds, and most chemical search engines have options to search by 2-D similarity. It is important to realize, however, that substructure searching can be more useful than similarity searching when there is specific interest in core SAR. Some relevant questions to ask include:

- Has this compound (or chemical analogs) been screened before? If so, how often has this compound been active? What percentage of assays has this compound been active in and against how many targets?
- Has the compound (or chemical analogs) been published in the scientific literature? If so, what claims were made about the compound? How was the compound tested? Based on how the compound was tested, is there a possibility that the results could be explained by non-specific chemical reactivity?
- Has this compound (or chemical analogs) been included in any scientific patents? If so, ask similar questions as above.

- How has the compound been synthesized? What are some possible bioactive contaminants? Are there alternative synthetic routes?

Compounds and chemical classes that show up repeatedly in unrelated screens and in different assays should raise suspicion about non-specific reactivity and other non-therapeutically interesting mechanisms of bioassay activity. Performing this exercise can prevent wasted follow-up on promiscuous compounds, or thoroughly investigated compounds, including reactive entities. These search activities can be time intensive, and in practice this step is optimal when the number of candidate compounds has been culled to a handful of chemical series.

Table 4: Examples of databases for examining compound natural histories.

Resource	Remarks
SciFinder	Useful for searching patent literature; very comprehensive; subscription service (http://www.cas.org/products/scifinder)
PubChem	Provides important information about bioassay promiscuity; open-access (https://pubchem.ncbi.nlm.nih.gov/)
Reaxys	Chemical database for searching scientific literature by chemical structure; subscription service (https://www.reaxys.com)
ChEMBL	Compounds and activities; open-access (https://www.ebi.ac.uk/chembl/)

Section Summary

The application of fundamental medicinal chemistry principles is key to identifying intractable reactive screening hits. Cheminformatic resources can help researchers efficiently flag screening actives containing well-known reactive functional groups, and other more subversive reactive substructures like PAINS. Freely available resources can allow researchers to check the 'natural histories' of the compounds that turn up as actives in their HTS. For both the HTS novice and experienced chemists, consulting experts in HTS triage is strongly advised when analyzing HTS results. As detailed in the next section, promiscuous or potentially reactive compounds should be followed up with rigorous experiments to prove that they are selectively hitting the target of interest.

Experimental-Based Strategies to Mitigate the Impact of Reactive Interference Compounds

Section Introduction

Non-specific reactive compounds have the potential to derail drug discovery and development projects if they are not identified promptly. Fortunately, there are many experimental tools available to researchers to mitigate this potential problem. In this section, experimental strategies to identify and triage reactive screening compounds are described. Each method has advantages and disadvantages, and a pragmatic approach is to employ several of these strategies. The use of these counter-screens is applicable to screening compounds derived from both target-based and phenotypic screens, and both real and virtual screens. While much of the text refers to enzymatic inhibitors, the counter-screens and overall concepts discussed herein are applicable to other types of targets and mechanisms of action.

Characterization of Assay Sensitivity to Known Reactive Compounds

During the assay development stage, it may be useful to characterize the effect of various reactive entities on the assay performance. As an example, workers at GlaxoSmithKline have developed a screening library of interference compounds that they use to probe the sensitivity of assays to compounds with different modes of

assay interference (T Jurewicz *et al*. unpublished results, SLAS 2015 Annual Meeting). This strategy allows them to fine-tune their assays to limit the interference of these compound classes. It also alerts them to potential compounds that will likely show up as spurious actives.

In practice, there are several ways this process can be accomplished. Assays can be challenged with commercially-available thiol-reactive compounds such as maleimides or quinones. Similarly, one can also test an assay readout and performance with established redox-active compounds like β-lapachone and NSC-95397, as well as H_2O_2 itself (42, 43). If an assay readout or target is particularly sensitive to H_2O_2 (and presumably redox-active compounds), then a strategy could be to adjust assay parameters to minimize H_2O_2 production and prioritize a redox-activity counter-screen early in the post-HTS screening tree, for example.

Use of Scavenging Reagents to Mitigate Impact of and to Identify Thiol-Reactive Compounds

One straightforward approach to mitigating the impact of thiol-reactive compounds in target-based assays is to include scavenging reagents in the assay buffer. For example, assays can include dithiothreitol (DTT), usually in 1-5 mM final concentrations, to accomplish two objectives. First, this agent helps to keep protein cysteines in the reduced form, which can be required for many protein systems. Second, thiol-reactive compounds can react with the free thiols on DTT. Therefore, DTT can serve as a potential scavenger for thiol-reactive compounds if present in sufficient quantities. Other biological reducing agents may have utility, including β-mercaptoethanol (BME), L-cysteine, *tris*(2-carboxyethyl)phosphine (TCEP; avoids the disulfide exchange possible with DTT) or even non-enzymatic proteins like bovine serum albumin (BSA).

This strategy can be employed from the beginning of the HTS to mitigate the impact of electrophilic compounds. In other words, particularly egregious thiol-reactive compounds would theoretically show reduced activity in the HTS, and would be less likely to be identified among the HTS hits. The ability of DTT to react with some electrophilic compounds can also be exploited in post-HTS assays. One strategy we and others have employed for enzymatic inhibition assays is to measure the IC_{50} of compounds in both the presence and absence of DTT (Figure 2) (25, 44). Thiol-reactive compounds will often have a significant increase in their IC_{50} values when sufficient amounts of DTT are added in otherwise identical assay conditions.

Sample steps | DTT counter-screen

1. Test assay with solvent controls (e.g. DMSO) at several concentrations of DTT (usually 0, 1, 5 mM final concentrations). For each test condition, prepare otherwise identical assay buffer with the appropriate levels of DTT.
2. If assay performance is unaffected by DTT, proceed to step 3. If the assay readout is not compatible with DTT or assay performance suffers, consider testing with other reducing agents (e.g. TCEP, GSH).
3. Test compounds of interest under otherwise identical conditions, ideally in parallel. Include a thiol-reactive positive control to 1) verify the target is sensitive to thiol-reactive compounds, 2) assess overall assay performance, and 3) gauge magnitude of activity differences between different test conditions.

Researchers should consider the following technical points:

- DTT has a limited half-life in aqueous solutions due to oxidation, and stock solutions should ideally be made fresh for each experiment.
- Prior to testing compounds, one should examine the effect of different concentrations of DTT (or comparable reagent) on the assay readout. Certain biological systems or assay readouts may be incompatible with such reagents.
- Significant changes in activity with the addition of DTT should raise concern about thiol-reactive compounds. There is no strict cut-off, but for conventional cell-free enzymatic assays, IC_{50} changes greater than 3-fold in several independent experiments should raise concerns. With particularly

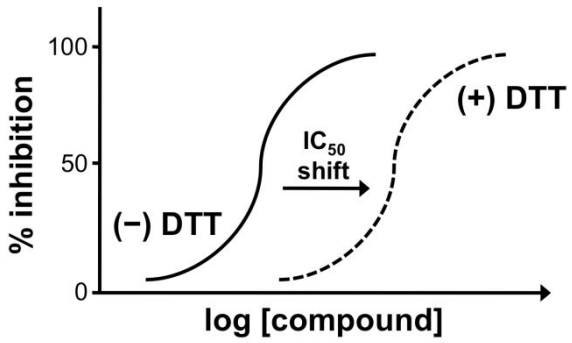


Figure 2: Illustrated effect of DTT on IC_{50} values of reactive compounds in target-based enzymatic assays. Compounds can be tested in the presence and absence of a protein-compatible scavenging agent like DTT. For DTT, this can typically be in the range of 1-5 mM final concentration. Thiol-reactive compounds should in principle show a higher IC_{50} value in the presence of DTT. In principle, other electrophilic scavengers can be used.

electrophilic compounds, we have seen IC_{50} changes greater than 10-fold when the [DTT] >> [test compound].

- The inclusion of DTT in an assay system does not absolutely prevent the reactivity-based activity or interference of thiol-reactive compounds. Certain compounds may have different reactivity with DTT than with proteinaceous thiols. Another possibility is that the DTT has been oxidized to a form that does not contain free thiols.
- Maleimides such as CPM can be highly useful thiol-reactive positive controls, provided the target of interest is sensitive to thiol-reactive compounds (25).

Kinetic and Mechanistic Methods to Identify Irreversible-Binding Compounds

Compounds can be tested for evidence of reactivity by kinetic and mechanistic methodologies. The premise is that reactive compounds generally function by irreversible inhibition, and therefore have distinct reaction characteristics compared to well-behaved, reversible inhibitors.

Assessment of activity time dependence

Reactive compounds typically show time-dependent effects (45-50). Testing can be relatively straightforward. The effect of a compound on a particular target is measured as a function of time. One approach is to vary the amount of time a compound is allowed to incubate with the target before starting an assay ('pre-incubation time'). A time-dependent effect can also be monitored after an assay has been initiated by using continuous reaction monitoring (or sampling assay aliquots), as a reactive compound may show increasing inhibition during the course of an assay as it continues to react with the target. This effect can vary with compound concentration as well. In the case of enzymatic inhibitors, the inhibitory effect of a reactive compound can increase the longer it is allowed to incubate with its reactive target (e.g. an enzyme). One should note, however, that fast-reacting and/or fast-binding compounds may not show time-dependent effects under certain experimental conditions (if, for instance, the incubation times are sufficiently long and the reaction rates are fast). It may not be experimentally feasible to sample short pre-incubation times – this can be due to the time it takes to add reagents, for example. Therefore, a word of caution is that the inability to observe a time-dependent effect does not necessarily exclude a covalent binding event if the binding and reaction is sufficiently fast.

Assessment of compound activity reversibility by dialysis methods

Covalent-modifying compounds often irreversibly modulate their target(s). In many cases, the desired profile of a lead compound includes a reversible mechanism of action. For many target-based assays, a test compound can

be incubated with the protein(s) of interest, then subjected to dialysis. The target can subsequently be tested for activity after this dialysis to examine for residual bioactivity. The key to interpreting the results is to understand the expected results for both reversible- and irreversible-binding compounds. If a test compound exerts its action by a reversible binding event, the compound should be effectively removed from the system when given sufficient time to equilibrate in a dialysis system (51). Conversely, irreversibly-binding compounds such as reactive screening actives (and also some tight-binding reversible inhibitors) will not be removed by dialysis.

Performing a dialysis experiment is relatively straightforward. The concentration of the test compound should be high enough that it will unequivocally modulate the target at the pre-dialysis concentration. For an enzymatic inhibitor with a Hill slope near 1.0, this may mean incubating the compound at a minimum of 10X its IC_{50} value. Testing of the dialyzed target originally treated with the test compound should be compared to a vehicle control (e.g. DMSO-only if the compound is delivered from a DMSO stock solution) subjected to an identical, parallel dialysis procedure (Figure 3). This controls for potential target denaturation or degradation over the course of the dialysis procedure. In principle, compounds that irreversibly modulate their target(s) will retain most of their activity compared to vehicle controls.

Sample steps | Dialysis counter-screen

1. Prepare stock solutions of compound and protein target.
2. Pre-determine the dose-response of the test compound versus the protein target. Determine the concentration of test compound that reliably results in > 90% target modulation (e.g. enzymatic inhibition). For a compound with a Hill slope of 1.0, this concentration should be greater than 10X IC_{50}, which corresponds to 91% inhibition.
3. Incubate compound with protein target for a short duration (e.g. 5-30 min) to ensure equilibration. This incubation can be done with the target at 10X final assay concentrations. This guards against diluting below 1X final assay concentrations during dialysis and prevents against certain proteins that are unstable in dilute solutions. The concentrated solution can still be diluted post-dialysis.
4. Transfer the solution from step 3 to a dialysis vessel with an appropriate molecular weight cut-off.
5. Dialyze compound and target in 1X assay buffer. The length of dialysis and number of buffer changes depends on multiple factors, including the volume of dialysis buffer and the surface area of the dialysis membrane.
6. Dilute dialyzed solution with 1X assay buffer to achieve desired concentration of protein target to perform the target assay.
7. Perform target assay.
8. Compare percent modulation to vehicle control and any positive and negative control compounds.

Researchers should consider the following technical points:

- Dialysis can be accomplished quite efficiently with many commercially available cassette systems. Many micro-cassettes allow for smaller volumes of dialysis buffer and fewer amounts of compound and target(s).
- Concentration factors should be accounted for when picking the test compound concentration to achieve a desired final compound concentration capable of near-maximal activity if the mechanism of action is indeed irreversible.
- The activity of the target system can diminish during the dialysis process, especially for certain enzymes sensitive to thermal denaturation or oxidation. Steps to help mitigate this denaturation include using cooler temperatures for dialysis, including carrier proteins in the dialysate, or using concentrated target solutions (see below).
- If dialysis is performed at cooler temperatures (usually done to mitigate target denaturation), remember to use pre-chilled dialysis buffer to avoid transient heating of the sample.
- When dialyzing samples at cooler temperatures (e.g. 4 °C), inspect the sample for signs of compound precipitation. Compounds with poor solubility can sometimes precipitate from solution.

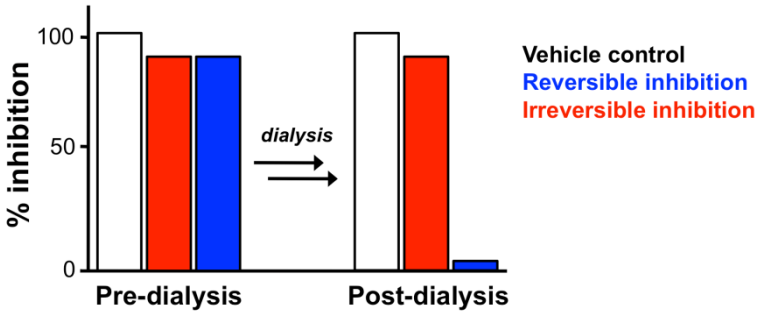


Figure 3: Assessment of compound inhibition reversibility by dialysis. Irreversibly-acting compounds (and some slow, tight-binding compounds) can modulate their target(s) even after dialysis. In this experiment, compounds are incubated with target(s) at high compound concentrations, enough that they should achieve near-maximal activity. This solution is then dialyzed. In principle, compounds that are reversibly-bound should pass through the dialysis membrane. After a sufficient period of time, the dialyzed sample is tested for activity and compared to a vehicle control. Reversibly-acting compounds should have activities comparable to the vehicle control, assuming equilibration was reached during dialysis. Shown in the above example is the expected result for an enzymatic assay testing reversible and non-reversible inhibitors.

- Consider the use of control compounds, both positive and negative, in this type of experiment. If a target is sensitive to thiol-reactive compounds, then a useful control is a non-specific thiol-reactive probe like a soluble maleimide. Such a compound should irreversibly modulate the target, even after dialysis.
- Keep in mind that test compounds have the potential to degrade during dialysis. Consider assessing the stability of the compound in buffer to control for compound stability.
- An additional control involves testing compounds prior to initiating dialysis. This can be accomplished by testing aliquots of the compound plus target solutions, along with the corresponding vehicle controls. This control verifies the original activity of the compounds when they are present at high concentrations in the test system.

Assessment of compound action reversibility by jump-dilution

Another classic experiment to test for irreversible target inhibition involves incubating the test compounds with the target(s) at higher concentrations, diluting the solution, and initiating the assay at the resulting lower concentrations (Figure 4) (51). This is the so-called 'jump-dilution' experiment (52-54). In this cell-free experiment, a test compound is typically incubated in a 100X-solution containing the target of interest (e.g. an enzyme), with a test compound concentration of 10-fold its IC_{50} value in the case of an enzymatic inhibitor with a Hill slope near 1.0. This solution is then rapidly diluted 100-fold into a reaction containing substrate. This dilution creates a 1X-solution of the protein, and dilutes the test compound from 10X the IC_{50} value to 0.1X the IC_{50} value. In well-behaved reversible inhibitors having a Hill slope of 1.0, a compound being tested at 10X its IC_{50} value will show 91% inhibition, while testing at 0.1X its IC_{50} value will show 9% inhibition. Thus, in general practice, reversible compounds will have a percent inhibition noticeably less than 50%, in contrast to reactive compounds, which will not show a reduction in inhibition because they are still bound to the target even after dilution.

Sample steps | Jump-dilution counter-screen

1. Determine the dose-response of the test compound versus the protein target. Calculate the IC_{50} value.
2. Prepare stock solutions of compound and protein target. Be sure to prepare a 1X and 100X solution of the protein target (the highly concentrated target solution is usually prepared with 1X assay buffer unless there are stability issues).
3. Incubate the test compound using 10X IC_{50} in a solution containing 100X protein target. Mix and incubate to allow for equilibration. Be sure to include a vehicle control
4. Dilute the solution from step 3 100-fold with 1X assay buffer.

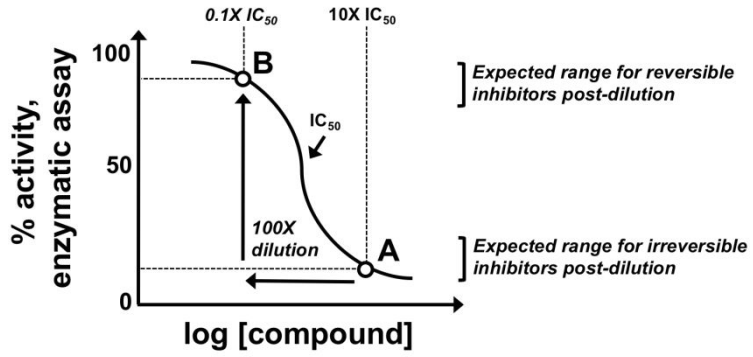


Figure 4: Assessment of compound activity reversibility by the jump-dilution method. Shown is an illustration for a compound being evaluated for reversible inhibition in an enzymatic assay, assuming a Hill slope near 1.0. At point (A), the test compound at 10X the IC_{50} value is initially incubated with the target enzyme at 100X the normal target concentration. After equilibration, this solution is diluted 100-fold with assay buffer. If a compound acts by a completely reversible process, then the expected activity should correspond to a final point (B) after equilibration.

5. Add substrate(s) and perform assay using standard procedures.
6. Compare activity of diluted test compound to vehicle control. Positive and negative controls should be included for comparison.

Researchers should consider the following technical points:

- Prior to performing jump-dilution experiments, it is advisable to have a solid understanding of the compound dose-response. For testing potential enzymatic inhibitors, this means knowing the IC_{50} value, the Hill slope, and the associated error in both.
- Check for compound precipitation prior to dilution. This is especially true for compounds with poor solubility or weak potencies. A compound with an IC_{50} of 10 μM will need to be incubated with the target at 100 μM in the aforementioned experiment. A straightforward way to check for compound precipitation is to centrifuge a sample and look for a pellet.
- Ensure the solubility of the proteins/biological target at the 100X concentrations. Look for evidence of protein/target precipitation after brief centrifugation. For example, certain protein complexes or targets sensitive to salt or pH conditions (like histones) may not be entirely soluble at these 100X concentrations.
- Consider the use of control compounds, both positive and negative, in this type of experiment. Targets sensitive to thiol-reactive compounds should retain most of their target modulation even after dilution.
- Testing compounds with Hill slopes that deviate significantly from 1.0 may require adjustments to the suggested dilution steps.
- Note that slow, tight-binding compounds may not show the predicted reversibility, depending on the experimental conditions. If this type of mechanism is suspected, one should carefully scrutinize and investigate the time parameters for each step in the experiment, such as post-dilution incubation time, before the reaction is initiated (55). The time-based effects can be conveniently monitored if a continuous assay readout is available. If this type of inhibition is suspected, we advise consulting an experienced enzymologist.
- Different dilutions or compound concentrations may be used (e.g. 50X, 10X for targets) but one should consider the expected changes in activity (based on the dose-response) and also the experimental errors associated with the assay of choice. For instance, lower magnitude dilutions can potentially make it more difficult to definitively detect reversibility.

Purification and/or Re-Synthesis of Compounds to Triage Reactive Impuritie s

As purchased, screening compounds can contain reactive impurities that can lead to assay interference or non-specific activity. These impurities can result from compound degradation, or be the remnants of reactive reagents used in the compound synthesis (56). Screening compounds are typically stored in frozen DMSO, which is a hygroscopic and a mildly reactive solvent. That being the case, prolonged storage of compounds in DMSO or frequent freeze/thaw cycles can cause degradation of screening compounds (57). These degradative transformations can also be catalyzed by trace metal contaminants that may remain from synthetic methods used in the preparation of the compound (58). Metal contaminants such as these can also react with proteins and may not even be detectable by standard measurements of compound purity.

There are several industry-inspired best practices designed to mitigate the risk of reactive impurities (Figure 5). Once a compound is found to be active in HTS, the standards of purity (e.g. LC-MS-PDA-ELDS) and chemical characterization (identity by spectroscopic methods: e.g. ^{1}H, ^{13}C NMR) become an important consideration (59). These quality control (QC) experiments are required by many funding agencies, including the NIH, which states in several funding announcements that compounds "must be analytically validated in terms of integrity and purity (e.g., use of a re-synthesized powder sample of high purity in the preliminary assays)". In follow-up assays, the compound of interest should be re-purchased from commercial vendors and subjected to HPLC purification and structural characterization in-house, re-assayed, and its performance compared to the original screening compound. Ideally, the IC_{50} values should be nearly identical between the library compound and the re-purchased compound. This step is critical because it allows for QC of the purity and structural identity of the compound of interest. Additionally, the most promising compounds should be re-synthesized in-house, subjected to LC and characterization, and then again re-assayed for comparison to prior samples. If there is a concern about a particular reactive intermediate, compounds can be synthesized by independent routes. Well-behaved compounds should have similar IC_{50} values for all batches tested (screening compound, commercial re-purchase, independent re-synthesis). The minimum purity for test compounds entering confirmatory assays should be 95%, as determined by HPLC/MS/PDA/(and, if available, evaporative light scattering detection (ELSD)).

Sample steps | Compound purification and re-synthesis

1. Active compounds should be repurchased as solid samples, purified by chromatography, and be subject to rigorous QC standards prior to having their activity confirmed.
2. Purified compounds that have reproducible IC_{50} values should be prioritized for re-synthesis and retest. Small, focused arrays of analogues made at the same time as the initial active compound can provide SAR that can be used in go/no-go decision making.

Researchers should consider the following technical points:

- QC should consist of LC/MS/PDA (ELSD is also helpful) and NMR (^{1}H, ^{13}C) assurance of ≥ 95% purity and identity. PDA = photodiode array
- Low levels of metal impurities (Zn, Pd, Fe, etc.) may cause false positives in some assays. If the assay is sensitive to trace reagent interference, it may be necessary to analyze the purified compounds for metals (detection of trace metals by typical spectroscopic methods is sometimes challenging), to apply resin-based chelators to remove any metals (60, 61), and/or to prepare the compounds by alternative routes that avoid the use of these potential interfering reagents.

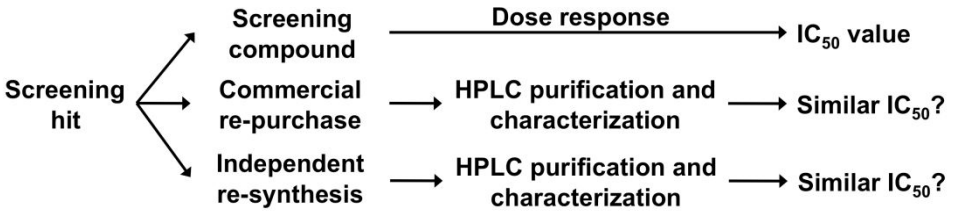


Figure 5: Schematic of purification and re-synthesis best practices. Hits derived from screening compound collections should be re-tested with a re-purchased commercial sample that is HPLC purified and characterized for structural identity. Promising compounds should then be re-tested with a sample that has been independently re-synthesized and HPLC purified. All the samples should have reproducible IC_{50} values.

Identification and Triage of Redox-Active Compounds

Several solutions exist to de-risking compounds for redox-activity. A two-fold strategy is recommended whereby 1) the effect of reactive oxygen species (ROS) such as H_2O_2 on the assay system is characterized, and 2) compounds being considered for progression are de-risked for their ability to act as redox-active compounds under the assay conditions.

Characterization of assay sensitivity to redox activity

This strategy can provide an initial gauge of how likely redox-active compounds are to show up among the assay hits. That is, if a particular assay is found to be sensitive to low micromolar concentrations of H_2O_2, then any active found should be rigorously evaluated for redox-activity in the post-HTS phase. Different targets may have different susceptibilities to oxidation. For target-based systems such as in vitro enzymatic or protein-protein interaction assays, this can be done by spiking the system with known amounts of H_2O_2 under assay-like conditions and then measuring the assay readout (25). Similarly, assays can be challenged with known redox-active compounds. For positive control compounds, some suggestions include using β-lapachone and NSC-95397 (42, 43). These approaches can also be performed with cell-based assays, as H_2O_2 is known to participate in many different cell signaling pathways, and H_2O_2 and related ROS can be cytotoxins at sufficient exposures (62). It is recommended therefore, that the effect of H_2O_2 on cell growth and cell signaling markers be measured in cell-based assays where redox-active compounds have the potential to be identified as actives. Even if a particular assay system is not overly susceptible to H_2O_2 modulation, compounds should still be evaluated for their ability to produce H_2O_2 because they can contribute to non-specific bioassay activity in other follow-on assays.

Testing compounds for redox activity

With respect to redox-active compounds, we also recommend that potential lead compounds be de-risked for their ability to act as redox-active compounds, even if this liability is not suspected. This can be done using the simple surrogate assays described below. The advantage of pursuing this strategy is that it can prevent wasted follow-up on intractable compounds early-on in a drug discovery campaign. Additionally, if no redox-activity is identified, it adds a layer of confidence that a candidate compound is displaying activity by a therapeutically relevant mechanism.

One assay to identify H_2O_2-producing compounds is a surrogate horseradish peroxidase-phenol red (HRP-PR) assay (43, 63). The experimental procedures outlined in these papers are well-described. The premise of this assay is very straightforward: phenol red produces an absorbance at 610 nm when it is oxidized by HRP in the presence of H_2O_2. Therefore, compounds that produce H_2O_2 *in situ* will lead to a color change (colorless → red) if present in sufficient concentrations. Compounds can be tested for their ability to produce H_2O_2 in both the presence and absence of the strong reducing agent DTT, as DTT can fuel redox-cycling at low millimolar

concentrations, and because some compounds are capable of generating significant amounts of H_2O_2 even in the absence of DTT (64). We have found the HRP-PR assay very robust, inexpensive, and relatively easy to set-up, as all of the key reagents are commercially available. We have found this robustness extends even to the assay buffer, meaning one can often test for redox-activity with the original HTS-like buffer. Another advantage of this assay is that the assay readout is proportional to the H_2O_2 concentration.

Sample steps | HRP-PR redox-activity counter-screen

1. Prepare compounds, reagents and instrumentation.
2. Add vehicle controls (e.g. DMSO) to the appropriate experimental plate wells. These vehicle controls will serve as the negative controls.
3. Add test compounds to the appropriate experimental plate wells.
4. Add H_2O_2 to the appropriate experimental plate wells. This will serve as the positive controls. Be careful not to contaminate other wells with H_2O_2.
5. Dispense assay buffer (e.g. the same buffer used in the original HTS) to all experimental plate wells.
6. Dispense assay buffer containing concentrated reducing agent to all experimental wells (except for the wells containing H_2O_2 – for these wells, simply add buffer without reducing agent). Note: steps 5 and 6 can be combined in one dispensing step depending on user preferences.
7. Dispense HRP-PR solution to all plate wells.
8. Mix plate contents by plate shaker for 1-2 min.
9. Incubate solutions at room temperature for 20 min.
10. Add NaOH solution to each experimental plate well.
11. Mix plate contents by plate shaker for 1-2 min, then incubate solutions at room temperature for 5 min.
12. Measure OD_{610} on plate reader.
13. Analyze data. The H_2O_2 wells will serve as the positive controls, while the vehicle controls will serve as the negative controls. Positive control compounds should show a dose-response H_2O_2 production.

Researchers should consider the following technical points:

- Additional useful technical details – including recommended dispensing volumes, specific reagents and stock solution compositions – are comprehensively discussed in the original references (43, 63).
- H_2O_2 can degrade over time. Prepare fresh H_2O_2 solutions daily from a stock solution of concentrated H_2O_2 stored under the manufacturer's recommended condition.
- Ideally, test compounds should be tested at several concentrations. We typically test between 1-250 μM compound concentrations in triplicate, with several intermediate test points. The number of replicates, dose-response points and test concentrations should be guided by the experimental question and project testing conditions.
- Redox-activity can occur in the presence and/or absence of DTT (or other strong reducing agents). We recommend testing compounds with and without a strong reducing agent like DTT.
- As an interference control, compounds active in this counter-screen should be tested for their ability to interfere with the assay readout in buffer alone, as colored compounds may show a false-positive readout if they absorb light significantly in the 610 nm range.
- In addition to H_2O_2 as a positive control, some suggested positive control compounds include using β-lapachone and NSC-95397 (42, 43).
- The threshold for redox-activity inevitably depends on the project. Evidence of a dose-response and H_2O_2 production several-fold above baseline values should be flagged for potential triage.
- The addition of catalase to the assay should attenuate the final concentration of H_2O_2 and decrease the colorometric readout. This can serve as an additional confirmatory step.

A second assay for detecting redox-active compounds utilizes a protein-free format to detect redox-cycling compounds. In this assay, compounds are incubated with DTT and the reagent resazurin (65). Redox-active

compounds identified by this assay catalyze the reduction of resazurin to resorufin by DTT. The reaction product resorufin can be quantified by measuring fluorescence intensity. Compounds testing positive in this assay are susceptible to producing H_2O_2 and superoxide (O_2^-) *in situ*, and are capable of oxidizing protein side chains. The advantages of this assay are that it is protein-free and like the HRP-PR assay, it is straightforward. Disadvantages of this assay are that, unlike the HRP-PR assay, the resorufin product can be formed independently of O_2^- or H_2O_2. Therefore, the assay may identify compounds that reduce resazurin but do not necessarily produce reactive oxygen species. Others have suggested that high hit rates and large numbers of false positives may result from this assay (63). Also, compounds cannot be tested in this format in the absence of DTT.

Finally, a lower-throughput option is to test for modification of specific cysteines (as well as other residues like methionine) in redox-sensitive proteins such as certain caspases and cathepsins (64-66). In this type of assay, compounds and redox-sensitive proteins are incubated together, and the protein is analyzed by either LC-MS or LC-MS-MS techniques. The advantage of the latter analytical technique is that it may identify protein residues particularly susceptible to oxidation. These are relatively low-throughput approaches compared to the two aforementioned assays. Using H_2O_2 and vehicle controls as positive and negative controls respectively are recommended, as well as known redox-active compounds. Before undertaking these experiments, it is often helpful to consult with researchers with expertise in proteomics to ensure the availability of the necessary instrumentation and to confirm that the assay conditions are compatible with protein mass spectrometry.

Identification of Unstable Compounds and Reactive Species in Assay Media

Compounds are not always stable in assay media (67). Certain compounds are themselves inert, but can undergo chemical or conformational transformations to form reactive moieties (Figure 6A). For example, we have described a series of succinimides that, when incubated in a basic solution, can undergo elimination to form reactive maleimides. Even reactive compounds can degrade into other reactive compounds, as we have shown with a series of *p*-hydroxyarylsulfonamides that decompose to form reactive quinones (25). Other compounds can oxidize in the presence of air to form reactive products, including certain catechols that can oxidize to form reactive quinones. In principle, many variables can affect the stability of a given compound under typical assay conditions, including:

- Time
- Temperature
- pH
- Ionic strength
- Gas (atmosphere)
- Concentration (compound and buffer components)
- Stirring
- Presence of certain ions

Assessing compound stability by chromatography

The stability of test compounds can be monitored in assay buffer over time, using HPLC-based methods (Figure 6B). Compounds are incubated in the assay buffer, and aliquots are analyzed by HPLC or HPLC-MS. Stability can be compared to a control sample in which the compound is incubated in a solvent such as methanol, or another non-reactive solvent. An advantage of HPLC and similar analytical methods include the small amounts of compound needed. We have found benefits to simultaneous reaction monitoring by both evaporative light scattering detection (ELSD) and UV absorption, as reactions monitored by UV can be subject to fluorescent impurities and wavelength-dependent fluorescence properties. Degradation results can also be checked versus a

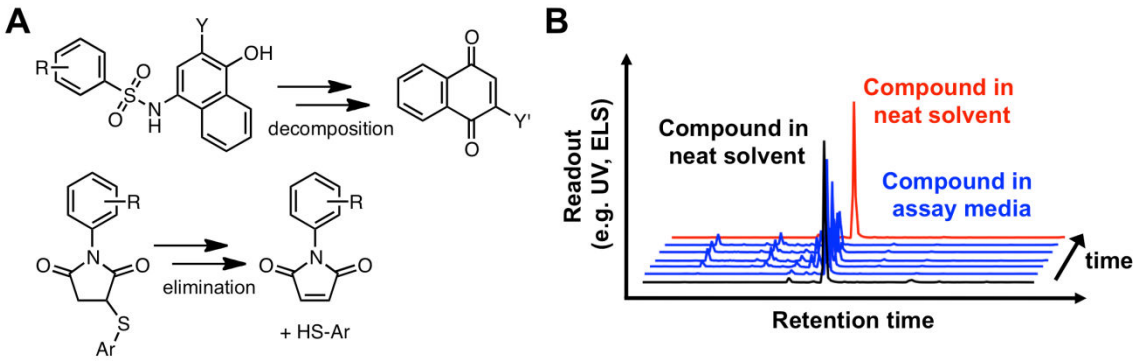


Figure 6: Assessment of compound stability in assay buffer. (A) Examples of compound classes showing buffer instability to generate reactive degradation product(s). Y denotes a leaving group such as a halogen or S-linked aromatic group; Ar denotes an aromatic group. (B) Schematic of a sample LC-MS experiment to assess compound stability in assay conditions.

related chemical analogue to draw conclusions about whether the stability is an isolated or series-wide phenomenon.

Sample steps | HPLC-based assay of compound stability

1. Make stock solution of test compound.
2. Dilute an aliquot of test compound in an inert solvent such as methanol. Run HPLC column to identify parent compound retention time.
3. Titrate concentration of compound and injection volumes to obtain adequate readout intensity without over-saturating detection moiety.
4. Run HPLC column of assay buffer to ensure solvent-instrument compatibility.
5. (Optional) Repeat steps 2-4 as needed to verify consistency of retention time and readout intensity. Optimize instrument settings such as injection volume, buffer gradients, columns, monitoring wavelength (if UV-Vis detection method), ionization settings (if MS detection method), etc.
6. Prepare two master solutions: one containing inert solvent and the other containing 1X assay buffer. Spike each sample with identical amounts of test compound to achieve the desired compound concentrations determined from the previous steps 1-5. Prepare enough solution to allow for multiple injections.
7. Obtain data from test compound in inert solvent.
8. Obtain data from test compound in assay buffer. Resample at multiple time-points.
9. Repeat step 7 at the last time point to control against degradation unrelated to assay buffer.

Researchers should consider the following technical points:

- Care should be taken to omit any buffer components incompatible with liquid chromatography, notably detergents. Many assay buffers now contain detergents like Triton X-100. However, many detergents interfere with proper column function, and should be omitted from the assay buffer.
- For more quantitative measurements, an internal standard can be added to the system to calibrate retention times and analyte concentrations. Samples can be spiked with internal standards for more rigorous quantification. If this strategy is performed, ensure that the internal standard is itself inert and its retention time does not interfere with the analyte.
- Compounds can be analyzed for stability at multiple time points. The minimum time should correspond to the length a test compound is exposed to assay buffer in the parent assay, though as a conservative measure we usually test compound stability for longer time periods.
- An advantage of monitoring HPLC by MS in addition to UV-Vis is the potential to identify the chemical structure of detected degradation products.

- Systems with a calibrated autosampler are ideal, as they allow precise injection volumes.

Assessing compound stability by NMR and other methods

Stability can also be assessed by relatively straightforward NMR experiments. Compounds undergoing chemical or conformational changes as a function of time in assay media may exhibit detectable changes via NMR. However, downsides to this method can be that the high compound concentrations required for NMR detection might not be achievable with lipophilic test compounds, and that deuterated buffer components may be required.

Assessment of Compound Reactivity with Known Nucleophiles

Potential lead compounds should be tested for evidence of reactivity with known biological nucleophiles. Many biologically-relevant nucleophiles are thiols, such as glutathione, coenzyme A, and protein cysteines. There are a variety of thiol-based nucleophilic probes available to test compounds for chemical reactivity, each with potential advantages and disadvantages (Table 5).

Table 5: Examples of nucleophilic probes to detect electrophilic compounds. Shown are the chemical structures of the various nucleophilic probes, select advantages and disadvantages of each test method, and citations to published examples.

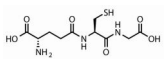
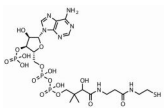
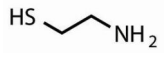
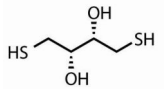
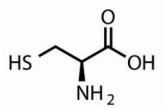
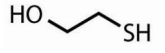
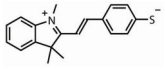
Probe	Structure	Potential advantages and formats	Potential disadvantages	References
Glutathione (GSH)		Inexpensive, HPLC-based, fluorescence-based	Non-proteinaceous	(25, 68)
Coenzyme A (CoA)		HPLC-based	More expensive than GSH, higher MW, non-proteinaceous	(25, 69)
Cysteamine		Inexpensive, HPLC-based	Non-proteinaceous	(70)
Dithiothreotol (DTT)		Inexpensive, HPLC-based	Short aqueous half-life, non-proteinaceous	(71)
L-cysteine		Inexpensive, HPLC-based	Non-proteinaceous	–
β-mercapto-ethanol		Inexpensive, HPLC-based	Odor, Non-proteinaceous	–
MTSI		High-throughput capability, fluorescence-based, HPLC-based	Oxygen-sensitive, self-dimerization, non-proteinaceous	(72)
Peptides	–	Protein-like, HPLC-based	Non-proteinaceous?	(16)
Proteins	–	Can perform tryptic digest to determine specific adduct sites, LC-based	Low-throughput, protein requirements, instrumentation requirements, not every peptide/adduct ionizable	(25, 73)
ALARM NMR	–	Protein-based, provides information about conformation changes (non-specific binding)	Expensive (requires non-commercially-available protein), Instrumentation requirements	(25, 44, 74)

Table 5 continued from previous page.

Probe	Structure	Potential advantages and formats	Potential disadvantages	References
ALARM MS	–	Protein-based, provides information about adduct structure (*m/z*)	Instrumentation requirements, not every adduct ionizable/stable	(44)

Chromatography-based methods to identify probe-compound adducts

The most straightforward methods to quantifying the reactivity of test compounds with nucleophilic probes involve incubating the test compound with the nucleophilic probe under assay-like conditions, and injecting aliquots into an LC-MS system to look for direct compound-thiol adducts (25, 68, 69). This experimental protocol is highly suited to small-molecule thiol probes including GSH, cysteamine, CoA, and even certain peptides. The disadvantages of these protocols can include the instrumentation required, the potential instability of some adducts to certain chromatographic methods, and the possibility that test compounds may react at different rates with proteinaceous nucleophiles than with the nucleophilic probe employed.

Sample steps | HPLC adduct counter-screen

1. Prepare stock solutions of compound, thiol probes, and assay buffers.
2. Dilute an aliquot of test compound in an inert solvent. Run HPLC column to identify parent compound retention time.
3. (Optional) Repeat step 2 for thiol probe. Note certain thiol probes may not be detectable by certain detection modalities due to small molecular weight, poor light absorption or poor ionization.
4. Titrate concentration of compounds and thiol probes and injection volumes to obtain adequate readout intensity without over-saturating the detector(s) employed.
5. Run HPLC column of assay buffer to ensure solvent-instrument compatibility.
6. (Optional) Repeat steps 2-5 as needed to verify consistency of retention time and readout intensity. Optimize instrument settings such as injection volume, buffer gradients, columns, monitoring wavelength (if UV-Vis detection method), ionization settings (if MS detection method), etc.
7. Incubate test compound and thiol probe in assay buffer.
8. Run HPLC column of a solution aliquot from step 7.
9. Analyze data for evidence of detectable compound-thiol adducts.

Researchers should consider the following technical points:

- Compounds are usually prepared as 10 mM stock solutions in DMSO. Thiol probes can be prepared as 1 M stock solutions in either water or assay buffer, ideally freshly to mitigate thiol oxidation.
- The ratio of compound to thiol probe can be adjusted, depending on the reactivity of the test compound and the specific experimental question. Usually, this thiol:compound ratio can begin at 1:1 or 2:1. To qualitatively assess whether a test compound can form a detectable adduct, the thiol:compound ratio can be increased.
- Similarly, the length of time and temperature that test compounds and thiols can be allowed to incubate together can vary depending on the specific experimental question. At a minimum, this incubation length and temperature should model the parent assay conditions. Increasing the time and temperature may allow for a greater formation for certain adducts.
- An advantage of monitoring HPLC by MS in addition to UV-Vis is the potential to identify the chemical structure of detected degradation products.
- Systems with a calibrated autosampler are ideal, as they allow precise injection volumes.
- Consider the use of control compounds, both positive and negative.

- Internal standards can be included if more rigorous quantification is desired. If used, such standards should not be reactive or co-elute with the test compound or adduct(s).
- For unstable compounds, consider the possibility of reactive degradation products.
- Note that some adducts may not be stable to certain HPLC conditions, giving misleading results.
- Similarly, certain adducts may be difficult to detect by certain detection modalities. Certain adducts may not be stable to specific MS methods, while certain adducts without a conjugated bond system may be relatively UV-lucent.
- Interpret negative results with healthy skepticism. While a very promising sign in most cases, the failure to detect compound-thiol probe adducts by HPLC or similar methods does not definitively exclude non-specific thiol reactivity.

Fluorescence-based methods to identify adducts

Thiol reactivity can also be assessed with fluorescence-based methods. For example, compounds that react with the probe MTSI produce a change in fluorescence (72). A similar, fluorescence-based method involves a competition-based assay where the compound competes with GSH for a maleimide-based probe (75). We recently described a similar method using CoA and the thiol-sensitive maleimide probe CPM (25). A disadvantage of these methods is that compounds can interfere with the fluorescent readout by a variety of pathways. Nucleophilic compounds can react with the maleimide-based detection component. Fluorescent or fluorescence-absorbing compounds can also interfere with the assay readout.

Sample steps | CoA/GSH-CPM counter-screen

1. Prepare stock solutions of compound, thiol probes, and assay buffers.
2. Add test compounds to experimental plate (96- or 384-well).
3. Add vehicle controls to experimental plate. These wells will be used for controls.
4. Add thiol source to appropriate wells. Mix.
5. Incubate plate from step 4 for > 10 min at 25-37 °C.
6. Add CPM to appropriate wells. Mix.
7. Incubate plate from step 6 for 10 min.
8. Read fluorescence intensity from the experimental plate (λ_{ex} 405 nm, λ_{em} 530 nm).
9. Analyze data. Compare fluorescence intensity of test compound wells with vehicle control wells.
10. Check putative thiol-reactive compounds for fluorescence quenching (25).

Researchers should consider the following technical points:

- Positive and negative controls are necessary. Several positive reaction controls should be included (vehicle controls + assay buffer + thiol source + CPM). Likewise, several negative reaction controls should be included on the same plate (vehicle controls + assay buffer + thiol source; vehicle controls + assay buffer + CPM).
- Positive and negative thiol-reactive control compounds are also recommended.
- A reduction in fluorescence intensity suggests compound-CPM reactivity and/or fluorescence quenching. An increase in fluorescence intensity suggests compound autofluorescence, or the creation of a fluorescent compound-reagent adduct.
- One can confirm fluorescence quenching by determining the absorption and the excitation/emission spectra of putative quenching compounds.
- Compounds are usually prepared as 10 mM stock solutions in DMSO. Thiol probes can be prepared as 1 M stock solutions in either water or assay buffer, ideally freshly to mitigate thiol oxidation. CPM can be prepared as a 1:1 MeOH:H_2O solution.

- More specifics about stock solutions, concentrations, reagent ratios, dispensing volumes and dispensing steps are described in detail elsewhere (25, 69). Other modified versions of this assay are also described (75, 76).
- The length of time and temperature that test compounds and thiols can be allowed to incubate together can vary depending on the specific experimental question.
- The threshold for thiol-activity inevitably depends on the project. Evidence of a dose-response and significant deviation from vehicle controls (e.g. >20%) baseline values should be flagged for potential triage or follow-up studies.
- Ideally, test compounds should be tested at several concentrations. We typically test between 0.1-100 μM compound concentrations in triplicate, with several intermediate test points. The number of replicates, dose-response points and test concentrations should be guided by the experimental question and project testing conditions.
- This method can be adapted to CoA or GSH as thiol sources. In principle, this method can be adapted for different fluorescence-based maleimide probes besides CPM.

Other methods

More advanced methods for detecting thiol-reactive screening compounds can involve the use of protein and protein-like (peptide) probes (16). These methods can involve MS or NMR detection methods. MS methods can involve the direct detection of the adduct ion. This can be done with potentially-reactive peptide probes or can involve the native proteins employed in the assay (77). These proteins can be run on a LC system and ionized whole using a LC-MS format. Alternatively, they can be subjected to tryptic-digestion and subjected to LC-MS-MS analysis. The advantages of this latter experiment include information about the specific residue(s) modified. A disadvantage of this approach is that not every peptide is equally prone to ionization, and the amount of time, resources and instrumentation can be prohibitive.

Assessment of Compound Reactivity with ALARM NMR/MS

The nucleophilicity of cysteine thiols can have significantly different reactivity profiles compared to small-molecule thiols. Therefore, it is possible that certain compounds may not form adducts with GSH, but can react with protein cysteine residues. One solution to identify protein-reactive compounds is ALARM NMR (Figure 7). This technology, developed by Huth and colleagues at Abbott, was adapted after an NMR-based screen to detect small-molecule modulators of the La antigen identified mostly thiol-reactive compounds (44, 74). The La antigen has two cysteine residues, C232 and C245, which are prone to react with electrophilic screening compounds. The formation of covalent adducts between the cysteine residues on the La antigen and test compounds leads to perturbations in several residues, namely L249, L294, and L296. ALARM NMR-positive compounds show characteristic peak shifts and signal attenuation for these particular residues. The inclusion of excess DTT in an otherwise identical reaction is used as a control for non-specific protein perturbations, as electrophilic compounds should react with the excess DTT and spare the protein cysteine residues. ALARM NMR can also detect protein denaturants. This is typically evidenced by complete loss of the characteristic signals for the ^{13}C-labeled amino acids (44). The protein is enriched with ^{13}C by including the labeled amino acid precursors during the protein synthesis. In our hands, this assay is medium throughput and robust. Throughput can be increased with the use of an autosampler, a cryoprobe, efficient ^{13}C-enrichment and optimized pulse sequences. However, unanswered questions about the method include its sensitivity and how to best quantify the results (especially for equivocal findings), answers that may unfold with additional studies.

This method has proven useful in our hands to detect thiol-reactive screening compounds (25). Benefits of this method include its NMR-based readout, which is completely orthogonal to either MS or fluorescent readouts used in other assays. This method also allows for the identification of promiscuous binders and possibly redox-active compounds, as these compounds would perturb the conformation of the La protein in both the presence and absence of DTT. The method is not without its drawbacks, namely the instrumentation required, the

expense of labeled amino acid precursors and the comparatively higher concentrations of protein and compound required.

ALARM NMR has also been adapted to ALARM MS. The advantages of this approach include the lack of any ^{13}C-labels as well as spectrometric information about compound-protein adducts, as the m/z shift resulting from compound-protein adducts can provide clues about the structural nature of the presumed adducts. Potential disadvantages of this approach include the requirement for protein-compatible LC-MS instrumentation and the possibility of compound-protein adduct instability in the LC-MS conditions.

Structure-Activity Versus Structure-Interference Relationships

The diagnostic importance of SAR in hit compound series has been a point of emphasis in recent literature (78). Closely related analogues of hits (10-25 compounds) prepared by synthetic chemistry should show > 10-fold difference in measured IC_{50} values. This exercise can provide confidence along three important lines of evidence that can make or break a compound series: 1) that the activity of the hit is real, 2) that analogues of a hit can be prepared, and 3) that the series has the potential for optimization.

What appear to be structure-activity relationships (SAR) may actually be structure-interference relationships (SIR). Therefore, the term SAR should be employed to describe 'activity' differences between compounds that are known to be non-reactive or have had their target confirmed. Structure-interference relationships can be observed in a compound series, but are typically related to reactivity differences between series members. For example, true SAR in a series for CDK2 inhibitors was confirmed by activity measurements with x-ray crystallography to demonstrate target engagement (79, 80). On the other hand, we have presented several examples of SIR in the context of HTS, in which structural modifications lead to apparent activity changes, but useful or specific target engagement could not be confirmed (25).

Orthogonal Assays

Employing orthogonal assays is highly recommended (if not mandatory), in part because reactive compounds can give misleading assay readouts. In some cases, when assays are interrogated by an orthogonal detection method, this apparent activity is lost. A straightforward example is the CPM-based thiol-scavenging assay described previously to indirectly quantify histone acetyltransferase (HAT) activity. In the HAT reaction, an acetyl group from acetyl-CoA is enzymatically transferred to a histone substrate, forming an acetylated histone and free CoA. In this assay format, electrophilic screening compounds can react with free thiols to give a false-positive readout (25, 69). Orthogonal assays that measured the actual acetylated histone products (a Western blot assay and a [^3H]-acetyl-CoA HAT assay) were critical in identifying thiol-trapping artifacts. Of course, other assay methods may also be susceptible to reactive interference. Therefore, one strategy to mitigate the selection of reactive interference compounds is the use of orthogonal assays.

Counter-Screens

Non-specific chemical reactivity can also be investigated by performing counter-screens versus related and unrelated targets.

An extremely important, yet usually simple measure to take is to run a counter-screen. This can take place either in parallel during a screening campaign or immediately afterwards once actives are identified. While this will be case-specific for each assay technology, a robust counter-screen typically involves testing the actives in the same type of assay but against a different protein that would not normally be expected to bind the same compound. Actives that hit both assays are likely to be promiscuous or to be interfering with the physics of assay signaling in some way. Care must be taken, however, as any given active can appear to be selective for a biological target over an individual counter-screen, but this is not conclusive proof that the compound will not reveal a reactivity profile when tested against a wider range of targets. Testing in a broad range of assays and proteins is therefore

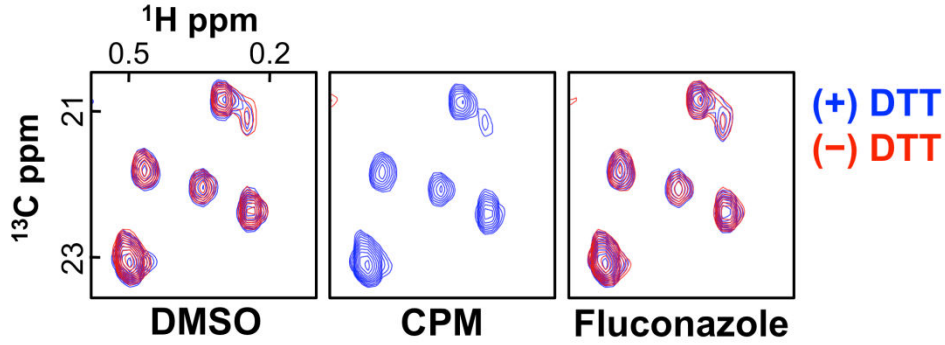


Figure 7: Demonstration of ALARM NMR to assess the thiol reactivity of screening compounds. Compounds are incubated with the ^{13}C-enriched La antigen in either the presence or absence of 20 mM DTT. Shown are the peaks for L249, L294, and L296. Signals were normalized to DMSO controls. Fluconazole is included as a negative thiol-reactive control compound. CPM, a thiol-sensitive maleimide probe, is shown as a positive control compound. Thiol-reactive compounds can cause peak shifts and/or signal attenuation in the absence of DTT, and this effect is significantly attenuated if DTT is included in the reaction mixture.

recommended (see below). It is hard to be definitive, but as a compound is optimized towards higher affinity, clear SAR should be revealed for the target of interest concomitant with increased selectivity over any other off-target activity that might have been observed in the screening hit.

Beyond these relatively straightforward counter-screens to detect highly promiscuous substances, compounds should also be shown to be selective against structurally homologous targets (isoforms). These assays should be routinely implemented in-house as they will frequently be used to prioritize hit compounds. Compounds that have been even further refined by optimization should be assayed against targets in the same class (e.g GPCRs, proteases, kinases, etc.) and, finally, a broad range of targets typically surveyed to provide evidence for lack of off-target toxicity. These assays are typically outsourced to contract research organizations and should be reserved for representative compounds selected from the best series of compounds. Broad activity in these assays may be indicative of non-selective reactivity.

Section Summary

Non-specific reactive compounds are a significant risk in early drug discovery and development. In certain cases, irreversible, covalent inhibition is desired. However, in most cases, reactive compounds are not initially sought. In this section, we have highlighted several experimental strategies to identify reactive compounds, including kinetic/mechanistic experiments and tools to specifically identify compound-thiol adducts. This list of experimental approaches is by no means exhaustive. Promising compounds arising from both virtual and real HTS campaigns should be assessed for chemical reactivity. We recommend testing for reactivity even in cases when the compounds of interest are considered benign. This de-risks the compound for later, more resource-intensive experiments.

Conclusions

Assay readouts are subject to various modes of interference, including chemical reactivity by test compounds. Reactive screening compounds often possess non-specific bioactivity and/or misleading readouts. As such, failure to triage these undesired, non-specific reactive entities can lead to wasted resources and also missed opportunities to pursue more tractable chemical matter with higher chances of downstream project success. Those who tout a compound as a chemical probe or possessing useful biological activity should demonstrate its lack of reactivity interference using a variety of methods (Table 6). Utilizing a two-pronged strategy of knowledge-based and experimental-based methods should enhance the likelihood of identifying reactive nuisance compounds. Within each of these two arms, researchers should aim to perform multiple – and in some

cases redundant – experiments to mitigate the risk of pursuing intractable reactive compounds and enhance confidence in biochemically (and therapeutically) useful modes of bioactivity and target engagement.

Table 6: Checklist for de-risking reactive compounds.

Cheminformatics
Have the screening hits been filtered for known reactive functional groups?
Have the screening hits been filtered for PAINS?
Have potential lead compounds been reported as active in other assays, patents or scientific papers? How have similar compounds behaved?
Reactive compounds
Have potential lead compounds been tested versus at least one nucleophilic probe?
Have potential lead compounds been tested by well-established kinetic experiments for reversibility?
Redox-active compounds
Has the assay system being tested for susceptibility to H_2O_2 and redox-active compounds?
Have potential lead compounds been de-risked with respect to redox-activity?
Impurities
Have potential lead compounds been tested by HPLC-purified and characterized commercial samples?
Have potential lead compounds been tested by HPLC-purified and characterized independently using re-synthesized samples?

Suggested Websites

1. SciFinder Scholar (http://www.cas.org/products/scifinder). Excellent resource for searching scientific and patent literature by chemical structure. It is highly recommended to search potential lead compounds and structural analogs for literature precedent.
2. PubChem (http://pubchem.ncbi.nlm.nih.gov/). An excellent open-access resource containing data from NIH-funded chemical screens. It is highly recommended to search potential lead compounds and structural analogs for their performance in both related and unrelated assays.
3. BADAPPLE (http://pasilla.health.unm.edu/tomcat/badapple/badapple). An open-access resource for assessing bioassay promiscuity, which can be used as a supplement to searching PubChem.
4. FAF-Drugs3 (http://fafdrugs3.mti.univ-paris-diderot.fr/). An excellent, free site for filtering relatively small sets of compounds.

Suggested Readings (alphabetical order)

1. Baell, J. B.; Holloway, G. A. J. Med. Chem. 2010, 53, 2719.
   Describes in detail the concept of pan-assay interference compounds. Many of these substructures are likely thiol-reactive. A must-read for any researchers engaged in early drug discovery and HTS.
2. Copeland, R. A. Evaluation of enzyme inhibitors in drug discovery: a guide for medicinal chemists and pharmacologists; New York: Wiley-Interscience, 2005.
   An excellent text describing kinetic and mechanistic experiments to elucidate the mechanism of action of test compounds. Many of the described experiments and approaches can be adapted to identify reactive compounds.
3. Dahlin, J. L.; Nissink, J. W. M.; Strasser, J. M.; Francis, S.; Zhou, H.; Zhang, Z.; Walters, M. A. J. Med. Chem. 2015, 58, 2091.
   Describes in detail multiple experimental strategies to identify thiol-reactive compounds. Illustrative of

the havoc that chemical reactivity can have on an HTS campaign if reactive compounds are not identified, and how apparent structure-activity relationships can actually be explained as structure-interference relationships.

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   Introduces ALARM NMR, a protein-based NMR assay to identify thiol-reactive compounds. Provides data suggesting differential reactivity between glutathione and a protein probe (La antigen).
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   Nicely describes a novel thiol-based fluorescent probe to identify reactive screening compounds adaptable to a high-throughput format.
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   Describes the prevalence of redox-active compounds in the context of a large-scale HTS, along with detailed protocols describing the HRP-PR assay.
7. Thorne, N.; Auld, D. S.; Inglese, J. Curr. Opin. Chem. Biol. 2010, 14, 315.
   Summarizes many of the common sources of compound-dependent assay interference encountered in HTS.
8. Walters, W. P.; Namchuk, M. Nat. Rev. Drug Disc. 2003, 2, 259.
   Describes the use of Rapid Elimination Of Swill (REOS) cheminformatics filters.

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Interference with Fluorescence and Absorbance

Anton Simeonov[1] and Mindy I. Davis[2]

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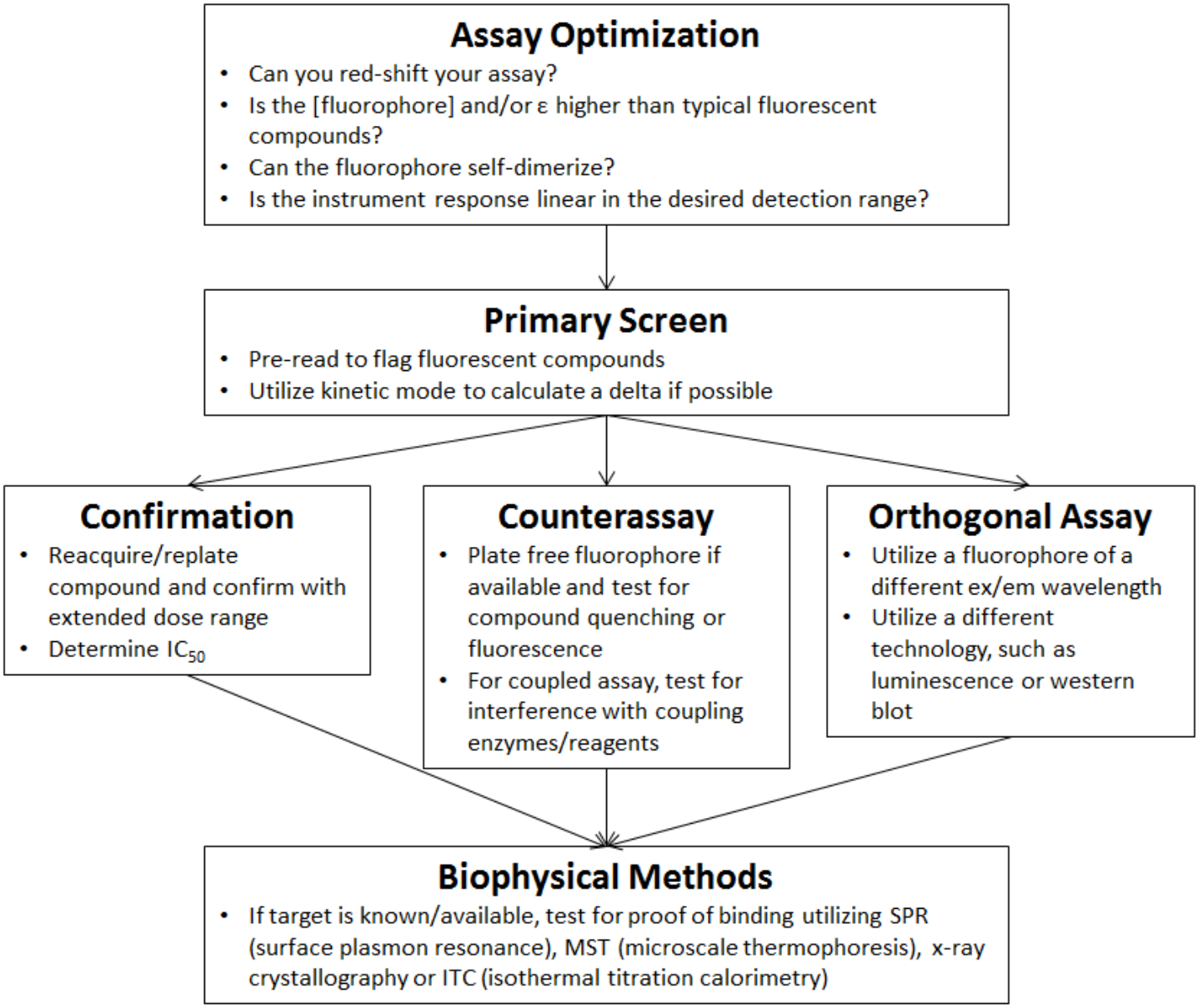
Abstract

Assays, both high-throughput and lower throughput, are an integral part of the drug discovery process and are used for identifying and validating potential drug candidates. Many biochemical and cell-based assays utilize fluorescence and to a lesser extent absorbance readouts so it is important to consider the impact on the assay quality of a given detection method. There are many fluorophores available that span a wide energy spectrum, and it is important to select the appropriate fluorophore and assay conditions for a given assay to minimize assay interference. Additionally, many of the small molecules found in libraries used for screening are themselves fluorescent, leading to potential false positives through interference. Similarly, for absorbance assays, colored compounds can interfere with the detection method depending on the concentration and extinction coefficient. Assays can be designed to minimize the interference from the library itself, and counterassays can be utilized to identify potential compounds that interfere with the detection method. Orthogonal assays using a different detection method can also be employed to further validate HTS hits. It is important to weed out assay artifacts as early as possible in the drug discovery process to avoid spending time and resources pursuing compounds that are not actually impacting the desired biology but rather are false positives.

Author Affiliations: 1 National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, Maryland, USA. 2 National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, Maryland, USA.

Corresponding author.

Flow Chart



Fluorescence Interferences

Typical Fluorophores used in High-throughput Screening

Fluorescence is the process by which a molecule absorbs a photon of light exciting an electron to a higher energy state. This electron often relaxes down to the lowest vibrational level of the excited state and then a photon of a longer wavelength (lower energy) is emitted. This shift in energy of the emitted photon relative to excited photon is called a Stokes Shift. Exceptions to this decrease in energy of the emission relative to the excitation can be found in the case of multiphoton excitation, which is beyond the scope of this chapter (1). Fluorescence has been a workhorse for many years in the design of assays for HTS. Examples include fluorescent enzyme substrates or products, coupled assays, fluorescence polarization assays, tracer assays and FRET ratio assays, where in many of these systems detection can be performed in either endpoint or kinetic mode. There is a veritable rainbow of fluorophores described that can be used in assay design (Figure 1). Table 1 shows eight representative

fluorophores commonly used in assays and their excitation (ex) and emission (em) wavelengths. It should be noted that the fluorophores are often not excited at the peak of the excitation spectrum but rather to higher energy to avoid overlap with the emission peak and likewise the emission peak is often detected off of the maximum at lower energy to avoid any bleedover from the excitation peak. There are many spectral viewers and tables of fluorophores available online that can be consulted to select the appropriate wavelength for excitation and emission for a given fluorophore (ex: http://searchlight.semrock.com/ and http://www.lifetechnologies.com/us/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html and http://aatbio.com/p3400.html). Additional details on particular fluorophore families can be found in two reviews (2, 3). In addition to using fluorescent molecules in biochemical assays, small molecule fluorophores, such as DAPI/Hoechst dyes, and fluorescent proteins such as GFP (green fluorescent protein), are frequently used in cell-based assay formats. For a given assay design, it is important to recognize the possible sources of false positives due to fluorescence interference as well as false negatives to minimize the cost in time and money for following up on assay artifacts or the cost for missing potential hits, respectively.

It is important to note that fluorophores can have highly conjugated planar systems that can cause issues in assay designs. For example, rhodamine 110 (ex= 497 nm, em= 520 nm), which is planar and has a low net charge, can form dimers, which are detected by the appearance of a higher energy peak (4). Some of the Alexa dyes have higher charge, which can limit the formation of these dimers. Dimer formation results in a decreased assay performance due to self-quenching of the fluorescence, spurious changes in fluorescence polarization as a function of probe concentration, and solubility issues. When a new peptide labeled with rhodamine is ordered, the spectrum can be measured +/- 20% acetonitrile, a condition which dissolves the dimers and should lead to a spectral shift if dimers are present (4). This effect of dimerization is also potentially problematic with some of the Bodipy dyes, such as Texas Red. Dye pairs with two rhodamines can also be used to develop assay systems for studying protein-peptide interactions, in which an increase in fluorescence upon disruption of the dimer is monitored (4).

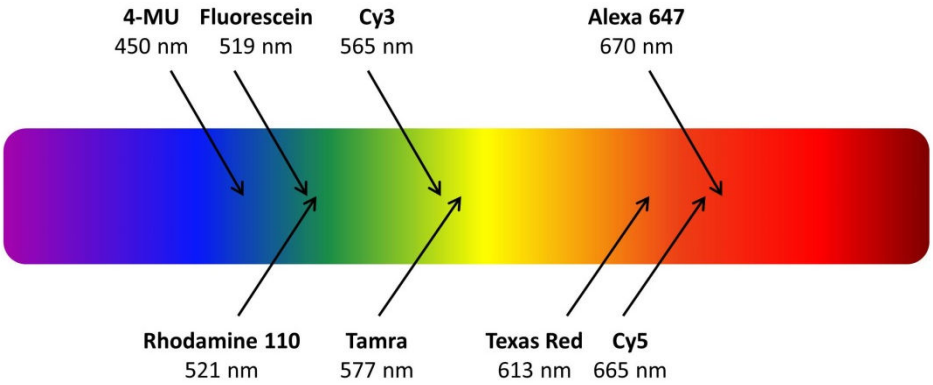


Figure 1: Fluorophores are available that span the UV and visible spectrum. Representative commonly used fluorophores are shown with their emission wavelengths indicated.

Table 1: Commonly Utilized Fluorescent Molecules and their Excitation and Emission Wavelengths.

Fluorophore	Excitation (nm)	Emission (nm)
4-MU[a]	356	448
Rhodamine 110[b]	497	521
Fluorescein, FITC, Alexa 488[b]	492	519
Tamra[c]	552	577
Cy3[b]	554	565
Texas Red[c]	594	613
Alexa 647[b]	650	670
Cy5[b]	648	665

Note: the superscript letters after the name of the fluorophore indicate the source of the excitation and emission wavelength information. a = AAT Bioquest, b = Semrock and c = Invitrogen.

Fluorescence Properties of Small Molecules

The features found in fluorophores, such as conjugated aromatic systems, can also be present in purchased and synthesized compound libraries. Just as luciferase reporter assays could have a hit list overpopulated by luciferase inhibitors, fluorescence assays can have a hit list overpopulated with compounds fluorescent in the region interrogated by the assay (Figure 2) (5, 6). These compounds will likely lead to dose-dependent interference and can thereby mimic a true active in the assay. The majority of assays used in HTS use plate readers with broad band filters. These broad band filters increase the likelihood that a fluorescent compound will interfere with the assay but have the benefit of allowing the same filter set to be used for fluorophores with nearby but not identical excitation and emission.

There are two main mechanisms by which a compound can directly interfere with a fluorescent assay: quenching and autofluorescence. A compound can absorb light itself and through a Beer's Law effect ($A=\varepsilon cl$) attenuate the intensity of, i.e., quench, the excitation or emission light from the assay (where A= absorbance, ε= extinction coefficient, C= concentration and l= pathlength). The unwanted interception of the light entering the assay system or leaving the assay system will affect the readout and could lead to false positives or negatives depending on the particular assay design. This effect is called the inner filter effect and is an intrinsic property of a

molecule. The efficiency of quenching is proportional to the extinction coefficient of the molecule and its concentration in the assay.

The second main mechanism is autofluorescence as some compounds found in libraries are themselves fluorescent. If the compound fluorescence overlaps with the region of detection in the assay, the compound can cause interference. The extent of interference will depend on the concentration of the fluorescent molecule in the assay and the concentration of the test compound and their relative fluorescence intensities in the assay conditions. An important consideration is quantum yield φ = (# of photons emitted)/(# of photons absorbed). Additionally, a compound that is very fluorescent can cause blow out of the signal in adjacent wells, sometimes referred to as a halo effect. Accordingly, fluorescence assays are usually run in black plates, which absorb some of the scattered fluorescence and limit scattering to adjacent wells. For a very weak fluorescent assay, like some HTRF (Homogeneous Time Resolved Fluorescence, Cisbio, http://www.cisbio.com/usa/drug-discovery/htrf-technology) assays, white assay plates can be used, such as was used for studying the glucokinase (GCK) and glucokinase regulatory protein (GKRP) interaction (7).

It is important to note that just because a compound is fluorescent or a quencher does not mean that it cannot also have important relevant biological activity. It just means that having an orthogonal method to confirm the activity that would not be subject to fluorescence interference is increasingly important. Examples of successful drugs that are fluorescent include the antracycline antibiotics doxorubicin, daunorubicin and idarubicin (8).

A detailed fluorescence analysis spanning a 4-log range was conducted on a large chemical library and Table 2 shows the percent of the library that was fluorescent in the different spectral regions at concentrations of test compounds relevant for typical HTS campaigns (6). About 5% of the library was more fluorescent than 10 nM of the UV-active fluorophore 4-methylumbellerone (i.e., 7-hydroxy-4-methylcoumarin), but by red-shifting the spectral window this rate of interference dramatically dropped (6). In a separate larger library screen, a similar % of the library (5.6%) was found to be auto-fluorescent in the blue spectral region (9). For three assays that utilized readouts in the blue-fluorescent spectral region, blue-fluorescent compounds, which make up just 5% of the tested compounds in the library made up nearly 50% of the identified actives, but when assays employing red-shifted readouts were utilized, the % representation of the blue-fluorescent compounds more closely mirrored their library composition (Figure 2) (6).

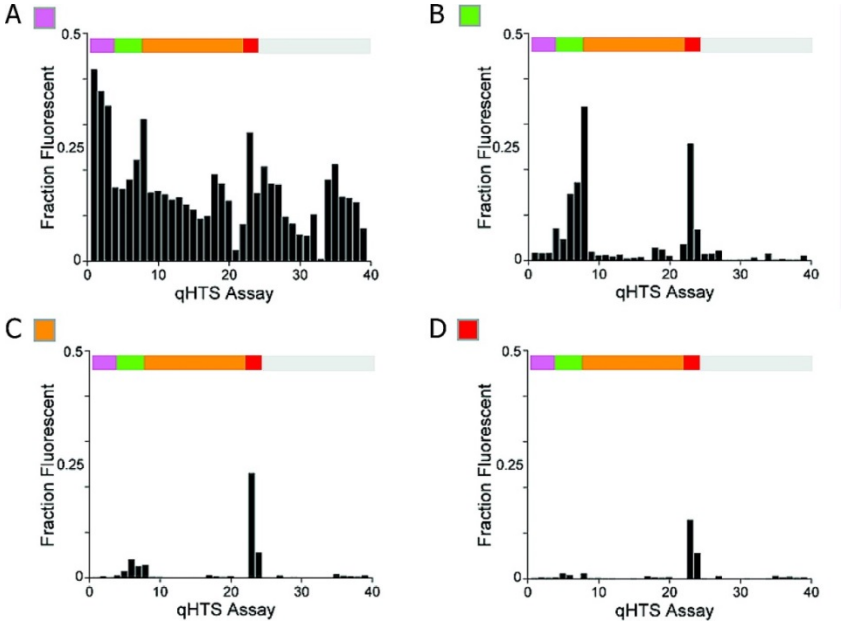


Figure 2: Enrichment of fluorescent compounds among the actives in wavelength-relevant regions. A fluorescence profile across 39 qHTS assays is shown, where the fraction of fluorescent compounds out of all actives identified in each screen is plotted for the library members fluorescent in 4-MU (A), fluorescein (B), rhodamine (C), and Texas Red (D) spectral regions. Assays are ordered by the spectral region being measured, ranging from blue (assay 1) to red (assay 24), and assays considered to be wavelength-neutral (assays 25–39, gray overhead bar) Reprinted with permission from (6). Copyright 2008 American Chemical Society.

Table 2: Number and Percentage of Compounds (out of 71 391 Tested) Displaying Fluorescence Equivalents Equal to or Exceeding 100 and 10 nM Standards. Reprinted with permission from (6). Copyright 2008 American Chemical Society.

Fluorophore standard	>10 nM	% of library	>100 nM	% of library
4-MU	3498	4.90	1306	1.83
AlexaFluor 350	3643	5.10	138	1.92
AlexaFluor 488	23	0.03	7	0.01
fluorescein	119	0.17	31	0.04
resorufin	7	0.01	3	0.004
rhodamine	7	0.01	3	0.004
Texas Red	8	0.01	1	0.001
AlexaFluor 647	0	0	0	0

Assay Design Strategies for Common Fluorescence-based HTS Assays to Minimize the Frequency of and Detect Fluorescence Interference

Fluorescent assays are widely used in HTS and direct fluorescence, coupled assays, fluorescence polarization, TR-FRET and DSF will be discussed. A common type of assay includes the consumption or generation of a fluorescent molecule. An example would be IDH1 (isocitrate dehydrogenase 1, an enzyme in the citric acid (TCA) cycle), which utilizes NADP and generates the fluorescent product NADPH, an example of an endogenous fluorophore (10). If the substrates or products of the enzyme reaction of interest are not endogenously fluorescent, a pro-fluorescent substrate can be used that only becomes fluorescent upon enzyme activity. For example, Ubiquitin-rhodamine is frequently used to assess the activity of deubiquitinases, which cleave off the Rhodamine fluorophore leading to an increase in fluorescence (11). These types of assays can be

run in either endpoint or kinetic mode, which will be described in more detail below. Using a standard curve, the amount of fluorophore present at the end of the reaction can be calculated and used to design a counterassay that can detect both autofluorescence and quenchers. The amount of fluorescent substrate and/or products is plated in assay buffer and the effect of compound on the observed fluorescence is measured.

In a typical multistep enzyme reaction, compound is added to enzyme and then after incubation, the reaction is started by the addition of one or more substrates. Prior to initiation of the reaction, a pre-read could be done to assess the intrinsic fluorescence of the added compound. If the assay is run in qHTS format (12) then an EC_{50} of the fluorescence can be obtained and compared to the IC_{50} of the reaction. When using a CCD plate reader, such as the Viewlux (PerkinElmer), this pre-read can be very fast and would not impact the overall speed of a large HTS campaign.

Because compound libraries tend to have greater interference in the blue-green spectral region, designing an assay with a red-shifted readout (beyond 500 nm) can dramatically limit the interference from a library and be a viable option as long as the detector used has effective and sensitive filters for the region of interest (6). This type of strategy has been used successfully for dehydrogenases that produce or use NADPH (ex= 340, em= 435 nm) or NADH, which in turn are substrates for diaphorase, an enzyme that converts resazurin to the fluorescent product resorufin (ex= 530, em= 590 nm) (10). This type of coupled assay can be run in kinetic mode if the product of the enzyme reaction is NADPH or NADH or in endpoint mode if the substrate is NADH or NADPH. The benefits of kinetic mode, when applicable, are described below. As mentioned above, care should be taken not to redshift to a fluorophore that will form dimers under the conditions of your assay (for example, rhodamine).

In some direct fluorescence assay designs, the concentration of the fluorophore is very high in the assay, which will decrease the likelihood of significant interference from the compound library. An example of utilization of a high concentration of fluorophore is an assay for glucocerebrocidase, a target for the treatment of Gaucher disease, in which the Km of the pro-fluorescent substrate was very high so its concentration used in the assay was high and the effect of compound fluorescence was negligible (13). This is an example of assay strength where even at low % conversion (5%), the amount of fluorophore generated is high (30 μM) so there was no observed interference from the compound library even though this assay was in the near UV region. This drop off in interference as the concentration of fluorophore increases is recapitulated in the results shown in Table 2. Additionally, in this case the authors developed two assays that used fluorophores from different spectral regions and interleaved the assays, which can further screen out compounds that interfere with a unique part of the spectral region.

Importantly, rather than using endpoint mode, kinetic mode can be used in which a change in fluorescence readout over time is measured. In most cases, the fluorescence of the test compound will not change in the assay over time so it will be subtracted out. Important exceptions to this would be fluorophores that photobleach in which case the fluorescence would decrease overtime due to exposure to the excitation light and, likewise, compounds that are exceedingly fluorescent can lead to imperfect subtraction. Kinetic mode is a very powerful method to eliminate many assay artifacts including subtle differences in initial dispense volumes (see Figure 3). For the cysteine protease cruzain, the use of kinetic mode decreased the number of compounds detected as hits due solely to detection interference (9). Additionally, a delta readout can increase the signal-to-background of an assay. For an assay for TGR (thioredoxin glutathionereductase) found in *Schistosoma mansoni* parasites, the signal to background was only 2.1 in endpoint mode but by utilizing kinetic mode a robust assay with a Z'= 0.76 was developed (14). For very fast reactions, the practical implementation of kinetic mode can be challenging due to the finite speed of dispensing reagent, transferring a plate to the reader and reading the plate. By utilizing a plate with a CCD detector, such as the Viewlux, all wells can be detected simultaneously and reactions as short as 5 minutes are feasible by leaving the plate in the detector and collecting readouts in continuous mode every 20-30 seconds. For slower reactions, the readouts can be done in discontinuous mode where after the first read

the plate is removed and incubated outside of the plate reader and then reloaded and read at a later time or times. The logistics of these different types of kinetic mode have been described (15).

Another common assay format is fluorescence polarization or FP (16). In this assay design the concentration of the fluorophore tracer can be very high which can affect the apparent IC_{50} of the test compound but this can be corrected for with the Kenakin equation as described in (17). Conversely if the concentration of the tracer is very low, such as in a Transcreener assay (BellBrook Labs) that may have tracer in the single digit nM concentration, the assay can be overly sensitive to autofluorescence interferences (even if red-shifted) because the concentrations tested of the library are significantly higher and even the tail of a compound's fluorescence spectrum can overwhelm the tracer signal. For this type of assay, which can be quite prone to interference when the concentration of tracer is quite low, as would be the case for studying a tight-binding pair, in addition to assessing the FP signal, the intensity could be observed and compounds in which the intensity is far greater than the basal would suggest the ability of that compound to interfere with the assay. Just as was done for glucocerebrosidase where two different fluorophores were used in a direct fluorescence assay, this technique was also used in an FP assay for peptidyl prolyl isomerase 1 (Pin1). This FP assay used a peptide tracer labelled with FITC (ex= 480, em= 540 nm) or with TAMRA (ex= 525, em= 598 nm) as described in Pubchem AID 504891 and 624312. Note that these excitation and emission wavelengths are off of the peak maxima for each fluorophore to avoid bleed-over as described above and also to accommodate available filter sets on the Viewlux plate reader. A compound that was a hit in both assays would be less likely to be the result of fluorescence interference. These two assays could be run either sequentially or in an interleaved fashion (18).

Assay designs in which the fluorophore of interest in the assay has a longer half-life, such as the long half-life of lanthanides, can allow the use of a delay during which the fluorescence of the test compound would dissipate as usual fluorescent half-lives are very fast and then the emission of the fluorophore in the assay can be measured after the compound autofluorescence has dissipated (Figure 4). A representative delay would be 50-100 μs. One type of assay that takes advantage of the longer life of lanthanides is the TR-FRET assay (time-resolved fluorescence resonance energy transfer). An example assay design would be for a protein-protein interaction where each protein is tagged with a unique tag such as GST and a 6x-His tag. Antibodies that are directed at these tags can be labeled with a unique fluorophore. One antibody will have what is called a donor fluorophore on it, this fluorophore will be excited by the instrument excitation energy and will transfer non-radiative energy to the acceptor fluorophore (which is not excited at the same wavelength as the donor) on the other antibody if and only if the two fluorophores are in close proximity, i.e., the proteins that they are targeting are close to each other (7). By monitoring not just the acceptor/donor intensity ratio but also the total intensity, compounds that are autofluorescent or quenchers can be flagged. If dose-dependent effects are seen in both the donor and acceptor channels and not just the acceptor channel, it is probable that the compound is interfering with the detection. There is a helpful paper on reducing compound interference in TR-FRET assays (19). There are two frequently used commercial vendors for TR-FRET assays that have a continually expanding array of assay kits available, as well as toolbox components to develop your own assay (HTRF, Cisbio, http://www.cisbio.com/usa/drug-discovery/htrf-technology; Lance, PerkinElmer, http://www.perkinelmer.com/Resources/TechnicalResources/ApplicationSupportKnowledgebase/LANCE/lance.xhtml). The HTRF assay for glucokinase (GCK) and glucokinase regulatory protein (GKRP) interaction is an example that was designed using toolbox reagents targeting the distinct tags on the two proteins (7).

Differential Scanning Fluorimetry (DSF) is another fluorescence-based technique that assesses whether a test compound stabilizes the protein leading to an increase in the melting temperature of the protein, thereby indicating direct binding of the compound to the protein. DSF employs a dye that is sensitive to the hydrophobicity of the environment, with fluorescence increasing with hydrophobicity when the protein melts and such hydrophobic regions are gradually exposed to the solvent, as reviewed in (20). DSF can be miniaturized to 1536 using an instrument such as the Roche Lightcycler enabling this technique to become HTS amenable.

Weak binders often need to be screened at high concentrations that may interfere with the detection in much the same way as described above for the other assay types.

It is important to note that for fluorescence-based assays, particularly in the blue/green spectral region, the presence of dust which is often fluorescent in this region can interfere with assay design. Limiting dust and blowing off any observed dust from plates being used for the assay is important. Additionally, running an assay in qHTS mode makes identification of outliers straightforward.

Absorbance Interferences

For HTS campaigns, fluorescence techniques are far more popular than absorbance based methods. This is due in part to the very short pathlength found in miniaturized assays and the effect of pathlength on the strength of the signal (Beer's law; $A=\varepsilon Cl$), causing miniaturized absorbance-based assays to irretrievably lose signal window. For example, in 1536 at a typical assay volume of 5 μL the pathlength of light is ~0.2-0.25 cm, while in a 384-well plate with a typical assay volume of 40 μL, the pathlength is approximately 0.5 cm. In order for the signal to be robust, either the ε or the concentration, or both, need to be high. Just as fluorescent compounds can interfere with fluorescence assays, colored compounds can interfere with absorbance based assays by the inner filter effect described above. If the ε is known for the compound of interest the effect on absorbance can be calculated from $A=\varepsilon Cl$ (Beer's Law).

An example of a successful miniaturized absorbance assay is DTNB (Ellman's reagent = 5,5'-Dithiobis(2-nitrobenzoic acid)) that uses mM concentrations of chromophore and since most compounds are assayed in the μM range, the assay chromophore dominates the readout and little interference will be observed (21). While there are instances where absorbance assays have been successfully employed, oftentimes it is beneficial to seek out fluorescence alternatives as seen in the following example. TGR (thioredoxin glutathionereductase) found in *Schistosoma mansoni* parasites and PRX2 (peroxiredoxin 2) enzymes are important components of the redox cascade in the parasite. A HTS assay was developed to screen for inhibitors of TGR/PRX2 (14). The initial assay design was developed to monitor the decrease in absorbance of NADPH at 340 nm but the authors found that switching to a fluorescent readout lead to an increase in signal strength and minimized quenching and inner-filter effects from the compounds in the library because many of the small molecules and buffer components absorbed in the 340 nm spectral region (14).

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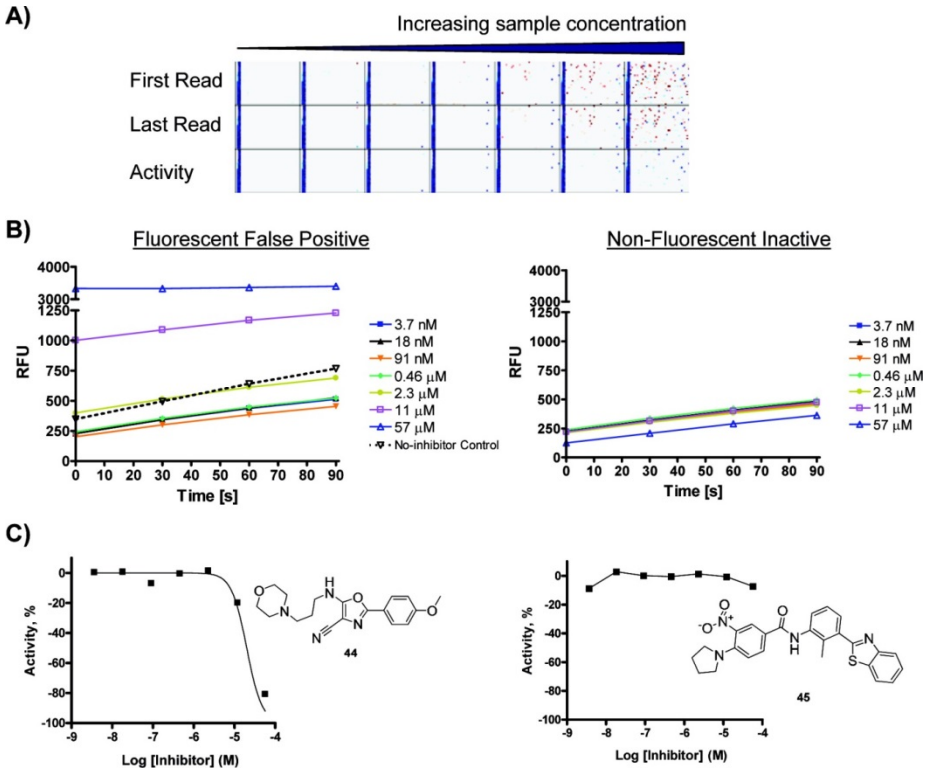


Figure 3: Fluorescence interference analysis. (A) Plate heatmaps associated with one seven-concentration compound series in 1536-well format. Shown are the first and last fluorescent reads and the activity calculated from the 60 s initial rates. Subtracting out the higher-than-average but steady fluorescence of some compounds (large number of red dots on heatmaps associated with first and last reads) leads to the significant reduction of interference (small number of blue dots in the activity heatmap). However, for compound 44, which is highly fluorescent (left plots in B and C), the drift in inherent fluorescence within the reaction time course (panel B, left plot) can lead to the erroneous calculation of concentration–response effect (C, left plot). A nonfluorescent inactive compound 45 (right plots in B and C) displays nearly overlapping reaction time courses (B, right plot) and as a consequence the corresponding concentration–response trend is relatively flat, resulting in the correct assignment of inactive phenotype (C, right plot) Reprinted with permission from (9). Copyright 2010 American Chemical Society.

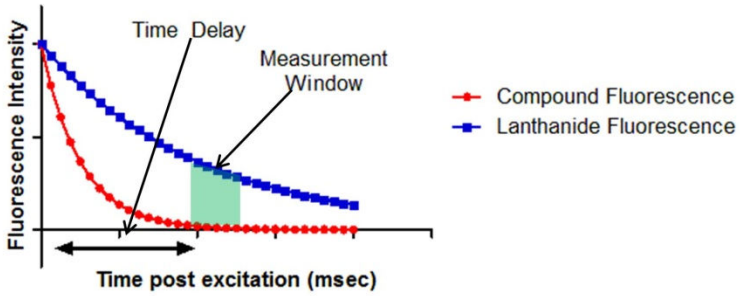


Figure 4: TR-FRET principle. By having a time delay between excitation and detection of emission (indicated by double-headed arrow), a measurement window (green trapezoid) can often be obtained in which the compound fluorescence (red) has decayed nearly to baseline and the longer-lived lanthanide (blue) has decayed significantly less.

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Interferences with Luciferase Reporter Enzymes

Douglas S. Auld[1] and James Inglese[2]

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Abstract

Here we describe the molecular basis underlying interference by inhibitors of reporter enzymes for biochemical and cell-based assays. This understanding is used to create strategies to mitigate the confounding effects reporter enzyme inhibitor interference has on interpreting high throughput screening results, and to aid in evaluating chemical probes derived from reporter gene assays. As well, example compounds that act as potent inhibitors of luciferases are described which can serve as useful tools when developing luciferase-based assays.

Introduction

This chapter records knowledge learned from studying inhibitors of luciferase enzymes commonly used as reporters in assays. First, an overview of how different types of assays – primary, secondary, counterscreens, and orthogonal, apply to the identification of assay interferences is described. Next, the prevalence of luciferase inhibitors in typical compound screening libraries is shown along with the mechanism by which compounds interfere with luciferases. Inhibitor profiles for ATP-dependent luciferases, such as firefly luciferase, as well as non-ATP-dependent enzymes, such as *Renilla* luciferase, are shown. Finally, methods and strategies that can rapidly identify luciferase inhibitors are defined.

Assay Artifact Definition

We define artifactual activity as an activity that arises due to a tested substance interfering with the assay signal in a manner that is not related to actual interactions with the biological target/pathway under study or any relationship that is of biological significance to the study. A classic example of such compound-mediated interferences include light scattering or 'concentration quenching' the latter of which occurs when a light absorbing compound or impurity, acting through a Beer's law effect, attenuates the excitation or emission light detected as described in equation 1, where the attenuation of bioluminescence light (RLU_0) by a compound with absorbance A will decrease the emitted light output to RLU according to equation 1 (1).

$$RLU = RLU_0 \times 10^{-\frac{1}{2}A} \quad \text{(eq. 1)}$$

Additional phenomenon resulting in artifactual activity include aggregation of a compound, redox behavior of a compound (2) inhibition of an enzymatic reporter, and complex forms of fluorescence interference by a compound observed in cell-based imaging assay formats (3). We do not include unwanted activity such as cytotoxicity or general transcriptional/translational effects as these have genuine biological mechanisms underlying the activity.

Role of Primary, Secondary, Orthogonal and Counterscreen Assays in the Identification of Assay Artifacts

Following the primary high throughput screen (HTS), where typically hundreds of thousands to millions of samples are rapidly screened, several types of additional assays are performed with different aims. To avoid confirmation error, the researcher's first goal is to provide evidence for on-target activity through follow-up assay

Author Affiliations: 1 Novartis Institutes for BioMedical Research. 2 National Center for Advancing Translational Sciences (NCATS), National Institutes for Health (NIH).

approaches (Figure 1). With this in mind, assays where a negative result is the desired outcome, particularly when this is due to a loss of signal, should be considered carefully before drawing conclusions about the underlying biological mechanism of the observed activity. For example, a typical counterscreen assay employed at the level of early assessment aims to test for direct interference with the detection reagent or cellular system; therefore a negative result in the counterscreen is the desired outcome. In this way, counterscreens only serve to deselect compounds (e.g., a screen for compound fluorescence serving to remove compounds from consideration in a fluorescent assay system) and fail to answer if interesting biological activity is also present. For instance, there is nothing wrong with developing a fluorescent compound provided that there are assays available to accurately measure the activity. Therefore, negative results in counterscreen assays are not as useful as an assay that reinforces *evidence for target activity*.

The most powerful assay for confirming activity of interest is an 'orthogonal' assay which provides *evidence for target activity*. These assays aim to replicate the exact biology as the primary assay but differ in that a single component is changed, such as the method of detection, which should be independent of the targeted biology. In an orthogonal assay a positive result is the anticipated outcome confirming that compound activity does not depend on the detection technology and is likely related to the target/pathway of interest (see flow chart in (4) and 'Orthogonal Cross-Validation Selection Strategy' Table 1 in (5) as examples). On the other hand, a negative result is a strong indication that the primary screen activity was dependent on the detection method, assay format or technique. Positive results in orthogonal assays do not imply that the desired mechanism of activity has been achieved. This requires additional work, for example by the determination of binding affinity and stoichiometry using biophysical techniques for target-based assays (6).

At the early stages of discovery, potentially the least reliable assays to ascertain genuine activity from the primary screen are the so-called secondary assays. There are several reasons for this. By their nature, secondary assays are often low-throughput (e.g. Western analysis, co-culture models, and experiments requiring manual manipulation) and as such require the premature, relatively uninformed selection of HTS actives from an often relatively large pool of candidates. Secondary assays can be less robust, subjected to observation bias, under powered, and are often far less understood in terms of interferences than typical HTS assays. Although, the aim in a secondary assay is to build proof for target activity, positive results often being the goal, negative results may eventually be dismissed if these assays are thought to be of lower quality or have reduced sensitivity. Therefore, before compounds are chosen for secondary assays, we suggest that there be strong positive evidence for target activity obtained in one or more orthogonal assay systems. Finally, throughout the confirmation process the researcher should avoid the "eureka" view and irrational exuberance that often follows the initial identification of potent 'hits'. Such an attitude can lead one to develop an erroneous narrative from which strong opinions are formed that can persist even when data is subsequently obtained demonstrating the initial findings to be false. Identification of artifacts at the earliest stage possible is crucial to prevent anchoring of misguided opinions.

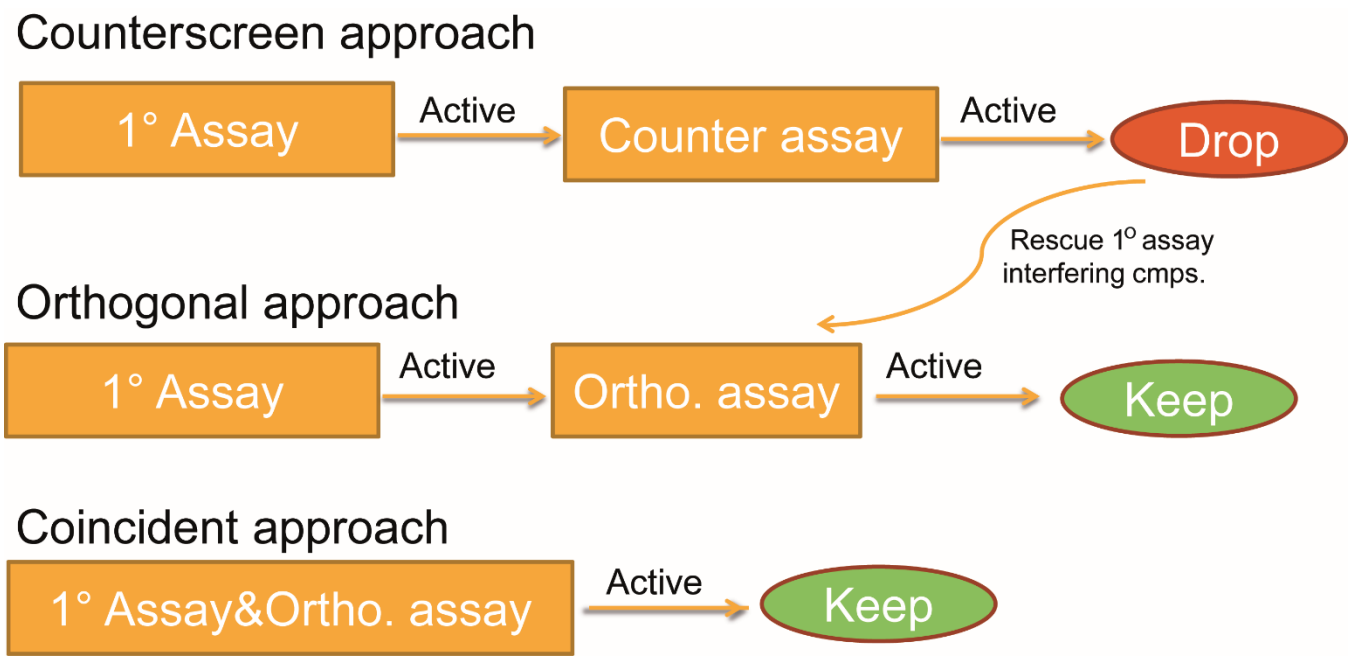


Figure 1: General strategies to identify assay interference compounds. For luciferase assays, counterscreens using the same reporter enzyme but under the control of an alternative promoter or a time course (e.g., for FLuc with D-luciferin for detection) are often used to identify compounds that interfere with luciferase detection, but these approaches only serve to deselect compounds that interfere with the detection. The biological activity of these compounds remains inconclusive unless an orthogonal reporter system is implemented where the same promoter (or biosensor for a pathway) is used in the same cell background but with a different reporter enzyme; ideally two luciferases with inhibitors that are defined by different SARs. In the orthogonal approach, compounds are selected if activity is found in both the primary and orthogonal assays. The coincident approach measures two orthogonal reporter enzymes under the control of a single response element in the same cells using a co-expression strategy, such as the incorporation of ribosome skipping sequences. The coincident approach represents an optimal orthogonal reporter system that can define whether any biological activity of interest is present in one experiment.

