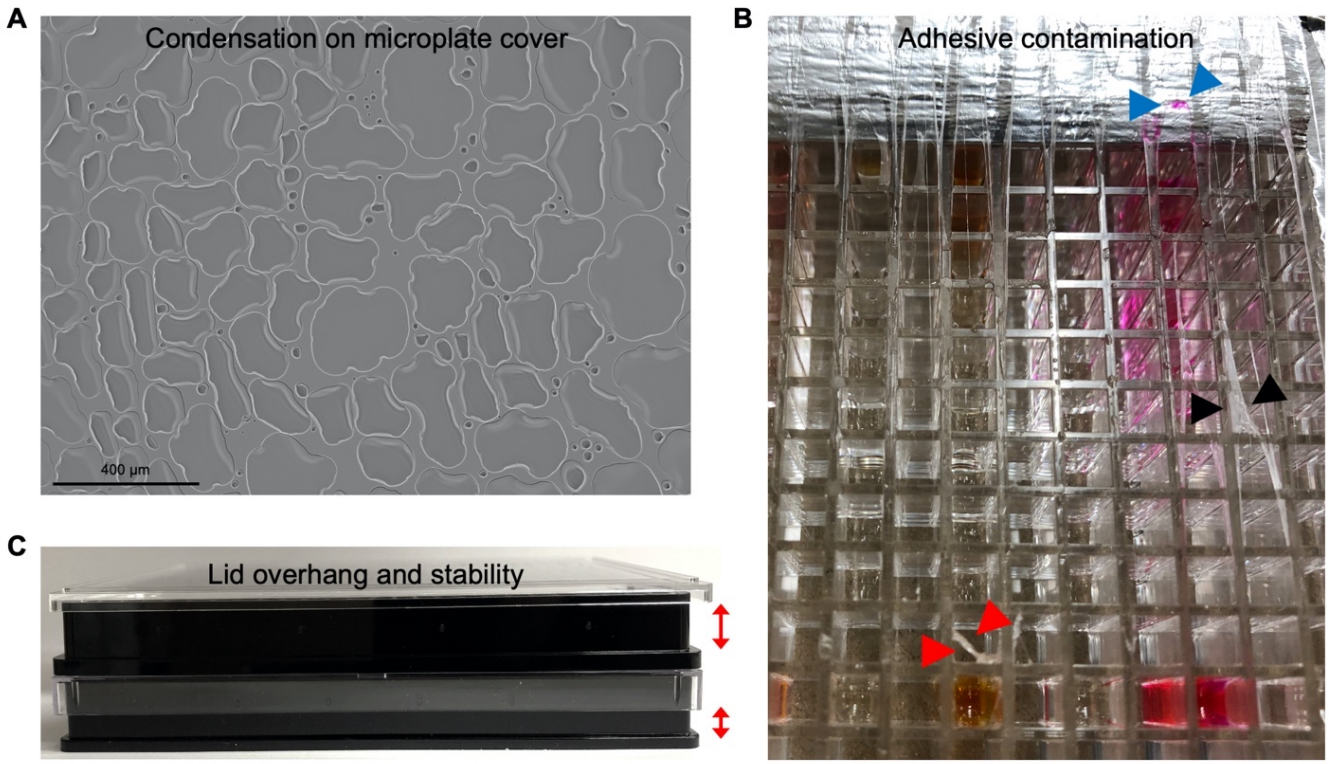


Figure 7. Microplate covering considerations. (A) Condensation interference. Water condensation can occur on microplate covers, which can interfere with certain imaging applications requiring light transmission, as in this case of live-cell imaging. Condensation can be mitigated by adequate equilibration (30 - 60 min) in a humidified incubator prior to image acquisition. (B) Sealant interference. When utilizing microplate seals with adhesives, be aware that sealants can adhere to the microplate (between black arrowheads) and even contaminate well contents (between red arrowheads). Also note that the well contents can adhere to the interior surface of the seal, even after a brief centrifugation (between blue arrowheads). Shown is a clear 384-well polystyrene microplate sealed for six months (RT, desiccator) with an aluminum-based adhesive seal. (C) Microplate lid stability. When utilizing some microplate lid designs, be aware that the lids may not descend as completely toward the microplate flange (see top plate, gap indicated by red arrows), which can make the lids susceptible to inadvertently opening if not handled carefully.

Considerations for choosing microplates in high-content screening applications

High-content screening (HCS, also used interchangeably with high-content imaging (HCI), high-content analysis (HCA), or image cytometry), encompasses a platform of automated computer-assisted fluorescent and transmission-light microscopy to rapidly capture images of cells or objects (16). Most HCS experiments begin with the process of seeding cells into microplate wells but may also include microscope slides, petri dishes, or other specialized vessels. Generating reliable and reproducible HCS data requires high-quality images, therefore careful considerations when selecting microplates is a key driver during experimental design.