Table 1: Properties of luciferases found as components in HTS assays.

Enzyme	Species	MW (kDa)	Em (nm)	ATP	Substrate	Stability	HTS
FLuc	***Photinus pyralis***	**62**	**550-570**	**Yes**	**D-luciferin**	**4.0 hr (cell)**	**B & C**
Ultra-Glo	***Photuris pennsylvanica*** **(mutant)**	**61**	**550-570**	**Yes**	**D-luciferin**	**>24hrs at 60°C[a]**	**B**
CBLuc	***Pyrophorus plagiophthalamus***	**60**	**537 or 613**	**Yes**	**D-luciferin**	**7.0 hr (cell)**	**C**
RLuc	***Renilla reniformas***	**36**	**480**	**No**	**Coelenterazine**	**4.5 hr (cell)**	**B &C**
RLuc8	***Renilla reniformas*** **(mutant)**	**36**	**480[5]**	**No**	**Coelenterazine**	**4.5 hr (cell)**	**C**
GRLuc	***Renilla reniformas*** **(mutant)**	**36**	**530**	**No**	**Coelenterazine**	**48 hr (cell)**	**C**
RFLuc	***Luciola cruciata*** **(mutant)**	**62**	**620**	**Yes**	**D-luciferin**	**3.0 hr**	**C**
NanoLuc	***Oplophorus gracilirostris*** **(mutant)**	**19**	**450**	**No**	**Furimazine[6]**	**6hr (cell) †**	**C**
GLuc	***Gaussia*** **princeps (mutant).**	**20**	**485**	**No**	**Coelenterazine**	**60 hr (cell media)**	**C**

Table 1 continued from previous page.

CLuc	***Cypridina noctiluca***	**62**	**465**	**No**	**Vargulin**	**53 hr (cell media)**	**C**
TLuc	***Metridia pacifica* (mutant)**	**15.7**	**480**	**No**	**Coelenterazine**	**1 hr (cell) †**	**C**

GRLuc, GLuc-Dura, and RFLuc are mutant forms of the native RLuc enzyme and TLuc (Turbo luciferase) are all commercialized by Thermo Fisher Scientific. GLuc is also available from New England Biolabs. NanoLuc and click beetle luciferases (CBLuc; both green and red emitting variants are available) developed by Promega Corp. The emission of RLuc8 can be blue-shifted to 395 nm using DeepBlueC (coelenterazine-400a). HTS refers to the type of HTS assay where the luciferase is commonly used, B – biochemical, C – cell-based. †Destabilized versions are available with half-lives of <30min in cells.

Interferences with Reporter Enzymes

Luciferases as Reporters

One of the most common reporter enzymes employed for constructing HTS assays are luciferases, which generate a bioluminescent signal through oxidation of a luciferin substrate. The two most common luciferases, which have been widely applied in HTS, are firefly luciferase (FLuc; EC 1.13.12.7) from the firefly *Photinus pyralis* and *Renilla* luciferase (RLuc) from the sea pansy *Renilla reniformis*. However, many new luciferases are now available which differ in substrate requirements, stability, emission spectrum and brightness (Table 1). Luciferases are attractive as assay reporters due to the high signal to background inherent to bioluminescence, largely due to a very low background signal as no excitation light is required to generate a signal (7).

Prevalence and Structure Activity Relationships of Luciferase Inhibitors

Studies of FLuc inhibitors among large compound libraries show that approximately 5% of the compounds inhibit FLuc at a screening concentration of 11μM (10). In a titration-based screen of the PubChem library against FLuc, 360,864 IC_{50} values were determined from a FLuc enzymatic assay (using K_M levels of D-luciferin and ATP). More than 10K compounds showed potent concentration-response curves and 168 compounds had potencies <100nM with many achieving single-digit nanomolar potency (4). This data is available in PubChem AID: 588342. Screening of the Novartis compound file at 10 μM showed a similar hit rate of 4% (at >30% inhibition; (8)). This hit rate is significant as HTS assays using FLuc as a reporter are found to have hit lists which are enriched with FLuc inhibitors. Such enrichments within hit lists can be as high as 98% (9-11).

Common chemotypes which act as inhibitors of FLuc have been published (4, 9, 11-13). The PubChem data (AID: 588342) can also be used as a resource. Here, we provide a summary of the major chemotypes found as inhibitors of FLuc. In general, potent FLuc inhibitors tend to be low molecular weight compounds (published data from PubChem shows a MW_{avg} = 325.8 ± 63.3 Da, maximum = 898.8 Da, minimum 122.17 Da; (4)) that have linear, planar structures, while compounds with larger branched configurations show weaker activity. One of the primary classes of inhibitors mimics the D-luciferin (D-LH_2) structure (2-(6-oxo-1,3-benzothiazol-2-ylidene)-1,3-thiazolidine-4-carboxylic acid; Figure 2) and contain either a benzothiazole, benzoxazole, or benzimidiazole core structure (Figure 2; (4, 9)). X-ray co-crystal structures support that simple benzothiazoles bind to the D-luciferin pocket of FLuc (4). Compounds which do not bear resemblance to the D-LH_2 substrate, such as CID:3238892 containing a quinoline core, are also found as enzyme inhibitors. Other inhibitor classes that do not appear to be structurally similar to either D-luciferin or ATP substrates include diaryl structures, such as resveratrol (12) and those containing a 3,5 diaryl-oxadiazole core (4, 9, 14). Compounds containing a *m*-carboxylate group, such as CID:1129835 (PTC124, Translarna™; Ataluren), have been shown to be potent inhibitors of the enzyme where the carboxylate group is appropriately positioned in the FLuc active site to form a highly potent adenylate adduct ((14, 15); and see below). Aryl carboxylates with different linkers that have been determined to undergo adenylate formation are shown in Figure 2 (and see (4)). Additionally, certain

compounds which bind to the luciferase pocket are large enough and have the appropriate geometry to gain access to at least part of the adenylate pocket (Auld, Inglese *et al.*, unpublished results).

Studies have shown overlap of FLuc inhibitors with some drugs often tested in now popular 'repurposing' studies (see as an example (5), SI Figures 3–5) and important drug target classes. For example, certain FLuc inhibitors are also found as protein kinase inhibitors (PKIs). From a library of 367 PKIs, 10 compounds were found as active against FLuc with IC_{50} <1μM. FLuc inhibitors were found among tyrosine kinase inhibitors, such as VEGFR-2/TIE-2, as well as GSKβ inhibitors (16). This emphasizes the need for orthogonal assays to determine relevant on-target activity from FLuc inhibitory activity.

Inhibitors for the commonly used luciferase RLuc have also been published (Figure 3). A prominent class of inhibitors includes those with an aryl sulfonamide core, some of which can achieve potencies of <0.1μM (8, 17). The well-utilized PKA inhibitor, H89, has this substructure and is an RLuc inhibitor which can confound the interpretation of this compound's activity in cell-based assays using RLuc as a reporter (18). However, in the same library of 367 PKI compounds where FLuc inhibitors were identified, none were potent inhibitors of RLuc, a finding perhaps related to the ATP-independent enzymatic mechanism of RLuc.

A newer luciferase isolated from the deep sea shrimp *Oplophorus gracilirostris* and optimized for stability and brightness by Promega scientists has also been described (Table 1) (19). The optimized luciferase is called NanoLuc™ due to the small molecular weight (19kDa) and superior brightness, partly afforded by the enzyme acting on a colenterazine analog, fumarizine (Figure 4). NanoLuc inhibitors have been described (see Figure 4 for examples) and there is some overlap among RLuc inhibitors (approximately 20%, Figure 4) which is not surprising given the similar substrates used by these enzymes. In contrast, overlap of NanoLuc inhibitors with FLuc inhibitors was found to be approximately ~6% (8, 20).

Mechanistic Understanding of Firefly Luciferase Inhibitors

The majority of compounds which have been investigated showing luciferase interference act as enzyme inhibitors and many are competitive with the luciferin substrate (4, 8, 9). Ring system analysis of chemotypes that interfere with fluorescent detection shows a different set of scaffolds compared to what is observed in FLuc inhibitors, supporting that FLuc inhibitors are not general light attenuating or light scattering compounds (4, 9). FLuc bioluminescence covers a range of the visible spectrum from 500-600nm and therefore compounds (typically richly colored, blue, black, or red dyes) could quench the luminescent light of FLuc but only at high (>10μM) concentrations; attenuation being dependent on the extinction coefficient of the compound at the detection wavelength (9). In contrast, RLuc signal, with an emission maximum near 480nm, can be attenuated by yellow/brown colored compounds that are much more prevalent in typical low molecular weight screening libraries, but again this only becomes significant at high compound concentrations (3, 21).

Firefly luciferase has a complex enzymatic mechanism involving both light producing oxidation of D-LH_2 as well as a few non-luminescent side-reactions (Figure 5). The light producing reaction requires ATP, which FLuc converts to a luciferyl-adenylate intermediate with the release of pyrophosphate (Figure 5a). This intermediate breaks down in the presence of molecular oxygen to form a short-lived dioxetanone intermediate which decays to oxyluciferin with the emission of a photon (Figure 5a). However, it has been known for nearly 50 years that FLuc also forms a dehydroluciferyl-adenylate product which acts as a potent inhibitor of the enzyme (L-AMP; Figure 5b, i) (22). The L-AMP inhibitor can be removed from the enzyme through the binding of CoASH which coverts this to a lower affinity L-CoA product. The L-AMP product is an example of a multi-substrate adduct inhibitor (MAI) which occurs in some enzymes in rare cases (23). The ability of FLuc to catalyze adenylate formation with certain long chain fatty acids, such as linoleic acid, has also been shown (Figure 5b, ii). These "dark reactions" play a role in the mechanisms of inhibition for certain compounds. A prominent example of a compound that undergoes adenylation by FLuc to form a potent MAI is the compound PTC124 (Translarna™; Ataluren), a compound containing a 3,5 diaryl-oxadiazole that was originally identified in an FLuc-based

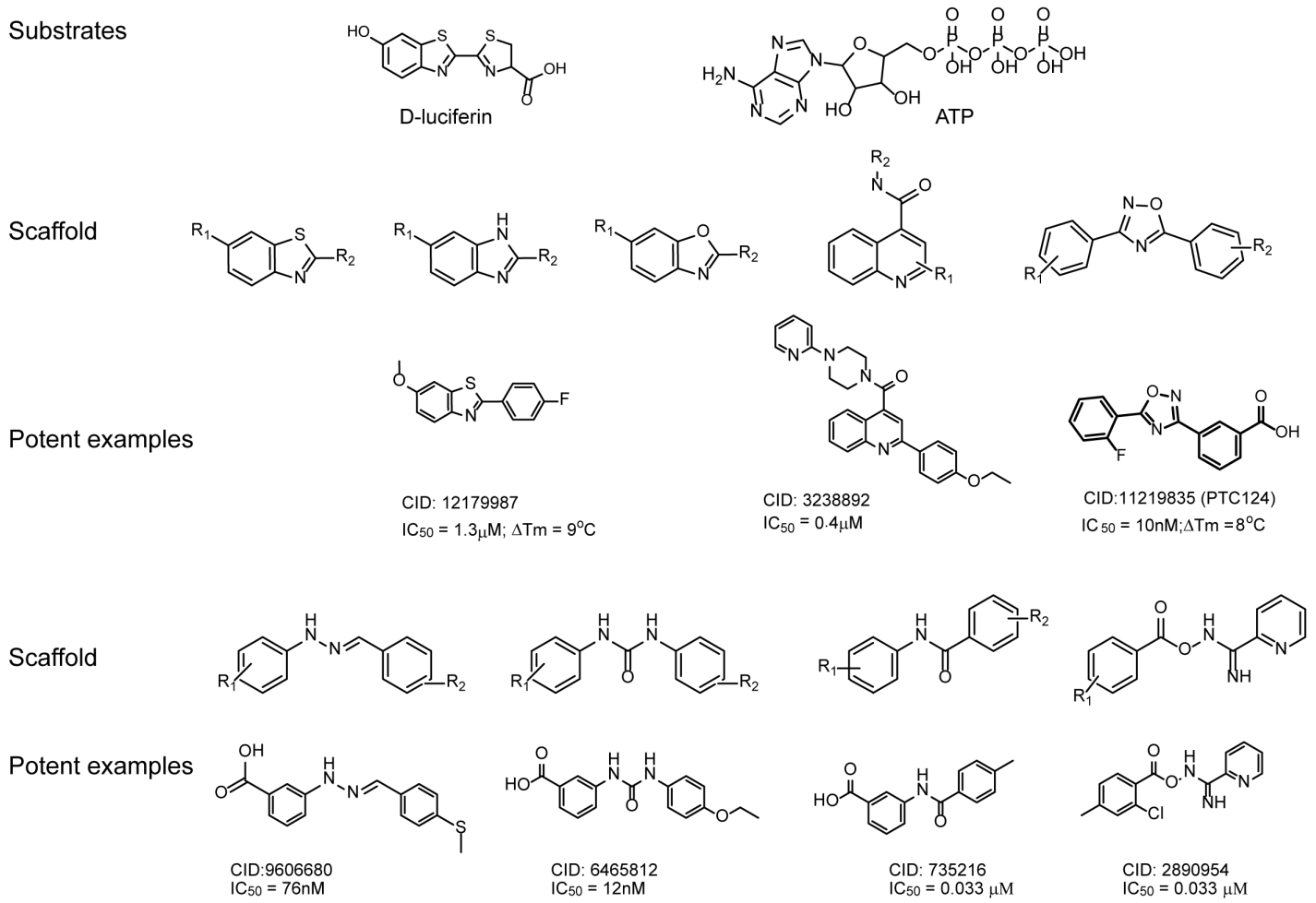


Figure 2: Example scaffolds and compounds that are inhibitors of FLuc. Pubchem CIDs are given as well as published potency values and thermal stabilization values when available.

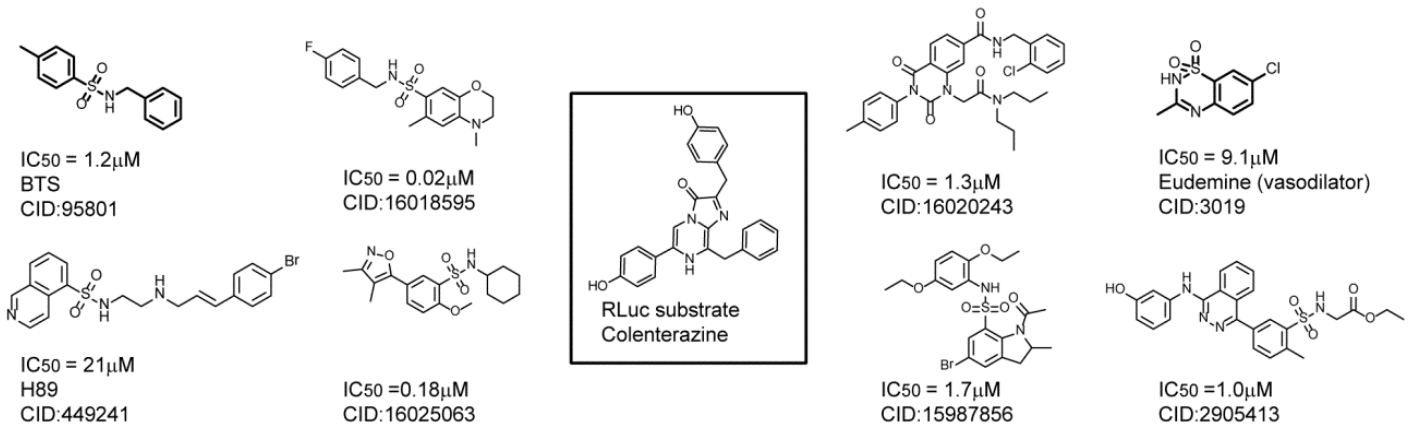


Figure 3: Example compounds that are inhibitors of RLuc. Pubchem CIDs are given as well as published potency values (1-4). The substrate for RLuc is shown boxed in the center.

reporter gene assay (RGA) (24). Mechanistic studies using a variety of analogs, including regioisomers of the aryl carboxylate and X-ray co-crystal structures, have demonstrated that the *m*-carboxylate of PTC124 is in the

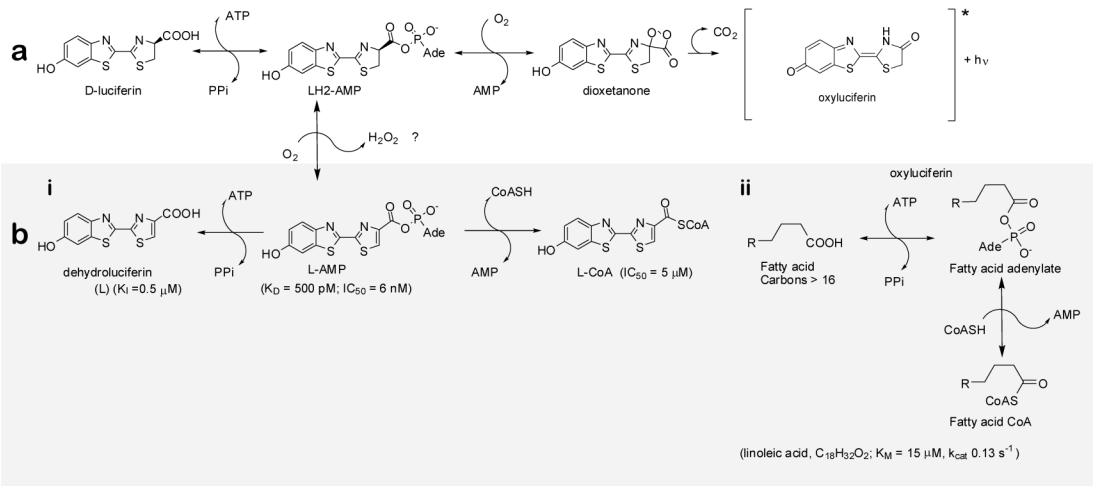


Figure 5: Reactions catalyzed by FLuc. (a) The light reaction catalyzed by FLuc. The substrates D-luciferin (D-LH_2) and ATP are used by FLuc to form a luciferyl-adenylate intermediate (LH_2-AMP). This intermediate then undergoes nucleophilic attack by molecular oxygen, and upon subsequent displacement of AMP, an unstable dioxetanone is formed, which spontaneously breaks down to form oxylucifein, and CO_2 with the emission of a photon (6). (b) Dark reactions catalyzed by FLuc are shown in the gray shaded area. (i) One of these involves a side-reaction in which oxidation of LH_2-AMP occurs to form the potent inhibitor L-AMP, which can undergo pyrophosphorolysis or thiolysis to yield less potent inhibitors, L or L-CoA, respectively (7). (ii) FLuc has also been reported to use certain fatty acids as substrates yielding fatty acyl-CoA metabolites. Kinetic constants for the synthesis of lineoic acid-CoA are taken from Oba *et al.* (9). Figure adapted with permission from Thorne *et al.*, (10).

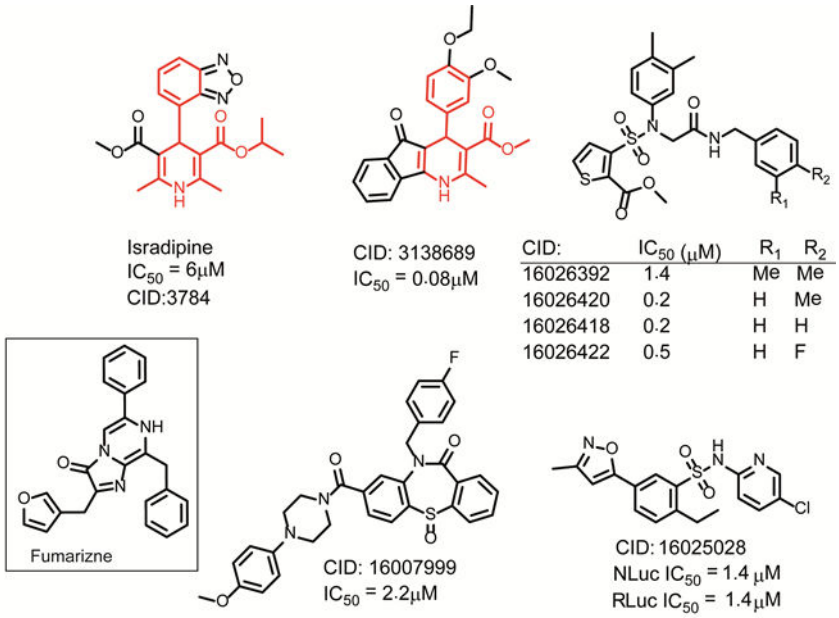


Figure 4: Example NanoLuc inhibitors. Common chemotypes acting as inhibitors of NanoLuc include those with a phenyl-1,4-dihydropyridine core as is found in the drug Isradipine. The aryl sulfonamide scaffold found in RLuc inhibitors is also found among NanoLuc inhibitors.

precise location once bound to the FLuc active site to attack the α-phosphate of ATP through a S_N2 displacement reaction, forming a phosphoanhydride yielding PTC124-AMP and PPi (Figure 6). The PTC124-AMP ligand binds with high affinity to the FLuc enzyme (K_D = 120pM). Both PTC124 itself and the MAI thermally stabilize the enzyme, but the MAI stabilizes FLuc by more than 10°C (15). X-ray co-crystal structures reveal that PTC124-AMP binds across the D-LH_2 and ATP sites in FLuc, filling the luciferin pocket and a

subspace of the ATP pocket reserved for the adenosine base (Figure 6d). Enzyme stabilization and potency correlate with the apparent activity derived in the FLuc-RGA but not an orthogonal RLuc-RGA and PTC124 is not an inhibitor of RLuc (14, 15). Near-attack conformation modeling and experimental results support that the *m*-carboxylate is optimal for MAI formation with *ortho* and *para* regioisomers showing to be far less optimal for MAI formation, leading to less activity in both the enzyme assay and the FLuc RGA (Figure 6f). The reporter-biased activity of PTC124 has been subsequently confirmed in a study that employed a wide variety of different reporters, demonstrating that PTC124 fails to show activity using alternative RGA systems (25). The basis for this confounding activity lies in the ability of this compound to initially act as a substrate of FLuc, participating in one of the dark reactions, leading to the formation of a potent MAI reminiscent of the naturally occurring L-AMP (Figure 6g). High intracellular ATP concentrations favor MAI formation leading to potent stabilization of FLuc in cells. Ligand-based stabilization leads to a rise in FLuc enzyme levels in compound-treated cells, resulting in an increase in signal relative to untreated cells after cells are lysed and detection reagents are added. Addition of detection reagents, such as BrightGlo, containing high concentrations of substrates, including CoASH, relieves the potent inhibition through formation of PTC124-CoA product, analogous to the L-AMP mechanism. Generally, it has been found that many FLuc inhibitors show increased signals in FLuc-based RGAs when certain detection reagents are used, due to ligand-based stabilization of the enzyme in cells (10). The effect of ligand-based stabilization on FLuc RGAs is described in more detail in the section below.

For coelenterazine non-ATP dependent enzymes, such as RLuc, the mechanisms of inhibition are likely simpler and many appear to be competitive with the coelenterazine substrate. However, to date there is no structural data on RLuc inhibitor complexes analogous to the X-ray co-crystal structures that have been determined for FLuc, so our current understanding on the binding mode of RLuc inhibitors is not well defined.

Role of Ligand-based Stabilization of Reporter Enzymes in RGA Responses

Protein conformational transitions exist in thermodynamic equilibrium and ligands may bind to a particular conformation, leading to stabilization of that protein state. Ligand-based stabilization of alternative protein conformations forms the basis for allosteric regulation of enzymes (26) and this fundamental property also forms the foundation of many biophysical techniques aimed at confirming ligand binding, including differential scanning fluorimetry (27, 28), chemical denaturation experiments (29), as well as methods for identifying the target of compounds (30, 31). In some cases ligand-binding events lead to significant stabilization of the protein structure, although some may decrease stability. However, studies of luciferase inhibitors have shown that many of these act to increase the thermal stability of the enzyme (Figure 7). For the studies of luciferase inhibitors, thermal stabilization is concordant with the potency against the enzyme with potent luciferase inhibitors leading to large ΔT_M values (~10°C). The half-life of the free enzyme in cells for common luciferases such as FLuc and RLuc is ~4 hr (see Table 1), therefore inhibitors which stabilize the luciferase can show significant increases in luciferase levels in cells relative to untreated wells within the typical incubation time of most RGAs employed in HTS (12-48 hr). This can lead to the counterintuitive finding where luciferase inhibitors lead to increases in luminescent signal in cell-based assays as many luciferase inhibitors are competitive with the luciferin substrate and detection reagents contain high concentrations of substrates (4, 9-11). Destabilized luciferases using proteasome targeting sequences, such as PEST, can show greater increases in signal, while more stable enzymes (e.g. NanoLuc) can show a muted activation response due to inhibitors or appear as inhibited in cell-based assays (8). Oftentimes the potency of the activation activity in the cell-based assay will mirror the potency of inhibitory activity in the luciferase enzyme assay (Figure 7).

Strategies to Mitigate Luciferase Inhibitor Interference

For biochemical assays that employ FLuc to detect ATP levels or use pro-luciferin substrates (e.g. CYP assays and caspases assays; Promega Corp.) one can check for direct interference with the formulated detection mix.

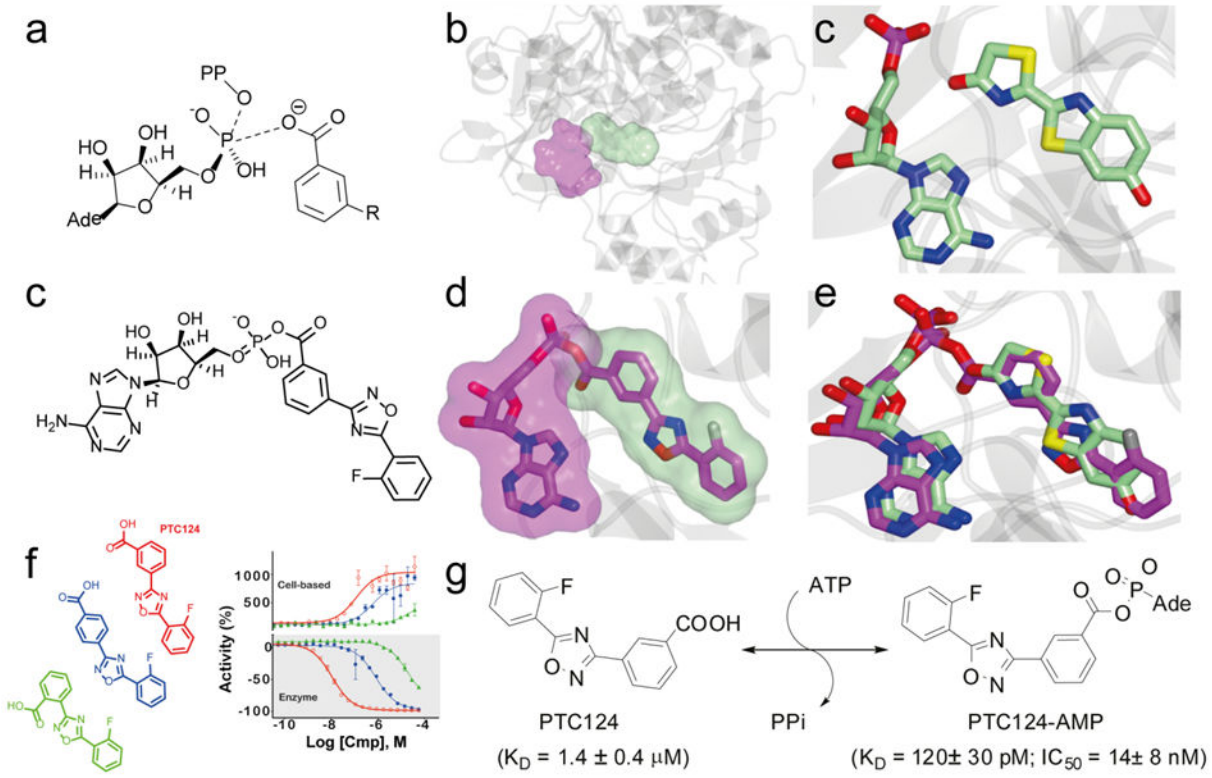


Figure 6: Mechanism and binding of PTC124 MAI. (a) The *m*-carboxylate of PTC124 is positioned to attack the α-phosphate of ATP releasing PTC124-AMP and PPi. (b) The volume of the AMP (purple) and D-LH$_2$ (green) active site pockets are shown. (c) Relative orientation of AMP and D-LH$_2$ in the FLuc active site. (c) Structure of PTC124-AMP MAI. (d) X-ray co-crystals showing the bound conformation of PTC124-AMP in the FLuc active site (PDB: 3IES). (e.) Overlay of the FLuc substrates with the MAI. (f) Structures of regioisomers of PTC124 are shown at left and experimental results for the three regioisomers shown at right, for a FLuc RGA (top) and the enzyme assay at K$_M$ levels of substrate (bottom). Data are from Auld *et al.* (5). (g) MAI formation results in a potent inhibitor, as expected from the product of AMP and PTC124 binding affinities.

For this purpose the enzyme assay can be run to completion or excess product can be added to the assay (in the absence of the enzyme) and detection reagents added to measure if any compounds inhibit the detection reaction (32). For ATP-dependent enzymes, such as protein kinases, orthogonal approaches can be employed through the use of orthogonal readouts, such as TR-FRET or FP, enabled using antibodies toward ADP (33). As well, the use of KinaseGlo and ADPGlo can provide orthogonal assays as the signals for kinase activity proceed in opposite directions, which can be used to test for interferences (34). Promega reagents use an optimized firefly luciferase variant derived from *Photuris pennsylvanica* (UltraGlo, Table 1) and this thermostable variant appears to be less susceptible to inhibitors relative to WT *Photinus pyralis* luciferase (35).

For cell-based assays one can identify luciferase inhibitors using several counterscreen approaches (4). In the case of FLuc, one can detect the activity using D-LH$_2$ alone (100μM is added to the wells) and the assay can then be read kinetically in live cells (36). In this assay format, FLuc inhibitors will often show inhibition at very early time points. Alternatively, hits can be assayed against the FLuc enzyme using the biochemical enzyme assay that has been described for which all the reagents are commercially available (4, 15). Enzyme assays for RLuc, NanoLuc, and TLuc are also easily constructed for use as counterscreens. For example, Inglese *et al.* used NanoLuc secreted into cell media as a source for a NanoLuc enzyme due to commercial unavailability of purified NanoLuc (37). Comparison to historical datasets using the same reporter can also be used, but one must be cautious when interpreting these results particularly when examining assays that measure an increase in FLuc signal, as the level of activation depends on the basal level of luciferase that is expressed, which is often different between RGA cell lines (4).

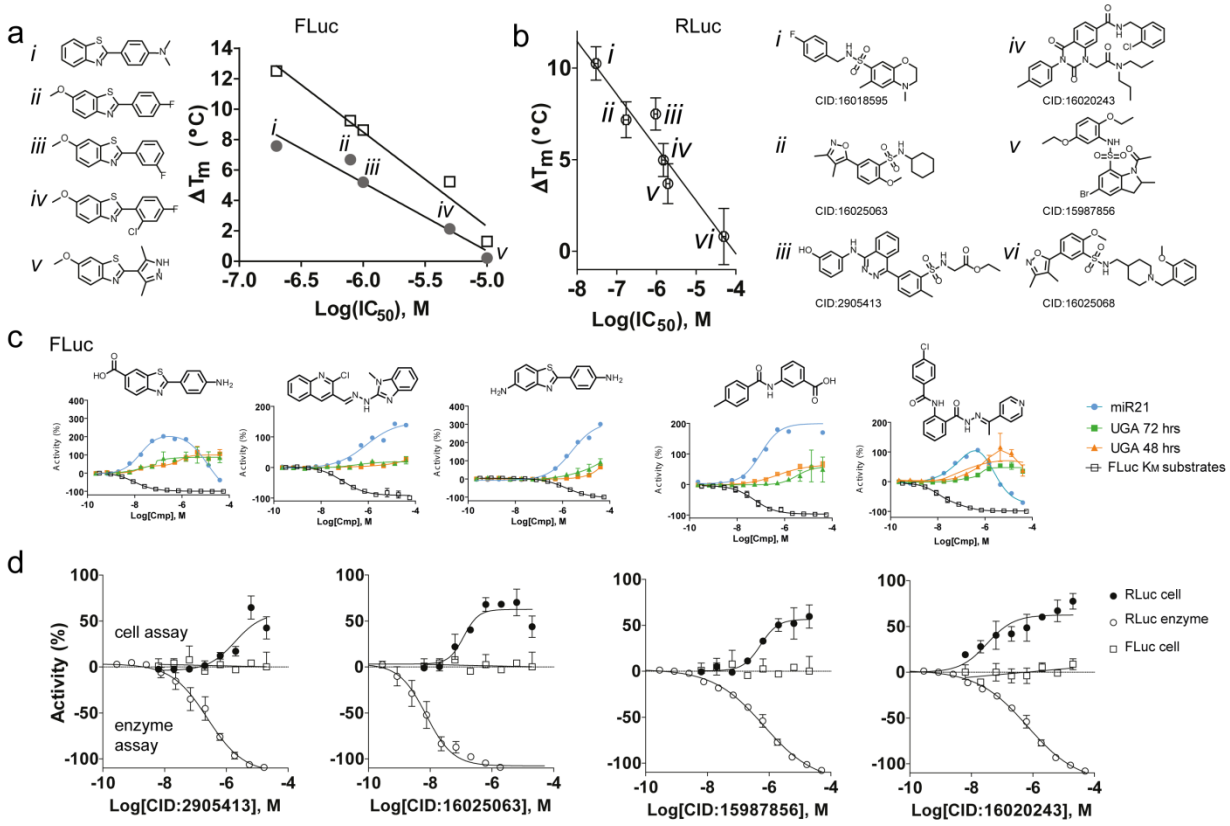


Figure 7: Thermostabilization of luciferases by inhibitors and effect on cell-based assays. (a). Representative simple benzothiazoles and example thermal shift data. The ΔT_M values obtained at 100 μM compound in the presence (open squares) or absence (solid circles) of 2 mM ATP are plotted against the potency of each benzothiazole (b) Structures and thermal melt data for RLuc inhibitors. DSF was used to measure the T_M values of representative RLuc inhibitors differing in potency against the enzyme. Correlation of ΔT_M values (taken as the difference between the T_M at 25 μM compound and apoenzyme) and the measured IC_{50}'s for the Renilla inhibitors (structures shown in panel B). Compound *vi* showed an estimated IC_{50} >50 μM in the enzyme assay. Data shown is from n = 3, error bars are the SD. (c) CRCs for select FLuc inhibitors comparing their activity in the cell-based assays and biochemical assay. FLuc inhibitors with different MOIs commonly exhibit inhibition of FLuc in the biochemical assay with K_M concentrations of substrates and produce activation or bell-shaped curves in cell-based assays. The data are the average of replicate determinations (n = 3 or 4, enzyme assay; n = 2 or 3 for cell-based assay), where error bars represent the SD. (d) Behavior of RLuc inhibitors in a FLuc/RLuc-based RGA. Graphs show data from both reporters in the RGA which was provided expression of both FLuc and RLuc in cells from bis-cistronic promoter (data at or above origin; solid circles RLuc activity; open squares FLuc activity, used as an indicator of cell viability in the assay) and the purified RLuc enzyme assay (curves, below origin). Data from RLuc-based RGA (n = 2) or enzyme assay (n = 4), error bars are SD. All compounds were inactive on FLuc up to 57 μM (FLuc inhibition values taken from PubChem, AID 588342). Figure was adapted with permission from references (4,10).,

Ideally, one should aim to employ an orthogonal reporter to rapidly identify luciferase inhibitors and determine if any activity of interest is present. This approach is superior to the counterscreen approaches mentioned above as these counterscreens can only deselect interfering compounds without being able to determine if these also have interesting activity (Figure 1). Proven orthogonal pairs include FLuc and NanoLuc or FLuc and RLuc (20), FLuc and beta-lactamase (5) and secNLuc and GLuc (37). As mentioned above, FLuc inhibitors overlap little (~6%) with NanoLuc inhibitors while the overlap with FLuc and RLuc is slightly greater (~10%; (8)). Additional bright luminescent reporter enzymes with a different inhibitor structure activity relationship (SAR) than FLuc include GLuc and TLuc (Table 1). A cell-based orthogonal reporter system has recently been described and is made possible for FLuc/RLuc and FLuc/NLuc as both of these have detection reagents which can be formulated to detect both luciferases from the same well (17, 20). In the optimized reporter system, two orthogonal reporters can be co-expressed in the same cell by incorporating a ribosomal skipping sequence between the two orthogonal reporter pairs which allows for rapid determination of reporter-biased responses using the available

dual detection reagents (17). In this so-called coincident reporter system, one seeks to find compounds that are active in both reporter signals; compounds showing activity in only reporter signal are likely reporter inhibitors.

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Assay Interference by Aggregation

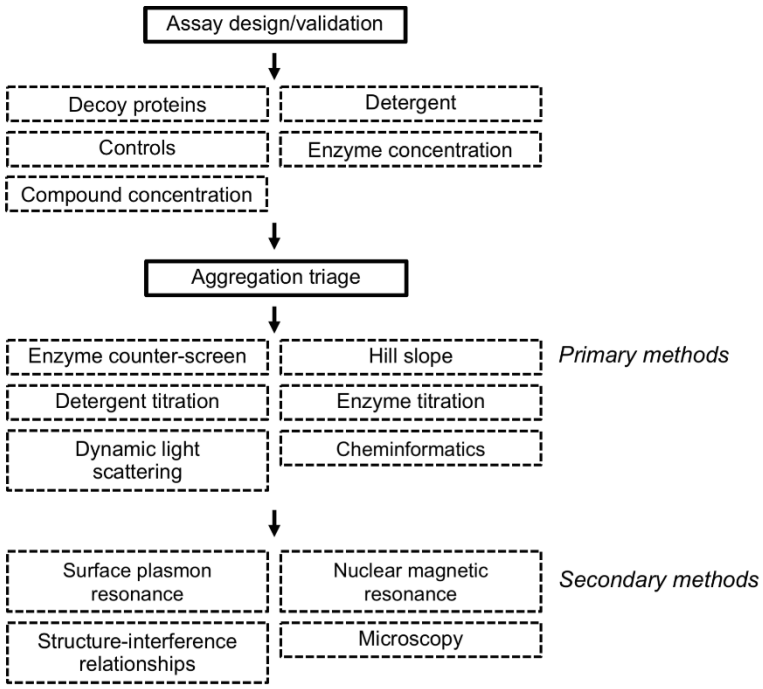
Douglas S. Auld, Ph.D,[1] James Inglese, Ph.D.,[2,3] and Jayme L. Dahlin, M.D., Ph.D.[4]

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Abstract

Aggregation is a common mechanism of compound-mediated assay interference encountered in high-throughput screening (HTS) and follow-up experiments. Compounds that form aggregates *in situ* can nonspecifically perturb biomolecules in biochemical and cell-based assays. Nonspecific bioactivity from aggregation can waste significant resources when unaccounted for in assay design and readout interpretation. This chapter describes two general principles: (a) experimental considerations to mitigate the impact of aggregation in bioassays, and (b) counter-screens and other strategies to identify aggregation among bioactive test compounds. This content should be useful for those performing bioassays utilizing small-molecules, including HTS and follow-up assays, chemical biology, and molecular pharmacology.

Flowchart



Abbreviations

BSA	bovine serum albumin
CAC	critical aggregation concentration
CRC	concentration response curve

Author Affiliations: 1 Novartis Institutes for Biomedical Research, Cambridge, MA, USA. 2 National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD, USA. 3 National Human Genome Research Institute, Bethesda, MD, USA. 4 Department of Pathology, Brigham and Women's Hospital, Boston, MA, USA; Email: jdahlin@bwh.harvard.edu.

Corresponding author.

Table continued from previous page.

CMC	critical micelle concentration
DLS	dynamic light scattering
HAT	histone acetyltransferase
HTS	high-throughput screening
IC_{50}	half-maximal inhibitory concentration
K_d	dissociation constant
MLSMR	Molecular Libraries Small Molecule Repository
NMR	nuclear magnetic resonance
qHTS	quantitative HTS
SIR	structure-interference relationships
SPR	surface plasmon resonance
TEM	transmission electron microscopy.

Introduction and Background

Introduction

Drug and chemical probe discovery often utilizes real and virtual high-throughput screening (HTS) to identify chemical matter for subsequent optimization. HTS readouts from biochemical and cell-based systems are subject to a variety of compound-mediated assay interferences, including aggregation (1). The apparent bioactivity derived from aggregation is often difficult to optimize, and if not recognized early in the discovery process, it can result in significant wasted resources and questionable conclusions derived from these experiments. This section provides an overview of the prevalence and mechanistic details of aggregation in HTS.

Mechanism of Assay Interference by Aggregation

Understanding the mechanisms of aggregation is important because it can inform data interpretation and counter-screen design. Aggregation occurs when susceptible test compounds form aggregates in solution (colloids) (**Figure 1**). For these compounds, aggregation occurs at a critical aggregation concentration (CAC), which is typically in the low-to-mid micromolar compound concentration range. Unlike simple compound precipitation, aggregates can dissolve when diluted below the CAC (2). These solid colloids are composed of up to 10^8 small-molecules and are several hundred nanometers in mean diameter (n.b. some polydispersity), present in mid-femtomolar concentrations when formed, and are therefore not typically observed by visual inspection like compound precipitation (2,3). For enzyme systems (the most well-characterized system for aggregators), bioactivity results from a *reversible* association between enzyme and aggregate surface (4,5). It is currently thought that this binding represents protein *ad*sorption, rather than *ab*sorption, with activity modulation occurring by partial protein unfolding (6). This nonspecific binding typically results in enzymatic inhibition. Furthermore, the affinity of aggregate-protein binding is quite strong, with K_d values in the picomolar range. By contrast, aggregates do not appear to have high affinities for other biomolecules such as DNA or peptides (3). However, aggregates may still interfere with protein-DNA/RNA binding assays through sequestration of the protein component. Individual aggregates can bind up to 10,000 individual enzymes (2). Interestingly, proteins bound to aggregates can retain their activity upon dissociation (“catch and release”) (5).

Notably, certain compounds may form “non-classical”, *smaller* aggregates capable of modulating protein structure and function. For example, the small-molecule JNJ525 showed apparent low micromolar activity in a TNFα-TNFR1/2 TR-FRET protein-protein interaction assay (7). Like conventional aggregators, this compound

showed detergent-sensitive activity and steep Hill slopes in the primary biochemical assay. Detailed mechanistic studies including x-ray crystallography revealed JNJ525 binds TNFα as an ordered 5-member aggregate, disrupting the TNFα-TNFα protein-protein interaction and preventing TNFα-TNFR1/2 association.

Importantly, aggregation can be dependent on multiple experimental factors, including the structure of the test compound, the assay conditions, and the susceptibility of a given target system to aggregates. For example, it is well known that the CAC for aggregators is compound-specific (e.g., some compounds may aggregate at 5 μM, whereas others may only aggregate at 50 μM compound concentrations). In a series of surface plasmon resonance (SPR) experiments, aggregates showed compound-dependent behavior versus the same target (8). In other words, not all aggregates behave the same way versus the same target. Furthermore, studies of the same aggregators versus different targets demonstrate proteins have different susceptibilities to aggregation (9,10).

Such varying responses to different aggregates may reflect differences in specific compound-aggregate affinities. For instance, a given aggregate may have protein-dependent affinities, as recent reports with dye-stabilized compound aggregates demonstrated up to 90-fold differences in apparent affinities between four unrelated proteins (3). These varying responses may also be explained by the nature of specific protein dynamic perturbations induced by a given aggregate. Depending on the tertiary structure of a given protein, aggregate adsorption may significantly perturb protein structural dynamics and function, while in other cases it may cause no appreciable changes.

Prevalence of Aggregation in HTS

Aggregation is a significant source of nonspecific bioactivity, particularly in HTS. In a seminal study screening a 70,563-member Molecular Libraries Small Molecule Repository (MLSMR) library for AmpC β-lactamase inhibition, 1204/1274 (95%) of the *primary actives* were identified as aggregators (10). Overall, 1.7% of the entire library was flagged as a likely aggregator in this HTS due to detergent-sensitive bioactivity. In another MLSMR HTS for cruzain inhibition, 1.9% of the 197,861-compound library and approximately 90% of the primary actives showed detergent-sensitive inhibition, behavior consistent with aggregation (11). Based on these studies, one could estimate that compounds capable of forming aggregates constitute less than 5% of HTS libraries. These studies also demonstrate that if an assay is not properly designed, the vast majority of primary actives can result from aggregation. The main consequences of enriching active compounds with aggregation is that significant resources are spent chasing aggregates in place of potentially more tractable chemical matter.

Section Summary

Aggregation represents a significant source of nonspecific bioactivity and compound-mediated assay interference in HTS and related experiments. Aggregation occurs at compound- and assay-specific critical compound concentrations, and promotes nonspecific activity through partial protein unfolding. Understanding the mechanism of aggregation allows for (a) strategies to mitigate the incidence of aggregation interference, and (b) the design of counter-screens to identify bioactivity due to aggregates.

Strategies to Mitigate Incidence of Aggregation in Biochemical Assays

Introduction

This section describes several experimental strategies and technical considerations to reduce the incidence of aggregation in biochemical assays (n.b., strategies for cell-based assays are less characterized). Strategies discussed include the use of detergents and decoy proteins to prevent aggregate formation, the choice of enzyme and compound concentrations to mitigate the effects of aggregates on assay readouts, and control compounds to characterize the effects of aggregation on assay readouts.

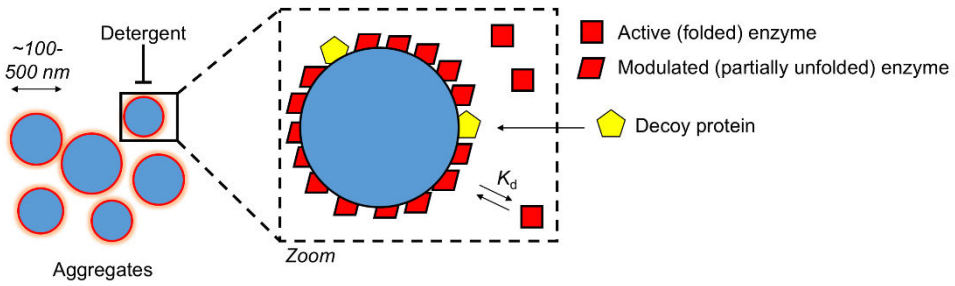


Figure 1. Current model of aggregation. Above the CAC, compounds form solid aggregates, typically 100-500 nm in mean diameter. Enzyme then adsorbs to the aggregate surface with high affinities (e.g., K_d = picomolar to nanomolar ranges). Adsorbed proteins are partially unfolded, which can modulate activity. Free, unperturbed protein occurs when the aggregates become saturated. When present in sufficient concentrations, detergents can disrupt aggregates. Decoy proteins such as BSA can compete with enzyme for adsorption to aggregate surfaces

Detergents

Perhaps the single-most effective strategy to prevent aggregation is to include detergents in assay buffers (4,9,12,13). These reagents act by disrupting colloid structure and can raise the CAC. With most aggregators, activity is dramatically attenuated by the inclusion of detergents (**Figure 2**). In many instances, the addition of detergent can also reverse nonspecific protein modulation by aggregation (4). Another benefit of including detergents is the prevention of nonspecific protein adsorption to container walls, thus preventing time-dependent losses in enzyme activity and decreasing the amount of required enzyme.

There are several choices of detergents to prevent colloid formation, with most being nonionic (**Table 1**). The most popular and well-characterized detergent with respect to aggregation is Triton X-100, which is often used at 0.01% (v/v) final concentrations. A common misconception is that using these "default" detergent conditions will completely eliminate assay interference and nonspecific bioactivity from aggregation. While this concentration is a helpful starting point, it does *not* absolutely prevent colloid formation for every compound. Detergents vary greatly in properties (**Table 1**) and detergents such as Triton X-100 can form very large micelles at concentrations at or above the critical micelle concentration (CMC) which are difficult to remove from proteins. Therefore, detergent concentrations that are well above the CMC should be avoided and one should verify that the complete assay system (e.g., target, reagents, readout, instrumentation) is compatible with the proposed detergent under the conditions to be tested.

A representative example of detergent effects on the distribution of HTS actives can be seen in a comprehensive study exploring the mechanistic basis of the actives from a β-lactamase HTS (15). Detergent sensitivity was used to establish the preponderance of actives that were a result of aggregation, with remaining mechanisms consisting of nonspecific reactive compounds, PAINS-like compounds, and *bona fide* AmpC β-lactamase inhibitors, the latter comprising < 2% of the HTS actives (**Figure 3**) (1,15,16).

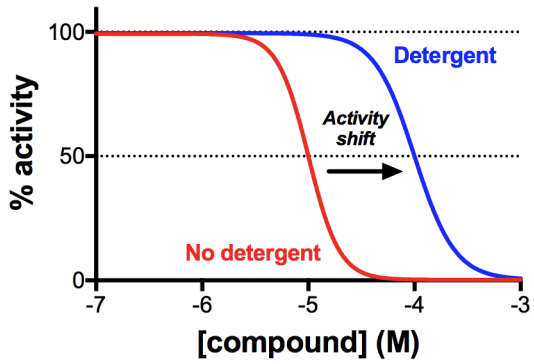


Figure 2. Detergent-dependent activity of aggregators. Shown is a simulation of a prototypical aggregator assayed without (red line; IC_{50} = 10 μM) and with detergent (blue line; IC_{50} = 100 μM). Sufficient amounts of detergent will typically attenuate bioactivity due to aggregation, as illustrated by the activity shift.

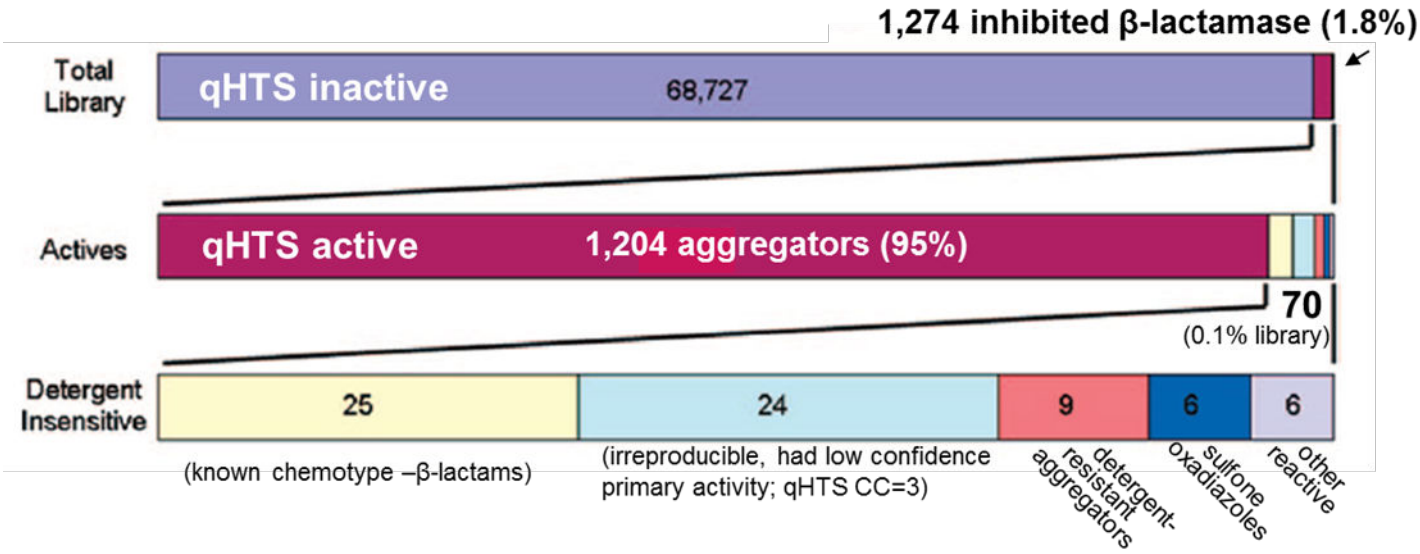
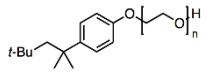
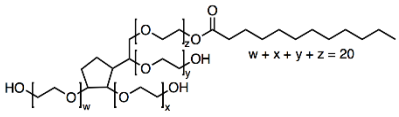
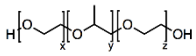
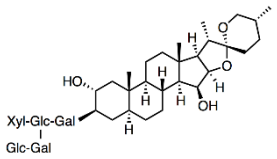
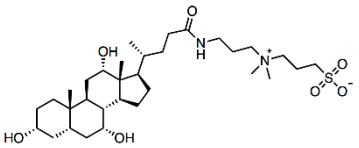


Figure 3. Mechanistic analysis of actives from a β-lactamase biochemical HTS dominated by aggregators. Using quantitative HTS (qHTS), ~70 K compounds were assayed for β-lactamase inhibition. The active compounds (1.8%) were analyzed for detergent-sensitive inhibition, which accounted for 95% of the primary qHTS actives. The remaining 5% of detergent-insensitive compounds (70 compounds) included β-lactam-based inhibitors, irreproducible samples, nonspecific reactive substances, and detergent-resistant aggregators. Detergent-resistant aggregators comprise only 0.7% of the aggregators identified. (adapted with permission from K Babaoglu *et al.* J Med Chem, 2008) (15)

Table 1. Example detergents used in biochemical assays. CMC, the critical micelle concentration, or the concentration at which the detergent begins to form micelles. Aggregation number, the number of detergent molecules in a micelle.

Detergent	Chemical structure	Ionic / nonionic	CMC[a]	Detergent aggregation number	Technical notes
Triton X-100		Nonionic	0.2-0.9 mM (20-25 °C) 0.01% = 0.16 mM	100-155	Often used at 0.01% (v/v) or higher; can produce H_2O_2 in aqueous solutions (14) (recommend preparing fresh buffers for experiments)
TWEEN-20		Nonionic	0.06 mM (20-25 °C) 0.001% = 0.8 mM		
Pleuronic F-68		Nonionic	0.04 mM (20-25 °C) 0.01% = 0.1 mM		
Saponin	Variable	Nonionic			
Digitonin		Nonionic	< 0.5 mM (20-25 °C)	60	
CHAPS		Ionic	6 mM 0.01% = 1.6 mM	10	

[a] Data obtained from manufacturer (Sigma-Aldrich)

Decoy Proteins

Another tactic to mitigate the effects of aggregation is the addition of decoy proteins. The current model for this phenomena involves pre-saturation of aggregates by relatively high concentrations of decoy protein, leaving the biomolecule of interest (e.g., target enzyme) unperturbed by the aggregates.

The prototypic example is the carrier protein bovine serum albumin (BSA). High concentrations of BSA can prevent nonspecific enzymatic modulation by several known aggregators when BSA is present in the assay before the addition of aggregators (17). Importantly, high concentrations of BSA do not routinely reverse enzyme modulation (17).

Several important technical points should be noted:

- A suggested starting concentration for BSA is 0.1 mg/mL.
- Because they do not reverse bioactivity from aggregation, BSA or other proteins should be present in the assay system *before* the addition of test compound.
- Carrier proteins have the potential to sequester monomeric test compounds, lowering the free test compound concentration. This effect may be pronounced at high protein:compound ratios.
- Verify high concentrations of aggregate decoy proteins such as BSA do not interfere with the assay readout or disrupt assay performance. For example, free thiols on untreated decoy proteins may react with assay reagents or electrophilic test compounds.

Enzyme Concentration

For enzymatic biochemical assays, another proposed tactic to mitigate the effects of nonstoichiometric bioactive compounds (such as aggregators) is to simply increase the concentration of target enzyme (18). This approach is based on a proposed stoichiometric model of enzyme inhibition for aggregators (**Equation 1**) (19):

$$\frac{[\mathrm{I}]}{K_\mathrm{d}} = \frac{\text{inh }\%}{100 - \text{ inh }\%} + \frac{\left[\frac{(\text{inh }\%)}{100}\right][\mathrm{E}]}{K_\mathrm{d}} \qquad \text{Equation 1}$$

In this model, enzyme inhibition will appear stoichiometric in cases when enzyme concentration significantly exceeds the K_d value of the inhibitor. This is not an unreasonable assumption for aggregators (recall that many aggregators appear to have K_d values in the low picomolar range). In other words, the IC_{50} value of an aggregator will increase linearly with increases in enzyme concentration. In this model, non-aggregating compounds are not expected to have such dramatic shifts in IC_{50} values unless they have low K_d values, which is unlikely when screening non-optimized chemical matter versus a novel target.

This technique is not without its drawbacks. Aside from consuming more enzyme per test compound, a potential downside to this tactic is that by increasing the amount of enzyme, reactions will proceed faster (18). A practical consequence is that it may be more difficult to sample reactions at low substrate turnover/product formation.

Compound Concentration

Another approach to prevent the formation of aggregates is to decrease the concentration of test compound. The logic of this approach is to lower compound concentrations below the CAC, so that observed bioactivity would more likely be due to other mechanisms of target engagement. This approach will only prevent colloid formation if the compound test concentrations are below the CAC for the specific experimental condition tested, and may not be effective for compounds with relatively lower CACs. Another drawback to this approach is the increased probability of missing less potent but potentially useful compounds which may only be identified at higher compound concentrations. This dilemma can be addressed by using qHTS should a range of compound concentrations be necessary to limit the false-negative occurrence or comprehensively profile the chemical library (20).

Controls/Reference Compounds

During assay development and optimization, it may also be advantageous to assess the effect of known aggregators on assay readout (**Table 2**) (21).

This tactic can help guide the optimization of experimental conditions to reduce the incidence of aggregation. For example, if aggregator controls show significant bioactivity, one may wish to modify the concentration(s) of detergent, aggregator decoy proteins, test compounds, and/or enzyme.

A second potential benefit of this approach is that it permits the actual characterization of aggregation on the assay readout. In conventional cell-free enzymatic assays, this effect may be as simple as enzyme inhibition marked by steep concentration-response curves (CRCs). This approach may be especially useful in more complex assays such as multi-step biochemical assays and cell-based assays, where the potential effect(s) of aggregation may not be intuitive. For example, some aggregators exhibit "bell-shaped" CRCs in cell viability assays, a phenomenon which reflects the concentration of bioactive, monomeric compound (**Figure 4**) (22). Taking the time to robustly characterize the effect of aggregation on assay readout may enhance bioactive compound triage. That is, when test compounds produce a similar readout to known aggregators, one may wish to triage such compounds or flag them for subsequent counter-screens (see section "STRATEGIES TO IDENTIFY AGGREGATION").

A third potential benefit of using aggregator controls is that it may help estimate the incidence of aggregation among bioactive compounds. If a variety of known aggregators are inactive, one might anticipate aggregators would not be particularly enriched among the primary active compounds. On the other hand, if most aggregator controls are active, one might anticipate a formidable proportion of the primary active compounds may owe their bioactivity to aggregation. This estimate may inform the design of the post-HTS screening tree (23). In the latter scenario, one may wish to perform a series of aggregation counter-screens earlier in the triage process, in order to rapidly discard nonspecific aggregators.

Aggregation can be dependent on the assay conditions, meaning even known aggregators will not form micelles in every assay. Therefore, if using this approach, it is advised to include several different controls to get a more comprehensive assessment of aggregation in a particular assay.

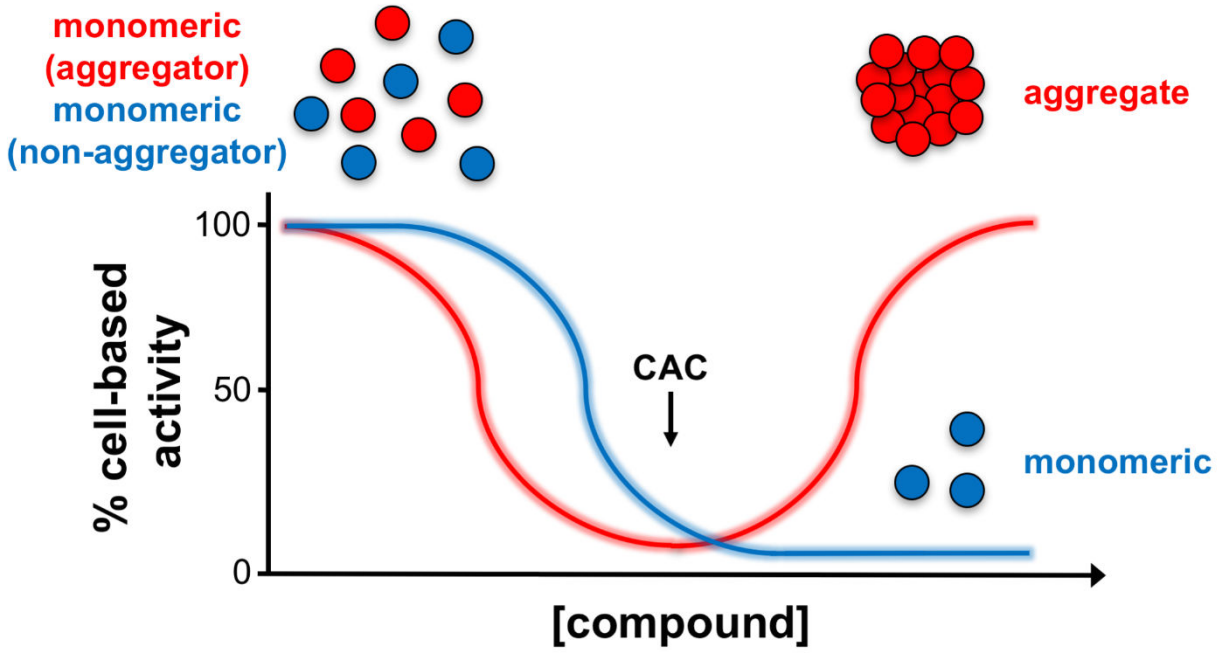


Figure 4. Cellular CRCs for aggregators. Some aggregators can exhibit bell-shaped ("U-shaped") CRC in cell-based assays. At low compound concentrations (below the CAC), monomeric compound is free to interact with the cellular components. Bioactive monomeric compounds will typically exhibit a standard dose-response. However, for aggregators at higher compound concentrations (at or above the CAC), the majority of compound forms an aggregate in the culture emedia (colloid), effectively sequestering bioactive compound and introducing aggregate-cell interactions.

Table 2. Suggested commercially available aggregation control compounds. Most of these compounds should form aggregates in the low micromolar range (e.g., 1-50 μM), though exact CACs will depend on experimental conditions including buffer composition. Additional examples can also be found in several seminal manuscripts (4,9,10,15,24). Additional examples of potential aggregation controls can be found online at PubChem (see Table 3 for relevant PubChem AIDs) and Aggregator Advisor.

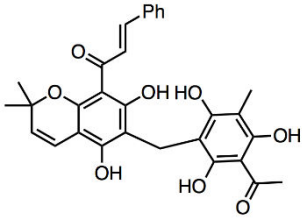
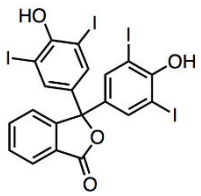
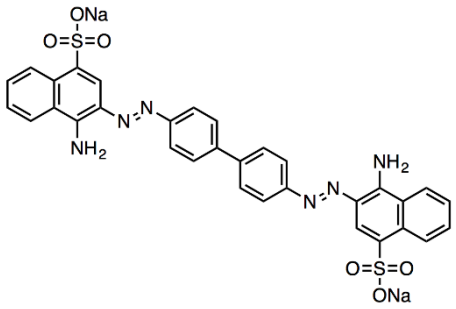
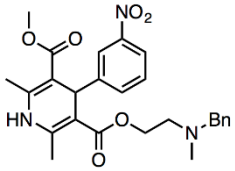
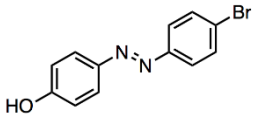
Compound	Chemical structure	Commercially availability	References
Rottlerin		Yes	(9)
TIPT		Yes	(9)

Table 2. continued from previous page.

Compound	Chemical structure	Commercially availability	References
Congo red		Yes	(9)
Nicardipine		Yes	(4)
4BPAP		Yes	(17)

Section Summary

Thoughtful assay design can potentially reduce the impact of nonspecific aggregation. Suggested strategies include the addition of detergents and decoy proteins, and careful consideration of enzyme and test compound concentrations. Assays can also be challenged with previously characterized aggregators to gauge the potential effect(s) of aggregation on assay readouts.

Strategies to Identify Aggregation

Introduction

Even with the aforementioned strategies in place, it is still possible to encounter aggregation. Alternatively, one may wish to investigate aggregation in previously identified bioactive compounds. This section describes several counter-screens and experimental strategies to identify aggregation among bioactive compounds. Primary methods to identify aggregators are discussed, including enzymatic counter-screens, CRC analyses, assay component titrations, and dynamic light scattering (DLS). Secondary methods to identify aggregation such as such as SPR, structure-interference relationships (SIR) , nuclear magnetic resonance (NMR), and microscopy are also discussed.

β-Lactamase Counter-Screen

The most popular and well-characterized counter-screen for aggregation is an AmpC β-lactamase enzymatic assay (25). The reasons for utilizing this particular enzyme are partly historic, as much of the published characterization of aggregation stems from an HTS targeting AmpC (9). In this assay, compounds are tested for inhibition of the *Escherichia coli* AmpC β-lactamase in the presence and absence of detergent (**Figure 5**). Most compounds should not show activity versus this target, regardless of detergent status, and non-aggregating, well-behaved inhibitors will show detergent-independent enzymatic inhibition. By contrast, aggregators will in many cases show detergent-dependent activity at low micromolar compound concentrations.

It is highly recommended to perform some variation of the AmpC assay as an aggregation counter-screen. This assay has several favorable characteristics: (a) it is highly robust; (b) it requires no specialized instrumentation aside from a standard absorbance plate reader; (c) the enzyme is relatively easy to produce and is also commercially available; and (d) there is an abundance of previously characterized control compounds.

Sample Steps | AmpC Counter-Screen

1. Prepare compound solutions. For testing compounds at 10 μM final concentrations, we prepare 500 μM working solutions for compatibility with the following assay protocol.
2. Prepare nitrocefin reagent stock solution. We prepare this solution as 5 mM nitrocefin in neat DMSO. Protect this solution from light. Stock solution is typically stable to multiple freeze-thaw cycles.
3. Prepare assay buffers. First prepare 10X stock solution without detergent. Then prepare two 1X buffer solutions (one without detergent, one with detergent).
4. Prepare enzyme stock solution. Prepare as 30X solution in assay buffer plus 0.0006% Triton X-100 (v/v). The minor amount of detergent prevents nonspecific enzyme adsorption to container walls.
5. Add 142 μL buffer to plate wells with multichannel pipette. We typically use 96-well plates (e.g., Corning Half-Area UV-transparent plates).
6. Add 5 μL of 30X enzyme solution to plate wells with multichannel pipette.
7. Add 3 μL of compound stock solution to plate wells with pipette. To do this step with a multichannel pipette requires "pre-plating" compound stocks in a separate microplate.
8. Mix by gently pipetting up and down three times. Ensure adequate mixing, as the neat DMSO has a tendency to pool at the bottom of plate wells if not sufficiently mixed.
9. Incubate solution from Step 8 for 5 min at room temperature.
10. Add 3 μL of nitrocefin reagent solution to solution from Step 9.
11. Mix by gently pipetting up and down three times. Ensure adequate mixing, as the neat DMSO has a tendency to pool at the bottom of plate wells if not sufficiently mixed.
12. Monitor reaction at 482 nm for 5 min using plate reader. Begin monitoring reaction as quickly as possible to best capture V_{max}.
13. Repeat Steps 5 through 12, substituting detergent-free buffer with detergent-containing buffer.
14. Analyze enzymatic activity using V_{max}.
15. Normalize percent activity to vehicle control (e.g., DMSO-only).
16. Compare activities between the detergent-free and detergent-containing reactions. Compounds are flagged as punitive aggregators if there is a significant attenuation* of bioactivity with the inclusion of detergent.
    * *Based on historical data in our lab, this is defined as > 20% reduction in activity. Other criteria can include > 3 SD from the negative control (untreated) wells.*

Researchers should consider the following technical points:

- The above protocol is an adaption of a previously published protocol (25).
- For general applicability, this protocol is based on multichannel pipettes rather than liquid-handling instruments.
- Compounds are typically tested at 10 μM final compound concentrations. However, it may be necessary to test at alternate concentrations, specifically if the CAC in the relevant assay system is potentially higher than 10 μM.
- Always include positive and negative control compounds. For suggested positive aggregation controls, refer to "Controls/reference compounds" section. We have historically used compounds such as lidocaine as negative aggregation control compounds.
- Recombinant *E. coli* AmpC can be prepared in-house, or purchased from a commercial source.
- Note different species of AmpC may have different susceptibilities to aggregation.

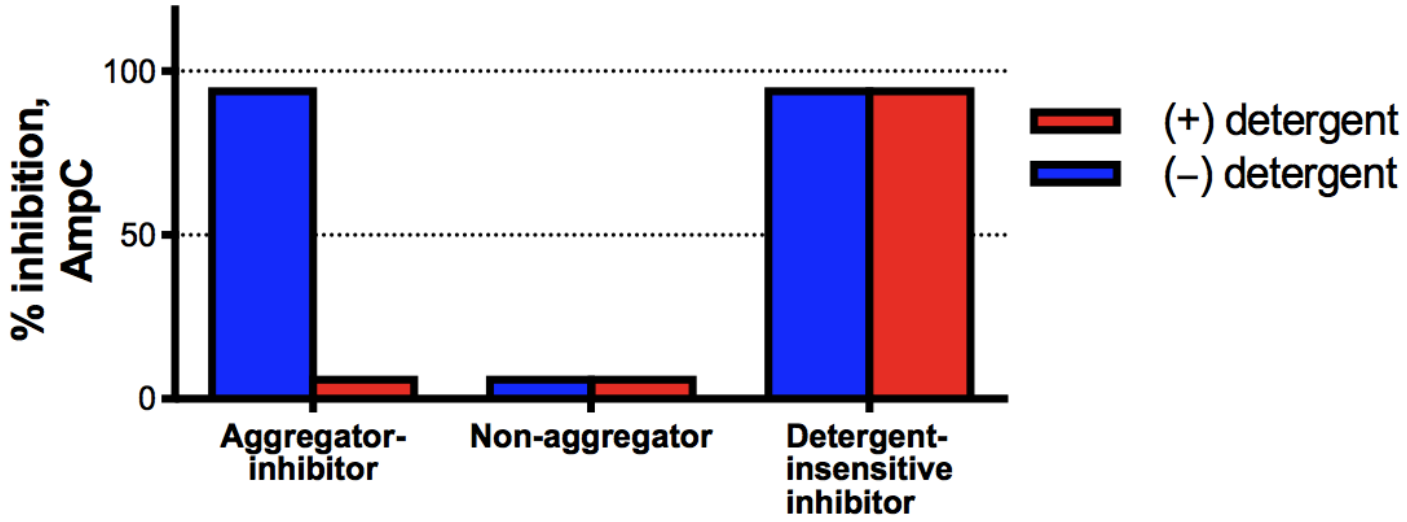


Figure 5. Representation of AmpC counter-screen for aggregation. Compounds are tested for inhibition of the AmpC B-lactamase in the absence (blue) and presence (red) of 0.01% Triton X-100 (v/v). Putative aggregators are most often identified by a significant attenuation of activity upon the inclusion of detergent (while not shown, some aggregators can activate AmpC activity). Non-aggregators typically show minimal activity in both the presence and absence of detergent. Detergent-insensitive inhibitors, which can include well-behaved compounds, show similar activity in the presence and absence of detergent.

- Purification tags may also alter the behavior of a protein with respect to aggregation.
- Different reaction buffers can likely be used for this counter-screen. For example, one may wish to test in the exact buffer as the original HTS. Always verify enzyme activity in any new buffer system.
- Large bubbles can be removed by brief centrifugation or manually by gentle blowing. Excessive bubbling often results from vigorous/turbulent dispensing and mixing, as well as excess detergent (10). To reduce bubbling, mixing should occur below the liquid level of the reaction well (25).
- Prepare detergent solutions fresh for each experiment, as Triton X-100 can produce H_2O_2 in aqueous solution (14).
- Pure detergents can take several minutes to an hour to completely dissolve when transferred to aqueous solutions. Verify detergent is completely dissolved before proceeding with experiments.
- Pure detergents can be highly viscous and difficult to pipette. Prepare 10% solution (v/v) in H_2O for easier, more precise dispensing.
- Depending on the enzyme source, purity, and method of protein quantification, a necessary experiment is to titrate the concentration of enzyme.
- Keep enzyme solutions on ice when not in use during the course of an experiment.

Detergent Titration Counter-Screen

As compound aggregates can have varying sensitivities to detergents and also different CACs, another tactic to identify aggregates is to perform a series of detergent titrations in the original assay system. Non-aggregating compounds will have minimal changes in bioactivity with increasing concentrations of detergent. By contrast, aggregates may demonstrate decreased potencies with increased concentrations of detergent (**Figure 6**).

Sample steps | Detergent Titration Counter-Screen

1. Prepare two or more buffer solutions: one containing no detergent, the other(s) containing varying levels of detergent (e.g., 1X, 5X, 10X)
2. Prepare parallel reaction solutions using the detergent and detergent-free buffers.

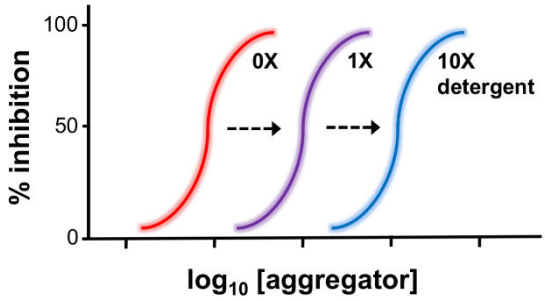


Figure 6. Representation of a detergent titration experiment to identify aggregation. For aggregators, the IC_{50} values can increase with increasing amounts of detergent. Shown is a hypothetical inhibitory aggregator (n.b. steep Hill slope).

3. Test compound in the detergent-free buffers.
4. Test compound in the detergent-containing buffers.
5. Compare activity in the presence and absence of detergent.

Researchers should consider the following technical points:

- Verify the target protein(s), test compound(s), assay reagent(s), and assay readout tolerate the specific detergent being used in this counter-screen.
- Because it can prevent protein adsorption to container walls, detergent-containing reactions may have more active enzyme, and consequently, may have greater overall bioactivity than otherwise identical detergent-free reactions.
- Aggregation is suspected when bioactivity is significantly attenuated by the inclusion of detergent. The exact magnitude of this effect will depend on the specific target and assay system. A rule of thumb is rightward shifts in IC_{50} > 3X should be considered likely aggregate-dependent effects and further analysis with higher detergent concentrations may be warranted if the compound is not immediately eliminated from further follow-up consideration. Depending on assay precision, IC_{50} values can fluctuate, and a useful tool for assessing the significance of changes in IC_{50} values is the minimum significant ratio (MSR). For detailed discussion, the reader is referred to Minimum Significant Ratio – A Statistic to Assess Assay Variability.

Hill-Slope Analysis

A salient feature of aggregation are steep CRCs (**Figure 7**) (4,10,19,27). This reflects the CAC, the high affinity of the protein-aggregate complex, and the protein-compound stoichiometry. Based on the Hill Equation (**Equation 2**), the "steepness" of a given CRC is quantified by Hill coefficient ("Hill slope").

$$\varnothing = \frac{[\mathrm{L}]^n}{K_d + [\mathrm{L}]^n} \qquad \text{Equation 2}$$

(where ∅ represents fraction of receptor bound to ligand, [L] represents free (unbound) ligand concentration, K_d represents apparent dissociation constant, n represents Hill coefficient)

For example, a well-behaved inhibitor with non-cooperative binding will have a Hill slope of 1.0, with approximately 90% of the bioactivity spanning across two orders of magnitude. In general, many aggregators will have Hill slopes greater than 1.5, some even being greater than 5.0. As a word of caution, a subset of aggregators can also have Hill slopes close to 1.0.

Calculating Hill slopes for CRC data is performed by regression analyses. Many labs will use commercial software packages such as GraphPad Prism. Many default curve-fitting algorithms will assume a fixed slope (Hill

slope equal to 1.0), so it is critical to select a "variable slope" curve-fit. For example, a sigmoidal CRC is often fit to a four-parameter variable slope equation (**Equation 3**) (28, 29).

$$Y = \frac{Low + (High - Low)}{1 + 10^{(\log EC_{50} - X)(Hil\ slope)}} \quad \text{Equation 3}$$

(where Y represents assay activity, Low and High represent the low and high bounds)

Depending on the shape and completeness of the CRC, additional constraints may need to be specified for an accurate curve-fit. For additional details on calculating the Hill slope, refer to Assay Operations for SAR Support.

There are some caveats to using Hill slope analysis for aggregation. First, aggregators with Hill slopes close to 1.0 have been described (10). Second, covalent modifiers can also have steep Hill slopes (10). Third, as mentioned previously, any situation where [E] >> K_d of the inhibitor will result in a steep Hill slope.

Enzyme Concentration Titration

Another relatively straightforward approach to identify nonspecific inhibition by aggregators is to perform a series of enzyme titration experiments (19,25). Based on the aforementioned stoichiometric model of aggregation inhibition, the IC_{50} value of an aggregator is expected to increase linearly with increases in enzyme concentration (for single-point experiments, percent inhibition should decrease when the concentration of enzyme is increased).

Sample Steps | Enzyme Titration Counter-Screen

1. Test compound at standard enzyme concentration (i.e., 1X) under standard conditions. Also perform vehicle control in parallel.
2. Determine activity of test compound from Step 1 relative to vehicle control.
3. Test same compound from Step 1, except at 5X enzyme concentration under otherwise standard conditions. Also perform vehicle control in parallel with this same enzyme concentration.
4. Determine activity of test compound from Step 3 relative to vehicle control.
5. Repeat Steps 3 and 4 with increasing concentrations of enzyme (e.g., 10X, 20X)
6. Plot IC_{50} versus enzyme concentration. A linear relationship is suggestive of stoichiometric inhibition, consistent with aggregation.

Researchers should consider the following technical points:

- If available, include a non-aggregator positive control compound. The activity of such a compound should not proportionally increase with additional enzyme (unless enzyme concentration significantly exceeds the K_d value of the inhibitor).
- Verify the effect of increased enzyme on reaction progress. With increased enzyme, reactions will proceed quicker and not approximate steady-state conditions due to increased product formation. Also verify assay readout is within the dynamic range of the detection system.

Centrifugation Counter-Screen

Aggregators will often show a pronounced decrease in bioactivity when reaction solutions are centrifuged (4). This process effectively pellets the aggregates, leaving only monomeric compound (and non-sequestered protein if present) in the supernatant (**Figure 8**). Notably, this counter-screen does not identify potential aggregators based on differential bioactivity in the presence and absence of detergents. For this reason, *this particular counter-screen may be especially useful in assay systems intolerant to detergents or decoy proteins.*

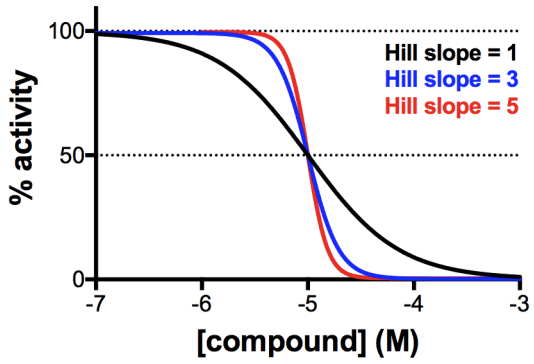


Figure 7. Hill slope analysis to identify aggregation. Shown is a simulation of compounds with identical 10 μM IC_{50} values. Aggregators characteristically have steep CRCs. A well-behaved compound (black line) typically has a Hill slope equal to 1.0, whereas many aggregators have higher Hill slopes (blue, red lines).

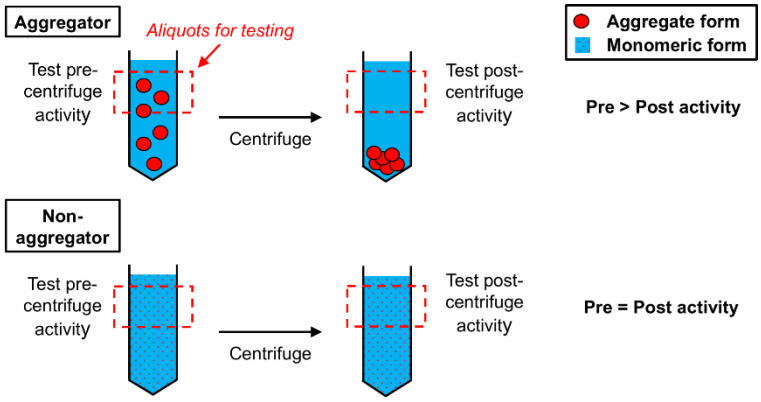


Figure 8. Centrifuge-based experiment to identify aggregation. Aggregates (top panel) can usually be removed by brief centrifugation of samples. For aggregators, post-centrifugation supernatant aliquots should show reduced bioactivity compared to pre-centrifugation samples. By contrast, non-aggregators (bottom panel) should have similar activity pre- and post-centrifugation.

Sample Steps | Centrifugation Counter-Screen

1. Incubate test compound in assay buffer for 5 min.
2. Remove supernatant aliquot from Step 1 for testing.
3. Centrifuge mixture from Step 1 at approximately 15,000 g for 30 min at 4 °C. Potential aggregates should form a sediment.
4. Remove supernatant aliquot from Step 3 for testing.
5. Test and compare bioactivity of aliquots from Steps 2 and 4 by adding remaining assay reagents (e.g., target enzyme(s), substrate(s), co-factor(s), etc.).

Researchers should consider the following technical points:

- Include a vehicle-only control (e.g., DMSO-only) to control for changes in enzyme activity during the centrifugation process.
- Also include a known non-aggregating positive control compound (if known).
- Both types of controls should be tested for bioactivity using pre- and post-centrifuged aliquots.
- For poorly soluble compounds, also consider simple precipitation of compound rather than colloid formation.

- As a control for the centrifugation process, store the pre-centrifugation aliquots in conditions similar to the centrifugation (e.g., 4 °C).

Pre-incubation Counter-Screen

A simple mechanistic counter-screen for suspected aggregators is to modify the compound-protein pre-incubation time. Aggregators will often show more potent activity when allowed to pre-incubate with the target protein (4,9). In an AmpC assay, the magnitude of this effect varied from several-fold to greater than 50-fold changes in IC_{50} values (9).

Notably, this counter-screen does not identify potential aggregators based on differential bioactivity in the presence and absence of detergents. For this reason, *this particular counter-screen may be useful in assay systems intolerant to detergents or decoy proteins, or to follow-up on those compound activities that may fall into the aggregator detergent-insensitive class* (see **Figure 4**).

The counter-screen premise is relative simple. For a conventional enzymatic assay, compounds are tested in two different reaction systems: (1) compounds and proteins are pre-incubated together for 5 min, then the reaction is initiated by addition of non-proteinaceous substrate, and (2) compounds and non-proteinaceous substrate are incubated together, then the reaction is initiated by addition of protein. In the first reaction, when aggregates and protein are allowed to incubate together for even 5 min, most of the protein will adsorb to the aggregates (in the absence of other strategies to mitigate aggregation such as detergents and/or decoy protein). In the second reaction, no such adsorption occurs between the non-proteinaceous assay components and aggregates when incubated together. When this second reaction is initiated by the addition of protein, there is a higher initial concentration of unbound (and unmodulated) protein.

This tactic is generally best when performed as a counter-screen, rather than in the HTS phase where one may consider reducing pre-incubation times to reduce assay interference from potential aggregation. Reducing pre-incubation times is difficult in many HTS settings due to instrumentation and scale. Furthermore, pre-incubation is often purposefully designed to allow for equilibration purposes.

1. Incubate test compound and protein for 5 min. Also perform vehicle control.
2. Initiate reaction by adding non-proteinaceous substrate to mixture from Step 1.
3. Determine activity from reaction from Step 2 relative to vehicle control.
4. In a separate reaction, incubate test compound with non-proteinaceous assay components for 5 min. Also perform vehicle control.
5. Initiate reaction by adding proteins (e.g., target enzyme) to mixture from Step 4.
6. Determine activity from reaction from Step 5 relative to vehicle control.
7. Compare activities from Steps 3 and 6.

Researchers should consider the following technical points:

- Include a known non-aggregating positive control compound (if known).
- The suggested length of pre-incubation is 5 min, though it is possible this interval may need to be extended for slower-forming aggregate-protein complexes.
- Recall the differential activity of aggregators will depend on the formation of aggregates during the pre-incubation phase. If sufficient amounts of detergent to dissolve aggregates are already present in the assay, minimal differential activity may be observed.
- For compounds showing increased bioactivity with protein pre-incubation, one should also consider the possibility of irreversible, time-dependent target modulation. Refer to Assay Interference by Chemical Reactivity for additional details on the identification of reactivity.

Dynamic Light Scattering

Dynamic light scattering (DLS) is a biophysical technique used to determine particle sizes in solution. Compounds are dissolved in solution, and if aggregation occurs, the size of aggregates can be quantified by measuring temporal fluctuations in light scattering and generating an autocorrelation function (**Figure 9**). There are several examples of DLS used in the identification and characterization of aggregates (2,4,6,24,30). Modern instruments utilize either cuvettes or microplates, the latter being amenable to higher-throughput counter-screens. This technique provides direct, physical evidence of aggregate formation, though it does not provide functional information about any detected aggregates. Another advantage is that the assay is not dependent on the particular target (protein-free), and in theory it can be adapted to model assay specific buffer conditions. Depending on available instrumentation and desired throughput, DLS can be a highly useful primary counter-screen for aggregation.

Structure-Interference Relationships

Structure-activity relationships (SAR) can often be explained by assay interference, so-called structure-interference relationships (SIR). Analysis for SIR in the context of HTS can help identify potential aggregators by analyzing specific bioactive chemotypes in-depth. This approach can be especially helpful for equivocal analyses, where it is unclear whether a bioactive chemotype is a function of aggregation or useful target engagement. If the apparent SAR appears to correlate with aggregation, then it can facilitate triage of related analogs and also inform the design of related chemotypes to prevent the formation of colloids. As with conventional SAR analyses, this approach is most helpful with multiple compounds. Bioactivity analysis should focus not just on the active compounds and close analogs, but also on structurally-related inactive compounds. If a chemotype is particularly important for a project, this analysis can be aided by SAR-by-commerce, in which closely related analogs (active and inactive) can be purchased and tested for activity in the original assay and aggregation counter-screens.

This approach was particularly helpful in the post-HTS triage of a 3-hydroxy-pyrrolidin-2-one core active against the fungal histone acetyltransferase (HAT) Rtt109 (**Figure 10**). In this case, clues that an apparent SAR actually reflected an aggregation-dependent SIR included: (a) little change in activity with minor aromatic substituent changes, (b) correlation of bioactivity with increased lipophilicity and molecular weight, and (c) a precipitous drop in activity with the removal of the *C3*-hydroxy group. Of course, these findings could also be explained by true bioactivity, which should also be considered in any such analysis. In this example the correct identification of aggregation as the primary source of bioactivity ultimately depended on correlation with other experimental data (AmpC counter-screen, steep Hill slopes, detergent titration, mechanism of action studies) (31). By determining the specific components contributing to aggregation, there was high confidence in discontinuing work on this chemotype.

Computational Approaches

Cheminformatics can also be utilized to help identify potential aggregators, especially in the context of the large datasets inherent to HTS. Since the factors contributing to aggregation are multifactorial, most current cheminformatics tools for aggregation triage are based on chemical similarity to known aggregators.

A helpful, open-resource for flagging potential aggregators is Aggregator Advisor (32). In this method, compound structures in the form of SMILES can be submitted to a database of known aggregators. Compounds with a high-degree of similarity to known aggregators are flagged. This page also contains an extensive list of aggregators ("Rogues' Gallery").

Datasets from several HTS campaigns in the presence and absence of detergents are also publically available *via* PubChem, which can facilitate additional data analyses and method development (**Table 3**).

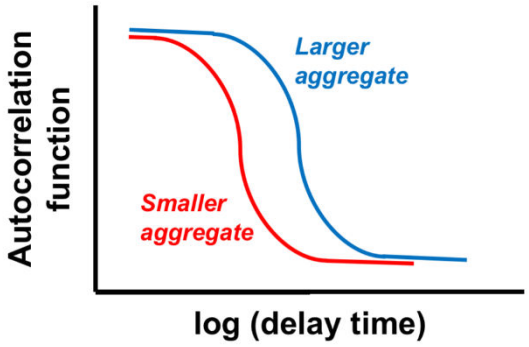


Figure 9. Dynamic light scattering for the characterization of aggregates. Due to their sizes, aggregates will scatter light. The size of aggregates can be characterized by DLS by plotting the autocorrelation function as a function of delay time. Larger aggregates will maintain a higher correlation for longer delay times relative to smaller aggregates (compare blue and red curves).

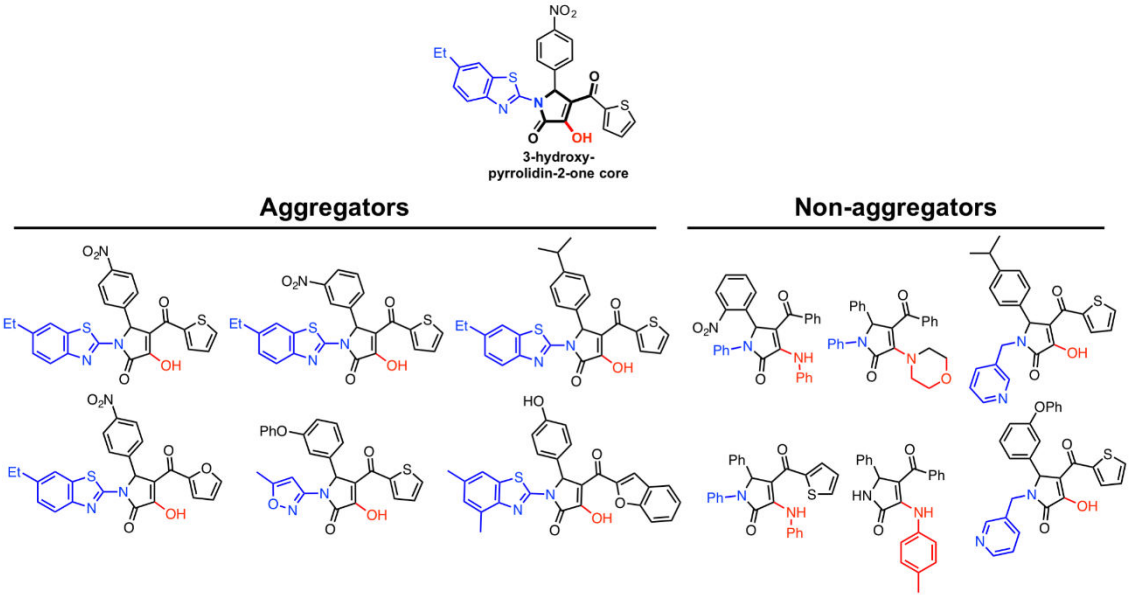


Figure 10. Structure-interference relationships of aggregators. Analysis of analogs can help identify bioactivity due to aggregation. In this example from an HTS for inhibitors of yeast Rtt10930, most compounds with the 3-hydroxy-pyrrolidin-2-one core are active at low micromolar compound concentrations versus Rtt109, and are also flagged as aggregators by the AmpC aggregation counter-screen. Compounds without the *C3*-hydroxy group were inactive versus Rtt109 and inactive in the AmpC counter-screen (*C3* substituents highlighted in red). Compounds with alkyl substituents at the *N1*-position were also inactive versus Rtt109 and inactive in the AmpC counter-screen (*N1* substituents highlighted in blue).

Table 3. PubChem HTS data relevant to aggregation.

PubChem Assay	PubChem Assay ID
qHTS Inhibitors of AmpC Beta-Lactamase (assay without detergent)	485341
qHTS Inhibitors of AmpC Beta-Lactamase (assay with detergent)	485294
qHTS Assay for Promiscuous and Specific Inhibitors of Cruzain (without detergent)	1476
qHTS Assay for Promiscuous and Specific Inhibitors of Cruzain (with detergent)	1478
Promiscuous and Specific Inhibitors of AmpC Beta-Lactamase (assay without detergent)	585
Promiscuous and Specific Inhibitors of AmpC Beta-Lactamase (assay with detergent)	584

Nuclear Magnetic Resonance

Two different nuclear magnetic resonance (NMR) techniques have been described to identify aggregates, one protein-free ("ligand-based") and the other utilizing a protein probe ("protein-based").

In one counter-screen, standard proton NMR can be used to identify aggregates without the need for target protein (33). Compared to monomeric (non-aggregating) forms, aggregates can exhibit characteristic chemical shift patterns due to the additional intermolecular effects from compounds in close proximity. These chemical shift perturbations revert to the monomeric chemical shift patterns upon dilution, with the addition of detergent, or after centrifugation (**Figure 11**). These chemical shift patterns include chemical shift change(s), additional peak(s), and change(s) in peak shape. The advantages of this technique include its lack of required protein, its use of relatively standard instrumentation (i.e., proton NMR), and a biophysical readout. The main disadvantage of this assay is its comparatively lower throughput. Like DLS and microscopy, it can detect aggregation but does not inform the functional consequence of these aggregates on bioactivity. For details on performing this counter-screen, see the original manuscript for an excellent experimental walkthrough (33).

Another technique, a La assay to detect reactive molecules by nuclear magnetic resonance (ALARM NMR), is sensitive to nonspecific protein perturbation by known aggregators (31). This assay is an industry-developed [^{1}H-^{13}C]-HMQC counter-screen originally developed to identify nonspecific thiol-reactive screening compounds by their ability to induce DTT-dependent conformational shifts in the La antigen (34,35). As they exert their bioactivity through non-covalent interactions, aggregators can perturb the La antigen conformation independent of DTT (**Figure 12**). An advantage of using ALARM NMR is its ability to identify nonspecific thiol-reactivity *and* aggregation. However, potential drawbacks of this method are the specialized instrumentation required, its medium throughput (approximately 50 samples per day), and the relatively high concentrations of compound required for testing (i.e., mid-micromolar). As the La antigen may not be sensitive to every aggregator (JL Dahlin, unpublished observations) and the experimental conditions may not approximate the relevant original screening conditions, we recommend following up critical results with additional counter-screens.

Surface Plasmon Resonance

Another biophysical technique for detecting aggregation utilizes surface plasmon resonance (SPR) biosensors. Aggregates can produce characteristic readouts in SPR experiments, most notably superstoichiometric binding (**Figure 13**) (8). An interesting finding from this original study is that the nature of any given aggregate-protein interactions can vary depending on the particular compound *or* the target protein identity. This technique can capture multiple modes of aggregation-target interactions, including stoichiometry and the kinetics of aggregate association and dissociation. Like many of the other aforementioned aggregation counter-screens, aggregation in this SPR setup is generally attenuated by the addition of detergent. The advantages of this technique include its general applicability to any target amenable to SPR, and its biophysical readout, which provides information about the kinetics of direct aggregate-protein interactions. The main disadvantages of this technique are its instrumentation requirements (i.e., non-standard in many academic labs), need for protein immobilization ("on chip"), and overall throughput. For details on performing this counter-screen, we refer to the original manuscript for several example aggregation readouts (8).

Microscopy

While not a routine tool due to the specific instrumentation required and the relatively low throughput, aggregates can also be directly visualized through specialized microscopy. The most common microscopy method for observing aggregates is transmission electron microscopy (TEM) (4,9,36,37). A general procedure involves preparing the compound aggregate in assay buffer, then transferring to a carbon-coated grid, followed by negative staining with uranyl acetate or ammonium molybdate. Aggregates can also be indirectly visualized

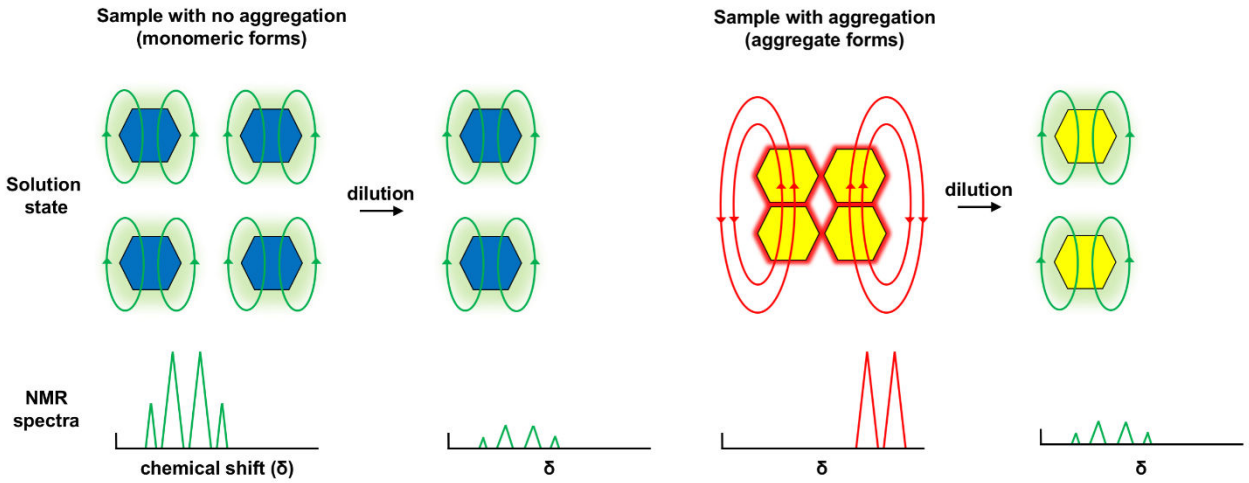


Figure 11. NMR-based detection of aggregation. (Left) Non-aggregating compounds (blue) exist in monomeric form, producing characteristic chemical shifts (depicted as green lines). Dilution of the sample does not produce chemical shifts, just dilution of signal intensity. (Right) Aggregating compounds (yellow) will exist as aggregates, producing a set of chemical shifts specific for the aggregate form (red lines). Dilution of the sample below the CAC disrupts the aggregate to form monomeric compound, which have a different chemical shift pattern (green lines).

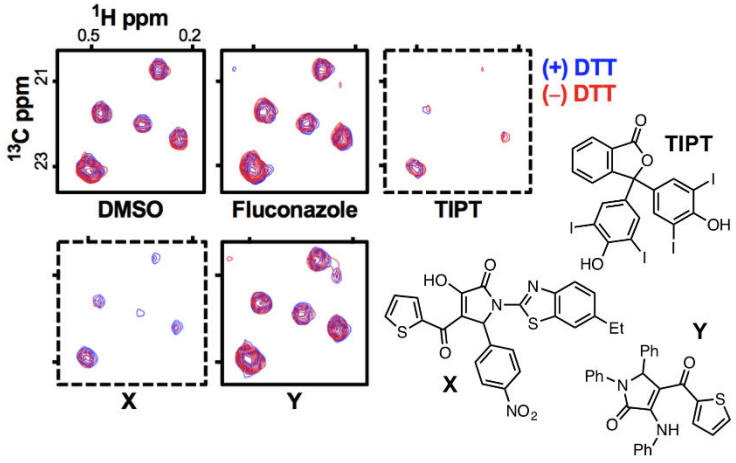


Figure 12. ALARM NMR readout sensitive to aggregation. Known aggregators TIPT and compound **X** perturb the La antigen conformation independent of DTT (9,31). Compound **Y**, a structural analog of **X**, is used as an aggregation negative control compound. Fluconazole included as additional non-reactive, non-aggregation control compound. Dashed border, ALARM NMR-positive.

with fluorescent microscopy when incubated with fluorescent proteins, which can adsorb to the surface of aggregates (4). The direct observation of adducts should be correlated with additional bioactivity and mechanistic data.

Section Summary

Bioactivity due to nonspecific aggregation can be identified through several strategies. Initial tests to evaluate for aggregation typically include enzymatic counter-screens, CRC analyses, DLS, and simple mechanistic experiments. More advanced and/or resource-intensive methods include SPR, NMR, and microscopy. In addition to triaging nonspecific compounds, these strategies can also be useful for de-risking compounds as they proceed along the discovery and development pipeline.

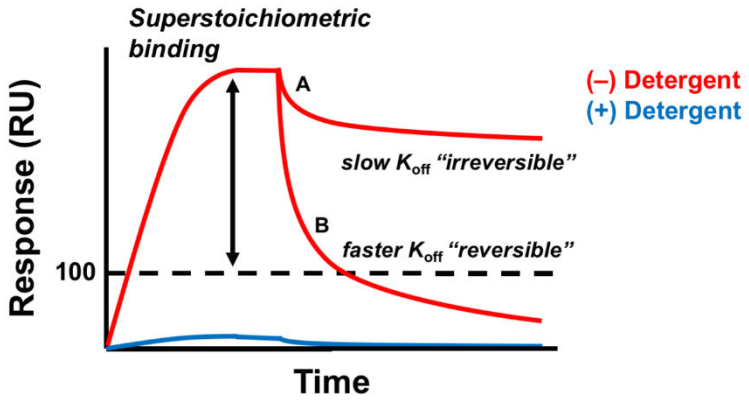


Figure 13. SPR characterization of aggregators. Shown is a simulation of a prototypical aggregator in a standard SPR experiment. In this example, response is normalized to a well-behaved inhibitor that binds the protein in a 1:1 ratio (RU = 100). Aggregators can produce superstoichiometric binding (characterized by large responses) and can display detergent-dependent protein binding (compare red and blue lines). Aggregates can display either slow dissociation mimicking irreversible binding (curve A) or faster dissociation (curve B).

Frequently Asked Questions

This section seeks to answer some common questions about aggregation in the context of drug and chemical probe discovery, including some common misconceptions (adapted from advisor.bkslab.org).

1 **Can aggregation affect cell-based assay readouts?**

Yes. There are several examples of aggregators modulating cell-based readouts. Interference and nonspecific bioactivity can result from direct interaction with membrane-bound receptors (38), a reduction in bioactive monomeric compound (37), or interactions with other extracellular factors (36). Aggregators have also been associated with cytotoxicity and changes in cellular histone acetylation (JL Dahlin *et al.*, forthcoming results). It is unclear if aggregates can form intracellularly. Studies with Evans blue suggest aggregates do not transverse individual cell membranes intact (e.g., by endocytosis) (22). Some known aggregators exhibit U-shaped curves due to aggregation at high compound concentrations which effectively prevents cell penetration (22). Other studies with Evans blue suggest certain aggregates may cross certain tissue structures such as tumor blood vessels via increased permeability (37). Compounds can potentially form aggregates *in vivo*, based on simulated intestinal fluid studies (39). Therefore, it is highly advised to consider the use of aggregator controls in cell-based assays, especially when testing compounds at micromolar concentrations. It is currently unclear which strategies, including the use of detergents and decoy proteins, can most effectively mitigate aggregation in cell-based experiments, especially without perturbing cell function.

2. **Several FDA-approved drugs are aggregators. How can this observation be reconciled with the recommendation to triage aggregators?**

Several drugs can form aggregates in enzymatic assays at micromolar concentrations (24). However, many of these drugs have been extensively optimized for potency and selectivity, and are typically used at nanomolar compound concentrations, well below their CACs.

3. **Can one predict aggregation based on chemical structure (e.g., *in silico*)?**

Several methods have been described to flag potential aggregators based on structure and other properties (24,30,32,40,41). As with any model, there is always the potential for false-positive and false-negative

predictions. While helpful for economically analyzing large numbers of compounds, these tools should not be used as sole substitutes for well-designed experimental counter-screens for aggregation.

4. **Do aggregators always inhibit enzyme activity?**

No. While the most common effect of partial protein unfolding by aggregation in enzymatic assays is inhibitory, it is possible to actually enhance target activity (10). For example, we have observed several reproducible cases of reported HAT inhibitors actually increasing AmpC activity (JL Dahlin *et al.*, forthcoming results). The mechanistic basis of this phenomenon is unclear, but it may be influenced by reaction volume and/or compound solubility.

5. **Can a compound aggregate in all assay conditions?**

No. Aggregation (i.e., CAC) depends on multiple factors, including assay buffer components, ionic strength, pH, and detergent (42). Other experimental factors such as pre-incubation time, temperature, mixing procedures, and organic solvents (e.g., DMSO) may also alter aggregation behavior. These factors, combined with the actual compound concentration, determine whether a compound aggregates or not.

6. **Do aggregators always show a complete concentration-response?**

Not necessarily. Aggregator CRCs can plateau before achieving a complete dose-response due to compound precipitation (19).

7. **What other HTS phenomenon can be attributed to aggregators?**

Gain-of-signal outputs have been observed, possibly the result of an aggregation phenomenon. In one case, an HTS TR-FRET assay identified a very large number of primary actives (a compound-dependent positive FRET signal). Given it was unlikely that a productive protein-protein interaction would be facilitated by these compounds, the possibility exists this effect was caused under a situation where aggregates created a surface upon which donor and acceptor labeled proteins adhered and resulted in a non-mechanism relevant proximity, thereby producing the positive FRET signal (J Inglese, personal observation ca. 2003).

Additionally, there is also the possibility for aggregates to interfere with antibody-based assays by sequestering immunoglobulin reagents.

Conclusions

Aggregation represents a significant source of compound-mediated assay interference and nonspecific bioactivity in biological assays which utilize small molecules. The incidence of aggregation by test compounds can usually be mitigated by careful assay design, such as the use of detergents and the optimization of enzyme and test compound concentrations. Aggregators can be identified by several strategies (**Table 4**), including enzymatic counter-screens, DLS, and various titration experiments. Cheminformatics and advanced strategies such as microscopy, NMR, and SPR can also identify aggregators.

Several recommendations are made to prevent wasted follow-up on aggregators in the context of HTS, chemical biology, and related fields.

1. Proactively design assays to mitigate the effect and the prevalence of aggregation.
2. Empirically test bioactive compounds for aggregation by at least one method (i.e., test regardless of whether aggregation is suspected or not).
3. Compounds with a high index of suspicion for aggregation should be evaluated by multiple methods.
4. Testing for aggregation should attempt to approximate the relevant biological assay conditions.

Following these strategies should triage intractable chemical matter and de-risk more promising bioactive compounds.

Table 4. Summary of aggregation counter-screens.

Assay	Pros	Cons	Notes
AmpC β-Lactamase	-Well-characterized -Robust -Medium-to-high throughput -Requires relatively standard instrumentation (i.e., plate reader)	-AmpC assay conditions might not be compatible with target assay conditions -Not all aggregators modulate AmpC	-AmpC commercially available -Provides additional information about target specificity -Assay subject to other forms of compound-mediated interference -Indirect evidence of aggregation
Detergent titration	-Utilizes original assay target and assay technique -Assays target modulation by aggregates	Detergents might not be compatible with target assay conditions	-Assay subject to other forms of compound-mediated interference -Indirect evidence of aggregation
Hill slope	Can analyze existing CRC data	Aggregators do not always exhibit steep Hill slopes	Indirect evidence of aggregation
Enzyme titration	-Utilizes original assay target and assay technique -Assays target modulation	May require significantly adjusting assay time points and other variables	-Assay subject to other forms of compound-mediated interference -Indirect evidence of aggregation
Centrifugation	-Utilizes original assay target and assay technique -Assays target modulation	High-force centrifugation generally lower throughput	Assay subject to other forms of compound-mediated interference
Cheminformatics	-Requires only chemical structure as input -High-throughput	-Training sets based on specific assay conditions, targets; applicability to other experimental variables unknown -Susceptible to false-positive and false-negative results	Experimental confirmation strongly recommended
DLS	-Biophysical evidence of aggregates -Medium throughput -Direct observation of aggregates	-Requires non-standard instrumentation -Does not assay target modulation	
NMR	-Biophysical evidence of aggregates -Does not require protein (ligand-based)	-Lower throughput -Does not assay target modulation -Requires non-standard instrumentation	Indirect evidence of aggregation
ALARM NMR	-Biophysical evidence of aggregates -Provides additional information about target specificity and nonspecific thiol reactivity	-Lower throughput -Requires relatively high protein and compound concentrations -Protein may not be universally susceptible to aggregates -Requires non-standard instrumentation	Indirect evidence of aggregates
SPR	-Biophysical evidence of aggregates -Can be adapted to target or surrogate proteins	-Lower throughput -Requires non-standard instrumentation -Does not assay target activity	Indirect evidence of aggregates

Table 4. continued from previous page.

Assay	Pros	Cons	Notes
Microscopy	-Lower throughput -Direct observation of aggregates	-Requires non-standard instrumentation -Does not assay target activity	

Suggested Web Resources

1 Aggregator Advisor (advisor.bkslab.org).

An excellent web-based cheminformatics resource for identifying potential aggregators. The input is test compounds in SMILES format, and the output is a similarity score relative to known aggregators.

2. PubChem (pubchem.ncbi.nlm.nih.gov).

A free resource for several HTS campaigns for aggregation.

Suggested Readings (alphabetical order)

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   A detailed follow-up mechanistic analysis of aggregators from a biochemical HTS campaign (accompanies Suggested Reading #3).

2. Feng, B. Y.; Shoichet, B. K. *Nat Protoc*, **2006**, *1*, 550.

   An easy to follow step-by-step guide describing the AmpC β-lactamase counter-screen for aggregation.

3. Feng, B. Y.; Simeonov, A.; Jadhav, A.; Babaoglu, K.; Inglese, J.; Shoichet, B. K.; Austin, C. P. *J Med Chem*, **2007**, *50*, 2385.

   A representative example of the prevalence of aggregation in a biochemical HTS campaign, demonstrating ~95% of primary actives were detergent-sensitive aggregators (accompanies Suggested Reading #1).

4. McGovern, S. L.; Helfand, B. T; Feng, B.; Shoichet, B. K. *J Med Chem*, **2003**, *46*, 4265.

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Assay Validation, Operations and Quality Control

Viswanath Devanarayan, PhD, Philip W. Iversen, PhD, Jeffrey R. Weidner, PhD, and Mary Jo Wildey, PhD

Basic Guidelines for Reporting Non-Clinical Data

Jayme L. Dahlin,[1] G. Sitta Sittampalam,[2] Nathan P. Coussens,[2] Viswanath Devanarayan,[3] Jeffrey R. Weidner,[4] Philip W. Iversen,[5] Joseph V. Haas,[5] Duane D. Bronson,[6] O. Joseph Trask, Jr.,[7] Todd R. Wiernicki,[5] and Steven D. Kahl[5]

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Abstract

Reporting experimental and assay results can occur in many different settings, including informal laboratory meetings, technical reports, collaborative interactions, updates to management groups and presentations at professional conferences. In order to convey the intended message and make a lasting impact, the data presented must be clear to the observer or reader. Understanding key concepts and methods for reporting data is also critical to preserve scientific findings. This chapter describes some basic guidelines for reporting non-clinical data with an emphasis on standard elements of graphs and tables and the use of these tools to describe data most appropriately. Several fundamental statistical and numerical descriptions such as significant digits, replicates, error and correlations are also included, as they constitute an integral part of communicating results. These guidelines form the foundation for non-clinical data reporting mechanisms, such as laboratory notebooks and reports. While these guidelines are general in nature and may not be inclusive of the requirements for publication within specific journals, they should provide a solid basis for reporting non-clinical data, independent of the presentation venue.

Abbreviations

AGM	Assay Guidance Manual
AUC	area under the curve
CRC	concentration-response curve
CV	Coefficient of Variation
EC_{50}	half-maximal effective concentration (relative or absolute, see *AGM* chapters on Assay Operations and Glossary for further definitions) (1-3)
HTS	high-throughput screening
LLOQ	lower limit of quantitation
Log	Log base 10 or Log_{10}
LsA	limits of agreement
MR	mean ratio
n	number of replicates
pEC_{50}	negative log EC_{50}
PK	pharmacokinetics
PMCC	product moment correlation coefficient
r	Pearson's correlation coefficient (equivalent to linear correlation coefficient)

Author Affiliations: 1 Brigham & Women's Hospital and Broad Institute of Harvard/MIT. 2 NCATS/NIH. 3 GlaxoSmithKline. 4 QualSci Consulting, LLC. 5 Eli Lilly and Company; Email: skahl@lilly.com. 6 ScreenAble Solutions. 7 Perkin Elmer, Inc.

Corresponding author.

Table continued from previous page.

ρ	correlation coefficient
SD	standard deviation
SE	standard error
SEM	standard error of the mean
ULOQ	upper limit of quantitation

Overview

Representation of data in graphs and tables is a key part of any scientific experiment. Creating appropriate figures requires familiarity with constructing them as well as knowledge of the data. The ability to convey results with clarity and accuracy does not require special skills but following some common guidelines can be very helpful in describing the data. This chapter outlines steps to make figures an effective communication tool for the scientist with a focus on assay development and high-throughput screening (HTS) applications. Certain figure types, such as flow charts and process diagrams, are not discussed in this chapter.

When considering graphs or tables for publications, grants, regulatory reports, etc. consult with the appropriate journals or technical documentation available for any specific requirements.

The main types of graphs for summarizing scientific data that are used for assay development or the HTS field include bar graphs, line graphs, scatter plots, frequency distributions, scatter box plots and heat maps. Other graph types such as spider or radar plots, pie charts, Pareto diagrams, and area charts are used less frequently and are not specifically discussed in this chapter.

Allocating most of the “graph ink” to data and minimizing extraneous ”chart junk” should be the goal of any effective graph (4). With that in mind, the main elements of many graphs include a title, axis scale with tick marks, axes labels, data, and a symbol key. When the graph is to be used in a printed format, a caption or legend and footnotes may be added. These graph components, and how to make better graphs, have been described in the literature (5-9). In addition, several books are often cited when describing graphing methods (4,10). At least one paper offers a five principal tutorial on how to visualize data (11). This chapter highlights some of the key graphing basics and considers graphs that may be used during everyday informal presentations of non-clinical data. In addition, tables are also briefly discussed.

Proper statistical treatment of the data is essential and consulting with a statistician familiar in assay development and HTS applications is highly recommended. Otherwise it is the author’s responsibility to ensure that the statistical methods support the types of conclusions being drawn and that the statistical methods are appropriate to the data based upon data type, distribution (e.g. normal vs. log-normal), and study design. More details are available in the statistics chapters of the Assay Guidance Manual (12).

Before creating graphs and tables, one should understand the numerical and statistical concepts described below. These concepts have a pivotal role in conveying data efficiently and effectively.

Rounding to Decimal Places

During the collection and manipulation of primary data, data should not be rounded until the end, when one wishes to summarize and report the findings in a table, etc. When rounding to decimal places, numbers are rounded by removing digits from the rightmost, farthest side, or the value which falsely suggests a high degree of precision. When the digit to be removed (immediately to the right of the rounding digit) is 0, 1, 2, 3 or 4, the rounding digit is “rounded down”. Similarly, when the digit to be removed is 5, 6, 7, 8 or 9, the preceding rounding digit is “rounded up”. When eliminating multiple digits from the right of a rounded digit, round from

the rightmost digit to the left, ensuring to capture any influence rounding carryover from preceding digits. See **Table 1** for rounding examples which include these carryover conventions.

Table 1. Rounding examples from thousands to three decimal places

	Rounding the Whole Number to:				Rounding the Decimal Number to:		
Result	**Thousand**	**Hundreds**	**Tens**	**Ones**	**Tenths**	**Hundredths**	**Thousandth**
1234.1234	1000	1200	1230	1234	1234.1	1234.12	1234.123
2345.2345	2000	2300	2340	2345	2345.2	2345.24	2345.235
3456.3456	3000	3500	3460	3456	3456.4	3456.35	3456.346
4567.4567	5000	4600	4570	4567	4567.5	4567.46	4567.457
5678.5678	6000	5700	5780	5679	5678.6	5678.57	5678.568

Although the authors subscribe to the common rounding rule which "rounds down" for digits less than 5 and "rounds up" for digits greater than 5, there are alternative "odd-even" rounding strategies for summarized values that have been published (13).

Rounding to Significant Digits

The number of significant digits or figures that are used to display a value is distinct from the number of decimal places that are used when expressing numbers. Demonstrating numerical expression consistency in tables, figures, legends, etc. is important when describing results and aids in the reporting and understanding of variability.

The following basic rules are used for rounding with significant digits:

1. Non-zero digits are always significant
2. A zero or zeros between two significant digits are significant
3. Final or trailing zeros in a number with no decimal are not significant (e.g. 1020)
4. Leading zeros in the decimal portion of a number are not significant; whereas final trailing zeros in the decimal portion of a number are significant

Expressing numbers using scientific notation can provide a useful method to demonstrate the number of significant digits associated with a value. As shown in **Table 2**, the defined digits which are located before the exponent part of the scientific notation expression are significant:

Table 2. Three significant digits for numbers viewed with scientific notation

Number	Scientific Notation	# Significant Digits	Significant Digits
1020	1.02×10^{3}	3	1,0,2
102	1.02×10^{2}	3	1,0,2
10.2	1.02×10^{1}	3	1,0,2
1.02	1.02×10^{0}	3	1,0,2
0.102	1.02×10^{-1}	3	1,0,2
0.0102	1.02×10^{-2}	3	1,0,2
0.00102	1.02×10^{-3}	3	1,0,2

As shown in **Table 3**, the number of significant digits for result values of different magnitudes (from 1 to 4), is depicted.

Table 3. The number of significant digits from one to four for several examples.

		Result with indicated # of Significant Digits			
Result	**Scientific Notation**	**1**	**2**	**3**	**4**
0.1234	1.234×10^{-1}	0.1	0.12	0.123	0.1234
1.234	1.234×10^{0}	1	1.2	1.23	1.234
12.34	1.234×10^{1}	10	12	12.3	12.34
123.4	1.234×10^{2}	100	120	123	123.4
1234	1.234×10^{3}	1000	1200	1230	1234

Presenting Rounded Data in Tables

When preparing summarized data tables, all result values should be expressed with the same number of significant digits. Once the desired number of significant digits has been established for the result value, use the same number of decimal places in the variability measurement (e.g. SD, SEM) (**Table 4**, Data Set **3**).

Consider the summary presented in **Table 4** that represents typical data generated for compound testing results and the associated error. Each data set in the table is discussed below.

***Data Set 1*:** Each Result Value and the paired statistical measurement (SEM) are expressed with the same number of decimal places, in this case three. Note that the table is cumbersome when values range several magnitudes and the number of significant digits is variable.

***Data Set 2*:** All Result Values have the same number of decimal places (three) with the paired statistical measurement (SEM) also having the same number of decimal places (three). Once again, the number of significant digits expressed for the result value is variable with anywhere from 2-7 significant digits. Summary tables with values and error like those shown are often seen when results are obtained from a database query with a set number of decimal places for the results.

***Data Set 3*:** All Result Values are shown with the same number of significant digits (three in this example). The paired statistical measurement (SEM) has the same number of decimal places as the result value. This is the preferred method of expression for results and error. If results are obtained from a database query, there may be some manipulation required with either the result value or the error.

Table 4. Expression of Result Values and Error in a Data Summary Table

	Data Set 1		Data Set 2		Data Set 3 (preferred)	
	Each Result Value and paired SEM have the same number of decimal places		**All Result Values and all paired SEM have the same number of decimal places (three)**		**Same number of Significant Digits for all Result Values; same number of decimal places for paired SEM**	
Compound	**Result Value**	**SEM**	**Result Value**	**SEM**	**Result Value**	**SEM**
1	0.0410	0.0067	0.041	0.007	0.0410	0.0067
2	2114.4	101.6	2114.437	101.642	2110	102
3	2635	238	2635.146	238.178	2640	238
4	389.3	35.2	389.358	35.261	389	35

Table 4. continued from previous page.

	Data Set 1		Data Set 2		Data Set 3 (preferred)	
5	0.188	0.008	0.188	0.008	0.188	0.008
6	1430.5	100.2	1430.561	100.263	1430	100

It is common practice to express assay results in summarized tables to two or three significant digits, but this can depend on the actual or perceived error associated with the process used in generating the result values. These error statistics (SD and SEM) are discussed further in a subsequent section.

Finally, expressing results using negative log transformations (e.g. pIC_{50}, pK_i, etc.) provide consistency in keeping all result values to the same number of significant digits. The concept of using negative log-transformed result values is discussed later in this chapter.

Logarithms

The logarithm (or log) of a number is the exponent that indicates the power to which another number (the base) is raised to produce the initial number. The "common" log or base-10 log is essentially the only one used in assay and screening applications and will be discussed exclusively in this chapter. The log base-10 (Log_{10}) of a number (n) can be described as shown in the equation below:

Equation 1. Base 10 Logarithm

$$\log_{10} n = \text{a}$$

where 10 is the base and a is the exponent or power to which the base is raised. For example, the base 10 log of 1000 is 3, since 10 raised to the power of 3 (or 10^3) is 1000. When the base is not indicated it means log base 10, by convention. **Table 5** shows some $\log_{10}$ values of several numbers.

Table 5. Log_{10} values of several numbers

Value	Log_{10}
1	0
10	1
100	2
1000	3
842	2.93
35	1.54
0.1	-1
0.01	-2
0.001	-3

Note that the log can be positive, negative or zero. However, log 0 is undefined since no number raised to the power of another number results in zero.

The antilog is the inverse log function. For instance, the antilog of -2 is 10^{-2} or 0.01. Software programs such as Microsoft Excel and others easily calculate log and antilog values using built in functions.

Additional information regarding the use of log values (geomean, pEC50, etc.) can be found elsewhere within this chapter. In addition, the rounding rules described above apply to log values as well.

Statistical Descriptors/Metrics

Arithmetic Mean

The ***mean*** (referred to as arithmetic mean) is the average of all results. To calculate the mean, add up all the result values in the data set and divide the sum by the number of values (n).

Geometric Mean

Use the ***Geometric Mean*** or ***Geomean*** when averaging data that have been calculated from log-normal values. The most common result types to which this often applies are potencies, affinities or inhibition constants (e.g. EC_{50}, IC_{50}, K_i, etc.) that have been determined from concentration response curves. The equation to calculate the Geomean for an EC_{50} is shown below:

Equation 2: Geometric Mean

$$\text{Geometric Mean} = 10^{\left[\left(\frac{\text{Log EC50}_1 + \text{Log EC50}_2 + \ldots \text{Log EC50}_n}{n}\right)\right]}$$

Median

The ***median*** is the middle value of all results in a ranked list. Half of the numbers in the data set will be above the median and half the numbers will be below the median. To calculate the median, rank order the result values in the data set (in ascending order) and determine the middle value. When there is an odd number of results, the median is the middle value. When there is an even number of results, the median is the average between the two middle values.

Examples for Arithmetic Mean, Geometric Mean and Median

The following examples serve to demonstrate the difference between and use of arithmetic and geometric means as well as median values.

The arithmetic mean and the median for three different sets of numbers are compared in Table 6:

Table 6. Arithmetic mean and median for three sets of numbers.

Result Values	Number of Results (n)	Arithmetic Mean	Median
18			
26	3	25	26
31			
18			
26	4	27.5	28.5
31			
35			
15			
18			
31	5	27	31
35			
36			

A key concept is that the mean of a data set can be influenced by outliers, whereas the median is relatively resistant to outliers and is a more robust statistic when several values exist. Consider the example shown in **Table 7** where a few values cause the elevated mean.

Table 7. Effect of an outlier on the arithmetic mean and median.

Result Values	Number of Results (n)	Arithmetic Mean	Median
16	7	53	22
18			
20			
22			
45			
72			
180			

Note: many of the parameters discussed in the *AGM* chapter on Assay Operations for SAR Support (1) refer to using the median instead of the mean.

Table 8 shows the geometric and arithmetic mean for several different sets of result values:

Table 8. Geometric mean and arithmetic mean for different sets of result values.

Result Values	Geometric Mean	Arithmetic Mean
4, 6, 9, 10, 12	7.6	8.2
123, 219, 228, 198, 267	201	207
0.25, 0.67, 0.17, 0.46, 0.91	0.41	0.49
2, 2, 2, 2, 2	2	2
1, 10, 100	10	37

The geometric mean is always less than the arithmetic mean unless all the result values are the same.

Standard Deviation and Standard Error of the Mean

Two statistical quantities that are often (incorrectly) used interchangeably are SEM and SD (14,15). ***SD*** is defined as:

Equation 3: Standard Deviation

$$s = \sqrt{\frac{\sum (X - \bar{X})^2}{n - 1}}$$

where s represents SD, X represents each data point, $\overline{X}$ represents the arithmetic mean of the population, and n represents the number of data points

The SD describes the variation, or dispersion, in measurements relative to the population mean. By contrast, ***SEM*** is defined as:

Equation 4: Standard Error of the Mean

$$s_{\bar{x}} = \frac{s}{\sqrt{n}}$$

where $s_{\bar{x}}$ represents SEM, s represents SD, and *n* represents the number of data points

The SEM describes the probability that the measured mean is different from the population mean. With increasing sample size (assuming constant deviation in measurement), the SEM will approach zero.

SD and SEM represent very different concepts. The SD describes the distribution of individual data points around a mean, while the SEM describes the precision of the mean estimate.

When presenting data, figure legends should explicitly state whether the error bars represent the SD or SEM, as well as the number and type of replicates. Since SEM is always smaller when compared to SD in replicate measurements, we find that SEM is often plotted, presumably to imply less variation in the data. SD should be plotted when trying to convey the variation in the data, while SEM should be plotted when trying to convey the differences in means. In addition, with small data sets, plotting the individual replicates tends to be the best way to demonstrate the variability in the results in a manner universally understood.

Consider the following three examples in **Figure 1** for a concentration-response curve (CRC) where the same data is plotted with SD, SEM, or individual run values at each concentration:

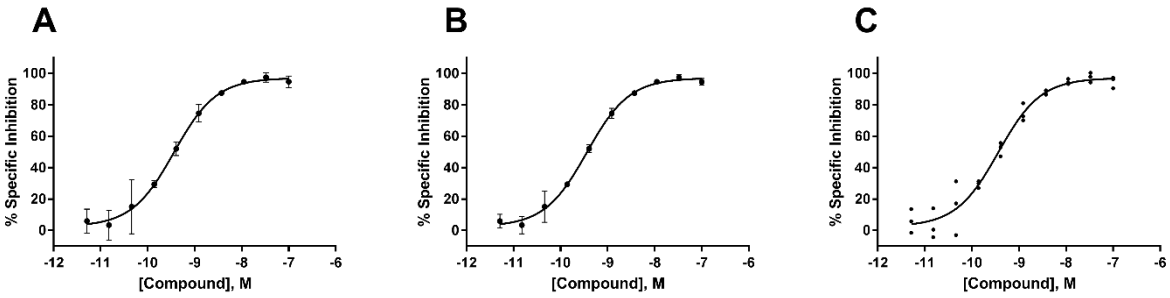


Figure 1. Concentration-response curve plotted with error.

A CRC (n = 3 inter-run replicates) is plotted with the error bars representing the SD (**A**) or SEM (**B**). In (**C**), all three independent values at each concentration are shown as data points on the graph. For **A** and **B**, the data points on the graphs are shown as the median of the independent data values from the three runs.

Confidence Interval

A confidence interval, computed from the statistics of the observed data, is an estimated range that is likely to contain the unknown parameter. For instance, the 95% confidence interval is a range of values that one can be 95% certain contains the true mean of the estimated parameter. The confidence interval for an estimated parameter is the estimate of that parameter plus or minus the quantile corresponding to the desired confidence level from the appropriate distribution (e.g., normal, t, chi-squared, etc.) times the standard error of the estimate. When the distribution is approximately normal, the confidence interval for a sample mean is given by **Equation 5**:

Equation 5. Confidence Interval

$$\bar{x} \pm t^* s_{\bar{x}}$$

where $s_{\bar{x}}$ is the standard error of the mean, as defined above, and t is the quantile from the Student's t-distribution corresponding to the desired confidence level and sample size. For example, $t = 2.26$, for 95% confidence and a sample size of 10 (9 degrees of freedom).

Example of Methods to Plot the Same Data

An example presented in an article that was published in four separate journals shows a set of data visualized using a scatter plot, box-and-whiskers, median and quartiles, mean ± SD, mean with confidence interval (see section below) and mean with SEM (16-19). To display variability, the author suggests showing raw data, the box-and-whisker plot, median and quartiles, or mean ± SD as the most effective methods. Using these principles, **Figure 2** shows all five of these plots representing data for a control compound in an assay performed over a period of time. It demonstrates the impact of each method and the resulting message about variability or error being conveyed. The scatter plot, showing all data points, is typically the preferred format, particularly with many journals.

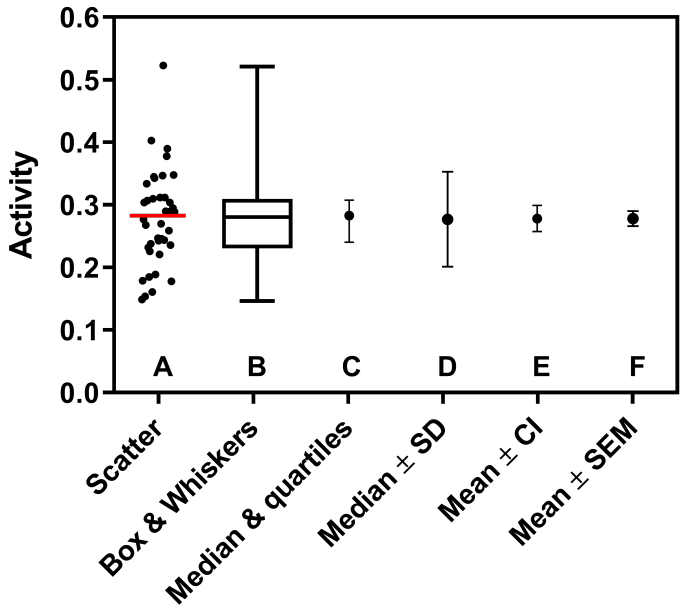


Figure 2. Summarized result for an assay control compound using six different methods.

The activity for an assay control compound from several different runs is plotted on the y-axis using different methods for expressing the variability. (**A**) Scatter plot showing all of the data values used, with the median indicated by the red line; (**B**) Box and whiskers plot, showing the range of data with the median indicated by the line; (**C**) Median and quartiles; (**D**) Mean with error bars (1 SD); (**E**) Mean with 95% confidence interval; (**F**) Mean with standard error of the mean. Adapted from reference (17).

Note that the box-and-whisker plot shows the distribution of a set of data by drawing a box (rectangle) that spans from the first to the third quartile, with a line at the median. The whisker on each end extends either to the most extreme data value or to a distance that is 1.5 times the interquartile range (IQR = third quartile – first quartile) from the end of the box, whichever is shorter. Data values that are more extreme than 1.5 times the IQR are plotted as individual points.

Signal-to-Noise (S/N or SNR), Signal-to-Background (S/B) and Z′-Factor

The **Signal-to-Noise Ratio (SNR** or **S/N)** can be defined as the ratio of the mean signal ($\overline{X}_S$) to the standard deviation of that signal (s_S). Although the S/N parameter incorporates signal variability, it only evaluates one signal in the assay. Typically, assays are optimized with controls that provide biologically relevant high and low signals that together define the dynamic range. The S/N ratio evaluates only one of the two relevant signals and thus does not allow the overall assay quality to be evaluated. In **Figure 3** S/N values are given for high and low assay controls in panels A-D. The S/N values are highest for the two signals in panel A due to the low variability.

Equation 6: Signal to Noise Ratio (SNR or S/N)

$$S/N = \frac{\bar{X}_S}{s_S}$$

The S/N is the reciprocal of the **coefficient of variation (CV)** which is a measure of precision relative to the mean. The coefficient of variation is often expressed as percent. Percent CV values are indicated for both high and low controls in the panels of **Figure 3**. The high and low controls of panel A have the lowest %CV values in **Figure 3** due to the low variability among data points. Alternatively, and to address the limitations noted above, S/N is sometimes defined as the ratio of the difference between high and low signals of the controls and the total variability of the signal from the controls. The "controls" here refer to whatever reflects the dynamic range of the assay signal; for example, they may be the positive control (at high concentration) and negative control (background), presence and absence of enzyme, etc.

Equation 7: Coefficient of Variation (CV)

$$CV = \frac{s}{\bar{X}}$$

The **Signal-to-Background Ratio (S/B)** is defined as the ratio of the high control mean signal ($\bar{X}_{HS}$) to the low control mean signal ($\bar{X}_{LS}$). While this metric is useful for describing the assay dynamic range, it does not incorporate the variability of the high (s_{HS}) or low (s_{LS}) signals. An assay with a reasonable S/B value can still be of poor quality if the variability of either signal or both are high. An example is provided in **Figure 3D**, which shows high variability among both high (%CV = 23) and low (%CV = 37) controls.

Equation 8: Signal to Background Ratio (S/B)

$$S/B = \frac{\bar{X}_{HS}}{\bar{X}_{LS}}$$

The **Z'-Factor** (20) is considered the best metric to describe assay quality because it incorporates the mean values of both high ($\bar{X}_{HS}$) and low ($\bar{X}_{LS}$) control signals as well as their variabilities (s_{HS} and s_{LS}, respectively). The Z'-factor value approaches 1 (an ideal assay) as the signal variabilities approach zero or as the dynamic range approaches ∞. An assay with a Z'-factor value above 0.5 is considered excellent. Below 0.5, the assay quality is considered progressively lower quality as the Z'-factor value approaches zero or becomes negative. Screening is essentially impossible when the Z'-factor value is less than zero. The data shown in **Figure 3** panels A-D have progressively higher variability as demonstrated by the CV values. Notably, the best quality assay is shown in panel A (Z' = 0.89), despite the modest dynamic range (S/B = 3.2). The low variability among both high and low controls is the primary driver of the excellent assay quality in this case. At the other extreme is the data in panel D, which has a Z' value of 0, despite having an S/B value more than 2-fold higher than that of panel A.

Equation 9: Z'-Factor

$$Z' = 1 - \frac{(3s_{HS} + 3s_{LS})}{|\bar{X}_{HS} - \bar{X}_{LS}|}$$

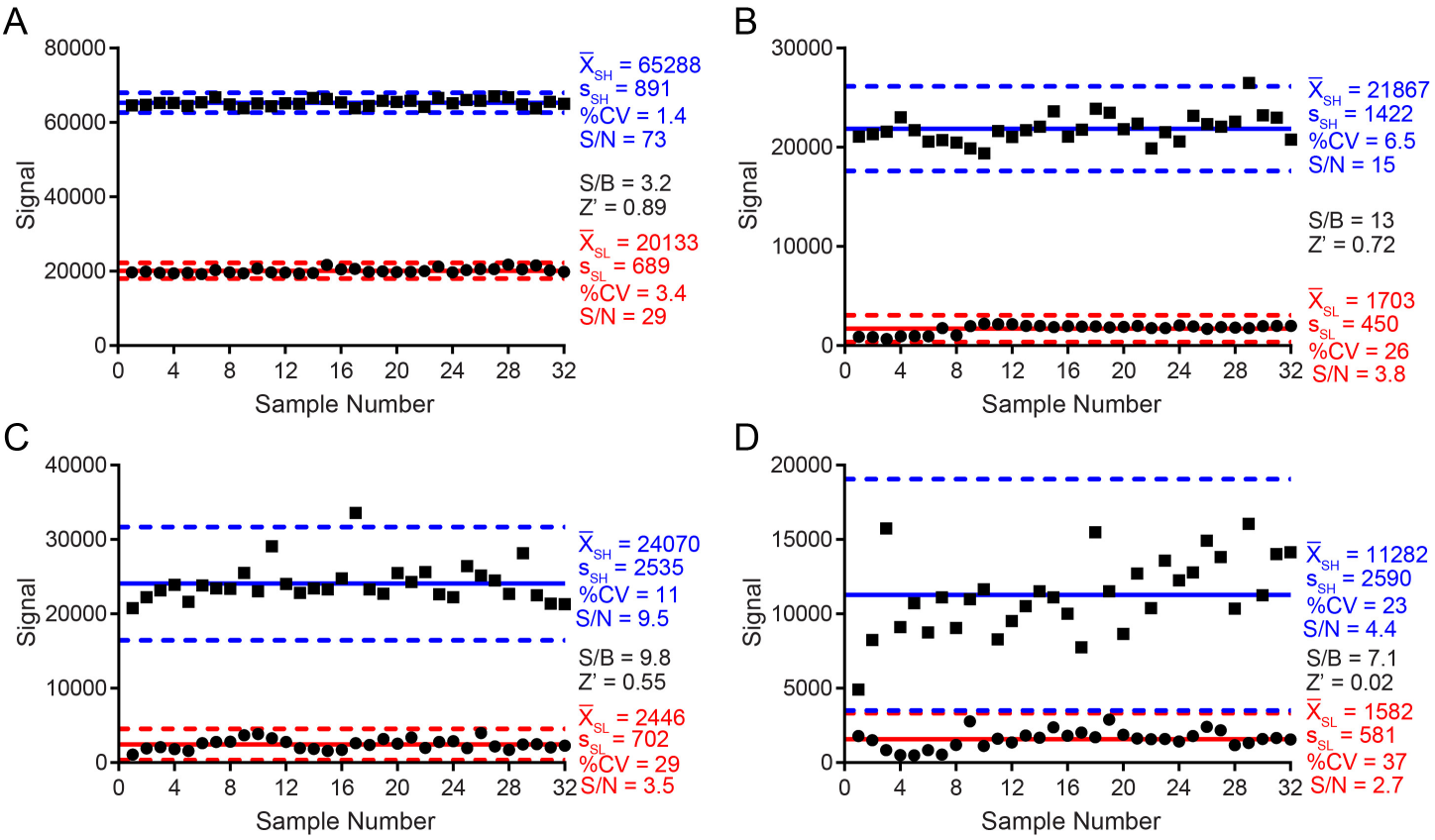


Figure 3. Commonly used metrics to describe features of assays, including S/N, CV, S/B and Z'-Factor.

Assays with varying data quality are shown with calculations of associated parameters. Solid lines represent mean signals and dashed lines represent 3 SD of the mean. (**A**) A high-quality assay has clear separation between high ($\bar{X}_{HS}$) and low ($\bar{X}_{LS}$) mean signals as well as low variability (s_{HS} and s_{LS}, respectively). Although the assay dynamic range can be considered modest (S/B = 3.2), the low variability of high and low signals (%CV = 1.4 and %CV = 3.4, respectively) results in an excellent Z'-factor value of 0.89. The low variability of the high signal results in a large S/N value of 73. (**B**) Despite the greater variability in the high signal (%CV = 6.5) compared to (**A**) that results in a lower S/N value of 15 (compared to 73), this assay has a larger dynamic range (S/B = 13), resulting in a similarly high Z'-factor value of 0.72. (**C**) Compared to (**B**), this assay has approximately 2-fold higher variability for the high signal than (%CV = 11 versus %CV = 6.5), with similar variability in the low signal. This results in lower values of S/N, S/B and Z'-factor. (**D**) Among the four assays, this assay has the highest variability in both high and low signals. Although the S/B value is not substantially lower than that in (**C**), the high variability results in low S/N and a Z'-factor near zero.

Replicates

There has been considerable focus on irreproducible research in science, identifying the issue as a crisis (21,22). Most journals have revised guidelines to address reproducibility within their respective submissions. However, there remains confusion around the definitions of basic scientific terms associated with reproducibility including replicates and repeats (23). Vaux *et al.* stated several fundamental principles related to statistical design with a focus on replicates and that replicates alone do not provide evidence of reproducibility (24). In addition, different disciplines (biology, chemistry, and statistics) may have different meanings for these terms, which adds to the confusion when involved in multi-functional projects or groups.

To minimize confusion and for the purposes of this chapter and the *AGM*, we define *replicates* (technical, independent and inter-run) as they apply to *in vitro* assay development and HTS disciplines. The definitions are followed by some specific examples. Keep in mind that these definitions may differ among various journals or funding agencies.

Technical replicates are measurements of the same sample occurring within **a single run** or experiment. Technical replicates can help to identify within-sample variation but are dependent replicates due to being tested under the same conditions. Technical replicates could be on the same plate or on different plates within the experiment, depending on the variability being assessed.

Independent replicates are measurements of distinct preparations of the same samples occurring within a **single run** or experiment. Comparison of enzyme lots or multiple batches of independently cultured and treated cell preparations are examples of independent replicates for *in vitro* assays. For the best estimate of between sample variations, independent replicates should be on the same plate to minimize the contribution of additional sources of error (e.g. between plate variation). If sample capacity of the plate is limited and multiple plates are required for the study, multiple independent replicates can be randomized to multiple plates with multiple technical replicates per plate.

Inter-run replicates are measurements of the same or different sample(s) **across multiple runs** or experiments. A compound tested in a CRC on three different days represents inter-run replicates. Each of the runs or experiments should have the same assay conditions and reagents.

Depending on the experimental design, the greater the number of measurements, the better the estimate of variation, and the better the estimate of the mean.

When presenting data, it is important to provide the *number* and *type* of replicates (24-26) and to include them in the figure legend. For written work, scientists should consider providing a statement in the experimental methods defining the nature of their technical, independent or inter-run replicates. In general, sample variation may be greater in magnitude than technical variation. The optimal type and number of replicates depends on the scientific question and the experimental methods.

In practice, the distinction between technical, independent and inter-run replicates is not always straightforward. For example, with cell-free/biochemical assays, one approach to modeling variation would be to utilize independently synthesized reagents (e.g., enzymes), though in practice this is either impractical or counter-productive (i.e., in the case of HTS where one is attempting to minimize imprecision). Steps can be taken to mitigate batch-to-batch variation in the context of large scale experiments, for example through the use of batch pooling strategies, which is described further in the AGM Chapter on Validating Identity, Mass Purity and Enzymatic Purity of Enzyme Preparations (27).

Consider the following examples describing typical assay or screening experiments and the type of replicates that would result from each format:

- ***Example 1.*** A compound is tested for inhibition of enzyme X in a 384-well microplate. Three concentration-response curves for this inhibitor are tested on the same plate, with compounds and reagents derived from the same stock solutions and tested at the same time. Variation in this experimental setup would be random, so the best description would be n = 3 technical replicates. Determine an average value at each concentration (mean or median, depending on the variability) and fit a single concentration-response curve for the entire data set. Preferably, fit all individual replicates using the curve fitting routine.
- ***Example 2***. A compound is tested for inhibition of enzyme X in a 96-well microplate. One concentration-response curve for this inhibitor is tested on the same plate. Each well is measured ten times with a plate reader. Variation in this experimental setup would be random, so the best description would be *n* = 10 technical replicates. In this case, unless the detection methodology is not very robust, this would probably constitute a poor choice of replication.
- ***Example 3***. A compound is tested for inhibition of enzyme X in a 384-well microplate. Three concentration-response curves for this inhibitor are tested on different plates, with compounds and reagents derived from the same stock solutions and tested at the same time. Variation in this experimental setup would still be random, so the best description would be *n* = 3 technical replicates. The most

appropriate approach for data analysis is to fit each curve independently and then report the average or geomean of the potency values with variability.

- ***Example 4***. A compound is tested for inhibition of enzyme X in a 384-well microplate. Three concentration-response curves for this inhibitor are tested, with compounds and reagents derived from the same sources, but tested on different days. These would be classified as inter-run replicates, since the same samples were tested, but in different runs. Therefore, $n = 3$ inter-run replicates. The most appropriate approach for data analysis is to fit each curve independently and report the mean and associated error of the results (e.g. EC50)..
- ***Example 5***. A compound is tested for inhibition of enzyme X in a 384-well microplate. Three concentration response curves are prepared for the inhibitor. Each curve is tested with a separate lot of enzyme from Vendor Y. All three curves are run on the same day, on the same plate. This would be an example of independent replicates, $n = 3$ independent replicates. Including technical replicates (multiple aliquots of the same enzyme) would help to determine if between sample differences are greater than the within sample variation. Determine a single response curve for each enzyme lot.
- ***Example 6***. A compound is tested for inhibition of enzyme X in a 384-well microplate. Three concentration response curves are prepared for the inhibitor. Each curve is prepared with a completely independent synthesis of the compound, i.e. each is a unique lot or batch. All three curves are run on the same day, on the same plate. This would be an example of independent replicates, $n = 3$ independent replicates. Determine a single response curve for each compound lot.

Figure 4 shows example curves that might be associated with data for technical replicates (**4A**), independent replicates (**4B**) and inter-run replicates (**4C**).

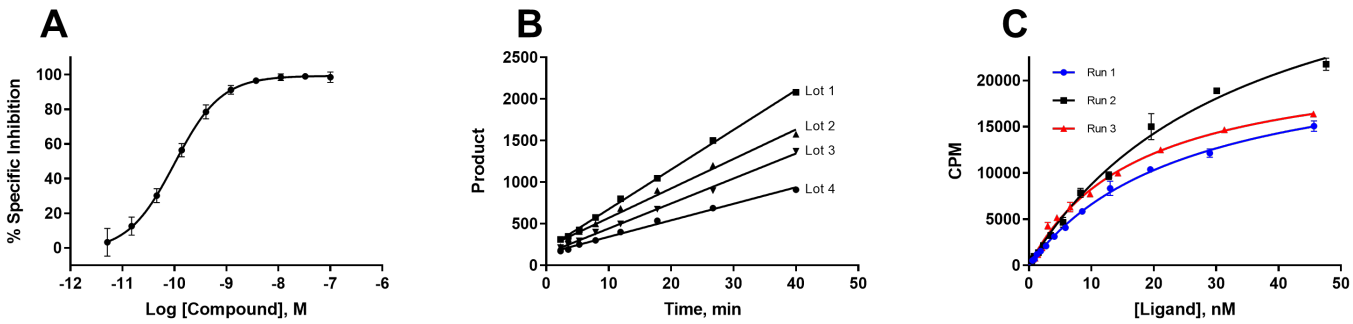


Figure 4. Comparison of technical replicates, independent replicates, and inter-run replicates.

(**A**) Three independently-prepared concentration-response curves (from the same compound stock) were tested on the same plate within a single experiment. This represents technical replicates ($n = 3$). Data points are the median result values with error bars indicated as SD. (**B**) Enzyme progress curves for four different lots of enzyme all tested within the same experiment. This represents independent replicates ($n = 4$). In this example, each lot was tested only once, so there were no technical replicates associated with the data. (**C**) Three independent saturation-binding experiments were performed on different days. All assay conditions and reagents were identical for each of the three runs. Within each run, there were three replicates for each concentration tested. This example represents inter-run replicates ($n = 3$) with $n = 3$ technical replicates within each run. Error bars represent SD.

There are no standard guidelines for how to treat technical versus independent replicates, and in practice it will depend on the scientific question and sources of error. In a system with both technical and independent replicates, if there is significant biological error, then it may be useful to analyze independent replicates (with any associated technical replicates) separately. In such a case of significant biological error, the source should be investigated or explicitly addressed.

A related concept is the number of independent experiments or inter-run replicates required to understand the variation of a process or an assay, which has been described for assay validation studies (28). Again, the

definition of a truly "independent" experiment may not always be straightforward. Independent experiments are typically performed on separate days using reagents that originate from the earliest possible source (enzyme stock, cell line stock, etc.).

There is not a consensus for the number of individual technical/independent replicates or independent experiments (inter-run replicates) required to understand the variation of a process or an assay. Several factors (time, reagent cost, etc.) may limit the number of practical replicates that can be conducted. In addition, journals may have their own guidelines on the number of replicates required for acceptable publications. Consider these factors with any study and consult a statistician to ensure an adequate level of replication.

Be cautious when terms such as "duplicate", "triplicate" or "quadruplicate" measurements are being used and be sure that the meaning of these terms is clearly understood. It can be very confusing when reading figure legends in publications, since there are so many variations on how to write up technical replicates, independent replicates and inter-run replicates, etc. Some examples from a few issues of the same journal are shown below. These examples represent the difficulty in interpretation that can happen when there are no standards or consistency.

- Plotted is the mean of triplicate reactions
- Data points represent the mean of triplicate measurements
- Data are mean ± SEM for 16 replicates
- Data are expressed as means ± SD of a representative experiment performed in quadruplicate out of three independent experiments.
- Data points represent the mean ± SD (n = 3) of three independent experiments.
- Shown are representative figures for n = 3 independent experiments
- Data shown are the mean ± SD; n = minimum of 2 wells
- Data points represent the mean and standard deviation of three independent replicates.

These definitions and guidelines presented for technical replicates, independent replicates, and inter-run replicates may help in interpreting and understanding data. It is important to provide additional information on replicates such as whether the replicates came from the same or independent sample stock, plates, experimental runs or from multiple runs. For example, simply stating that the "*data represents 16 replicates*" is not useful. An example of a representative legend can be found in the Figure Legends section of this chapter. In addition, a couple of direct examples from a journal that has figure legends that fully describe the data points and error in graphs are shown below:

- Concentration-response curves, fitted according to the Hill equation, are shown for three technical replicates from the same assay plate. Error is represented by SD.
- Results are expressed as the geometric mean of 5 independent measurements all made on separate days. Error = SD.

Random and Systematic Error

This section describes random and systematic error. Understanding the type of error that may exist in an assay is an important counterpart to experiment replication and the assessment of variability in an assay.

Random errors are unpredictable and have no defined pattern. Random errors are fluctuations that are not replicated in subsequent experiments. Sources of random error may include the precision limitations of detection or liquid handing instrumentation, changing temperatures in a laboratory and fluctuations in the assay methods. An example of random error is measuring the same sample in an instrument with three different result values. As described above, technical or biological replicates can provide an estimate of variation for understanding random error within an assay. Another example of random error occurs when measuring radioactive experiments such as scintillation proximity assays or SPA as described in the *AGM* chapter Receptor

Binding Assays for HTS and Drug Discovery (29). The variability associated with random error from radioactivity can be reduced by increasing the read time (30).

Systematic errors are persistent (unless addressed) and can be associated with instrumentation, technique or the experimental design itself. An improperly calibrated instrument can lead to constant variation. Often systematic error results in values that are proportional or scaled to the true value. Examples of systematic error in HTS can include an incorrect wavelength setting/emission filter on a detector, faulty liquid and compound dispensing instrumentation, positional effect within a detector (31), time drift in a stack of microplates being read, and edge effects associated with evaporation. Systematic errors may be difficult to identify, but a thorough knowledge of equipment and the experimental methods being used are a critical part of detecting and minimizing the effects. One group has written extensively about minimizing the impact of systematic errors in HTS data (32-35) including those associated with assay-specific and plate-specific spatial biases (36).

Some examples of plate drift and spatial effects, which are systematic errors, are shown in the HTS Assay Validation chapter of the *AGM* (28).

Correlation of Data

Pearson's Correlation

Pearson's correlation measures the strength of the *linear* relationship between two sets of variables and is therefore equivalent to a linear correlation. An underlying assumption is that both variables are normally distributed (bell-shaped, symmetrical distribution of data). Only extreme or obvious departures from this assumption are problematic. Other names for Pearson's correlation include product moment correlation coefficient (PMCC) and Pearson's *r*. Pearson's correlation returns a value (the correlation coefficient, *r*) between -1 and 1 where:

$r = -1$ indicates a perfect negative linear relationship

$r = 1$ indicates a perfect positive linear relationship

$r = 0$ indicates no linear relationship

The larger the absolute value of the correlation coefficient, the stronger the linear relationship. The meaning of the correlation coefficient size varies in the scientific literature, but one suggested range (37) is shown in **Table 9**.

Table 9. Meanings for Pearson's correlation coefficient from Reference (37).

Absolute size of correlation (positive or negative)	Interpretation of correlation
0.90 to 1.00	Very high correlation
0.70 to 0.90	High correlation
0.50 to 0.70	Moderate correlation
0.30 to 0.50	Low correlation
0.00 to 0.30	Negligible correlation

The correlation coefficient number alone is not adequate for demonstration of statistical relevance. Different patterns of Y vs X relationships can have the same correlation coefficient (11,38) as shown in **Figure 5**. Therefore, it is always important to plot the data for any reported correlation and consult with a statistician if the resulting correlation coefficient does not match an expected visual representation of the data.

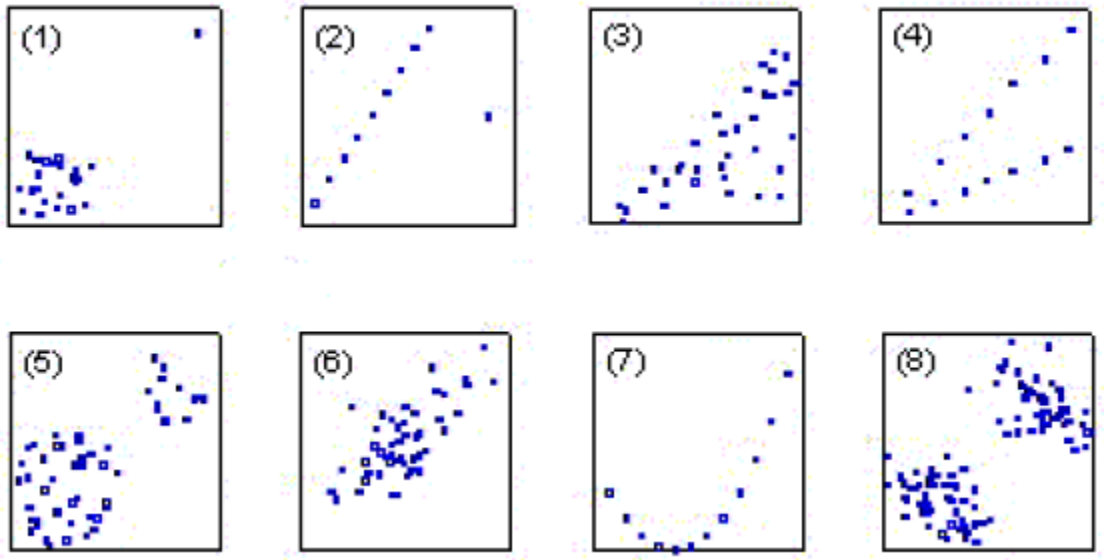


Figure 5. Example plots of 8 different data sets with the same Pearson correlation coefficient value (r=0.7).

All the graphs shown have Pearson correlation coefficients equal to 0.7. The example in graph 6 is the one most typically perceived when the correlation coefficient is 0.7. (Reprinted from (38) with permission.)

Spearman's Correlation

Spearman's correlation is a nonparametric approach to correlation, which means the variables are not assumed to have a normal distribution. It measures the strength of the rank-ordered relationship between two variables. Computationally, Spearman's correlation is the linear correlation of the ranks of each variable. That is, the values for each variable are separately assigned ranks 1 to n, and Spearman's correlation is calculated on the ranks. The result is a value (the correlation coefficient, ρ) between -1 and 1 and can be interpreted in a similar fashion to Pearson's correlation shown in **Table 9**.

Correlation Example

This subsection describes a common analysis made in the drug discovery process using the correlation concepts described in the previous sections.

Consider the EC_{50} results in **Table 10** for two cell-based assays associated with different species, Assay 1 and Assay 2. The goal of the analysis is to determine whether there is a correlation between the performance of the two assays, such that one assay could be used for predictive purposes over the other assay, if needed.

Table 10. EC50 values for two cell-based assays used for correlation analysis.

	EC_{50}, nM	
Compound	**Assay 1**	**Assay 2**
1	0.33	0.29
2	0.41	0.74
3	0.52	0.24
4	0.83	0.42
5	1.1	1.2
6	1.2	0.68

Table 10. continued from previous page.

	EC_{50}, nM	
7	1.6	1.9
8	2.2	1.2
9	2.5	2
10	3	4
11	6	2
12	7	6
13	11	12
14	16	7
15	19	22
16	25	8
17	26	18
18	30	27
19	35	12
20	40	1

Performing a linear regression using GraphPad Prism software with the data yields the graph in **Figure 6** and the Pearson's correlation coefficient (r = 0.62). The x-axis is a linear scale.

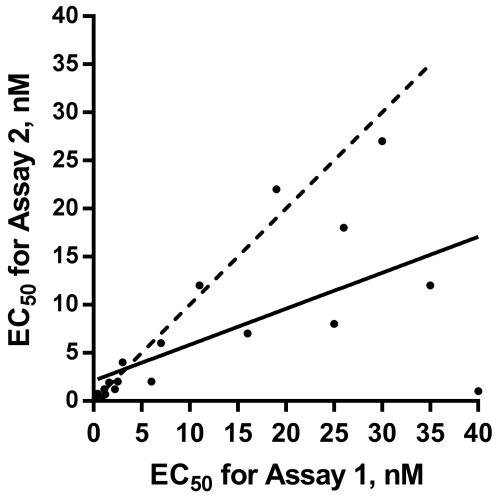


Figure 6. Linear correlation using EC_{50} values.

EC_{50} values in nM (from **Table 10**) determined from two different CRC assays were plotted on a linear x-axis (Assay 1) and a linear y-axis (Assay 2). Shown is the regression line (solid line) determined from the analysis along with the line of identity (dashed line, defined as unity or x=y). The Pearson's correlation coefficient is r=0.62.

Note that the data points are tightly clustered at the lower end of the regression line. Pearson's correlation for such highly clustered data would give disproportionately higher weight to a few data points. Since the potency data was originally derived from nonlinear regression of log-transformed concentrations (x-axis), using log transformed potency values would be the correct method for determining correlations. The use of a log transformation on potency values provides a symmetric (approximately normal) distribution, and thus all points

would more equally contribute to the calculated value of Pearson's correlation. It is recommended to perform the log transformations of the potency values using the molar scale to avoid issues with negative and positive log values in the same scale. Furthermore, some scientists prefer to express the data in terms of the negative $\log_{10}$ EC_{50} using molar units, which is referred to as pEC_{50} in the literature. The same correlation coefficient will be obtained whether using log-transformed or pEC_{50} values. The data in **Table 10** are now updated in **Table 11** to include the results in terms of $\log_{10}$ EC_{50} (Molar) and pEC_{50}.

Table 11. Results for Assay 1 and Assay 2 in Table 10 converted to Log EC50 and pEC50 values.

	EC_{50}, nM		EC_{50}, Molar		Log EC_{50} Molar		-Log Molar = pEC_{50}	
#	**Assay 1**	**Assay 2**	**Assay 1**	**Assay 2**	**Assay 1**	**Assay 2**	**Assay 1**	**Assay 2**
1	0.33	0.29	3.3E-10	2.9E-10	-9.48	-9.54	9.48	9.53
2	0.41	0.74	4.1E-10	7.4E-10	-9.39	-9.13	9.38	9.13
3	0.52	0.24	5.2E-10	2.4E-10	-9.28	-9.62	9.28	9.62
4	0.83	0.42	8.3E-10	4.2E-10	-9.08	-9.38	9.08	9.37
5	1.1	1.2	1.1E-09	1.2E-09	-8.96	-8.92	8.95	8.92
6	1.2	0.68	1.2E-09	6.8E-10	-8.92	-9.17	8.92	9.16
7	1.6	1.9	1.6E-09	1.9E-09	-8.80	-8.72	8.79	8.72
8	2.2	1.2	2.2E-09	1.2E-09	-8.66	-8.92	8.65	8.92
9	2.5	2	2.5E-09	2.0E-09	-8.60	-8.70	8.60	8.69
10	3	4	3.0E-09	4.0E-09	-8.52	-8.40	8.52	8.39
11	6	2	6.0E-09	2.0E-09	-8.22	-8.70	8.22	8.69
12	7	6	7.0E-09	6.0E-09	-8.15	-8.22	8.15	8.22
13	11	12	1.1E-08	1.2E-08	-7.96	-7.92	7.95	7.92
14	16	7	1.6E-08	7.0E-09	-7.80	-8.15	7.79	8.15
15	19	22	1.9E-08	2.2E-08	-7.72	-7.66	7.72	7.65
16	25	8	2.5E-08	8.0E-09	-7.60	-8.10	7.60	8.09
17	26	18	2.6E-08	1.8E-08	-7.59	-7.74	7.58	7.74
18	30	27	3.0E-08	2.7E-08	-7.52	-7.57	7.52	7.56
19	35	12	3.5E-08	1.2E-08	-7.46	-7.92	7.45	7.92
20	40	1	4.0E-08	1.0E-09	-7.40	-9.00	7.39	9.00

The linear regressions using the Log_{10}-transformed EC_{50} values (Molar) or the pEC_{50} values from **Table 11** are shown in **Figures 7A** and **7B**.

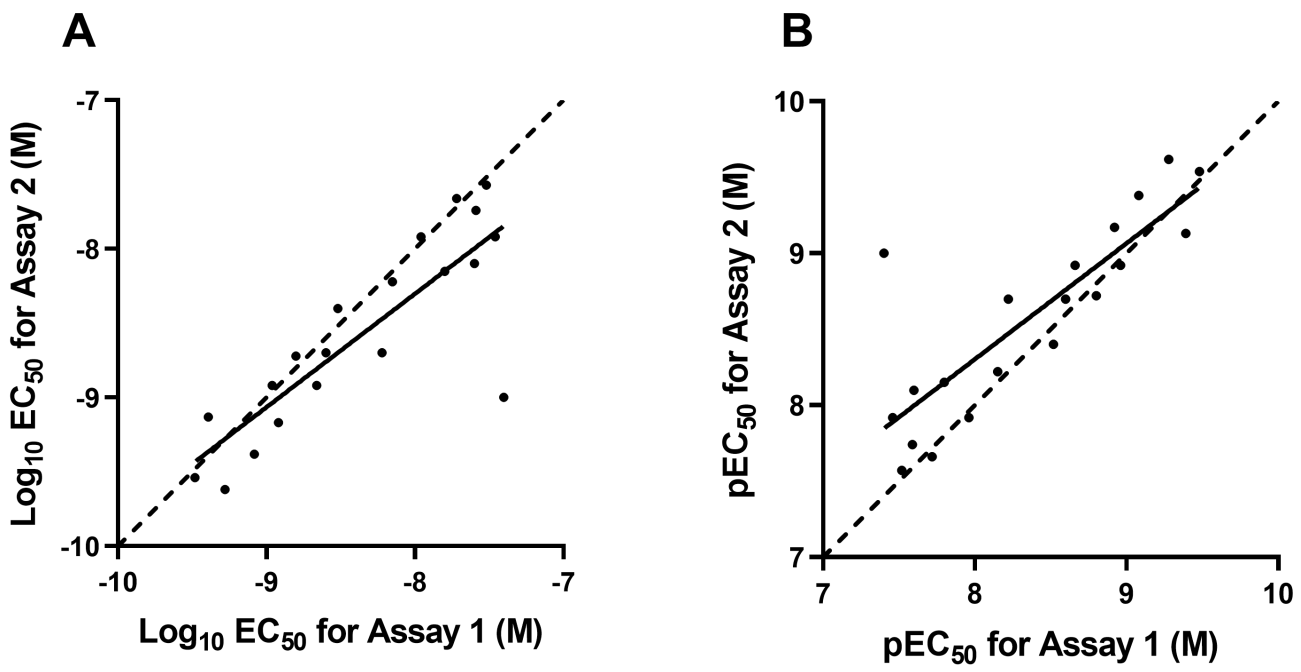


Figure 7. Linear correlation using Log_{10} transformed EC_{50} and pEC_{50} values.

(**A**) EC_{50} values (Molar) determined from two different CRC assays (**Table 11**) were Log_{10}-transformed and plotted on a log-transformed linear x-axis (Assay 1) and a log-transformed linear y-axis (Assay 2). (**B**) A similar plot was created using pEC50 values (-Log Molar) for Assay 1 and Assay 2. Shown in both plots are the linear regression line (solid line) and the line of identity agreement, x=y (dashed line). Note that the correlations are mirror opposites, since the pEC_{50} (Plot **B**) is the -Log Molar EC_{50} (Plot **A**). The Pearson's correlation coefficient is 0.83 in both plots.

The Pearson's correlation coefficient for this example is now 0.83, and adequately displays the linear relationship between the two assays, since the data points are more evenly dispersed across the data range on the log-transformed linear scale.

To determine Spearman's correlation coefficient for this example, the compound result values (either EC_{50} or log transformed) are ranked for each assay from one-to-*n*. The analysis can be performed in GraphPad Prism, JMP, or another suitable statistics software. **Figure 8** shows a plot of the ranks for each assay. Spearman's ρ is 0.80 for this data set.

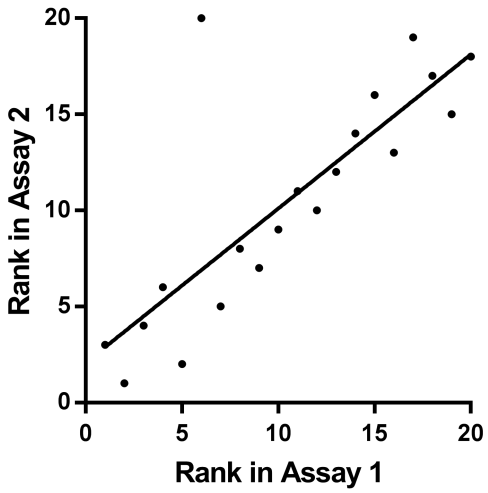


Figure 8 Plot of the ranked values for each assay in Table 10.

Spearman's correlation coefficient for ranked data from **Table 10**, $\rho = 0.80$.

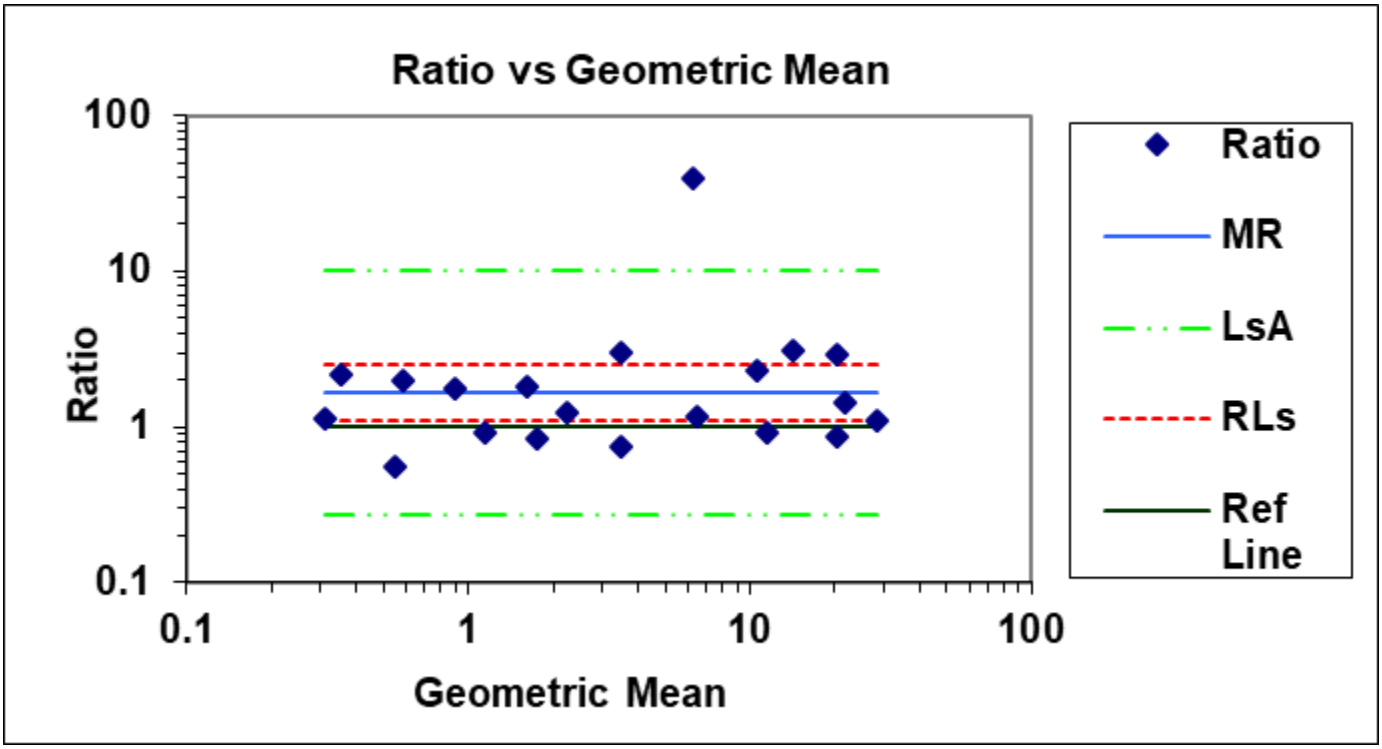
Concordance Correlation Coefficient

Another parameter that will be useful to evaluate and report for comparing variables that are expected to yield identical results is the Concordance Correlation coefficient (39). This correlation measures the degree of agreement between the values of two variables in relation to the 45-degree line (line of agreement). Mathematically, it is approximately equivalent to first evaluating the Pearson's correlation, which measures the degree of closeness of the values to the best straight line (least-squares regression line), and then penalizing this correlation value based on the degree of the departure of this best straight line from the 45-degree line. Therefore, the Concordance Correlation value can never be greater than the Pearson's correlation value. This calculation is also available in the Replicate-Experiment analysis Excel template referenced below.

Agreement Between Two Variables

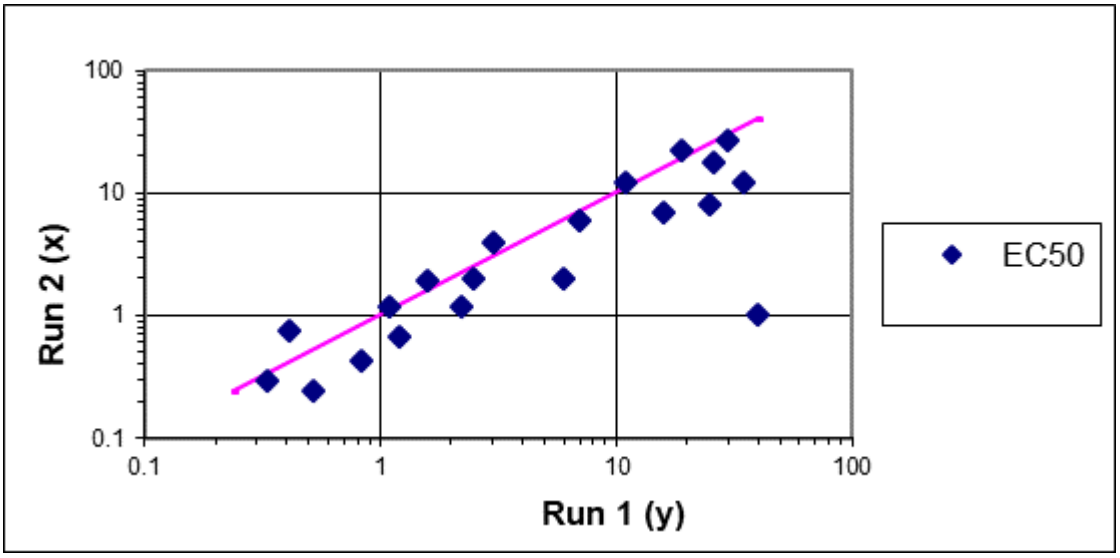
If the interest is more in assessing the agreement between two variables, then the Bland-Altman method (40) demonstrated in the Replicate-Experiment template available in the Replicate-Experiment Study section of the *AGM* chapter on HTS Assay Validation (28) could be used, followed by correlation assessments. Examples of such scenarios include comparison of two assays that are expected to give similar results, comparison of results from the same assay from different laboratories or assay reagents. From this analysis, additional information such as the limits of agreement (LsA), mean ratio (MR), minimum significant ratio (MSR), and other statistical parameters are estimated.

The Replicate-Experiment analysis for the data in **Table 10** is shown in **Figure 9**.



N		Value	95% LCL	95% UCL
	Mean Ratio (MR)	1.654	1.086	2.519
20	Agreement Limits		0.274	9.991

N	MSR (N)
1	6.041
2	3.567
3	2.825
4	2.458



Concordance Correlation Coefficient			Pearson	Spearman
Value	95% LCL	95%UCL		
0.49	34.18	62.12	61.88	79.44

Figure 9. Agreement between Assay 1 and Assay 2 using data from Table 10.

Results from the Replicate-Experiment template available in the *AGM* for the comparison of Assay 1 and 2 data obtained from **Table 10 or 11**.

P-values

In 2016, the American Statistical Association published an official statement on p-values (41). A list of the key principles from that statement is shown below:

- P-values can indicate how incompatible the data are with a specified statistical model.
- P-values do not measure the probability that the studied hypothesis is true, or the probability that the data were produced by random chance alone.
- Scientific conclusions and business or policy decisions should not be based only on whether a p-value passes a specific threshold.
- Proper inference requires full reporting and transparency.
- A *p*-value, or statistical significance, does not measure the size of an effect or the importance of a result.
- By itself, a *p*-value does not provide a good measure of evidence regarding a model or hypothesis.

We refer the reader to the main article and a discussion article for further information (41,42).

A key topic related to p-values is multiple comparisons or multiple testing. When multiple groups from the same experiment are compared pairwise or in relation to a control group, the unadjusted p-values can overstate the overall significance. Appropriate adjustments should be made using methods such as Bonferroni, Dunnett, or Tukey depending on what is being compared (i.e., comparisons vs. a control group or all possible pairwise comparisons between groups) (42). In situations with a very large number of comparisons, such as with 'omics data, the False Discovery Rate (FDR) method is often recommended (43).

Calculation of Upper and Lower Quantitation Limits

Assays where the analyte concentration levels are calibrated from a reference standard curve entail special statistical considerations and evaluation of additional parameters during the development, optimization and validation, as explained in the Immunoassay Methods chapter in this manual (44). This includes accuracy, intermediate precision, sensitivity, dynamic range with the lower and upper quantitation limits, dilution linearity, parallelism, stability, etc. The lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ) are defined as the lowest and highest concentrations of the analyte that can be reliably measured by the assay based on pre-specified criteria around accuracy, imprecision and sometimes total error (see Table 8 in the Immunoassay Methods chapter of the *AGM* (44)). Accuracy is typically defined in terms of percent relative bias of the measured analyte concentration from the calibration curve relative to its spiked nominal level. Imprecision (also referred to simply as precision) is defined by the percent coefficient of variation. Total error is defined as the sum of the absolute value of relative bias plus the imprecision (%CV). The criteria for total error, accuracy, and imprecision vary on a case by case basis. For example, accuracy and imprecision are typically expected to be < 15% for PK assays of small molecules, < 20% for PK assays of large molecules, < 25 to 30% for biomarker assays. For biomarker assays, a white paper from the cross-industry working group (45) proposed the criteria for LLOQ and ULOQ to be based on 30% total error, 25% accuracy, and 25% imprecision.

As mentioned above, a Microsoft Excel-based template for the analysis of pre-study validation data for these types of assays associated with the calculation of assay performance characteristics such as accuracy, precision, sensitivity, etc., is available in the Replicate-Experiment Study section of the *AGM* chapter on HTS Assay Validation. An example output from this Excel-based tool is provided in **Figure 10**. This is a graph of the Bias, Imprecision (%CV) and Total Error for each validation sample. If the LLOQ and ULOQ are defined as the lowest and highest concentrations where the bias and imprecision are less than 25% and the total error is less than 30%, then in this example dataset, the LLOQ is 5.9 pg/mL and the ULOQ is 550 pg/mL.

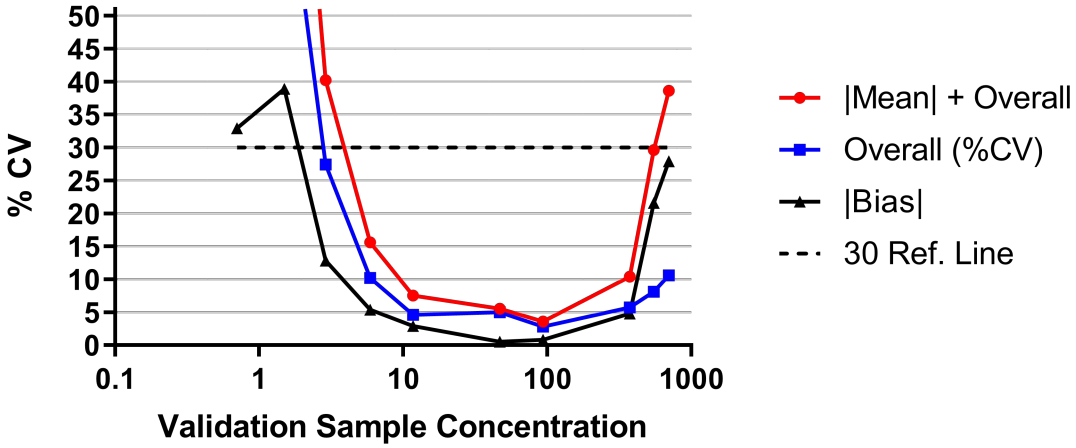


Figure 10. Profile for Total error, precision and bias.

Profile of the % relative error (bias), % imprecision (coefficient of variation; CV) and % total error for the samples used in a validation experiment. Additional assay parameters such as the sensitivity and dynamic range are derived from this analysis. For practical purposes the useful calibration range is below the 25-30% CV line.

Area under the Curve

The area under a concentration response curve or kinetic time course can be quantified and used as a metric to compare data, which is referred to as the area under the curve (AUC). This metric provides a basis for

comparing data when it is not possible to determine the values of EC_{50} and E_{max}. However, even when these parameters are determined, the AUC can be preferable because it incorporates both potency and efficacy within a single metric (46). **Figure 11** illustrates the concept of AUC.

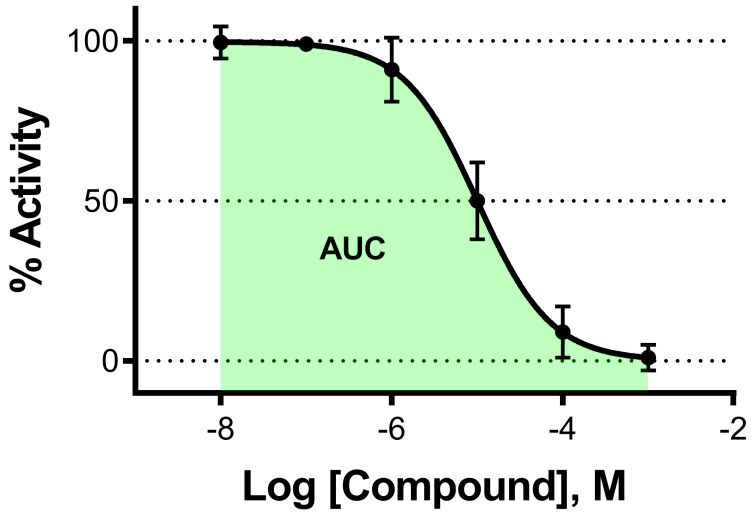


Figure 11. CRC plot illustrating the quantification of area under the curve (AUC).

Area under the curve (AUC) is shown as the green shaded region. The advantage of calculating AUC is that both potency and efficacy are incorporated into a single metric.

Graph Elements

Title

A graph in written format typically has a figure number and a legend title (see Figure Legends section below) whereas a graph used in a presentation or discussion may have only a title. This title can provide the speaker with a visual cue, but it should be succinct so that it does not distract the listener or reader from the message being delivered, often during a short time period. Examples of succinct titles for a standard CRC like those shown previously in **Figure 1** might be:

Concentration-Response Curve for Compound X

CRC for Compound X

or simply, *Compound X*

Much of the context for a graph in a discussion/presentation format can be provided during the presentation.

Figure Legends

Figure legends (also referred to as captions) are crucial components of scientific figures in the literature. Remember, figures and their accompanying legends should function as stand-alone material. In other words, readers should be able to interpret the *overall message of the figure without having to consult the primary text*. High-quality figure legends should contain the following: title, description of techniques used, summary of results, and definitions. Note that depending on the publication source, certain components may be emphasized or de-emphasized. Figure legends typically comprise 100 to 300 words in total.

Titles should be brief and either descriptive ("Inhibition of enzyme X by compound Y") or declarative ("Compound Y is a nanomolar inhibitor of enzyme X"). The choice of descriptive versus declarative titles may depend on journal formats or author preference. For multi-panel figures, the title should encompass a common message for all of the panels.

The techniques used should be briefly described ("Inhibition of enzyme X was determined by radiolabeled substrate incorporation") and should be minimized to only what is necessary to understand the figure. This should include the number and type of replicates as well as independent experiments, whether the results are pooled or representative and any statistical tests utilized (e.g., SD or SEM).

Depending on the nature of the data and figure, a brief statement about the key results should be included in the figure legend ("Compound Y is 5-fold more potent than the previously reported Compound Z").

Lastly, figure legends should define any abbreviations, symbols, coloring, or scaling. Uncommon features, such as broken axes, may need to be explicitly noted (see below).

Other important miscellaneous notes:

- For multi-panel figures, it may not be possible to describe each panel in detail. In such cases, it may be most effective (and efficient) to summarize several related panels in one statement.
- Utilize consistent verb tense. Past tense is used most often for describing completed experiments ("Inhibition of enzyme X was determined by radiolabeled substrate incorporation"), while present tense can be used for declarative statements ("Compound Y is a nanomolar inhibitor of enzyme X").

To illustrate these concepts, consider the graph in **Figure 12** and several examples of a figure legend:

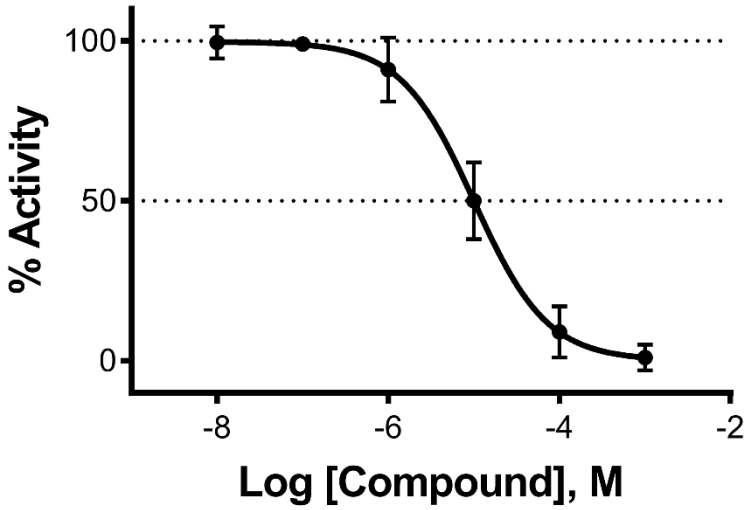


Figure 12. CRC plot with three example figure legends shown below.

Lower-quality example figure legend:

Figure 12. Effect of compound X versus enzyme Y.

Better-quality example figure legend:

Figure 12. Inhibition of enzyme Y by compound X. Enzymatic activity was determined using a radiolabeled substrate assay in triplicate. Compound X inhibits enzyme Y with an IC_{50} value of 10 μM.

High-quality example figure legend:

Figure 12. Inhibition of enzyme Y by compound X. Enzymatic activity of enzyme Y was determined using radiolabeled substrate assay. Compound X inhibits enzyme Y with an IC_{50} value of 10 ± 2 μM. Data are mean ± SD from three technical replicates.

Axis Scale

Most graphs will have a single horizontal axis (x-axis) which corresponds to the independent variable and a single vertical axis (y-axis) which corresponds to a dependent variable. The axis scale can vary depending on the type of data being displayed and the same data on different scales may not convey the same message. The main types of scales used in assay and screening applications include linear (or arithmetic) or logarithmic (or log). In addition, when data is converted to log values, the data points can be plotted on a linear scale.

- **Linear scale** – a linear scale will show equal spacing between the scale units or tick marks. With a linear axis, the baseline often begins with zero. Log-transformed data can be plotted on a linear scale as well.
- **Log scale** – a log scale will have unequal spacing between scale units or tick marks. Major tick marks will have a consistent ratio between them, such as ten. By definition, logarithmic axes do not contain negative numbers. In addition, zero cannot be plotted on a logarithmic axis. To use a logarithmic scale, the actual data values are plotted.

Consider the data values from the curves in **Figure 2**, shown in **Table 12** with the concentration (x-axis) listed in the native format (Concentration, [nM]) and in the log-transformed format (Log M).

Table 12. CRC data with x-axis values in native and log-transformed formats.

Concentration, [nM]	Concentration, [Molar]	Log M	Replicate 1	Replicate 2	Replicate 3
100	1.00E-07	-7.00	97.3	90.5	96.4
33.3	3.33E-08	-7.48	94.3	97.9	100.4
11.1	1.11E-08	-7.95	93.3	94.3	96.6
3.70	3.70E-09	-8.43	86.6	86.9	89.2
1.23	1.23E-09	-8.91	72.7	70.3	81.0
0.412	4.12E-10	-9.39	53.1	47.3	55.7
0.137	1.37E-10	-9.86	27.0	31.4	30.0
0.0457	4.57E-11	-10.34	31.4	-3.0	17.2
0.0152	1.52E-11	-10.82	14.1	-4.2	0.5
0.00508	5.08E-12	-11.29	13.7	-1.5	5.8

The data in **Table 12** can be plotted on a log scale (**Figure 13A**), linear scale (**Figure 13B**) and using log-transformed data on a linear scale (**Figure 13C**).

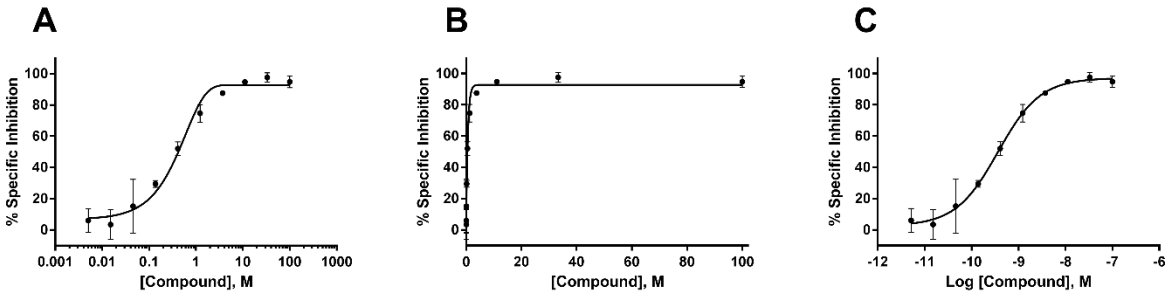


Figure 13. CRC data plotted with three different x-axis scales.

Data from **Table 12** was plotted using a log x-axis scale (**A**), linear x-axis scale (**B**) or log-transformed data on a linear x-axis scale (**C**). Data points for all graphs are the mean of three technical replicates. Error bars represent SD. The same nonlinear equation (4-parameter Hill equation (47)) was used for each curve fit.

Comments about each sub-figure are shown below:

Figure 13A. This figure uses the actual concentration values (Molar) with an x-axis log scale. The resulting curve fit from GraphPad Prism is ambiguous (compare the curve fit of **13A** and **13C**, which both use the same nonlinear curve fitting routine, but difference x-axis scales). This type of scale is not typically used with CRC data.

Figure 13B. This figure uses a linear scale for the actual concentration values. As a result, the data points on the graph will be clustered at one end of the scale and the curve fit is also ambiguous. This type of scale is seldom used with CRC data that has a concentration range over several log scales.

Figure 13C. This figure uses log-transformed concentration values on a linear scale and is the most common method for plotting CRC data. The common sigmoidal curve resulting from the nonlinear regression analysis is shown.

In a different example, consider the data in **Table 13** from a standard radioligand binding saturation analysis (29). The goal of this type of experiment is to determine a plateau of binding activity that results from varying the concentration of radioligand (x-axis) and measuring the binding response (pmol/mg).

Table 13. Data from a saturation binding analysis.

nM	Log nM	pmol/mg
0.30	-0.516	0.385
0.53	-0.274	0.587
0.98	-0.008	0.908
1.7	0.238	1.38
2.7	0.432	1.93
4.4	0.646	3.13
6.9	0.842	3.70
11	1.05	4.57
18	1.26	5.00
29	1.47	5.35
47	1.67	5.68
75	1.87	5.77

Like **Figure 13**, the data in **Table 13** was plotted using the concentration vales on the x-axis with a log scale (**Figure 14A**), a linear scale (**Figure 14B**) and a linear scale using log-transformed data (**Figure 14C**).

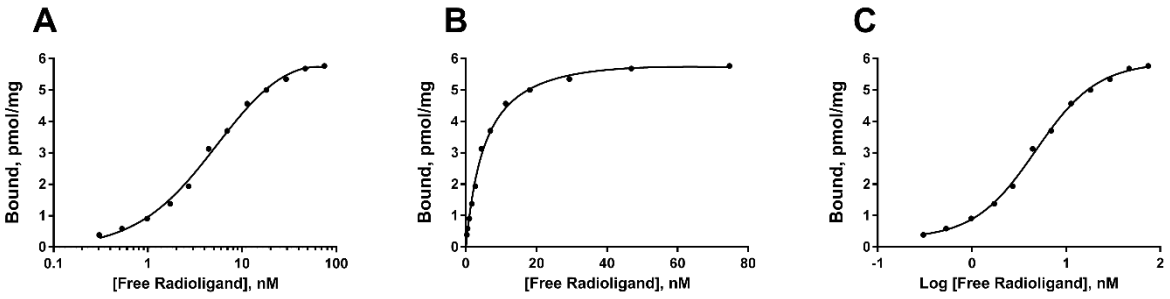


Figure 14. Saturation binding data plotted with three different x-axis scales.

Data from **Table 13** was plotted using a log x-axis scale (**A**), linear x-axis scale (**B**) or log-transformed data on a linear x-axis scale (**C**). Data points are from a single measurement at each concentration level and were fit in GraphPad Prism with nonlinear regression routines as follows: (**A**) and (**B**) using a one-site hyperbolic function; (**C**) using a four-parameter log inhibitor versus response function.

Figure 14A. Using the actual concentration values with an x-axis logarithmic scale can yield information about the level of saturation achieved.

Figure 14B. A linear scale is used with the actual concentration values. The binding activity does not appear to change after ~30 nM concentration on the x-axis.

Figure 14C. Log-transformed concentration values on a linear scale are possible in software programs, however, this format is seldom used.

The graph in **Figure 14B** is most commonly used when evaluating saturation binding experiments. However, the graph in **Figure 14A** may be a better example of demonstrating that the binding activity reaches a significant asymptote.

Therefore, the type of scale chosen for each graph should be carefully evaluated to ensure that information is conveyed as desired.

Axis Scale Range

In most cases, the vertical (y) axis should begin at zero. Not having the origin begin at zero can distort the relative magnitudes between data values. This concept is shown in **Figure 15A** (scale begins at 1.6) and **Figure 15B** (scale begins at zero) for the same data values.