There are several commercial choices for "image compatible" microplates for HCS applications, with most microplates constructed with black walls to reduce fluorescent signal crosstalk from adjacent wells and fabricated with an optically clear bottom (COC/COP, glass, or quartz) for transmission of fluorescent light to the cells or objects of interest when using inverted microscope systems. The ideal microplate bottom surface should be uniform with a bottom thickness of approximately ~170 µm (equivalent to number 2 cover slip) to match microscope objective lenses with high numerical apertures (NA; e.g., 0.17 mm). While imaging-compatible microplates are readily available from commercial suppliers, there remain a few key considerations when

selecting microplates for HCS applications that include the cell model being investigated, the microplate type, design and composition materials as previous described in the chapter, and the optics on HCS imager including the autofocus mechanism to identify cells or objects in the microplate wells. The *biology* is the most important criteria of HCS experimental design, and this will dictate which cell model is used and the subsequent microplate selection, both of which may or may not be ideal for HCS imaging, therefore understanding the limitations and challenges to generate high-quality images is important to gain insights into the biological question.

Even with the advances in manufacturing and fabrication of microplates and improved HCS imaging instrument technologies, there are still challenges and obstacles to consider when matching microplates with the optics of the HCS imagers. Ultralow attachment (ULA) microplates are designed to force cells into the center of the well bottom to physically form 3D spheroid-like structures (12). One of the challenges in ULA microplate use is not all cells form spheroid-like structures and may not remain in the center of the well bottom due to manipulations or handling microplates during treatment, staining, or other automation manipulations. The outcome may lead to subsequent challenges in image capture of objects in ULA microplates versus flat-bottom microplates (Figure 8).

HCS cell models

Since the cell model is likely the most important criteria of the biological experimental design to study disease or address a biological question, the selection of the microplate type often is usually not considered early during the experimental design process. Furthermore, not all cell types are amendable to miniaturization in microplate format or can even be sustained on conventional microplate materials. In HCS applications, cell models are categorized as adherent, suspension, microtissue, *ex vivo*, or even small organisms (e.g., *Caenorhabditis elegans*, *Danio rerio*). Some cell models may require additional growth enhancements for attachment, survival, or to promote a biological response in the artificial environment of microplates. These enhancements may include growth factors, PDL, ECM, or other matrices. Both 2D and 3D cell models are widely used in HCS imaging applications and although the selected microplate dictates the type of assay, the choice of microplate will have a direct consequence on how the cell model is captured and interrogated by HCS imaging system.

Adherent immortalized cell lines are the most widely used cell model in HCS today. Stem cells and primary cells alone or combined with more sophisticated complex cell model systems (including mixed cell cultures such as primary human tumoroids and other normal and diseased tissue cell models) are also being used by HCS practitioners. After the selection of the microplate and secondary surface treatments (if applicable), the cell seeding process is an essential step to allow cells to naturally adhere, spread, proliferate, or remain in stasis over hours to days in optimized media and environmental conditions. For basic 2D imaging, the "flatter" the cell in z-dimension and wider in x- and y-dimensions (e.g., U-2 OS cells), the easier it is for an HCS imaging device to find and focus on the cells during the automated image acquisition step.

When using *suspension* cells in HCS applications, there are two methods to consider when choosing microplates: [1] the coating on bottom of the plate to enhance cell binding, and [2] the geometric shape of the microplate wells. The simplest coating method is PDL, PLL, or equivalent; these synthetic polymer agents help but do not guarantee cell adherence to the bottom of the microplate well. Adherence can be assisted by gentle centrifugation (i.e., 5 min, less than 1000 rpm, greater than room temperature) and is recommended during any subsequent staining process to minimize cell loss. There are other sources of chemical treatments offered by commercial microplate suppliers that have the promise to enhance cell binding such as covalently-bound coatings (e.g., streptavidin, nickel chelate, protein A, lectin wheat germ agglutin, etc.), but these need to be carefully evaluated to make sure they do not adversely perturb the relevant biology. The microplate design can also assist in forcing suspension cells to the bottom of the microplate well; U-bottom and V-bottom microplates are recommended for labeling suspension cells and for preparing higher-throughput flow cytometry experiments. Standard U-bottom and V-bottom microplates are not recommended for HCS imaging due to the thickness at the bottom of

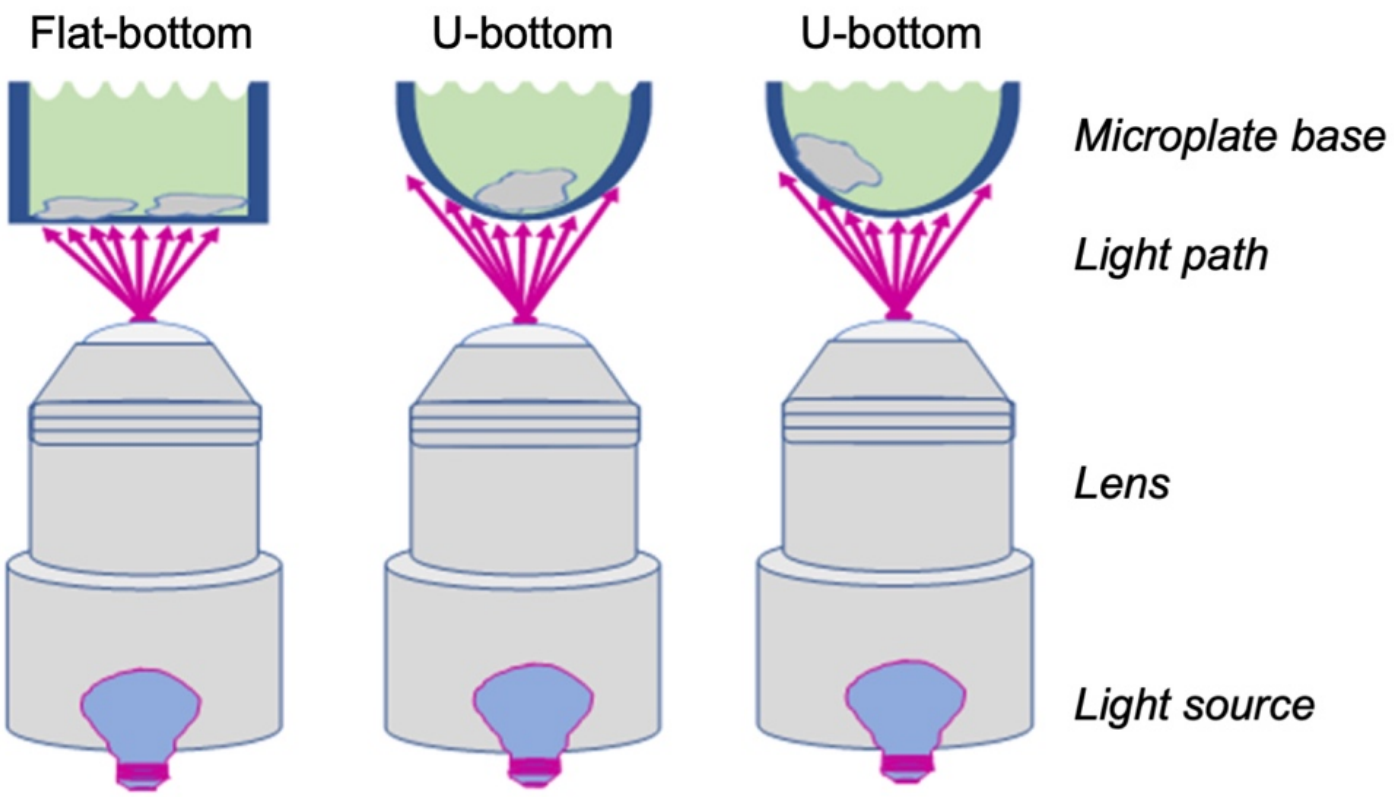


Figure 8. Objective lens light scatter on microplate well bottoms. Left: cellular objects in flat-bottom microwell plate; light scatter detects all objects within the well's field of view. Center: cellular objects in the center of the U-bottom microplate well; light scatter detects objects only in the center of the well. Right: cellular objects on the left curvature part of the U-bottom microplate well; due to the position, light scatter results in an out-of-focus image.

the well and the curved shape are not ideal for microscope objective lens to capture high-quality images of cell objects to allow proper image segmentation (Figure 8). Additionally, there are a few other specialized microplates with geometric designs to force suspension or adherent cells into designated growth areas including micropatterning (e.g., CYTOO and ForCyte Biotechnologies).