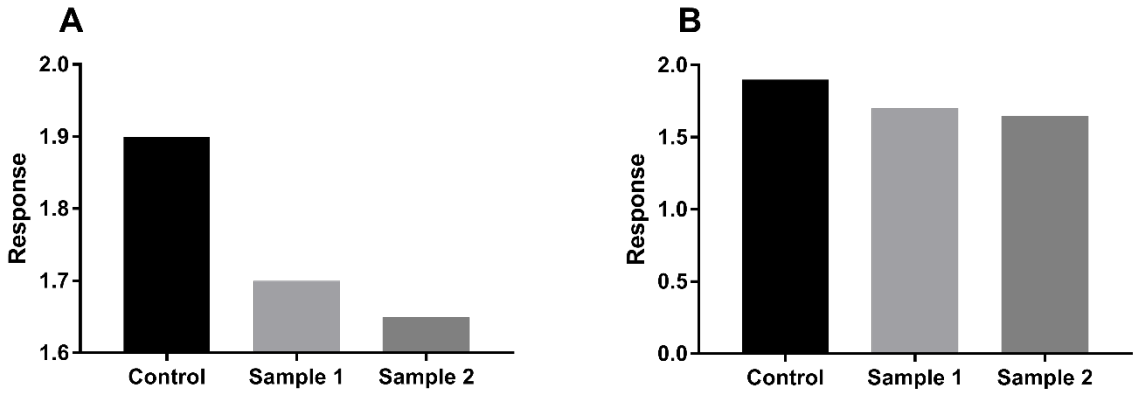


Figure 15. Example bar graphs with non-zero and zero y-axis origin.

(**A**) Y-axis scale begins at a value of 1.6 and skews the relative difference between the three samples. (**B**) Y-axis scale begins at zero and the relative differences between the three samples are properly depicted.

Starting the y-axis scale at zero may be preferred, but consider the example shown in **Figure 16** where the same data is plotted with a y-axis scale that begins at zero (**Figure 16A**) and a y-axis scale that does not begin at zero (**Figure 16B**).

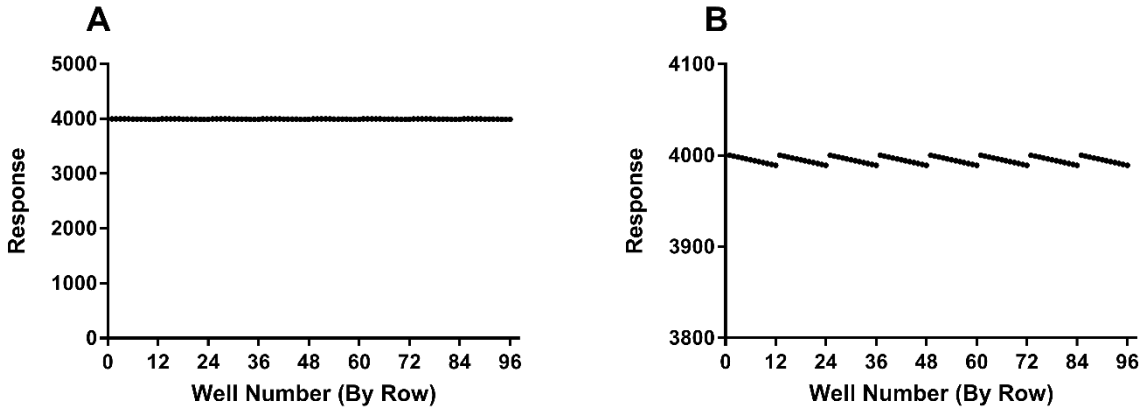


Figure 16. Example line graphs with non-zero and zero y-axis origin.

(**A**) Y-axis scale begins at a value of zero; the data appears to be uniform. (**B**) Y-axis scale begins at a value of 3800 and a repetitive pattern is observed. This is an example of systematic error.

While this data has been exaggerated to demonstrate a point, these types of patterns can exist with assays that involve automated liquid handlers, plate transfers, and detection equipment and usually suggest a causal relationship that can be investigated. If identified, these effects can be corrected and quality control chart monitored to reduce the overall noise and variation in the assay (1). Viewing data by rows or columns with a non-zero y-axis scale may be necessary to identify potential issues or patterns.

An example where a reduced scale may be required is demonstrated in **Figure 17**. Here a single obvious extreme outlier (data point in red) enlarges the scale, which masks the effect of the remaining data including the variability of n=3 technical replicates. Changing the scale so that the obvious outlier is not shown may yield the desired curve. An explanation for excluding the data point should be described in a figure legend, text, orally, etc.

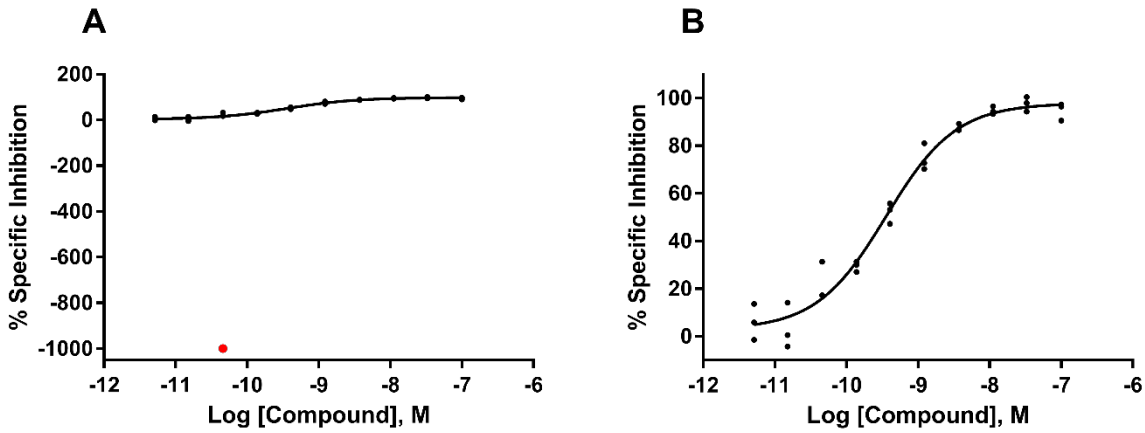


Figure 17. Effect of including all data when an extreme outlier exists.

(**A**) One extreme outlier (in red) creates a large y-axis scale that obscures the remaining data points. (**B**) The extreme outlier is omitted and a standard sigmoidal CRC curve is observed compared to the previously flattened CRC shown in **A** (n=3 technical replicates).

Alternatively, the use of a broken axis (see **Figure 19**) may be an acceptable alternative to show both the expected CRC and the outlier.

Finally, two different samples that are tested in the same experiment should have the same scale range so that relative differences are obvious to the observer. **Figure 18** demonstrates how conclusions could be misinterpreted when the y-axis scales for like-treated samples are not kept the same. In this example, Sample 1 and Sample 2 are two different preparations of the same protein tested in the same assay with the same conditions and reagents. **Figure 18** panels **A** and **C** may give the appearance that Sample 1 and Sample 2 have similar activities, since y-axis scales, relative to each maximum, were used in the two graphs. **Figure 18** panels **B** and **D** demonstrate the difference in relative activities for the two samples, when the same y-axis scale is used in each graph.

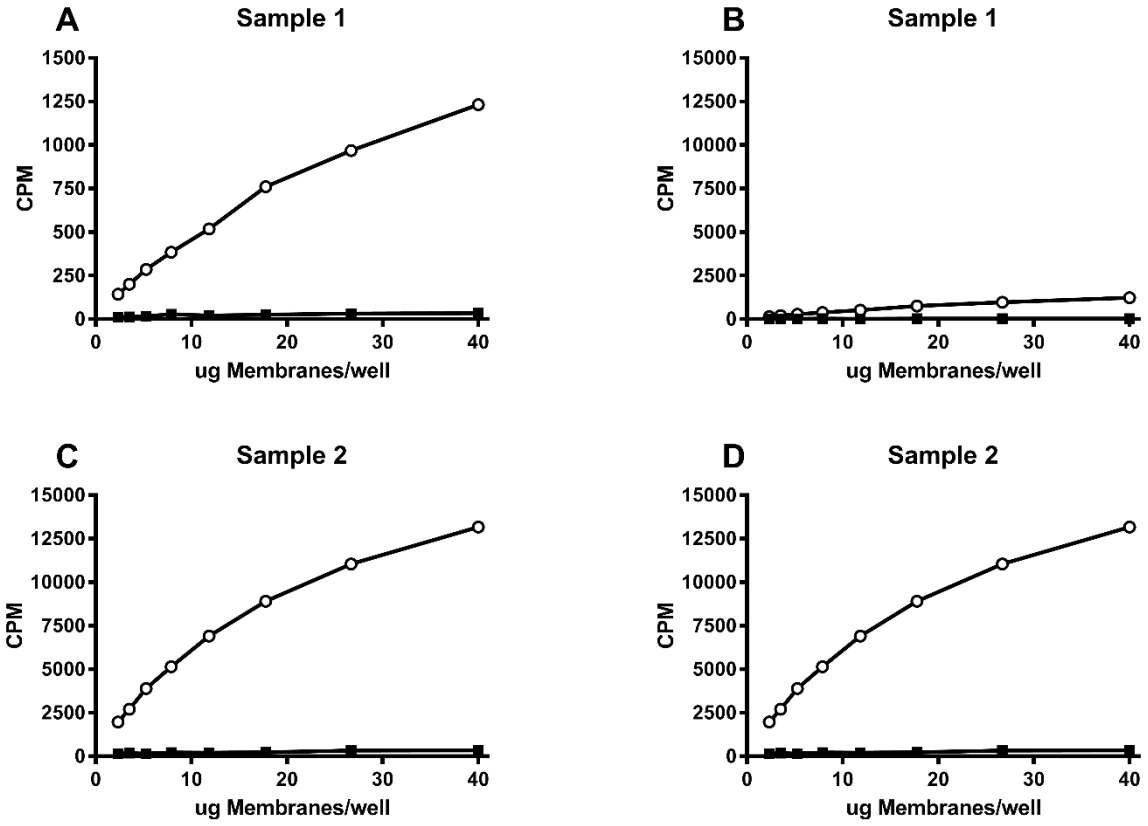


Figure 18. The effect of using different y-axis scales on similarly treated samples.

Sample 1 and Sample 2 are two different protein preparations that were treated in the same experiment with the same reagents and conditions. Nonspecific binding (solid squares) and total binding in the absence of competitor (open circles) were measured. In **A** and **C**, the y-axis scale range is determined by the maximum observed response for each sample. In **B** and **D**, the same y-axis scale range is used for both samples. Panels **B** and **D** give an accurate representation of their relative activities compared to each other (n=1).

Therefore, the scale range should be carefully evaluated to ensure that information is conveyed appropriately. In some cases, the activity of Sample 1 should be depicted as shown in **Figure 18A** to indicate that there is potentially a useable signal with that sample, depending on the situation.

Generally, it is acceptable to extend the scale range a few percent on either side of the axis to avoid data points on the plot frame (if using one) or on the axis extremes.

Broken Axes

If a broken axis is used to emphasize a specific point regarding the data being plotted, a note to indicate the broken axis in the figure legend, text, discussion, etc. should be included. This alerts the observer to this non-standard technique and can prevent misinterpretation of the data. **Figure 19** utilizes a broken y-axis to capture all data points of a CRC experiment (same data presented in **Figure 17**). It demonstrates the value of a broken axis so that key CRC information is retained while including all data points.

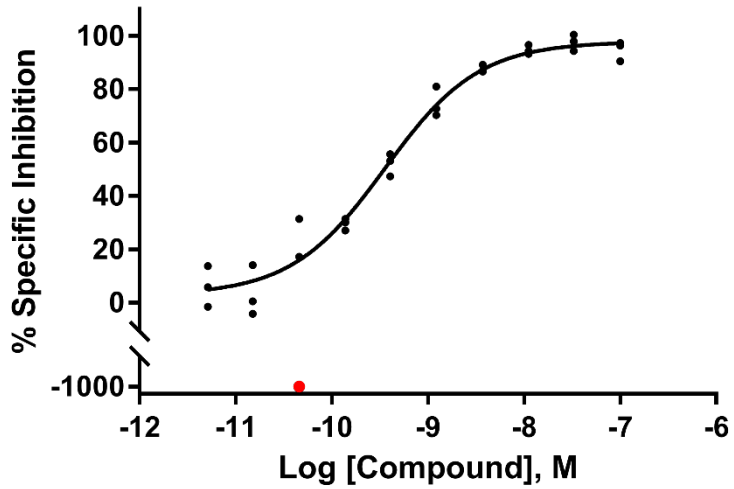


Figure 19. Using a broken axis to include all data points in a CRC.

A broken y-axis is used to include an outlier while still maintaining an appropriate concentration response curve for analysis. See the graphs in **Figure 17** to compare other possible ways to present this data (n=3 technical replicates).

Tick Marks

The most common placement of tick marks in a graph are on the outside of the axis; however, tick marks inside the axis are acceptable in many cases.

The number of tick marks being used on a graph axis should be chosen to represent the scale range adequately, without clutter, including extraneous tick marks that distract the reader. Placing tick marks at round integer numbers to correspond with the range is most frequently practiced. Minor tick marks and unlabeled tick marks should be avoided whenever possible.

The examples shown in **Figure 20** demonstrate three different axis scales for the same data range. In **Figure 20A**, the tick marks are integers and evenly spaced across the scale range, which represents a desirable presentation of the axis. In **Figure 20B**, there are unnecessary minor tick marks included. In **Figure 20C**, there are too many numbered major tick marks and the labels are uneven integers.

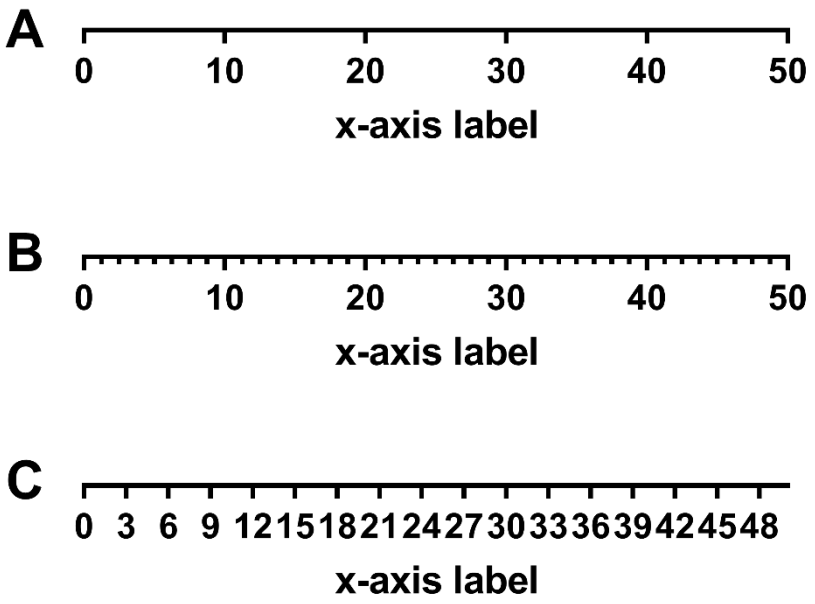


Figure 20. The effect of the number of tick mark labels on a graph axis.

(**A**) A standard, visually acceptable axis with even integers spaced across the entire length of the axis. (**B**) This axis has unmarked minor tick marks which do not add information necessary to understand a graph. (**C**) This axis has too many tick mark labels and the labels are non-standard integers.

When the data values being plotted are large, resulting in axis labels with several zeros (**Figure 21A**), divide the numbers by a constant factor and indicate the manipulation in the axis label as shown in **Figure 21B**. The message and interpretation of the data is the same with the y-axis scale depicted in **Figure 21B** being clearer.

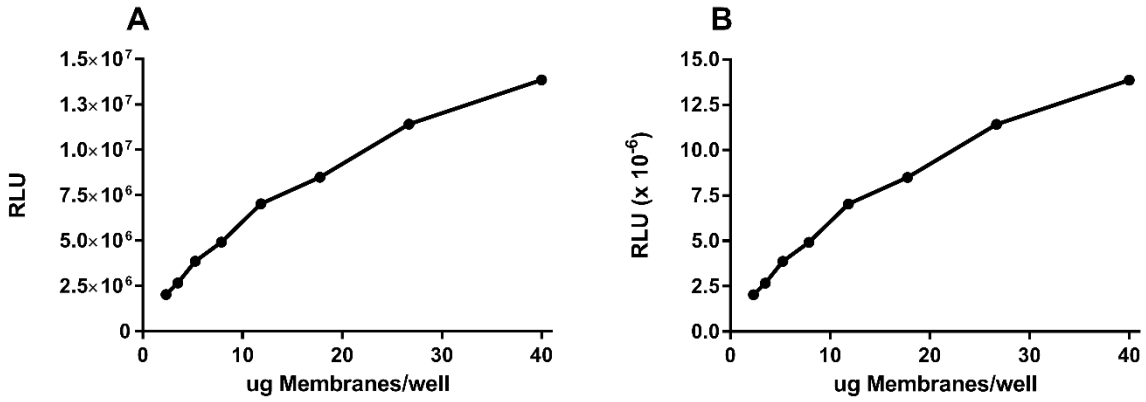


Figure 21. Y-axis scale with large numbers.

Plotted are raw data values using exponential notation on the y-axis scale (**A**) and a transformed scale on the y-axis using a multiplier expression indicated in the x-axis label (**B**). In this example, data points are connected with straight lines. The connected segments imply an appropriate continuous trend between the data points.

Axes Labels

Accurate, unambiguous axes labels are important to avoid confusion regarding the data being plotted in a graph. As an example, all of the following labels were intended to represent log-transformed concentrations plotted on the x-axis. They were found within published journal articles for CRCs. Some comments are listed after the label:

Log [Compound]: Doesn't specify the concentration units

Log [Compound] (M): Acceptable

Log [Compound], M: Acceptable

Log Compound Concentration (M): Acceptable (less standard)

Log [Compound]/M: Unclear, without further information on whether the x-axis values are a ratio

[Compound] in Log (M): Acceptable (less standard)

Log_{10} (Concentration (M)): Acceptable (less standard)

[Compound], M: Doesn't specify that that the concentrations are log-transformed

Compound [Log M]: Non-standard

Log Compound, M: Suggests log of a compound name rather than a compound concentration

[Compound], Log M: Possibly suggests that the units are Log M

Compound, Log [M]: Acceptable (less standard)

This example illustrates a couple of important principles:

1. Be consistent with the labels throughout a set of graphs
2. Make sure that the label describes accurately what is being plotted

Y-axis labels should follow the same principles as those listed above. An important example is a scale from zero to 100 that could be a percent scale or actual data values. The label should always indicate percent if that is the intention of the data being plotted.

Types of Graphs

Many graph and figure types exist so that your data can be tailored to a specific purpose or audience, regardless of whether it is in written or spoken format. A table for choosing when to use common data presentation techniques has been previously published (48). One online source even describes 44 types of graphs that can be chosen to present information (49). This section focuses on bar graphs, line graphs, scatterplots, frequency distributions, and heat maps.

Bar Graph

Bar graphs contain horizontal or vertical bars with lengths proportional to the value of the data. A bar graph is usually used to show the relative differences between categories of data. See **Figure 15** for an example.

If a bar graph has too many bars, it becomes cumbersome and can be difficult to interpret (**Figure 22A**). A scatterplot or heat map may be better choices for this level of data. Too few bars (**Figure 22B**) and the data in the plot could be displayed in a table or described within text and still be effective.

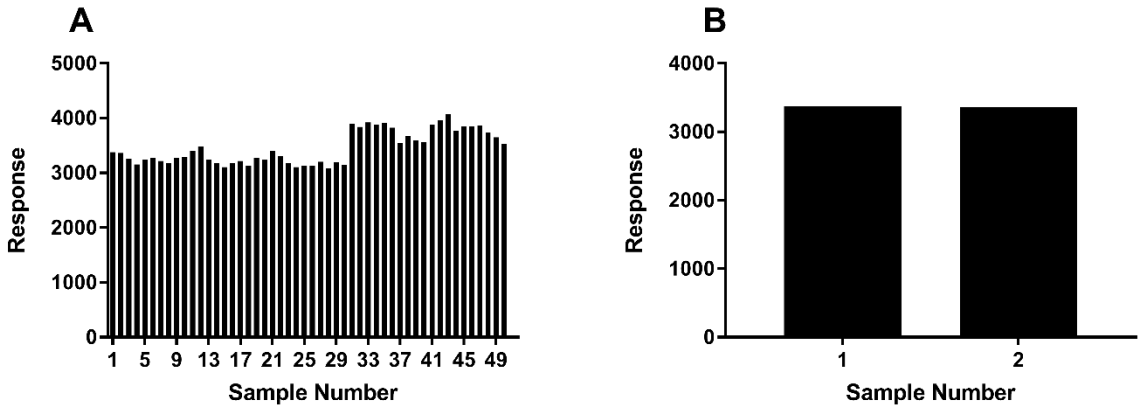


Figure 22. Bar graphs with too many and too few values.

A representation of a bar graph with too many data points or bars (**A**) or too few bars (**B**).

With respect to bar graphs, the following is also recommended:

- Use colorblind-accessible color combinations whenever possible. Red/green pairings are most problematic but refer to ColorBrewer, an online diagnostic tool for evaluating the robustness of individual color schemes, for more information.
- Ideally, keep text on the baseline axis in the horizontal direction, without overlap.
- The error (SD or SEM) should be addressed as discussed above (see **Figure 1** and **Figure 3**).

Line Graph

A line graph is a series of data points connected by a line or line segments. The lines may be connected data points (**Figure 1B**), a linear regression fit (**Figure 6 or Figure 7**) or a nonlinear regression fit (**Figure 14**). For the purposes of definition, linear regression uses a linear model to determine the relationship between a dependent variable and one or more independent variables. By contrast, non-linear regression is a form of analysis modeled by a non-linear function that utilizes model parameters and one or more independent variables.

Like a bar graph, if there are too many lines the reader is distracted from the message of the data in a line graph. This is especially true if the curves being shown have similar activities (**Figure 23**). Figures with too many curves often force the preparer to include a legend within the graph, which can be distracting and, in many cases, is considered chart junk.

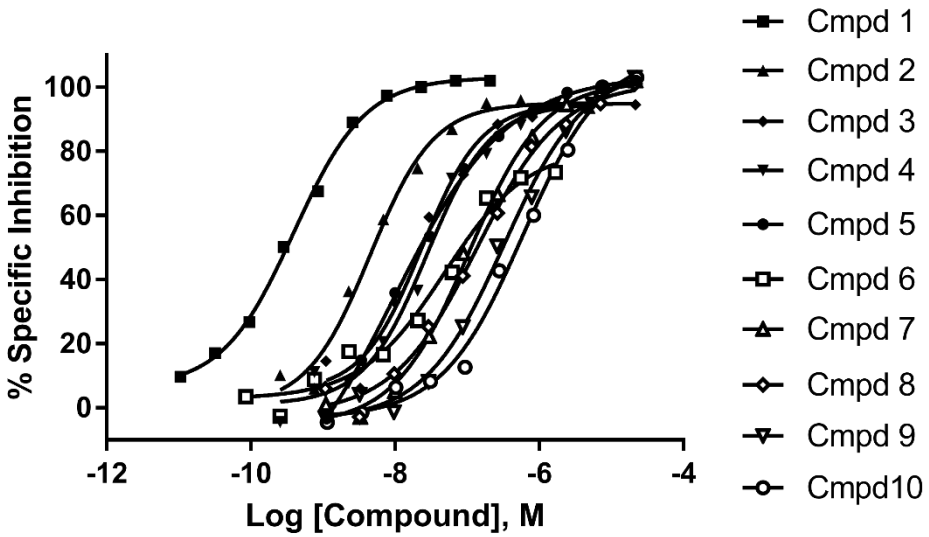


Figure 23. Multiple compound CRC graph.

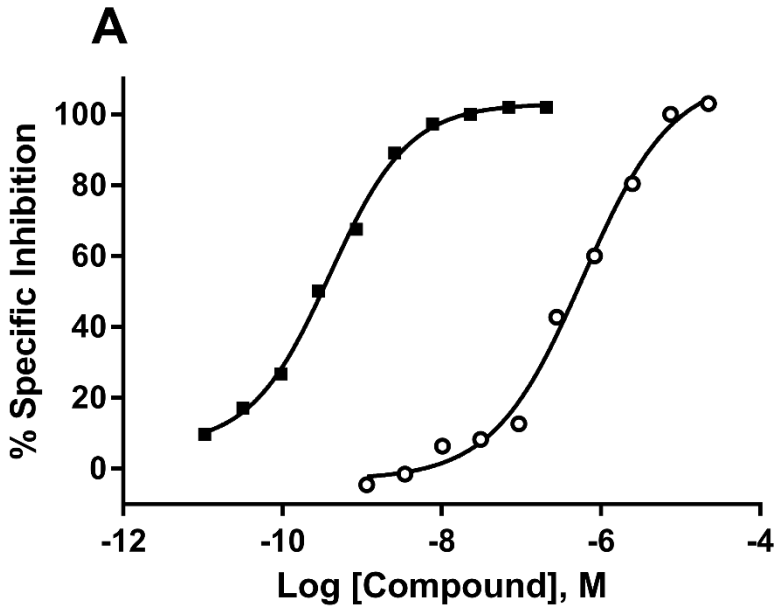
In this graph, the CRC for several compounds are shown with nonlinear regression curve fits. Each line has a unique symbol and the symbol legend appears at the right. There are too many compounds being shown on this graph to decipher the individual information.

Considerations include:

- Use colorblind-accessible color combinations whenever possible. (See above for more info.)
- Use colors only when necessary – avoid excessive use.
- The thickness of lines should be such that they are clearly visible but do not obscure individual data points.
- The method used to fit a set of data points to a line should always be conveyed with the graph (via legend, text, discussion, etc.).

In place of **Figure 23**, showing a representative curve for one or two compounds along with a table of calculated results (e.g. pIC_{50}) for the other compounds tested may provide an improved approach for presenting this type of data. An example of this concept is shown in **Figure 24** where the two compounds with the largest difference in activity are displayed in the graph (**A**) and the activity (pIC_{50}) of all the compounds tested is displayed in the table (**B**).

Figure 24 represents an additional reason for using negative log-transformed activity values (pIC_{50}) when comparing compounds. The larger the pIC_{50} value, the more potent the compound. Other advantages include the following: the nonlinear curve fit routine solves for the log IC_{50}, the error associated with the pIC_{50} is symmetric and normally-distributed and expressed with the same level of significance (see significant digits section) as well as making the determination of the geometric means easier.



B

Compound	pIC_{50}
1	9.41
2	8.34
3	7.63
4	7.56
5	7.37
6	7.32
7	6.98
8	6.87
9	6.48
10	6.22

Figure 24. CRC and table for several test compounds.

(**A**) Concentration response curves for two compounds in a study (n = 1). The curves represent the most potent compound (Compound 1, closed squares) and least potent compound (Compound 10, open circles) in an experiment that tested ten compounds. (**B**) Table showing the pIC_{50} values for all ten compounds tested in the experiment.

Scatter Plot

A scatter plot (also referred to as a scatter gram) is a graphic visualization of two-dimensional data using dots to represent data values. Scatter plots have x and y-axes and each data point is a coordinate on the plot. Scatterplots are often used to demonstrate the activity of the wells on a microplate, such as plate uniformity studies (28) and large data sets. In addition, they are the type of graphs used in the correlation plots discussed earlier (**Figure 6 and 7**).

Color coding data points can provide additional information within a scatter plot as shown in **Figure 25** for plate data with the color-coded max and min plate controls. However, color-coding individual data points can be tedious and the data may be better represented with a heat map (**Figure 28 and 29**).

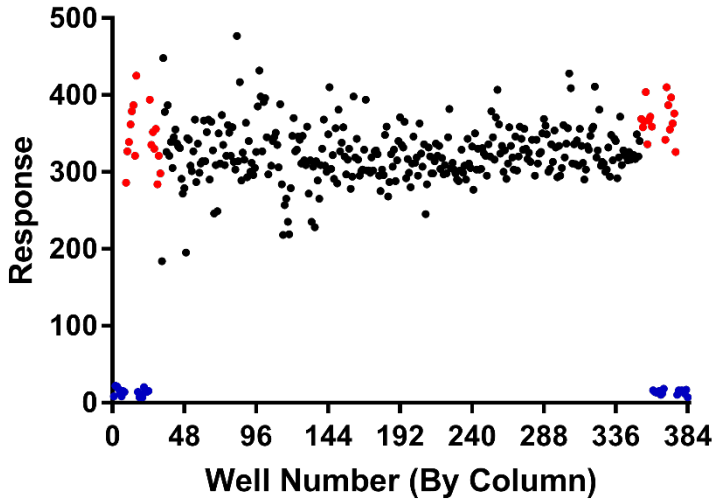


Figure 25. Scatterplot example.

Individual well data from a 384-well plate that includes positive controls (red) and negative controls (blue). Wells are aligned by columns on the plate.

With respect to scatter graphs, the following is also recommended:

- Use colorblind-accessible color combinations whenever possible. (See above for more info.)
- Data points that are too large obscure the detail of the pattern or trend being depicted in the scatterplot.

Frequency Distribution

A frequency distribution typically uses bars or rectangles and can include a further analysis for normality, such as a Gaussian curve, embedded in the plot. The x-axis represents a range or group of ranges ("bins") and the y-axis represents the frequency at each range or bin. An example of a frequency distribution is shown in **Figure 26**. In this example, the three panels demonstrate frequency distributions using an appropriate number of bins (**A**), too many bins (**B**) and too few bins (**C**).

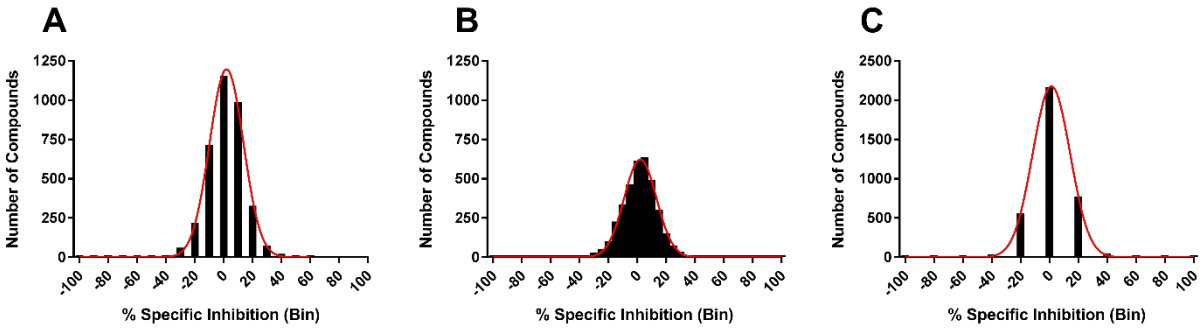


Figure 26. Frequency distribution.

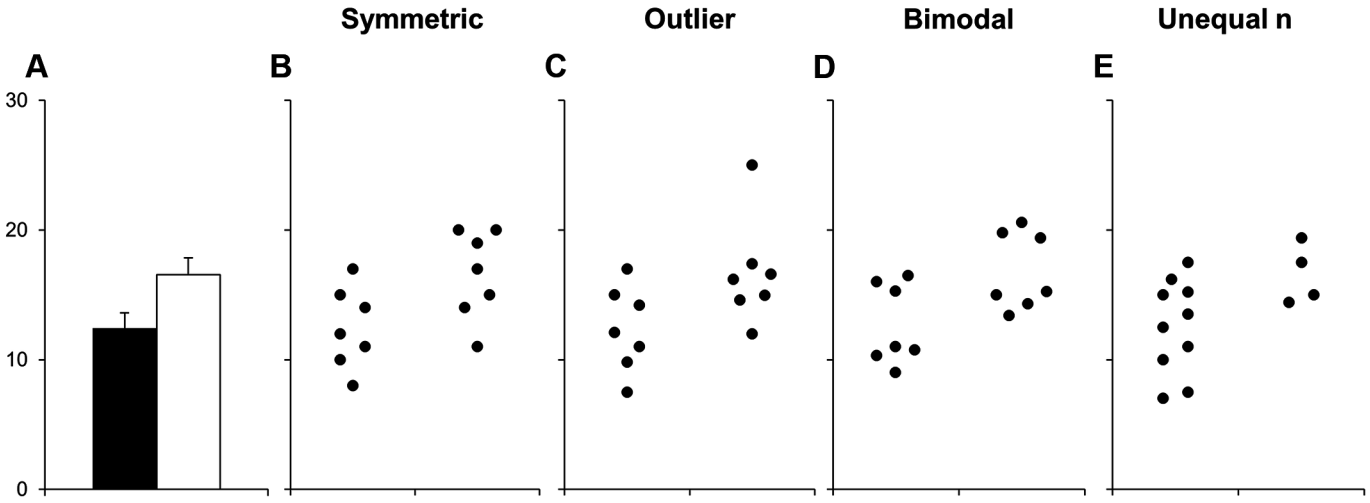
Activity is divided into bins of percent specific inhibition (x-axis). The number of compounds in each bin (y-axis) are represented by the bars. To test for normality, a Gaussian distribution (red line) was fit to the frequency data. (**A**) Represents an appropriate number of separation bins (at 10% inhibition intervals), while (**B**) has too many separation bins (at 5% inhibition intervals) and (**C**) has too few separation bins (at 20% inhibition intervals).

With respect to frequency distributions, the following is also recommended:

- The number of bins ultimately depends on the number of data points, and determining the number of bins (as demonstrated above), may be a trial and error process to achieve a desired graphical result.

Alternative Plots

While widely used, bar graphs have significant limitations. Since bar graphs only display summary statistics (generally mean and SD or SE) it is possible to generate identical bar graphs from different data sets due to outliers, bimodal distributions, differences in sample sizes, confounding variables or other reasons (50). Alternative graphical methods which display all of the data and the distribution information, such as Univariate Scatterplots, Box plots (which graphically overlay the summary statistics) or Violin plots are preferable. Since most preclinical studies use relatively small sample groups (n < 15) this is very feasible and Weissgerber et al (51) have provided an online tool which allows scientists to easily generate and download these plots using their own data. (Figure 27).



Test		p value		
T-test: Equal var.	0.035	0.050	0.026	0.063
T-test: Unequal var.	0.035	0.050	0.026	0.035
Wilcoxon	0.054	0.073	0.128	0.103

Figure 27. Many different distributions can lead to the same bar graph.

The full data may suggest different conclusions from the summary statistics. The means and SE values for the four example datasets shown in B-E are all within 0.5 units of the means and SE values shown in the bar graph A. ρ values were calculated in R statistical software (version 3.0.3) using an unpaired t-test, an unpaired t-test with Welch's correction for unequal variances, or a Wilcoxon rank sum test. In B, the distribution in both groups appears symmetric. Although the data suggest a small difference between groups, there is substantial overlap between groups. In C, the apparent difference between groups is driven by an outlier. D suggests a possible bimodal distribution. Additional data are needed to confirm that the distribution is bimodal and to determine whether this effect is explained by a covariate. In E, the smaller range of values for group 2 may simply be due to the fact that there are only three observations. Additional data for group 2 would be needed to determine whether the groups are actually different. *var*, variance. (Adapted from Weissgerber *et al. (50)* under a creative commons license. Figure and figure legend used with permission.)

Heat Maps

A heat map is a representation of data values using a color scale or grayscale. The resulting graph can be in a matrix format, which makes them popular for data generated using microplates. **Figure 28** shows a heat map for the same data that was previously graphed in a scatter plot (**Figure 25**).

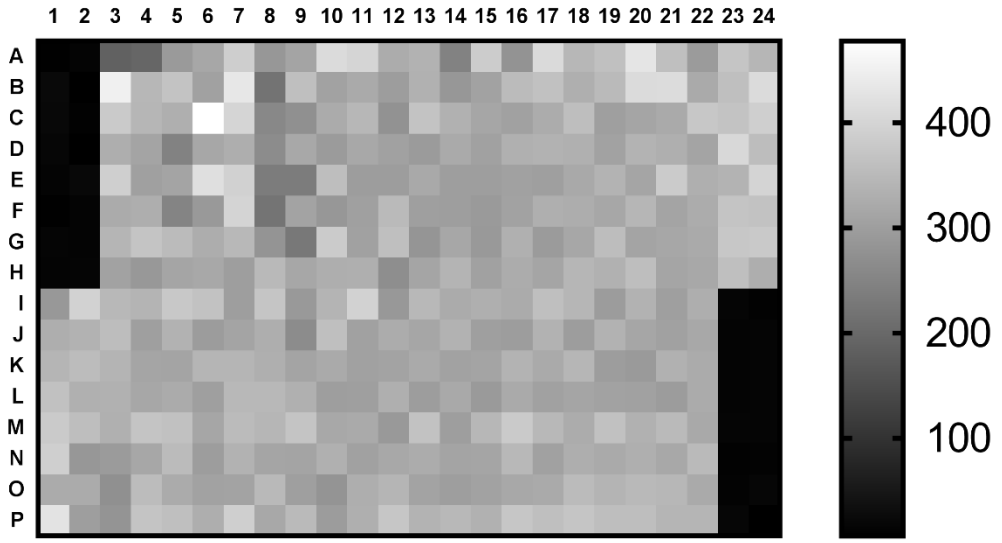


Figure 28. Heat map for the 384-well plate data shown in Figure 21.

The scale at the right of the heat map shows the colors associated with the signal range. Note that in this example, the positive controls are in wells A1-H2 and I23-P24. The negative controls are in wells I1-P2 and A23-H24.

Heat maps can be used to view multiple plates at a time and to assist with efficient identification of data patterns, position effects and trends (52).

With respect to heat maps, the following is also recommended:

- Individual data points can be framed with a thin border. Frames are typically avoided when there is a large amount of data points such that the frames would obscure the interpretation of the data.
- Heat maps can be displayed in color or grayscale. The choice of color versus grayscale often depends on the data being presented. For data with large dynamic ranges, two colors may be appropriate, whereas for data with small dynamic ranges, grayscale may be sufficient (**Figure 29**). As stated previously, the use of red and green coloring schemes should be avoided. (This is perhaps a relic of certain microarrays that utilized red and green fluorescent proteins to assay gene expression.)
- As with other plot formats, the scaling of the color can be adjusted to better illustrate key scientific points, such as plate positional effects where relatively minor systematic errors may be significant (**Figure 29**).
- Outliers can be displayed on heat maps as a separate color and then noted in the legend.
- Consider providing a supplemental file listing the data in numerical format. Data points can also be printed within each matrix point, though this can add considerable visual artifacts that detract from interpreting the figure (**Figure 29E**).
- The use of multiple colors ("rainbow schemes") can make the perception of gradients difficult (**Figure 29F**) and are generally more appropriate for categorical or grouped data.
- Another approach to emphasizing positional effects is to express data as deviation from the mean or median values (**Figure 29G**). This is also amenable to analyzing data for up- or down-regulation, such as gene expression or protein abundance.

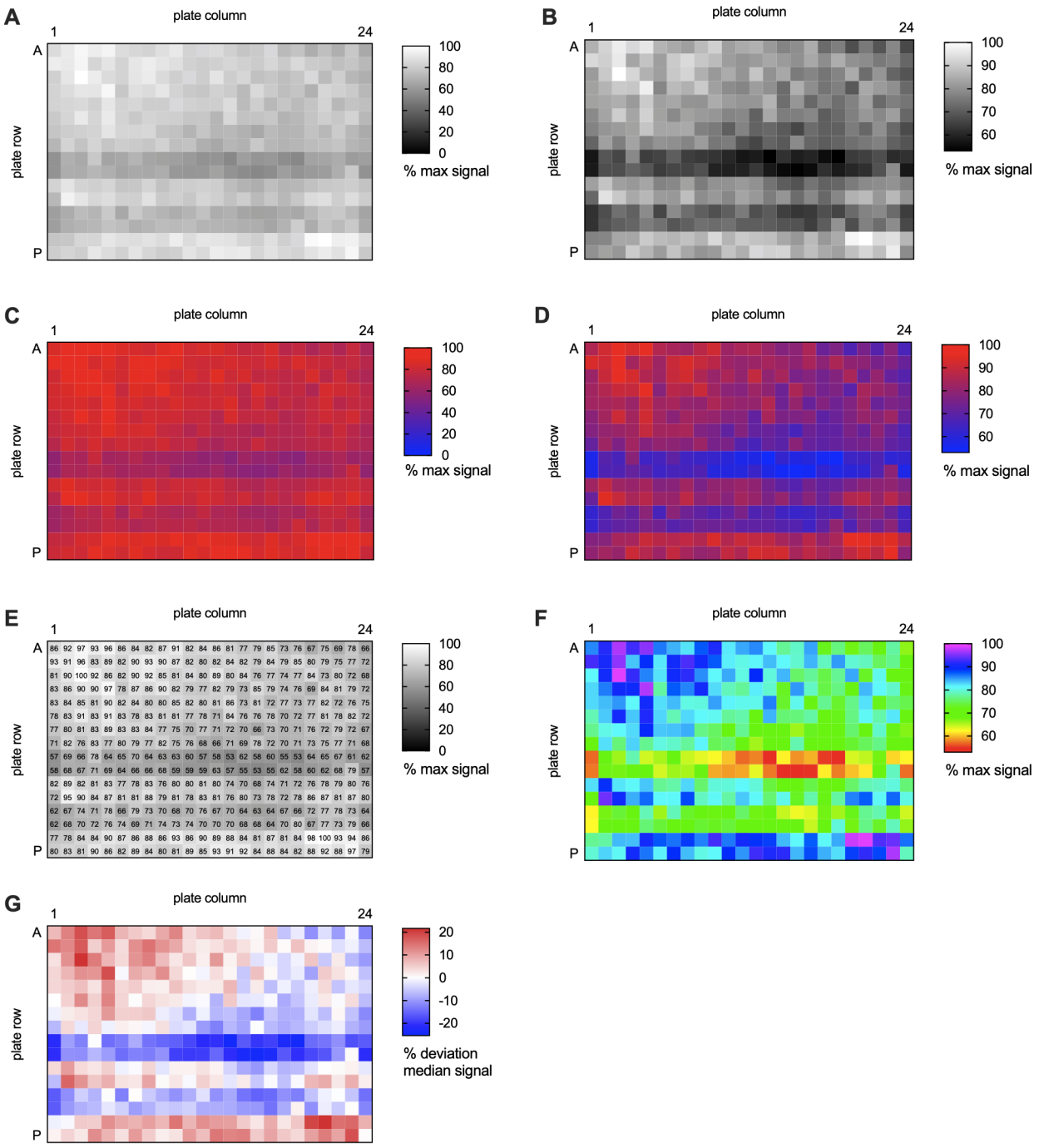


Figure 29. Effect of color schemes on heat maps.

Data are normalized fluorescence intensity measurements from a uniformity plate (i.e., all wells should have equal, 100% max signal) to assess for microplate positional effects. Clearly, rows I, J, M, and N show decreased signal, which is due to a clogged liquid dispensing nozzle. Panels A and B demonstrate the effect of varying scales; a coloring scheme starting at the minimum value (panel B) better highlights the systematic errors in this particular data. The same data can be plotted in color (panels C and D), depending on the desired aesthetics. Individual values can be printed within each matrix point (panel E), but this generally adds noise and should be done only when necessary. The same data can be plotted in a rainbow coloring scheme (panel F), though the meaning of individual colors is not necessarily intuitive. Finally, the same data can be plotted as a function of deviation from the mean or median (panel G). (Unpublished data courtesy of JL Dahlin.)

Three-Dimensional Graphs

In most cases, 3-dimensional (3D) graphs are distracting to the reader and can be difficult to interpret due to the added complexity or distortion that can occur. They are often used in business publications and newspapers but should be avoided in scientific data presentations. A simple, classic example is shown in **Figure 30** using a standard bar graph and a 3-D bar graph generated with Microsoft Excel. In the example, both samples 2 and 4 have median values above 100% of the control in the standard bar graph. However, in the 3-D bar graph, it appears that samples 2 and 4 do not reach the 100% of control level based on the axis scale lines for the graph. While this is a matter of perspective, it can lead to incorrect analysis and conclusions.

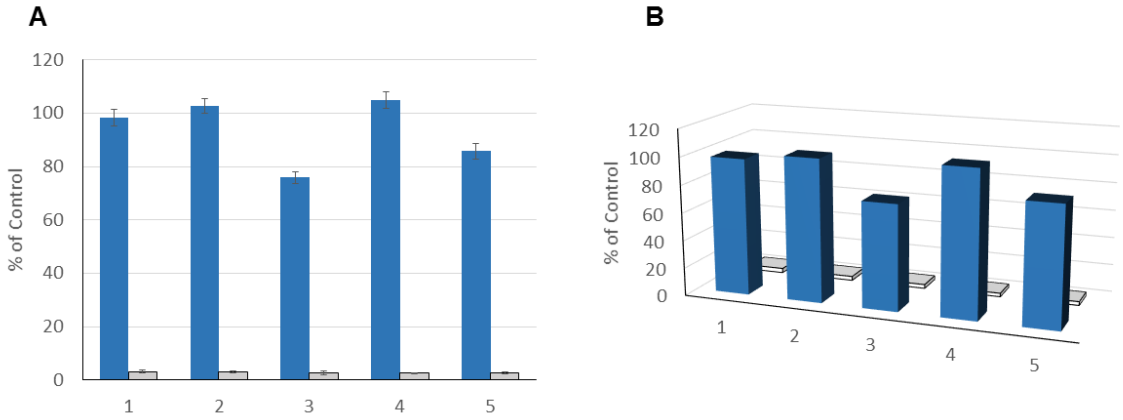


Figure 30. Standard and 3D bar graphs.

(**A**) (Typical bar graph) and (**B**) (3-D bar graph) are from the same data. Five different samples were tested for activity against a control. Blue bars are total activity and grey bars are nonspecific activity. Bars are the median of 8 technical replicates from the same run.

Graphing/Statistical Software Programs

This chapter asserts no preference in software used for creating graphs or statistically analyzing data. Specific programs cited are at the discretion of the authors, based on experience. Some notable software programs for graphing or statistical analysis include, but are not limited to, the list below.

GraphPad Prism

JMP

KaleidaGraph

MATLAB

Microsoft Excel

Minitab

Origin

R Statistical Package

SAS

ScreenAble

SigmaPlot

SPSS

Stata

Tables

It is important to note that graphs and tables may lead to completely different interpretations, even with the same data set. Each has a purpose, depending on how the data will be used and who the consumers of the data will be. Large tables do not work well in a presentation setting and graphs may not provide the level of detail required for a further calculation or show exact values and differences when analyzing data.

Tables should not contain so much data that they are hard to follow. Likewise, tables should not have print that is so small that it is difficult to read. Conversely, simple tables with only a few values may be less effective.

Considerations for tables include:

- Include a legend/caption where needed to describe or define any abbreviations, etc.
- Include units with the table (e.g. as part of the header title for the column)
- Use a minimum number of significant digits that are consistent throughout the table
- Shading rows or columns can help improve readability
- Use lines to separate sections; do not overuse lines within the table
- Include only essential data
- Be clear, concise and legible
- There should be adequate space within the table for clarity
- Use caution with word wrapping, especially when it only occurs in a couple of table cells

Some examples of tables appear earlier in this chapter.

Interactive Data Visualization

Visual inspection of graphic displays can identify natural groupings of data points suggestive of correlations in the underlying data. Modern computer graphics interfaces often have the capability to use a computer's mouse or other pointing device to select one or more data points in a graphic for further investigation. Some of the more commonly encountered types of these tools are discussed below.

Data Hints

The data or chart "hint" is often used to describe the action that occurs when the mouse hovers over, or is clicked on, single points (e.g. scatterplot) or specific sub-plot regions of a multi-plot display. Typically, this is used to display an informational child window near the selected point/region. An example of a chart hint is shown in **Figure 31**. If a data grid is visible in the application, then a useful side effect would be to select (highlight) the row of interest.

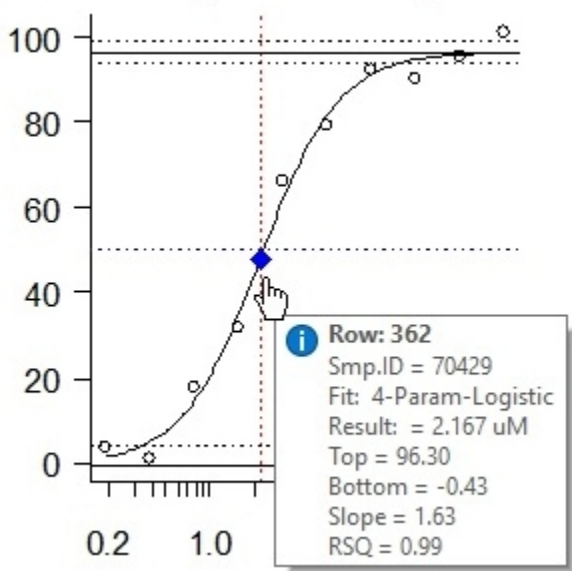


Figure 31. The result of a chart hint action on a multi-panel CRC plot.

In this example, hovering over the curve provides a value for each of the parameters listed as a quick reference to the underlying data row contents. The availability of hints for graphics is typically indicated by a hand with pointing finger cursor.

Brushing Data

Data *brushing* refers to using the pointer in a click-and-drag fashion to define a region that encompasses one or more data points of interest on a chosen graphic. The selected points/region are subsequently "highlighted" (e.g. using color or plot characters) in the selection plot device in order to focus subsequent User attention. Typically, graphic plots using other variables/responses are similarly updated and the relevant data grid rows are selected. For maximal utility, this should be a two-way process and the ability to highlight graph points from row selections of data displayed in grids following complex sorting operations should also be available.

Context Menus

Many graphics displays allow the development of menu functionality for common operations such as copying, printing and file creation to be readily available to the user. The content of these menus can be programmatically linked to the type of plot and the data under consideration. Plot scaling can be keyed to mouse behavior (e.g. mouse wheel) and, for 3-dimensional graphics, useful behaviors such as rotational direction and speed can be intuitively linked to gestural pointer actions. **Figure 32** demonstrates the concept of a context menu.

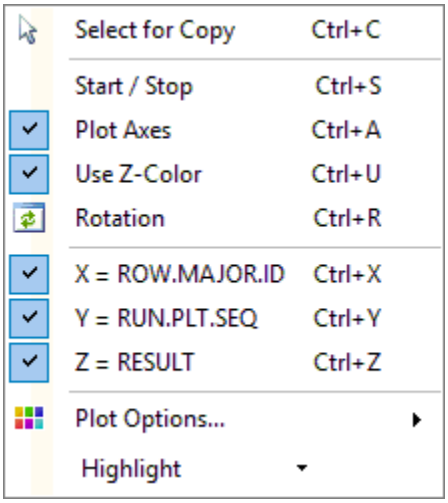


Figure 32. Example of a Context Menu associated with a 3D graphics display.

Several options for rotational control, plotting of axes etc. can be accessed using either the pointer or keyboard shortcut combinations. Options with checkboxes are those that have an on/off toggle function.

Databases

A well designed and properly implemented Relational Database Management System (RDBMS) (53) is a valuable tool not only for the storage of information but also for promoting both reproducibility of experiments and consistent, appropriate data reporting. A database offers a number of advantages when compared to flat files such as spreadsheets:

- **Data Safety and Integrity.** Built in features of the database automatically back up data and track any changes made to the data. In the event of errors, changes can be rolled back
- **Data Consistency.** Much of the data in spreadsheets is repeated and subject to errors in data entry such as typographic errors or cut and paste errors. A structured data model eliminates this redundancy and provides tools to validate data entries. Additionally, having all the data in one place simplifies the identification of systematic shifts or random errors in the data.
- **Change Management.** Results can be recalculated automatically from the original primary data to explore new models or allow for changes in methodology. Explanations of any changes along with a record of when and who made the change(s) are also systematically captured.
- **Data Analysis and Reporting.** Structured reports ensure that data is always presented in a consistent manner with proper mathematical and statistical treatment, regardless of the sophistication of the user, while still allowing for export and exploration of the data to other tools. Best practices, such as those described in this chapter, can be incorporated into these reports. Reporting of both primary and derived data has also become more common for both publication and funding and has been demonstrated to enhance both transparency and reproducibility.

There are numerous commercial and open source implementations of RDBMS technologies. All of them feature some implementation of standardized Structured Query Language (SQL) commands for obtaining results for data reporting purposes. For maximal data integrity and control of data flow, the chosen system should also feature a procedural language for sophisticated development and implementation of the automated processes discussed above. Most commercially available Laboratory Information Management Systems (LIMS) use RDBMS technology to provide much of their functionality and represent a path to obtain the benefits of a relational database without the need to directly manage one.

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Development and Applications of the Bioassay Ontology (BAO) to Describe and Categorize High-Throughput Assays

Uma D. Vempati[1] and Stephan C. Schürer[2]

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Abstract

Ontologies are important tools to organize information and formalize knowledge in a subject domain. In this chapter, the authors introduce Bioassay Ontology (BAO), the first ontology to describe and categorize chemical biology and drug screening assays and their results. BAO has been applied to standardize, organize and annotate assays and screening results to enable analysis and information extraction across multiple and diverse data sets, with the goal to infer new knowledge about the molecular mechanism of action of small molecules in the assay model systems. The authors provide an overview of BAO and present several applications, including the annotation of a large number of HTS assays and an assay annotation tool (BioAssayAnnotationTemplate). BAO has also been used in a software tool (BAOSearch) to enable query and exploration of assay experiments and results via a simple user interface. BAO is an evolving ontology with a growing nymber of applications and user community.

Introduction

High-throughput screening (HTS) of small molecules is one of the most important strategies to identify novel entry points projects for drug discovery projects (1). For several decades, HTS and ultra-high throughput screening (uHTS) have primarily been in the realm of the pharmaceutical industry, where huge amounts of data have been generated using these technologies. In 2003, NIH made HTS accessible to public sector research via the Molecular Libraries Program (MLP), the aim of which is to advance translational research (2). MLP projects leverage innovative assay technologies to develop compounds, termed chemical probes, which are effective at modulating specific biological processes or disease states via novel targets. The program has established publicly funded screening centers, along with a common screening library (the Molecular Libraries Small Molecule Repository, or MLSMR) and a data repository, PubChem (3). One of the visions of the MLP is to generate a complete data matrix screening the same compound library across a large and diverse biological space, thereby producing the most comprehensive public chemical biology data resource.

The ability to identify suitable leads for novel probes or drug development candidates requires carefully designed screening campaigns. Such campaigns are typically a series of assays performed to select a suitable class of compounds that show the desired biological effect, and to establish that the observed effect is based on a perturbation of the biological system by the small molecule (and not, for example, due to interference with the assay system). It is particularly desirable to establish the molecular mechanism of action (MOA) of a lead compound. A screening campaign typically includes primary screens (screening a large number of compounds very fast), confirmatory screens (replicates), concentration response screens, and various types of secondary assays, such as orthogonal or counter assays. If suitable compound series are identified, SAR (structure activity relationship) studies are performed, and a project can then move forward onto lead optimization. In the context of the MLP, it should be noted that the NIH Chemical Genomics Center (NCGC) developed an innovative screening protocol combining primary and concentration response screening, called quantitative HTS (qHTS) (4). Using the qHTS approach, an entire compound library is screened at several concentrations, enabling the

Author Affiliations: 1 Center for Computational Science, University of Miami Miller School of Medicine. University of Miami, Florida. 2 Department of Molecular and Cellular Pharmacology, University of Miami Miller School of Medicine. University of Miami, Florida.

identification of SAR directly from the primary screening results; thus, the discovery process is accelerated, while also producing a more information rich data matrix. For assays run at the MLP and deposited to PubChem, the results and conclusions from a screening campaign are in most cases deposited as a separate "assay" called the "summary assay". However, in PubChem, it is not easy to retrieve all assays from a campaign together with their particular purpose (primary, confirmatory, secondary) and various other important characteristics, because relationships between assays to describe campaigns and other critical metadata are not captured in a way that would allow systematic querying.

Similarly to the HTS datasets produced in the pharmaceutical industry, the public sector screening data are considered a valuable resource, which has received wide-spread attention. However, their diversity and quantity also present enormous challenges to organizing, standardizing, and integrating the data to maximize their scientific, and ultimately, their public health impact. In response to calls for HTS standards (5), we have taken the initiative in defining a knowledge model and terminology to describe bioassays, such as those produced by the MLP and deposited into PubChem. A recent report describing Minimum Information About a Bioactive Entity (MIABE) (6) further emphasizes the need for controlled terminologies and standardized approaches to describe chemical biology assays and their outcomes. We are working towards establishing a community standard to formally describe probe and drug discovery assays and screening results. Our approach is similar to the one taken in other fields such as microarray experimentation, where minimum information specifications (Minimum Information About a Microarray Experiment or MIAME 2.0), multiple data models (MicroArray Gene Expression Object Model or MAGE-OM) and the MGED (Microarray and Gene Expression Data) ontology (7) have been developed and incorporated into Web Services such as the Gene Expression Omnibus (8) to facilitate data exchange. Having such standards in place and implemented in an appropriate informatics infrastructure (9) will facilitate a) the re-use of assay results beyond an individual project (screening campaign) for the purpose of identifying compounds based on established or hypothesized molecular MOA; and, b) the evaluation of screening results in the context of all available information, for example to remove promiscuous compounds, or to perform global analyses of chemical versus biological space. Comprehensive and standardized assay annotations will also facilitate the design of screening campaigns and aid in the development and implementation of new assays. Here, we focus on the Bioassay Ontology (BAO, http://bioassayontology.org/) to formally describe and categorize biological screening assays and their results.

Ontologies have been used in biology to organize information within a domain, and to a lesser extent, to annotate experimental data. The most successful and highly-used biomedical ontology is the Gene Ontology (GO) (10), which consists of terms to describe gene product biological processes, cellular localization and molecular function. Several hundred ontologies are hosted by the Open Biological and Biomedical Ontologies (OBO) Foundry (102, counted on 4/15/2011) (11) as well as the National Center for Biomedical Ontologies (NCBO, 263 ontologies counted on 4/15/2011) (12), centered on domains ranging from African traditional medicine to Zebrafish anatomy. However, no public ontology to describe assays in the drug and probe discovery domain exists. Moreover, there is no ontology to describe the screening outcomes. The most obvious reason for this is that HTS datasets have not been publically accessible until recently, with the establishment of PubChem. We created the BioAssay Ontology (BAO) for the purpose of standardizing, organizing and semantically describing these biological assays and screening results. The BAO is under active development, and we refer the reader to our website (http://bioassayontology.org/) and the BAO Wiki (http://bioassayontology.org/wiki), which contain the most current information related to this project.

In this article, we briefly discuss the main concepts required to describe important details of bioassay experiments and screening results. Then, we report the development of the ontology (BAO) and provide an overview/outline of the BAO with examples of how some of the concepts are implemented in the BAO; we also discuss related resources incorporated into the BAO. Subsequently, we describe applications of the BAO. Most importantly, the annotation of assays including the annotation process, statistics of the distribution of assays in PubChem based on the main BAO concepts, and an example of a screening campaign using the BAO are

described. We also show how BAO annotations can be leveraged to analyze data across many assays to derive novel insights that are not readily obtained from any individual screening campaign. The BAO supports inferences within the functionality of Web Ontology Language (OWL) 2.0, raising the possibility of formally reasoning across the data, with several possible applications. To simplify assay annotations and to enable a large community to annotate assays, we generate a prototypical annotation template. Finally, we briefly describe BAOSearch, a Semantic Web software application to explore screening assays and results in the context of BAO. Figure 1 shows a high level overview of the contents of this chapter

The BAO to organize screening assays, results and campaigns

Organizing bioassays into interpretable categories

One of the goals of the BAO was to organize assays by several main concepts describing their most important characteristics, and enabling the meaningful categorization of assays, for example, by biology or technology. Such a classification of assays enables the identification of related or similar assays, either for the purpose of data analysis or assay development. These main categories were determined based on competency questions such as:

i. What is known about the biological target of the assay?
ii. Was the assay performed on a purified protein or in a cellular system (assay format)?
iii. What are the characteristics of the perturbing agent (e.g. small molecule)?
iv. How was the perturbation converted into a detectable signal?
v. What was the physicochemical method on which the detection is based?

To answer such questions across a large and diverse assay space, we organized the BAO into the main hierarchies describing assay format, design, target, and detection technology (described in Overview of BAO). For example, cell-based assays can be divided into the following broad categories, based on the assay design: reporter-gene, viability, cell morphology, redistribution (protein/second messenger/metal/dye redistribution), membrane potential, binding, second messenger (cAMP, calcium), and enzymatic assays. To illustrate this further, assays utilizing luciferase technology can be classified into subcategories, such as luciferase reporter gene, viability, adenosine triphosphate (ATP)–coupled, luciferin-coupled, and luciferase enzyme activity assays. All of the main BAO concepts can also be further specified. For example, to describe a permanent cell line, it will be relevant to describe growth mode (adherent or in suspension), cell line culturing components (including the culture medium and assay medium), cell line modifications (such as transfection or viral transduction), and transfection attributes (DNA construct and transfection agent) used to modify a cell-line. Having such information about cell lines used in assays would make it possible to delineate the role of the different attributes to the outcome of an assay, and thus, would facilitate assay design and optimization.

Describing screening outcomes/results

Another important question to analyze assay results is: How were the results reported/quantified? Although the BAO covers many assay types, including endpoint and kinetic assays and the corresponding outcomes, we specifically focused on describing the most important HTS types, such as single concentration response type and concentration response assays. Typical endpoints of assays include IC_{50}, EC_{50}, and percent inhibition, percent activation, respectively (described in the Overview of BAO section). Further specifications about assays endpoints, for example the curve fit method or normalization, are also described.

Describing screening campaigns

To describe screening campaigns, we need concepts to describe the assay stage (primary, confirmatory, secondary), the measurement throughput quality (single concentration, concentration response, single measurement, and multiple replicates), a campaign name, and relationships to define how one assay is related to

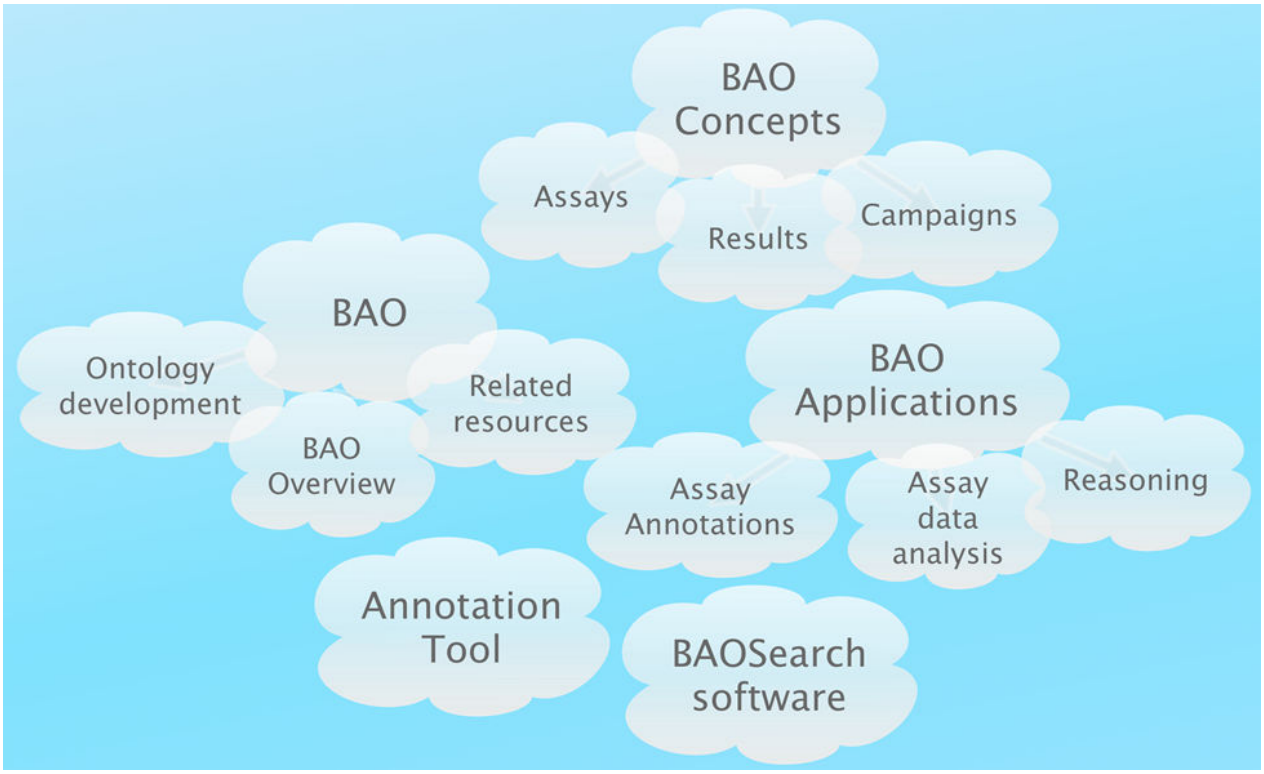


Figure 1. Chapter overview: Development and applications of the Bioassay Ontology (BAO) to describe and categorize high-throughput assays.

another in a screening campaign, including typical confirmatory and counterassays. These annotations are needed to define business rules to aggregate assay results across an entire screening campaign. Moreover, in combination with the assay categories above, the overarching goal is to enable the meaningful aggregation of assay outcomes across many campaigns to identify compounds with known or likely molecular mechanism of action (MMOA).

Ontology development and overview of the BAO

Ontology Development

During the last 10 years, tremendous progress has been made in developing Semantic Web (13) technologies with the goals being the formalization of knowledge, linking information across different domains, and integrating complex, diverse, and large data sets. Semantic Web technologies can solve many data integration problems, because they formally define semantics and support descriptive formal knowledge representations (14). Ontologies, like controlled vocabularies and thesauri, describe what things mean by linking terms to a human-readable definition. As such, ontologies are used for sharing knowledge in a common language, as well as to organize that knowledge. Beyond controlled vocabularies and thesauri linking terms to human-readable definitions and Uniform Resource Identifiers (URIs), ontologies can be used to formalize knowledge in explicit (logical) axioms defining classes, their properties and relationships. Thus, ontologies enable a computational system to reason formally on the content, with the potential to discover new knowledge by inference or to identify possible sources of errors.

The BAO was constructed using Protégé version 4.1 (15) in the OWL 2.0 (16), which supports description logic (DL). A number of available plugins were used throughout the development process, including OWLViz2 (17) and OntoGraf (18) for visualization, and DL reasoning engines HermiT (19) and Pellet (20). Our ontology

development follows established ontology engineering methodologies using a combination of top-down (domain expert-driven) and bottom-up (data-driven) approaches (21). The BAO is instantiated in a well-specified syntax and designed to share a common space of identifiers. The ontology has a formally specified and clearly delineated content. All terms in the ontology have textual definitions. The BAO is developed using version control and the derived products; for example, an annotation tool or BAO annotated content (described in Application of the BAO) are developed to correspond to a specific BAO release. In the development of the BAO, we also make use of existing ontologies and database (described in Integration of BAO with Existing Ontologies and Databases). The BAO (v1.4b1158) has SROIQ(D) (22) expressivity and consists of 1035 classes, 47 object properties (relations), 9 data properties, and 27 individuals (not including individuals from annotated assays or endpoints).

Overview of BAO

In the following, we use 'single quotes' to denote BAO classes and *italic* font to denote the relationships in the BAO. The main class hierarchies in the BAO include 'format', 'design', 'meta target', 'detection technology' and 'endpoint', which were described in a recent publication (23). 'Format' and 'perturbagen' have direct relationships to 'bioassay'; 'meta target', 'detection technology', 'design', and 'endpoint'are linked to 'bioassay' via 'measure group'. 'Measure group' is a concept created to group experimental outcomes into sets, and thus enables the modeling of multiplexed and multi-parametric assays. Each BAO class hierarchy includes multiple levels of sub-classes and is also linked to a corresponding specification class hierarchy (Figure 2). Specification classes contain additional details and attributes about a specific BAO assay component class. In addition, the BAO includes general assay specifications such as footprint (plate density), assay conditions (including pH, incubation time), readout content, quality measurement (including z-factor, z'-factor, signal to noise, coefficient of variation), measurement throughput quality, and assay stage to describe campaigns, among others.

As the BAO is under active development, we want to refer readers to the BAO website (http://bioassayontology.org/) for the most current classes and their exact definitions. Here, we briefly define the main class hierarchies. 'Assay format' is a conceptualization of assays based on the biological and/or chemical features of the experimental system, and includes several broad categories: 'biochemical format', 'cell-based format', 'cell-free format', 'tissue-based format', 'organism-based format', and 'physicochemical format'. 'Assay design' describes the assay methodology and implementation of the way in which the perturbation of the biological system is translated into a detectable signal. Specifically, the 'assay design' is organized into various types of reporters, including 'binding reporter', 'conformation reporter', 'enzyme reporter', 'inducible reporter', 'membrane potential reporter', 'morphology reporter', 'redistribution reporter', and 'viability reporter' (Figure 2A). Each of these have several subclasses and attributes (specifications); for example, luciferase reporter gene assays ('luciferase induction') that were analyzed below are a subclass of reporter gene assays, and several assay kits are available to quantitate that, as described in the corresponding specifications. The BAO describes the physicochemical method of signal detection used in bioassays as 'detection technology'. It includes 'spectrophotometry', 'fluorescence', 'luminescence', 'radiometry', 'label free', and 'microscopy'. Assay 'meta target' describes what is known about the biological system and/or its components interrogated in the assay (and influenced by the perturbagen). 'Meta target' can be directly described as a 'molecular target' (e.g. a purified protein or a protein complex), or indirectly by a 'biological process' or event (e.g. 'regulation of transcription'). In the case of cell-based assays, the BAO defines important attributes of a 'permanent cell line' in an assay (Figure 2B). The BAO cell line specifications include: 'growth mode', which describes whether it grows 'adherent' or in 'suspension'; a 'cell line culturing component' that includes the 'culture medium' and 'assay medium'; a 'cell line modification' that describes the type of modification that the cell line underwent, e.g. 'transfection', 'viral transduction', etc.; and 'transfection attributes', namely the 'DNA construct' and the 'transfection agent' used to modify a cell-line.

An assay 'endpoint' describes a quantitative or qualitative result of the bioassay. The main classes that we identified are concentration- and response-type endpoints. Simple examples include 'IC_{50}', 'EC_{50}', and 'percent

inhibition', 'percent activation', respectively. In the BAO, the concept 'endpoint' is described using DL by classifying them (among others) into 'response' and 'perturbagen concentration' endpoints, and formalizing how these are related. Endpoints are linked to what type of change they quantify, such as 'inhibition' or 'activation', and the BAO also formalizes relationships between different types of endpoints; for example, the fact that an IC_{50} corresponds to the perturbagen concentration at which 50 percent response (and specifically inhibition) is observed. This allows the querying of screening results by a mechanism of action, such as inhibition or activation, or a query based on type of results (IC_{50}, EC_{50} vs. percent inhibition, activation) and the retrieval of semantically equivalent results (described in Inference and Reasoning Using the BAO) (23).

In addition to annotating individual assays, the BAO is also suited to defined screening campaigns. As mentioned above, the BAO includes classes to define a campaign name (purpose), an 'assay stage' ('primary', 'confirmatory', 'secondary') and a 'measurement throughput quality' including 'single concentration single measurement' (which we also define as HTS), 'single concentration multiple replicates', 'concentration response single measurement' (defined as qHTS), and 'concentration response multiple replicates'. Relationships to define how one assay relates to another in a screening campaign include '*is confirmatory assay of*', '*has confirmatory assay*', '*is counter assay of*', '*has counter assay*', '*uses orthogonal technology to*', and '*uses same technology as*', etc. Relationship characteristics are also defined; for example, the inverse relationships are '*is confirmatory assay of*' and '*has confirmatory assay*'. Based on these classes and relationships, it is possible to define business rules to aggregate results within one screening campaign, and potentially across many campaigns, to prioritize compounds of a known or hypothesized MMOA. Table 1 shows examples of sets of assays comprising screening campaigns (partial view) and their (informal) BAO annotations and relationships.

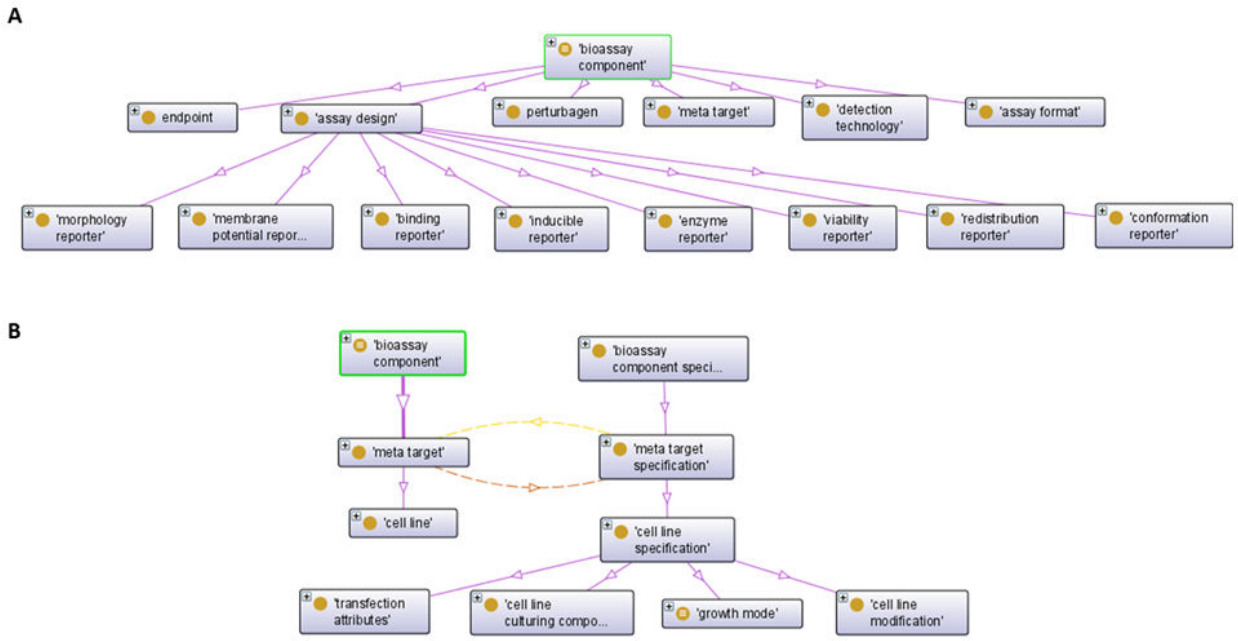


Figure 2. (A) Illustration of the major BAO class hierarchies, including the top level classes of 'assay design.' (B) The 'cell line' concept and its corresponding specification class hierarchy.

Table 1. Partial (informal) view of BAO annotations and relationships to describe assays related to the screening campaigns, "Identification of inhibitors of Kruppel-like factor 5" and "Novel allosteric modulators of the M5 muscarinic receptor."

Screen. campaign	"Identification of inhibitors of Kruppel-like factor 5"		
AID	1700	1973	1825
Assay Stage	Primary Assay	Confirmatory Assay	Secondary Assay
Relationship	has confirmatory assay 1973, has counter assay 1825	has primary assay 1700	has primary assay 1700
Assay Measurement Throughput Quality	Single concentration single measurement	Concentration response multiple replicates	Single concentration single measurement
Endpoint	% Inhibition	IC_{50}	% Inhibition
Assay Format	Cell-based format	Cell-based format	Cell-based format
Assay Design	Luciferase induction	Luciferase induction	Viability reporter, ATP content
Meta Target	Regulation of transcription	Regulation of transcription	Cell death
Detection Technology	Luminescence	Luminescence	Luminescence
Measured Entity	Luciferase	Luciferase	ATP
Screen. campaign	"Novel allosteric modulators of the M5 muscarinic receptor"		
AID	2665	2194	2206
Assay Stage	Primary	Confirmatory	Secondary
Relationship	has confirmatory assay 2194, has counter assay 2206	has primary assay 2665	has primary assay 2665
Assay Measurement Throughput Quality	Single concentration single measurement	Concentration response multiple replicates	Concentration response multiple replicates
Endpoint	Maximal activation	EC_{50}	EC_{50}
Assay Format	Cell-based format	Biochemical format	Cell-based format

Table 1. continued from previous page.

Assay Design	Calcium redistribution	Radioligand binding	Calcium redistribution
Meta Target	Muscarinic acetylcholine receptor M5	Muscarinic acetylcholine receptor M5	Muscarinic acetylcholine receptor M1
Detection Technology	Fluorescence intensity	Scintillation counting, filter assay	Fluorescence intensity
Measured Entity	Calcium	(3H)-NMS	Calcium

Integration of BAO with existing ontologies and databases

We analyzed existing biomedical ontologies with respect to their coverage of concepts required to describe HTS assays performed for probe and drug discovery projects. Although no resource existed to describe the experiments and data types, including biochemical and cell-based assays of various technologies and assay designs using small molecules, a number of existing ontologies and bioinformatics databases contain information that are needed to define parts of concepts related to HTS. We leverage these existing resources in the BAO. For example, GO (10) 'biological process' and Cell Line Ontology (CLO) (24) 'cell line' classes, along with additional parameters, are used in the BAO 'meta target' and 'meta target specifications'. CLO is currently being extended as a collaborative effort to cover cell lines relevant for biological screening (25). Organism names associated with targets were imported from NCBITaxon (26). Protein target names and IDs were referenced from UniProt (27). From Unit Ontology (UO) (28), 'concentration unit', 'time unit', and 'length unit' terms were imported into the BAO. The Ontology for Biomedical Investigation (OBI) also includes relevant information, although it describes different types of experiments (29). We are also currently working on importing the disease terminology from the human disease (DOID) ontology, which would facilitate linking targets studied in a bioassay directly to a disease (30).

We have used standard practices that allow modular reuse of external ontologies. Towards this, we have used two methods: OntoFox (31) and the module and axiom extraction facilities built into the OWL3 API (32). We have added the additional general concept inclusion axioms (GCIs) to the BAO to bridge the host concepts and the imported axioms, and we checked the safety and the satisfiability of the knowledge base using the HermiT reasoned in Protégé.

Application of the BAO

Expert annotation of PubChem assays using the BAO

Annotation Process

Using the BAO, a large number of assays were annotated by domain experts (Ph.D. biologists with expertise in HTS). To aid in manual annotation, assays were first clustered based on textual descriptions. We achieved this by initially generating a text fingerprint from all words used in title, description, protocol, and source using the Pipeline Pilot 8.0 (Accelrys, San Diego, CA) text analytics component collection. The assay "documents" were then clustered based on the fingerprints using the Tanimoto similarity metric. This method grouped together similar assays very effectively; for example, assays from the same screening campaign, by center, or assays with the same procedure or assay design, such as NCGC toxicity assays, were grouped together. Following cluster preprocessing, assays were manually annotated by assay format, design, detection technology, and other BAO categories, which included over 100 distinct concepts. The assay annotations from each assay were cross-checked, and then loaded into a Resource Description Framework (RDF) triple store. Screening outcomes (endpoints) were loaded into a relational database.

Statistics of PubChem Assay Categories

Using BAO annotations, assays can be readily categorized. We performed statistics on the 1,008 PubChem assays that we manually curated so far (Figure 3). For categorizations based on 'assay stage' there were: 286 primary assays, 425 confirmatory assays, 242 secondary assays, and 55 summary assays. In terms of 'assay format,' the majority of assays were of 'cell-based format' [548] or 'biochemical format' [372]. There are also a few assays having 'organism-based format' [13], 'cell-free format' [12], and 'tissue-based format' [8]. The 'assay design' was curated as 'binding reporter' [241], 'enzyme reporter' [227], 'inducible reporter' [212], 'redistribution reporter' [117], 'viability reporter' [91] and a few 'membrane potential reporter' [59] and 'conformation reporter' [5] types. Assays were annotated with the following 'detection technologies': 'fluorescence' [553], 'luminescence' [342], 'spectrophotometry' [39], 'radiometry' [11], 'label free technology' [6], and 'microscopy' [1]. A high level 'bioassay type,' based on compound properties and processes that are interrogated in the assay, was assigned to PubChem assays as follows: 'functional' [722], 'binding' [193], and 'ADMET' [33]. The types of assay classifications given above are relevant to interpret screening results; for example, biochemical assays provide direct evidence of the mechanism of action (e.g. inhibition of an enzyme), while activity in cell-based assays implies that a compound is cell permeable.

Annotating Screening Campaigns Using the BAO

In addition to the above annotations, the BAO can be used to group assays into screening campaigns and to organize them by assay stage, measurement throughput quality, and various assay relationships, among other categories. Table 1 provides a partial (informal) view of the BAO annotations and relations of a screening campaign to identify inhibitors of Kruppel-like factor 5 performed at the Scripps Research Molecular Screening Center (33).

Cross Assay Data Analyses Using the BAO

In the following example, we leveraged BAO annotations of PubChem assays to elaborate on compounds that are promiscuously active in assays that employ a luciferase-based design, which is one of the most widely used assay technologies. We were also interested in suggesting possible mechanisms underlying their promiscuous activity (34). Luciferase-assays were classified into five subcategories:

1. Reporter gene assays: use the luciferase gene downstream of a promoter of interest. The amount of luciferase expressed is quantified by the intensity of light (luminescence) produced in the presence of substrates, ATP and luciferin.
2. Viability assays: estimate the proportion of living cells in an assay by measurement of ATP content in a luciferase-catalyzed reaction
3. Adenosine triphosphate (ATP)-coupled assays: measure the residual amount of ATP (e.g, after a kinase reaction) by a coupled luciferase reaction.
4. Luciferin-coupled assays: measure the amount of luciferin generated after detoxification by cytochrome P450 enzyme activity
5. Luciferase enzyme activity assays: quantify the luciferase enzyme activity by the amount of light produced in a biochemical reaction.

Figure 4 illustrates the promiscuity of compounds in these five types of assays tested at either a single concentration or concentration-response. The promiscuity index (PCIdx) was simply defined as the ratio of the number of assays in which a compound was found active, and the number of assays in which it was tested.

Centered on this analysis, compounds can be classified based on their likely mechanism of promiscuity, and this mechanism can be related to their chemical structure. For example, we looked specifically at compounds most frequently active in luciferase reporter gene assays and analyzed their promiscuity in the other types of assays using luciferase (Figure 5). Two clusters emerged, one in which compounds were also active in the majority of

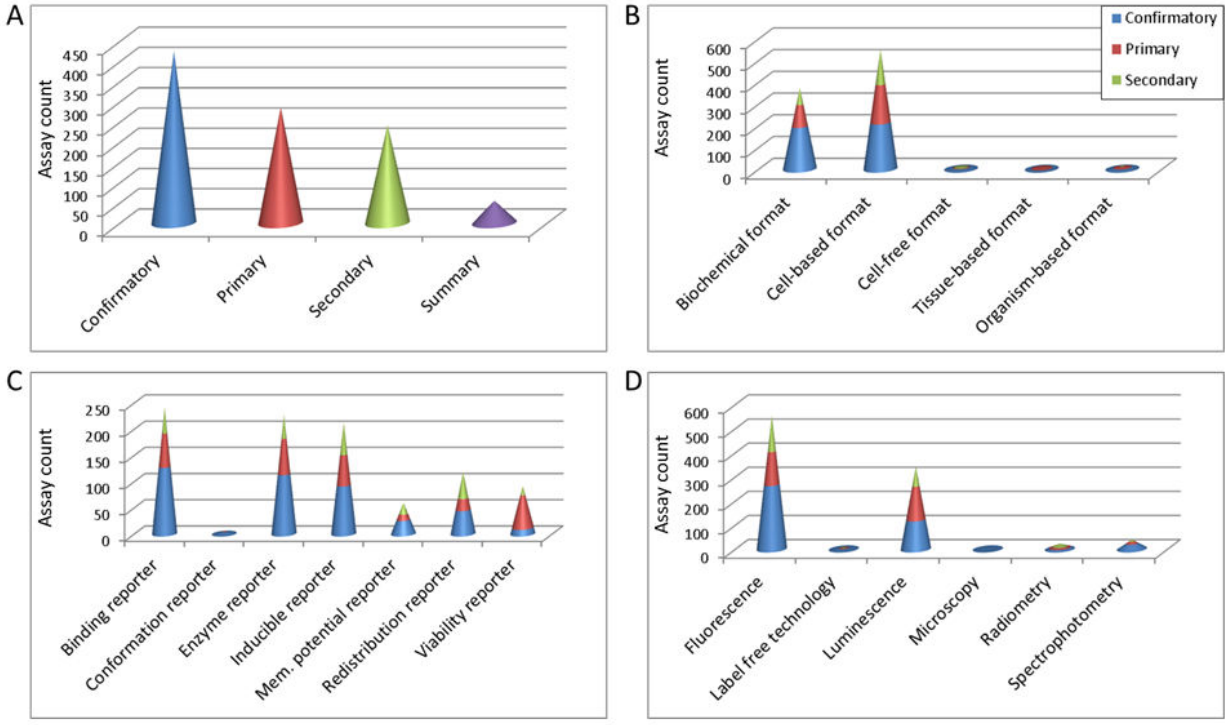


Figure 3. PubChem Assay counts based on BAO annotations of assays by 'assay stage' (A) 'assay format' (B), 'assay design' (C), and 'detection technology' (D) of 1,008 assays.

viability assays and which are therefore likely toxic, and another where compounds are active in luciferase enzyme inhibition assays and whose activity is therefore likely related to interference with the luciferase enzyme. Several chemotypes were identified, which were consistent with the respective proposed mechanisms (34).

In other examples, as the BAO allows the annotation of important reagents used in an assay, it may thus be applied to identify and analyze the influence of subtle differences in the assay conditions, e.g., the presence of 'detergent', 'reducing agent', etc. In PubChem AIDs 584, 585, 1002 and 1003, compounds were screened to identify AmpC beta-lactamase inhibitors, both in the presence and absence of 0.01% Triton X-100. These assays were performed at the NCGC, where they used the aggregation profiling approach to identify sensitivity of aggregate formation of compounds to detergent (35, 36). In another campaign, the investigators screened for inhibitors of caspase-1 (AIDs 888, 929) and used reagents with different redox potential to eliminate false positives that could result from compound-generated reactive oxygen species (37, 38). In annotating PubChem assays using the BAO, we capture these types of reagents, facilitating later identification and analysis of the effects of those reagents on screening outcomes.

Inference and reasoning using the BAO

In addition to identifying assays and subsequently analyzing their results based on BAO class annotations (as described above), the BAO also provides the potential to infer annotations and to identify assays and screening results that are semantically equivalent to a query of interest, but involve query terms not explicitly matched, and therefore could not be easily identified by a classical (relational) search. These types of functionalities are made possible by the open-world reasoning/inference capabilities of the system. For example, the BAO formally (using DL) defines an 'IC_{50}' endpoint as the 'perturbagen concentration' that results in '50 percent inhibition'. As described above, both 'IC_{50}' and 'percent inhibition' are specified as quantifying 'inhibition' (mode of action). These formal descriptions, among others, enable the retrieval of both IC_{50} or percent inhibition results in queries for active compounds, as specified by response and screening concentration cutoffs (23). BAO inverse

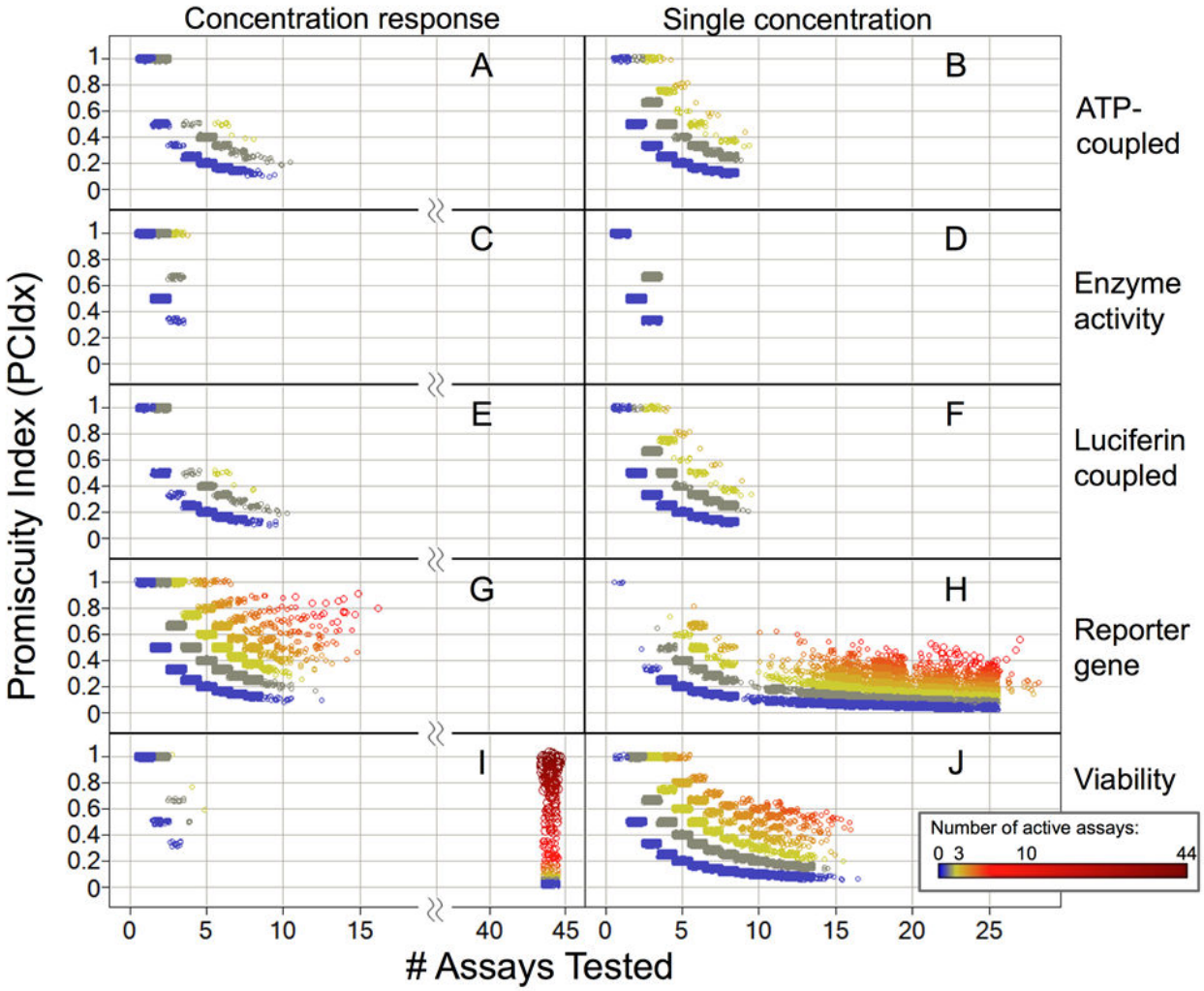


Figure 4. Compound promiscuity by luciferase assay technologies. For each compound, the Promiscuity Index versus number of tested assays is depicted. (**A, B**) Adenosine triphosphate (ATP)–coupled enzyme activity (e.g., kinase activity, not viability). (**C, D**) Luciferase enzyme activity. (**E, F**) Luciferin-coupled enzyme activity (e.g., P450). (**G, H**) Luciferase reporter gene assays. (**I, J**) Cell viability assays (ATP coupled). (**A, C, E, G, I**) Concentration–response assays. (**B, D, F, H, J**) Single-concentration assays. Color and size indicate the number of assays (of the particular luciferase assay type) in which a compound was active. In total, 87,615 data points with at least one active assay are shown: (**A**) 3,457, (**B**) 5,619, (**C**) 2,313, (**D**) 3,646, (**E**) 3,457, (**F**) 5,619, (**G**) 14,200, (**H**) 36,685, (**I**) 1,413, (**J**) 11,206. Figure reprinted from reference (34) with permission from Sage publications.

relations linking perturbagens, endpoints, and measure groups further enable the identification (by inference) of assays that are active in a certain number of assays. Several SPARQL (Simple Protocol and RDF Query Language) query examples are available from our website (http://www.bioassayontology.org/).

In other possible applications, reasoning can be leveraged to identify potential annotation errors, because BAO axioms do not allow all possible combinations of annotations. In a simple example, 'cell–based format' may be associated with specific subclasses of 'assay design' such as 'inducible reporter', 'viability reporter', or 'redistribution reporter', while biochemical assays may be associated with 'enzyme activity'. Because the various subclasses of 'assay design', as well as cell-based and biochemical assays are disjointed, errors could readily be identified via reasoning.

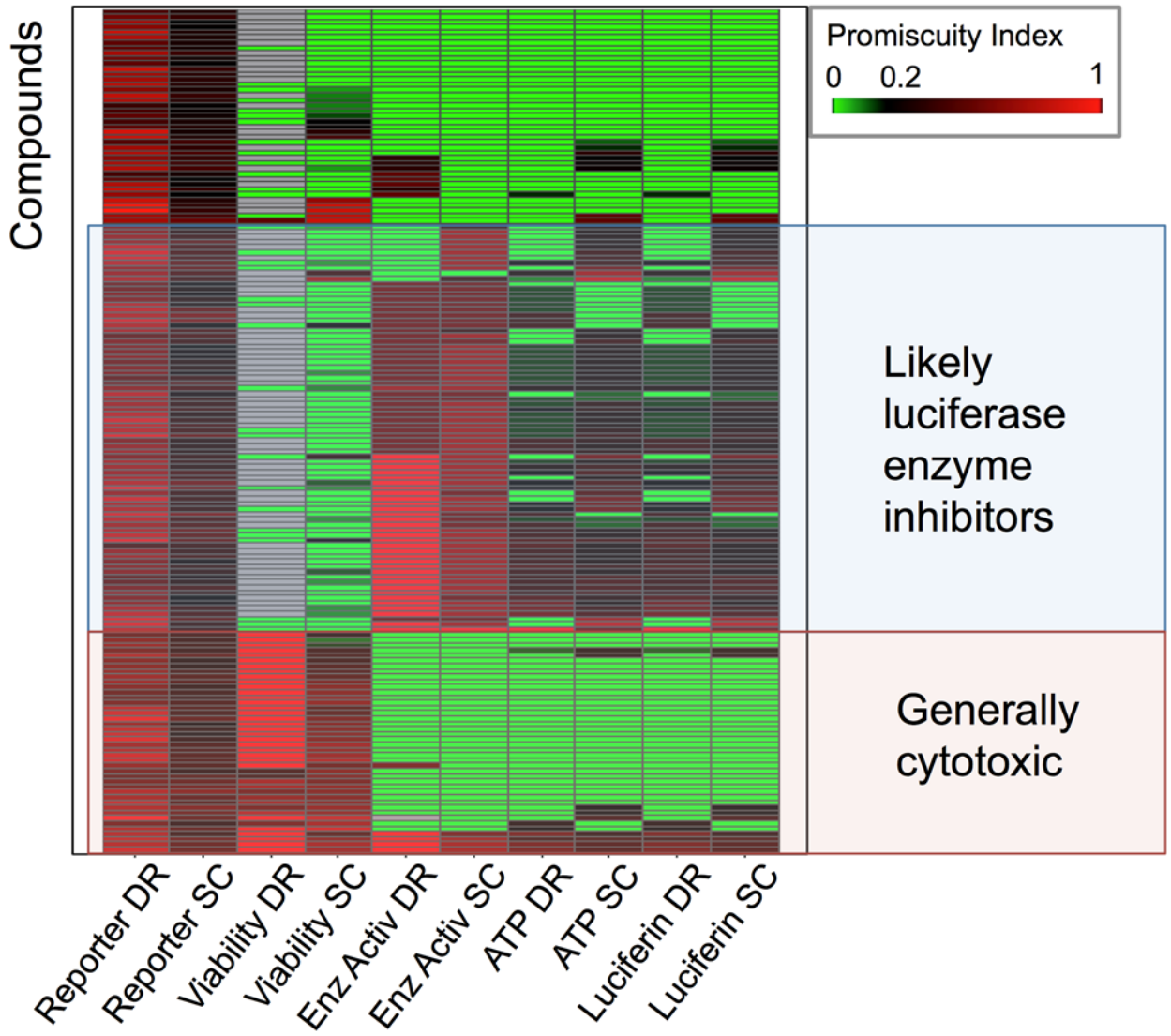


Figure 5. Heat map of the 161 most promiscuous compounds in luciferase reporter gene assays, which are active in at least 5 concentration-response and 5 single concentration (luciferase reporter) assays. DR and SC denote "dose response" and "single concentration," respectively. Shown are the promiscuity indices of all compounds in the different luciferase assay categories for both concentration-response and single concentration assays, respectively, clustered by their PCIdx profiles. Two groups of promiscuous reporter gene compounds were apparent, suggesting the mechanism for reporter gene assay promiscuity; this mechanism is one in which compounds were also active in viability assays (red shade) and the other where compounds were also active in luciferase enzyme assays (blue shade). Figure reprinted from reference (34) with permission from Sage publications.