The use of microtissue, organoids, and spheroids has grown and gained traction in the field to better represent and recapitulate an *in vivo* response within microplate well environment (17). Microtissues typically require ECM substrates or matrices to support and maintain growth and typically form 3D structures or scaffolds. The microplate type and design may vary from ultralow attachment (ULA) to flat-bottomed microplates containing hydrogel or Matrigel matrices. *Ex vivo* tissue is typically comprised of whole intact tissue slices from organs. While this cell model should best represent the biological function of the organ, the longevity outside of the body is only a few hours or days before succumbing to cell death in the artificial microplate environment. This is not necessarily the fault of the microplate, but rather due to the lack of nutrients and other signaling cascades to sustain tissue viability outside of the body. *Ex vivo* tissue such as brain or liver slices are typically plated into 6-well, 12-well, or 24-well microplate formats due to their size and because higher-throughout experiments are often not feasible. When moving to these larger microwell plate formats, the properties of the microplate construction changes from thin bottom thicknesses (as available in 96-well and 384-well formats) to thicker bottom thicknesses that are not compatible with higher NA objective lenses (e.g., 0.17 mm). If using a higher magnification objective lenses with high NA with microplates having thick bottoms, then the image quality is less optimal, which can manifest later as poorer object segmentation and "blurrier" images (Figure 9).

In vivo imaging of small organisms such as *C. elegans* and *D. rerio* are imageable in microplate wells. One of the biggest challenges using adult organisms in HCS is the reduction of organism movement during image acquisition. Several different methodologies have been used to solve this technical challenge, from chemical treatment to specialized microplates to force organisms into compartments (so they remain upright or semi-stationary). Some specialized microplates have reflective mirrors built into the wells to allow investigators to observe the transverse side of the organism (e.g., ZF microplate, Funakoshi, Japan; PSI SideView microplate, ViewPoint, France).

General considerations when matching a cell model with microplates for HCS imaging

1. When possible, use optically-clear, thin-bottom microplates.
2. Determine the resolution (magnification) requirements to answer the biological question.
3. Decide if cells are "plateable" – do they readily attach?
4. Are PDL, ECM, or other matrices required for cell attachment, for cell health, or for the biological process being studied?
5. Beware of new or undefined microplate composition materials modulating cellular health (e.g., proliferation, cytotoxicity) or relevant bioactivity.
6. Do the microplate well geometric dimensions affect the biology being studied? Consider surface area to support growth and gas exchange needs.
7. Are cells or cell objects imageable by HCS imagers? Does the microplate well have clear bottoms for inverted microscope HCS imagers to transmit light?
8. Are the cell objects plated in a manner to allow adequate image analysis segmentation?

Microplate type and design for HCS imaging

Commercially available HCS imagers are equipped with a microscope stage that supports ANSI/SLAS standard microplate formats. To determine if a given microplate is compatible with a particular HCS imager, practitioners can consult the microplate manufacturers (often posted on microplate supplier website) to determine more exact microplate specifications such as the length, width, and height of the microplate; the well positions; well bottom elevation; the microplate shirt height; outside flange dimensions; bottom thickness; well volume; and distance between wells. These standardized microplate dimensions are critical for proper alignment to capture images of cells or other objects within the microplate well, as this information is applied to the HCS imager instrument software setup to optimize autofocusing and image capture. Some of the most important ANSI/SLAS standard microplate dimensions for microplate HCS imaging instruments are the bottom thickness and skirt height (Figure 10). The bottom thickness informs the selection of the microscope objective lenses, both the numerical aperture and working distance. The microplate skirt height determines if the outside well boundaries from the microplate geometry will physically interfere with the microscope objective lens.

Autofocus mechanism and fluorescent probe effects in HCS imaging

1. *Autofocusing*. HCS imaging instruments use an autofocusing mechanism to determine the location of the cells or objects in the microplate well for rapid image capture. Often this is at the bottom of the microplate wells. The two most common methods of HCS autofocusing are [1] image-based autofocus, otherwise known as contrast-based image focus, and [2] laser-based autofocus (see also Assay Development Guidelines for Image-Based High Content Screening, High Content Analysis and High Content Imaging). Both methods are dependent on the microplate dimensions, the type of materials used in the fabrication of the microplate, the liquid media interface within wells, and the preparation of microplates for the HCS assay for the following reasons: [1] some polymer based plastics may reflect light differently than the glass – the gold standard for optical systems; and [2] contaminates or impurities in plastics or well artifacts can modulate biological affects or identify objects that are not of biological

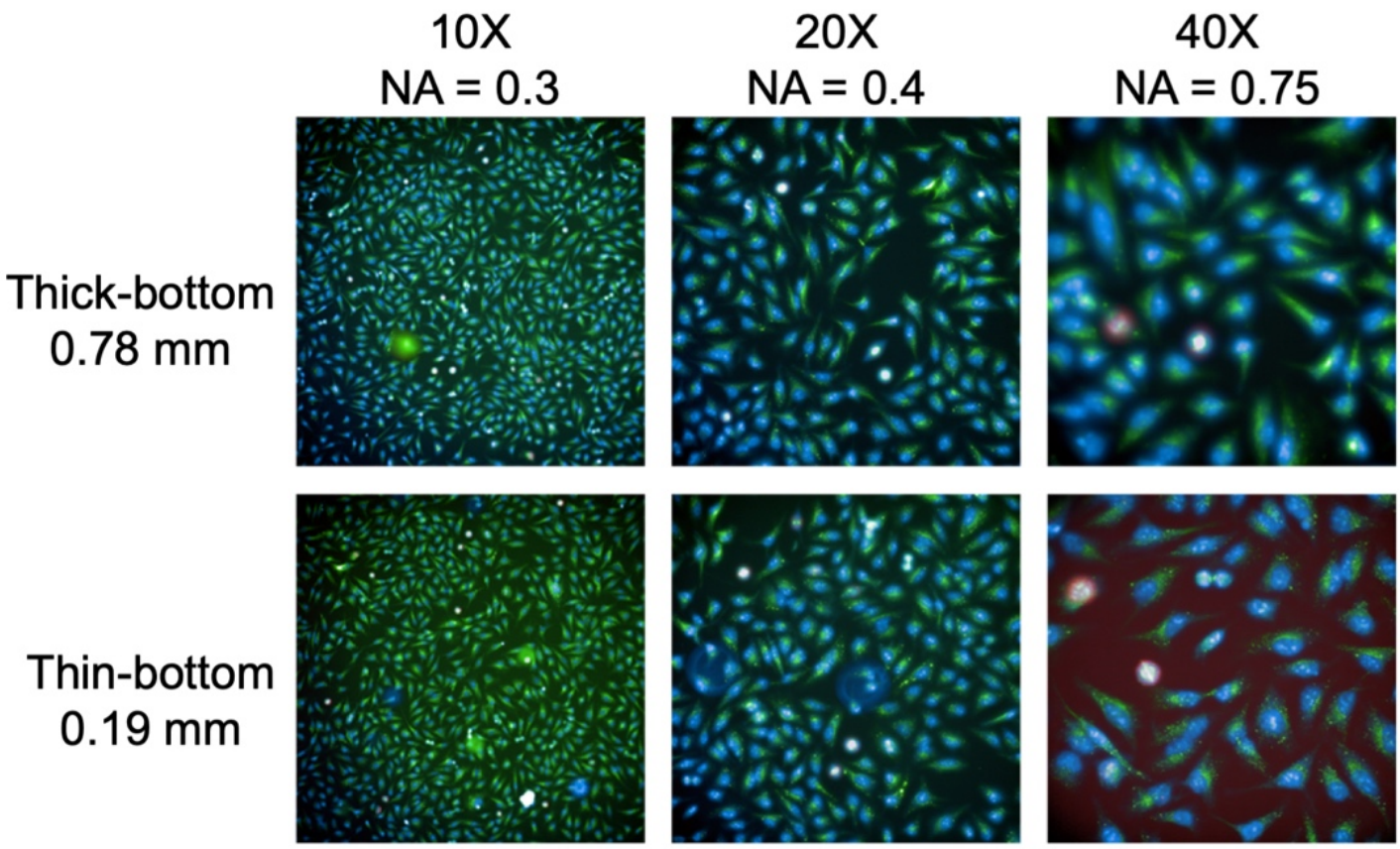


Figure 9. Microplate well bottom thickness compatibility with microscope air objective lenses. HCS images of cells seeded into thick (0.78 mm) and thin (0.19 mm) microplate bottoms. Left: Plan-Neofluar objective lens with a 0.3 NA with the recommended 0.17 mm coverslip thickness/microplate well bottom. However, due to the low magnification, the images of both thin and thick bottom microplate wells are indistinguishable. Center: LD Plan-Neofluar with correction collar has a 0.4 NA with coverslip thickness/ microplate well bottom of 0.55 mm. Note adjusting the correction collar moves the barrel lens elements inside the objective lens to the appropriate setting for proper focus of the image which is ideal for thin and thick microplate bottom thickness. The caveat is correction collar lenses have lower NA, thus lower light throughput. Right: Plan-Neofluar with a 0.3 NA with the recommended 0.17 mm coverslip thickness / microplate well bottom. Note the higher NA with fixed thickness of 0.17 mm allows higher light throughput for reduced exposure times and higher resolution on HCS imaging device. In this example, 190 µm microplate bottom-thickness images (bottom) are in focus and the 780 µm microplate bottom-thickness images (top) are out of focus. Therefore, match the objective lens coverslip thickness setting with a compatible microplate bottom thickness.

interest (18-20). Image-based autofocus, unlike laser-based autofocusing, is more likely to fail when objects are not detectable in the field of view or from artifacts in different z-plane space. However, image-based autofocusing is capable of finding objects when present anywhere in z-dimensional space to the limits of the working distance of the objective lens.