Assay Annotation Tool to facilitate adaptation of the BAO

To enable biologists to report sufficiently annotated and standardized screening data using the BAO, we developed a proto-typical Excel-based annotation tool called the BioAssay Annotation Template (BAT) (39, 40). To create the BAT, we first generated a BAO-derived dictionary file in which the major BAO class hierarchies are flattened out. The resulting BAO-derived dictionary file includes all the leaf nodes of the BAO with a label/name that reflect the original BAO hierarchy. The IDs of these terms correspond to the original BAO leaf nodes (Figure 6). This flattened version of the BAO was then used in RightField (40), an open-source tool for adding ontology terms to Excel spreadsheets, to generate an annotation template by mapping BAO terms to corresponding Excel cells and exposing them as drop down menus. The Excel-based BAT includes the metadata field names to be annotated with the corresponding BAO terms. It is organized by the main BAO categories. For many of the

annotation fields, the template specifies a range of allowed terms from the BAO, which are presented as a drop-down list/menu. To illustrate the use of BAT to annotate data, we illustrate the annotation of a PubChem assay (partial view, Table 2).

As the BAO is version controlled, any BAT corresponds to a specific BAO release. Because the BAO leaf node IDs correspond to the dictionary terms, annotations made based on a previous version of the BAT can (in most cases) be mapped to an updated version. This is critical, because the terminology will likely be optimized over time and new terms may be added corresponding to new assay designs, technologies, etc. We envision the BAO to be established as a standard to describe probe and drug discovery assays, and we will maintain the BAO in the public domain. BAO-derived products, such as dictionary files and an annotation tool (conceptually similar to BAT) will thus enable the community to annotate assays using the same centrally and publically maintained terminology, identified by BAO IDs, since the derived products correspond to a specific recent release of the BAO.

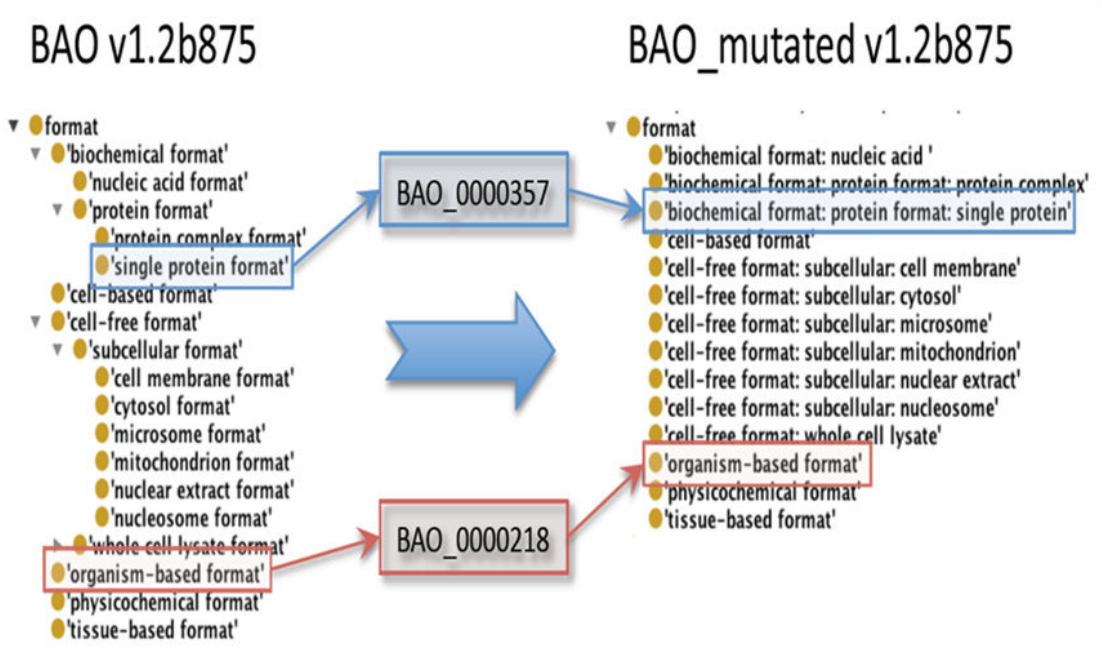


Figure 6. Flattening of the BAO class hierarchy generates a BAO-derived dictionary file, which contains only the most specific leaf nodes for the main BAO categories. The BAO leaf node IDs were maintained in this process. The labels/names in the flat dictionary file reflect the original class BAO hierarchy.

Table 2. Partial view of annotation of a PubChem assay (AID1700) using BAT

BAO Concept	BAO Annotation Terms
id specification: bioassay id: **pubchem aid**	1700
bioassay specification: **screening campaign name**	Identification of inhibitors of Krüppel-like factor 5
bioassay specification: **assay title**	Primary cell-based high throughput screening assay to identify inhibitors of Krüppel-like factor 5 (KLF5)
bioassay specification: **assay stage**	primary
bioassay specification: **bioassay type**	functional
bioassay specification: **assay measurement throughput quality**	single concentration single measurement
bioassay specification: **assay measurement type**	endpoint assay
bioassay specification: **content readout type**	single readout
bioassay specification: **assay readout method**	regular screening
bioassay specification: pubchem specification: **assay source**	The Scripps Research Institute Molecular Screening Center
assay format	cell-based format
assay format detail: **assay phase characteristic**	heterogeneous assay
meta target: **biological process target**	regulation of transcription
meta target: **permanent cell line**	IEC-6
meta target detail: cell line specification: **cell modification**	stable transfection
meta target detail: cell line specification: transfection specification: **dna construct**	luciferase gene with Kruppel-like factor 5-promoter
assay design	luciferase induction
assay design detail: **measured entity**	luciferase
assay design detail: **signal direction**	signal decrease
assay design detail: assay kit information: **name**	SteadyLite-HTS reagent
assay design detail: assay kit information: **manufacturer**	Perkin Elmer
perturbagen	small molecule
perturbagen detail: **compound library**	MLSMR
detection technology	luminescence
detection technology detail: **detection instrumentation**	ViewLux CCD imager
detection technology detail: **detection instrumentation manufacturer**	Perkin Elmer
endpoint	percent inhibition
endpoint detail: **pubchem tid**	1
endpoint detail: response endpoint: **response unit**	percent
endpoint detail: **screening concentration**	5
endpoint detail: screening concentration: **concentration unit**	micromolar
endpoint detail: curve fit specification: **number concentration values**	1
endpoint detail: curve fit specification: **number replicates**	1
endpoint detail: **endpoint mode of action**	inhibition

BAOSearch software application

We have developed a prototype Semantic Web software application, BAOSearch (41). BAOSearch is a web-based application for querying, browsing and downloading small molecule bioactivity data by using concepts defined in the BAO. BAOSearch is available at http://baosearch.ccs.miami.edu/. BAOSearch is a multi-tier, web-based, AJAX-enabled application written primarily in Java and built following a RESTful Web Services paradigm (Representational State Transfer (REST)), which is a software architecture for multi-platform on-demand content delivery (42, 43); all data are stored in MySQL. SPARQL Database (SDB (44)) is used for the triple-store to store asserted and inferred assay annotations encoded in OWL. Numerical data associated with screening results and chemical structure information are stored in the associated relational schema and accessed using Hibernate (45) in order to enable fast searching of these types of data. The free-text search functionality is enabled using the Lucene text search library (46, 47). This is done using a modified version of the VIVO indexer (48, 49), which pulls new annotations from the triple-store, adds associated information from the BAO based on the subsumption hierarchies, and then processes this information using the Lucene library to build the free-text search indexes. This is illustrated in Figure 7, where a query was run (by concept search) for identifying biochemical assays having IC_{50} endpoints of less than 10 micromolar concentration. The endpoints that satisfy the set search criteria were categorized based on the major BAO concepts, namely, 'assay design', 'detection technology', 'assay format' and 'meta target', as displayed in the results page.

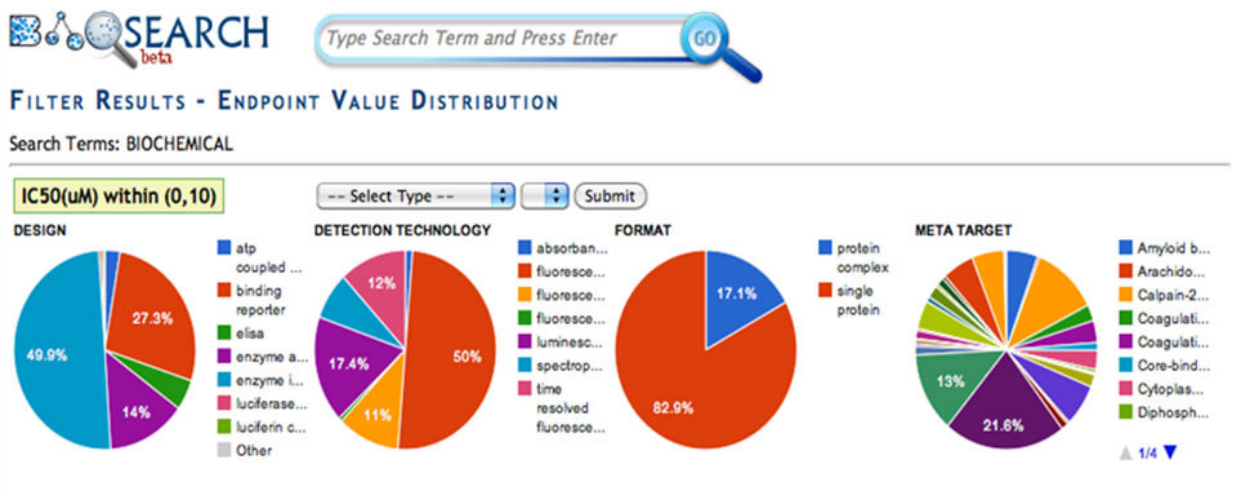


Figure 7. BAOSearch facilitates querying, browsing and downloading of small molecule chemistry and bioactivity data by using concepts defined in the BAO.

Summary and conclusion

Large amounts of data are generated by HTS in private and public organizations. Nevertheless, large scale screening capabilities have so far not translated to increased numbers of approved drugs (50). One likely reason is that available data is used inefficiently. It remains a challenge to effectively translate increasing amounts of data into actionable knowledge; at the very least, this is the case for the current public domain data. To address this challenge, we have developed the BioAssay Ontology (BAO) to describe biological assays and their outcomes by concepts that are relevant to interpret, analyze and integrate screening data. The BAO addresses: 1) development of standardized terminology and uniform standards to report HTS and lower throughput probe and drug discovery assays and results; and 2) a semantic description of bioassays and their results to formalize domain knowledge and to facilitate semantic integration with diverse other resources (10, 25, 26, 28). We have used the BAO to annotate PubChem assays, provide statistics, and show that BAO concepts are useful to categorize and analyze screening results (34). Beyond individual assay and results, the BAO also describes screening campaigns. The BAO supports inferences within the functionality of OWL 2.0, raising the possibility to formally reason across the data with potential to discover novel insights or detect possible errors sources and inconsistencies by inference. We are developing the BAO into a community standard to describe assays and their results, with the goal of enabling the integration of diverse datasets and facilitating the interpretation and global comparison and analysis of assay experiments and screening outcomes. The BAO also includes information to enable linking external content, such as pathway databases. The overarching goal of the BAO is to better organize the available assay space in the context of bioinformatics and chemical biology resources, and in doing so, to enable inference or prediction of MMOA of small molecules based on the corpus of available screening data. The BAO will thus enable the biological research community to better utilize increasingly large and diverse chemical biology screening datasets to develop and test scientific hypotheses. We also see potential for the BAO to facilitate cross-disciplinary collaborations, for example, among screening biologists, chemical biologists, engineers, medicinal chemists, and cheminformaticians, because the BAO provides formal definitions (including relationships) of concepts that relate to several disciplines.

The BAO is openly available and under active development. For the most current release and a wide variety of information related to the BAO project, we refer to our website (http://www.bioassayontology.org/) and Wiki (http://www.bioassayontology.org/wiki). We continue to annotate assays develop software tools related to the BAO. Our BAOSearch Semantic Web application makes it very easy to query, search, explore, and download

BAO-annotated assays, standardized screening results, and chemical structures. It is available at http://baosearch.ccs.miami.edu/.

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Glossary of BAO terms (partial list)

As the definition of terms will evolve, be optimized over time, and BAO terms will evolve over time, we are maintaining a glossary of BAO terms on our BAO Wiki: http://bioassayontology.org/wiki/index.php/BAO_Glossary. Here, we provide a current partial list of the most important BAO terms.

Absorbance wavelength — In absorbance measurements, it is the wavelength at which light is absorbed by a biological entity or a dye.

Assay biosafety level — The level of biocontainment required to isolate hazardous biological agents in an enclosed facility; it ranges from the lowest biosafety level of 1 to the highest at level 4.

Assay design — The underlying method (technology) and assay strategy used to determine the action of the perturbagen in the assay system.

Assay footprint — This describes the physical format, such as plate density, in which an assay is performed; this is generally a microplate format, but it can also be an array format.

Assay format — Assay Format is a conceptualization of assays based on the biological and/or chemical features of the experimental system.

Assay kit information — ManufacturerInformation about the name of the company that created the kit/component used in the assay.

Assay kit informationName — The title of the kit/biologicals used in the assay.

Assay measurement throughput quality — Describes the quality of the measurements performed on each sample, such as single concentration, single repetition, concentration-response, multiple repetitions, etc.

Assay measurement type — This describes whether a change in an assay is measured once at one fixed end-point or over a period of time at several time points.

Assay medium — The cell culture broth used while performing an assay on cells; it is optimized for each assay type. Some interfering additives such as serum, growth factors, buffers, amino acids, antibiotics, etc. might be eliminated in this medium.

Assay phase characteristic — Refers to whether all assay components are in solution or in solid phase, which determines their ability to scatter light.

Assay readout method — This describes the difference in the number of detection parameters used in HTS versus HCS assays.

Assay serum — The serum used in assay medium while performing an assay on cells; it is optimized for each assay type.

Assay source — The screening center at which the assay was performed. Most of the assays deposited in PubChem were performed at one of the MLPCN screening centers.

Assay stage — It describes the purpose of the assay in an assay campaign. Assay stage also relates to the order of assays in a screening campaign. For example, the primary assay, which is performed first, identifies some hits. The primary hits are then confirmed in a confirmatory assay. Subsequent secondary assays are run to eliminate compounds that are not of interest or to confirm hits using an alternate design/technology, or to further characterize compounds.

Assay temperature — The temperature at which the assay was performed.

Assay title — The name of a bioassay, which is associated with each assay ID (AID).

Background control — It is the reading obtained from wells to which the sample or one of the reagents was not added. This is subtracted from all of the experimental readings prior to further analysis.

Bioassay type — Categorization of bioassays based on the property or process that the assay is interrogating, e.g. ADMET, functional, etc.

Biological process target — A biological process is a process of a living organism. It is a recognized chemical reaction or molecular function with a defined beginning and an end.

b-score — It is calculated by first computing a two-way median to account for row and column effects of the plate; then, this is divided by their median absolute deviation to standardize for plate-to-plate variability.

Buffer — A mixture of a weak acid and its conjugate base or a weak base and its conjugate acid.

Cell modification — The type of alterations performed on the cell line, which include plasmid transfection, viral transduction, cell fusion, etc.

Coefficient of variation — Measure of the signal dispersionCV=100x[StdDev(sample)/Avg(sample)]

Cofactor — A nonprotein component of enzymes required for the enzyme's activity, e.g., coenzyme A (CoA).

Compound library manufacturer — The name of the company that synthesized a compound library.

Compound library — The name of a perturbagen collection from one source, which can be either commercial or academic, e.g., MLSMR, LOPAC, etc.

Concentration unit — This describes the most common unit used in calculating volumetric stoichiometrymolarity. Units includemolar, millimolar, micromolar, nanomolar.

Content readout type — It is the throughput and information content generated in an assay. Categorizes multiplexed (i.e. multiple targets measured simultaneously), multiparametric assays, high content (image-based) and regular (plate reader) assays.

Coupled substrate incubation — It is the interval of time between the addition of a coupled enzyme substrate and the reaction stopping procedure in a coupled enzyme assay.

Coupled substrate — It is an enzyme substrate whose concentration is regulated by the activity of a different enzyme.

Culture medium — The liquid broth used to grow cells, which is optimized for each cell type and includes additives such as growth factors, buffers, amino acids, antibiotics, etc.

Culture serum — Cultured cells require serum or growth factors for growth by cell division. Each cell type is grown in a medium supplemented with a variable concentration of serum, which is optimized for its growth.

Curve fit model — A mathematical model/equation to which the data is fitted (using the curve fit method/ algorithm). The Hill equation is commonly used to estimate the number of ligand molecules that are required to bind to a receptor to produce a functional effect.

Curve fit specification — input dataIt identifies whether the data has been normalized (by Z and Z' factors, signal to background, signal to noise, and coefficient of variance), or if it was an immediate readout obtained from a HTS assay.

Curve fit specification — number concentration valuesIt is the number of concentrations at which the perturbagen is screened to obtain the curve fit endpoint.

Curve fit specification — number replicatesIt is the number of replicate measurements (repetitions) per concentration value used in the curve fit procedure.

Detection instrumentation manufacturer — The name of the company that created the detection instrumentation.

Detection instrumentation — Lists the type of equipment used for measurement/readout from an assay, e.g. FLIPR, ViewLux plate reader, PHERAstar, etc.

Detection technology — The physical method (technology) used to measure/readout the effect caused by the perturbagen in the assay environment.

Detergent — A surfactant used in biological assays to lyse the cells and tissues by solubilizing the membrane lipids, and to unfold the proteins by disrupting the bonds.

Disposition — This describes the status of a tissue, and whether it is from a normal or a diseased tissue.

DMSO — It stands for dimethyl sulfoxide, which is a common organic solvent used to solubilize the chemical compounds prior to their addition to an assay; it is often used as a control.

DNA construct — A brief description of the recombinant DNA created by the insertion of a gene or siRNA encoding DNA of interest into a vector. It includes information on the type of vector, the type of promoter, the selectable markers, etc.

Dye — Molecules used as optical probes, which have fluorescence/colorimetric properties.

EC number — A unique identifier of an enzyme based on the chemical reaction that it catalyzes. It is created by the Enzyme Commission.

Emission wavelength — For fluorescence measurements, it is the wavelength at which an excited fluorophore emits fluorescence.

Endpoint mode of action — This refers to the effect of the perturbagen on the target of an assay, and whether it brings about inhibition, activation, cytotoxicity, etc.

Endpoint — An abstract concept representing a measurement or parameter quantifying or qualifying a perturbation.

Entrez gene accession number — A unique identifier of a gene, e.g., GeneBank ID.

Enzyme activity measurement — The different methods by which an enzyme function can be measured, such as product formation or substrate depletion.

Enzyme reaction — The interval of time during the enzyme activity measurement in a kinetic assay.

Excitation wavelength — For fluorescence measurements, it is the wavelength at which a fluorophore is excited.

Growth mode — Refers to the manner in which cells grow in culture, either in suspension (without being attached to a surface) or adherent (cells requiring a surface, such as tissue culture plate).

High control — The high control contains the substrate titration without inhibitor to reflect the maximum activity at each substrate concentration.

Inducer — An inducer is a molecular entity that is required to initiate a biological process, e.g., interferon-gamma is an inducer of the transcription factor STAT-1.

Inhibitor reagent — A known blocker of a target used to lower the activity of that target prior to screening for selective activator compounds of that target.

Interaction — The molecule or entity to which the protein binds in a binding reaction, e.g. protein, DNA, RNA, etc.

Low control — The low control contains the substrate titration without enzyme or substrate and without inhibitor. The low controls should reflect the signal expected for no enzyme activity at each substrate concentration.

Measure group — It is an abstract concept to group and link multiple (different) sets of experimental results to one bioassay. Measure groups thus facilitate the description of multiparametric (or multiplexed) assays that measure more than one effect of the perturbagen on the system that is screened. They would also facilitate the definition of profiling assays, e.g. assays that test compounds against multiple (protein) targets.

Measured entity — A molecular entity, which is the output of a biological reaction or process that is quantitated either directly (by the presence of a tag or probe) or indirectly in a coupled reaction.

Metal salt — An ionic compound (principally consisting of Na, K, Li, Ca, and Mg as cation) that is used as an additive in an assay.

Mode of perturbagen introduction — The reagent used to introduce siRNA into the cell, e.g. lipofectamine, Fugene, etc.

Molecular target — It is either a protein or a nucleic acid of interest, which is modulated by a perturbagen in an assay.

Monoclonal antibody — Highly specific antibodies produced by the clones of a single hybrid cell, which is generated by the fusion of a B cell with a tumor cell. They recognize a unique epitope of an antigen.

NCBI taxonomy ID — The unique identification number pertaining to the classification of an organism. It is obtained by referring to the NCBI taxonomy database.

Negative control — The control is used to determine the base-line to compare the effect of the test perturbagen. Often the negative control is the solvent (e.g. DMSO) in which the perturbagen was dissolved.

Normalized percent inhibition — Data normalization obtained by dividing the difference of mean of the positive controls and sample measurement by the difference of the mean of the positive controls and mean of the negative control.

Occluder — A compound that impedes interaction of promiscuous perturbagens with co-expressed proteins; it is used to limit non-specific interactions of the perturbagen.

Organism name — Entity from which protein / cell line was derived.

Percent control — A measure of the compound activity calculated by dividing the raw measurement obtained with the compound by the mean of the measurements with the controls multiplied by 100.

Permanent cell line — Immortalized cell, which has undergone transformation and can be passed indefinitely in culture.

Perturbagen delivery — This describes whether the perturbagens are tested individually or as mixtures/pools.

Perturbagen incubation — The interval of time between the addition of a perturbagen and the measurement of change, as observed by a detection/readout in a bioassay.

Perturbagen library type — A collection of perturbagens, e.g., compounds, siRNA, miRNA, etc., which are sold by different vendors, or generated in academic institutions.

Perturbagen origin — The source of perturbagen, whether it is purified from a natural source or it is a synthetic compound, etc.

Perturbagen — The agents used to alter the activity of the target of interest in a bioassay.

pH — Assay pH approximates the negative logarithm (base 10) of the molar concentration of dissolved hydronium ions in a solution.

PMIDA PMID — (PubMed Identifier or PubMed Unique Identifier) is a unique number assigned to each PubMed citation.

Polyclonal antibody — Antibodies produced by immunizing a mammal such as a mouse, rabbit or goat with an antigen. They are a mixture of antibodies produced from different B cells raised against any of the different epitopes of that antigen.

Positive control — It is a chemical compound or reagent used in each plate of an assay to normalize the response of the test perturbagens (by plate).

Potentiator — A known activator of a target/pathway, used prior to screening the compounds for selective inhibitor indications (e.g., forskolin is a potentiator for Gi/o pathway of G-proteins).

Pressure — It is the force per unit area applied in a direction perpendicular to the surface of an object in an assay.

Primary cell line — Cells that are cultured from a subject (tissue or tumor) and which are not immortalized. They can undergo only a limited number of cell divisions prior to reaching senescence.

Protein form — It describes whether changes were introduced into a protein sequence and the type of changes, e.g., fusion, mutation, deletion, post-translational modifications.

Protein preparation method — The method by which a protein is purified from its source.

Protein purity — The steps involved in isolating a protein from either its natural or recombinantly expressed sources.

PubChem AID — It is the unique identification number of a bioassay.

PubChem assay comment — This is the PubChem assay statement that can be assigned by depositors to a bioassay.

PubChem assay depositor — The organization or individual that added the assay to PubChem.

PubChem assay description — This contains the background information and rationale for performing a bioassay, and is found on the PubChem website associated with each assay ID (AID). It also includes the assay design and some information on the related bioassays.

PubChem assay protocol — This includes the methodology used to perform a particular bioassay and is found on the PubChem website associated with each assay ID (AID). It includes the assay components and the order in which they were added, the incubation times, detection method and the kit used, etc.

PubChem assay provider — The investigator who provided the assay to be run at the screening center.

PubChem summary comment — The statement deposited by the assay provider, which summarizes the screening campaign with respect to the identification of lead compounds and chemical probes.

PubChem TID — It is an endpoint ID of a bioassay deposited at PubChem.

Redox reagent — Redox reagents either accept or donate electrons to facilitate the oxidation-reduction reactions, e.g. dithiothreitol (reducing agent), hydrogen peroxide (oxidizing agent).

Response unit — Units of endpoints/results, which are obtained from testing perturbagen at a single concentration, e.g., percent, count, ratio. Response endpoints quantify the magnitude of the perturbation.

Screening campaign name — A title used to describe a set of assays that are performed to achieve a screening campaign goal, e.g., to identify leads for a drug development program.

Screening concentration — It is the concentration of the perturbagen used in an assay to elicit the biological effect or perturbation.

Signal direction — It is the trend of measured readout signal, whether it increases or decreases in perturbagen treated wells, as compared to the untreated or carrier-treated wells in an assay.

Signal to noise — Measure of signal strength: S/N=[Avg(pos control)-Avg(neg control)]/[StdDev(pos control) +StdDev(neg control)]

Signal window — Difference of signal between positive and negative controls; assay dynamic rangeSW={Avg(pos control)-Avg(neg control)-3x[StdDev(pos control)+StdDev(neg control)]}/StdDev(pos control)

Substrate incubation — The interval of time between the addition of an enzyme substrate and the measurement of change, as observed by a detection/ readout in a bioassay.

Substrate — The substance on which the enzyme acts to generate a product.

Tracer — A radioisotope used as a tag in a biological assay, e.g. 3-H thymidine used as a tracer to detect DNA-replication.

Transfection agent — The agent used to introduce the nucleic acid of interest into cells, e.g., lipofectamine, Fugene, etc.

UniProt ID — It is a unique identifier of a protein sequence.

z factor — It describes the assay dynamic range considering range and data variation: Z=1-{3x[StdDev(sample) +StdDev(neg control)]/[Avg(sample)-Avg(neg control)]}. It is not to be confused with z-score.

z-prime factor — It describes the assay dynamic range considering range and data variationZ'=1-{3x[StdDev(pos control)+StdDev(neg control)]/[Avg(pos control)-Avg(neg control)]}

Z-score — Random and systematic error can induce either underestimation (false negatives) or overestimation (false positives) of measured signals. Z-score indicates how many standard deviations an observation is above or below the mean. For calculating this value, the average of the plate values is subtracted from individual raw values, and the difference divided by the standard deviation estimated from all measurements of the plate.

Data Standardization for Results Management

Robert M. Campbell,[1] Julia Dymshitz,[2] Brian J. Eastwood,[3] Renee Emkey,[4] David P. Greenen,[5] Julia M. Heerding,[6] Dwayne Johnson,[7,*] Thomas H. Large,[8] Thomas Littlejohn,[9] Chahrzad Montrose,[10] Suzanne E. Nutter,[11] Barry D. Sawyer,[12] Sandra K. Sigmund,[13] Martin Smith,[14] Jeffrey R. Weidner,[15,†] and Richard W. Zink[16]

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Abstract

Raw data collected in screening assays should be appropriately analyzed to derive activity expressed as potency or efficacy values of tested compounds (IC_{50} or EC_{50} values). In this chapter the authors discuss standardized approaches to processing radioligand binding, enzyme and functional assays used in HTS and lead optimization. Detailed account is given in processing normalizing raw data and curve fitting. A glossary is also included defining the terms used for consistency in processing raw data in a standardized manner.

Introduction

Definitions of Result Levels

Raw data: Individual measurements as produced by the instruments used in the experiment.

Normalized well level: Individual data values that have been transformed to provide a consistent, biologically relevant context. This is often done using vehicle and maximally efficacious compound controls. (Inhibition, Stimulation, etc; in most cases Inhibition and Stimulation are expressed as a % of the dynamic range of the assay)

Aggregate: median (preferred) or mean normalized well level data when replicates exist in a single run (Inhibition, Stimulation, etc.). This level provides for a consistent determination of n as it applies to *in vitro* results.

Derived data: Results calculated from groups normalized or aggregate well level data based upon the fit of this data to a mathematical model. (IC_x, Relative IC_x, EC_x, K_i, K_b, etc.)

Summarized data: Statistical summarization of results across multiple runs. (geometric mean ICx, Relative IC_x, K_i, K_b or average Inhibition, Stimulation, etc.)

Absolute IC_{50}, Relative IC_{50} or Relative EC_{50}

For assays described in this chapter, absolute IC_{50} (abs IC_{50}), relative IC_{50} (rel IC_{50}) and relative EC_{50} (rel EC_{50}) are predominantly used to derive a value that can be used to compare results within and across runs in the same assay, as well as between different assays. Abs IC_{50} and rel IC_{50} are used when different assumptions are

Author Affiliations: 1 Eli Lilly & Company, Indianapolis, IN. 2 Eli Lilly & Company, Earlwood Manor, UK. 3 Eli Lilly & Company, Earlwood Manor, UK. 4 Amgen Inc., Boston, MA. 5 Eli Lilly & Company, Indianapolis, IN. 6 Eli Lilly & Company, Indianapolis, IN. 7 Eli Lilly & Company, Indianapolis, IN. 8 Sunovian Pharmaceuticals Inc., Boston, MA. 9 Eli Lilly & Company, Indianapolis, IN. 10 Eli Lilly & Company, Indianapolis, IN. 11 Eli Lilly & Company, Indianapolis, IN. 12 Eli Lilly & Company, Indianapolis, IN. 13 Eli Lilly & Company, Indianapolis, IN. 14 Eli Lilly & Company, Indianapolis, IN. 15 AbbVie, Chicago, IL. 16 Eli Lilly & Company, Indianapolis, IN.

* editor

† editor

applied; the selection of either is at the discretion of the scientist but should be applied consistently and not changed for a defined assay.

For consistency, rel IC_{50} is used for inhibition assays while rel EC_{50} is used for stimulation assays, even though there is no fundamental difference between them. Because of their relative simplistic composition, biochemical *in vitro* assays can be easily labeled as either "Stim" or "Inh", while every biochemical whole cell assay can be either as "Stim" or "Inh" depending on multiple factors. Therefore, the guideline for defining whole cell biochemical assays is to use the label that better reflects the perceived pharmacology, regardless of the direction (increasing or decreasing with test substance concentration) of the raw signal. How an assay is defined can also drive which result type label to use. For instance, if an assay categorized by cell cycle modulation is attempting to inhibit the cell cycle, the rel IC_{50} should be used.

Guidelines for Curve Fitting

- Three or four parameter logistic curve fits are acceptable.
- Under appropriate conditions, the top may be fixed to 100 (maximum or compound control level) and the bottom may be fixed to 0 (minimum or vehicle control level).
- It is recommended that the Hill Coefficient not be preset to any fixed number, unless supported by a statistician.
- Cubic spline curve fits *are not recommended*, unless supported by a statistician.
- The Fitting Error of the IC_{50}/EC_{50} should not exceed 100%, unless supported by a statistician. (*It should be noted that this "standard error" is a measure of "goodness of fit" of the data to the curve fitting equation and not the "standard error" of aggregate data values*).

Normalizing Data using a Positive Control Curve

In some cases (such as where there is a non-linear standard curve for the analyte), it is preferable to use a reference curve to define the dynamic range of the assay. In those cases, the fitted top of the reference curve is substituted for the max while the fitted bottom of the reference curve is substituted for the min in normalization calculations. This may be particularly useful in agonist assays where the use of a reference agonist curve is strongly recommended. It is still preferable to define the dynamic range on each plate so that individual plate drift is assessed and single plates can pass/fail. Additionally, the upper and lower asymptote of the reference curve should be established by the data in order to use them for dynamic range determination.

Application of a Standard Curve

Use of a standard curve is required wherever possible when the raw data is not a linear function of the biological response. For example, optical densities, fluorescence units and luminescence units often cannot be directly used for calculations of activity as they are often non-linear functions of the concentration of the relevant biological product. A standard curve is used to convert the raw data to concentration of biological substance. The calculated concentrations are then used to calculate the *Normalized Result*, as discussed in most thoroughly in the Immunoassay chapter. The standard curve data should be generated with an appropriate number of points and concentration range, fit by an appropriate concentration-response model so that bias and precision are within acceptable limits. All raw data within the scope of the assay can be converted to the biological response.

Data Types and Associated Rules for Radioligand Binding Assays: Inhibition Mode

Normalized Results

For radioligand binding methods, the use of Inhibition is recommended to quantify the ability of individual concentrations of a substance to inhibit the total specific binding of radioligand. The use of % bound *for normalization is discouraged*, but it's recommended that biologists calculate and track changes to % bound as a measure of assay performance.

Calculation:

$$\text{Inhibition} \quad (\%) = 100 - \left[\frac{(\text{Measured Binding} \quad - \text{Min})}{(\text{Max} - \text{Min})}\right] x100$$

Max = maximum binding

Min = non-specific binding

Derived Results: Absolute IC_{50} and Relative IC_{50}

Absolute IC_{50} = the molar concentration of a substance that reduces the specific binding of a radioligand to 50% of the maximum specific binding.

Relative IC_{50} = the molar concentration of a substance that reduces the specific binding of a radioligand to 50% of the range of the binding curve (Top – Bottom) for that particular substance.

Notes:

- For incomplete curves, the response data should span above 50% for an IC_{50} to be used for the determination of a K_i.
- The Top and Bottom parameters should be within +/- 20% of the Top and Bottom dynamic range control values.

Derived Results: K_i

The equilibrium dissociation constant of a test compound (Ki) should be calculated using the standard Cheng-Prusoff equation:

$$K_i = \frac{IC_{50}}{1 + \frac{[R]}{K_d}}$$

[R] = concentration of radioligand used in the assay

K_d = the equilibrium dissociation constant of the radioligand in the assay

Notes:

- K_i carries the same prefix as the IC_{50} from which it is derived.
- For competitive binding mechanisms, a K_i is recommended to be reported for radioligand binding assays, from IC_{50} values generated using 3 or 4-parameter curve fitting methods.
- For uncompetitive or complex (ill-defined) binding mechanisms, an IC_{50} is preferred, because one of the main assumptions for the use of the Cheng-Prusoff equation is based on a competitive, bimolecular interaction.

Data Types and Associated Rules for Enzymatic Assays: Inhibition Mode

Normalized Results

Inhibition with a Unit of Measurement (UOM) of % based on complete enzyme inhibition (dynamic range of the assay)

Calculation:

$$\text{Inhibition}\quad (\%) = 100 - \left[\frac{(\text{Activity of Enzyme with Test Cmpd \& Substrate} - \text{Min})}{(\text{Max} - \text{Min})}\right] x100$$

Max = observed enzyme activity measured in the presence of enzyme, substrate(s) and cofactors utilized in the method.

Min = observed enzyme activity measured in the presence of substrate(s) and cofactors utilized in the method, and (a) in the absence of enzyme, or (b) in the presence of a fully inhibited enzyme.

Derived Results: Absolute IC_{50}, Relative IC_{50}

Relative IC_{50} = the molar concentration at which 50% of maximal inhibition for that substance is observed.

Absolute IC_{50} = the molar concentration of a substance that reduces the enzymatic activity to 50% of the total enzymatic activity.

Data Types and Associated Rules for *In Vitro* Functional Assays

Antagonists

Normalized Results

Inhibition with a UOM of % should be calculated for responses to individual concentrations of test substances.

Calculation:

$$\text{Inhibition}\quad (\%) = 100 - \left[\frac{(\text{Response in presence of Test Cmpd \& Ref Agonist} - \text{Min})}{(\text{Max} - \text{Min})}\right] x100$$

Max = response in presence of some concentration of a reference agonist challenge dose.

Min = (a) response in the presence of diluents and in the absence of test substance and agonist; or (b) response in the presence of maximally effective antagonist and challenge dose of agonist.

Derived Result: Rel IC_{50}

Relative IC_{50} = the molar concentration of a substance (antagonist) that reduces the efficacy of the reference agonist or the constitutive activity of the biological target by 50% of the antagonist curve (Top-Bottom) for that particular test substance.

Derived Result: K_b

Calculation of K_b by Schild analysis isn't standard practice due to throughput and cost disadvantages. Consequently, the Cheng-Prusoff equation is typically used to reduce the data and subsequently assigned the label of K_b.

Calculation: Use standard Cheng-Prusoff equation for functional assays.

$$Kb = \frac{IC_{50}}{1 + \frac{[A]}{EC_{50}}}$$

[A] = the concentration of the reference agonist that is being inhibited

EC_{50} = the Relative EC_{50} of the reference agonist determined in the same run of the assay.

If the slope of the curve for the reference agonist deviates significantly from 1, the use of the modified Cheng-Prusoff equation is recommended.

Other Derived Results:

Schild K_b

Schild K_b is measure of affinity for a competitive antagonist that is calculated using the ratios of equi-active concentrations of a full agonist (most typically EC_{50} concentrations are used) in the absence and presence of one or more concentrations of the antagonist. Schild K_b offers a true evaluation of a test compound's ability to mechanistically perform as an antagonist. This process exposes toxic effects and compound precipitation as false positive activity, and therefore, should be used when time and cost are not limitations.

E_{min}

The maximum activity of an antagonist test substance relative to a reference agonist. This is obtained by first generating a fitted top from a %Inhibition curve and then converting that to the corresponding %Stimulation of the reference agonist curve. The E_{min} value for antagonist mode should equal the relative efficacy for agonist mode for competitive inhibitors. In order to make use of E_{min}, the selected agonist concentration (i.e. EC_{80}) should produce an activity above the expected E_{min} value (Figure 1).

Notes:

- K_b carries the same prefix as the IC_{50} from which it is derived.
- The use of Abs IC_{50} is discouraged.
- Because partial antagonists exist, a full response curve with defined Top & Bottom can be achieved even if the %Inh doesn't exceed 50%.
- A concentration response curve for the reference agonist should be determined in each experimental run if a K_b is to be determined. The frequency within the run depends on assay variability. A statistician should be consulted concerning this frequency during the assay validation process.

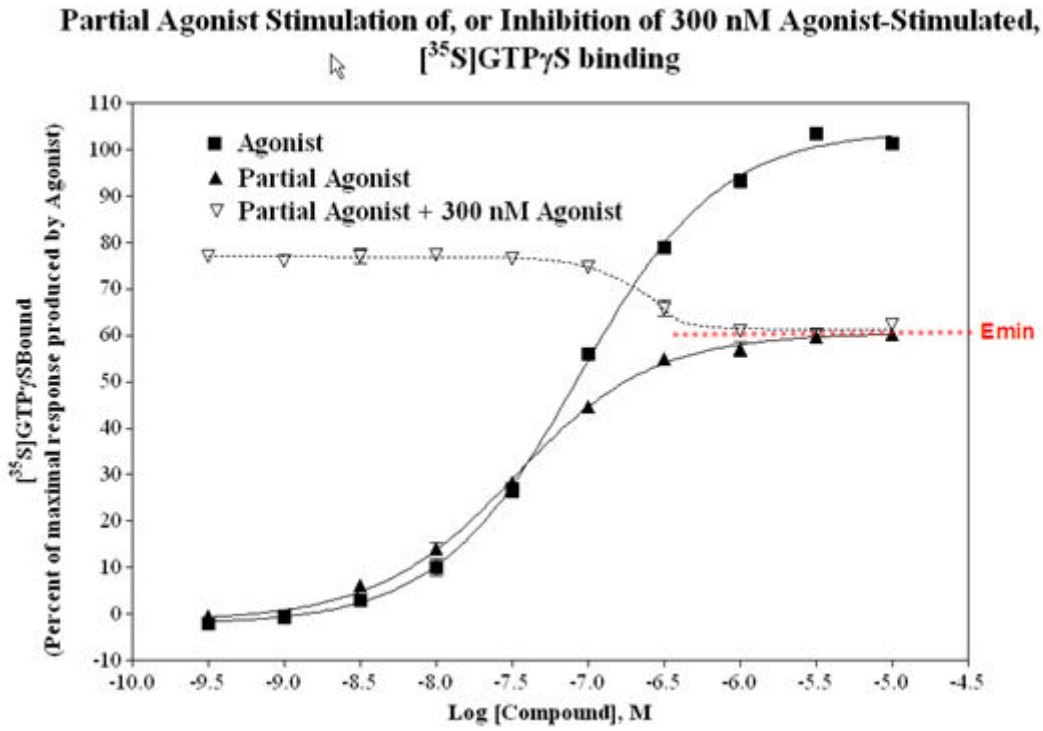


Figure 1: Partial agonist stimulation or inhibition of 300 nM agonist-stimulated [^{35}S]GTPγS binding

Agonists

Normalized Data

Stimulation with a UOM of % should be calculated for responses to individual concentrations of test substances.

Calculation:

$$\text{Stim (\%)} = \left[\frac{(\text{Response in presence of Test Cmpd} - \text{Min})}{(\text{Max} - \text{Min})}\right] x100$$

Min = the fitted Bottom of a 4 parameter logistic curve fitting equation applied to data generated from the positive control (reference agonisit).

Max = (a) the maximum activity of a positive control agonist determined by the fitted Top of a 4 parameter logistic curve fitting equation applied to a concentration response curve from the positive control; or (b) the maximum activity of a positive control in Max wells, which should represent the empirically-derived saturating concentration of the positive control.

Derived Results: Relative EC_{50} and Relative Efficacy

Relative EC_{50}= the molar concentration of a substance that produces 50% of that test substance's maximum stimulation.

Relative Efficacy = the maximum activity of a test substance relative a reference agonist. The UOM for Relative efficacy is %.

Calculation:

$$\text{Rel Eff (\%)} = \left[\frac{\text{Fitted Top of Test Cmpd}}{\text{Fitted Top of Reference Agonist}} \right] x100$$

Other Derived Results:

Fold Activity and Fold Activity Max

The fold activity (or fold activity max) result is useful when comparing *test compounds evaluated across multiple functional assays* because varying levels of efficacy can be observed amongst the different or same reference agonists.

The intended use of this calculation is to provide additional information to reduce or define differences between assays, so that differences between compound activities can be further quantified. For example, a compound run in an assay normalized to a *reference agonist with low efficacy* would appear to be more efficacious when compared to another compound run in a separate assay normalized to a *reference agonist with high efficacy.* Comparing folds activities, which looks at the magnitude of compound-induced activity relative to baseline, enables a scientist to make a conclusion that is not influenced by differences in reference agonist responsiveness. Also, the fold activity result of a control compound can be useful to quality control chart, tracking changes in assay responsiveness over time.

Calculation:

$$\text{Fold Act \& Fold Act Max} = \frac{\text{Raw data response in presence of Test Cmpd}}{\text{Min}}$$

Min = Raw basal activity of constitutive receptor.

Relative AUC

Relative AUC (Area Under the Curve) is defined as the ratio of the area under the fitted concentration-response curve for the test compound to the area under the fitted concentration-response curve for the reference compound. Specifically, areas are calculated as the area under the curve that lies above the horizontal line y = 0%. The area calculation corresponds to the shaded region in the figure below, where the contribution to the area as one move along the concentration axis is proportional to the log of the concentration distance covered, **not** the linear concentration distance covered. One should calculate the area using an exact formula when it is available, as is the case for the 4PL and 3PL models. Otherwise, one may use an approximation method, such as the trapezoid rule. In either case, for the calculated value of relative AUC to be meaningful, the areas for both the test and reference compounds should be computed in the same concentration range. Likewise, the comparison between two relative AUCs is only meaningful when each is computed in the same concentration range. If the same concentration range was not used for assaying the test and reference compounds, the equations for the fitted curves may be used for extrapolation in order to compute the components of the relative AUC over the same concentration range.

Rel AUC is useful with functional assays in which compounds are measured with varying efficacies (agonists and partial agonists) and potencies. Because Rel AUC measures the area of activity, *both efficacy and potency data are essentially combined, generating a value that provides an overall assessment of activity and selectivity between tested compounds.* However, Rel AUC should not be a substitute but rather a supplement to individual efficacy and potency data during the analysis process. Figure 2 illustrates the "area of activity" that is used in the calculation as Rel AUC.

Calculation:

$$\text{Rel AUC} = \left[\frac{\text{AUC of Test compound}}{\text{AUC of Reference compound}} \right]$$

Notes:

- A four-parameter curve fit should be used for the Ref Agonist.
- The maximum and minimum asymptotes should be defined by the data for the Ref Agonist
- Calculation of Rel Eff assumes that both the test compound and positive control each have a defined Top asymptote.

Orphan Receptors – Stimulation Mode

Assays exist for which there are no identified positive control or reference agonist compounds. An example of this situation is an assay that utilizes an "orphan" target as a bio-entity. An "orphan" target is a bio-entity that has a primary sequence suggesting it is a member of one of the super families of biological targets; however, *no ligand for this "receptor" has been identified*. Generally, it is the aim of the research effort to identify ligands for this "orphan" so that a protocol for a validated assay can be developed. Until at least enough data is gathered to identify a ligand for these types of bio-entities, assays utilizing them will be considered "validated" at only the hit to lead level. During this period, responses to individual concentrations of test substances can be normalized by one of the following formulae, which either make use of a known nonspecific activator or simply use basal activity of the constitutive receptor.

Orphan Receptors Normalized to Nonspecific Activator

Stimulation with a UOM of % should be calculated for responses to individual concentrations of test substances.

$$\text{Stim (\%)} = \left[\frac{(\text{Response in presence of Test Cmpd} - \text{Min})}{(\text{Max} - \text{Min})} \right] x100$$

Max = fully activated by nonspecific activator.

Min= constitutive basal activity of the receptor (no activation)

Orphan Receptors Normalized to Constitutive Receptor

Responses to individual concentrations of test substances that increase the measured activity of the orphan target are normalized to the basal level of activity of the target measured in the absence of the test substance. These responses can be expressed as either a percent of the basal activity or as a fold of the basal activity using one of the following

Calculation:

$$\text{Fold Act \& Fold Act Max} = \frac{\text{Raw data response in presence of Test Cmpd}}{\text{Min}}$$

Min = Raw basal activity of the constitutively active receptor

Notes:

- Results from this equation can generate percents much greater than 100.
- Expression of Fold Act or Fold Act Max should only be determined until either a nonspecific activator or ligand is identified; and should only be used to rank order compounds tested in the same assay.

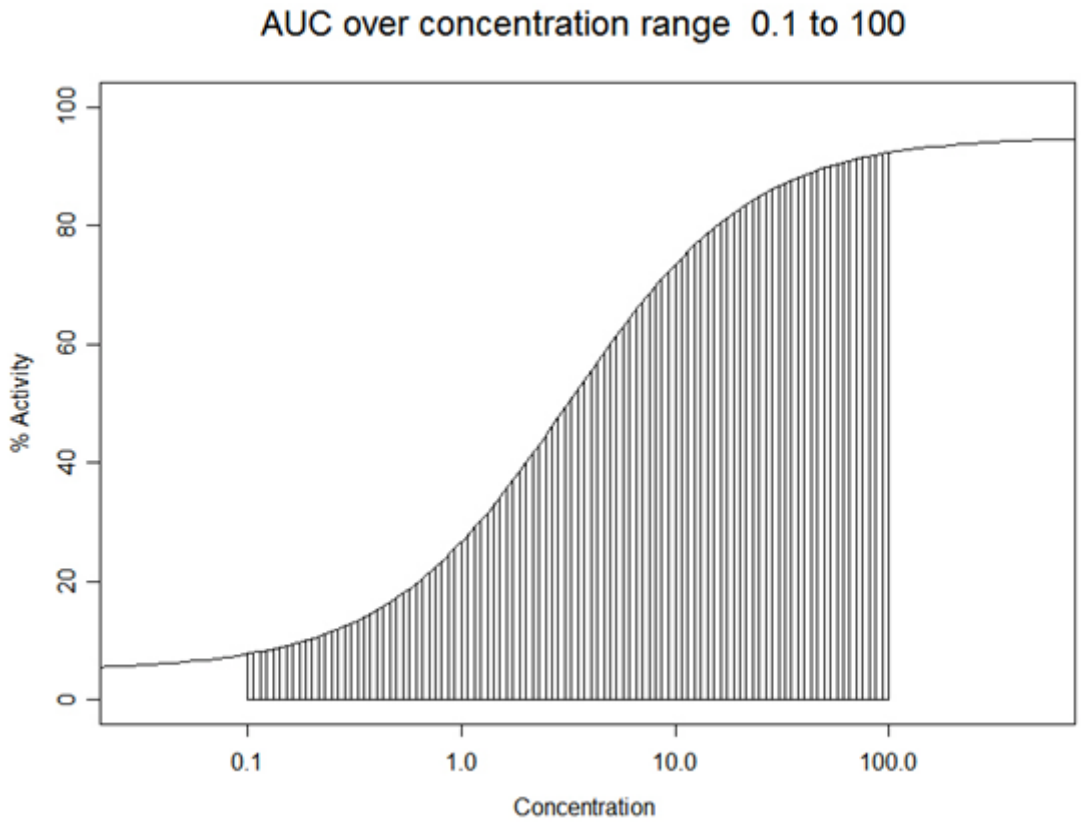


Figure 2: The "area of activity" that is used in the calculation as Rel AUC.

- The calculated Fold Act or Fold Act Max value is expected to be greater than 1 for an agonist. If the calculated value is less than 1, the test compound could be an inverse agonist.

Potentiators

Potentiation assays measure the ability of an *test substance to augment the response produced by a relatively low concentration of an active substance in some biological system*. Currently, these assays are run in *one of two modes*. The following paragraphs address the most frequently used mode.

The first mode involves the addition of one or more concentrations of a test substance in the presence of a fixed concentration of the known active substance called the "Reference Agonist". In this mode, potentiation is the response produced by the combination of substances minus the response produced by the specific concentration of Reference Agonist alone. *But, how does one normalize this response?*

It is recognized that potentiation assays might be executed when no known potentiator exists. However, no potentiation assay should be run without the existence of a known Reference Agonist. Therefore, the response to the specific concentration of the Reference Agonist plus the test substance (potentiation) should be normalized to the fitted Top of a concentration response curve of the Reference Agonist, determined at least once in every run of the assay. The frequency of the determination of the concentration response curve of the Reference Agonist for the purpose of normalizing other responses in any potentiation assay would be depend upon other factors such as plate variability and run-to-run reproducibility.

Normalized Data

Stimulation with a UOM of % should be calculated for responses to the Reference Agonist.

Potentiation with a UOM of % should be calculated for responses to individual concentrations of test substances.

Calculation:

$$\text{Pot (\%)} = \left[\frac{(\text{Response in presence of Test Cmpd \& Challenge Dose - Min})}{(\text{Max - Min})} \right] x100$$

Min = Response in the presence of challenge dose EC_{10} of reference agonist

Max = Response in the presence of full agonist dose

Notes:

- This provides for a Potentiation equal to 0% when the response to the combination of test substance and Reference Agonist is equal to the response to the Reference Agonist alone (e.g. a test substance that is not a potentiator).

Derived Data

Relative EC_{50}= the molar concentration of a substance that produces 50% of that test substance's maximum stimulation.

Relative Potentiator Efficacy: There is little if any discussion in the scientific literature addressing a standard term or calculation of efficacy of a potentiator. It is suggested that this result type be termed Relative Potentiator Efficacy (or Rel Pot Eff) to distinguish it from the Relative Efficacy of an agonist. It is equal to the fitted Top of the potentiation curve minus the normalized response to the specific concentration of Reference Agonist alone divided by 100 minus the normalized response to the specific concentration of Reference Agonist alone. Figure 3 illustrates the above decisions.

Inverse Agonists

According to multiple models of drug-receptor interaction, receptors have been demonstrated to exist in equilibrium between two states. These two states are R*, the active form of the receptor, and R, the inactive form.

Agonists exhibit higher affinity for the active form of the receptor. When an agonist binds to a receptor, it stabilizes the active form of the receptor, shifts the equilibrium toward the active state and produces a response in the biological system under investigation. Substances that produce this effect possess positive intrinsic activity.

Antagonists exhibit equal affinity for both forms of the receptor. When an antagonist binds to a receptor, it stabilizes the initial equilibrium between the active and inactive forms of the receptor. Therefore, no observable change in the activity of the biological system occurs. Substances of this type possess zero intrinsic activity.

Inverse agonists exhibit higher affinity for the inactive state of the receptor. When an inverse agonist binds to a receptor, it stabilizes the inactive form of the receptor, shifts the equilibrium toward that state and produces an opposite response in the biological system. These substances possess negative intrinsic activity.

Receptors have been demonstrated to exist in a constitutively active state both *in vitro* and *in vivo*. *In vitro*, the constitutive activity observed in assays utilizing transfected cell lines is generally attributed to the over expression of the receptor at levels hundreds to thousands of times higher than occur in vivo. Under these conditions, the total number of receptors in the active state is sufficiently high to produce a measurable response even when no exogenous substance has been added to the system. *The addition of an inverse agonist to the system*

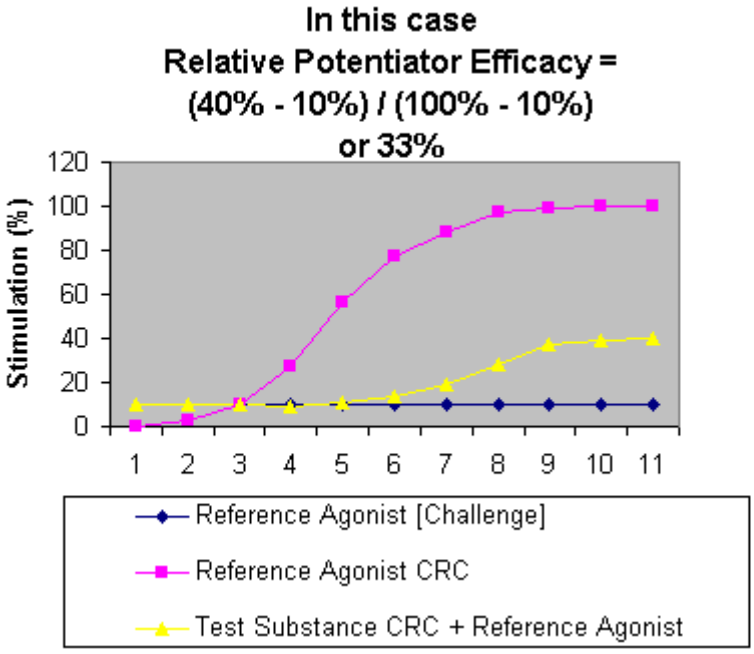


Figure 3: Relative Potentiator Efficacy example plot.

produces a decrease in the measured response. The magnitude of the decrease is related to the amount of negative intrinsic efficacy of the inverse agonist.

The possibility for confusion exists when one desires to quantify results for potential drug candidates that are inverse agonists. Some of the questions that arise are:

1. Because the measured response is a decreased activity produced by an inverse agonist, is the normalized result type Inhibition or Stimulation?
2. What is the algorithm for normalized results?
3. What is the algorithm for fitting concentration response curves?
4. Is the result type describing potency of a test substance a Relative EC_{50} or a Relative IC_{50} or another measure?
5. How is the result type describing potency of a potentiator differentiated from the potency result type for an agonist?
6. Is Relative Efficacy a negative number?

There are no absolute answers to these questions provided by the current literature; however, there is a consistent theme.

1. The most frequently used normalized result type is Inhibition with a unit of measure of % activity.
2. The dynamic range for inverse agonists is the difference between activity in the absence of, or fully inhibited, biological target and the constitutive activity. *Use of the "absence" method is preferable in early development of inverse agonist assays because it eliminates the dependency on a pre-existent known inverse agonist to compare responses of test substances to*. However, as with other functional assays, as soon as an appropriate inverse agonist has been found, it should be utilized as a positive control in the assay for the purpose of calculating relative efficacies.

Assays Normalizing Data to an Inverse Agonist Control

Normalized Data

Inhibition with a UOM of % should be calculated for responses to individual concentrations of test substances.

Calculation:

$$\text{Inh (\%)} = \left[\frac{(\text{Response in presence of Test Cmpd - Min})}{(\text{Max - Min})}\right] x100$$

Min = Response activity in presence of constitutively active receptor alone

Max = Response activity in presence of positive control and receptor

Derived Data

Relative EC_{50} Inverse = the molar concentration of a substance that produces 50% of the range of inverse agonist curve (Top – Bottom) for that particular test substance.

Rel Efficacy Inverse = 100 x (Fitted Top of the test substance expressed as %/Fitted Top of the Positive Control Reference Inverse Agonist expressed as %)

Calculation:

$$\text{Rel Eff Inv (\%)} = \left[\frac{\text{Fitted Top of Test Cmpd}}{\text{Fitted Top of Reference Inverse Agonist}}\right] x100$$

Notes:

- Because inverse agonist response curve profiles look similar to profiles generated by toxic compounds, it's advised that a confirmation assay be used to provide more evidence that a given test compound is an inverse agonist.
- Hill Coefficient and Rel Efficacy Inverse values are positive.
- The calculated Fold Act or Fold Act Max value is expected to be greater than 1 for an agonist. If the calculated value is less than 1, the test compound could be an inverse agonist.

Assays Normalizing Data to No Receptor Control (Orphan Receptor)

Normalized Data

Inhibition with a UOM of % should be calculated for responses to individual concentrations of test substances.

Calculation:

$$\text{Inh (\%)} = \left[\frac{(\text{Response in presence of Test Cmpd \& Receptor - Min})}{(\text{Max - Min})}\right] x100$$

Min = Response activity in the presence of the constitutively active receptor alone

Max = Response activity in the absence of the receptor

Derived Data

Relative EC_{50} Inverse = the molar concentration of a substance that produces 50% of the range of inverse agonist curve (Top – Bottom) for that particular test substance.

Notes:

- Because inverse agonist response curve profiles look similar to profiles generated by toxic compounds, it's advised that a confirmation assay be used to provide more evidence that a given test compound is an inverse agonist.
- Hill Coefficient and Rel Efficacy Inv values are positive.
- The calculated Fold Act or Fold Act Max value is expected to be greater than 1 for an agonist. If the calculated value is less than 1, the test compound could be an inverse agonist.

Assays Normalizing Data to Reference Agonist:

Normalized Data

Stimulation with a UOM of % should be calculated for responses to individual concentrations of test substances.

Calculation:

$$\text{Stim (\%)} = \left[\frac{(\text{Response in presence of Test Cmpd} - \text{Min})}{(\text{Max} - \text{Min})}\right] x100$$

Min = the fitted Bottom of a 4 parameter logistic curve fitting equation applied to data generated from the Reference Agonist

Max = the maximum activity of a Reference Agonist determined by the fitted Top of a 4 parameter logistic curve fitting equation applied to a concentration response curve from the positive control.

Notes:

- % Stimulation values will be negative for inverse agonist test compounds.

Derived Data

Relative EC_{50} Inverse = the molar concentration of a substance that produces 50% of that test substance's inverse agonism.

Relative Efficacy = the maximum activity of a test substance relative to a Reference Agonist. The UOM for Relative efficacy is %.

Calculation:

$$\text{Rel Eff Inv (\%)} = \left[\frac{\text{Fitted Bottom of Test Cmpd}}{\text{Fitted Top of Reference Agonist}}\right] x100$$

Notes:

- Rel Efficacy and Hill Coeff values for inverse agonists will be negative.
- Calculation of Rel Eff assumes the test compound has a defined Bottom asymptote and Reference Agonist have a defined Top asymptote.

- Because inverse agonist response curve profiles look similar to profiles generated by toxic compounds, it's advised that a confirmation assay be used to provide more evidence that a given test compound is an inverse agonist.
- The calculated Fold Act or Fold Act Max value is expected to be greater than 1 for an agonist. If the calculated value is less than 1, the test compound could be an inverse agonist.

Reference

1. Neubig RR, Spedding M, Kenakin T, Christopoulos A. International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification. XXXVIII. Update on terms and symbols in quantitative pharmacology. Pharmacol Rev. 2003;55(4):597-606. doi: 10.1124/pr.55.4.4. PubMed PMID: WOS:000186910700007.

Glossary

Abs IC_{50}

Absolute IC50; the molar concentration of a substance that inhibits 50% of the dynamic range of the assay. In contrast to Rel IC_{50}, Abs IC_{50} is not the inflection point of the curve. It's determined to be the concentration at which 50% inhibition is realized.

Bottom

The lower asymptote of a logarithmically derived curve. The Bottom value can be determined with real values or predicted using the logarithm applied to the result data set.

CRC

Concentration-response curve mode. The mode to describe an assay performed with multiple concentrations of a given test substance, which might then render a logarithmically-derived graph curve.

E_{min}

The maximum activity of an antagonist test substance relative to a reference agonist. This is obtained by first generating a fitted top from a %Inhibition curve and then converting that to the corresponding %Stimulation of the reference agonist curve. The E-min value for antagonist mode should equal the relative efficacy for agonist mode for competitive inhibitors.

Fold Activity

The ratio of biological activity in the presence of an exogenous substance to that in its absence. It is the test compound's observed response (raw data value) divided by the median of the same plate's Min wells. This result type is used exclusively with single point assays. If the value is greater than 1, the test compound is likely an agonist. If the calculated value is less than 1, the test compound could be an inverse agonist.

Fold Activity Max

The maximum observed Fold Activity in a concentration response curve whether it was excluded or not. It is the test compound's observed response (raw data value) divided by the median of the same plate's Min wells. If the value is greater than 1, the test compound is likely an agonist. If the calculated value is less than 1, the test compound could be an inverse agonist.

Fold Activity Max (FA)

The maximum observed Fold Activity in a concentration response curve whether it was excluded or not. The (FA) indicates that his result type is summarized. Because activity can be detected at different test substance concentrations, the summarized value must be viewed with this knowledge.

Hill Coeff

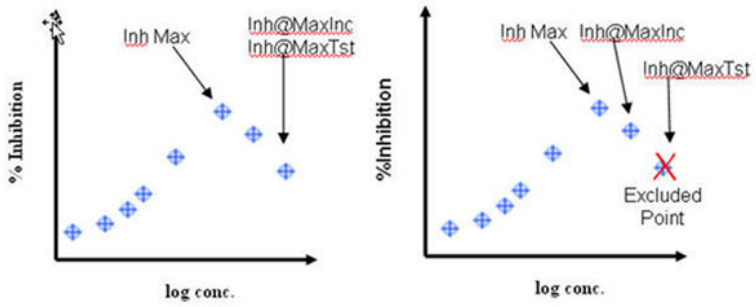
Derived slope a three or four parameter logistic curve fit. Should not be fixed to any given value without consultation with a statistician. It should not be a negative value except for inverse agonist assays.

Inh

Activity determined for a single point inhibition assay. Unit of Measure is always %.

Inh @ Max Inc

Inhibition observed at the highest included (i.e. not excluded) concentration of a substance tested in a concentration response mode method version regardless of whether it was included in the parametric fit to produce derived results. (See Illustration below)



Inh @ Max Tst

Inhibition observed at the maximum concentration of a substance tested in a concentration response mode method version regardless of whether it was included in the parametric fit to produce derived results. (See Illustration below)

Inh Max

Maximum inhibition produced by any concentration that was included for the application of a curve fit algorithm (See Illustration below).

Inh Max (FA)

Maximum inhibition produced by any concentration that was included for the application of a curve fit algorithm. This result type differs from Inh Max by allowing summarization to occur; the FA is defined as 'for averaging'. Because this result type could yield an average value from multiple test substance concentrations, the value should be used with this knowledge and therefore with caution.

K_i

Result from the Cheng-Prusoff equation or from a slightly modified derivation. This label is used primarily with binding assays (see standard texts for formula) and represents the affinity of a compound for a receptor. Documentation of the formula and any changes to the Cheng-Prusoff should be noted in the assay protocol.

K_b

Result from the Cheng-Prusoff equation or from a slightly modified derivation. This label is used primarily with functional antagonist assays (see standard texts l for formula) and represents the affinity of a compound for a receptor. This label doesn't represent results mechanistically determined via the Schild analysis; rather the label Schild Kb is used in those calculations.

Pot

Potentiation result type for single point mode. Many potentiation assays involve the addition of one or more concentrations of a test substance in the presence of a fixed concentration of the known active substance called the Reference Agonist. In this mode, potentiation is the response produced by the combination of substances minus the response produced by the specific concentration of Reference Agonist alone.

Pot @ Max Inc

Potentiation observed at the highest included concentration of a substance from an analysis of a concentration response curve.

Pot @ Max Tst

Potentiation observed at the maximum concentration of a single substance tested in a concentration response mode method version regardless of whether it was included in the parametric fit to produce derived results.

Pot Max

The maximum potentiation observed for a substance in a single run of a potentiation concentration response mode method regardless of whether it was included in the parametric fit to produce derived results.

Rel AUC

Defined as the ratio of the area under the fitted concentration-response curve for the test compound to the area under the fitted concentration-response curve for the reference compound.

Rel EC_{50}

Relative EC50; the molar concentration of a substance that stimulates 50% of the curve (Top – Bottom) for that particular substance. It can also be described as the concentration at which the inflection point is determined, whether it's from a three- or four-parameter logistic fit.

Rel EC_{50} Inv

The Relative EC_{50} of an inverse agonist.

Rel Eff

The maximum activity of a test substance relative to a standard positive control agonist. The result is expressed as percent from the following formula: 100 x Fitted Top of the test substance divided by the Fitted Top of an Agonist control. The agonist control should have a four parameter curve fit with defined lower and upper asymptotes but can have the Bottom fixed to zero in certain cases. The test compounds should have a four parameter curve fit but can have a three parameter fit with the bottom fixed to zero if the data warrants it.

Rel Eff Inv

The maximum activity of a test substance relative to a standard positive control inverse agonist. The result is expressed as percent from the following formula: 100 x Fitted Top of the test substance divided by the Fitted Top of the Inverse Agonist control. The inverse agonist control should have a four parameter curve fit with defined

lower and upper asymptotes but can have the Bottom fixed to zero in certain cases. The test compounds should have a four parameter curve fit but can have a three parameter fit with the bottom fixed to zero if the data warrants it.

Rel IC_{50}

Relative IC_{50}; the molar concentration of a substance that inhibits 50% of the curve (Top – Bottom) for that particular substance. It can also be described as the concentration at which the inflection point is determined, whether it's from a three- or four-parameter logistic fit.

Rel Pot Eff

The fitted top of the potentiation curve minus the normalized response to the specific concentration of Reference Agonist alone divided by 100 minus the normalized response to the specific concentration of Reference Agonist alone.

Stim

Activity determined for a single point stimulation assay. Unit of Measure is always %.

Stim @ Max Inc

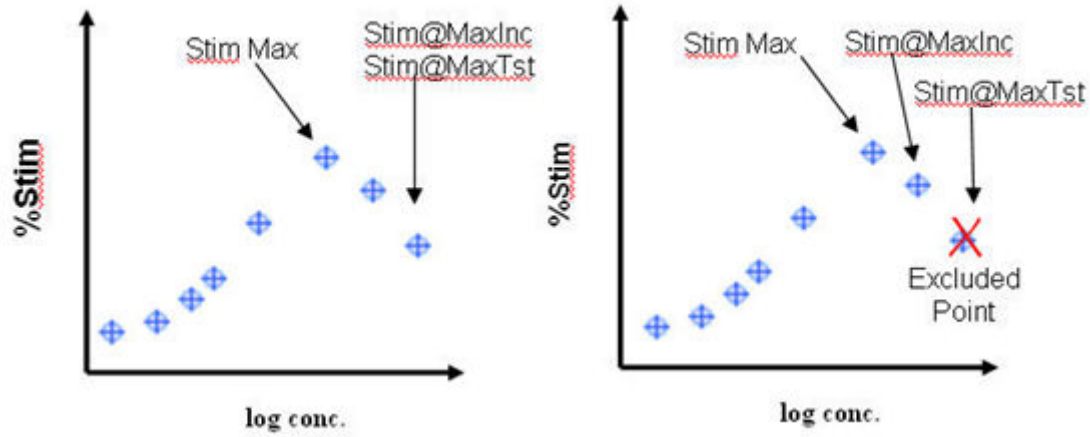
Stimulation observed at the highest included (i.e. not excluded) concentration of a substance tested in a concentration response mode method version regardless of whether it was included in the parametric fit to produce derived results. (See illustration below)

Stim @ Max Tst

Stimulation observed at the maximum concentration of a substance tested in a concentration response mode method version regardless of whether it was included in the parametric fit to produce derived results. (See illustration below)

Stim Max

Maximum stimulation produced by any concentration that was included for the application of a curve fit algorithm (See illustration below)



Stim Max (FA)

Maximum stimulation produced by any concentration that was included for the application of a curve fit algorithm. This result type differs from Stim Max by allowing summarization to occur; the FA is defined as 'for

averaging'. Because this result type could yield an average value from multiple test substance concentrations, the value should be used with this knowledge and therefore with caution.

Schild K_b

A measure of affinity for a competitive antagonist that is calculated using the ratios of equi-active concentrations of a full agonist (most typically EC_{50} concentrations are used) in the absence and presence of one or more concentrations of the antagonist. See pp. 335-339, Pharmacologic Analysis of Drug-Receptor Interaction, 3rd Ed. by Terry Kenakin.

SP

Single point mode. Assay performed with once concentration of test substance. Common result types used include Inh and Stim. Result values should always include the concentration of the test substance used to determine the activity.

Stephenson's K_p

A measure of affinity for a partial agonist that is calculated through the comparison of equi-active concentrations of a full agonist in the absence and presence of a single concentration of the partial agonist. See pp. 284-286, Pharmacologic Analysis of Drug-Receptor Interaction, 3rd Ed. by Terry Kenakin.

Top

The upper asymptote of a logarithmically derived curve. The Top value can be determined with real values or predicted using the logarithm applied to the result data set.

HTS Assay Validation

Philip W. Iversen, PhD,[1,*] Benoit Beck, PhD,[2] Yun-Fei Chen, PhD,[1] Walthere Dere, MS,[3] Viswanath Devanarayan, PhD,[4] Brian J Eastwood, PhD,[1] Mark W. Farmen, PhD,[1] Stephen J. Iturria, PhD,[5] Chahrzad Montrose, PhD,[1] Roger A. Moore, MS,[6] Jeffrey R. Weidner, PhD,[8,†] and G. Sitta Sittampalam, PhD[7,‡]

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Abstract

Assays employed in HTS and lead optimization projects in drug discovery should be rigorously validated both for biological and pharmacological relevance, as well as for robustness of assay performance. This chapter addresses the essential statistical concepts and tools needed in assay performance validation developed in the pharmaceutical industry, specifically for higher throughput assays run exclusively in 96-, 384- and 1536-well formats using highly automated liquid handling and signal detection systems. In this context, it is assumed that the biological and pharmacological validation of the assay system has already been established, and the high through put performance characteristics requires validation. This is an essential chapter for these who design and implement HTS and lead optimization assays to support SAR projects in pre-clinical drug discovery.

1. Overview

The statistical validation requirements for an assay vary, depending upon the prior history of the assay. Stability and Process studies (Section 2) should be done for all assays, prior to the commencement of the formal validation studies. If the assay is new, or has never been previously validated, then full validation is required. This consists of a 3-day Plate Uniformity study (Section 3) and a Replicate-Experiment study (Section 4). If the assay has been previously validated in a different laboratory, and is being transferred to a new laboratory, then a 2-day Plate Uniformity study (Section 3) and a Replicate-Experiment study (Section 3) are required. An assay is considered validated if it has previously been assessed by all the methods in this section, and is being transferred to a new laboratory without undergoing any substantive changes to the protocol. If the intent is to store and use data and results from the previous laborarotory, then an assay comparison study (Section 4) should be done as part of the Replicate-Experiment study. Otherwise only the intra-laboratory part of the Replicate-Experiment study (Section 4) is recommended.

If the assay is updated from a previous version run in the same facility then the requirements vary, depending upon the extent of the change in methodology, equipment, operator and reagents. Major changes require a validation study equivalent to a laboratory transfer. Minor changes require bridging studies that demonstrate the equivalence of the assay before and after the change. See Section 5 for examples of major and minor changes.

Author Affiliations: 1 Eli Lilly & Company, Indianapolis, IN 46285. 2 Axiosis, Inc., Brussels, Belgium. 3 GSK Biologicals, Brussels, Belgium. 4 Charles River Laboratories, 5 Monsanto, Ankeny, IA. 6 Retired. 7 National Institutes of Health. 8 QualSci Consulting, LLC.

Corresponding author.

* Editor

† Editor

‡ Editor

These techniques are intended to be applied to ≥ 96-well primary and functional assays. Discussion with a statistician is necessary for alternatives for assays that require significant time, resource or expenditure to scientifically balance validation requirements with these constraints.

2. Stability and Process Studies

2.1. Reagent Stability and Storage Requirements

It is important to determine the stability of reagents under storage and assay conditions.

- Use the manufacturer's specifications if the reagent is a commercial product.
- Identify conditions under which aliquots of the reagent can be stored without loss of activity.
- If the proposed assay will require that the reagent be frozen and thawed repeatedly, test its stability after similar numbers of freeze-thaw cycles.
- If possible, determine the storage-stability of all commercial and in-house reagents.
- If reagents are combined and aliquoted together, examine the storage-stability of the mixtures.

2.2. Reaction Stability Over Projected Assay Time

Conduct time-course experiments to determine the range of acceptable times for each incubation step in the assay. This information will greatly aid in addressing logistic and timing issues.

2.2.1. Reagent Stability During Daily Operations; Use Of Daily Leftover Reagents

The stability studies will require running assays under standard conditions, but with one of the reagents held for various times before addition to the reaction. The results will be useful in generating a convenient protocol and understand the tolerance of the assay to potential delays encountered during screening.

If possible, reagents should be stored in aliquots suitable for daily needs. However, appropriate stability information pertinent to storing leftover reagents (particularly expensive ones) for future assays should be obtained.

New lots of critical reagents should be validated using the bridging studies with previous reagent lots.

2.3. DMSO Compatibility

Test compounds are delivered at fixed concentrations in 100% DMSO, thus solvent-compatibility of assay reagents should be determined., The validated assayshould be run in the absence of test compounds and in the presence of DMSO concentrations spanning the expected final concentration. Typically, DMSO concentrations from 0 to 10% are tested. Note that the DMSO compatibility of the assay should be completed early, since remaining validation experiments such as the variability studies, should be performed with the concentration of DMSO that will be used in screening. For cell based assays, it is recommended that the final % DMSO be kept under 1%, unless demonstrated by other experiments that higher concentrations can be tolerated.

3. Plate Uniformity and Signal Variability Assessment

3.1. Overview

All assays should have a plate uniformity assessment. For new assays the plate uniformity study should be run over 3 days to assess uniformity and separation of signals, using DMSO concentration to be used in screening. For the transfer of validated assays to other laboratories, the plate uniformity study can be performed over 2 days to establish that the assay transfer is complete and reproducible (see Section 1 for the definition of an assay

transfer). Uniformity assays shoul be performed at the maximum and minimum signal or response levels to ensure that the signa window is adequate to detect active compounds durin the screen.

The variability tests are conducted on three types of signals.

- *"Max" signal*: This measures the maximum signal as determined by the assay design. In in-vitro assays that measure receptor-ligand binding or enzyme activity measurement with substrate conversion to products, maximum signal represents a readout signal in the absence test compounds. For cell-based agonist assays this parameter would be the maximal cellular response of an agonist; for potentiator assays "Max"signal is measuredwith an EC_{10} concentration of a standard agonist plus maximal concentration of a standard potentiator. The observed response is as per validated assay protocol, and may exceed 10% in some cases. For inhibition assays the "Max" signal would be signal response obtained with an EC_{80} concentration of a standard agonist Again the observed response is as per protocol, and may not be 80%. For inverse agonist assays "Max" signal would be the untreated constitutively active response of the cells in the presence of DMSO alone.
- *"Min" signal*: This measures the background signal as determined by the assay s design. In in-vitro assays that measure of receptor-ligand binding or enzyme activity measurement with substrate conversion to products, minimum signal represents a readout signal in the absence test compounds, labeled ligand or the enzyme substrate. For cell-based agonist assays this is the basal signal. For potentiator assays this is an EC_{10} concentration of agonist. For inhibitor assays, including receptor-binding assays, this is an EC_{80} concentration of the standard agonist plus a maximally inhibiting concentration of a standard antagonist (preferred) or unstimulated reaction.
- *"Mid" signal*: This parameter estimates the signal variability at some point between the maximum and minimum signals. Typically, for agonist assays the mid-point is reached by adding an EC_{50} concentration of a full agonist/activator compound; for potentiator assays it is an EC_{10} concentration of agonist plus EC_{50} concentration of a potentiator; and for inhibitor assays it is an EC_{80} concentration of an agonist plus an IC_{50} concentration of a standard inhibitor to each well. In receptor-ligand binding or enzyme activity measurement based assays, this parameter represents the mid-point signal measured using EC_{50} concentration of a control compound.

  N.B *If calibration of the analytical signals is required for compound activity measurements, the Max, Min and Mid signals correspond to calibration curve responses, and not the raw plate reader counts. It is a requirement that the raw signals lie within the range of the calibration curve. Not more than 1-2% of the wells should be outside the calibration range (i.e. above the fitted top or below the fitted bottom of the calibration curve).*

Two different plate formats exist for the plate uniformity studies: an **Interleaved-Signal** format where all signals are on all plates, but varied systematically so that o on all plates, on a given day, each signal is measured in each plate. This approach can also be used in concentration-response curve *(*CRC*)* plate formats where a reference compound is tested at multiple concentrations with production control wells (Max and Min). Uniform signal plates for "Max" and "Min" are also included where each signal is run uniformly across entire plates. The Interleaved-Signal format can be used in all instances and requires fewer plates. The CRC format is usually easier to run, since it closely conforms to the production process for the assay, including automated pipeting. It is also useful for detecting non-uniform signals, but usually takes more plates in total and the data analysis is more complex. The uniform-signal plates should be interpreted with caution if signals vary across plates on a given day. See Section 3.3.5 for examples and more information.

3.2. Interleaved-Signal Format

3.2.1. Procedure

The following plate layout is recommended for which the Excel analysis templates have been developed. These layouts have a combination of wells producing "Max", "Min", and "Mid" signals on a plate with proper statistical design. Use the same plate formats on all days of the test. Do not change the concentration producing the mid point signal over the course of the test. See Section 3.2.4 for a further discussion about midpoint accuracy. The trials should use *independently* prepared reagents, and preferably be run on *separate* days. Data Analysis Templates are available (from the online eBook) for 96- and 384-well plates.

Plate 1

Row	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
1	H	M	L	H	M	L	H	M	L	H	M	L
2	H	M	L	H	M	L	H	M	L	H	M	L
3	H	M	L	H	M	L	H	M	L	H	M	L
4	H	M	L	H	M	L	H	M	L	H	M	L
5	H	M	L	H	M	L	H	M	L	H	M	L
6	H	M	L	H	M	L	H	M	L	H	M	L
7	H	M	L	H	M	L	H	M	L	H	M	L
8	H	M	L	H	M	L	H	M	L	H	M	L

H=Max, M=Mid, L=Min

Plate 2

Row	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
1	L	H	M	L	H	M	L	H	M	L	H	M
2	L	H	M	L	H	M	L	H	M	L	H	M
3	L	H	M	L	H	M	L	H	M	L	H	M
4	L	H	M	L	H	M	L	H	M	L	H	M
5	L	H	M	L	H	M	L	H	M	L	H	M
6	L	H	M	L	H	M	L	H	M	L	H	M
7	L	H	M	L	H	M	L	H	M	L	H	M
8	L	H	M	L	H	M	L	H	M	L	H	M

H=Max, M=Mid, L=Min

Plate 3

Row	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
1	M	L	H	M	L	H	M	L	H	M	L	H
2	M	L	H	M	L	H	M	L	H	M	L	H
3	M	L	H	M	L	H	M	L	H	M	L	H
4	M	L	H	M	L	H	M	L	H	M	L	H
5	M	L	H	M	L	H	M	L	H	M	L	H

Plate 3 continued from previous page.

6	M	L	H	M	L	H	M	L	H	M	L	H
7	M	L	H	M	L	H	M	L	H	M	L	H
8	M	L	H	M	L	H	M	L	H	M	L	H

H=Max, M=Mid, L=Min

3.2.2. Summary Signal Calculations and Plate Acceptance Criteria

Calculations and acceptance criteria are described below. The overall requirement is that the raw signals are sufficiently tight and that there is significant separation between the "Max" and "Min" signals to conduct screening.

1. Outliers should be flagged with an asterisk in the plate input section. The outliers should be "obvious", and the rate of outliers should be less than 2 percent (i.e. on average less than 2 wells on a 96-well plate, 8 wells on a 384-well plate).
2. Compute the mean (AVG), SD, and CV (of the mean) for each signal (Max, Min and Mid) on each plate. Note that the CV is calculated taking into account the number of wells per test compound per concentration to beused in the production assay. For example, if in the production assay duplicate wells will be run for each concentration of each test substance, then $CV = (SD/\sqrt{2})/AVG$. More generally, if there will be *n* wells per test compound per concentration, then $CV = (SD/\sqrt{n})/AVG$. The acceptance criterion are that the CV's of each signal be less than or equal to 20%. Note that the Min-signal often fails to meet this criterion, especially for those assays whose Min- signal mean is very low. An alternate acceptance criterion for the Min- signal is $SD_{min} \leq$ both SD_{mid} and SD_{max}. All plates should pass all signal criteria (ie all Max, and Mid- signals should have CV's less than 20% and all"Min" signals should either pass the CV criteria or the SD criteria).

For each of the "Mid"-signal wells, compute a percent activity for agonist or stimulation assay relative to the means of the (Max and Min- signals on that plate, i.e.,

$$\%\text{Activity} = \frac{\text{well}_{\text{mid}} - \text{AVG}_{\text{min}}}{\text{AVG}_{\text{max}} - \text{AVG}_{\text{min}}} \times 100$$

.

For inhibition assays compute percent inhibition for each mid-signal well, where %Inhibition = 100 - %Activity.

3. Compute the mean and SD for the "Mid"-signal percent activity values on each plate. The acceptance criterion is $SD_{mid} \leq 20$ on all plates.

4. Compute a Signal Window (*SW*) or Z' factor (*Z'*) for each plate, as described below. The acceptance criterion *SW* ≥ 2 or *Z'* ≥ 0.4 on all plates (either all *SW*'s ≥ 2 or all *Z'* ≥ 0.4).

The formula for the signal window is

$$SW = \frac{\left(\text{AVG}_{\text{max}} - 3\text{SD}_{\text{max}}/\sqrt{n}\right) - \left(\text{AVG}_{\text{min}} + 3\text{SD}_{\text{min}}/\sqrt{n}\right)}{\text{SD}_{\text{max}}/\sqrt{n}}$$

,

where *n* is the number of replicates of the test substance that will be used in the production assay. Instead of the SW the Z' factor can be used to evaluate the signal separation, where the only difference is the denominator ($AVG_{max} - AVG_{min}$) is used instead of SD_{max}. The complete formula is

$$Z' = \frac{\left(\mathrm{AVG}_{max} - 3\mathrm{SD}_{max}/\sqrt{n}\right) - \left(\mathrm{AVG}_{min} + 3\mathrm{SD}_{min}/\sqrt{n}\right)}{\mathrm{AVG}_{max} - \mathrm{AVG}_{min}}$$

If one assumes that the SD of the "Max", signal is at least as large as the SD of the "Min" signal, then the Z' factor will be within a specific range for a given signal window, as illustrated in Figure 1. Note that Z' values greater than 1 are possible only if $AVG_{max} < AVG_{min}$, and so the templates also check that all Z' values are less than 1.

The recommended acceptance criterion is Z' factor ≥ 0.4, which is comparable to a SW ≥ 2. Either measure could be used.

3.2.3. Spatial Uniformity Assessment

A scatter plot (see examples below) can reveal patterns of drift, edge effects and other systematic sources of variability. The response is plotted against well number, where the wells are ordered either by row first, then by column, or by column first, then by row. The overall requirement is that plates do not exhibit material edge or drift effects. In general drift or edge effects < 20% are considered insignificant. Effects seen only on a single or few plates, and not a predominant pattern, are also considered insignificant. Some guidelines for detecting and dealing with these problems follow.

3.2.3.1. No drift or edge effects

Figure 2 (A, B) shows two plots (of the same data) show an example where there are no edge effects or drift.

3.2.3.2. Drift

Use the Max and Mid signals to assess for drift. Look for significant trends in the signal from left-to-right and top-to-bottom. If you observe drift that exceeds 20% then you have material drift effects. In Figure 3 (A, B) the mean of column 1 is 10.6, while the mean of column 10 is 13.8, and the overall mean is 12.2. The drift is 26% [(13.8-10.6)/12.2], and therefore the cause of this drift should be investigated.

3.2.3.3. Edge Effects

Edge effects can contribute to variability, and spotting them can be a helpful troubleshooting technique. Edge effects are sometimes due to increased evaporation from outer wells that are incubated for long periods of time. Edge effects can also be caused either by a short incubation time or by plate stacking – these conditions allow the edge wells to reach the desired incubation temperature faster than the inner wells. Edge effects are demonstrated in Figure 4.

3.2.4. Inter-Plate and Inter-Day Tests

The normalized Mid signal should not show any significant shift across plates or days. "Significant shift" depends to a certain extent on the typical slopes encountered in dose response curves. Thus plate-to-plate or day-to-day variation in the mid point percent activity needs to be assessed in light of the steepness of the dose-response curves of the assay. For receptor binding assays, and other assays with a slope parameter of 1, a 15% difference can correspond to a two-fold change in potency. The template will translate the mean normalized Mid signal to potency shifts across plates and days. There should not be a potency shift >2 between any two plates within a day, or >2 between any two average day Mid point % activities. For functional assays whose slopes may not equal 1 you can enter a "typical" slope into the template. This should be derived from the slope of a dose-response curve for the substance used to generate the Mid point signal.

For these calculations to have utility the Mid-point % Inhibition/Activity should be "near" the midpoint. Values within the range of 30-70% are ideal. Studies with mean values outside this range should be discussed with a statistician, especially before any studies are repeated solely for this reason. Also note that the conditions used to obtain the midpoint should not be changed over the course of the plate uniformity study.

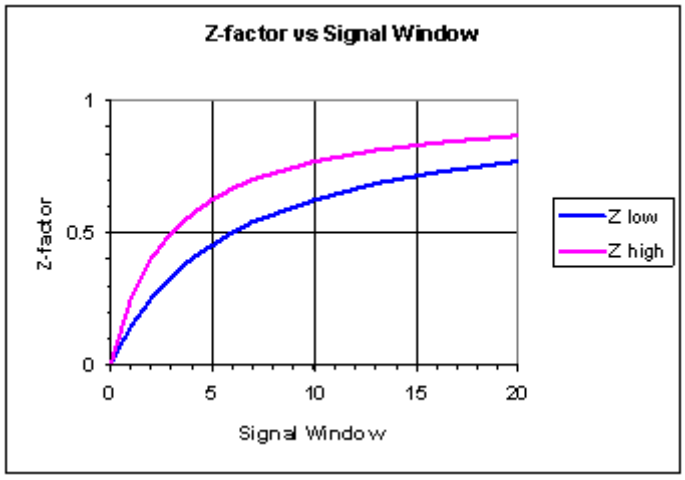
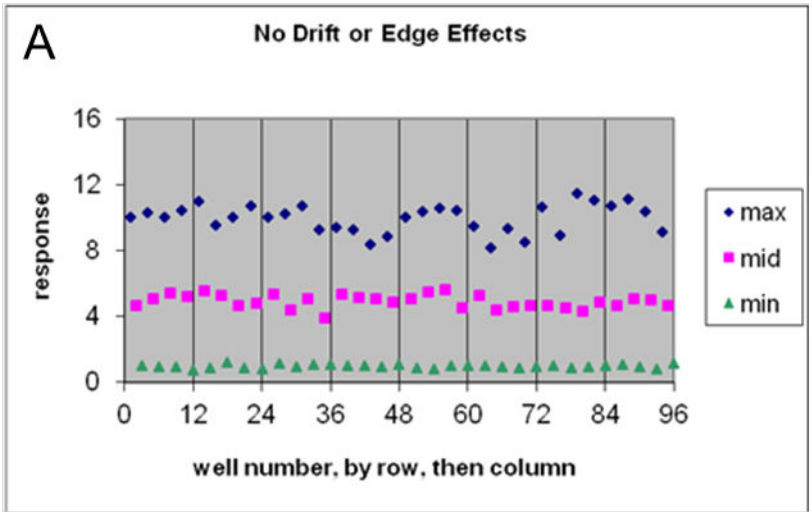


Figure 1: Z-Factor interval versus Signal Window.



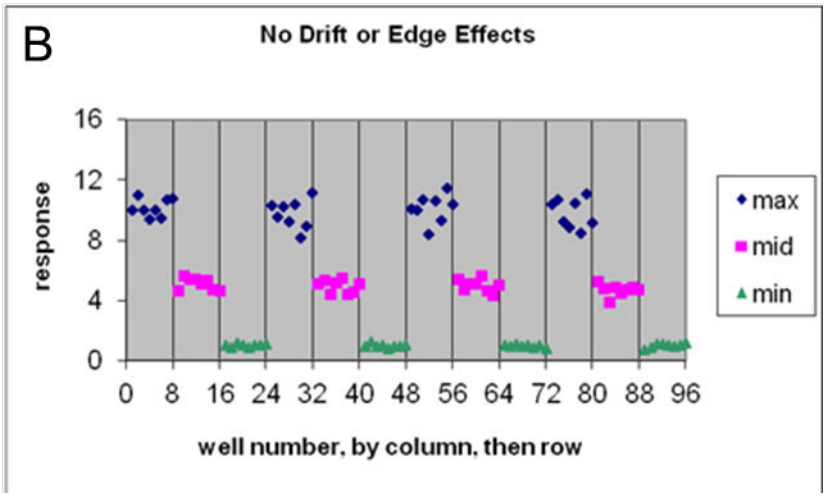
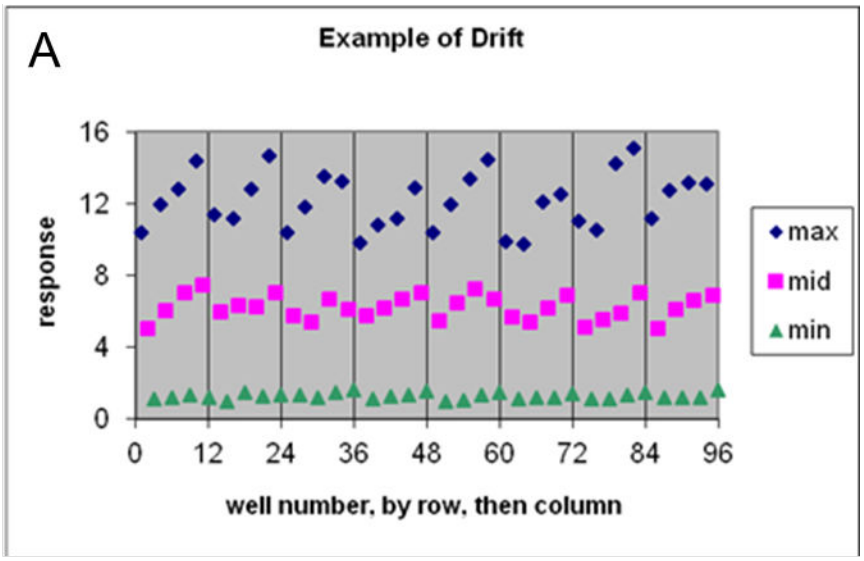


Figure 2 A and B: Example with no drift or edge effects. The data in both plots come from the same set.



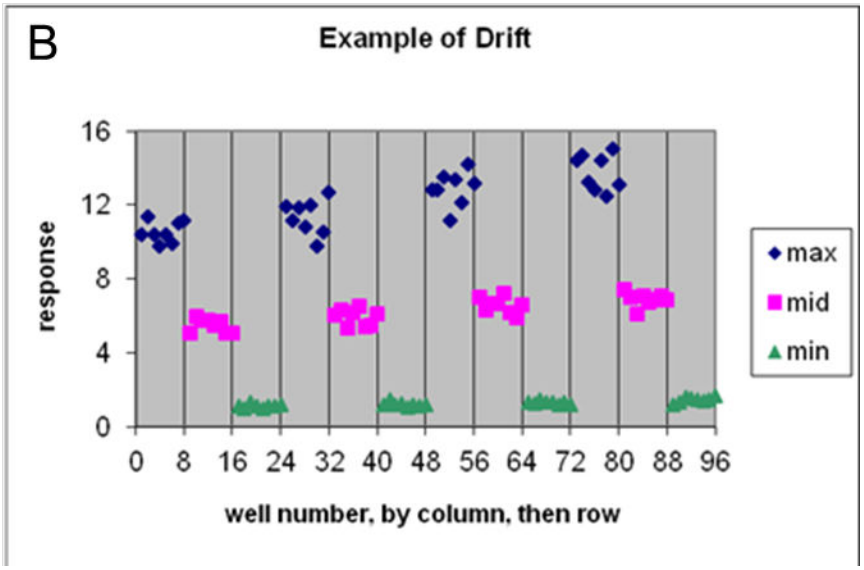


Figure 3 A and B: Example of material drift effects. The data in both plots come from the same set.

3.2.5. Summary of Acceptance Criteria

1. **Intra-plate Tests:** Each plate should have a
   CV_{max} and $CV_{mid} \leq 20\%$,
   $CV_{min} \leq 20\%$ or $SD_{min} \leq \min(SD_{mid}, SD_{max})$,
   Normalized $SD_{mid} \leq 20$,
   $SW \geq 2$ or $Z' \geq 0.4$.
2. **No material (or insignificant) edge, drift or other spatial effects.** Note that the templates do not check this criterion.
3. **Inter-plate and Inter-Day Tests:** The normalized average Mid-signal should not translate into a fold shift
   > 2 within days,
   > 2 across any two days.

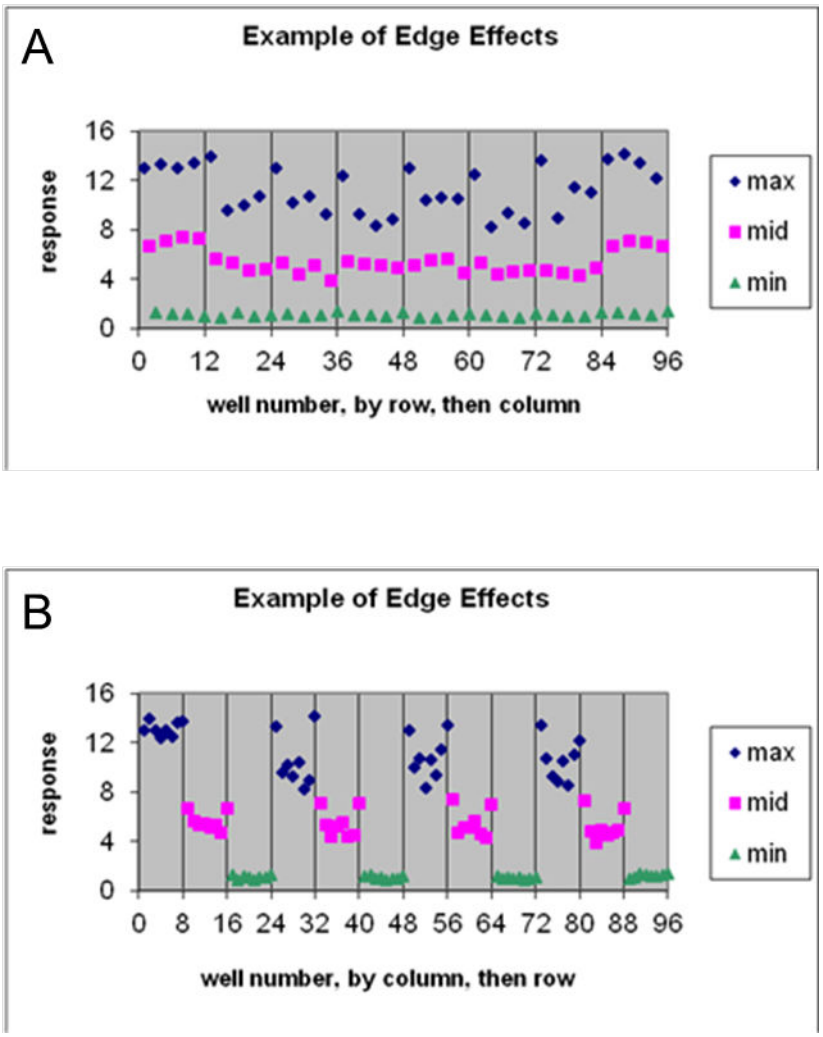


Figure 4 A and B: Example of edge effects. The data in both plots come from the same set. N.B., Because of the vertical axis scale, problems in the Min and even Mid signals may not be visible. Adjusting the scale to highlight the edge effect may be necessary to properly examine these signals.