2. *Fluorescent probes*. Unless the fluorescent probe is contaminated, falls out of solution, or forms precipitants, rarely do fluorescence probes interfere with autofocusing process in labeled cells in microplates. However, HCS practitioners need to be cautious of possible interference in unique cases (e.g., fluorescent compounds or lint in bottom of microwell microplate; Figure 11).

HCS imaging instrument optics and microplate specifications

Microplates selection should always be matched with the objective lens numerical aperture, working distance, depth of focus requirements, and minimal planes for z-steps to identify objects in the microplate well. Note some of these details can be found on the microscope objective lens (Figure 12A). HCS imaging instruments are

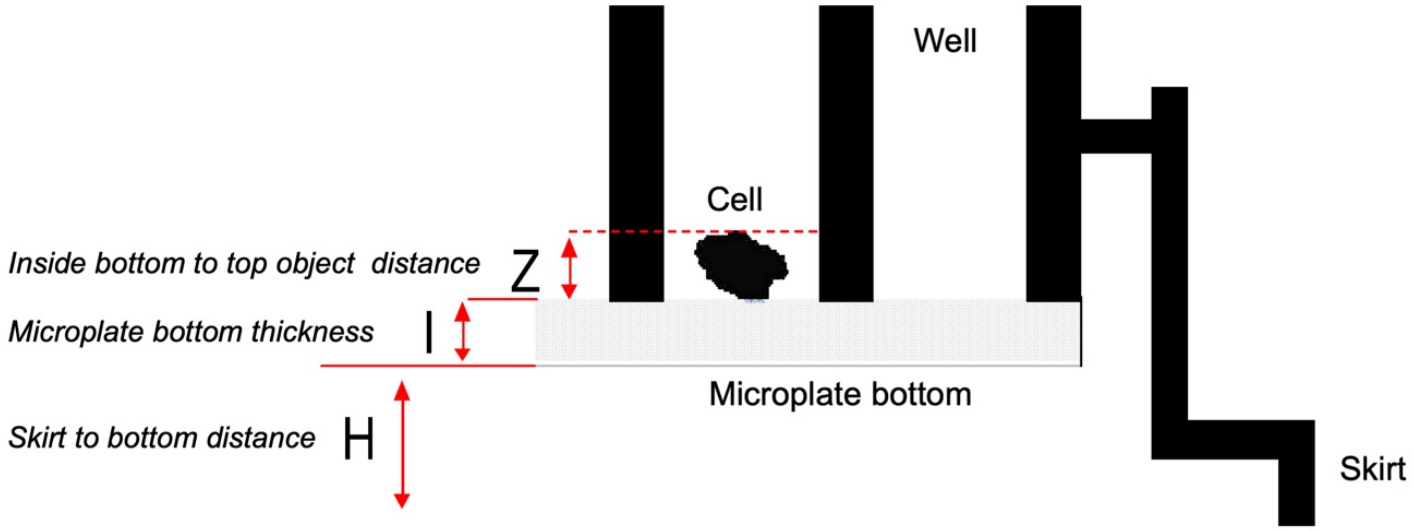


Figure 10. Illustration of the cross-section of a black wall HCS imaging microplate wells. "H" is the distance from the skirt to the microplate well bottom; "I" is the thickness of the microplate well bottom; Z" is the distance from the inside bottom surface of the microplate well to the top of the cellular object inside the well. The combination of H + I will determine if high numerical aperture objective lenses can be used; and the combination of H + I + Z will determine the recommended working distance for the objective lens.

commonly equipped with commercially available microscope objective lenses (e.g., Leica, Nikon, Olympus, Zeiss) to enable multiplexing fluorescent probes. These objective lenses can also reduce chromatic aberrations using plan achromat lenses that use normal working distances. However, HCS instruments can also include long-working objective lens equipped with correction collars to more flexibly dial-in and match the microplate bottom thickness.

Designing, developing, and validating experiments need to consider all aspects of the microplate specification along with these five key features of the objective lens (Figure 12A):