3.2.6. 384-well Plate Uniformity Studies

384-well plates contain 16 rows by 24 columns, and one 384-well plate contains the equivalent of four 96-well plates. Two different formats of interleaved plate uniformity templates have been developed. The first layout expands the 96-well plate format into four squares (Figure 5).

The second is useful for assays using certain automation equipment such as Tecan and Beckman (Figure 6). For these instruments column 1 of the 96-well plate corresponds to columns 1 and 2 of the 384-well plate, and is laid out in 8 pairs of columns.

The analysis and acceptance criteria are exactly the same as for 96-well format Plate Uniformity Studies. See Section 3.2.5 for a summary of the acceptance criterion.

3.3. Concentration-Response Curve (CRC) plus Uniform-Signal Plate Layouts

CRC plus Uniform-Signal plate layouts are an alternative format to conduct the plate uniformity studies. Their main advantage is easier execution since it is more amenable to automated pipetting per the production process

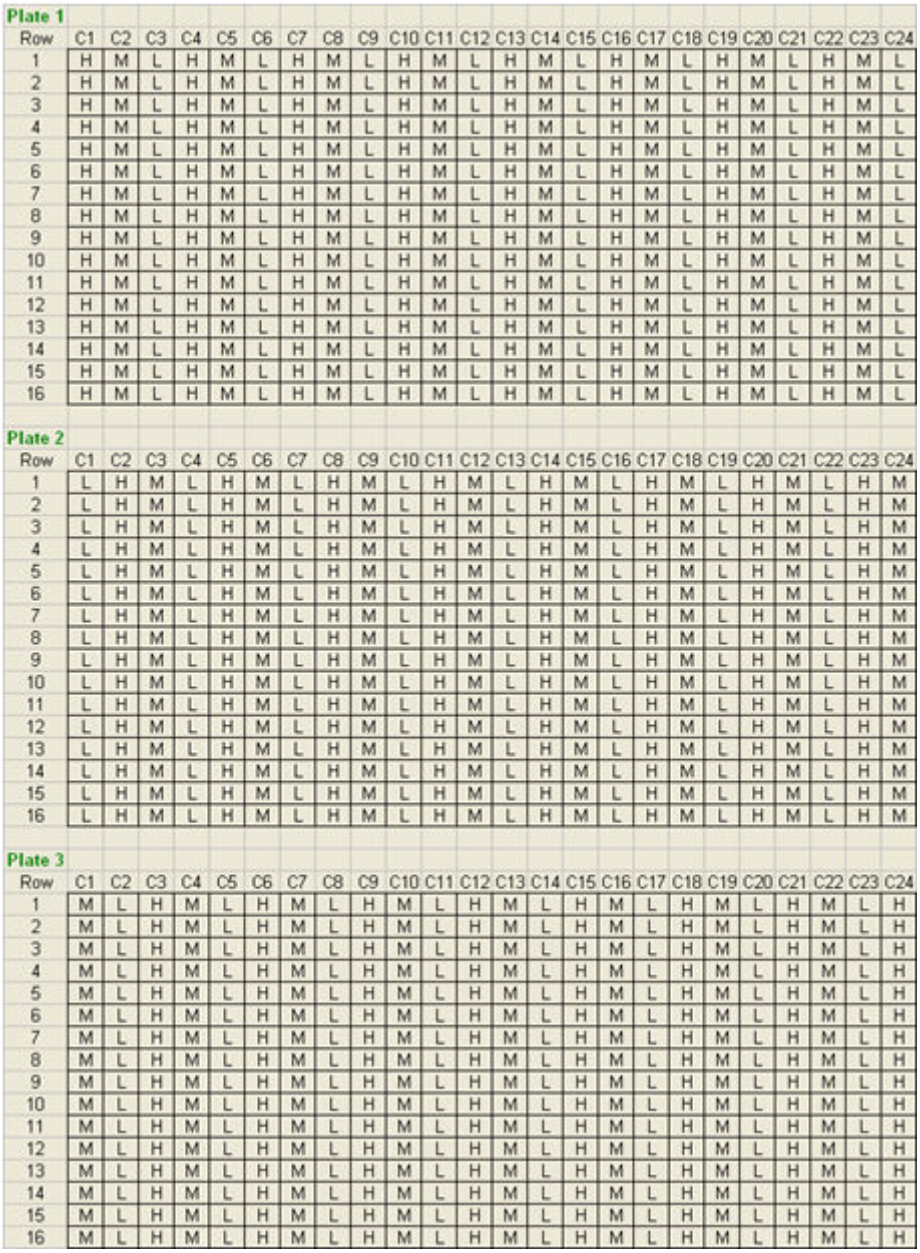


Plate 1

Row	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20	C21	C22	C23	C24
1	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L
2	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L
3	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L
4	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L
5	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L
6	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L
7	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L
8	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L
9	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L
10	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L
11	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L
12	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L
13	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L
14	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L
15	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L
16	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L

Plate 2

Row	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20	C21	C22	C23	C24
1	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M
2	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M
3	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M
4	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M
5	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M
6	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M
7	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M
8	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M
9	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M
10	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M
11	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M
12	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M
13	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M
14	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M
15	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M
16	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M

Plate 3

Row	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20	C21	C22	C23	C24
1	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H
2	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H
3	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H
4	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H
5	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H
6	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H
7	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H
8	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H
9	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H
10	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H
11	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H
12	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H
13	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H
14	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H
15	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H
16	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H	M	L	H

Figure 5: Standard Interleaved 384-well Format Plate Layouts.

for the assay. In the uniform-signal plates, all wells on each plate are exactly the same, and together with heat maps provide for a straightforward assessment of spatial properties. Optionally, the opposite production controls (Max and Min) can be included on the uniform-signal plates to assess with-in plate Z factors. The CRC plates have a single reference compound serially diluted across every row or every column, along with Max and Min-control wells, per the planned production layout.

3.3.1. Procedure

Max and Min signals are prepared as defined in Section 3.1. Two plates are run for each signal. Two CRC plates are also run, for a total of six plates per day. The number of days required is the same as the Interleaved-Signal layout: three days for new assays, two days for transfers of previously validated assays.

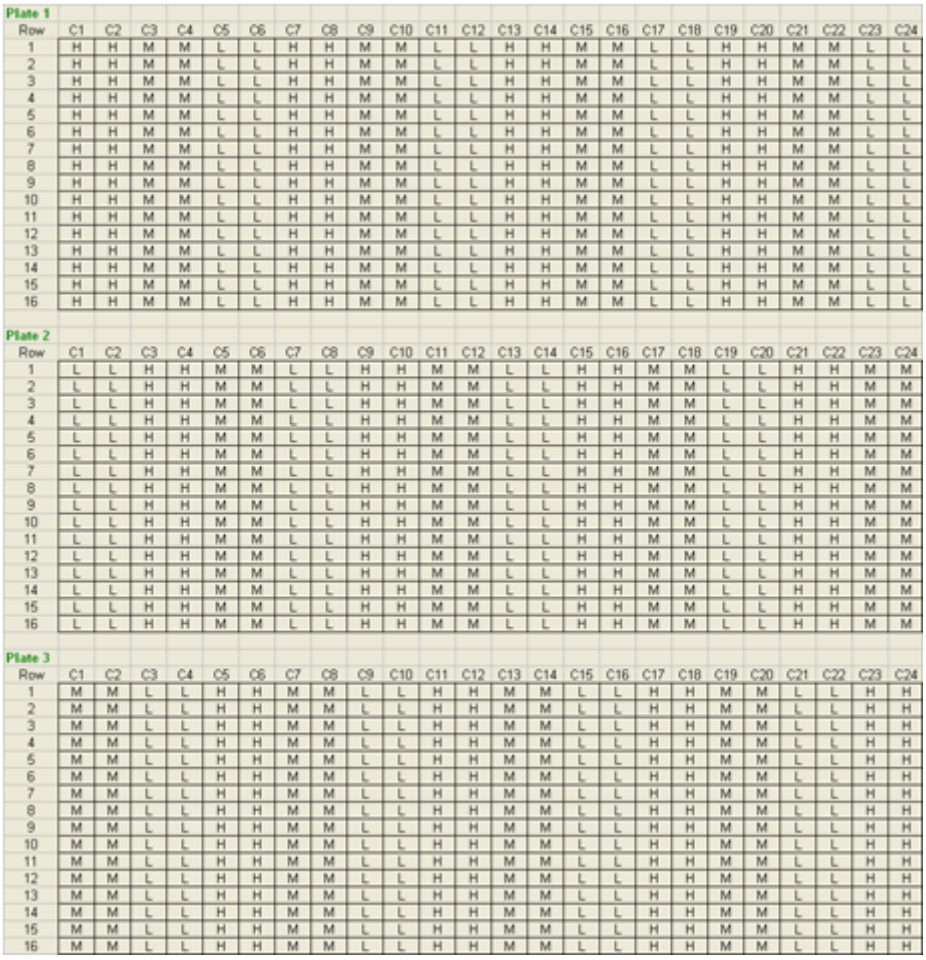


Plate 1

Row	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20	C21	C22	C23	C24
1	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L
2	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L
3	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L
4	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L
5	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L
6	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L
7	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L
8	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L
9	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L
10	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L
11	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L
12	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L
13	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L
14	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L
15	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L
16	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L

Plate 2

Row	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20	C21	C22	C23	C24
1	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M
2	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M
3	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M
4	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M
5	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M
6	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M
7	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M
8	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M
9	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M
10	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M
11	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M
12	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M
13	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M
14	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M
15	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M
16	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M

Plate 3

Row	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20	C21	C22	C23	C24
1	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H
2	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H
3	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H
4	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H
5	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H
6	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H
7	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H
8	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H
9	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H
10	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H
11	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H
12	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H
13	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H
14	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H
15	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H
16	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H	M	M	L	L	H	H

Figure 6: HHMMLL 384-well Plate Uniformity Plate Layouts

3.3.2. Summary Calculations and Plate Acceptance Criterion

The calculations will be performed by the Excel template provided. Details of the calculations are as follows:

1. Compute the mean (AVG), standard deviation (SD) and Coefficient of Variation (CV) for the Max and Min plates (as per the Interleaved-Signal format the CV's should reflect the number of wells per test-condition envisioned in the production assay). Also compute these quantities using the Max and Min control wells on the CRC plates. Requirements are the same as for Interleaved-Signal format: The CV of each plate should be less than 20%. Alternatively, the Min plates should have SD ≤ smaller of SD_{mid} and SD_{max}.
2. For the CRC plates, determine which concentration has mean activity across all plates that is closest to 50%. Designate this concentration as the "Mid" signal. Then compute the percent activity for agonist or stimulation assays, and percent inhibition for antagonist or inhibition assays (including binding assays). In this format the calculation is

$$\%\text{Activity} = \frac{\text{well}_{\text{mid}} - \text{AVG}_{\text{min}}}{\text{AVG}_{\text{max}} - \text{AVG}_{\text{min}}} \times 100$$

3. where AVG_{min} is the average of the on-plate Min control wells, and AVG_{max} is the average of the on-plate Max control wells. Percent Inhibition = 100 - %Activity.

4. Compute the SD of the normalized signals on each Mid signal. The acceptance criterion is $SD_{\%mid} \leq 20$.

5. Compute the Z' factor and/or the SW for each plate where Max and Min controls are present, and for the Max and Min uniform plates, if the opposite production control wells were included. Z' factor and SW can also be calculated within a day across plates, pooling all of the Max and Min wells, but this is only useful if the raw signal levels are consistent between plates. The formulas are the same as in Section 3.2.2. The acceptance criterion is either all Z' ≥ 0.4 or all SW ≥ 2.

3.3.3. Spatial Uniformity Assessment

Heat maps or similar types of plots can be generated for the raw plate data. The criterion for acceptance is the same as for the interleaved format: No drift or edge effects that exceed 20% of the mean. Also as in the Interleaved-Signal format, the presence of these effects should be apparent as the predominant effect, and not seen just in single isolated plate for the assay to be failed by this criterion.

Using the Max and Min uniform plates, Figure 7 illustrates a spatially uniform result, an edge effect, and a drift effect. Uniform Mid signal plates were used in this example instead of CRC plates. Day 1 shows an acceptably uniform result. Day 2 shows an assay with a significant edge effect (25% from the mean edge value to the mean of the interior), and Day 3 shows an assay with significant drift (25% change in mean value from left to right as compared to the average in the middle). If patterns are similar or worse than those depicted in Day 2 or Day 3 then the assay ***does not pass*** the spatially uniform requirement.

3.3.4. Inter-Plate and Inter-Day Tests

The Inter-plate and inter-day tests are the same as in Section 3.2.4, except the definitions of % Activity and % Inhibition defined above (Section 3.3.1) are used in the tests. In addition, EC/IC_{50}s can be calculated from the CRC data, and fold shifts in mean EC/IC_{50} between plates and days can be examined directly to assess variability. In addition, a preliminary Minimum Significant Ratio (MSR) can be calculated from the CRC plates, as follows. An EC/IC_{50} can be calculated for each row on each plate. For higher density plates (384-wells and above), there could be more than one CRC curve fit per row. Compute the standard deviation (S) of all the Log IC_{50}s across plates and days (Alternatively, a variance components analysis could be used to separate within-plate, within-day and between-day variability). The MSR is 10^(2*sqrt(2)*S), and this value is the smallest ratio between two IC_{50}s that is statistically significant. A common criterion is for the MSR to be less than 3. This MSR should be considered a preliminary and optimistic estimate of the assay MSR, because it is based on a single compound and because the IC_{50}s derived from different rows on the same plate, or even different plates on the same day, will most often not reflect all of the sources of variability in the assay.

3.3.5. Impact of Plate Variation on Validation Results

Uniform-Signal plates in the CRC format make the assumption that plate variation within each run day is negligible. If this assumption is not correct, then some of the diagnostic tests described here will be misleading. In that case, one should either include opposite production control wells (Max and Min) on the uniform-signal plates, or use the Interleaved-Signal format instead. In particular, Z' factors and/or Signal Windows computed across plates or across days may be incorrect in either direction, and the Inter-plate and Inter-Day tests could unnecessarily fail acceptable assays.

The following example illustrates the problem. The raw signals of one day of an Interleaved-Signal format Plate Uniformity Study are shown in Figure 8. The Max and Mid raw signals vary across the 3 plates (Figure 8, Plates 1-3), but note that the % Activity is very stable across the 3 plates (Figure 9, Plates 1-3). The maximum fold shift across plates is 1.2.

The Midpoint Percent Activity plot (Figure 9) shows what happens when on-plate Max and Min controls are not used. The three left-hand panels show the plates, normalized to their own controls while, to mimic the Uniform-

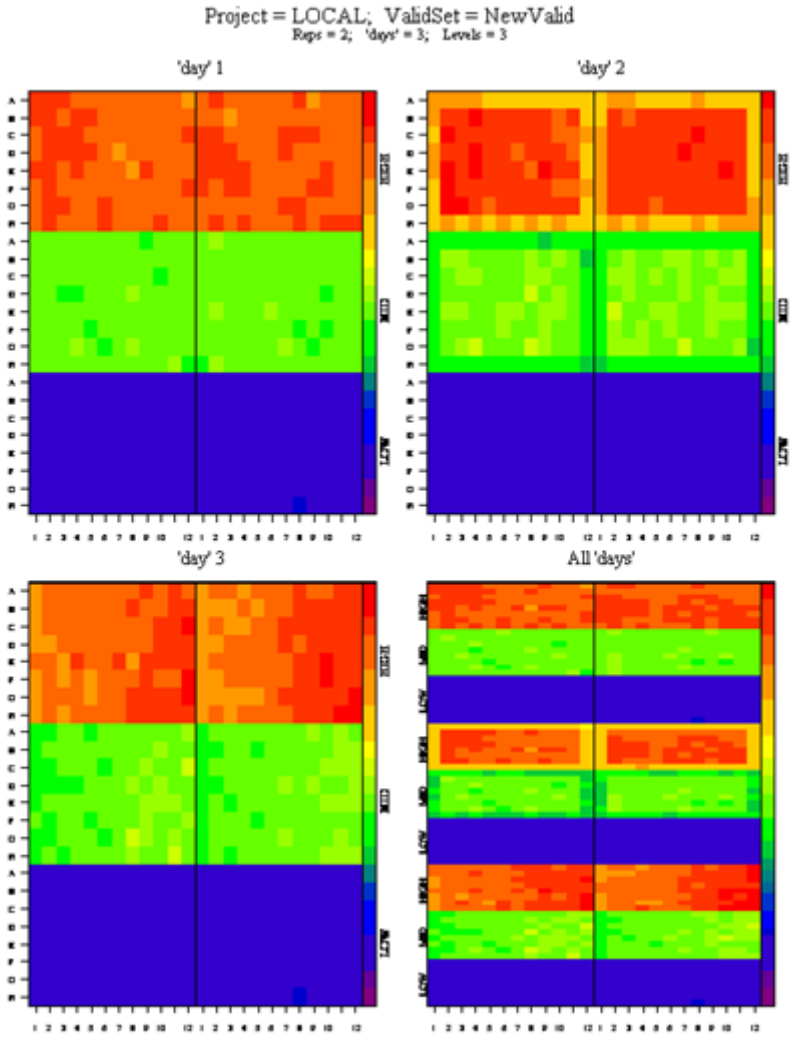


Figure 7: Illustrates a spatially uniform result (Day 1), an edge effect (Day 2), and a drift effect (Day 3). Mid signal plates were used in place of CRC plates.

Signal protocol with its off-plate controls, the three right-hand panels of Figure 9 show each plate's mid signal normalized to the plate 3 controls:

Plate 4 shows the plate 1 mid signal data normalized to the plate 3 Max and Min signals,

Plate 5 shows the plate 2 mid signal data normalized to the plate 3 Max and Min signals and

Plate 6 is the same as plate 3.

In the presence of variation in the uniform-signal plates, off-plate controls do not effectively normalize the assay. As Figure 9 shows, plate-to-plate variation in the raw signals can induce the appearance of significant mid-point variation when in fact there is little variation in signals properly normalized to on-plate controls. In this example using off-plate controls, Plates 1-3 have a max fold shift of 2.0 which does not pass the inter-plate acceptance criterion.

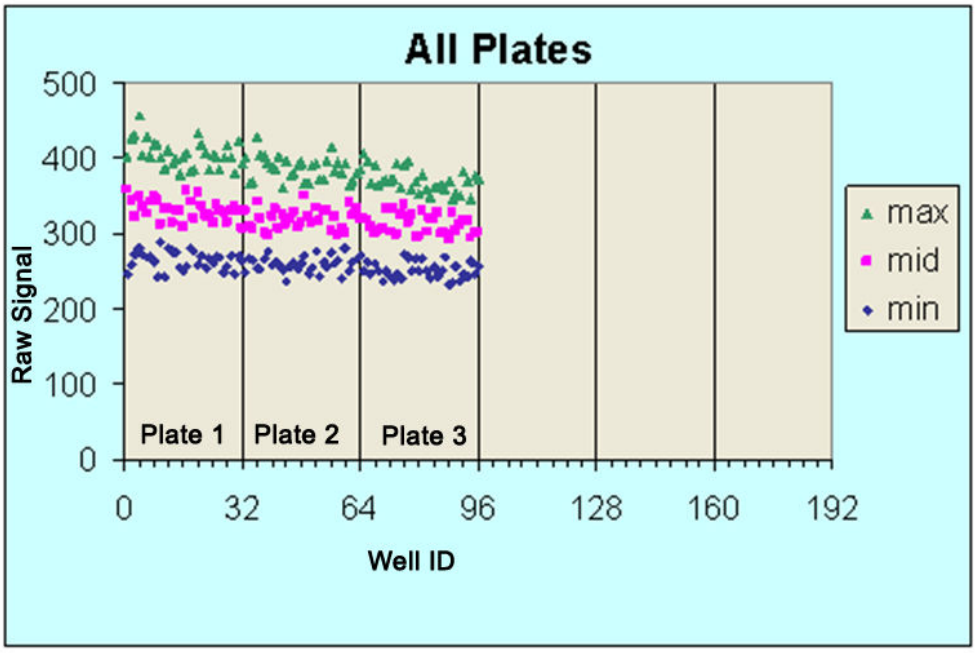


Figure 8: Raw data values for 3 plates of an Interleaved-Signal Plate Uniformity Study. Plates 1-3 show the actual plate values obtained on one day of the test.

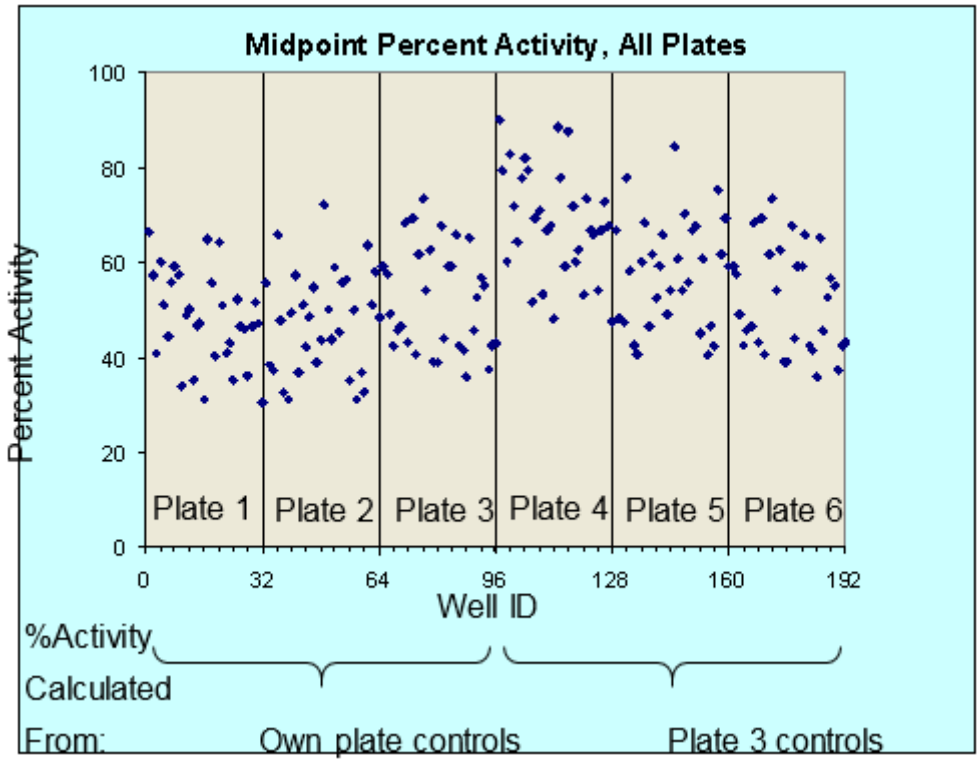


Figure 9: Normalized midpoint values for 3 plates of an Interleaved-Signal Plate Uniformity Study. Plates 1-3 show the actual plate midpoints normalized to the on-plate controls. Plates 4-6 show the same mid points all normalized to the Plate 3 Min and Max controls. Notice the shift in activity for plates 4 vs. 1 and 5 vs. 2.

Table 1: **Reference for the number of replicates necessary for assays with high variability.**

CV using 1 well	Number of wells so that CV < 20%	Number of wells so that CV < 20%
<10	1	1
10.1-14.1	2	1
14.2-17.3	3	1
17.4-20.0	4	1
20.1-22.3	5	2
22.4-24.4	6	2
24.5-26.4	7	2
26.5-28.2	8	2
28.3-30.0	9	3
30.1-31.6	10	3
31.7-33.1	11	3
33.2-34.6	12	3
34.7-36.0	13	4
36.1-37.4	14	4
37.5-38.7	15	4
38.8-40.0	16	4

Table 3: **MSR and LsA evaluation of Replicate-Experiment study using both replicates, and then re-calculated using just the first replicate.**

Replicates Used	Assay 1		Assay 2	
	MSR	LsA	MSR	LsA
Rep 1 Only	2.27	0.44-2.27	3.30	0.28-3.08
Both Reps	1.71	0.57-1.67	2.15	0.44-2.03

Table 2: **MSR and LsA evaluation of Replicate-Experiment study using the first 2 replicates, first 3 replicates, and all 4 replicates.**

Reps	MSR	LsA
2	3.62	0.35-4.59
3	3.32	0.43-4.74
4	2.44	0.53-3.16

4. Replicate-Experiment Study

4.1. Overview

It is important to verify that the assay results are reproducible, i.e. that the variability of key end points of the assay are acceptably low. In addition, if the assay is to report results previously reported by another assay, then it is necessary to verify that the two labs produce equivalent results. In this section, we define how to quantify assay variability and determine assay equivalence. It is important to read the entire section below to understand the rationale for the statistical methods employed in calculating reproducibility of potency and efficacy. We strongly recommend consultation with a statistician before designing experiments to estimate variability described below.

4.1.1. Rationale

Replicate-Experiment studies are used to formally evaluate the *within-run* assay variability and formally compare the new assay to the existing (old) assay. They also allow a preliminary assessment of the *overall* or *between-run* assay variability, but two runs are not enough to adequately assess overall variability. Post-production methods (Section 3) are used to formally evaluate the overall variability in the assay. Note that the Replicate-Experiment study is a diagnostic and decision tool used to establish that the assay is ready to go into production by showing that the endpoints of the assay are reproducible *over a range of potencies*. It is not intended as a substitute for post-production monitoring or to provide an estimate of the *overall* Minimum Significant Ratio (MSR).

It may seem counter-intuitive to call the differences between two independent assay runs as "within-run" variability. However, the terminology results from how assay runs are defined. Experimental variation is categorized into two distinct components: *between-run* and *within-run* sources. Consider the following examples:

- If there is variation in the concentrations of buffer components between 2 runs, then the assay results could be affected. However, assuming that the same buffer is used with all compounds within one run, each compound will be equally affected and so the difference will only show up when comparing one run to another run, i.e. in two runs, one run will appear higher on average than the other run. This variation is called *between-run* variation.
- If the concentration of a compound in the stock plate varies from the target concentration then all wells where that compound is used will be affected. However, wells used to test other compounds will be unaffected. This type of variation is called *within-run* as the source of variation affects different compounds in the same run differently.
- Some sources of variability affect both within- and between-run variation. For example, in a FLIPR assay cells are plated and then incubated for 24-72 hours to achieve a target cell density taking into account the doubling time of the cells. For example, if the doubling time equals the incubation time, and the target density is 30,000 cells/well, then 15,000 cells/well are plated. But even if exactly 15,000 cells are placed in each well there won't be exactly 30,000 cells in each well after 24 hours. Some will be lower and some will be higher than the target. These differences are *within-run* as not all wells are equally affected. But also suppose in a particular run only 13,000 cells are initially plated. Then the wells will on average have fewer than 30,000 cells after 24 hours, and since all cells are affected this is *between-run* variation. Thus cell density has both within- and between-run sources of variation.

The total variation is the sum of both sources of variation. When comparing two compounds across runs, one must take into account both the *within-run* and *between-run* sources of variation. But when comparing two compounds in the same run, one must only take into account the within-run sources, since, by definition, the between-run sources affect both compounds equally.

In a Replicate-Experiment study the between-run sources of variation cause one run to be on average higher than the other run. However, it would be very unlikely that the difference between the two runs were exactly the same for every compound in the study. These individual compound "differences from the average difference" are caused by the within-run sources of variation. The higher the within-run variability the greater the individual compound variation in the assay runs.

The analysis approach used in the Replicate-Experiment study is to estimate and factor out between-run variability, and then estimate the magnitude of within-run variability.

4.2. Procedure

All assays should have a reproducibility comparison (Steps 1-3). If the assay is to replace an existing assay and combine the data, then an assay comparison study should also be done (Steps 4 and 5).

1. Select 20-30 compounds that have potencies covering the concentration range being tested and, if applicable, efficacy measures that cover the range of interest. The compounds should be well spaced over these ranges.
2. All compounds should be run in each of two runs of the assay.
3. Compare the two runs as per Section 4.3-4.6.
4. Assay comparison: Same compound set should be run in a single run of the previous assay.
5. Compare the results of the two assays by analyzing the first run of the new assay with the single run of the previous assay.

4.3. Analysis (Potency)

For the reproducibility comparison, paste potency values from the two runs into the Run-1 and Run-2 data columns. All tests are analyzed and computed by the spreadsheet, which also provides additional plots and diagnostics to assist in judging the results.

For the assay comparison study, paste the potency values for the first run of the new assay into the Run-1 column and the potency values for the (single) run of the previous assay into the Run- column. Potency values should be calculated according to the methods of Section 3. A template for the Replicate-Experiment data analysis is available for download from the online eBook.

The points below describe and define the terms used in the template and the acceptance criterion discussed in the Diagnostic Tests section below.

1. Compute the difference in log-potency (= first – second) between the first and second run for each compound. Let $\bar{d}$, and s_d be the sample mean and standard deviation of the difference in log-potency. Since ratios of EC_{50} values (relative potencies) are more meaningful than differences in potency, we take logs in order to analyze ratios as differences. (Note: hypothetical potency values of 1 and 3, 10 and 30, 100 and 300 have the same ratio but not the same difference),
2. Compute the **Mean-Ratio**: $MR = 10^{\bar{d}}$. This is the geometric average fold difference in potency between two runs.
3. Compute the **Ratio Limits**: $RLs = 10^{\bar{d} \pm 2s_d/\sqrt{n}}$, where n is the number of compounds. This is the 95% confidence interval for the Mean-Ratio.
4. Compute the **Minimum Significant Ratio**: $MSR = 10^{2s_d}$. This is the smallest potency ratio between two compounds that is statistically significant.
5. Compute the **Limits of Agreement**: $LsA = 10^{\bar{d} \pm 2s_d}$. Most of the compound potency ratios (approximately 95%) should fall within these limits.
6. For each compound compute the **Ratio** (=first/second) of the two potencies, and the **Geometric Mean** potency: $GM = \sqrt{\text{first} \times \text{second}}$.

Items 2-6 can be combined into one plot: the Ratio-GM plot. An example is in Figure 10. The points represent the compounds; the blue-solid, green long-dashed and red short-dashed lines represent the MR, RLs and LsA values respectively.

Figure 10 shows the desired result of pure chance variation in the difference in activities between runs. The blue solid line shows the geometric mean potency ratio, i.e. the *average* relationship between the first and second run. The green long-dashed lines show the 95% confidence limits of the mean ratio. These limits should contain the value 1.0, as they do in this case. The red short-dashed lines indicate the limits of agreement between runs. They

indicate the *individual compound* variation between the first and second run. All or almost all, the points should fall within the red dashed lines. The lower line should be above 0.33, while the upper line should be below 3.0, which indicates a 3-fold difference between runs in either direction. The MSR should be less than 3.0, as it is in this example.

4.4. Diagnostic Tests and Acceptance Criterion (Potency)

1. If the MSR ≥ 3 then there is poor **individual** agreement between the two runs. This problem occurs when the *within-run* variability of the assay is too high (Figure 11A). An assay meets the MSR acceptance criterion if the (within-run) **MSR < 3.**
2. If Ratio limits do not contain the value 1, then there is a statistically significant **average** difference between the two runs. Within a lab (Step 3) this is due to high *between-run* assay variability. Between labs (Step 4), this could be due to a systematic difference between labs, or high between-run variability in one or both labs. See Figure 11B for an illustration. Note that it is possible with a very "tight" assay (i.e. one with a very low MSR) or with a large set of compounds to have a statistically significant result for this test that is not very material, i.e., the actual MR is small enough to be ignorable. If the result is statistically significant then examine the MR. If it is between 0.67 and 1.5 then the average difference between runs is less than 50% and is deemed immaterial. However, in Figure 2(b) the MR=2.01, indicating a 101% difference between runs, which is too high to be considered "equivalent". Note that there is no direct requirement for the MR, but values that are this extreme are unlikely to pass the Limits of Agreement criterion in step 3 below.
3. The MR and the MSR are combined into a single interval referred to as the **Limits of Agreement**. An assay that either has a high MSR and/or an MR different from 1 will tend to have poor agreement of results between the two runs. An assay meets the Limits of Agreement acceptance criterion if both the upper and lower limits of agreement are between 0.33 and 3.0. Note that assays depicted in both Figure 11 A and B do not have Limits of Agreement inside the acceptance region and thus do not meet the acceptance criterion.

4.5. Analysis (Efficacy)

The points below describe and define the terms used in the template and the acceptance criterion discussed in the Diagnostic Tests section. Note that the methods described here are intended for functional full/partial assays and non-competitive antagonist assays. Some potentiator assays, as well as assays normalized by fold stimulation may best be analyzed with the techniques described in the potency section rather than the methods described here. Consult a statistician for the best method of analysis.

1. Compute the difference in efficacy (= first – second) between the first and second run for each compound. Let $\bar{d}$, and s_d be the sample mean and standard deviation of the difference in efficacy.
2. Compute the **Mean-Difference**: $MD = |\bar{d}|$. This is the average difference in efficacy between the two runs.
3. Compute the **Difference Limits**: $DLs = \bar{d} \pm 2s_d/\sqrt{n}$, where n is the number of compounds. This is a 95% confidence interval for the Mean-Difference.
4. Compute the **Minimum Significant Difference**: $MSD = 2s_d$. This is the smallest efficacy difference between two compounds that is statistically significant.
5. Compute the **Limits of Agreement**: $LsAd = \bar{d} \pm 2s_d$. Most of the compound efficacy differences should fall within these limits (approximately 95%).
6. For each compound compute the **Difference** (= first-second) of the two efficacies, and the **Mean** efficacy (average of first and second).

Items 2-6 can be combined onto one plot: the Difference-Mean plot (not shown). The plot is very similar to the Ratio-GM plot except that both axes are on the linear scale instead of the log scale.

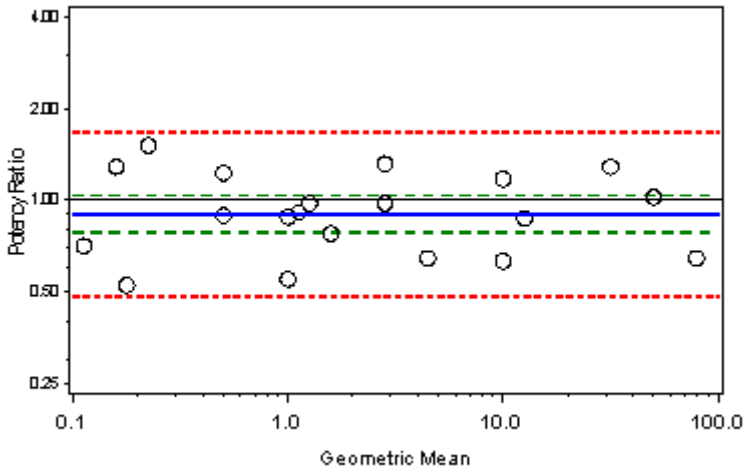


Figure 10: Potency Ratio versus GM Potency. This is a typical example for an acceptable assay: The MR=0.90, RLs=(0.78-1.03) [contains the value 1.0], MSR=1.86 [under 3.0], LsA=(0.48-1.67) [between 0.33 and 3.0]. The blue-solid, green long-dashed and red short-dashed lines represent the MR, RLs and LsA values respectively

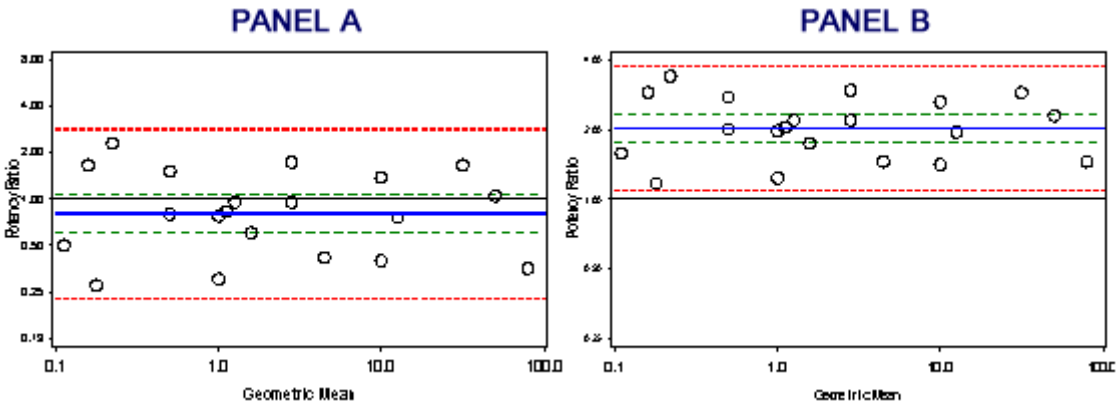


Figure 11: Potency Ratio vs. GM Potency. (A) Shows a case where the within-run variability is too large (MR= 0.8, RLs= (0.61-1.07), MSR= 3.54, and LsA= (0.23-2.84), and (B) shows a case where the LsA are outside the acceptable range because the Mean Ratio is too large, i.e., there is a tendency for the activity values in run 1 to be larger than in run 2 (MR= 2.01, RL= (1.75-2.32), MSR= 1.86, and LsA= (1.08-3.75). In both cases the reason(s) for these conditions should be investigated.

4.6. Diagnostic Tests (Efficacy)

Generally the same two problems discussed under potency need to be judged for efficacy as well. However, a general acceptance criterion for efficacy has not been established as there is not a consensus on efficacy standards and for most projects potency is the primary property of interest. As guidelines, the MD should be less than 5 (i.e., less than 5% average difference between runs) and the MSD should be less than 20 (e.g., 20% activity). More importantly, the MD and MSD should be used to judge the appropriateness of any efficacy CSF's a project may have. For example, if the CSF for efficacy is >80%, and the MSD is 30%, then the assay will fail too many efficacious compounds - a 90%-active compound would fall below the CSF 25% of the time. A more appropriate CSF in this situation would be 70 or even 60%.

4.7. Summary of Acceptance Criteria

1. In Step 3, conduct reproducibility and equivalence tests for potency comparing the two runs in the new lab. The assay should pass both tests (MSR < 3 and both Limits of Agreement should be between 0.33 and 3.0).
2. In Step 5, conduct reproducibility and equivalence tests for potency comparing the first run of the new lab to the single run of the old lab. The assays should pass both tests to be declared equivalent (Limits of Agreement between 0.33 and 3.0).
3. For full/partial agonist assays and non-competitive antagonist assays, repeat points 1 and 2 for efficacy. Use the informal guidelines discussed above, and project efficacy CSFs to judge acceptability of results.

4.8. Notes

1. If a project is very new, there may not be 20-30 unique active compounds (where active means some measurable activity above the minimum threshold of the assay). In that case it is acceptable to run compounds more than once to get an acceptable sample size. For example, if there are only 10 active compounds then run each compound twice. However, when doing so, (a) it is important to biologically evaluate them as though they were different compounds, including the preparation of separate serial dilutions, and (b) label the compounds "a", "b" etc. so that it is clear in the test-retest analyses which results are being compared across runs.
2. Functional assays need to be compared for both potency (EC_{50}) and efficacy (%maximum response). This may well require a few more compounds in those cases.
3. In binding assays, it is best to compare K_i's, and in functional antagonist assays it is best to compare K_b's.
4. An assay may pass the reproducibility assessment (Steps 1-3 in the procedure [Section 4.2.]), but may fail the assay comparison study (Steps 4-5 in the procedure [Section 4.2]). The assay comparison study may fail either because of a MR different from 1 or a high "MSR" in the assay comparison study. If it's the former then there is a potency shift between the assays. You should assess the values in the assays to ascertain their validity (e.g. which assay's results compare best to those reported in the literature?). If it fails because the Lab Comparison study is too large (but the new assay passes the reproducibility study) then the old assay lacks reproducibility. In either case, if the problem is with the old assay, then the team should consider re-running key compounds in the new assay to provide comparable results to compounds subsequently run in the new assay.

5. How to Deal with High Assay Variability

5.1. High Variation in Single Concentration Determinations

Table 1 can be used as a reference to determine the number of replicates necessary for assays with high variability. For a given CV of the raw data values based on 1 well, it shows the number of replicates needed for the CV of a mean to be less than or equal to 10 or 20%. This table does not indicate how the $IC_{50}/K_i/K_b$ variability will be affected (See Section 5.2 for high variation in $IC_{50}/K_i/K_b$ responses).

Adding replicates to reduce variability will also reduce the capacity (i.e., throughput) of the assay to test compounds. Further optimization of the assay could reduce variability and maintain or increase its capacity. The decision to further optimize or add replicates will have to be made for each assay.

5.2. Excess Variation in Concentration-Response Outcomes (EC_{50}, IC_{50}, K_i, or K_b)

If in Section 4 the assay fails either test (MSR > 3 or Limits of Agreement outside the interval 1/3-3) then the variability of the assay is too high. The following options should be considered to reduce the assay variability:

1. Optimizing the assay to lower variability in the signal (see Section 6) of the raw data values. Check that the concentration range is appropriate for the compound results. Adding more concentrations and/or replicates may improve the results. A minimum of 8 concentrations at half-log intervals is recommended. In general, it is better to have more concentrations (up to 12) rather than more replicates.
2. Consider adding replicates as discussed below. Note that the impact of adding replication may be minimal, and so the Replicate Experiment Study should be used to assess whether increasing the number of replicates will achieve the objective.
3. Adopt as part of the standard protocol to re-run results. For example, each compound may be tested once per run on 2 or more runs. Then averaging the results will reduce the assay variability (**Note:** In such cases the individual run results are stored in the database, and tthe data mining/query tools are used to average the results).

To investigate the impact of adding replicate wells in the concentration-response assay conduct the Replicate-Experiment study with the maximum number of wells contemplated (typically 3-4 wells / concentration). To examine the impact of replication compute the MSR versus number-of-replicates curve. To construct this curve, make all data calculations using only the first replicate of each concentration to evaluate the MSR and Limits of Agreement for 1 well per concentration. Then repeat all calculations using the first two replicates per concentration, and so on until you are using all replicates. If the assay does not meet the acceptance criterion when all replicates are used then replication will not sufficiently impact the assay to warrant the replication. If it does meet the criterion using all replicates, ascertain how many replicates are needed by noting the smallest number of replicates that are required to meet the Replicate-Experiment acceptance criterion. Two examples below will help illustrate the steps.

Example 1: A binding assay was run using 1 well per concentration and the Replicate-Experiment study did not meet the acceptance criterion. To examine if replication would help, a new Replicate-Experiment study was conducted using 4 wells per concentration. Using only the first replicate from each concentration, the results were normalized, curves fit, and K_i's were calculated for each concentration-response curve. The MSR and LsA were evaluated. The entire calculation steps were repeated using the first 2 replicates, first 3 replicates and all 4 replicates, with the results listed in Table 2.

From Table 2, it is evident that i all 4 replicates are needed to meet the MSR acceptance criterion, and no amount of replication (up to 4 replicates) will meet LsA acceptance criterion.

Example 2: In a second study, a pair of uptake inhibition assays (the project had two targets, each measured by one assay) the Plate Uniformity Study indicated two replicates would be required to meet the Plate Uniformity Signal acceptance criteria in Assay 2. However, plate uniformity criteria concerning replication do not readily translate to dose-response requirements, and so the requirements were investigated in both assays. The Replicate-Experiment Study was conducted using two replicates. The calculations were performed using both replicates, and then re-calculated using just the first replicate. The MSR and LsA are summarized in Table 3.

Using two replicates both assays meet all acceptance criterion. Using only a single replicate, Assay 1 still meets all criteria, while Assay 2 does not. Note that in this instance both assays benefited from increased replication. However, Assay 1 is a very tight assay and hence this benefit is not really needed. So in this example, the replication requirements were the same for both single dose screening and concentration -response studies, but in general this will not be the case.

6. Bridging Studies for Assay Upgrades and Minor Changes

6.1. Overview

Sections 3 and 4 cover the validation of entirely new assays, or assays that are intended to replace existing assays. The replacement assays are "different" from the original assay, either because of facility changes, personnel differences, or substantively different reagents, detection and automation equipment. Assay upgrades and changes occur as a natural part of the assay life cycle. Requiring a full validation for every conceivable change is impractical and would serve as a barrier to implementing assay improvements. Hence full validation following every assay change is not recommended. Instead bridging studies or "mini-validation" studies are recommended to document that the change does not degrade the quality of the data generated by the new assay.

The level of validation recommended has 3 tiers: A small plate uniformity study (Tier I), to an assay comparison portion of the Replicate-Experiment study (Tier II), to a full validation package of Sections 3 and 4 (Tier III). Examples of changes within each Tier are given below, along with the recommended validation study for that Tier. Note that if the study indicates the change will have an adverse impact on assay quality (i.e. the study indicates there are problems), then the cause should be investigated and a full (Tier III) validation should be done. If the results from that study indicate the assays are not equivalent, but the new assay has to be implemented, then the results should not be combined into one set.

The following applies principally to changes in biological components of the protocol. If changes are made to the data analysis protocol then these can ordinarily be validated without generating any new data, by comparing the results using the original and new data analysis protocols on a set of existing data. Discuss any changes with a statistician. If changes are made to both the data analysis and biological components of the protocol, then the appropriate Tier should be selected according to the severity of the biological change as discussed below. The data analysis changes should be validated on the new validation data and any additional validation work may be needed as judged by the statistician.

6.2. Tier I: Single Step Changes to the Assay

Tier I modifications are single changes in an assay such as a change to a reagent, instrumentation, or assay condition that is made either to improve the assay quality or increase the capacity without changing the assay quality. Changes can also be made for reasons unrelated to assay throughput or performance (e.g. change of a supplier for cost savings). Examples of such changes are

- Changes in detection instruments with similar or comparable optics and electronics. E.g.: plate readers, counting equipment, spectrophotometers. A performance check for signal dynamic range, and signal stability is recommended prior to switching instruments.
- Changes in liquid handling equipment with similar or comparable volume dispensing capabilities. Volume calibration of the new instrument is recommended prior to switching instruments. [Note that plate and pipette tip materials can cause significant changes in derived results (IC_{50}, EC_{50}). This may be due to changes in the adsorption and wetting properties of the plastic material employed by vendors. Under these conditions a full validation may be required].

The purpose of the validation study is to document the change and not reduce the assay quality (see Figure 12).

6.2.1. Protocol

Conduct a 4-plate Plate Uniformity Study using the layouts in the "2 Plates per Day" tab of the Plate Uniformity Template (the layouts are the same as Plates 1 and 2 of Section 3.2). Plates 1 and 2 should be done using the existing protocol, and Plates 3 and 4 done using the new protocol on the same day using the same reagents and materials (except for the intentional change). Use the 2-Day / 2-Plates per Day template to conduct the analysis.

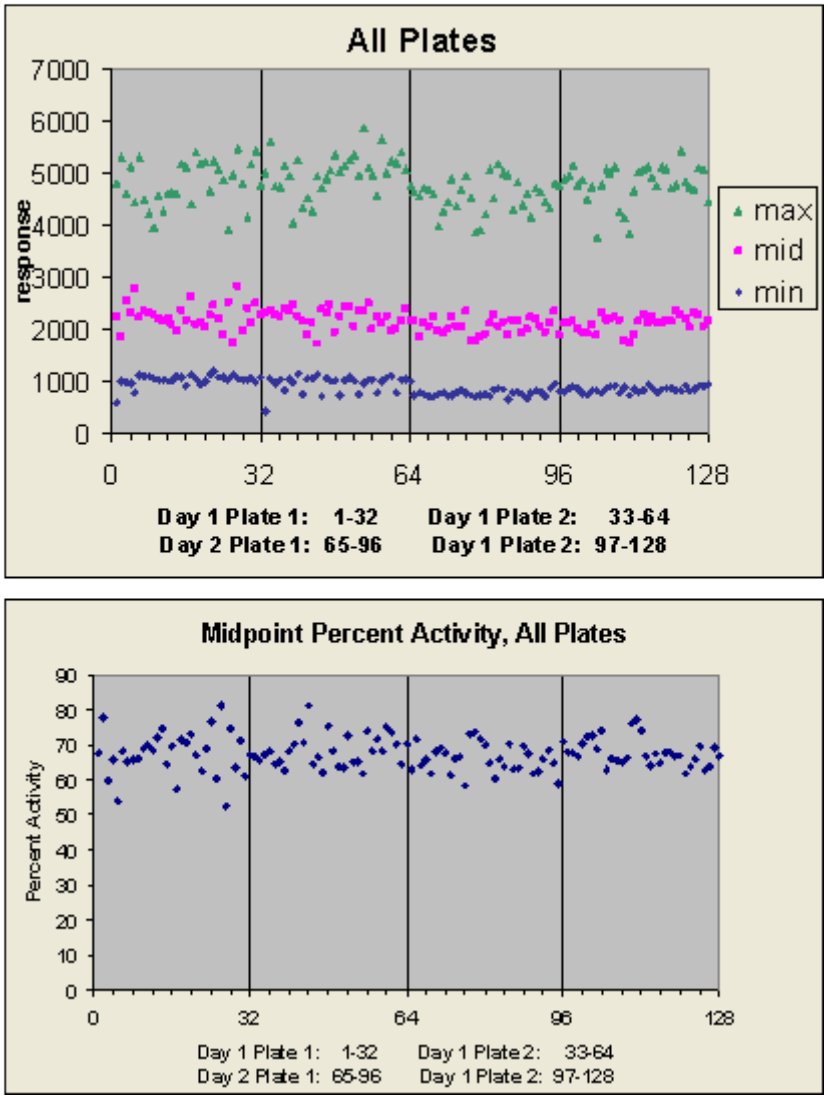


Figure 12: Tier I validation study comparing manual pipetting (plates 1 and 2) versus multidrop pipetting (plates 3 and 4) in GTPγS assay.

6.2.2. Analysis

The main analysis is a visual inspection of the "all plates" plots to ensure that the signals have not changed in either magnitude and/or variability. The mean and SD calculations for each plate can help, but visual inspection is usually sufficient.

6.2.3. Example

An assay was changed by replacing a manual pipetting step with a multidrop instrument. A 4-plate Plate Uniformity study was run as per the protocol, with the manual pipetting done in plates 1 and 2, and the multidrop in plates 3 and 4. The results show that the mean percent activity is the same and the multidrop's varability superior (i.e. lower) to the manual pipetting (Figure 12).

6.3. Tier II: Minor Assay Changes

Tier II changes are more substantive than Tier I changes, and have greater potential to directly impact EC_{50}/IC_{50} results. Examples of such changes are

- Changes in dilution protocols covering the same concentration range for the concentration–response curves. A bridging study is recommended when dilution protocol changes are required.
- Lot changes of critical reagents such as a new lot of receptor membranes or a new lot of serum antibodies.
- Assay moved to a new laboratory without major changes in instrumentation, using the same reagent lots, same operators and assay protocols.
- Assay transfer to an associate or technician within the same laboratory having substantial experience in the assay platform, biology and pharmacology. No other changes are made to the assay.

6.3.1. Protocol and Analysis

Conduct the assay comparison portion of the Replicate Experiment Study discussed in Section 4, i.e. compare one run of 20-30 compounds of the assay using the existing assay to one run of the assay under the proposed format and compare the results. If the compound set used in the original validation is available then run the same set again in the new assay protocol, and compare back to Run-1 of the original Replicate-Experiment Study. The acceptance criterion is the same as for the assay comparison study: Both Limits of Agreement should be between 1/3 and 3.0.

6.4. Tier III: Substantive Changes

Substantive changes requiring full assay validation: When substantive changes are made in the assay procedures, measured signal responses, target pharmacology and control compound activity values may change significantly. Under these circumstances, the assay should be re-validated according to methods described in Sections 3 and 4. The following changes constitute substantive changes, particularly when multiple changes in factors listed below are involved:

- Changes in assay platform: e.g.: Filter binding to Fluorescence polarization for kinase assays.
- Changes in assay reagents (including lot changes and supplier) that produce significant changes in assay response, pharmacology and control activity values. For example, changes in enzyme substrates, isozymes, cell-lines, label types, control compounds, calibration standards, (radiolabel vs. fluorescent label), plates, tips and bead types, major changes in buffer composition and pH, co-factors, metal ions, etc.
- Transfer of the assay to a different laboratory location, with distinctly different instrumentation, QB practices or training.
- Changes in detection instruments with significant difference in the optics and electronics. For example, plate readers, counting equipment, spectrophotometers.
- Changes in liquid handling equipment with significant differences in volume dispensing capabilities.
- Changes in liquid handling protocol with significant differences in volume dispensing methods.
- Changes in assay conditions such as shaking, incubation time, or temperature that produce significant change in assay response, pharmacology and control activity values.
- Major changes in dilution protocols involving mixed solvents, number of dilution steps and changes in concentration range for the concentration-response curves.
- Change in analyst/operator running the assay, particularly if new to the job and/or has no experience in running the assay in its current format/assay platform.
- Making more than one of the above-mentioned changes to the assay protocol at any one time.

Substantive changes require full validation, i.e. a three day Plate Uniformity Study and Replicate Experiment Study. If the intent is to report the data together with the previous assay data then an assay comparison study should be conducted as part of the Replicate Experiment study.

7. References

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Assay Operations for SAR Support

Benoit Beck,[1] Yun-Fei Chen, Walthere Dere, Viswanath Devanarayan, Brian J. Eastwood, Mark W. Farmen, Stephen J. Iturria, Phillip W. Iversen,[2] Steven D. Kahl, Roger A. Moore, Barry D. Sawyer, and Jeffrey Weidner*

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Abstract

Most biological assays measure potency of compounds as an activity coefficient, frequently known as molar concentration of inhibitor at 50% response (IC_{50}) or an agonist concentration at 50% biological activity (EC_{50}). Since biological response responses are non-linear and generally sigmoidal in shape, appropriate curve fitting is important. In addition, the variability in measured IC_{50}/EC_{50} should be monitored to ensure reliable and robust data analysis of the assay curves to support expensive SAR operations in drug discovery. This chapter addresses in detail significant concepts in curve-fitting techniques and statistical concepts and tools required SAR support.

A. Determination of EC_{50}/IC_{50}

Models and Curve Fitting Guidelines

For competitive binding assays and functional antagonist assays the most common summary measure of the dose-response curve is the IC_{50}, the concentration of substance that provides 50% inhibition. For agonist/stimulator assays the most common summary measure is the EC_{50}, the concentration giving 50% of that compound's maximal response. Substantial variation in the methodology used to derive these values exists, and this variation has been shown to substantially impact overall assay variability. This section discusses important issues to consider and provides some guidelines on how to proceed. They are a based on the Data Standardization for Results Management chapter of this book. Consult that document for the specifics for each assay type. Consult a statistician to see if these guidelines are appropriate for your assay, and if other outcomes such as AUC or a threshold dose should be used.

Before fitting a concentration -response curve to obtain the EC_{50}/IC_{50}, each well should be converted to either percent activity or percent inhibition with respect to positive and negative controls (note: for simplicity all text below is stated for determining IC_{50}'s; determining EC_{50}'s is identical). Percent activity of all replicate wells from a given run, (including multiple plates per run) for a given concentration, should be averaged either by taking the mean, or preferably, taking the median. *Outliers less influence the median values when there are 3 or more replicates*. Thus only one averaged point per concentration per run is used to fit the dose-response equation to the data. This is because replicate wells on either the same or different plates are often correlated with each other and, thus, do not provide true replication of the experiment.

The four parameter logistic model (4PL), also called the *Hill-Slope model*, is the most common equation fit to in vitro concentration-response data. One form of the equation is

$$y = bot + \frac{(top - bot)}{1 + (x/IC_{50})^{slope}}$$

Author Affiliations: 1 Eli Lilly & Company, Indianapolis, IN. 2 Eli Lilly & Company, Indianapolis, IN.

* Editor

where, *y* is the percent activity and *x* is the corresponding concentration. The fitted IC_{50} parameter is the **relative** IC_{50}, and is defined as the concentration giving a response half way between the fitted top and bottom of the curve. Some software, such as ActivityBase, also provides the **absolute** IC_{50}, which is defined as the concentration giving exactly a 50% response. The relative IC_{50} is recommended for most assays (see notes below and Glossary for definitions). You should also report the **fitting error**, which is usually called the standard error by most software packages (we use the term fitting error to differentiate it from the standard error of the mean [SEM] derived from multiple determinations of a compound).

The 4PL model is the best model for concentration -response data, but there are cases where it should not be used. In some cases, due to the potency of the compound falling outside the concentration range, the data may not fully describe the bottom or top asymptote of the curve. In those cases, respectively, the bottom (3PLFB) or top (3PLFT) can be fixed to improve the curve fit. If you observe a substantial reduction in the % Fitting Error, and a better concentration-response plot of the fitted curve with respect to the actual data then you should switch to either the 3PLFB or 3PLFT model as appropriate.

Examples

All examples below are from receptor binding data fitting % Activity versus concentration (expressed by ActivityBase as log-concentration in the plots). For this type of assay, the top, bottom and slope parameters should in theory by 100, 0 and –1, respectively.

Figure 1 is a concentration-response best fit by the 4PL model. Both asymptotes are defined by the data, and the fitting error is approximately the same with all 3 models. Note that even though the fitting error is smallest with the top fixed (8.63% versus 9.51%), the reduction is not small enough to warrant the fixed top model, nor is there any material change in the IC_{50}. The fixed bottom model is clearly inappropriate as the data clearly defines a bottom >0.

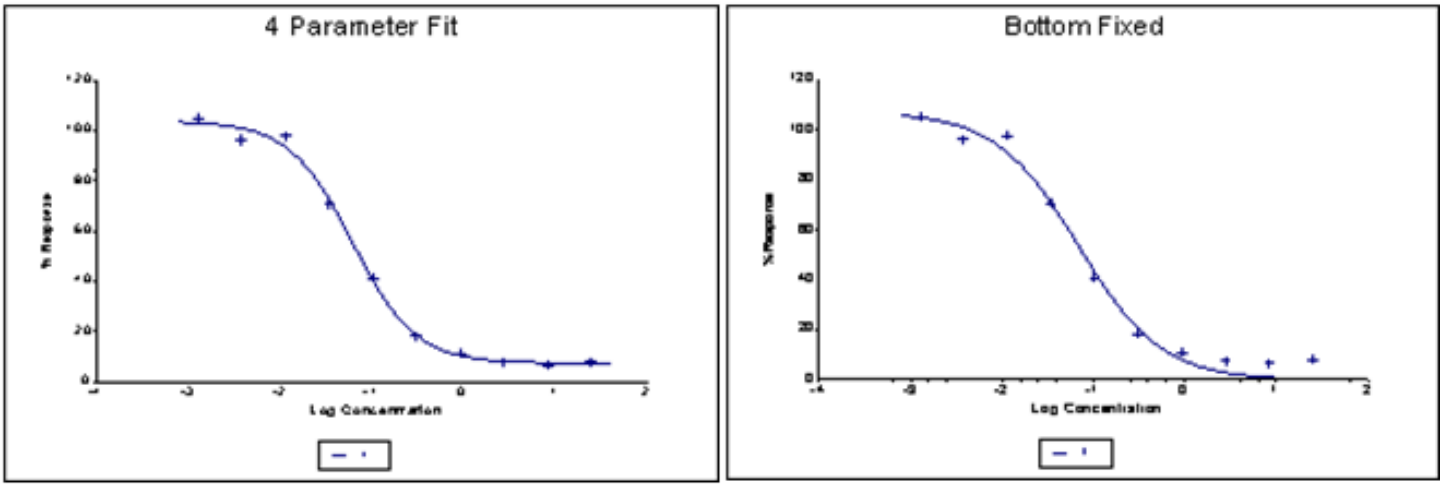
The fitting error is expressed here as a percentage of the fitted parameter value. For example, if the IC_{50} is 0.061 and its fitting error is 0.0058, then the % Fit Error is 9.51%.

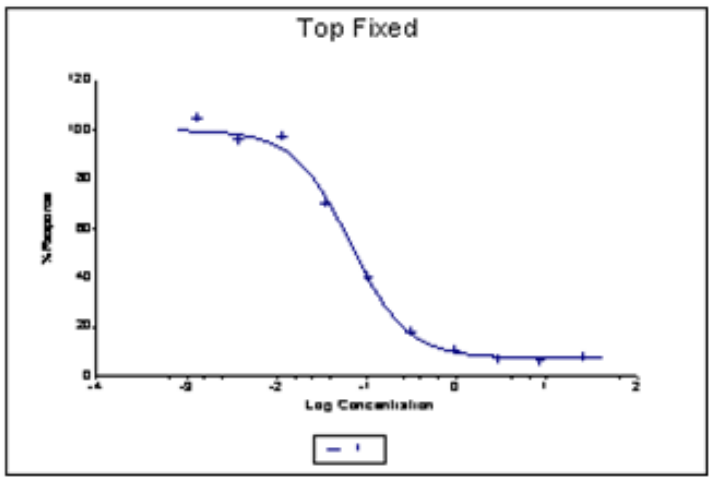
Figure 2 is best fit by the fixed top (3PLFT) model. The data does not define a top asymptote, and the fitted top (128.32) and slope (-0.58) from the 4PL model are inappropriate for this (binding) data. By fixing the top at 100% the fitting error is reduced from 57.54 to 21.55%, and the IC_{50} increases by more than two-fold. Thus the 3PLFT model should be selected over the 4PL.

Figure 3 is best fit by a fixed bottom (3PLFB) model. Note that the data does not define the bottom asymptote, and the fitted bottom (41.54) and fitted slope (-1.83) from the 4PL are inappropriate for binding data. The fixed bottom model reduces the fitting error from 80.19% to 20.85%, while the IC_{50} increases by more than two-fold. The fitted IC_{50} (20.88 nM) is inside the dose-range (0.001-25 nM), and so it is appropriate to report this value. Note in this case Activity Base was unable to fit a fixed top model.

Figure 4 illustrates the definition and *effect of outliers* (Figure 4 A). Outliers are single, vertically isolated points that are clearly inappropriate. The point is "obviously" erroneous. The effect of the outlier in this case is to bias the estimate of the bottom upwards, pulling it away from the other points of the data. In general, outliers can bias either the top, bottom or slope parameter depending upon where they occur in the concentration -response curve. It is appropriate to remove the outlier (Figure 4 B) and refit the points. Fixing top or bottom did not materially improve the curve fit (not shown).

Figure 5 illustrates the effect of *high assay variation*. No single point stands out as "obviously erroneous", and therefore it would be inappropriate to remove any points from the curve fit. Fixing top or bottom does not materially improve the curve fit, and so the 4PL model should be used. Note that the estimates themselves are not implausible, but the fitting error is 33.83%, which is caused by the relatively high assay variation.





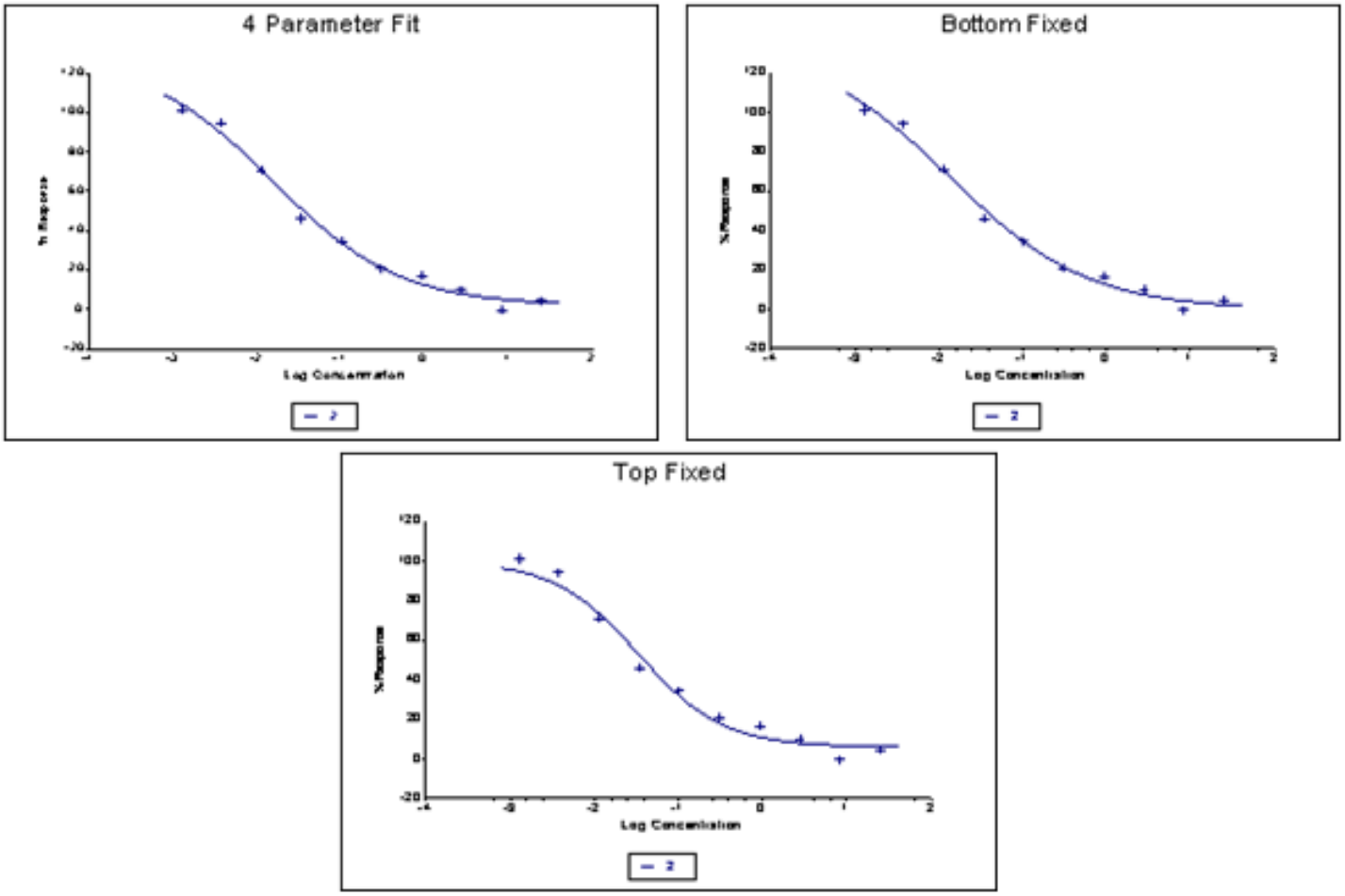
	Bottom	Top	Rel IC50	Slope
4-Param	7.38	103.65	0.061	-1.27
%Fit Err	22.08	2.36	9.51	-10.74
Bottom=0	0.00	107.10	0.069	-0.96
%Fit Err		4.95	19.94	-15.05
Top=100	7.79	100.00	0.066	-1.39
%Fit Err	22.07		8.63	-10.21

Figure 1: Curve fit results for a dose-response best fit by a 4PL model.

Notes:

1. This equation can be fit to the data using Activity Base, Bravo Curve fit, JMP, Graphpad Prism or Sigma Plot. Note that the form of the equation varies from one software package to the next. Some, such as Graphpad Prism, fit Log-IC_{50} instead of IC_{50}, and the equation looks quite different, but the results are the same as that shown above.
2. The terms **absolute** and **relative** IC_{50} are not universal. Both are usually just called the "IC_{50}", and it's left unstated which value is actually used.
3. If your software toll reports Log-IC_{50} then convert both the estimate and the % fitting error (%FE) according to the formulas

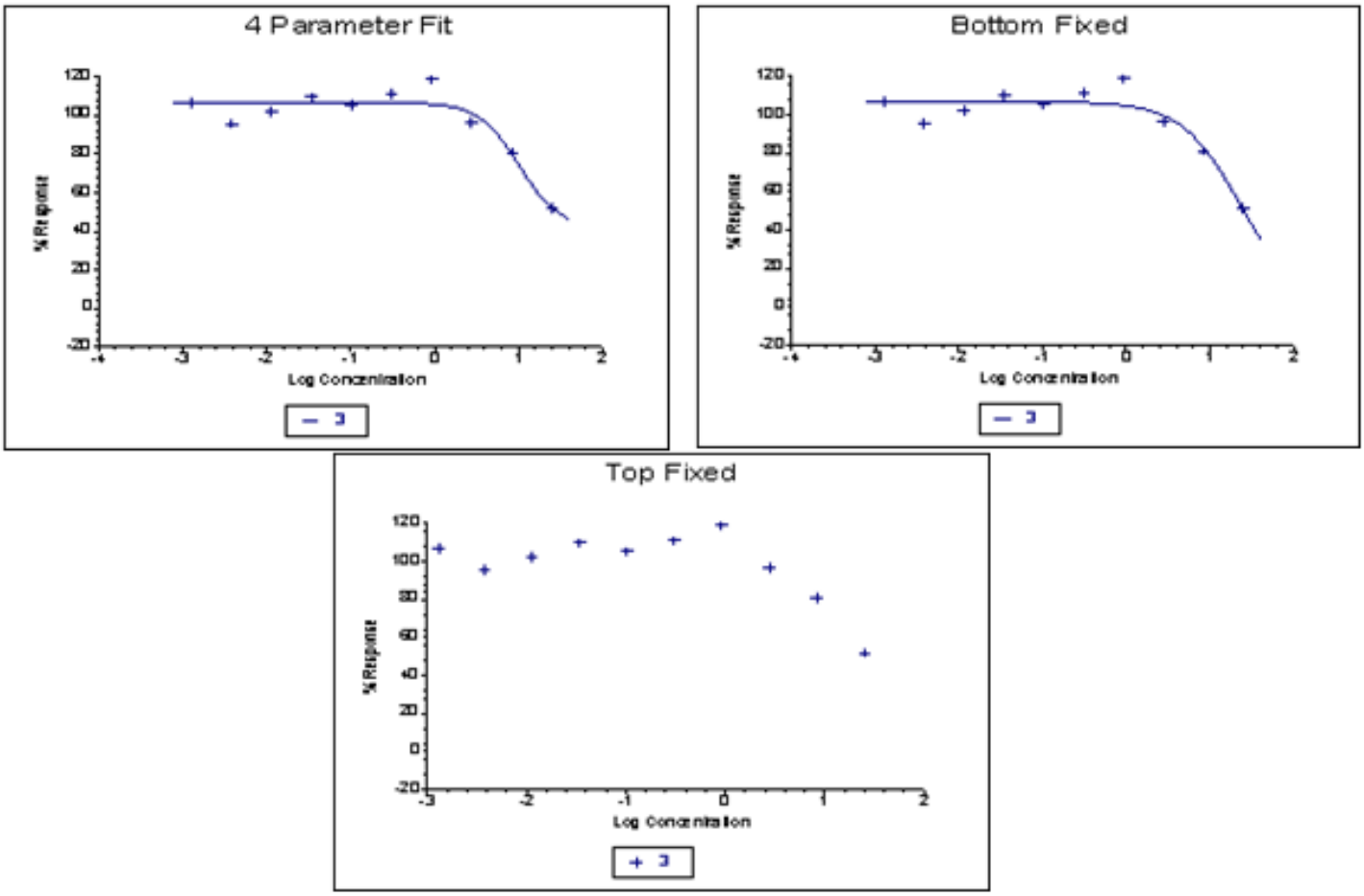
$IC_{50} = 10\text{^Log-}IC_{50}$ and $\%FE(IC_{50}) = FE(\text{Log-}IC_{50}) \times \ln(10) \times 100$



	Bottom	Top	Rel IC50	Slope
4-Param	2.01	128.32	0.015	-0.58
%Fit Err	202.85	14.82	57.54	-22.56
Bottom=0	0.00	134.33	0.014	-0.53
%Fit Err		12.42	57.21	-12.91
Top=100	6.21	100.00	0.034	-0.87
%Fit Err	57.08		21.55	-16.09

Figure 2: Curve fit results for a dose-response best fit a by a 3PLFT model.

4. There should be at least one point on both sides of the reported IC_{50}, i.e. the reported IC_{50} should lie inside the concentration -range used in the assay. The intent of this rule is to make the IC_{50} estimate an interpolation of generated data and not an extrapolation of generated data. Cases not satisfying this rule should not have an IC_{50} reported or reported with a comment that indicates the value is extrapolated. If a value is reported, it should be "$<X_{min}$" or "$>X_{max}$", as appropriate, where X_{min} is the lowest concentration and X_{max} is the largest concentration included in the analysis.
5. It is a good idea to remove obvious outliers and then refit the curve without the outliers. Note that if it isn't obvious, it isn't an outlier. See examples 4 and 5 above to distinguish high variability from outliers.
6. For competition assays, such as radioligand binding assays and competitive inhibition assays, the fitted slope should be within 2 (slope) fitting errors of the value 1, and slope estimates outside this range indicate assay problems that need to be investigated.

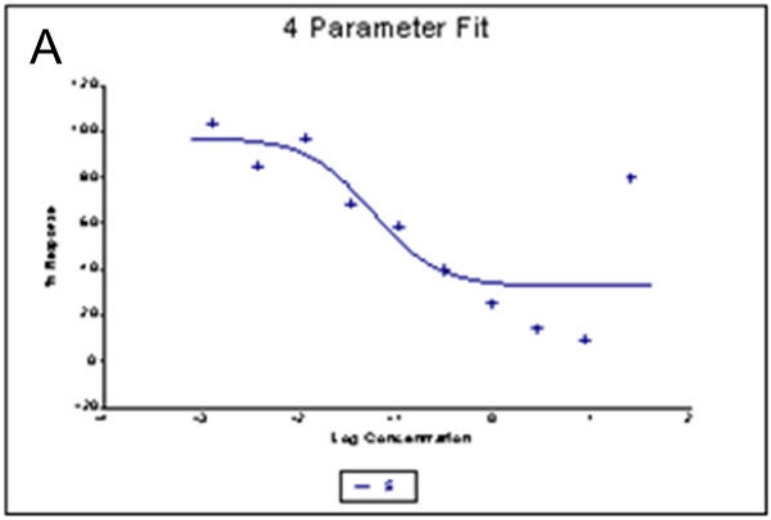


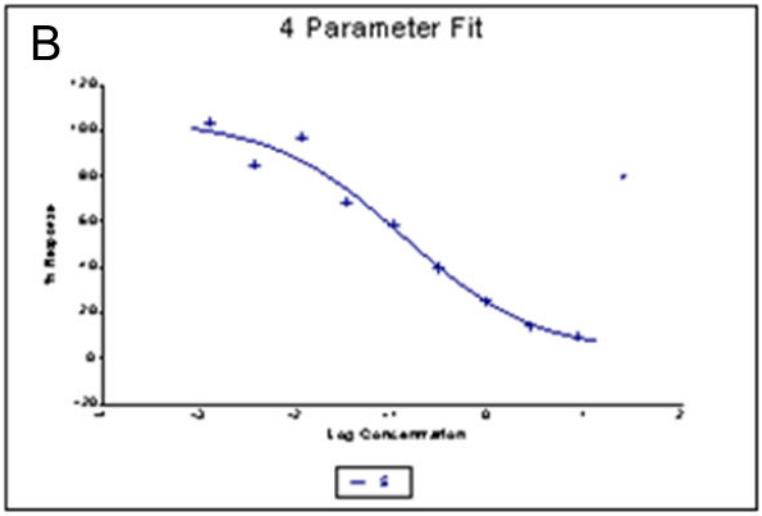
	Bottom	Top	Rel IC50	Slope
4-Param	41.54	106.79	10.17	-1.83
%Fit Err	81.91	2.90	80.19	-95.59
Bottom=0	0.00	106.94	22.88	-1.25
%Fit Err		2.77	20.85	-30.49
Top=100	#N/A!	#N/A!	#N/A!	#N/A!
%Fit Err	#VALUE!		#VALUE!	#VALUE!

Figure 3: Curve fit results for a dose-response best fit by a 3PLFB model.

B. Production Monitoring

Production assays can be monitored in two basic ways: running control (reference) compounds and retrospective studies of compounds that have repeat evaluations that accumulate as part of the normal SAR process. Of the two methods, running control compounds allows problems to be identified prospectively and corrected, whereas retrospective studies are limited to verification of past activity. However, retrospective studies can be useful supplements, especially when conducted prior to important milestones where demonstration of "*valid biological assays*" is a requirement. Below are comments on the setup/selection of controls and the analysis of retrospective studies, and the use of bridging studies to verify that changes to assay protocols have no effect on the assay results.



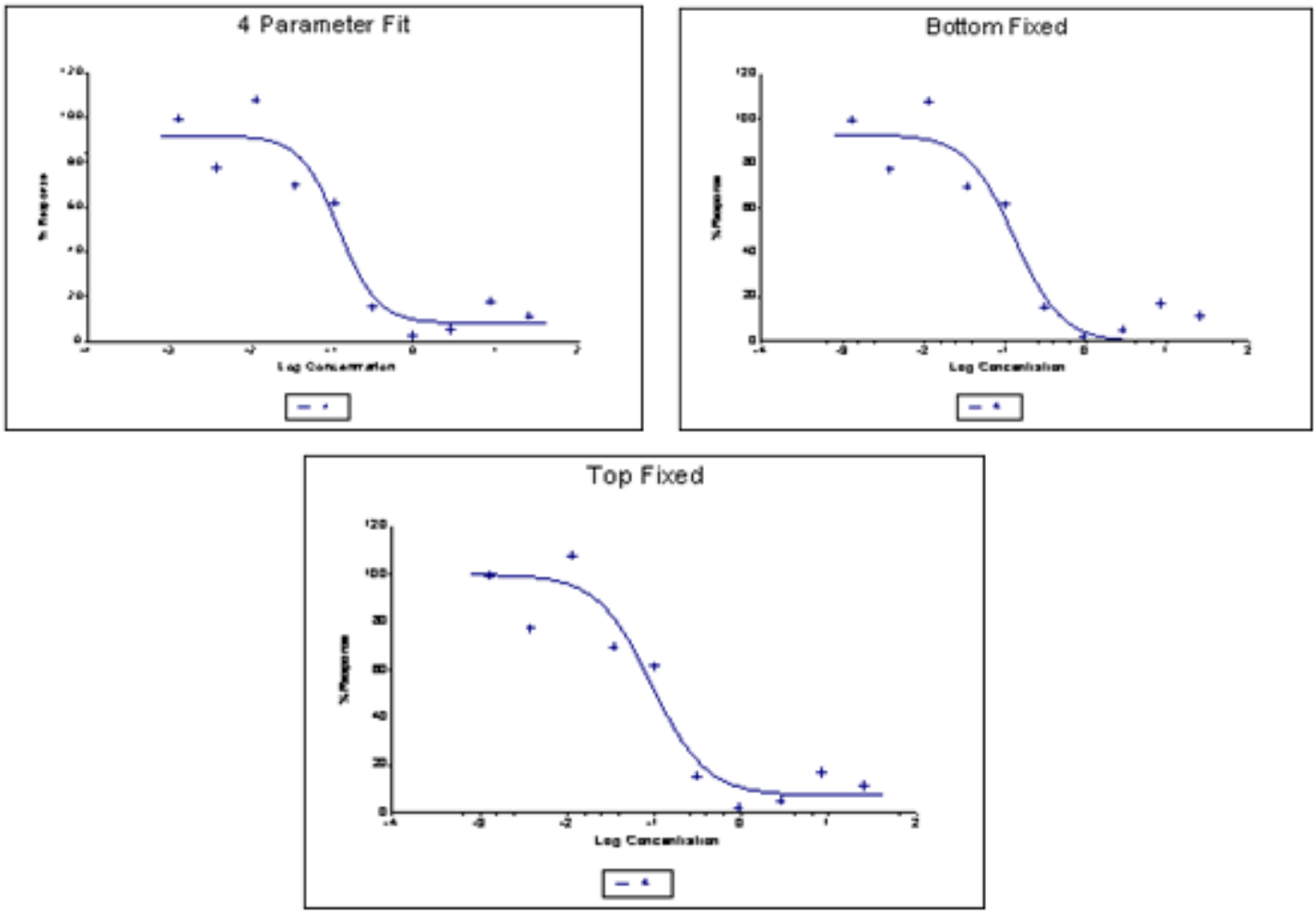


All Data	Bottom	Top	Rel IC50	Slope
4-Param	32.63	97.21	0.056	-1.31
%Fit Err	40.09	20.73	114.10	-132.18
Outlier Rem	Bottom	Top	Rel IC50	Slope
4-Param	2.25	104.78	0.130	-0.63
%Fit Err	611.06	11.29	53.38	-40.98

Figure 4: Curve fit results for a dose-response containing an outlier. (A) The effect of the outlier in this case is to bias the estimate of the bottom, pulling it away from the other points of the data. (B) Results once the outlier is removed and the curve refit to the points.

Control Compounds

Key assays in a project and assays where problems are suspected should have *two control compounds, a primary and a secondary* (this is referred to as **Close Monitoring**). All other assays should have at least a primary control (**Regular Monitoring**). Both compounds need to be run once per run, unless plate variability is suspected. In that case the primary control compound needs to be run once per plate. The purpose of the primary control is to ensure that there isn't any "assay drift", i.e. that the same compound has a stable $K_i/K_b/EC_{50}$ over time, and that



	Bottom	Top	Rel IC50	Slope
4-Param	8.12	91.76	0.117	-1.86
%Fit Err	84.04	8.30	33.83	-58.17
Bottom=0	0.00	92.98	0.130	-1.51
%Fit Err		8.94	34.55	-44.50
Top=100	7.62	100.00	0.093	-1.40
%Fit Err	92.45		33.77	-40.93

Figure 5: Curve fit results for a dose-response with high assay variability, but no outliers.

the assay reproducibility (MSR, is stable over time. (MSR: Minimum Significant Ratio; see HTS Assay Validation).

The purpose of the secondary control is to examine the stability of results over a concentration -range. If problems do develop, then it is important to examine whether the entire concentration -range is *equally affected* (a small problem) or whether the concentration -range is *differentially affected* (a big problem). Also, two controls permit direct calculation of both the within-run and overall MSR's, and a check that the MSR is consistent over a range of potencies.

The activity of the primary control should be at or near the most potent compound available, and ideally should be the Lead compound. There should also be sufficient stock of a single lot of the compound so that it can be run on a continuous basis for some period of time. Since the control compound is supposed to be representative of the test compounds, it should receive the same sample handling as all the test compounds, and not be specifically prepared and added to the assay outside of normal test compound procedures.

For the secondary control, IC_{50} should be *>100 fold less* potent than the primary control. Otherwise it has the same requirements as the primary control. As the SAR develops the potency traditionally improves. So when the "best" compounds are more than 100-fold more potent than the primary control then select a new primary control. If the assay has a secondary control then the old primary control becomes the new secondary control, and the existing secondary control is dropped. If there is no secondary control, then it is suggested to run both primary controls over the first 6 runs of the new primary control.

A scatter plot for control compound for the values of log-K_i/K_b/EC_{50} versus run date should be updated after every run and checked for problems. For assays with two control compounds the difference in log-K_i/K_b/EC_{50} versus run date should be plotted, and for agonist and non-competitive antagonist assays the efficacy versus run date should also be plotted. Outliers and trends in the values of log-K_i/K_b/EC_{50}, either up or down (assay drift) should be checked visually, and problems investigated and corrected as they occur. Runs with significant numbers of outliers should be repeated.

After 6 runs compute the overall MSR of the assay based on the control compounds according to formula:

$$MSR = 10^{2\sqrt{2}s}$$

where *s* is the standard deviation of the log-K_i/K_b/EC_{50} values. This MSR is the total or *overall* MSR (whereas the one computed in a test-retest study encompasses only the within-run variability), and should be less than or equal to 7.5. This standard comes from practical experience obtained thus far with assays in the company, and not theoretical statistical considerations. Note that this is a minimum standard that all assays should meet, and in practice chemistry requirements may indicate a smaller MSR (as low as 2-3) is required for some or all assays. The Project/Program Team should discuss this issue with a statistician to set appropriate MSR's for their assays.

After each run, *a running MSR plot* should be maintained (i.e. computed from the last 6 runs) and checked to ensure the continued good reproducibility of the assay. The attached template (available from the online eBook) can be used to generate this with MSR values calculated from the last 6 runs.

Examples

Figure 6 illustrates results for an assay *with a single control*. Figure 6A shows the potency versus run date scatter plot, Figure 6B shows the moving MSR chart. The MSR points are based on the last 6 runs of the assay, i.e. the first point is computed using runs 1-6, the second point uses runs 2-7, etc. The Mean Summary section indicates the highest/lowest/last IC_{50}'s in the period were 22.63, 4.42 and 11.25 μM respectively (chart units are in nM). The overall average was 10.17 μM. The potency has no apparent temporal trends, and no unusual observations. Figure 6B shows the trends in MSR over time, which appears to increase until mid Feb-2002, and then decrease. However, the magnitude of the increase trends is quite small and well within the variation of an estimate based on a sample of size 6. The highest/lowest/latest MSR's are 6.8, 2.7 and 2.7 respectively. The overall MSR is 4.4, which is not the average of the 6-run MSR's but instead is a single estimate derived using the entire sample (18 data points in this case). This is a stable assay with moderate assay variation ($3 < MSR < 5$).

Figure 7 illustrates an assay *with two controls.* In Figure 7A, the red and blue lines represent the two compounds, and are positioned using the left axis. The green line is the potency ratio between the two compounds and is positioned using the right axis. Figure 7B shows the moving MSR values both within run and overall. The Overall-Overall MSR is the value to be reported. The within-run MSR's are only for comparison backwards to the test-retest study results, and for times when compounds are compared within the same run of an assay. As

with Figure 6, there are no apparent temporal problems, i.e. this is a stable assay with an overall MSR of 2.0. This assay is less variable than the assay in Figure 6.

Figures 8 and 9 illustrate problems with a *shift in compound potency*. Figure 8 illustrates a steady degradation in potency over time, whereas Figure 9 illustrates a more sudden shift in potency at a particular point in time. In Figure 8 the assay variability appears to be shrinking, while in Figure 9 it appears to be stationary. Repetitive freeze-thaw cycles of a compound may cause a slow degradation in potency whereas a change in lot of a key assay ingredient may result in a sudden potency shift.

In both cases it is important to identify the cause and correct it as soon as possible.

Figure 10 illustrates an *assay with stable potency, but in June the assay variability increased.* The moving MSR was stable around 3, but after June increased to over 10, and remained there. This also is most likely caused by a change in the assay process around that time. Again it is important to identify and correct the cause as soon as possible. Note however that a single outlier will cause the MSR chart to increase for the next 6 runs, and so it usually takes more time to correctly distinguish a change in assay variability from a single outlier result.

Retrospective Studies

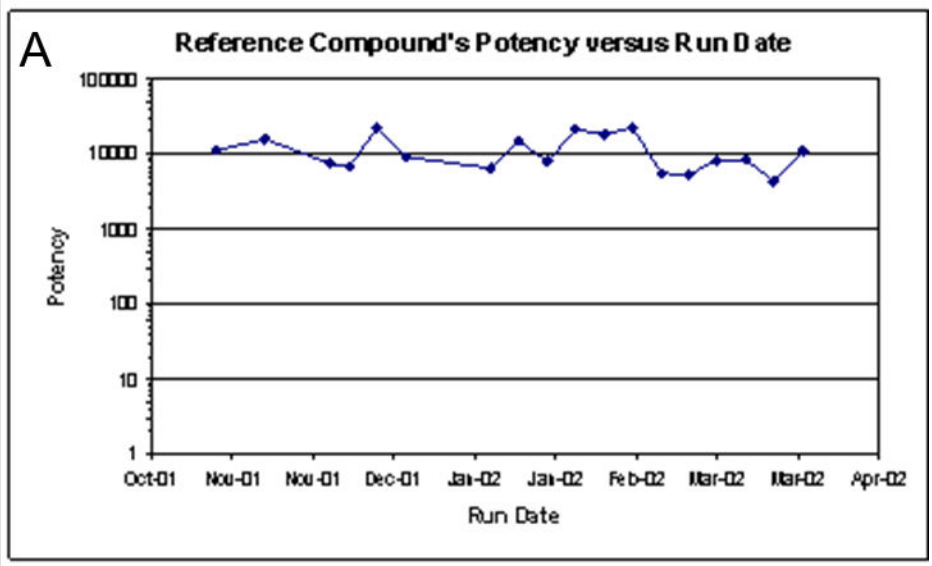
During the course of project/program development numerous compounds are repeatedly evaluated and stored in archival databases. This data can be mined to examine the reproducibility of assay results. This work should always be done by a statistician as the repeated compounds are not a random selection of all compounds, and may be biased with respect to time of evaluation, potency, structure and "assayability" (the latter term is meant to reflect conditions such as solubility, quenching, stickiness to plastic and other practical problems). In spite of these potential problems retrospective studies can be a very useful exercise, particularly in establishing the acceptability of older assays that have never been formally evaluated for reproducibility. In addition, the MSR can be examined over various subsets such as potency range, structure and run date to check that the control compound MSR's are representative of the test compounds with respect to potency range, structure and run date.

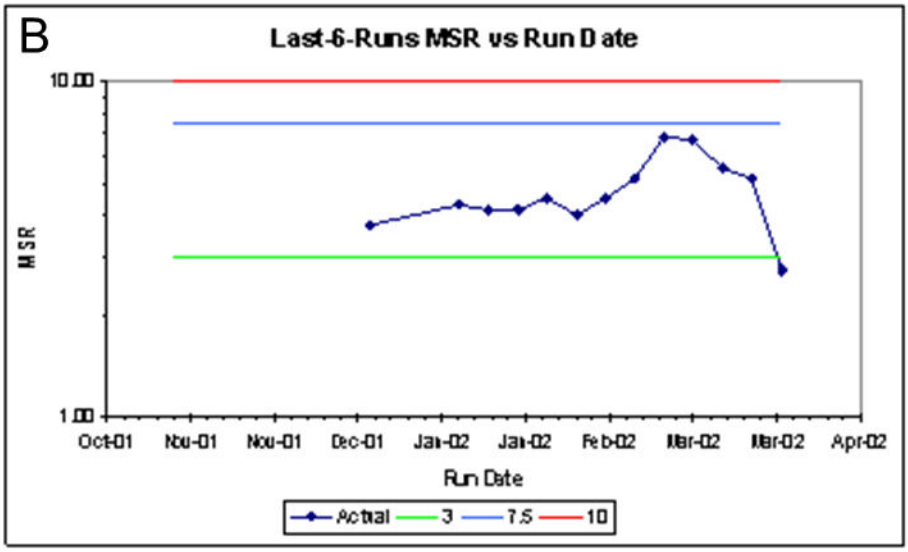
Bridging Studies

If a key aspect of an assay changes, such as an equipment change or lot of a reagent, then a test-retest study should be conducted to verify equivalence of the two protocols. A judgment should be made on a case-by-case basis of whether the full protocol outlined in the HTS Assay Validation chapter needs to be made, or only a single run under old and new conditions (i.e. one might do just Step 4 of the procedure, or one might do both Steps 3 and 4 depending upon the severity of the protocol change). Also in cases of specific modifications such as replacing equipment for a particular step in the assay an experiment can be designed to validate that the replacement is equivalent to the original in the conduct of that step of the assay.

Dimethylsulfoxide: biological compatibility and compound storage.

Dimethylsulfoxide (DMSO) is a universal solvent for all compounds tested in high, medium and low throughput screens (HTS, MTS and LTS). Compounds are initially dissolved in 100% DMSO and further diluted in 100% DMSO screening and IC_{50} or K_i determinations. So manyassays may require an additional dilution step in water or assay buffer to reduce the DMSO concentration to a level that is acceptable for the assay, depending upon the specific capabilities of the equipment being used. It is extremely important that the DMSO compatibility of biological reagents such as enzymes, receptors, protein/peptide reagents and cells be established to ensure that the screening assays are not adversely affected. In general, the final DMSO concentrations in cell-based assays are <0.2% and are <1% in biochemical assays. It is highly recommended that the tolerable DMSO concentration be determined individually for each validated assay.



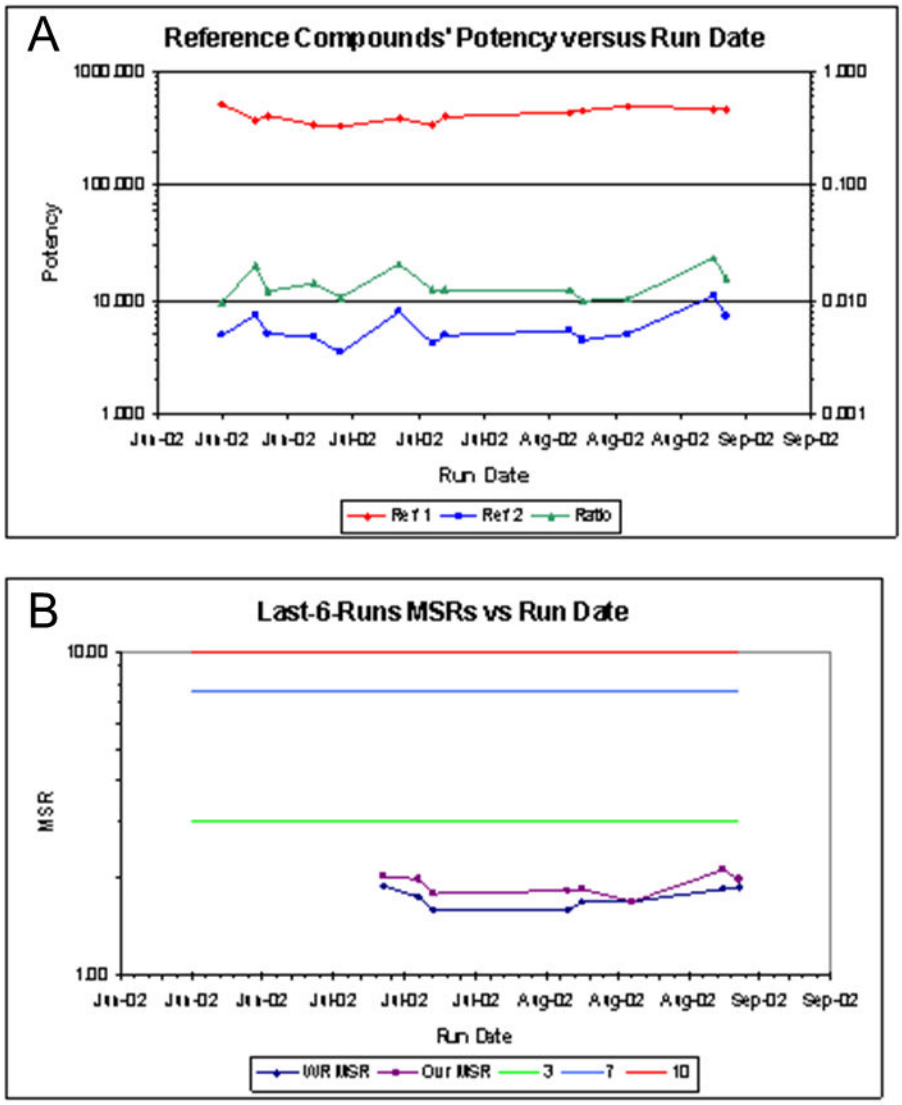


Mean Summary				MSR Summary	
High	22630.00			High	6.8
Low	4420.00			Low	2.7
Overall	10172.76			Overall	4.4
Current	11250.00			Current	2.7

Figure 6: Potency, MSR chart, and summary statistics for an assay with one control.

DMSO is also used as a cryoprotectant in the freezing of cell cultures at ATCC. The product is cell culture grade and has been tested to ensure cell viability. Each lot is also tested for the absence of bacteria, fungi, and endotoxin.

When solubilized compounds are stored in DMSO, it is important to understand the stability of these compounds under various storage conditions and freeze-thaw cycles. A detailed study of these effects was published recently (1). It is believed that the degradation of DMSO solubilized compounds is mainly due to moisture absorbed from the air. This can happen during frequent freeze-thaw cycles of compounds stored frozen in DMSO, or frequent exposure to air during repeated access for biological testing (cherry-picking).



		Mean Summary				MSR Summary	
	Ref 1	Ref 2	Ratio			WR	Overall
High	505.26	10.84	0.02		High	1.9	2.1
Low	324.19	3.42	0.01		Low	1.6	1.7
Overall	409.51	5.53	0.01		Overall	1.8	2.0
Current	462.99	7.21	0.02		Current	1.9	2.0

Figure 7: Potency, MSR chart, and summary statistics for an assay with two controls.

Recommended storage conditions for DMSO solubilized compounds:

- Polypropylene plates.
- Storage temperature: 10 degree C or room temperature.
- Inert gas atmosphere: argon flush.
- Minimal exposure to moist environments

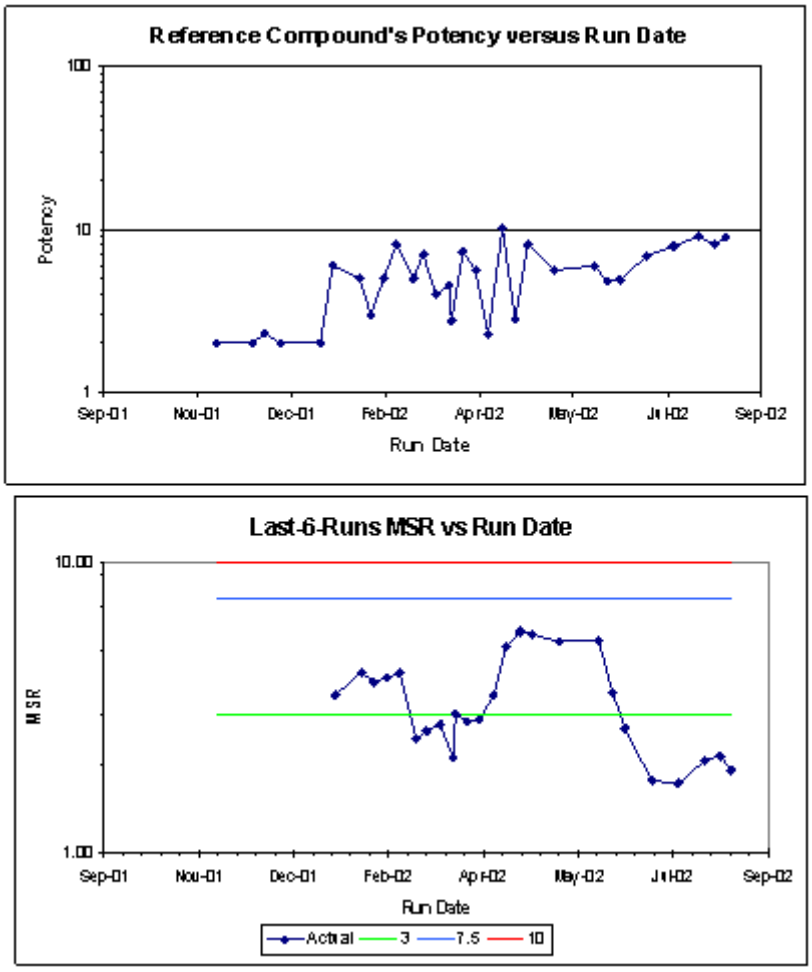


Figure 8: Potency and MSR chart illustrating assay drift.

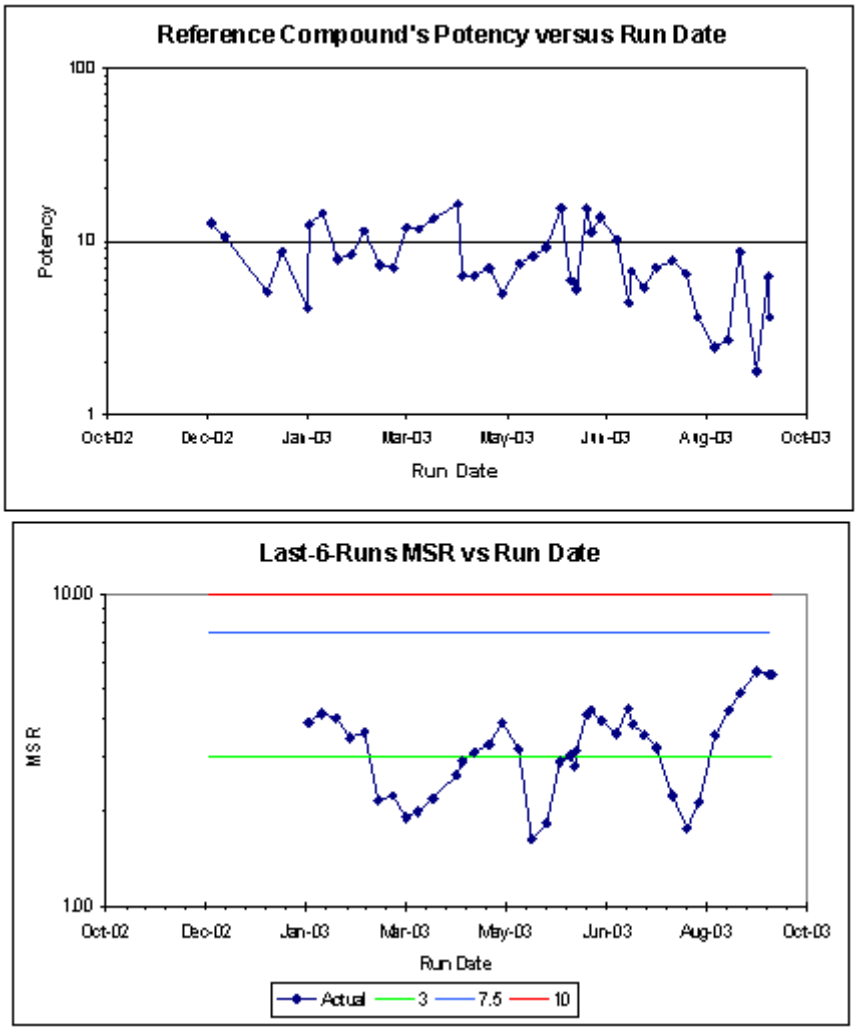


Figure 9: Potency and MSR chart illustrating sudden change in potency.

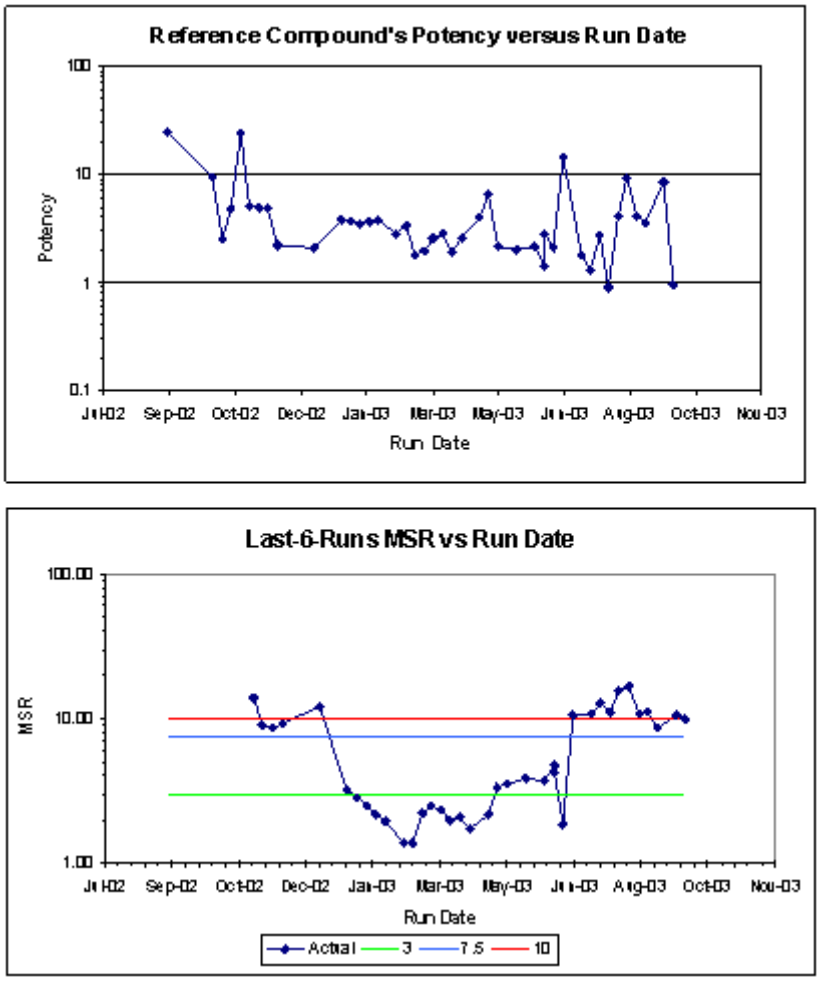


Figure 10: Potency and MSR chart for change in assay variability.

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2. Kozikowski BA, Burt TM, Tirey DA, Williams LE. Kuzmak, BR, Stanton, DT, Morand, KL, and Nelson, SL The Effect of Room-Temperature Storage on the Stability of Compounds in DMSO. J Biomol Screen. 2003;8:205–209. PubMed PMID: 12844442.

Minimum Significant Ratio – A Statistic to Assess Assay Variability

Joseph V. Haas, MS,[1] Brian J. Eastwood, PhD,[2] Philip W. Iversen, PhD,[3] Viswanath Devanarayan, PhD,[4] and Jeffrey R. Weidner, PhD[5]

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Abstract

The MSR is a statistical parameter that characterizes the reproducibility of potency estimates from *in vitro* concentration-response assays. Biological activity expressed as potency of compounds is an important parameter in screening and drug discovery. Standard potency estimates are expressed as IC_{50}, EC_{50} or simply as AC_{50} values derived from concentration-response (CRC) assays designed to measure activation, inhibition or modulation of targets and pathways of pharmacological significance. Concepts of Minimum Significant Ratio (MSR) discussed below address how the reproducibility of potency values is derived quantitatively using standard or control compounds of known activity and test compounds that have been tested in multiple runs. MSR values inform the scientists the extent of reliability of assays designed to support new compound selection through Structure-Activity Relationships (SAR) of chemical libraries.

1. Introduction

The chapter on HTS Assay Validation (Section 4) provides a detailed discussion of the Minimum Significant Ratio (MSR) in the context of the Replicate-Experiment study. The MSR is a statistical parameter that characterizes the reproducibility of potency estimates from *in vitro* concentration-response (CRC) assays and can be estimated from the Replicate-Experiment study (1). The chapter on Assay Operations for SAR Support also discusses the MSR (Section B) in two contexts: running control (reference) compounds and retrospective studies of compounds that have repeat evaluations that accumulate as part of the normal SAR process. This chapter provides additional detail on the calculation and interpretation of the MSR and further demonstrates the value of this parameter to characterize the reliability of an *in vitro* assay.

2. Common Types of MSR

2.1. Overview

Potency measurements from concentration-response assays are usually log-normally distributed and as such, $\log_{10}AC_{50}$ (log concentration at half maximal activity) is preferred for statistical analysis and modeling, including estimation of the MSR. *The MSR is defined as the smallest ratio between the potencies of two compounds that is statistically significant* and is calculated as $MSR = 10^{2\sqrt{2}s}$, where s is an estimate of the standard deviation of a log potency for one compound. The variability estimate s can be estimated in different ways depending on available data and associated analysis method (e.g., within-run, between-run, and other sources of variability). The common ways to estimate assay variability are detailed below.

2.2. Replicate Experiment MSR

As detailed in the chapter on HTS Assay Variability (Section 4), the Replicate-Experiment MSR is a diagnostic and decision tool used to establish that an assay is ready to go into production. It is estimated from two

Author Affiliations: 1 Eli Lilly and Company; Email: haas_joseph_v@lilly.com. 2 Eli Lilly and Company; Email: eastwood_brian_j@lilly.com. 3 Eli Lilly and Company; Email: iversen_philip_w@lilly.com. 4 Charles River Laboratories; Email: Viswanath.Devanarayan@crl.com. 5 AbbVie; Email: jeffrey.weidner@abbvie.com.

independent runs of 20-30 compounds and is calculated as MSR = 10^{2Sd}, where s_d is the standard deviation of the paired differences in log potency across the two runs. A template for the Replicate-Experiment data analysis is available for download from the online eBook.

Two runs are not considered adequate to estimate between-run variability so the Replicate-Experiment MSR focuses only on within-run variability. Use of the standard deviation of the paired differences factors out the between-run variability. The chapter also specifies the Limits of Agreement (LsA) as an assessment of between-run reproducibility, but is necessarily a preliminary assessment as it is based on only two runs.

Variability due to reagents is limited and sample management variability is not typically represented in the Replicate-Experiment, so the resulting MSR is considered to be the most optimistic estimate of the reproducibility of an assay. The Replicate-Experiment MSR should be < 3 to move an assay into production.

2.3. Control Compound MSR

As discussed in the chapter on Assay Operations for SAR Support (Section B), concentration-response assays should include a control compound in every run for process monitoring over time. This enables prospective identification and correction of problems (e.g., drifts or shifts in the AC_{50}) as well as estimation of assay reproducibility via the MSR and determination of whether the MSR is stable over time. A minimum of six runs is considered adequate to estimate between-run variability, and the Control Compound MSR can be calculated from six or more runs as MSR = $10^{2\sqrt{2}s}$, where s is the standard deviation of the $\log_{10}AC_{50}$ values across runs, assuming one AC_{50} result per run. The attached template (available from the online eBook) can be used to generate this with MSR values calculated from the last 6 runs.

While the Control Compound MSR captures between-run variability, compounds chosen for QC monitoring are typically the most potent compound available to a project team and are generally well-behaved. Additionally, common sources of sample variation may not be represented. As such, while the Control Compound MSR is more representative of the reproducibility of an assay due to inclusion of between-run variability, it is still somewhat optimistic because it is based on a single well-behaved compound. Given the < 3 criteria for the Replicate-Experiment MSR, it is not unusual to observe a Control Compound MSR as high as 4, depending on the level of between-run variability.

If a control compound is independently repeated in each run (e.g., on each plate), within-run variability can also be estimated and incorporated into the calculation of the MSR in addition to between-run variability. The MSR is calculated as $10^{2\sqrt{2}s}$, but s is the square root of the sum of the run date and residual variance components from the fit of a random effects model (2).

2.4. Database MSR

Retrospective studies are also discussed in the chapter on Assay Operations for SAR Support (Section B). These studies make use of potency results for compounds with repeat evaluations in multiple runs, including the control compound, to estimate the reproducibility of an assay. It is likely that numerous repeat evaluations will accumulate during the course of a project with results stored in databases for ready access to the data for estimation of the MSR.

The Database MSR captures both within-run and between-run variability from multiple compounds, and is considered the most representative estimate of assay reproducibility (subject to the caveats below). A minimum of six runs is desired. The MSR is calculated as MSR = $10^{2\sqrt{2}s}$, where s is the square root of the sum of the run date and residual variance components from the fit of a mixed effects model with compound as a fixed effect and run date as a random effect (2).

It is important to note that the database MSR reflects variability from all parts of the compound testing process including, but not limited to:

- Compound synthesis
- Compound handling
- Assay preparation
- Assay operation
- Data analysis

As such, Database MSRs can be in the 4-5 range even when an assay had a Replicate-Experiment MSR < 3. When this occurs, it does not necessarily imply there is a problem with the assay itself. There are many potential sources of variability being captured by a single number – differences in structural classes, properties of individual compounds (solubility, quenching, stickiness to plastic, etc.), temporal effects, etc. Diagnostic work is often needed, and the Database MSR can be calculated and compared over various subsets, such as potency range, structural class, time periods, etc. Database MSRs can also be calculated with and without the control compound to determine how well the control compound represents the test compounds. Plots of residuals from a one-way analysis of variance (ANOVA) with compound as the fixed effect (2) are also effective tools for identifying possible causes of an elevated Database MSR. For example, plotting residuals versus run date can be useful for identifying temporal effects.

2.5. Robust Database MSR

2.5.1. Overview

As mentioned in Section 2.4, various compound specific problems (solubility, quenching, stickiness to plastic, etc.) can contribute to assay variability and/or produce extreme values. Extreme values can also arise due to errors in the compound testing process (compound handling, assay preparation, assay operation, etc.). A high occurrence of such outliers is not typically expected, but some number will undoubtedly arise over the course of a project where large numbers of compounds are tested. Although the mixed model described in Section 2.4 is the preferred method for calculating a Database MSR, even a small number of outliers can inflate the variability estimates and artificially inflate the MSR.

2.5.2. Diagnostic Plots and Exclusion of Data

One approach to dealing with outliers and high variability for individual compounds is to identify them with diagnostic plots and exclude them from the calculation of the MSR. Figure 1 is a plot of residuals versus run date for compounds with repeat evaluations in multiple runs of an assay. The residuals are from a one-way analysis of variance (ANOVA) of the $\log_{10}AC_{50}$s with compound as the fixed effect (2). Five compounds are identified in Figure 1 as having large residuals. Figure 1 also identifies some unusual runs where all the residuals lie above or below the 0 reference line. Normally you should expect a random scatter about this line. The Database MSR = 10.0 with all data included compared to 5.8 without the five compounds identified in the residual plot. See Section 2.5.5 for additional diagnostic plots for this assay.

2.5.3. Robust Estimates of Variability

Another approach to dealing with outliers and high variability for individual compounds is to use variability estimates that are less sensitive to the presence of extreme values. At the simplest level, estimating assay variability amounts to calculating the standard deviation of log potencies from compound repeats. Let $\boldsymbol{X_i}$ represent repeat AC_{50}s for a control compound

$$SD=\sqrt{\frac{\sum(x_i-mean(x_i))^2}{n-1}} \quad MSR=10^{(2\sqrt{2}SD)}$$

Even a small number of extreme values can artificially increase or decrease the mean, and to an even greater extent artificially increase the SD due to squared term. The median absolute difference is a robust estimator of variability that is less sensitive to extreme values (3).

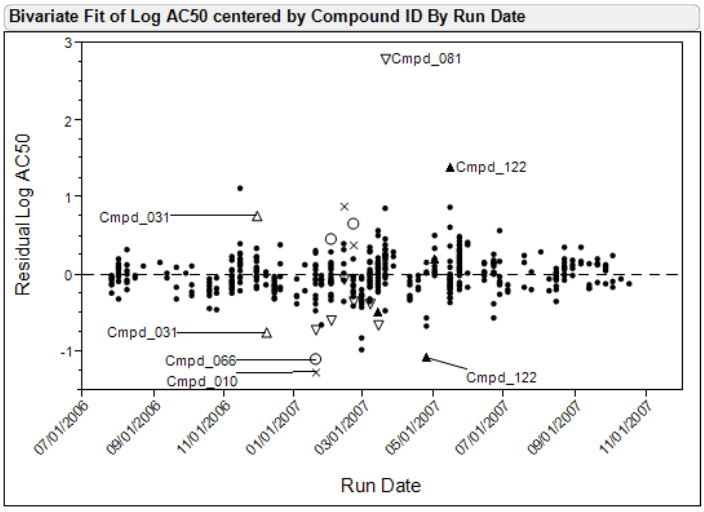


Figure 1: Compound $Log_{10}AC_{50}$ Residuals vs. Run Date

$$MAD = median(\ |x_i - median(x_i)|\ ) \quad MSR = 10^{(2\sqrt{2}(MAD/0.6745))}$$

The MAD makes use of the median rather than the mean, which is less sensitive to outliers, and uses the absolute value instead of the square. These differences make the MAD more robust to outliers compared to the standard deviation. The 0.6745 scale factor is needed for the MAD to be consistent estimator of the standard deviation under the normal distribution.

The median and the MAD can also be used to calculate a Robust Database MSR as an alternative to the Database MSR calculated via the mixed model in Section 2.4. The resulting MSR is robust to the presence of a small number of outliers and provides a more accurate representation of the reproducibility of an assay. For the example assay in Section 2.5.2, the Robust Database MSR = 5.9, which is very similar to the Database MSR of 5.8 calculated without the five compounds with large residuals.

2.5.4. Calculation of the Robust Database MSR

There are many robust methods that could potentially be used to calculate a Robust Database MSR. One obvious approach is M-estimation (3) applied to the mixed model fit described in Section 2.4. M-estimation involves using iteratively reweighted least squares (IRLS) with a weighting function (e.g., Tukey's bi-square) applied to the mixed model residuals at each iteration. The weighted residuals of iterations are used as weights in the next iteration until the process converges. This has the effect of down-weighting potencies with large residuals and results in variability estimates (and hence MSRs) that are not artificially inflated by outliers.

M-estimation in the context of a mixed model is computationally intensive and can have convergence issues due to the presence of both fixed and random effects in the model. To avoid these issues, especially in an industrial setting where there are assays with large numbers of repeat evaluations and/or where there are large numbers of assays, a method of moments approach using medians and MADs is recommended for the Robust Database MSR.

The small sample properties of the resulting Robust Database MSR dictate a minimum of 100 observations for adequate performance. The 100 or more observations can be repeat evaluations of a single control compound across ≥ 100 runs or a combination of within- and between-run repeats of the compound across at least six runs to generate a Robust Control Compound MSR. The Robust Database MSR is calculated by adding test compounds with repeat evaluations in at least two runs, including within-run repeats where available.

For a CRC assay with 100 or more observations from compounds with two or more AC_{50}s across at least six or more runs, the Robust Database MSR is calculated as below.

Steps 1-11 are performed on each compound, step 12 is the summary across compounds, and step 13 is the Robust Database MSR calculation.

1. Compute within-run $Median_{(W\text{-}R)i}$ as median of $logAC_{50i}$ for each run i. If a run has no within-run repeats, $Median_{(W\text{-}R)i}$ is the single $logAC_{50}$ for that run.
2. Compute $Median_{All}$ as the median of all $logAC_{50}$s for a given compound
3. Compute between-run $MAD^2_{B\text{-}R}$ as $(median(|Median_{(W\text{-}R)i} - Median_{All}|) / 0.5725)^2$
4. Compute within-run $MAD^2_{(W\text{-}R)i}$ for each run as $(median(|logAC_{50i} – Median_{(W\text{-}R)i}|) / 0.5725)^2$
5. Compute $N_{(W\text{-}R)i}$ as # of within-run repeats for each run
6. Compute within-run $MAD^2_{W\text{-}R}$ as $\Sigma_i((N_{(W\text{-}R)i} - 1) \times MAD^2_{(W\text{-}R)i}) / \Sigma_i(N_{(W\text{-}R)i} - 1)$
7. Compute $N^2_{(W\text{-}R)i}$ as # of within-run repeats squared for each run
8. Compute N_{Run} as # of runs for a given compound
9. For each compound, compute N_0 as $(\Sigma_i(N_{(W\text{-}R)i} - 1) - \Sigma_i N^2_{(W\text{-}R)i} / \Sigma_i(N_{(W\text{-}R)i} - 1)) / (N_{Run} - 1)$
10. For compounds without within-run repeats, compute $RobustVar_{Cmpd}$ as $N_0 \times MAD^2_{B\text{-}R}$
11. Otherwise, compute $RobustVar_{Cmpd}$ as $MAD^2_{W\text{-}R} + ((N_0 \times MAD^2_{B\text{-}R}) - MAD^2_{W\text{-}R}) / N_0$
12. Compute RobustVar across all compounds as the median of the pair-wise averages (i.e., Walsh Averages) of $RobustVar_{Cmpd}$ as described in (4)
13. Compute the Robust Database MSR as $10^{2\sqrt{2}s}$, where s is the square root of RobustVar

The calculation of the Robust Database MSR is somewhat complicated. An alternative when Steps 1-13 is prohibitive is a one-way ANOVA with compound as the fixed effect (2). The same MSR formula (Step 13) is used, but s is the root mean square error from the one-way ANOVA. The diagnostic plots discussed in Section 2.5.2 would also be needed to identify and exclude outliers from the calculation of the MSR.

2.5.5. Automated Calculation of the Robust Database MSR

Due to lack of convergence issues and fast computational time, the Robust Database MSR described in Section 2.5.4 is amenable to automation. The Robust Database MSR can be calculated for all assays publishing to a database and regularly re-calculated (e.g., weekly or even nightly) as assays publish new potency results to the database. This provides consumers of *in vitro* potency data an up-to-date estimate of the reliability of an assay.

What follows are figures and tables from an implementation of the automated Database MSR for the sample assay discussed in Section 2.5.2. The Robust Database MSR is calculated as described in Section 2.5.4, and the Non-Robust Database MSR is calculated by method of moments. Method of moments was chosen over the preferred mixed model approach for the Non-Robust Database MSR for computational speed and consistency with the robust method of moments approach. For balanced datasets, the method of moments approach is equivalent to the mixed model and is a reasonable approximation to the mixed model for the unbalanced datasets that typically arise over the course of a project.

Figure 2 displays AC_{50}s for the twelve compounds with the most repeat evaluations for an assay, including the control compound in the lower left panel. This is intended as a quick visual assessment of reproducibility of potency for the compounds with the most repeat evaluations.

Figure 3 is a plot of compound $log_{10}AC_{50}$s in order of decreasing potency for all compounds with repeat evaluations. The compounds identified as having extreme residuals in Section 2.5.2 are also marked in the plot. The entire collection of repeat evaluations for this assay consists of 510 evaluations of 182 compounds in 55 runs. The non-robust MSR = 10.6 and the robust MSR = 5.9. Note that the robust MSR is considerably smaller than the non-robust MSR, as the former is less sensitive to the extreme values seen in Figure 3.

Figure 4 is a plot of compound $\log_{10}AC_{50}$s in order of decreasing potency for the last twelve months of repeat evaluations, which consists of 457 evaluations of 165 compounds in 44 runs. The non-robust MSR = 11.6 and the robust MSR = 6.01 with the robust MSR considerably smaller due to being less sensitive to the extreme values.

Several diagnostic plots can also be included in the Automated Database MSR output. Figure 5 is a plot of the Non-Robust (red line) and Robust (black line) Database MSRs in twelve-month periods (only one in this particular example) that is useful for assessing the stability of the MSR over time. Numbers of observations are captured as tick marks on the x-axis. Figures 6 & 7 plot the residuals from a one-way ANOVA with compound as the fixed effect versus compound and run date respectively. The plot of residuals versus compound shows that the variability in $\log_{10}AC_{50}$ can be different for different compounds. The plot of residuals versus run date can identify unusual runs as noted in Section 2.5.2.

3. Other Considerations

3.1. Precision of Replicate AC_{50}s for Individual Compounds

The MSR estimates the overall reproducibility of AC_{50}s for an assay, but not the precision of specific compound AC_{50} estimates. When repeat evaluations are available for a compound, it is appropriate to report the results for the compound as the geometric mean of the replicate AC_{50}s ($10^{MeanLog}$, where MeanLog is the arithmetic mean of the $\log_{10}AC_{50}$s) and 95% confidence interval for the mean (10^{LCL} to 10^{UCL}, where LCL and UCL are lower and upper confidence limits of the 95% CI for the arithmetic mean of $\log_{10}AC_{50}$s). The geometric mean is appropriate for the log-normally distributed AC_{50}s and the confidence interval is the range within which the mean compound potency is expected to lie. The width of the CI (upper limit divided by lower limit) depends on the number of replicates and underlying assay variability, and the CI can be wide if the number of replicates is small. It is also appropriate to report the geometric mean potency and standard error of the mean, where the latter is calculated as SEM = (geometric mean of AC_{50}s) x (SE of $\log_{10}AC_{50}$s) x ($\log_e 10$). The relative standard error is also a useful estimate of the precision of replicate AC_{50}s and is calculated as (SE of $\log_{10}AC_{50}$s) x ($\log_e 10$).

3.2. Value of Additional Replication

As mentioned previously, an adequate number of repeat evaluations can be expected to accrue over the course of a typical project. Even so, the vast majority of compounds are typically only tested once in an assay as shown in the histogram of compound replicates in Figure 8, which is based on actual experience in a large pharmaceutical company. As such, project teams may also elect to repeatedly evaluate a small set of compounds at regular intervals to increase confidence in the Database MSR estimate and provide additional process monitoring over time beyond what is provided by including a control compound in every run.

A project team may also elect to institute repeat testing of all important compounds (e.g., repeat all compounds in the lead optimization phase n=3 times) when the Database MSR is large (i.e., approaching or exceeding 5). In this paradigm, geometric mean potencies are compared between compounds which has the effect of reducing the MSR, since the MSR for the comparison of means is MSR = $10^{2\sqrt{2}(s/\sqrt{n})}$, where n is the number of times each compound has been tested (assumed to be the same across compounds). The preferred approach to dealing with a highly variable assay is to thoroughly investigate potential sources of variability and eliminate them, but depending on assay costs, replication can be an effective way to manage variability.

3.3. Effect of Compound Lot on Database MSR

Although a single lot represents most compounds in a typical project, important compounds will likely require the synthesis of multiple lots. Multiple lots are presumed to be thoroughly characterized by chemists to ensure equivalence between lots, so multiple lots should not be expected to have substantively different potencies.

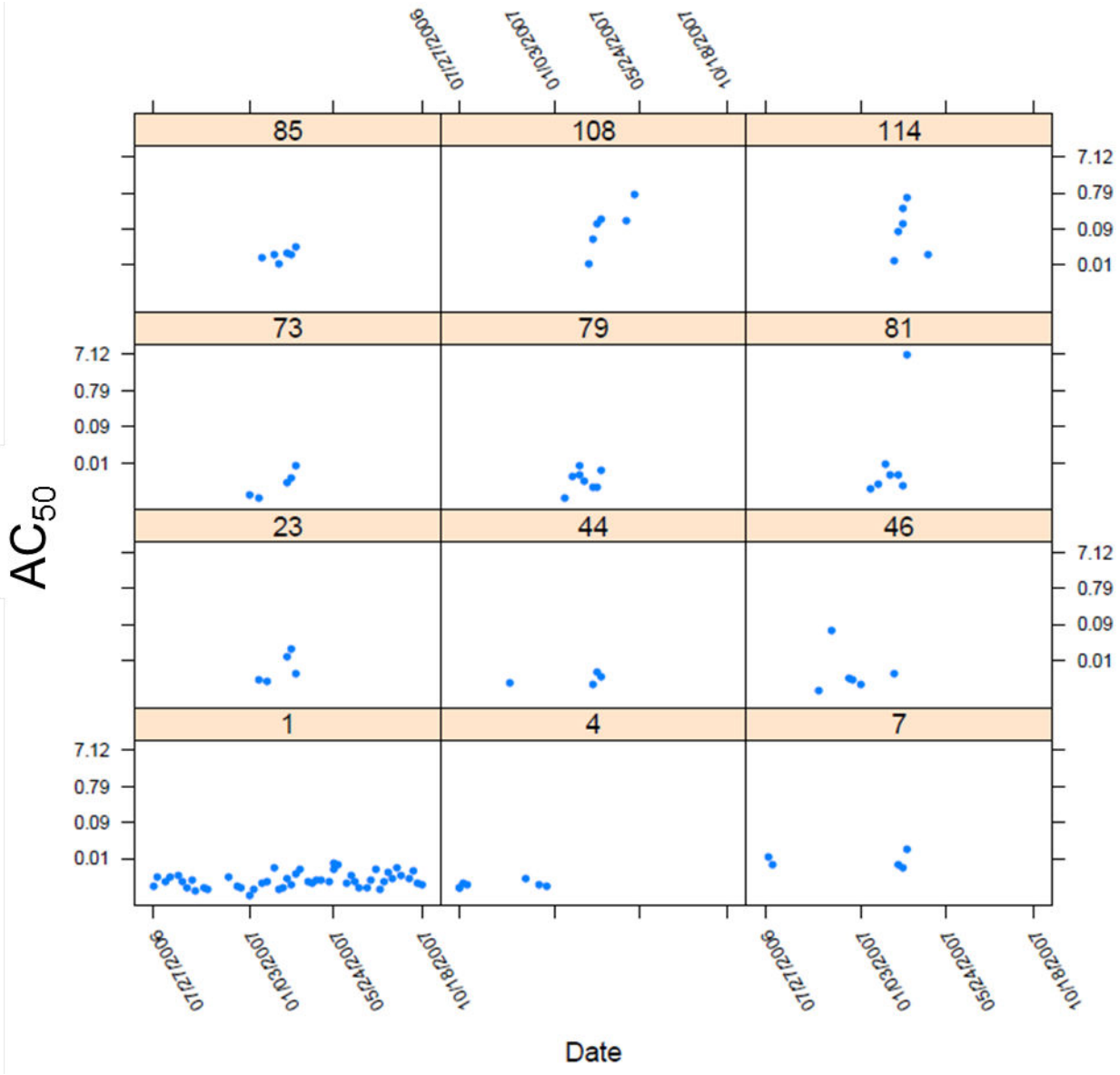


Figure 2: Twelve Compounds with the Most Repeat Evaluations

Figure 9 compare MSRs – with and without accounting for sample lot – of 15 assays. The results indicate no substantive differences in calculated MSRs. Note also that the MSR sample size can increase or decrease based on distribution of lots for multiple lot compounds.

While multiple lots are not typically expected to be substantively different, the potential for large impact is always there, so it should at least be considered for assays with large MSR.

4. Conclusion

The MSR is a statistical parameter that characterizes the reproducibility of potency estimates from *in vitro* concentration-response assays. It is also a valuable tool for assessing the reliability of an *in vitro* assay throughout its life cycle. The Replicate-Experiment MSR is a diagnostic and decision tool that establishes an assay's readiness to go into production. Once an assay is in production, the Control Compound MSR enables prospective identification and correction of problems (e.g., drifts or shifts in potency), as well as assessment of the stability of the MSR over time. The Database MSR is considered the most representative estimate of assay reproducibility

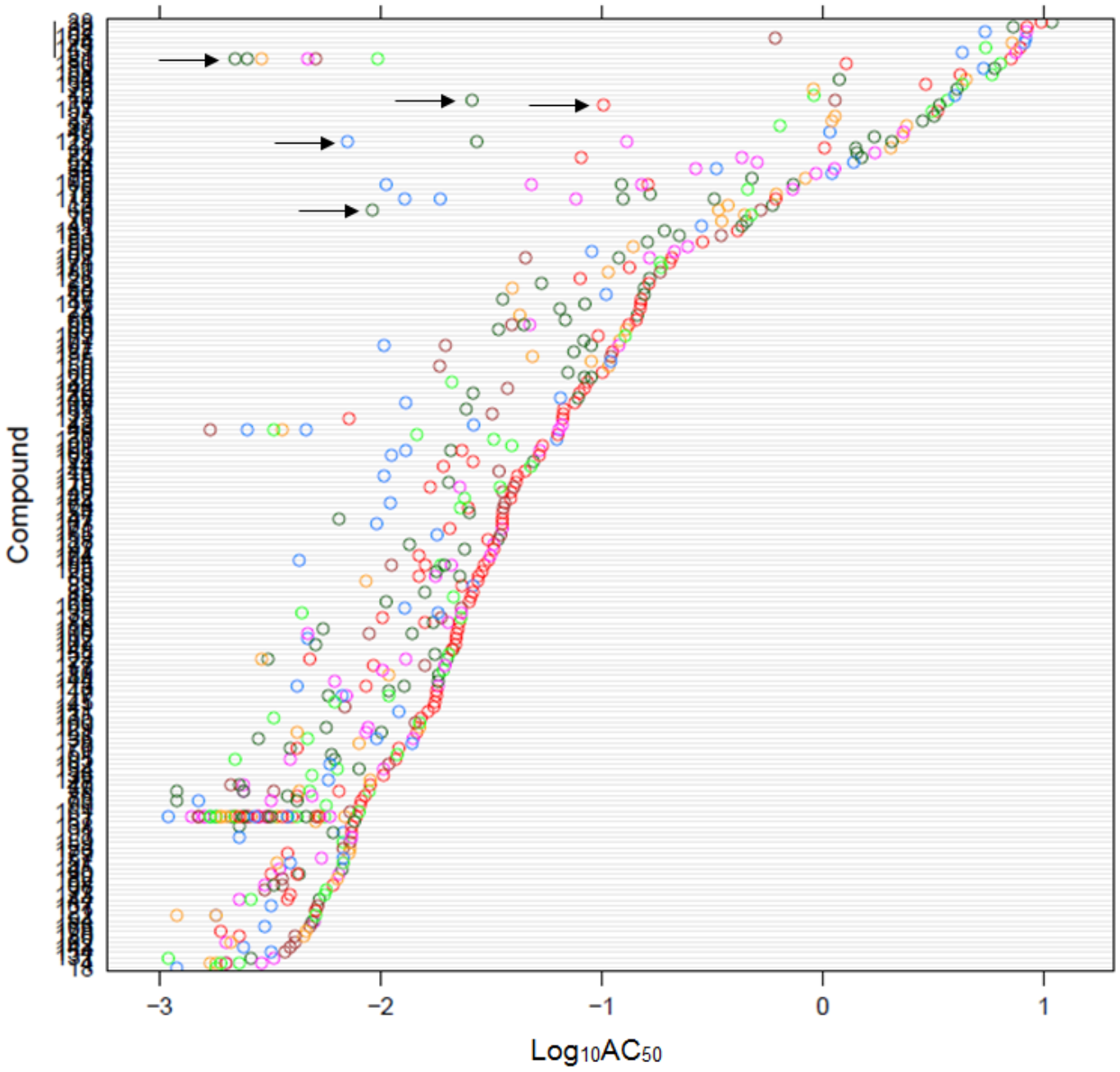


Figure 3: Compound $Log_{10}AC_{50}$s in Order of Decreasing Potency for All Compounds with Repeat Evaluations

and is calculated from compounds with repeat evaluations that accumulate during the course of a project. The Database MSR can be calculated automatically for assays publishing to a database giving consumers of *in vitro* potency data up-to-date estimates of assay reliability. Outliers can arise over the course of a project for a variety of reasons and even a small number can artificially inflate the Database MSR. Diagnostic plots are needed to identify and exclude these values or a Robust Database MSR can be calculated that is less sensitive to the presence of a few extreme values.

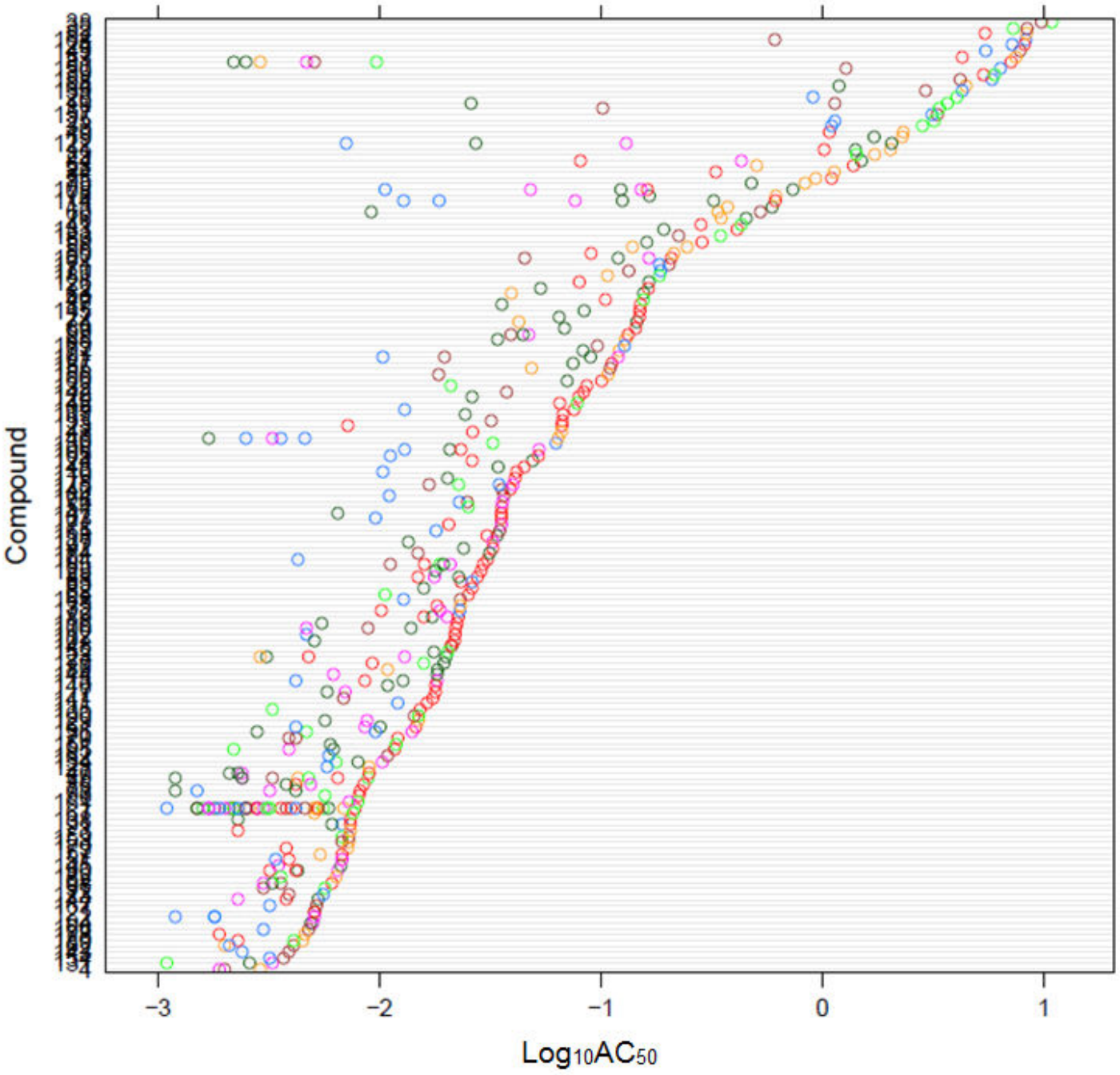


Figure 4: Compound $Log_{10}AC_{50}$s in Order of Decreasing Potency for Last Twelve Months of Repeat Evaluations

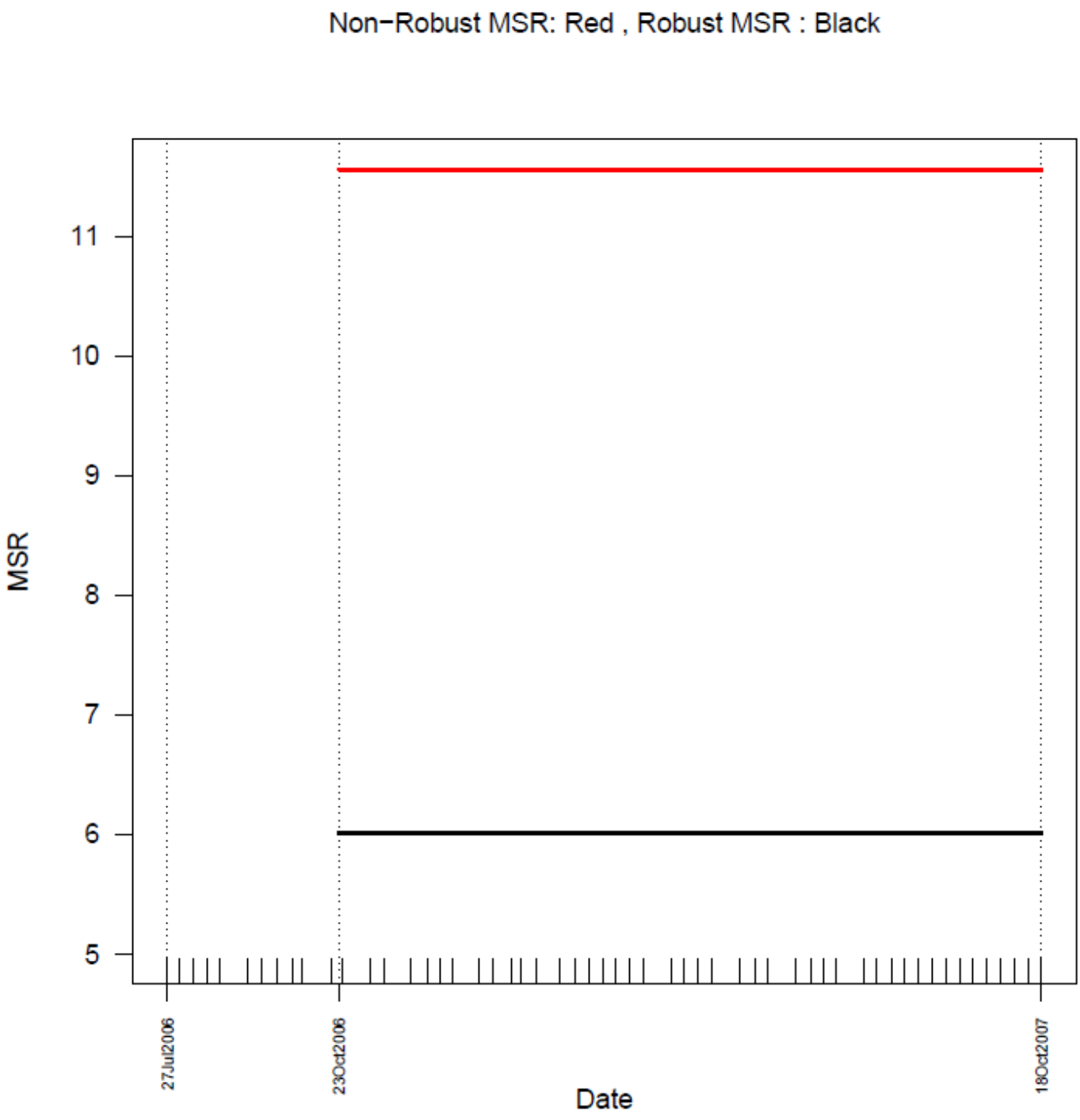


Figure 5: Non-Robust and Robust Database MSRs in Twelve Month Time Periods

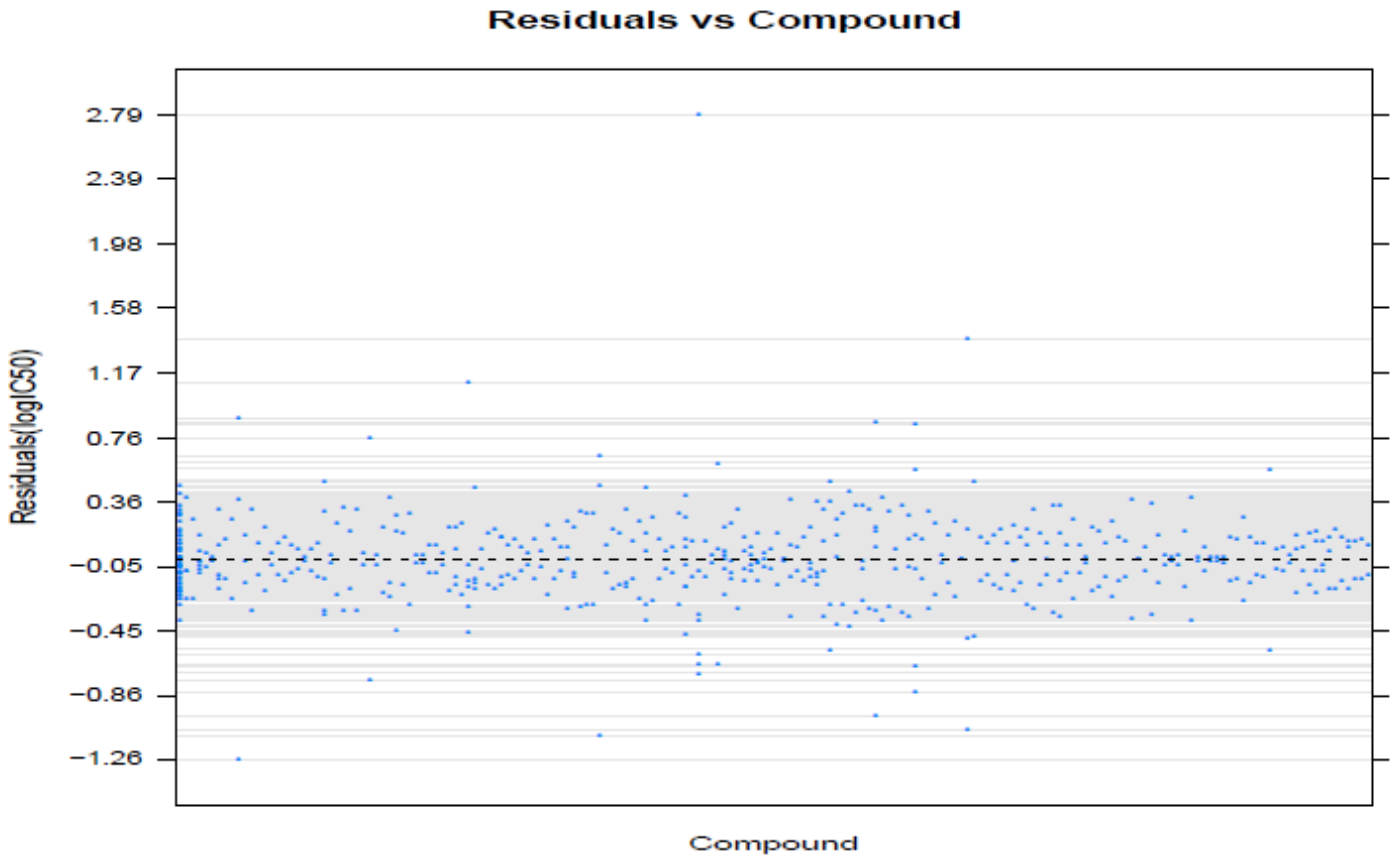


Figure 6: One-Way ANOVA Residuals vs. Compound for All Available Data

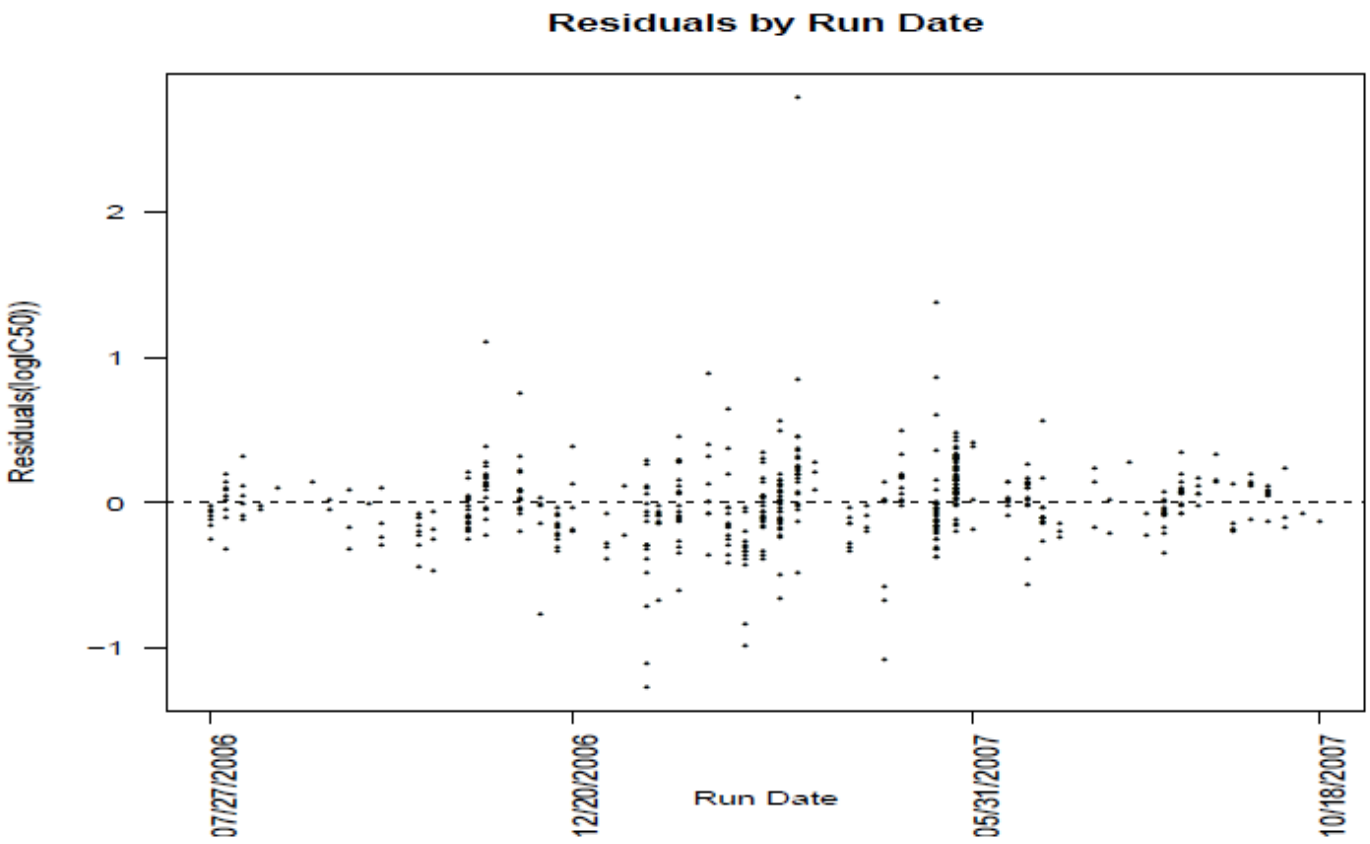


Figure 7: One-Way ANOVA Residuals vs. Run Date for All Available Data

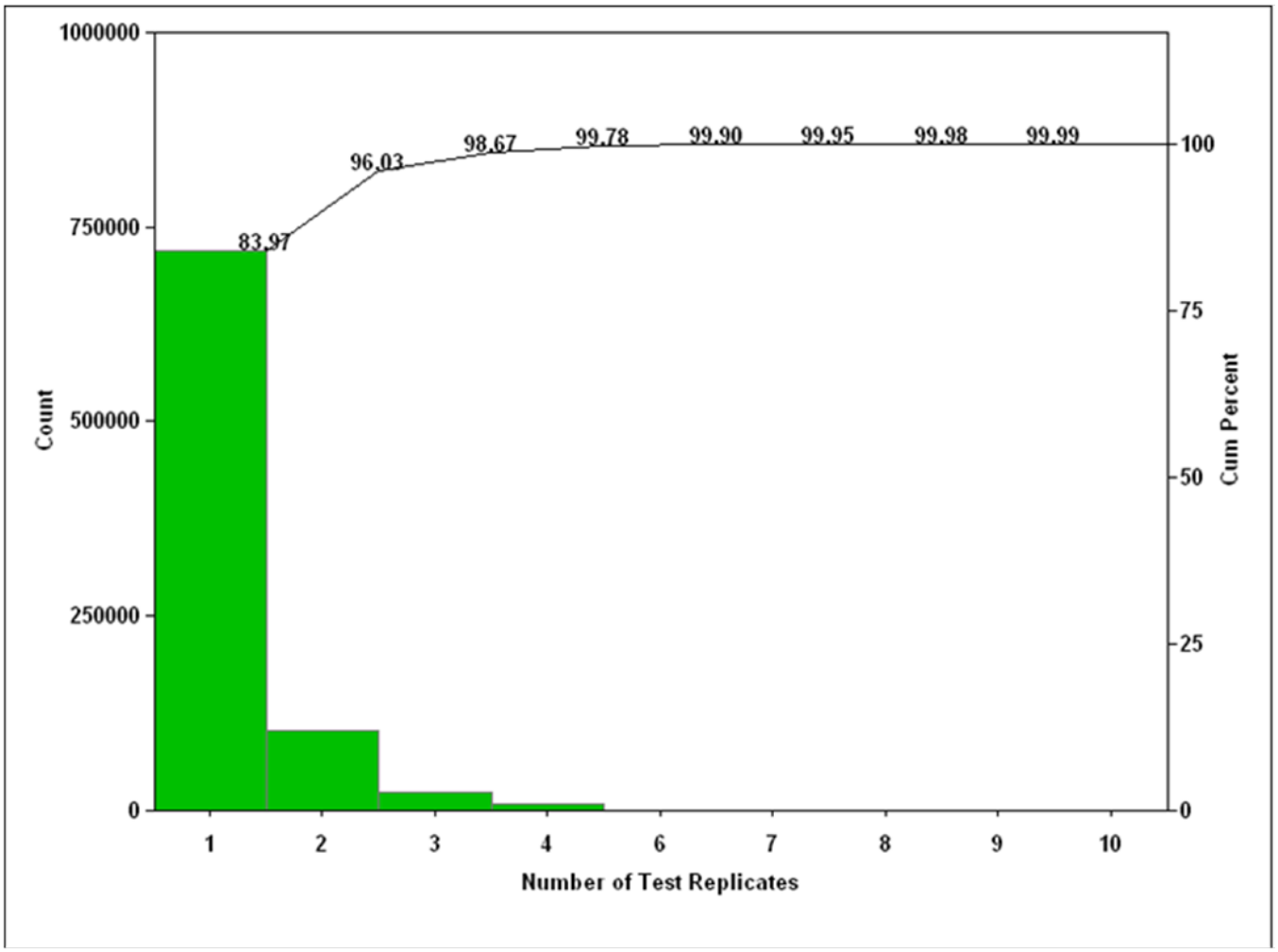


Figure 8: Data from Lots Created in 2008 and Run in CRC Assays in 2008-09

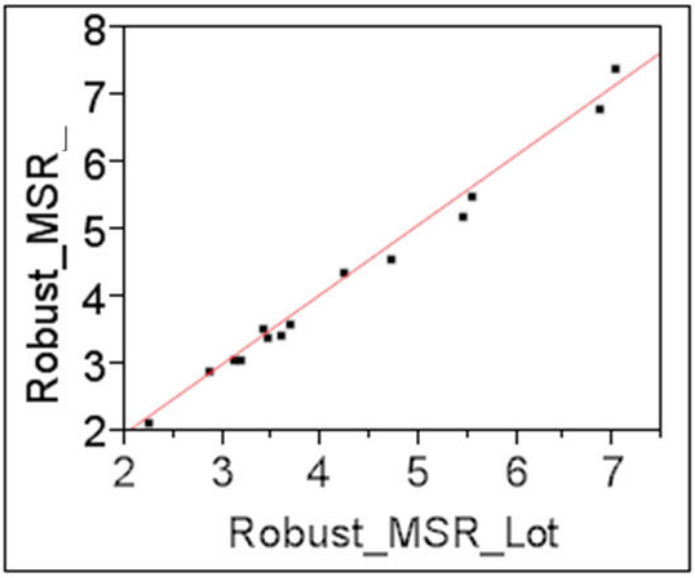


Figure 9: Robust MSR (Compound) vs. Robust MSR (Compound & Lot) for 15 Sample Assays with Line of Agreement

5. References

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Assay Technologies

Marcie Glicksman, PhD, James McGee, PhD, Owen McManus, PhD, and G. Sitta Sittampalam, PhD

HPLC-MS/MS for Hit Generation

Stefan Jon Thibodeaux, M.S.,[1,*] David A Yurek, M.S.,[2,†] and James E McGee, Ph.D.[3,‡]

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Abstract

Mass Spectrometry based screening has allowed researcher's to employ native substrates for screening, screen previously intractable targets, and eliminate the need to use coupled reactions. Recently, many instrumentation advances have been made to increase throughput, thus allowing routine access to this technology. This chapter details the different instrumental set-ups which will enable mass spectrometry based screening, and issues and solutions likely to be encountered during assay development.

Introduction

The use of LC-MS/MS as an assay platform for hit generation has enabled the development of assay methodologies that were previously difficult or impossible using conventional means of detection (2-4). These instruments are able to identify and quantify low levels of analytes in complex matrices (5). Their ability to detect unmodified natural substrates and products is also an advantage as modified substrates may impact the quality of hits (6, 7). Additionally, the near universality of detection gives LC-MS/MS a wide range of applications. Historically, LC-MS/MS detection techniques were too slow for screening applications. However, with the clever use of valves and pumps, several researchers are beginning to exploit LC-MS/MS and have implemented this strategy for routine screening (8-10). Liquid chromatography techniques encompass no chromatography (flow injection), to isocratic, and to gradient methods using short columns, and to staggered injections on multiple columns. Early versions of LC-MS/MS screening utilized Gilson's 8-probe liquid handler and staggered injection systems (8, 9). A few years ago, a company employing a unique autosampler, (RapidFire™, Agilent) was spun out of Pfizer with LC-MS/MS screening as their mainstay (10). In the past few years, other manufacturers have offered autosamplers capable of the high sampling rates required for this technique. This manual will focus on four systems which are proven to deliver the through-put necessary for hit generation for a 100,000-200,000 compound screen in a few weeks. Also, the technical details ranging from assay buffer components to data processing will discussed.

Assay Components

Ideally, only buffers and detergents which are compatible with the mass spectrometer should be used in the bio-chemical assay mixture. However, enzyme systems commonly require the presence of non-volatile buffers, proteins, and/or detergents for optimal activity. The levels to which non-volatile components can be tolerated in the bio-chemical system is directly related to the chromatographic system employed (Table 1). For example, the flow injection mode is most sensitive to ion suppression and requires the most sample clean-up or only mass spectrometry friendly components. Whereas chromatographic separation methods can tolerate less stringent sample clean up and less mass spectrometry friendly components can be employed.

Author Affiliations: 1 Eli Lilly and Company; Email: thibodeaux_stefan@lilly.com. 2 Eli Lilly and Company; Email: yurek_david_a@lilly.com. 3 Eli Lilly and Company; Email: Mcgee_james_e@lilly.com.

* Author

† Author

‡ Editor

The buffers of choice for the biochemical assay are ammonium acetate and ammonium bicarbonate as these buffers are compatible with the mass spectrometer due to their volatility. If these buffers are not compatible with the enzyme assay, then conventional buffers can be used. However, for flow injection significant sample cleanup would need to occur. Detergents represent the most troublesome assay components as these are normally retained on reverse phase resins. Typically, replacing or avoiding SDS and polymeric detergents such as TWEEN, Triton, and NP50 is the best course of action. Polymeric surfactants are mixtures of several molecules of varying polarities and masses, which interfere by eluting in a broad time window and flooding the system with a large number of ions that will greatly suppress the desired signals or cause "cross talk"(11). If these cannot be replaced with the more mass spec friendly detergents, then these should be used at the lowest effective concentration. When detergents must be used, single molecule forms are best e.g. CHAPS, octyl β-D-glucopyranoside or dodecyl β-D-glucopyranoside. Then, only one component needs to be separated from the analytes to enable detection.

Table 1: Volatile HPLC Modifiers used in LC-MS/MS

Volatile Modifiers	Abbreviation	Concentration Used	pKa	pH range	Comments
Acids					
Trifluoro-Acetic Acid	TFA	0.02-0.1%	0.3	1-2	Forms ion-pairs in gas phase, suppresses positive ionization, gives strong negative ion
Formic Acid	FA	0.1-1%	3.8	2-3	
Acetic Acid	HOAc	0.1-1%	4.8	3.8-5.8	
Propionic Acid		1-5%		2.5-4.5	Used as post-LC additive to break ion-pairing
Heptafluoro-Butyric Acid	HFBA	0.10%		1.5-3.5	Forms ion-pairs in gas phase, suppresses positive ionization, gives strong negative ion
Bicarbonate	HCO3	10-50 mM	6.3, 10.3	6.8-11.3	Prepare fresh daily
Bases					
Ammonia	NH3	10 mM	9.2	8.2-10.3	Prepare fresh daily
Triethylamine	TEA	10 mM	10.7	9.7-11.7	Prepare fresh daily
Piperidine		1-10 mM	10.0	9-11	
1-Methyl-Piperidine		1-10 mM	10.2	9.3-11.3	
Morpholine		1-10 mM	8.4	7.4-9.4	
4-Methyl-Morpholine		1-10 mM	8.4	7.4-9.4	
Pyrrolidine		1-10 mM	11.3	10.3-12.3	
Common Buffers					
Ammonium Acetate	AmOAc	10-200 mM		7.0	
Ammonium Formate	AmF	10-200 mM		6.3	
Ammonium Bicarbonate	AmHCO3	10-100 mM		8-10	Prepare fresh daily
Triethyl-Ammonium Bicarbonate	TEAB	10-100 mM		8-10	Prepare fresh daily

Mass Spectrometers

The choice of mass spectrometer will have significant impact on the ability to perform MS based screening. A triple quadrupole is the preferred instrument because it delivers the best combination of sensitivity, selectivity, scan speed, and dynamic range. A triple quadrupole instrument is usually run in multiple reaction monitoring (MRM) mode. MRM works by selecting the ion of interest in the first quadrupole, fragmenting the ion of interest in the second quadrupole, and finally selecting one of the fragments in the third quadrupole for detection by the detector. Typically, one unique product ion is selected for each parent, but more than one product ion can be selected as a confirmatory ion. Also, quadrupole-time-of-flight or orbit trap instruments are being used for screening. Quadrupole-time-of-flight or orbit trap instruments provide the ability to obtain accurate mass measurements of the ions of interest. Due to this capability a narrow mass range window can be used to obtain specificity rather than fragmenting the molecule as in a triple quadrupole instrument. This can be a significant advantage when dealing with small molecules which have poor fragmentation efficiency or do not fragment to produce a dominant ion (12). The mass range, resolution, and speed of the quadrupole-time-of-flight or orbit trap enable the use of small proteins as substrates that may generate multiple enzymatic products. It is not necessary to have standards for the products as the entire mass range is captured in the TOF scan, enabling all the products to be measured simultaneously. For example, a small protein which undergoes multiple phosphorylations could be analyzed directly to determine if inhibitors give different phosphorylation patterns. The drawbacks to the use of Q-Tof instruments for screening are the large data files produced and the limited data processing software available. However, instrument companies are activity working to remove these barriers.

The short LC columns or flow-injection used in mass spectrometry based screening places additional demands and restraints on the mass spectrometry system. The reduced chromatography used in these rapid assays typically does not separate substrate and the product, therefore; the use of unique MRM transitions or exact masses for identification and quantitation are needed. Generally, no problems are seen if the compounds are not isobaric as unique molecular ions are observed. Occasionally, cross-talk between analytes may occur if the collision cell of the MS is not sufficiently cleared between analyte transitions (seen only in older instruments), or if an analyte converts to the other analyte in the source. If analytes have the same molecular ion and the fragment selected for analyte A also appears as a fragment for analyte B, then a pure sample of A will produce a peak in B and vice versa. If A and B are separated in time, this is not a problem. However, analytes are normally not separated in these rapid assays and unique fragments are then required. This may necessitate the use of lower sensitivity fragments to assure uniqueness. Typically, sensitivity is not an issue for these screening assays. As an illustration, a major fragment at 271 is seen in the analysis of several prostaglandins that would be optimal for sensitivity; if the analyte did not co-elute with other components also producing this fragment. Selection of other less abundant fragments allows unique identification of specific prostaglandin analytes. The isomeric prostaglandins PGE2 and PGD2 may be identified by their fragmentation pattern (13). Also the 5-, 12-, and 15-HETEs are good examples of isobaric molecules which can be identified and quantitated by their fragmentation patterns even through signal intensity is lost compared to the most abundant fragment (14).

To deal with the inherently variable process of generating and measuring ions in a mass spectrometer, internal standards are used. The best internal standards are isotopically labeled analogues with a mass shift of +4 or greater. If an isotopically labeled product is not available, then a structural analog should be used. The internal standard should be added as soon as possible, (normally when the reaction is quenched) and its concentration should be half way between the maximum and minimum concentration of the product. The internal standard normalizes the analyte response to a known amount of material that has undergone identical sample handling. Therefore, once the internal standard is added, the ratio of analyte to internal standard is fixed regardless of decomposition, sample handling differences, and variances inherent in the mass spectrometry process. If the substrate and product for a reaction are close in structure and the calibration curves are parallel then the

substrate can be used as in a ratio with the product. If the substrate is completely different than the product or the product and substrate calibration curves are not parallel, then an internal standard should be used.

The high flow rates used to obtain the through-put necessary to enable an MTS campaign place increase desolvation demand on the mass spectrometer. The source temperature is usually set to the highest allowed value and the source gas flow rates are set very close to the maximum allowed. Also, the high volume of samples injected on the mass spectrometer is greater than the typical sample work load. These special circumstances necessitate a more frequent cleaning schedule compared to that typically recommended by the instrument vendor. When running 100 or more 384-well plates a week, the instrument should be cleaned every two weeks or earlier depending upon the signal degradation. This can be monitored by plotting the response of the control wells over time. When the area counts decrease twenty-five percent or more then cleaning should occur.

For typical bioanalytical assays, linearity of triple quadrupole instrument response to analyte concentration may be established over >4 orders of magnitude. For screening assays, only 2-3 orders of magnitude are needed as activity of the reaction is measured from 0-100% inhibition. It is important to monitor the analytes in the linear range of the instrument response because calculation of percent inhibition assumes a linear function. Also, the signal to noise ratio for the product should be sufficient to measure 99% inhibition of the enzyme. The response of the non-inhibited wells should therefore be at least 100-fold signal to noise.

The mass spectrometer can ionize molecules using four basic modes of ionization electrospray positive, electrospray negative, atmospheric pressure chemical ionization positive and atmospheric pressure chemical ionization negative. While any ionization mode may be used, typically, electrospray ionization is used; as most compounds will have a fixed or inducible charge or be able to form adducts with an appropriate modifier. The topic of electrospray ionization is beyond the scope of this article. Here are a number of reviews and books on this topic (15-18)

Sample Preparation

Normally, some clean-up of screening samples is required prior to chromatography (Figure 1). At the very least, a stop reagent that halts the reaction and engenders stability to the final mixture is required, as the samples may sit in the autosampler or freezer for an extended time. The stop reagent may consist of a low or high pH buffer, organic solvent, quenching reagent, derivatizing reagent, etc; similar to normal screening methods. Again, volatile reagents should be used to avoid contamination of the MS system. The internal standard(s) is normally added in the stop reagent (see MS parameters section).

The most common stop reagents are acetonitrile or methanol with or without modifier, and water with formic or trifluoroacetic acid. Acetonitrile (ACN) or methanol ratios of 1:1 to 1:4 sample to solvent are generally used. The high levels of ACN or methanol will cause the proteins in the sample to precipitate, and require the plates to be centrifuged or filtered prior to analysis. If the biochemical system cannot be stopped with ACN or methanol due to chromatographic issues, then water with formic or trifluroacetic acid at 1-10% (v/v) should be tried. This type of sample preparation leaves the salts and buffers in the sample. Therefore, the system must include a divert valve or “dump valve” between the column and the MS to remove the salts to waste and preserve the cleanliness of the MS. Alternatively, the analytes may be captured from the assay mix by liquid/liquid or solid phase extraction, followed by drying and reconstitution or dilution with water. Analysis of the subsequent samples often does not require a “dump valve” system as these methods result in cleaner samples with salts, proteins, and detergents typically removed. Liquid/liquid extraction methods may be automated using robotics, but require easily achieved modifications to the normal robotics and use of solvent resistant plates. Venting of the robot to remove organic solvent(s) vapors is necessary for safety (Figure 2; see instrument set-up section). Also, organic solvents used in liquid/liquid extraction are not tolerated by the plates normally used for the assays Table 2. Of the common polymers used, only polypropylene is resistant to some of these solvents (see manufacturer’s specifications). However, Teflon-like plates are available in 96 and 384 formats. These Teflon-like plates are

typically used for combinatorial chemistry applications, and are more expensive than the plates normally employed in screening. However, in many cases, they may be washed and re-used. Also, reagent reservoirs containing solvents on the robot deck must also be made of solvent-resistant materials (e.g. metal, glass, Teflon). As pipette tips are constructed from polypropylene, they tend to be resistant to solvents, especially as they are only in contact with the solvent for a short time. However, compatibility should be checked prior to use. Although 96 and 384-well plate based SPE phases are available, these tend to be cost prohibitive when considering the number of samples to be processed in a typical screen.

Derivatization of the analytes can make them amenable to extraction or detection. This is beneficial when the analytes are difficult to chromatograph due to sticking to surfaces, broadening of peaks, or poor retention. However, this does add further complexity to the screen and may affect the assay statistics if the reactions are not optimized or complete. If derivatization is required for sensitivity or to overcome some other property, both substrate and product should react to the same level of completion or a stable-labeled internal standard will be necessary.

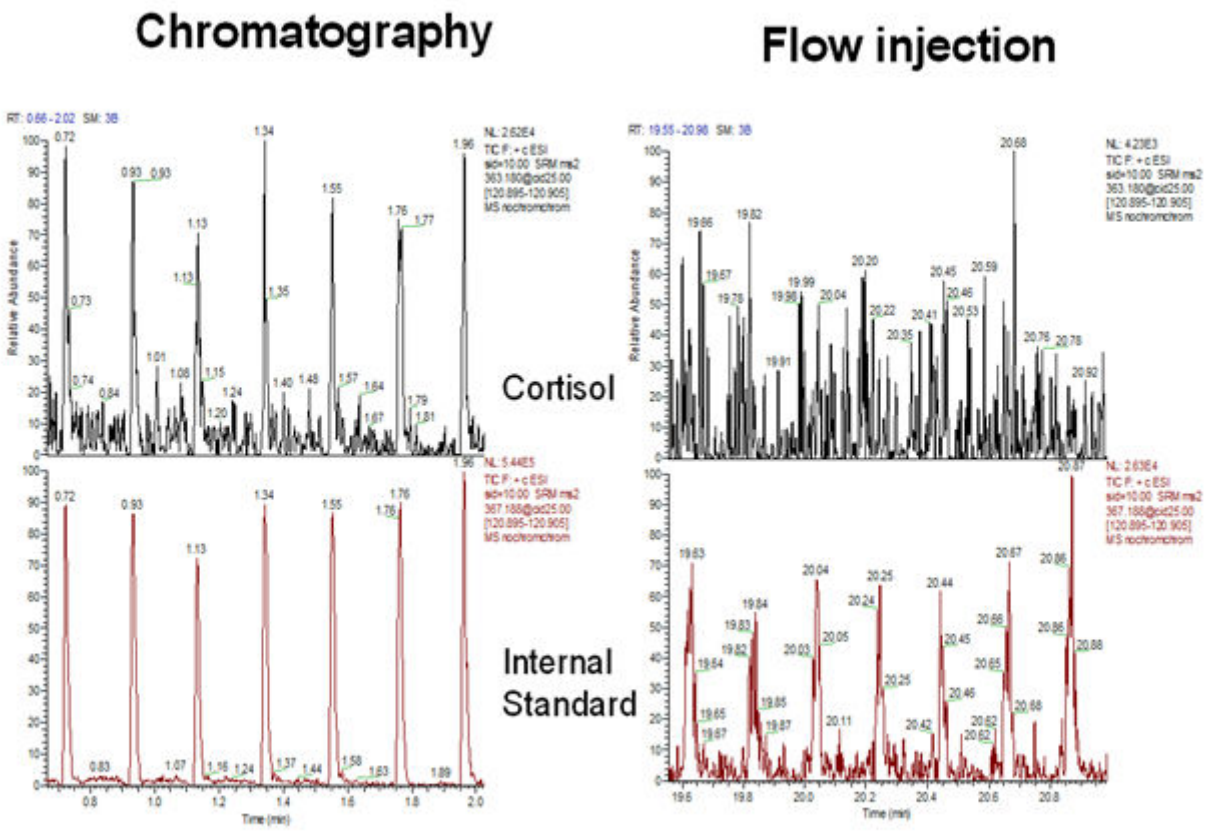


Figure 1: Comparison between ballistic chromatography and flow injection for 10 ng/mL of cortisol. Intensity of internal standard for flow injection was $2.6e^4$ compared to $5.4e^5$ for the ballistic chromatography setup.

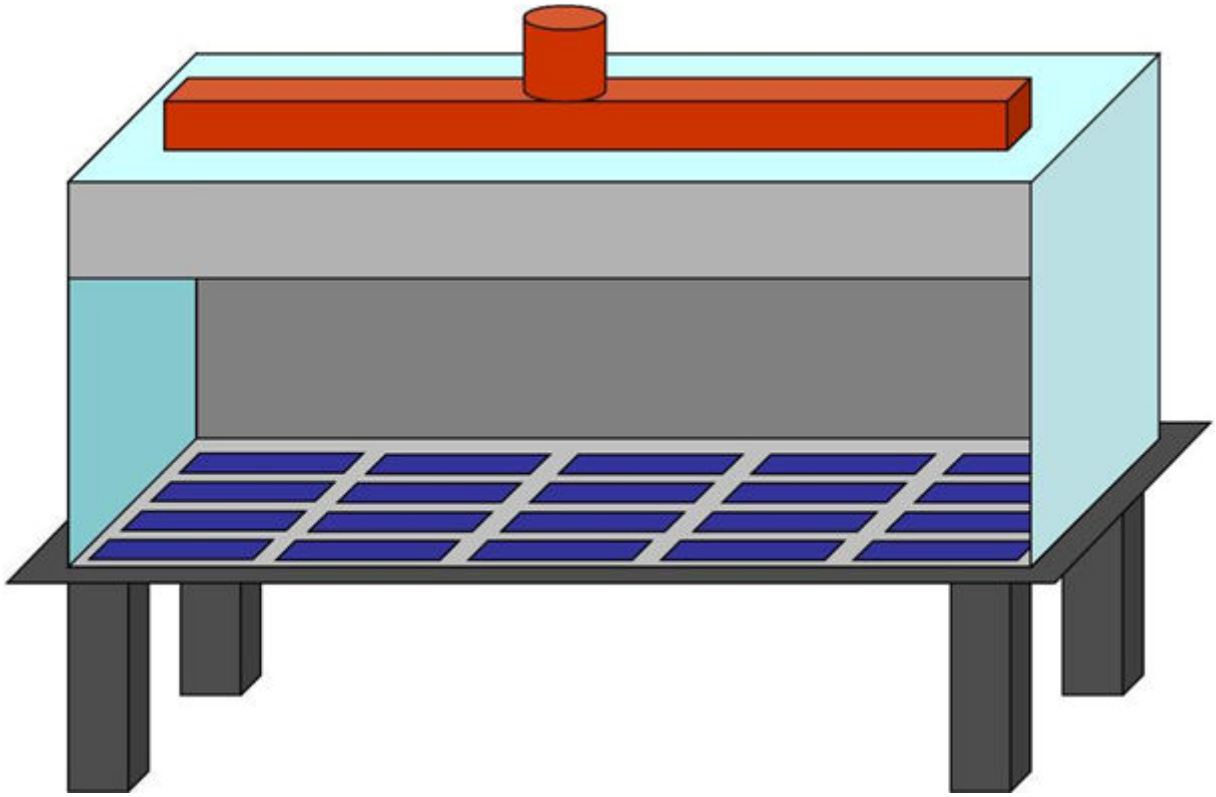


Figure 2: Schematic for Modified BioMek FX for Organic Solvents

Table 2: Extraction Solvents

Extraction Solvent	Solvent Strength	Polarity	BP	Dielectric Constant	% of Solvent in Water	% of Water in Solvent
Heptane	0.01	0	98	1.92	0.0003	0.01
Toluene	0.29	2.4	110	2.4	0.67	0.046
MTBE	0.47	2.5	55	4	4.8	1.5
Diethyl ether	0.38	2.8	35	4.3	6.89	1.3
Methylene Chloride	0.42	3.1	40	9.08	1.6	0.17
Chloroform	0.4	4.1	61	4.81	0.815	0.072
Ethyl Acetate	0.58	4.4	77	6.02	8.7	9.8
Butanol	0.7	3.9	117	17.8	7.8	20.1

Chromatography

In the most common situation, chromatography is required to avoid ion suppression from the sample matrix (Figure 2). The goal is to keep the analysis time as short as possible while providing acceptable separation of the analytes from the suppressing contaminants. A chart of sample analysis cycle time and daily throughput is shown in Table 3. In the realm of analysis times <20 seconds/sample, assay screens of 50-100K samples become feasible. However, even an increase of a few seconds/sample will raise the total screen time significantly. This restricts the use of chromatography to ballistic or snap gradients – essentially "on-line SPE" with little or no separation of the analytes from each other, requiring unique MRM transition channels for each analyte or unique parent masses for accurate mass instruments. In these methods, the sample is loaded with a retaining

mobile phase while unretained components are diverted to waste (via a dump valve), followed by a very rapid mobile phase switch to elute the analytes to the MS.

The two most used types of chromatography for medium through-put screening are reverse-phase and HILIC. Reverse-phase chromatography uses a hydrophobic stationary phase, usually a C18 alkyl chain bonded to a silica particle. The C18 alkyl chain can be replaced by a C4, C8, phenyl, or cyano, but the same methodology applies. The initial mobile phase is water with or without some amount of organic, plus a modifier. Typical modifiers and their concentrations are located in the table (Table 1). The elution solvent is then acetonitrile or methanol with the same or possibly different modifiers. The analytes are retained on the alkyl phase due to its hydrophobic nature and then elute with the higher percentage of organic solvent. This type of chromatography is excellent for the majority of analytes which will be screened by mass spectrometry. Reverse phase chromatography is also versatile due to the ability to change modifiers which adjust the retention of the analytes. Two of the most significant advances with this type of chromatography are the increase in pH range and the ability to use 100 percent aqueous solvent systems. The bonded phase is typically only stable to pH 7; however, with the introduction of hybrid particles, pH stability up to twelve is possible. This allows for the use of high pH mobile phases to retain molecules that are not well behaved at lower pH. Also, increase in pH can dramatically improve peak shape and can sometimes dramatically effect sensitivity in the mass spectrometer. Using 100% aqueous phases may cause phase collapse for older resins, resulting in non-reproducible retention times. However, resins are currently manufactured which are compatible with 100% aqueous phases. For a more in-depth discussion of reverse-phase chromatography, please refer to the following references (19-26).

HILIC (Hydrophilic Interaction Chromatography) is very good for retention of polar analytes. This type of partition chromatography can use the same solvents as reverse-phase chromatography but in the reverse order. The compounds are loaded in high organic (typically 95% acetonitrile) and eluted by increasing the water content. The samples are also typically in a high percentage of organic solvent, which couples well with stopping the reaction with acetonitrile and then direct analysis of the samples. The stationary phases for HILIC are quite different from reverse-phase chromatography. Typically HILIC stationary phases are bare silica, diol, amino, amide or specialty stationary phases which have dual functional groups. Unlike reverse-phase columns where exchanging one manufacturer's C18 column for another may provide minimum changes in peak shape or retention, changing from one HILIC packing material to another may produce significant changes in retention, peak shape, and carry-over. For this reason, typically 5 or 6 different HILIC stationary phases are tested using the same mobile phase composition. For a more in depth discussions on HILIC chromatography please refer to these reviews (23, 25, 27, 28).

Table 3. Sample Analysis Rates

Cycle Time (sec)	Samples/Minute	Samples/Hour	384-well Plates /Hour	Samples/Day	384-well Plates /Day	Days for 50K Screen*
3	20	1200	3.13	28800	75	2.1
5	12	720	1.88	17280	45	3.5
8	7.5	450	1.17	10800	28.13	5.6
10	6	360	0.94	8640	22.5	7.0
12	5	300	0.78	7200	18.75	8.4
15	4	240	0.63	5760	15	10.5
20	3	180	0.47	4320	11.25	14.0
25	2.4	144	0.38	3456	9	17.4
30	2	120	0.31	2880	7.5	20.9

Table 3. continued from previous page.

Cycle Time (sec)	Samples/Minute	Samples/Hour	384-well Plates /Hour	Samples/Day	384-well Plates /Day	Days for 50K Screen*
60	1	60	0.16	1440	3.75	41.9

* 320 samples and 64 controls/plate, continuous operation

Instrument Set Up

In this section, several arrangements of instruments are described. Although other arrangements are possible, these have proven robust in our hands.

Modified Gilson 8-probe

For extracted or generally clean samples, an isocratic system, developed employing a Gilson 8-probe that uses a mobile phase and column allowing for slight separation of the void volume and analytes (8). The setup is pictured in Figures 3 and 4. The Gilson 8-probe was modified by addition of a 10-port column-switching valve that is used to route pump flow to each of the eight sample injection valves in sequence, each sample injection valve linked directly to the corresponding positions on the switching valve. The two remaining positions on the switching valve (9 & 10) are used to by-pass the Gilson, routing flow directly to the column. During operation, the eight samples are loaded to the corresponding sample injection valve loops (load position) with the switching valve in the by-pass position (#10). Then, all sample valves are switched to the inject position. Because there is no flow through the sample valves, the samples are locked in the loops. Each sample valve is then selected in turn by rotating the switching valve through positions #1-8 using a time program of contact closures from the Agilent HPLC pump. Position #9 is passed over to wait at #10 for the next eight samples. As each sample injection valve is selected, the isocratic flow (2 ml/min) sweeps the sample from its loop to the column, where it is separated, and on to the MS, where it is detected. Each sample valve is selected for 6 seconds. The analytes are partially retained on the column, separating slightly from the void volume. The pressure spike observed on switching the 10-port valve causes a blip in the data trace from the MS and timing of its occurrence is adjusted away from the appearance of the peaks to enable facile integration.

During the rotation of the switching valve, the Gilson's 8-probes and injection ports are washed using a modified washing system. The needles are washed at the wash station, both inside and out, using the syringe working solvent in the normal manner. The injection ports are washed using a modified washing system incorporating a HPLC pump, a two-position switching valve, and an 8-way splitter. Flow of wash solution from the pump (16 ml/min) proceeds through the two-position switching valve that directs flow to the pump solvent reservoir or the injection ports, thus recycling solvent when not in use. When the injection ports are selected, flow proceeds through the 8-way splitter creating a flow of 2 ml/min to the exit/waste ports of the sample valves and up through the needle entrance of the ports, back-flushing the ports. The overflow from the ports is collected in the drainage moat surrounding the ports and exits via a drain tube to a waste bottle that is under constant negative pressure using an exhaust trunk. This method reduces carryover to essentially zero and also clears any plugging of the injection ports.

For high-throughput operation, the Agilent HPLC pump, degasser, and column compartment, and the AB Sciex API-4000 are controlled by the Sciex Analyst software. To facilitate the rapid analysis, data was acquired into a single data file for each iteration of the Gilson 8-probe, yielding 8 samples per file (Figure 5). The movement of the column switching valve used to select the individual sample injection valves on the Gilson 8-probe was controlled by the inject start contact closure from the 8-probe and a time program of contact closures from the Agilent HPLC pump. The washing pump was set up to run continuously, with its switching valve controlled via the AB Sciex software. The Gilson 8-probe was run in stand-alone mode using Trilution software, but with a Ready-Out contact closure signal required from the LC-MS/MS. The 8-probe also sent an inject start contact

closure to the MS to start the acquisition. Thus, if the autosampler failed, the MS would not start. Also, if the LC-MS/MS failed, the autosampler would not proceed. This dual "handshake" saves samples in the event of equipment failure. To prevent failures due to leaks in the plumbing that are missed by the leak detection resistors on the Agilent equipment, the low pressure setting on the pump is set to 34 bar. Also, as PEEK tubing will fail after prolonged exposure to ACN, all lines up to the column were plumbed with stainless steel. Data handling utilized a method written in the vendor software with analytes 1-8 representing samples 1-8 of each iteration of the 8-probe. After integration, the results were transferred to Excel and transposed with a template to 384-well format.

Off-the-shelf MTS system

For analyses requiring a ballistic gradient, the Shimadzu Prominence system offers a practical alternative. A typical set up utilizes a 10-port two position valve to select pumps and route flow to either waste or the MS. During equilibration and sample injection, flow proceeds from the loading LC pump to the autosampler, to the 10-port valve, through the column, and exiting to waste. Flow from the eluting LC pump proceeds through the same 10-port valve to the MS. After the sample is injected, the analytes are retained on the column while unretained materials are sent to waste. The valve switch moves the column from the loading pump flow to the eluting pump stream and sweeps the analytes into the MS. After the analytes have eluted, the valve is switched again and the column re-equilibrated with the loading pump flow.

Figure 6 illustrates the layout of the pumps, autosampler, 10 port valve and mass spectrometer. This arrangement provides for instantaneous delivery of the elution and re-equilibration solvents to the column through the 10 port valve. Also, solvent B is continuously pumped to the MS, either bypassing the column or through the column.

Figure 7a depicts the wiring diagram which starts both the pumps and the mass spectrometer simultaneously. With each injection, the autosampler sends a start signal to the pumps to begin the pump's time programs and to the mass spectrometer. However, the mass spectrometer ignores any further start signals from the autosampler until the time programmed into the mass spectrometer method has elapsed. The time program for pump A controls the switching valve and the pump's shut off time. The time program for pump B controls its shut off time. The flow rates for pump A and B are 2.5 mL min^{-1} until 2.9 minutes, with a drop to 0 mL min^{-1} at 3 min. With this set-up the pumps will stop flowing if they do not receive a start signal before 3.0 minutes. Therefore, after the 12th plate has run or if an error occurs, the pumps will shut off, allowing for unattended operation of the system.

Figure 7b depicts the wiring diagram enabling a pump error to stop the autosampler while running in local mode. After the wiring is connected, the External Signal Functions (EXT-S) parameter in each pump is set to 2. Without this configuration, the autosampler would continue to inject samples even though the pumps are not working. There is no need the change the wiring connections and the EXT-S settings when switching between MTS mode and normal bio-analytical mode.

Commercially-built System for MTS

A commercially available instrument was built to perform rapid ballistic gradients with a computerized interface to facilitate automated mass spectrometry based screening. Therefore, this instrument has significant advantages in ease of use, speed, and robustness over the previous two platforms. The instrument consists of three isocratic pumps and one peristaltic pump, four valves, a vacuum system for sample loading, column holder, refractive index detector, and a plate changer. Another vital component is the software to integrate the data which is further discussed in the data handling section. Unlike most sample inlet systems, this commercially built instrument uses vacuum to remove sample from the sample plate. The sample is pulled through the tubing, through the injector loop, and to an optical window. A refractive index change is detected when the sample

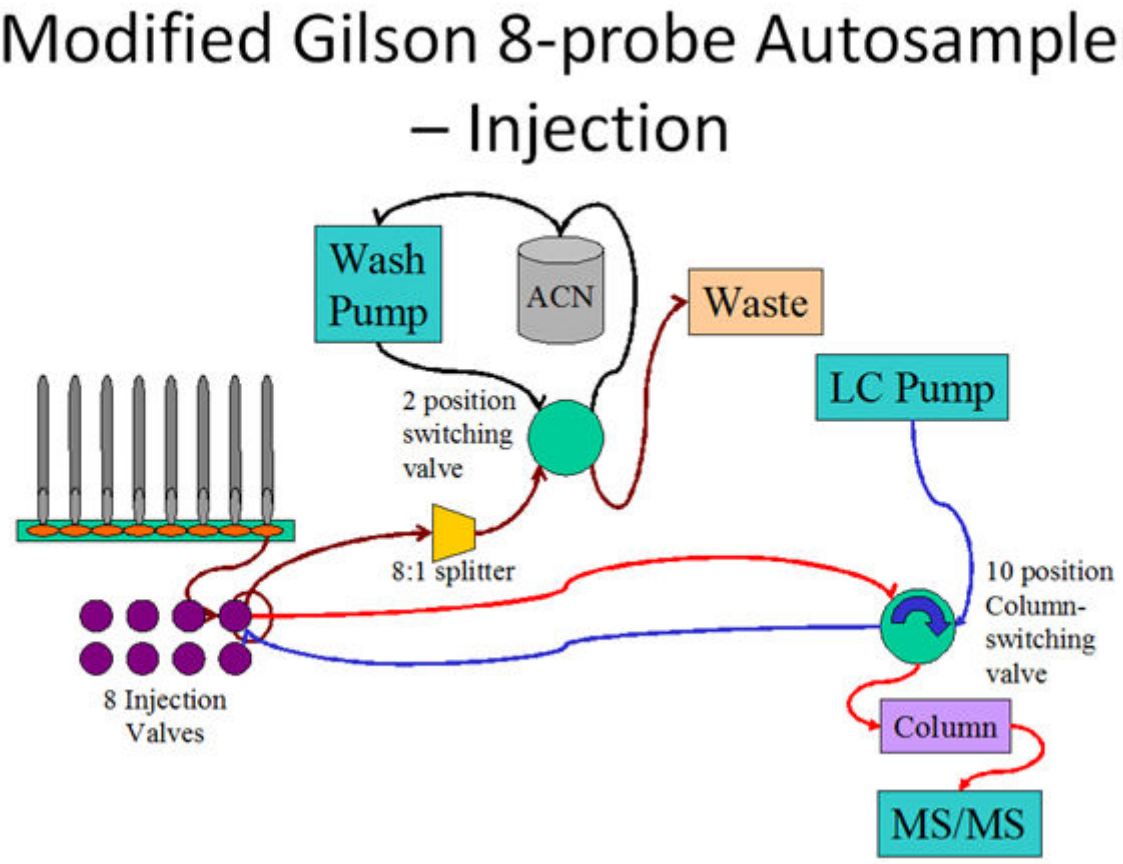


Figure 3: Schematic of Modified Gilson 8-probe Autosampler in the injection position. All peek lines have been replaced metal.

liquid appears, which then triggers the injection valve to switch and load the column with the sample. If the sample is not loaded by the time specified in the method, the valve will still inject and that well will be missed. If a specified number of missed injections occur, the system will shut down. The three isocratic pumps work in concert with the values to provide low carryover and rapid sample clean up. Pump 1 is used to load the compound onto the column, wash the sample, and re-equilibrate the system. Pump 2 is used to wash the sample flow path. The solvents from pump 2 do not interact with the sample. This allows for the possibility of using a very aggressive wash in pump 2 if needed to reduce carryover; however, this solvent is usually the same as the elution solvent. Pump 3 elutes the sample from the column, with flow either to the mass spectrometer or through the column to the mass spectrometer. When the system switched from load to elute, pump 2 is then flushing the system while pump 3 is eluting the sample. The column compartment on this instrument can hold up to 6 columns. When performing method development these can range from cyano to C18 phase. In production mode these are the same stationary phase. If during a run the backpressure is above the limit specified and there is another column of the same packing material available, then the instrument will automatically change to the new column and resume the analysis. The standard packing materials supplied in their catalog are cyano, C4, C8, C18, silica, HILIC and Hypercarb columns. Custom packed columns can be obtained; however, by modifying the plumbing the system can be made to accept a multitude of columns. This is especially useful when trying to decide on the best column or if a high pH compatible stationary phase either for reverse-phase or HILIC is necessary. The one drawback to modifying the plumbing is the loss of the automated column switching capability. But the ability to use more column types is a worthwhile trade off. This system is best illustrated by the Agilent Rapidfire™ (2, 4, 29-31).

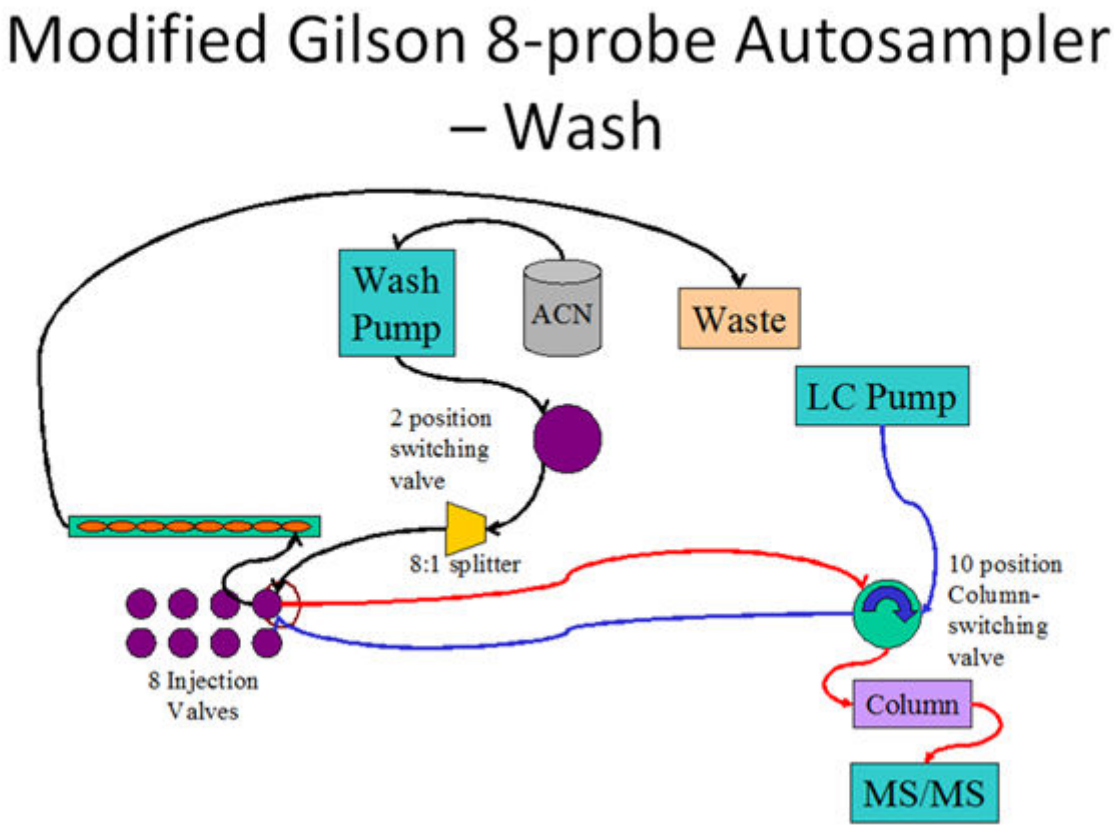


Figure 4: Schematic of Modified Gilson 8-probe Autosampler in Wash position. All peek tubing has been replaced with steel.