1. *Magnification power*. Selection of the objective lens magnification power enlarges the object size, but *it does not necessarily improve the resolution power*.
2. *Numerical aperture (NA)*. The NA of the objective lens is an important consideration because it indicates the resolving power of a lens (e.g., the size of the finest detail which is directly dependent on the proportional to λ/NA, where λ is the wavelength of the light transmitted). The higher the NA, the more light that enters through the objective lens to the specimen, resulting in a signal increase (intensity) that can be detected by a camera, a photomultiplier tube, or another detection device. Reducing the exposure times helps to reduce photobleaching and phototoxicity. *A lens with a larger NA will be able to visualize finer details than a lens with a smaller NA.*
3. *Working distance (WD)*. The WD is commonly referred to the distance between the interface of the objective lens and the in-focused objects (usually cells) in the microplate well. *As the WD decreases, the magnification power of the objective lens increases.*
4. *Depth of field*. Depth of field (a.k.a. depth of focus) is the tolerance or range of the optical path to keep objects in focal clarity within the desired image plane(s) captured. *High NA objective lenses have a smaller depth of focus range than low NA objective lenses.*
5. *Minimal Z-plane interval spacing*. Lens and HCS instrument manufacturers provide specifications of the minimal z-axial intervals for resolving power based on the Nyquist theorem. Do not over-sample 3D objects; only use the minimal or higher z-intervals to capture objects. In HCS confocal and widefield systems, the spatial resolution of a 3D object (voxel) is typically about one-half of the x-y lateral

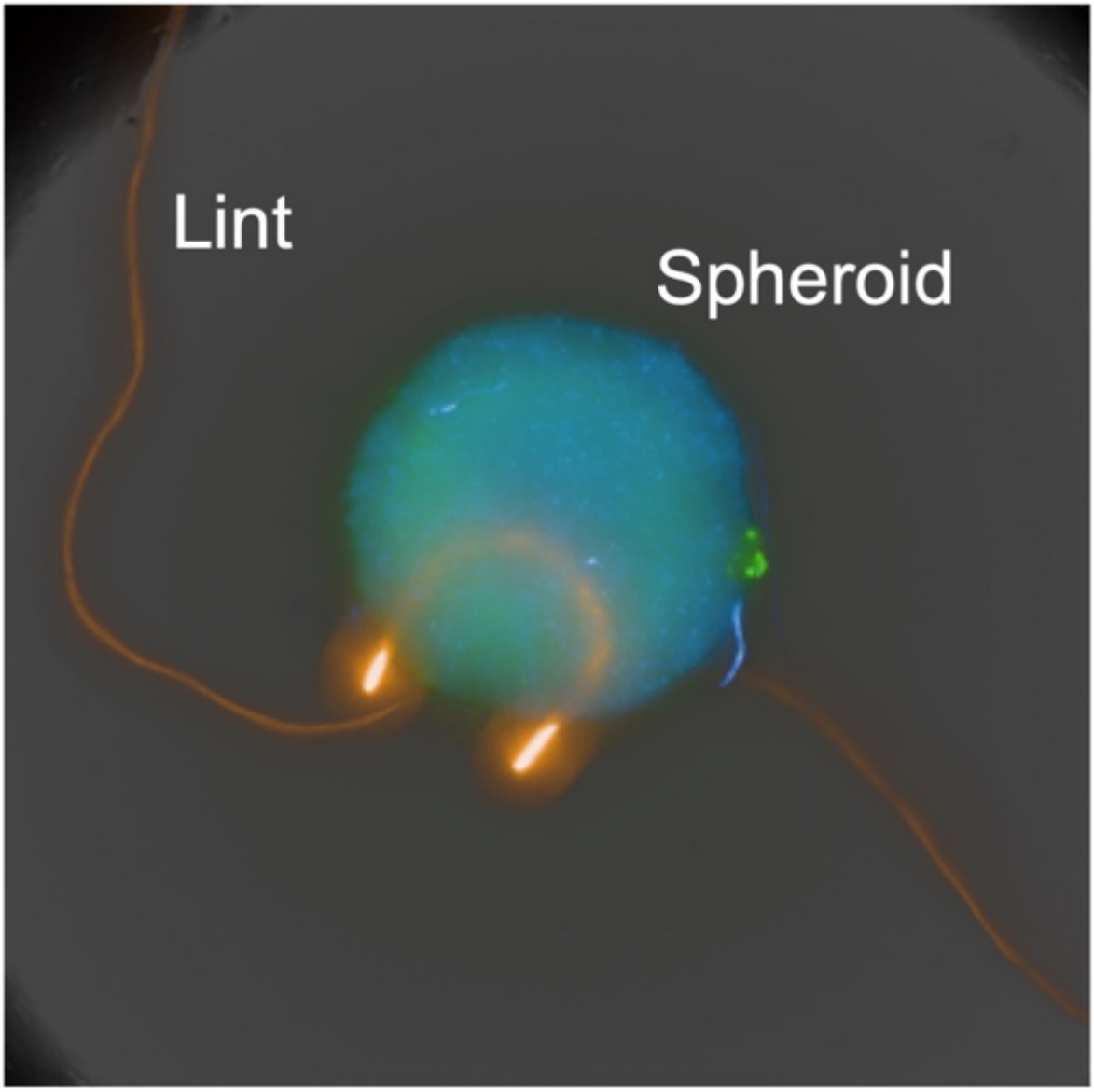


Figure 11. Laboratory contaminant interference with HCS autofocusing. 3D spheroid image from 96-well ULA microplate well labeled with Hoechst nuclear dye (blue) captured with 5X objective lens. Artifact lint is pseudocolor in orange.