Staggered Injection System

The staggered injection system is the only true HPLC system of the four discussed. These systems are typically composed of four HPLC pumps, two or four auto-samplers, four columns, a column switcher, and advanced software to integrate and control these components (32-34). The injections are staggered in time so that the compounds are eluting at different times into the mass spectrometer. These systems allow true gradient elution instead of only a ballistic gradient. Therefore, this type of system may be able to successfully overcome more complex matrixes than the systems previously described. The price paid for this increased chromatographic resolving power is speed. The fastest this system can inject is about one sample per minute; therefore, the best through-put this system can achieve is 4-5 samples per minute. The biggest shortcoming to this system is all the parts that have to keep running to achieve this through-put. Recent versions of software controlling this system will compensate for loss of one channel by redistributing the samples across the other three channels. This system (like the Shimadzu system previously discussed) can be used to perform other functions besides MTS screening making it a versatile option for any lab thinking about screening on infrequent basis. This system is best illustrated by the Aria System from Thermofisher.

Data Handling

In years past, processing MTS data was a challenge using vendor software; however, this is now not the case. The most complete solution is provided using the Rapidfire® because that system has its own data integration software capable of integrating data from Thermo Fischer Scientific, AB Sciex and Agilent mass spectrometers. Using the Agilent platform integration software it is quite easy to process data from samples contained in 10 to 20 plates collected in a single file. After straight-forward data integration, the software can display the data in

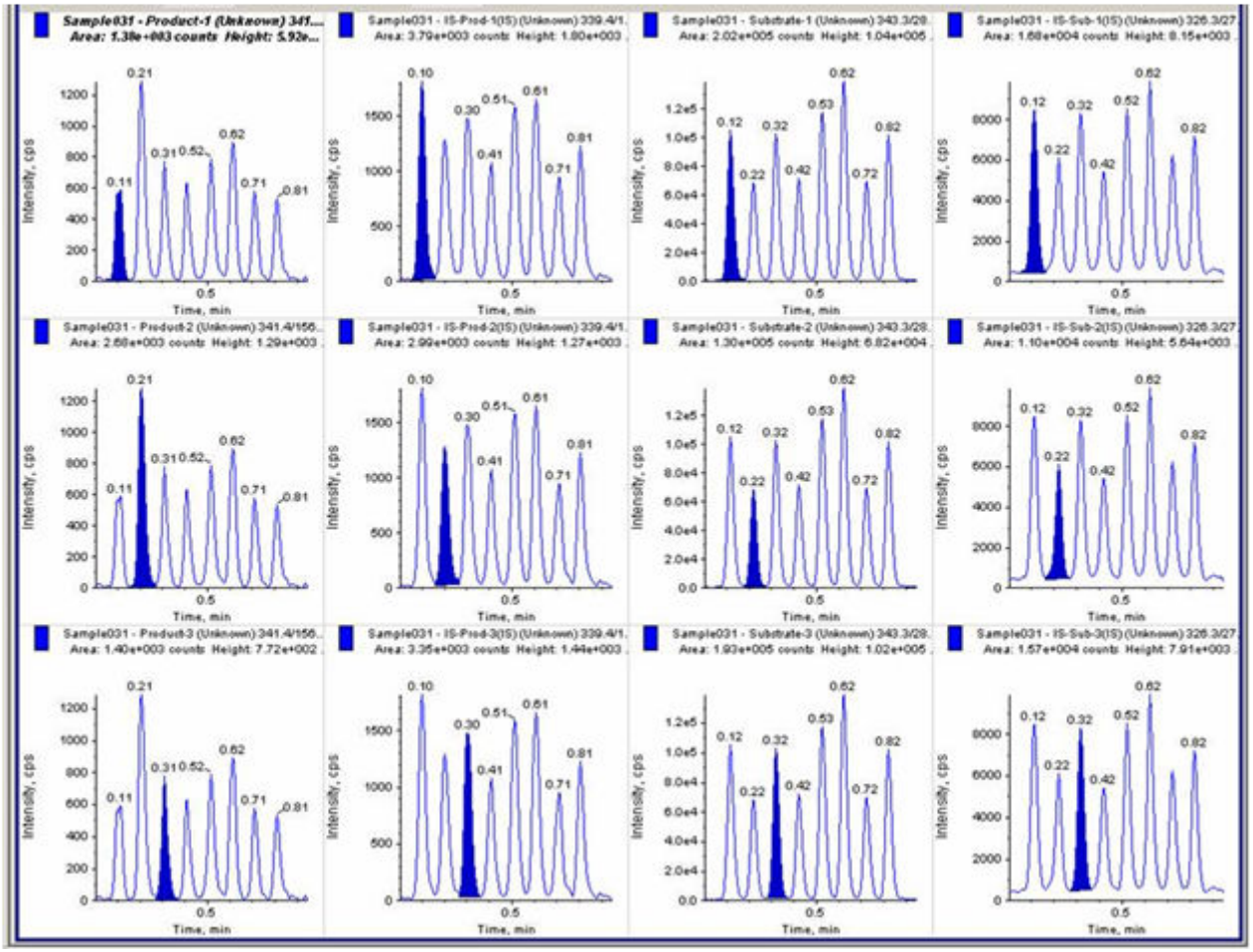


Figure 5: Example of data obtained with Gilson 8-probe. Gilson 8-probe was connected to AB SCIEX 4000 and the data was processed using Analyst®.

either plate format or column format. The software will do an excellent job processing data in screening mode; however, if calibration curves are needed, then the software has limitations depending upon the MS instrument used. If the Rapidfire® is connected to an Agilent mass spectrometer, then making in-plate calibration curves will be straight-forward as the system is fully integrated into the Agilent Mass Hunter software. If the Rapidfire® is connected to a Thermo Fischer Scientific or AB Sciex mass spectrometer, obtaining a calibration curve requires data to be imported into a program like Excel or JMP for further processing or will require using the mass spectrometry vendor's software to process the data.

All three brands major brands of mass spectrometers (Agilent, ThermoFisher Scientific and AB Sciex) may be used to obtain data from the Gilson 8-probe or the Shimadzu autosampler options described. Using the Gilson allows the normal vendor quantitation software to be used for data integration of the eight peaks per data file, with further processing in Excel or similar programs. The Shimadzu set up requires use of vendor specific software to process the data as the entire plate data is contained in a single file. For the Agilent, this will be done using their Mass Hunter software. For the ThermoFisher Scientific instrument, data processing is performed using GMSU/QuickCalcCalculation, a separate software package from their standard package. For AB Sciex, data processing is performed using their Multiquant™ software package, also purchased separately. As opposed to the Rapidfire® integration software, these vendor solutions work better if each 384/96 well plate is contained in a single file. These software packages also work better if an internal standard (or the substrate which can act as the internal standard) is used as an injection marker for processing the data. After the data is processed and exported to a spread sheet, percent conversion or area ratio can be calculated. Mass spectrometry data running

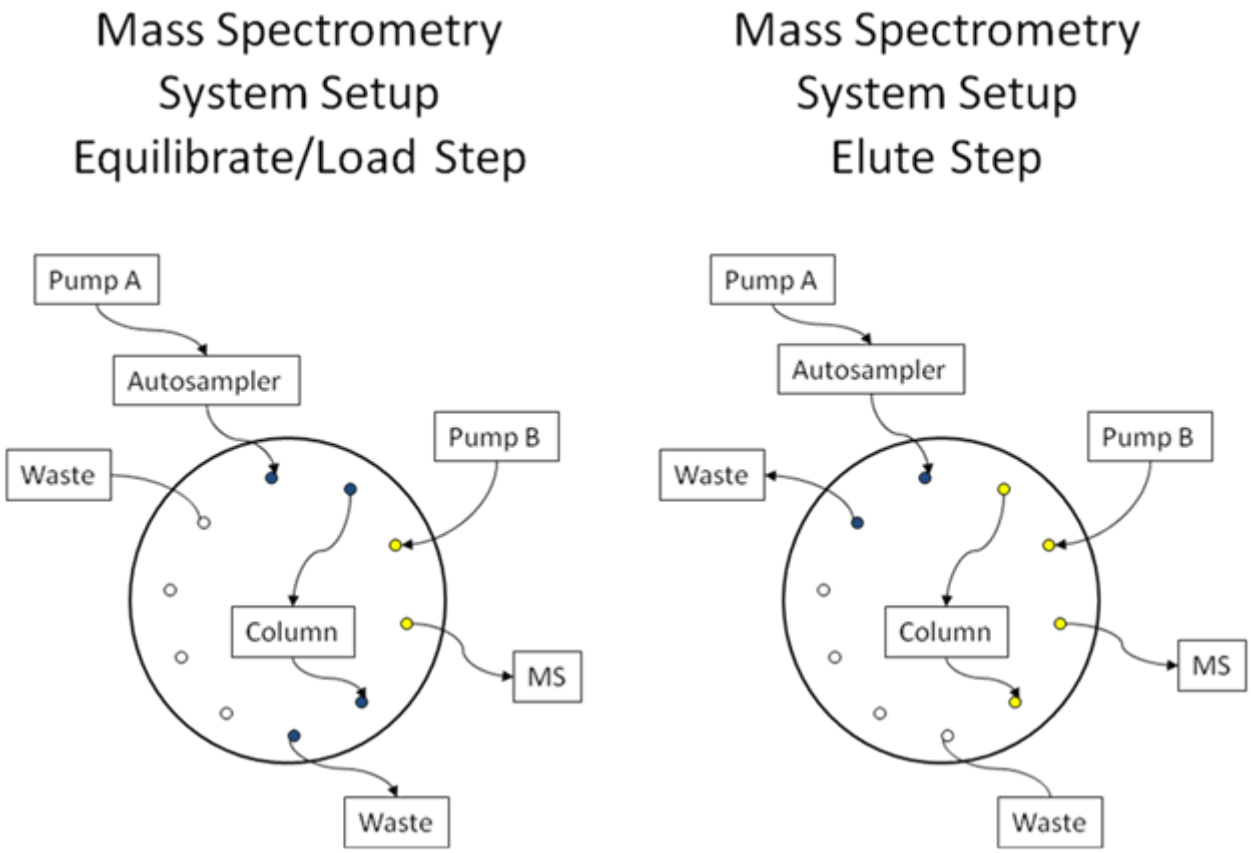


Figure 6: Schematic of MS-MTS system using the 20 AC Shimadzu auto-sampler, 20 AD Shimadzu pumps, 10 port value, and a mass spectrometer.

in MTS mode is inherently more variable when only the product of the reaction is considered; however, because the product of the reaction is made from the substrate, a percent conversion calculation may be used to normalize the data. This should only be done if the instrument response slopes for the substrate and product standard curves are parallel. If the lines are not parallel an internal standard must be used. The calculation for percent conversion is:

% Conversion = Product/(Product + Substrate)

If the biochemical system allows for percent conversion to be used, this will result in the least amount of assay variance as it negates the variance caused by the robotics systems used to add substrate to the assay plates. Addition of internal standard will only account for the variance occurring after its addition at the stoppage of the reaction.

Other Notes

Some time is generally required for the screening lab personnel to become accepting of this new technique and its ability to pinpoint problem areas in assay methods and practice. In this process, robust experiments with adequate controls are needed that allow the data to speak for itself without equivocation. MRM transitions may be included that monitor each component of the reaction, allowing any errors or trends to be seen and facilitating rapid assay development. This will quickly highlight the new LC-MS/MS techniques as rapid assay development tools.

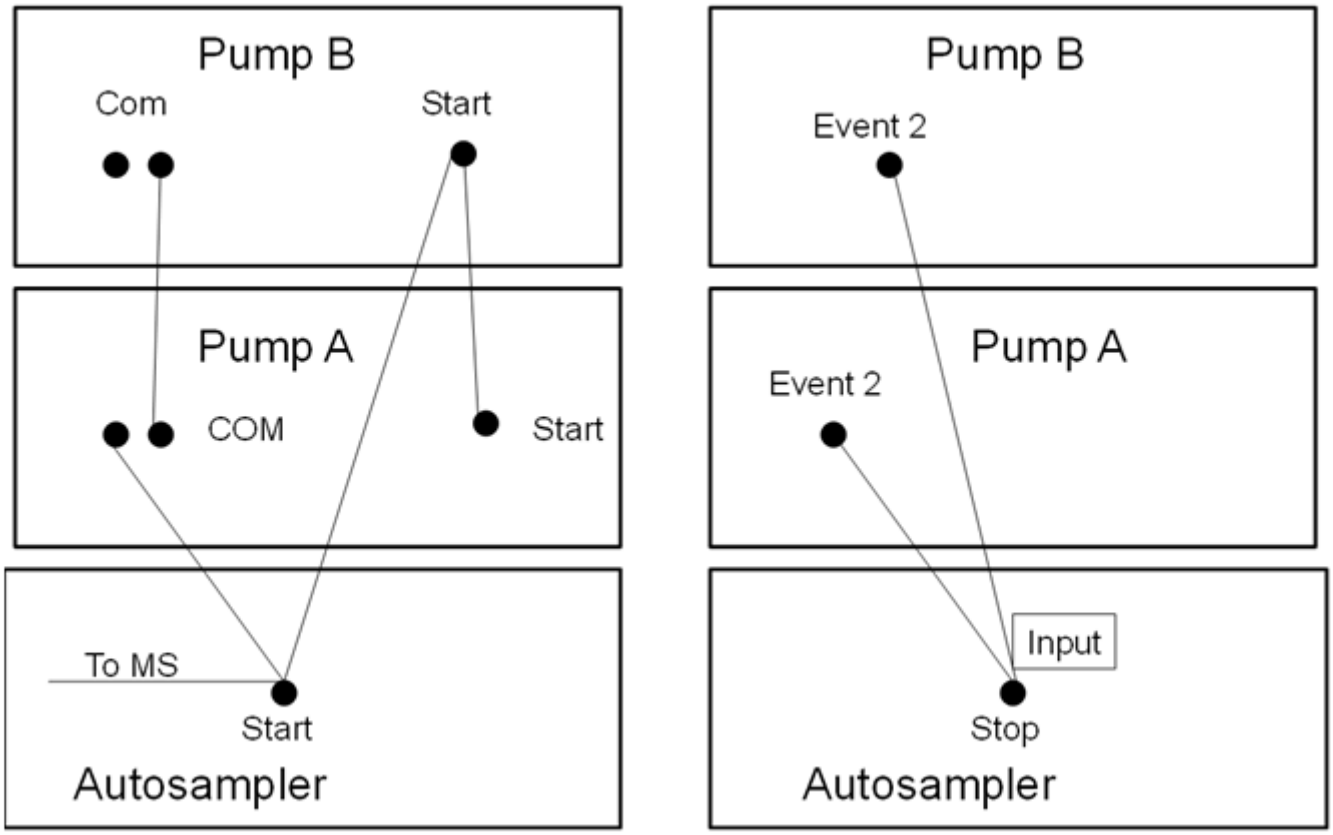


Figure 7: Schematic of wiring. A) Wiring setup for starting the pumps and auto-sampler simultaneously. B) Wiring setup for sending a stop signal to the auto-sampler if the pumps' pressure fall below minimum pressure setting, or detects a leak.

Collaborative efforts initiated with analytical departments containing the equipment and assay personnel are recommended as the purchase of the LC-MS/MS equipment requires significant capital expenditure, and its operation requires the incorporation of trained, experienced staff with the screening lab personnel.

Example Initiation of a New Screen

The following is a typical example of what occurs when developing a new MTS assay:

1. Order product or products of the reaction and obtain substrate or substrates.
2. Infuse the substrate/s and product/s into the triple quadrupole instrument to obtain the MRM transitions. Ascertain from infusion if molecule will ionize in positive mode only or negative mode only or both. Usually three MRM channels are obtained per analyte and tested.
3. From the structure or from literature discern if reverse-phase or HILIC chromatography will retain and elute the compounds.
4. Using a traditional HPLC system run a quick gradient from 100 percent water to 95 percent organic in reverse-phase mode or 95 percent organic to 10 percent organic in HILIC mode. Determine if peak is eluted from column with good peak shape and low carry-over. Determine minimum amount of organic tolerated for reverse-phase ie. 95/5, 90/10, 80/20 water/organic.
5. Inject the substrate at 1 μM concentration to determine if cross talk into the product channel occurs. If there is less than 0.5% cross talk then proceed to next step. If significant cross talk is observed determine the reason.
    a. Substrate is not pure and contains some product. This analysis needs chromatographic resolution between the product and substrate.

   b. The MRM channel chosen is not specific enough. Re-infuse the analytes and obtain more MRM channels to test.
6. Run a calibration curve in the assumed range of the reaction. We usually prepare our calibration curves by serial dilutions either 1:2 or 1:3 dilutions in water for reverse-phase and 1:3 water/ACN for HILIC. This will generate an ideal state/sensitivity.
7. Determine if the reaction can be stopped with organic and at what ratio or does the reaction have to be stopped with water which will usually contain 10% formic acid in a 1:1 ratio.
8. Rerun calibration curve in buffer system stopping with conditions developed in step 7.
9. Now decrease the chromatographic run time by doing a step gradient i.e. hold for 6 seconds to wash and then elute at 90/10 organic/water for reverse-phase and 10/90 for HILIC.
10. Rerun calibration curve with substrate and product to determine new limit of quantitation, linearity, and parallelism of substrate and product.
11. If Substrate and product response are parallel, use percent conversion for screen. If slopes are not parallel, then obtain an analog IS or stable labeled IS.
12. Analytical assay is now ready to test biochemical samples.
13. Run minimum and maximum experiments with calibration curves to determine if the product concentration is in the previously established linear range.
14. Run standard biochemical assay development experiments.

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Impedance-Based Technologies

Kim E. Garbison,[1] Beverly A. Heinz,[2] Mary E. Lajiness, Jeffrey R. Weidner,[3,*] and G. Sitta Sittampalam[4,†]

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Abstract

Activation of endogenous receptors (GPCR, tyrosine kinase and nuclear receptors) results in changes in the cytoskeleton and related morphological changes in cells. Changes in electrical impedance associated with these morphological changes in the presence of agonists and antagonists of these receptors can be used to evaluate hits from HTS assays. In this chapter, the basics of impedance assays and the use of two instruments available for these applications are discussed. Examples of GPCR activation and a sample protocol are also provided for the benefit of beginners and experienced investigators.

Introduction

Note: The content of the Assay Guidance Manual will be updated quarterly with contributions and new chapters to ensure the manual stays relevant to the current technologies and best practices used in the rapidly changing field of drug discovery and development. This chapter is currently in the process of being updated to reflect the current state of the field with respect to impedance-based technologies. Therefore, it is possible that the most up-to-date information may not yet be included, but will be added in forthcoming chapter updates.

Impedance-based assay systems for cell-based assays measure changes in electrical impedance relative to a voltage applied to a cell monolayer. They allow measurement of activation of all receptor types including G−protein coupled receptors, tyrosine kinase receptors, and some nuclear receptors. Upon activation of cell surface receptors, signal transduction pathways are initiated causing cellular morphological changes. Production of intracellular effectors results in changes in the cellular cytoskeleton which are reflected as changes in the flow of current across, and between the cells in the monolayer. This change in the flow of current around and through cells is represented in a single well by an overall change in the impedance within that well (Figure 1). Impedance-based assay technologies are universal and require no labels or special reagents. They may be used with either transfected or endogenous receptors and for primary cells.

Overview of ACEA Biosciences RT-CES®

Cells are seeded into the ACEA RT-C ES® 96-well plate which contains electrodes in the bottom of each well. After monitoring cell impedance through an equilibration period, the plate is removed from the instrument and compound additions are made, either manually or with external liquid handling instrumentation. The plate is returned to the incubator and the connection with the instrument is re-established. Monitoring of impedance continues. Responses are reported as "Cell Index", a parameter derived from impedance measurements.

Features:

Author Affiliations: 1 Eli Lilly & Company, Indianapolis, IN. 2 Eli Lilly & Company, Indianapolis, IN. 3 AbbVie, Chicago, IL; Email: jeffrey.weidner@abbvie.com. 4 National Institutes of Health, Rockville, MD; Email: gurusingham.sittampalam@nih.gov.

* Editor

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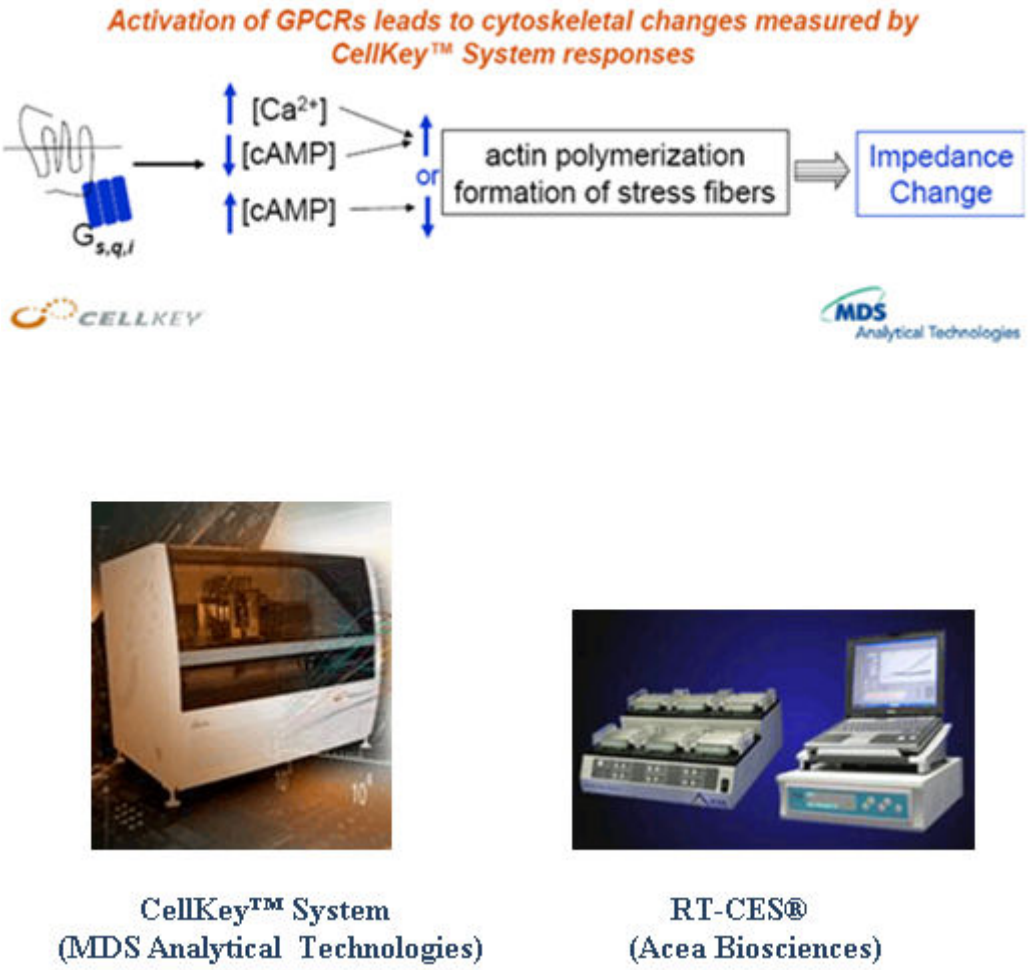


Figure 1: Two example impedance-based assay platforms are the RT-CES® from Acea Biosciences and the CellKey™ System from MDS Analytical Technologies.

- System is set up in an incubator, so cells may be continuously monitored over long periods (eg. for growth curves and proliferation assays).
- With optional RT-CIM® module, cell invasion and migration assays may be performed

Overview of CellKey™ System

The CellKey™ System is based on a label-free technology called Cellular Dielectric Spectroscopy (CDS), which is capable of measuring complex impedance changes in cell monolayers. Impedance (Z) is related to the ratio of voltage to current (Z=V/I) as described by Ohm's law. Cells are seeded onto a CellKey™ microplate that contains electrodes at the bottom of each well. The CellKey™ instrument applies voltage across the electrodes producing electrical currents that flow around and between cells (extracellular current, iec) and through cells (transcellular current, etc) (Figure 2A).

The CellKey™ System measures changes in impedance that occur in each well upon stimulation of cell surface receptors. Contributors to the impedance as measured in each well are changes in cell adherence to substrate, changes in cell shape and volume, and changes in cell-cell interactions. These dynamic cellular changes affect the flow of extracellular and transcellular current and hence the magnitude and characteristics of the impedance measured. In practice, the extracellular current is what contributes most of the signal.

Changes in impedance are captured in real time and are quantified. In addition, the unique response profiles produced after receptor activation are indicative of the G-protein coupling type (Figure 2B). Applications of this technology include receptor panning, signal pathway identification and deconvolution, hit identification, enhanced selectivity screening, and pharmacological profiling for potency and efficacy.

Features:

- Built in liquid handling allows continuous monitoring of responses from the moment of compound addition.
- May be integrated with lab automation.
- Both adherent and suspension cells may be used as well as primary cells.
- Assays may be run at either 37°C or room temperature.
- Response profiles have been correlated to G-protein coupling status.
- Small sample 96-well plates are available with 1/20 the well surface area as the standard 96-well plate, allowing a 75% or greater reduction in cells required as well as savings on test compounds.
- Easy to use software for data analysis

Sample Protocol for CellKey™ (MDS Analytical Technologies)

Also see CellKey™ Assay Development Guide provided by MDS Analytical Technologies and the CellKey™ Operator's Manual.

1. Assay Buffer: Hank's Balanced Salt Solution (HBSS) with Ca^{2+} and Mg^{2+}, 20 mM HEPES, 0.1% BSA. If BSA is included, it should be fatty acid-free (Sigma #A0281 or #A6003) to avoid activation of endogenous fatty acid receptors on cells. (Other buffers such as Tyrodes, PBS, or culture media may also be used.)
2. Harvest, count, and resuspend cells to yield the appropriate density determined during assay optimization. Cells should be just confluent at the time of assay. Seed cell plate with 150 µl/well.
    - Adherent cells are usually plated the day before the assay in culture medium and incubated at 37°C, 5% CO_2 overnight. The CellKey™ 96-well microplates may be treated with surface coatings such as collagen or poly-d-lysine to improve adherence if desired.
    - Suspension cells are harvested, washed three times in assay buffer, counted and resuspended to the appropriate concentration in assay buffer. 135 µl of cell suspension is dispensed into each well. Cells are allowed to settle for a minimum of 30 minutes at room temperature before assay.
3. Prepare 10X agonist and antagonist compounds in assay buffer in 96-well plates.
4. Load cell plate, pipet tips, compound plate, and reservoir with assay buffer into the CellKey™ System.
5. Default protocols are available for fluid exchange and stimulation/data acquisition, depending on whether cells are adherent or suspension, or if agonist or antagonist responses are to be measured. These protocols may be edited or used as written. These protocols control the fluidic parameters (tip height, volumes, speed, etc) as well as assay temperature.
6. Stimulation and data acquisition may be performed at either 37°C or room temperature.
7. Fluid exchange is performed on adherent cells plated in culture media. It may be done using the CellKey™ System or off-line with other liquid handling instrumentation. It consists of aspiration of culture media, washing the wells 3 times with assay buffer, and leaving 135 µl of assay buffer in the wells. There is no need to perform fluid exchange on suspension cells.
8. Generally, cells are equilibrated for 20 minutes after fluid exchange. If antagonists are to be added, they are pipetted by the CellKey™ System immediately after fluid exchange so pre-incubation can proceed during the equilibration period. 15 µl of 10X antagonist is added to 135 µl well volume (Figure 3).

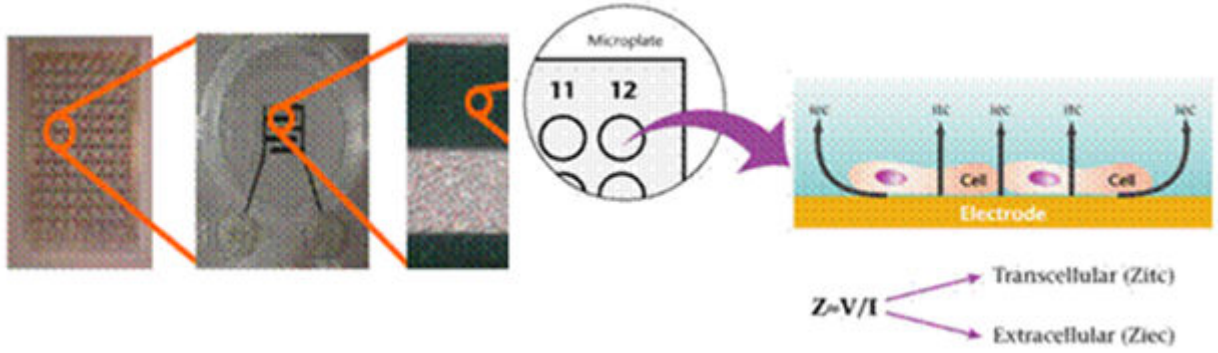


Figure 2A: CellKey™ Standard 96W microplate (left), and individual assay well with close-up view of interdigitated electrodes (center left), and live cells plated on and between the electrode fingers (center right) and diagram highlighting flow of extracellular (iec) and transcellular (itc) current (right).

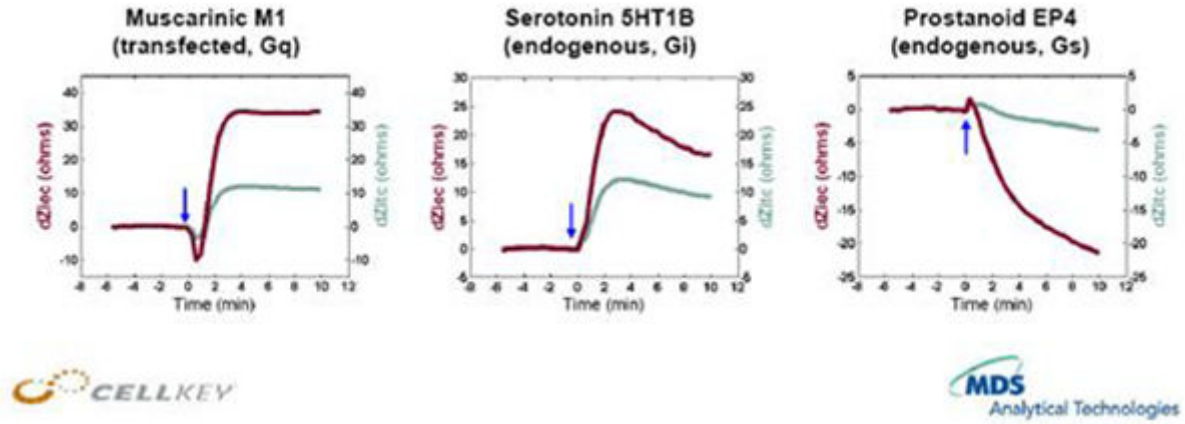


Figure 2B: Typical CellKey™ Response Profiles for G_q, G_i and G_s coupled GPCR

9. The agonist stimulation protocol is initiated after equilibration. Generally 15 µl of 10X agonist is added to 135 µl in the plate. If an antagonist has been added previously, well volume = 150 µl, and 16.5 µl of 10X agonist is added.
10. For data collection, default settings in the CellKey™ System software are provided for most adherent cells (for example HEK293 and CHO). Details are found in the Operator's Manual. Response time is usually 5-15 minutes following a 2 minute baseline data collection. Quantifying responses is typically done by calculation of the maximum change from baseline.
11. In addition to quantifying the response, qualitative MOA information may be obtained by examination of kinetic response profiles.

CellKey™ System Results and Data Analysis

1. Kinetic responses for a selected individual well or all wells are displayed on the screen. dZiec (extracellular current) responses are quantified by the instrument software (Figure 4A and 4B) by either, subtraction of the minimum impedance reading from the maximum impedance reading, the maximum impedance reading from baseline, or the impedance reading at a specified point in time during the stimulation period. Data is exported to Excel and saved.

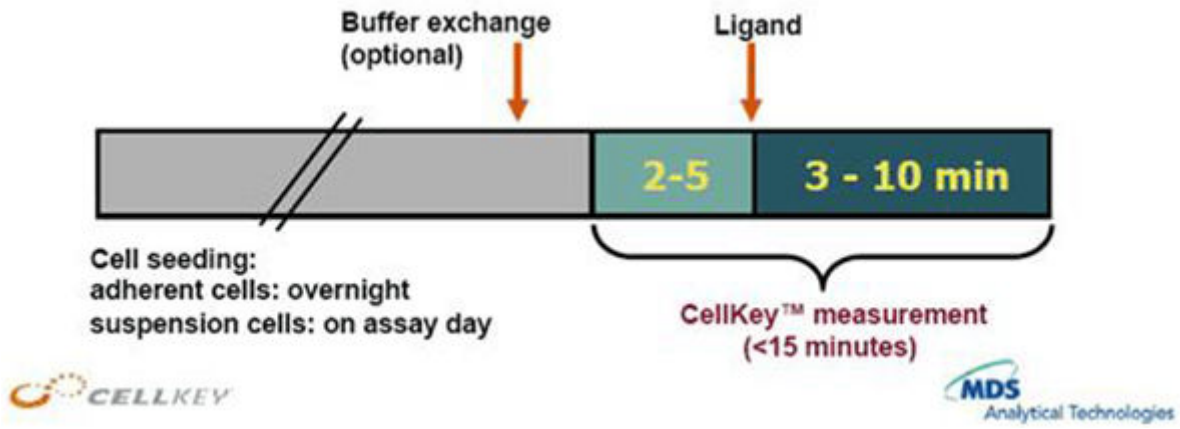


Figure 3: CellKey™ System Assay

2. Maximal – minimal dZiec responses can be plotted vs compound concentrations to generate concentration response curves and calculate EC_{50} / IC_{50} (Figures 5 and 6). (Please refer to Data Standards for Results Management.)

Helpful Hints for Performing CellKey™ Assays

- Allowing cell plates to rest for at least 15 minutes before placing in the incubator for overnight incubation allows cells to settle evenly to the bottom of the well and improves variability.
- Evaporation control: Media evaporation during overnight incubation can lead to "edge effects" due to effects on cell growth. Use of MicroClime™ Environmental Lids (Labcyte Inc. #LLS-0300) or BREATHseal™ (Greiner Bio-One #676051) is recommended on cell plates to reduce evaporation.
- Changes in buffer components such as DMSO, BSA and cations, can lead to responses in cells reflected by changes in impedence. Buffer constituents should be consistent between the cells in the microplate and compounds added. If the final DMSO concentration will be higher than 0.1%, it is necessary to include the same concentration of DMSO in the equilibration buffer.
- In order to avoid changes in compound concentration due to evaporation before or during incubation in the instrument, a pre-scored plate seal such as the EZ Pierce Plate Sealing films (Sigma) should be used on the compound plates.
- Make sure compound plate is warmed to at least room temperature before running the assay to avoid temperature differences between cell plate and compounds.
- For characterization of receptor-mediated response profiles, best results are obtained at 28°C rather than 37°C. This may be due to slower kinetics at the lower temperature.
- CellKey™ Small Sample 96-well plates are available that have the footprint of a 96-well plate but the well area of a 1536-well plate. This allows a reduction of at least 75% in cells required, and also reduces volume of compound needed. CellKey™ System Technical Note 1 details special considerations when using these plates.
- CellKey™ System microplates may be coated with collagen (Sigma C9791) or poly-d-lysine (Sigma P6407) or any other coating material of interest. If using poly-d-lysine, be sure to rinse plates before plating cells as it is toxic to the cells when free in solution. Use the following procedure for coating plates:
    1. Pipet 50 µl/well of 50 mg/ml poly-d-lysine (in sterile water).
    2. Incubate plate at room temperature for 1 hour.
    3. Aspirate contents and pipet in 100 µl sterile water to wash wells.

Aspirate water out and immediately plate cells. (Do not allow to dry as crystals may form causing the electrode on the bottom of the wells to lift.)

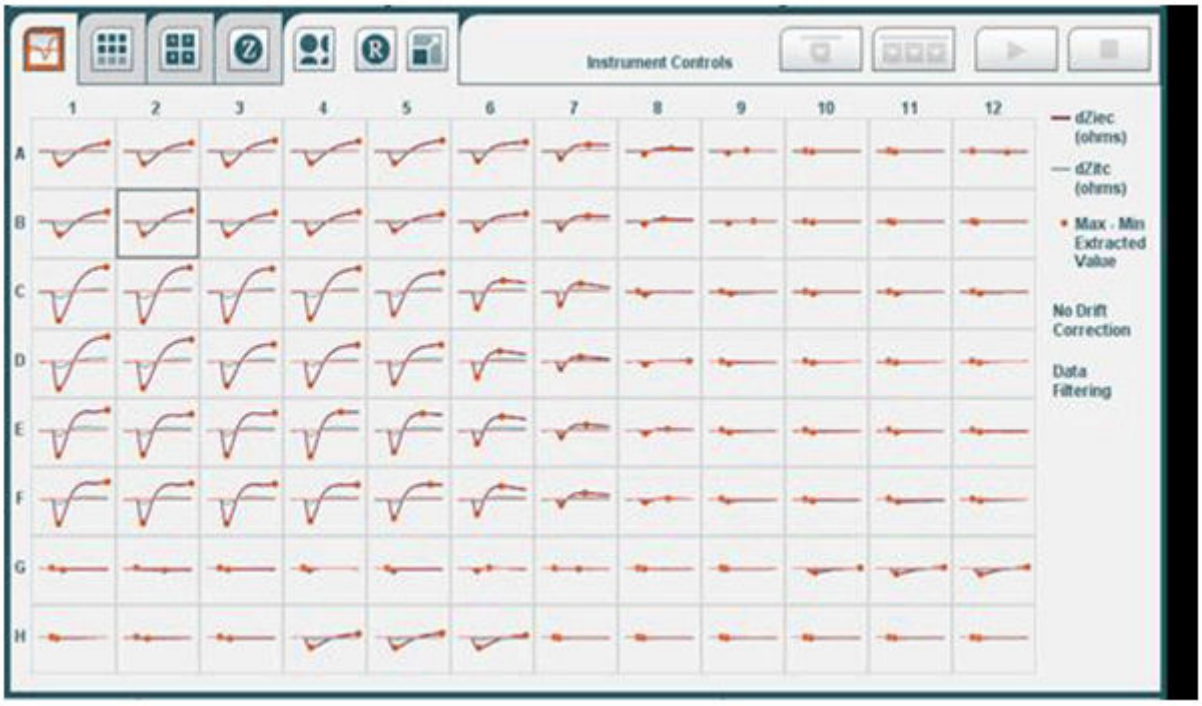


Figure 4A: Kinetic profiles from 96-well plate

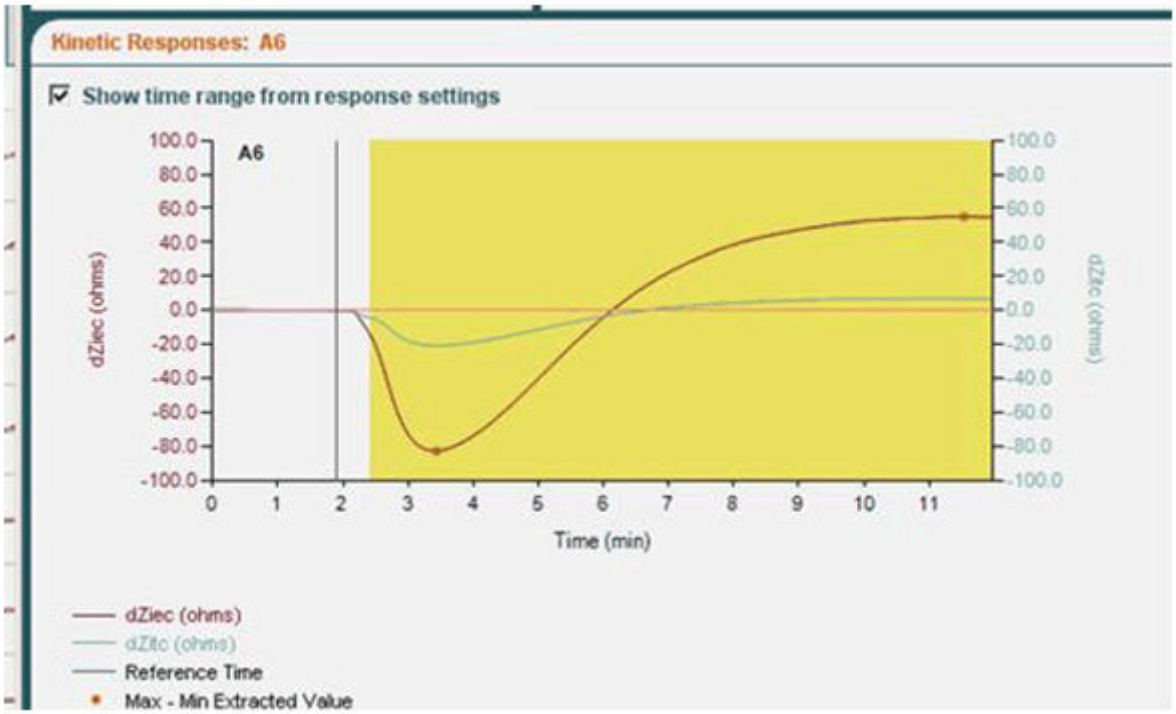


Figure 4B. Kinetic Profile from individual well

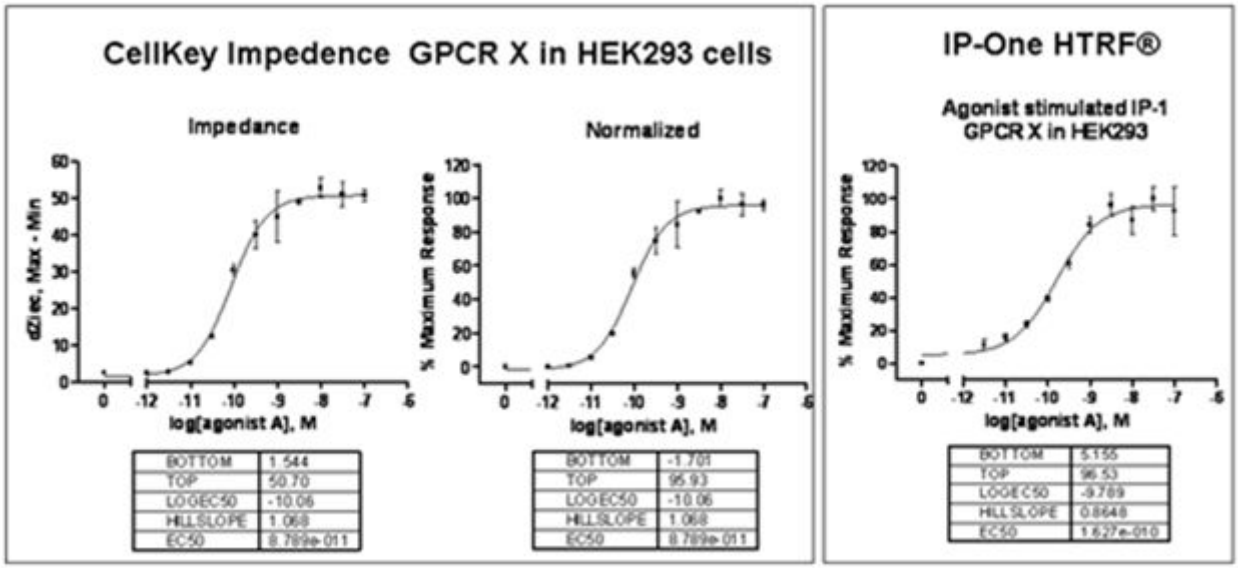


Figure 5. Example: Agonist concentration response curve EC50 for agonist stimulated increase in impedance for a Gq coupled GPCR correlates with the EC50 for IP-1 accumulation using IP-One HTRF®.

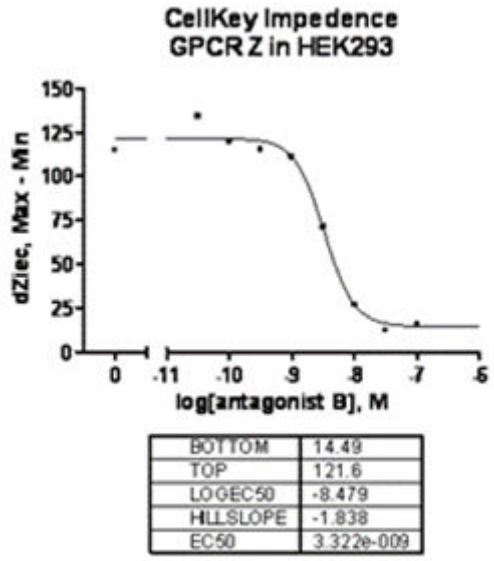


Figure 6. Example: Antagonist concentration response curve

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Nathan P. Coussens, PhD, Sarine Markossian, PhD, and G. Sitta Sittampalam, PhD

Microplate Selection and Recommended Practices in High-throughput Screening and Quantitative Biology

Douglas S. Auld, Ph.D.,[1] Peter A. Coassin, B.S.,[2] Nathan P. Coussens, Ph.D.,[3] Paul Hensley,[4] Carleen Klumpp-Thomas,[5] Sam Michael,[5] G. Sitta Sittampalam, Ph.D.,[5] O. Joseph Trask, B.S.,[6] Bridget K. Wagner, Ph.D.,[7] Jeffrey R. Weidner, Ph.D.,[8] Mary Jo Wildey, Ph.D.,[9] and Jayme L. Dahlin, M.D., Ph.D.[7,10]

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Abstract

High-throughput screening (HTS) can efficiently assay multiple discrete biological reactions using multi-well microplates. The choice of microplate is a crucial yet often overlooked technical decision in HTS and quantitative biology. This chapter reviews key criteria for microplate selection, including: well number, well volume and shape, microplate color, surface treatments/coatings, and considerations for specialized applications such as high-content screening. This chapter then provides important technical advice for microplate handling including mixing, dispensing, incubation, and centrifugation. Special topics are discussed, including microplate surface properties, plate washing, well-to-well contamination, microplate positional effects, well-to-well and inter-lot variability, and troubleshooting. This information and best practices derived from academic, government, and industry screening centers, as well as microplate vendors, should accelerate assay development and enhance assay quality.

Key Abbreviations

ADE	acoustic droplet ejection
ALPHA (“Alpha”)	amplified luminescent proximity homogeneous assay
COC	cyclic olefin copolymer
COP	cyclic olefin polymer
ELISA	enzyme-linked immunosorbent assay
FRET	fluorescence/Förster resonance energy transfer
HCS	high-content screening
HTS	high-throughput screening
NA	numerical aperture
PP	polypropylene
PS	polystyrene
RFMP	radio frequency microwave plasma

Author Affiliations: 1 Novartis Institutes for BioMedical Research, Cambridge, MA, USA. 2 Aurora Microplates, Whitefish, MT, USA. 3 Molecular Pharmacology Branch, Frederick National Laboratory for Cancer Research, Frederick, MD, USA. 4 IonField Systems, Moorestown, NJ, USA. 5 National Center for Advancing Translational Sciences, Rockville, MD, USA. 6 PerkinElmer, USA. 7 Chemical Biology and Therapeutics Science, Broad Institute, Cambridge, MA, USA; Email: jdahlin@bwh.harvard.edu. 8 QualSci, USA. 9 Merck, Kenilworth, NJ, USA. 10 Department of Pathology, Brigham and Women's Hospital, Boston, MA, USA; Email: jdahlin@bwh.harvard.edu.

Corresponding author.

Introduction to Microplates in High-Throughput Screening and Quantitative Biology

The discovery of the microtiter plate, as we know it today, was first conceived and designed in the laboratory of Dr. Gyula Takatsy in 1950 in Hungary (1). The original plate, made of plexiglass with 6 x 12 "cups" or wells (72 total wells), was intended for serological testing of the influenza virus. This plate design and the spiral loop as a quantitative liquid transfer device (a precursor to micropipettes) were seminal developments at that time because the intent was to semi-automate serial dilutions of viruses to determine titer and potency, and to test drugs that inhibit viral activity. In 1955, Dr. Takatsy redesigned the plate with 8 x 12 wells (96 total wells) to accommodate the original loop handles for liquid delivery with knitting needles which was more adaptable for use by technicians. In 1961, Dr. John Sever at the National Institutes of Health widely adopted this technique for serological investigations. He reported the use of the 96-well plate along with a "modified pipette dropper" and a centrifuge carrier for the plate, further popularizing the use of microplates for biological testing (2). In the 1970s, the 96-well microtiter plates were adapted for immunological testing assays (i.e., ELISA) followed by subsequent commercialization and standardization of the plate dimensions, materials, and well designs. As this format became extensively utilized for HTS and automated drug discovery methodologies in the 1990s, the applications for biological measurements rapidly expanded. The detailed history has been elegantly described in articles by Tom Astle and Roy Manns (3,4).

Further refinement of the microplate design took place to standardize its use for HTS and encourage all plate and instrument manufacturers to agree to specified dimensions for compatibility with screening instruments and automation. This effort was pioneered by the Society for Biomolecular Screening in 1998 in collaboration with American National Standards Institute (ANSI) which became the SBS/ANSI standard (Figure 1). This ensured that microplates will have standardized footprint dimensions, plate height, flange dimensions, chamfers, side wall rigidity, and well positions. Some commonly modified plate dimensions include flange height, well number, and well depth. Despite this standardization, for many instruments (microplate readers, robotic handlers, pin tools, acoustic dispensing) users still may need to specify *exact* plate dimensions for optimal use. Most manufacturers can provide specific technical blueprints for microplates.

From a practical standpoint, microplates are also important because they can represent the largest portion of a HTS budget, though this can be offset by expensive reagents. From a budgetary perspective, multiple competing factors must be considered when selecting the final microplate. While many institutions have preferred vendors with lower negotiated prices, a more expensive but optimal microplate may actually enable less reagent per well, yielding more cost savings when all assay costs are considered. An Excel template is available to assist with microplate cost estimation.

The cost of a microplate is typically determined by several factors: material, type of plate bottom (solid, clear, or permeable), necessary molds and overall manufacturing process, well density, barcodes, and finally any surface treatments/coatings. This combination of factors can determine if a microplate has a retail cost of around $4 for a standard 6-well cell culture plate, to $50 per plate for a 1536-well plate with a collagen coating, to over $300 per plate for a 96-well permeable support plate. Although increasing plate density drives the cost of the part higher (injection molding fixtures become more complex and therefor more expensive), it is still typically more cost effective to miniaturize an assay as the overall cost per well will decrease as less reagents will be required due to the lower well volume and less plates will be required to run an experiment as more samples can be placed within the plate. We note the microplate manufacturing process can be the primary cost factor. For example, the process of applying a permeable membrane to the bottom of a 96-well support structure is incredibly difficult and laborious, driving the cost of the part far higher as compared to a solid-bottom 96-well plate manufactured via injection molding. Although cost will always be a factor when determining which well plate to use for an assay, the primary factor should always be to make a selection based upon the requirements of the assay and the

desired measurement technique to be utilized. *In our experience, assay performance and optimized microplate measurement should rarely if ever be sacrificed due to the cost the plate*.

Desired microplate properties

Microplates to be used for biological assays should have certain properties:

- Dimensional stability under multiple temperature and humidity conditions
- Flatness
- Chemical and biological compatibility with assay reagents (DMSO-stable, does not denature proteins)
- Low-binding surfaces/surface energy (lower adsorption of chemicals or biologicals)
- Low autofluorescence
- Supports cell viability, attachment, and growth
- Supports relevant optical detection modes
- No leaching of solvents, metals, or chemicals (e.g., bisphenol A, heavy metals from catalysts, solvents)

Microplate manufacturing

It can be helpful to understand some of the basic microplate manufacturing processes. For many microplates, the manufacturing process involves an injection of liquid polymer into a mold ("injection molding"). Depending on the plate type, several pieces can be fused together, such as a glass bottom and the main polymer frame. For clear-bottom microplates, the main polymer frame are injected molded and fused with a pre-made clear bottom film in a process known as *overmolding*. Incomplete fusing and poor quality control has been known to create conduits between adjacent wells (unpublished observations; see also subsequent section Troubleshooting and Special Topics – Well-to-Well Contamination). Pigments to color the microplate are often added by the manufacturers. The raw materials used to create microplates are often an overlooked source of microplate performance by the end-user scientists. Manufacturers will usually acquire raw materials from an outside vendor, and any changes in this sourcing or the manufacturing techniques are not usually provided to consumers. In our experience, we have encountered significant changes in assay behavior for a given microplate across different manufacturing lots that can reasonably be attributed to a seemingly innocuous change in the manufacturing process. Raw materials for microplates can have highly variable quality and characterization, and while medical-grade plastics have higher quality standards, these are often not used for microplates.

Section summary

Microplates are a critical yet often overlooked component for enabling robust, reproducible biological assays. This chapter will describe key technical factors in microplate selection, certain technology-specific guidelines (notably high-content imaging), best practices for microplate usage in biological assays, troubleshooting and special topics intended to maximize microplate usage. The goal of this chapter is to disseminate high-yield common as well as tribal (tacit) knowledge from microplate manufacturers and academic/industry/government experts, which should enhance the quality of biological assays performed with microplates.

Authors' note: While microplates are also used for applications besides biological assays (compound storage, sample storage, sample purification, etc.), for brevity such applications will not be discussed.

Technical Factors in Microplate Selection

This section describes major decision points in microplate selection for *in vitro* assays. Once a particular assay technology and approach is chosen, scientists should select microplates that meet the specific technical requirements of their project.

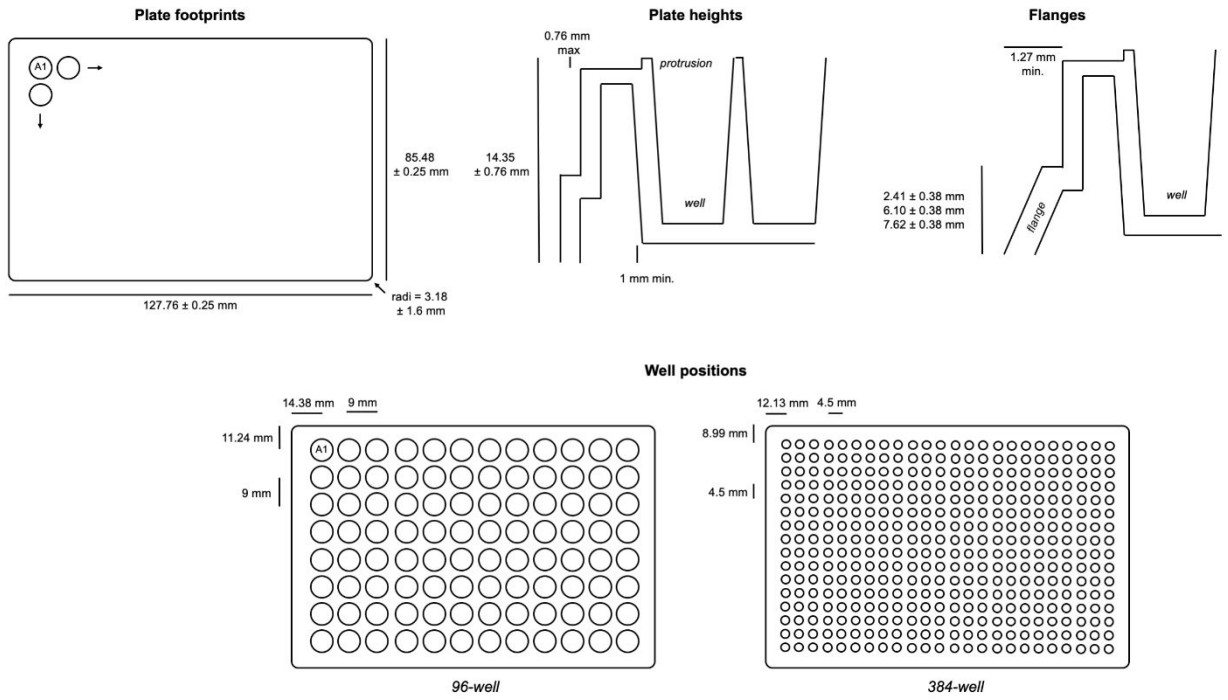


Figure 1. Schematic of standard microplates. Standard microplates used in quantitative biology will typically conform to the following dimensions. Adapted fromANSI/SLAS standards (www.slas.org/resources/information/industry-standards, accessed 14 May 2019). Scientists should consult the technical drawings from plate manufacturers for specialized applications such as incorporation of automation and microplate reader optimization.

Overview of the microplate selection process

The selection of an optimal microplate for an assay can be a tortuous process due to multiple options (and competing trade-offs) in microplate choices at each step of the assay development workflow. Not only does the assay development scientist have a series of decisions to make on plate choices based on the biology, assay technology, and desired throughput, but there are multiple microplate manufacturers offering similar products where differences in the microplate plastic, quality control of a coating, and even the "whiteness" of the plate can significantly affect assay performance. A query with "Select Sciences" (search date 21 Oct 2019) resulted in approximately 700 microplate types across 26 vendors (5). Many HTS laboratories will have a preferred vendor and as a result, have several different microplate options readily available in the lab which can serve as starting points. In addition, high-quality publications and protocols are careful to detail the specifications of the microplate (catalog and lot numbers) and detection systems, which can be a critical pairing in order to achieve and maintain the desired assay performance metrics.

The following section highlights one path for the selection of microplates that has been helpful for several of our labs with diverse discovery approaches (Figure 2). *In practice, there are likely other optimal decision trees for microplate selection*. The basic process will be outlined, and many of the individual criteria will be discussed in more detail in subsequent sections. Microplate selection is an iterative process with multiple comparisons between plates often needed before selecting an optimal plate. Many screening labs have minimum quality criteria, using parameters such as Z-score, Robust Z-score, B-score, Z-factor, Z'-factor, signal-to-noise, signal-to-background, and percent CV to characterize the assay performance and maintain it at a level necessary to increase the possibility of identifying the desired chemical matter (6-8). In practice, a balance must be struck between understanding the quality of an assay system at each stage of assay development or validation and the time to complete the microplate selection. Using one lot/batch of a microplate for the selection process can be critical to mitigate lot-to-lot variances.

In our experience, deciding on a cell-free or cell-based assay is the highest-yielding initial decision point during the microplate selection process (Figure 2). Microplates supporting cellular assays are typically tissue culture treated (sometimes abbreviated "TC treated" in catalogs), sterilized, and might have requirements for clear-bottomed wells and special coatings aimed at visualization and enhancing or preventing cell attachment. Cell-free assays, on the other hand, can require a range of surface conditions from non-binding surfaces which minimize protein adsorption (e.g., enzymatic, homogenous proximity assays) to high-binding surfaces which enhance adsorption (e.g., ELISA). Another key consideration for identifying the optimal microplate is the assay/detection technology, where multiple plate types can be tested against multiple assay/detection technologies using the performance criteria as part of the decision matrix. The expected throughput of the assay must also be factored and while the preferred density for screening assays might be 1,536-well (Figure 2), assay performance or automation may only support 384- or 96-well formats. In these situations, alternative strategies for prosecuting a screening deck might be necessary (9). The assessment of plate density can also raise questions about the well shape and volume, which will also impact the shaking efficiency and well meniscus that can be critical components in certain detection systems.

Whether the well density has been established by good performance statistics or performance is sub-optimal with the preferred plate density, consideration should be given to the physical components of the microplate itself. For example, the color of white is not standardized in opaque microplates and there are blue-white and grey-white hues. The hue of the color can make a difference in the performance of an assay and often varies from vendor to vendor and sometimes even from lot-to-lot by the same vendor. Additionally, there are several options for plate material, including polystyrene (PS), polypropylene (PP), cyclic olefin copolymer (COC), and cyclic olefin polymer (COP). The assay biology itself and sometimes the preferred detection system might influence the choice of microplate material.

The microplate surface treatment must be well understood to maximize the overall success of an assay and to minimize variability and misleading data interpretations. There are numerous microplate surface modifications available to enhance a preferred biological feature, such as cell attachment for a cell-based assay or surface adsorption of a capture molecule for an ELISA. Alternatively, surface modifications can be selected to minimize an effect, such as protein adsorption by an enzyme or other assay component. One critical point to be aware of while investigating surface treatments is the within-well, intra-plate, and inter-plate uniformity of the surface treatment. It is recommended to have a QC workflow either within the assay development lab or with the vendor to ensure that the variability of the surface coatings are understood and within the tolerance limits. Such a workflow can include an investigation of uniformity (see also Assay Guidance Manual chapter HTS Assay Validation), reference compound performance, and assay robustness metrics (e.g., Z', signal-to-background ratios).

There are additional components of the assay workflow that can have an impact on the selection of an optimal microplate for an assay and screen. Two of the most impactful components of an assay system are the assay temperature sensitivity and the microplate lid. To minimize rework of the microplate selection process, it is recommended to decide on the temperature at each step of the assay and the acceptable tolerances before beginning the selection process. The same recommendation is made for the selection of a microplate lid. There are a variety of lids available on the market, not all of which are compatible with all plate types and with all types of automation. It is also a good idea to select the lid type with the end automation platform in mind to avoid costly delays in revalidating/redeveloping an assay due to an incompatible lid type.

Finally, many microplate vendors provide plate selection guides to facilitate choosing an optimal microplate within their product line. These guides typically provide a detailed overview of the plate types, surface treatments, and typical applications.

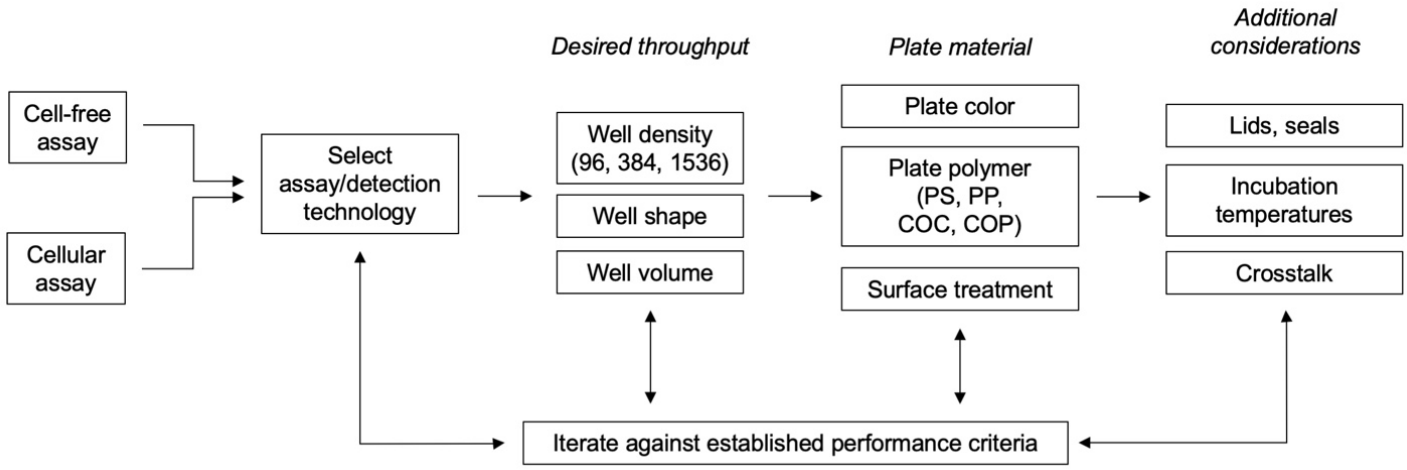


Figure 2. Flowchart for a microplate selection process. This flowchart provides a potential workflow to identify an optimal assay microplate for screening. In practice, the selection of a microplate is usually an iterative process that must balance competing factors such as automation compatibility, cost, availability, and performance.

Microplate well density

Microplates for HTS applications are available with various numbers of individual wells, usually from 96 to 1,536 wells per plate, each with distinct advantages and disadvantages (Figure 3, Table 1). Microplates with 6, 12, 24, and 48 wells per plate are also used for certain lower-throughput and/or specialized applications including HCS (e.g., *ex vivo*) and experiments which may require manual manipulations of the microplate well contents. In general, the reaction volume per well decreases with increasing numbers of wells per plate. Overall, the cost per well is relatively constant as higher-density microplates are more expensive (Table 1). However, miniaturization reduces the quantity of reagents required for each well, which reduces the cost per test point. Additionally, working with higher-density plates reduces the total number of plates required to perform a screen, which decreases the labor required, processing time, as well as wear and tear of the equipment. Thus working with higher-density plates can be a cost-effective strategy. However, miniaturization can present new technical challenges for the assay as well as the need for instrumentation capable of dispensing compounds and reagents into higher-density formats. The overall throughput will ultimately depend on a variety of technical factors including instrumentation (e.g., plate reader speed, with well-by-well reads slower than whole-plate reads) and properties of the assay itself such as readout stability and reagent availability (e.g., precious or difficult to culture cells).

Micro-surfaces. Note that surface area only refers to gross surface area and does not account for microscopic effects due to surface asperities (i.e., unevenness of surface, roughness, ruggedness). Some of these asperities are due to the microplate production process (molds for microplates are machined so that the microplates will retain the inverse of the mold surface features). It has been observed that such surface asperities can affect certain biological assays. For more information, see the subsequent section "Troubleshooting and Special Topics – Microplate Surfaces and Associated Properties".

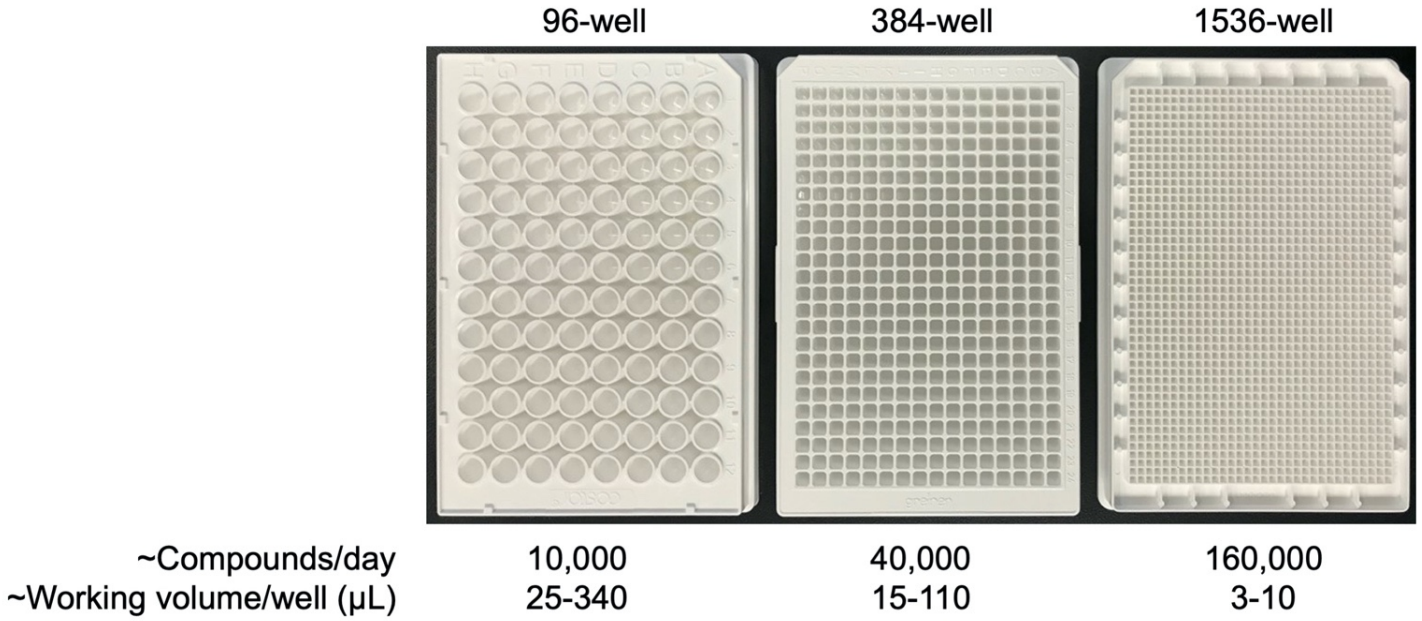


Figure 3. Typical *in vitro* assay microplate densities. Examples of the plate density of 96-, 384-, and 1,536-well microplates. Per microplate, increasing well density enables more compounds to be tested per experiment.

Table 1. Comparison of microplates with circular, F-bottom wells. Exact characteristics will vary based on individual microplates. For sample comparison of cost per microplate well, data was taken from a major microplate manufacturer for otherwise identical black, F-Bottom, TC-treated, sterile plates with lids.

Number of wells	Typical gross well surface area (GWSA, mm^2)[a]	Typical working surface area (WSA, mm^2)[b]	Typical well volumes (µL)	Typical well working volumes (µL)	Typical working volume/ plate (mL)	Cost/ microplate (USD)	Cost/ well (USD)	Pros	Cons
96	259	34	250-382	25-340	12-33	$2.74	$0.03	Supported by most instruments and manufacturers; adaptable to both manual and automated workflows	Increased reagent per well; required reagent volumes can present challenges for supply
96 half-area	173	15	125-175	15-75	1.5-17			Reduction in reagent use compared to conventional 96-well microplates	More limited product catalog
384	140	10	100-152	15-145	6-56	$5.43	$0.02	Supported by most instruments and manufacturers; adaptable to both manual and automated workflows	Overall volumes can be limiting and expensive for custom reagents for uHTS

Table 1. continued from previous page.

Number of wells	Typical gross well surface area (GWSA, mm^2)[a]	Typical working surface area (WSA, mm^2)[b]	Typical well volumes (µL)	Typical well working volumes (µL)	Typical working volume/ plate (mL)	Cost/ microplate (USD)	Cost/ well (USD)	Pros	Cons
384 low-volume	48	5	10-30	4-25	1.5-9.6			Reduction in reagent use compared to conventional 384-well microplates	More limited product catalog
1,536	26	2	10-15	3-10	5-15	$20.20	$0.01	Cheapest per well Least reagent per well	Specialized instrumentation Mixing can be limited to diffusion More limited product catalog Difficult to work with in a manual mode

[a] Does not consider micro-surface areas; spherical well shape using formula GSWA = $2\pi rh + \pi r^2$
[b] Does not consider micro-surface areas; spherical well shape using formula WSA = πr^2

Microplate color

The choice of microplate color and base material is usually based on the type of assay readout, most often to maximize the signal-to-background ratio (Table 2). Microplates generally come in four colors: clear, white, grey, and black:

- Clear microplates are typically used for absorbance (colorimetric)-based readouts.
  - Note that most conventional polystyrene microplates are not suited for UV light transmission. UV-transparent microplates are often made from COC or COP which can typically measure wavelengths as low as 230 nm.
- White microplates are most often used for luminescence-based readouts. The white color (often from titanium dioxide, TiO_2) can reduce well-to-well crosstalk, while enhancing the luminescence signal by better reflecting the light.
  - Example assay technologies include AlphaScreen (and related technologies such as AlphaPlex, AlphaLISA; PerkinElmer), time-resolved fluorescence (TRF), time-resolved fluorescence-resonance energy transfer (TR-FRET), homogeneous time-resolved fluorescence (HTRF; Cisbio), and bioluminescence resonance energy transfer (BRET; e.g., NanoBRET, Promega).
  - Can be used with fluorophores that have longer half-lives (µsec to msec), as the background autofluorescence has usually diminished when the assay readout is measured.
  - White microplates can be "dark-adapted" to reduce background phosphorescence by the plate materials. This is typically done by placing the microplate in reduced light conditions for ten minutes before reading the microplate.
- Black microplates are well-suited for fluorescence-based readouts, which have higher signal intensities than luminescence and therefore do not need the reflective contributions from the white color. Similar to white microplates, the black color (often from carbon) can reduce well-to-well crosstalk, while also reducing background autofluorescence and phosphorescence from test compounds, reaction mixture components, and the microplate itself.

    - Example technologies include fluorescence intensity (FI), FRET, and fluorescence polarization (FP).
- Other less common colors include grey and yellow microplates for specific assay technologies. Grey is an attempt to compromise between white and black microplates, and proprietary grey plates have been developed for certain applications such as Alpha technology. Yellow plates have been developed for certain TRF applications to reduce background autofluorescence. An intra-run comparison between specialized and conventionally-colored microplates is usually the best approach to objectively determine if there are any benefits to such boutique microplates.
- Suboptimal choice of microplate color will often manifest as [1] lower signal-to-background ratios compared to the optimal microplate color, and/or [2] well-to-well crosstalk when highly active and inactive samples are adjacent to one another.

Microplates can also have clear-bottom wells to facilitate optical measurements from the bottom, including high-resolution microscopy. For many cell-based assays, it can be highly advantageous to use a clear-bottom microplate to enable spot-checking of the cells for proper cell density and morphology.

Table 2. Comparison of microplates by color. The optimal choice of microplate color is usually based on the assay technology to maximize the signal-to-background ratio.

Microplate color	Recommended assay formats – top read	Recommended assay formats – bottom read	Notes
Clear	Colorimetric/Absorbance	Colorimetric/Absorbance	Consider COC/COP when needing near-UV light transmission
White	Luminescence AlphaScreen/AlphaPlex/AlphaLISA Time-resolved fluorescence (TRF)		Reduces well-to-well crosstalk Enhances luminescence by reflecting light
White with clear bottom		Colorimetric/Absorbance Luminescence	Enables cell visualization in cell-based normal and confocal assays
Black	Fluorescence intensity (FI) Fluorescence-resonance energy transfer (FRET) Fluorescence polarization (FP)	Fluorescence intensity (FI) Fluorescence-resonance energy transfer (FRET)	Quenches background/nonspecific fluorescence Reduced well-to-well crosstalk
Black with clear bottom		Fluorescence intensity (FI) Fluorescence-resonance energy transfer (FRET)	Enables cell visualization in cell-based normal and confocal assays
Grey	AlphaScreen/AlphaPlex/AlphaLISA		Reportedly optimized for Alpha-based assays

Microplate well shape

The selection of a microplate well shape usually follows the choice of assay technology. Microplate wells typically come in round, square, or rounded-square geometries (Figure 4).

- Round wells have less total area compared to square wells, which can be advantageous for minimizing reaction volumes. Also, the shape of a round well promotes mixing in a circular vortex, whereas vortex mixing in a square well (described below) is not optimal. The round corner-less shape may reduce certain intra-well effects and the lack of corners eliminates wicking effects (see below).
- Square-shaped wells with flat bottoms maximize the area for light transmission and hold a larger volume of liquid than round wells. Depending on the surface tension and viscosity of the liquid sample dispensed into a square well, the geometry can result in a slow wicking of liquid up along the 90° angles of the well

corners towards the top of the plate. For example, organic solvents including methyl *tert*-butyl ether, acetonitrile, and methanol are known to be susceptible to wicking. This can result in sample accumulation near the top of the wells, facilitating evaporation and possibly cross-contamination between wells. Wicking can also make it difficult to retrieve the entire volume of liquid within a well. The use of square wells with rounded corners (Figure 4) can reduce the phenomenon of wicking, which does not occur in round wells (10). See also subsequent section Troubleshooting and Special Topics – Well-to-Well Contamination.

The bottoms of wells can be flat (F-bottom), conical (V-bottom), rounded/spherical (U-bottom) or flat with a rounded edge (C-bottom):

- Flat-bottom wells (F-bottom) allow for the maximum transmission of light and are well-suited for bottom-reading applications, including total fluorescence intensity measurements from plate readers or high-content imaging from automated microscopes and screening systems. Additionally, flat bottom wells are ideal for adherent cell cultures grown as monolayers.
- Conical bottom (V-bottom) wells enable maximal retrieval of small sample volumes or the concentration of small particles. This well shape was originally designed for hemagglutination assays, where a small pellet of red blood cells could be concentrated at the tip of the V-shaped cone (11).
- Rounded/spherical bottom (U-bottom) wells are intended to facilitate mixing, washing and coating as well as the retrieval of solutions from wells. Rounded wells may also decrease trapped air at the bottom of the well, enabling a more consistent meniscus level from well-to-well. This well shape is often used with suspension cells and spheroid or organoid cultures (12).
- Curved bottom (C-bottom) wells combine features of both the flat bottom and round bottom wells, with a flat bottom suitable for optical measurements, but with rounded corners to facilitate mixing and washing (11). Also, this well shape is well-suited for use with suspension cells, spheroids, or organoid cultures (12).

Microplate well volume

Microplates wells have a usable "working volume" and are manufactured as normal and low-volume variants (Figure 5). At a certain point, assay performance declines by simply reducing the reaction volume and specialized microplates can enable a lower working volume. Lower-volume variants are usually constructed by tapering the well along the vertical dimension, and/or reducing the well depth. When utilizing low-volume microplates (especially those with reduced well depths), users should carefully ensure their compatibility with instrumentation. For example, pin tools would need to have their depth reduced for low-volume microplates to avoid damaging the pins, and certain microplate readers may benefit from different read heights to accommodate altered sample heights.

Microplate polymers

The most common microplate materials are the polymers polystyrene (PS), polypropylene (PP), COP, and COC (Figure 6). Each of these polymers has advantages and disadvantages (Table 3). For example, COP has many favorable properties including low autofluorescence, UV transparency, and mechanical stiffness (13).

The choice of microplate polymer can impact assay quality due to properties such light transmission, autofluorescence, water absorption, and gas exchange. Light absorption by a polymer decreases sensitivity in fluorescence assays by attenuating the intensity of the excitation illumination, while autofluorescence from a polymer can decrease sensitivity and dynamic range by contributing additional light to the assay emission signal. The common polymer PS is generally not suitable for transmitting UV light, while COC/COP are often used for such purposes. The autofluorescence from a microplate can also vary depending on the plate material will also vary based on the pigments used in generating the color.

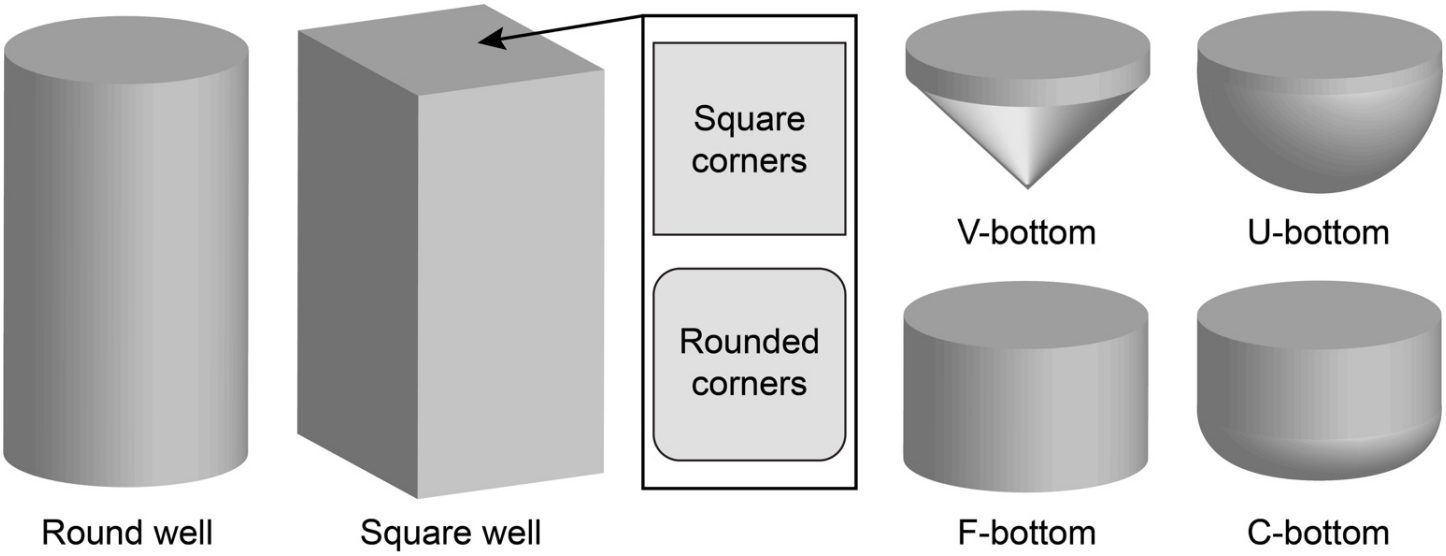


Figure 4. Schematic of microplate well shapes. Microplate wells generally have either a round or square shape. Square-shaped wells can have either straight or rounded corners. Wells can have a variety of bottom shapes including conical (V-bottom), rounded/spherical (U-bottom), flat (F-bottom) and flat with rounded corners or curved bottom (C-bottom) that endow the well with different properties.

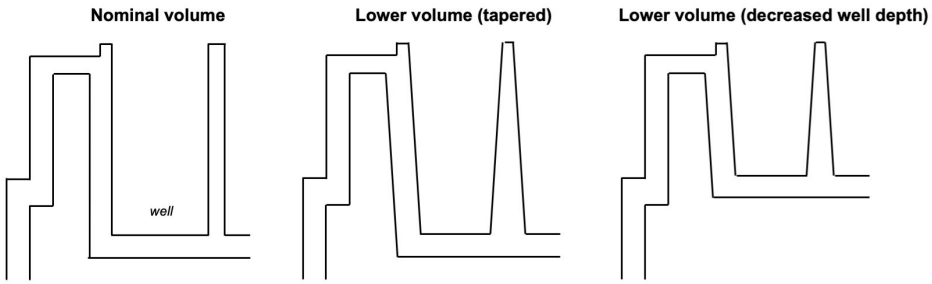


Figure 5. Comparison of microplate volumes. Lower-volume variants are available from some plate manufacturers. Volume reduction is usually obtained by tapering the well shape along the vertical plate axis and/or reducing the well depth.

Polymers can also have different propensities to absorb water and exchange gases (13). Finally, polymers have different mechanical and thermal properties (Table 3). The mechanical properties are important considerations during the manufacturing process, but more relevant for end-users, day-to-day handling where overly brittle or flexible polymers can crack or warp with aggressive handling, respectively. Thermal properties can play a role when sealing plates with heat sealers, as excessive or prolonged heating can result in warping of the plate (small degrees of bending or torsion can cause significant perturbations) and also issues with imaging or handling of the plates by automated systems.

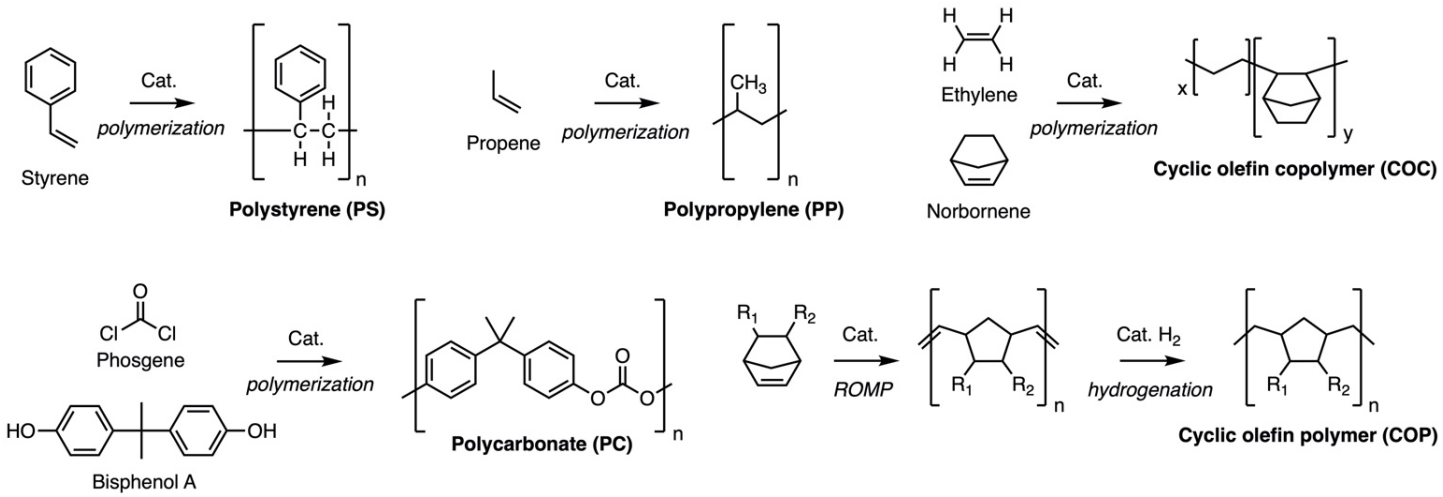


Figure 6. Chemical structures and general syntheses of common microplate materials. For the most common microplate materials, monomers are polymerized with the aid of catalysts to yield the desired polymer.

Table 3. Comparison of thermal and mechanical properties of microplate polymers. Note that COP behaves mechanically similar to PS during molding. Adapted from Niles and Coassin (13). COP, Zeonor 1420R; PS, high-impact, injection mold-grade.

	COP	PS	PP
Flexural modulus (GPa)	2.2	3.3	0.55-2.41
Flexural strength (Mpa)	94	103	23-50
Tensile modulus (Gpa)	2.4	3.4	0.5-7.6
Tensile strength (Mpa)	61	80	12-43
Glass transition temperature (°C)	136	90-104	44-148

Microplate surfaces and treatments

The surface properties of microplates are fundamentally determined by the chemical composition of the plate polymer. Olefins like PP, COP, and COC consist of saturated hydrogen and carbon only. These materials tend to be very hydrophobic and present high contact angles with water when untreated by standard plasma and corona discharge systems (reminder: hydrophilic surfaces have lower contact angles with water droplets, while hydrophobic surfaces repeal water droplets and produce higher contact angles). These hydrophobic surfaces have a low surface energy and low protein adsorption. PS is an example of an unsaturated hydrocarbon-based material that has aromatic substituents which confer partial positive and negative charges, and as a result, untreated PS will adsorb more protein to the surface than untreated olefins. Polycarbonate microplates (now rarely used due to high leachables like bisphenol A ("BPA") and heavy metals; and high fluorescence backgrounds) are an example of a polymer with aromatic functionality and heteroatom contributions due to the presence of oxygen in the polymer.

The primary purpose of cell culture treatment to microplates is to make the microplate surface more amenable to mammalian cell culture. Enhancing wettability and providing surface charge enables cell adhesion molecules to "attach" firmly to the surface *via* charge neutralization. There are two primary techniques that have been used for decades to surface-modify plastics: [1] radio frequency microwave plasma (RFMP) and [2] corona discharge *via* high voltage in regular atmosphere. Both of these processes generate covalently-modified surfaces. The RFMP technique is generally preferred for scientific applications because of the uniformity of the surface treatment. RFMP is a vacuum-based process whereby electric fields create plasma (charged gas) from a carrier

gas (commonly CO_2, O_2, or NO_2). These gases are released at very low pressures into a reaction chamber, and collisions between the charged gas molecules and the microplate surface result in covalent attachments of the gas to the surface, effectively creating chemical functionality. If CO_2 is used as the carrier gas, a carboxyl surface with a negative charge is generated; if O_2 is used, an ether and hydroxide surface that is polar but neutral is generated; and if NO_2 or NH_3 are used, an amine surface is generated with a positive charge. The CO_2 or NH_2 that are incorporated into polymer surfaces can also be exploited to attach molecules with an appropriate conjugation chemistry. This primary surface treatment is essential if a secondary treatment is necessary.

Additional chemical functionality can be conferred to the surface with the use of special chemicals and modified plasma instruments (Table 4). Epoxide groups, tri-amino-hydrocarbons, non-fouling (e.g., resists protein adsorption) low-adhesion molecules, diethyl ether, and azides are a few relevant examples. It should be noted that surface treatments alter the surface but not the bulk polymer. These treatments are durable but have limitations and must be protected from excessive heat. It can sometimes be helpful to think of the polymer as a very viscous liquid that can slowly rotate and invert the charged functionality away from the microplate surface, which can effectively eliminate the original surface treatment modification (14).

Secondary treatments can typically include liquid-based biologicals such as poly-D-lysine (PDL), poly-L-lysine (PLL), laminen, collagen, hydrogels, or cellular extracts. These surface treatments are used because they can enhance adherence for specialized cells and can be critical for more biologically relevant morphology, growth, migration, and differentiation of many cell types. In practice, these biological materials do not adsorb well to untreated polyolefin or polystyrene plates. Therefore, when commissioning a secondary treatment (often done for special cell-based assay applications), it is important to make sure that the original microplate product is "TC-treated" first. Another important consideration is whether to have the secondary treatment performed locally or by a manufacturer.

There are anecdotal reports that fresher secondary treatments perform better, presumably because over time the microplate reverts to the neutral/unmodified polymer surface (PA Coassin, unpublished observations). As this phenomena has not been widely documented (i.e., treatments most susceptible to degradation, magnitude of degradation), scientists should define early-on in the project the acceptable performance criteria for surface-treated microplates (e.g., less than 20% deviation of historical max control signal), and then include periodic experiments to detect shifts in assay performance over time. Approaches for detecting performance shifts and drifts are discussed in subsequent sections ("Special Topics and Troubleshooting – Microplate Lots).

Table 4. Comparison of microplate surface treatments. For cell-free assays, surfaces can be treated to enhance or attenuate biomolecule binding, as well as functionalized with reactive functional groups (e.g., maleimides, amines) or affinity reagents (e.g., streptavidin) to anchor specific biomolecules. For cell-based assays, surfaces must be tissue-culture treated to functionalize the surface; following this, additional secondary treatments such as the addition of extracellular matrix (ECM) coatings can be performed to enhance adherence for specialized cell types.

Surface treatment	Recommended assay formats	Notes
Non-treated (medium binding)	Immunoassays	Usually PS which has a hydrophobic surface that passively/nonspecifically adsorbs biomolecules
Non-binding surfaces	Homogenous assays (including enzymatic assays)	Treated PS with nonionic, hydrophilic surface to reduce polymer-biomolecule interactions; net effect is the reduction of nucleic acid and protein binding
High-binding	Immunoassays	Suitable for binding biomolecules with ionized groups
Streptavidin	ELISA	Contains streptavidin which can bind biotin-containing reagents
Sulfhydryl-binding surfaces		Contains thiol-reactive maleimides on polymer surface

Table 4. continued from previous page.

Surface treatment	Recommended assay formats	Notes
Tissue culture (TC)-treated	General adherent cell culture	Creates a net-negative surface charge (*via* carboxyl groups)
TC-treated, Poly-(D ± L)-lysine (PDL, PLL)	Specialized adherent cell cultures	Non-biological reagent, therefore does not significantly modulate surface receptors Creates a net-positive surface charge; can be useful for difficult-to-attach cell lines (e.g., neuronal)
TC-treated, ECM coatings	Specialized adherent cell cultures	Critical for biologically relevant adherence, morphology, growth, migration, and differentiation in many cell types Collagen I: HUVEC, hepatocytes, muscle cells, osteoclasts

Barcoding

Depending on the expected throughput, plates can be labelled manually or with barcodes. Many modern screening instruments are equipped with barcode readers. Barcoding enables unambiguous plate identification and reduces errors from manual labeling methods. Barcodes are typically placed on a short side of the microplate and can be left-, center-, or right-aligned. Modern barcoding can consist of an alphanumeric text, linear barcode, or matrix (2D) barcode. Many vendors offer pre-barcoded plates, which can be especially advantageous for microplates to be used in sterile conditions. Otherwise, microplates must be removed from the sterile packaging in order to be barcoded. Barcoding can also be performed on-site with appropriate instrumentation, but is often nonsterile.

Additional considerations for barcodes include:

- When using any barcode system, ensure that the barcode format is readable by the relevant instruments.
- Stability of the barcode to storage and handling conditions. Barcodes might not adhere to microplates in excessive heat or cold, or if exposed to certain solvents which might dissolve the adhesive. A common mistake is to attach non-cryogenic labels to a batch of microplates prior then place them into a freezer, only to lose all barcoding information.

Microplate covers (lids, seals)

During various assay steps, microplates can be sealed, covered with a lid, or left uncovered. Microplate covers are generally made of clear polystyrene, while plate seals are typically made of aluminum or polyolefin acrylates. A special lid used on GNF robotic systems is designed from a solid block of stainless steel and fitted with an autoclavable sealing rubber gasket designed to increase the plate contact area with the weight of the steel lid ensuring even contact across the plate edges (15). Seals can be generated by an adhesive (e.g., silicone), or by heat (e.g., Agilent PlateLoc). Usual advantages of microplate covers are the prevention of evaporative losses, well-to-well contamination (*via* spillage or wicking), and biological and environmental contamination. Disadvantages of using seals/lids include additional costs and the procedural steps to accommodate their placement and removal. Therefore, the use of lids or seals for microplates must consider multiple factors:

- *Length of incubation time*. If assays are performed in a short time-scale (minutes to several hours), microplate covers might be superfluous as there would be minimal evaporation.
- *Reaction volume*. When using lids or seals, generally avoid filling microplate wells with greater than 50% of the well volume, which mitigates contact between the well contents and the lid.
- *Condensation*. The well contents may condense at the top of lids or seals, which can affect assay performance and even facilitate well-to-well contamination. A brief centrifugation can usually recover condensates from lids or seals prior to their removal. For certain applications, such as live-cell imaging

where lids are used, condensation can interfere with image acquisition (Figure 7A). This can be mitigated with humidified incubators within the instrument combined with a short (30 - 60 min) equilibration period prior to imaging.

- *Number and timing of assay steps*. If using lids or seals, carefully consider the timing of the placement and removal steps. For biochemical assays, it might only be necessary to cover microplates after the initiation of the biological reaction, whereas for cell-based assays it is generally recommended to cover microplates whenever there is not active dispensing.
- *Compatibility with automation and instrumentation.* Certain instruments might be able to remove and replace microplate lids. This is generally a manual process for microplate seals.
- *Detection.* For certain bottom-reading applications, the presence of lids or seals might not interfere with the readout detection (e.g., fluorescence imaging), whereas it may interfere with other applications that require light transmission between the top and bottom of the microplate (e.g., phase-contrast microscopy).
- *Sealants*. Microplate sealants have the potential to chemically modulate bioactivity or interfere with light-based readouts (Figure 7B).
- *Continuous versus end-point assay formats.* Continuous or kinetic assays with multiple detection steps might necessitate the repeated removal and addition of microplate covers, especially if multiple plates are being analyzed in a given experiment. To prevent this logistical burden, consider bottom-read options.
- *Prevention of biological contamination.* Depending on the assay, biological contamination might not be a major concern, such as simple biochemical assays. However, for cell-based assays, covering microplates is practically essential.
- *Prevention of environmental contamination.* Environmental contamination from airborne particulates (dust) might be a concern for certain light-based readouts (see also subsequent section, "Special Topics – Environmental Contaminants").
- *Permeability.* When sealing microplates, consider whether there is a need for gas and/or water permeability. Certain materials designed for such permeability, while other seals might prevent adequate gas exchange between the incubator and the microplate wells, which can modulate cell functions by perturbing media pH and oxygen content.
- *Lid stability*. Note that certain lid designs might not be as secure as others and therefore might be inadvertently opened (i.e., important for sterility) if not handled with care (Figure 7C).
- *Photo protection*. For light-sensitive applications (such as labile fluorophores or AlphaScreen), seals or opaque lids can be used to protect the well contents from light in addition to the usual light-reduction strategies.

Section summary

A key factor in obtaining robust assay performance is the optimal choice of a microplate. During assay development and optimization, scientists should select microplates that meet their technical requirements. Key parameters in microplate selection include microplate well number, well shape, plate color, plate material, and surface coatings or treatments.

Technology-Specific Considerations – High-Content Screening

When selecting microplates, consideration must be given to the specific assay technology. This section describes detailed considerations for high-yield specialized applications, specifically high-content imaging. *Other specialized microplate applications will be described in updated versions of this chapter or in relevant Assay Guidance Manual chapters.*