resolution. *Use the recommended minimal (or higher) z-plane intervals to best capture objects in 3D.* Acquiring images with lower z-plane intervals will not improve the overall rendering of large 3D objects, and can be counter-productive because it will create longer acquisition times, increase the potential for phototoxicity or photobleaching, generate unnecessary data, and slow down the subsequent image analysis process. For example: if the minimal z-plane specification of the objective is 1 µm, then use a 1 µm or higher step size; adjusting the image acquisition to 0.5 microns with the intention of capturing a better image by over-sampling will *not* improve image analysis segmentation. Caution: oversampling images greater than the volume of a 3D object could result in out of focus image planes, which may affect subsequent image analysis segmentation.

Overall, high NA objective lenses are designed and engineered with short working distances (WD). The most commonly used high NA objective lenses equipped on HCS imaging instruments are engineered with 0.17 mm working distances, which are ideal match for number 1.5 microscope coverslip equivalence or microplates with approximately 170 µm bottom thicknesses. Some objective lenses are engineered with a correction collar with a

tunable adjustment on the lens to correct for microplates bottom thicknesses. However, the NA in these lenses are typically lower, and these adjustable microscope objective lenses are useful for both thin bottom or thicker microplate bottoms that exceed 500 µm. In cases where microplate bottom thickness are unknown or outside of the specifications of the objective lens, then it is recommended that all microplates or objective lenses be tested for overall compatibility. Objective lenses should be appropriately paired with numerical aperture to match the microplate bottom thickness (Figure 12B).

Interference from foreign materials

Since HCS imaging instruments are equipped with very sensitive charge-coupled devices (CCDs) or complementary metal–oxide–semiconductor (CMOS) camera detectors to capture dim fluorescent signals, HCS practitioners should be cautious of any debris, lint, or other foreign matter that may obscure the light path and the autofocus processes. Such contaminants can be introduced during screening operations from "dirty" work areas and from liquid handing devices during compound/reagent delivery, microplate washing, or other workflow processing steps. This usually occurs when dispensers inadvertently inject salt solutions to the bottom of the microplate, or when concentrated salt solutions form as a result of well-to-well contamination. Interferences may also be introduced while performing live-cell imaging when plate seals are not used, and media or buffer overflows the edge of the well height and spills onto side or bottom of microplate (Figure 13). Salts and other foreign materials on the bottom of HCS microplates can interfere with image acquisition and even damage lenses when immersion-based objective lenses are used. Another type of interference that can affect the optical path is scratches on the bottom surface of the microplate wells. This is more common in microplates with low or non-existing skirt heights, or glass bottom microplates that are more prone to scratching then polymer-based plastic microplate bottoms (n.b., recall discussion of hardness related to COC/COP). The interior well bottom can also be damaged by processes such as the scratch wound assay, micropatterning, or other automation manipulations that employ apparatuses in close proximity to the bottom surface. The effects of such artifacts can be mitigated by [1] maintaining a clean work environment, [2] systematically examining the bottom of microplates by visual inspection, [3] gently removing dust and lint with air blowing, and [4] for salt residue contamination, applying copious amount of dH_2O to the area and drying with a clean lint-free paper cloth (e.g., Kimwipe).

Microplate Usage

In addition to the choice of microplate, another key aspect of robust assays is determining the optimal use of microplates. In other words, even with the perfect microplate, assays will only be as robust as the experimental design and processes they are subjected to during an experiment. This section describes several topics in microplate usage, including microplate storage, mixing, dispensing, wicking, and other miscellaneous best practices.

Microplate storage and shelf-life

Microplates are generally very stable laboratory consumables. In order to maximize the shelf-life of microplates, they should not be exposed to extreme conditions, such as extreme heat or cold. Always consult the vendor documents for recommended storage conditions. Key issues to consider include:

- *Sterility*. Plates used for tissue culture should be stored such that the sterile packaging is preserved.
- *Warping*. Over time, especially when subjected to temperature extremes or mechanical stresses, microplates may warp, which can adversely affect their performance. For example, warped plates may interfere with automation processes or assay detection if the microplate wells have even slightly-altered positions (different read heights, x-y coordinates).
- *Degradation*. Certain microplates with special chemical treatments have limited stability. In such cases, assays should be timed such that they can be performed within the validated stability period. If new

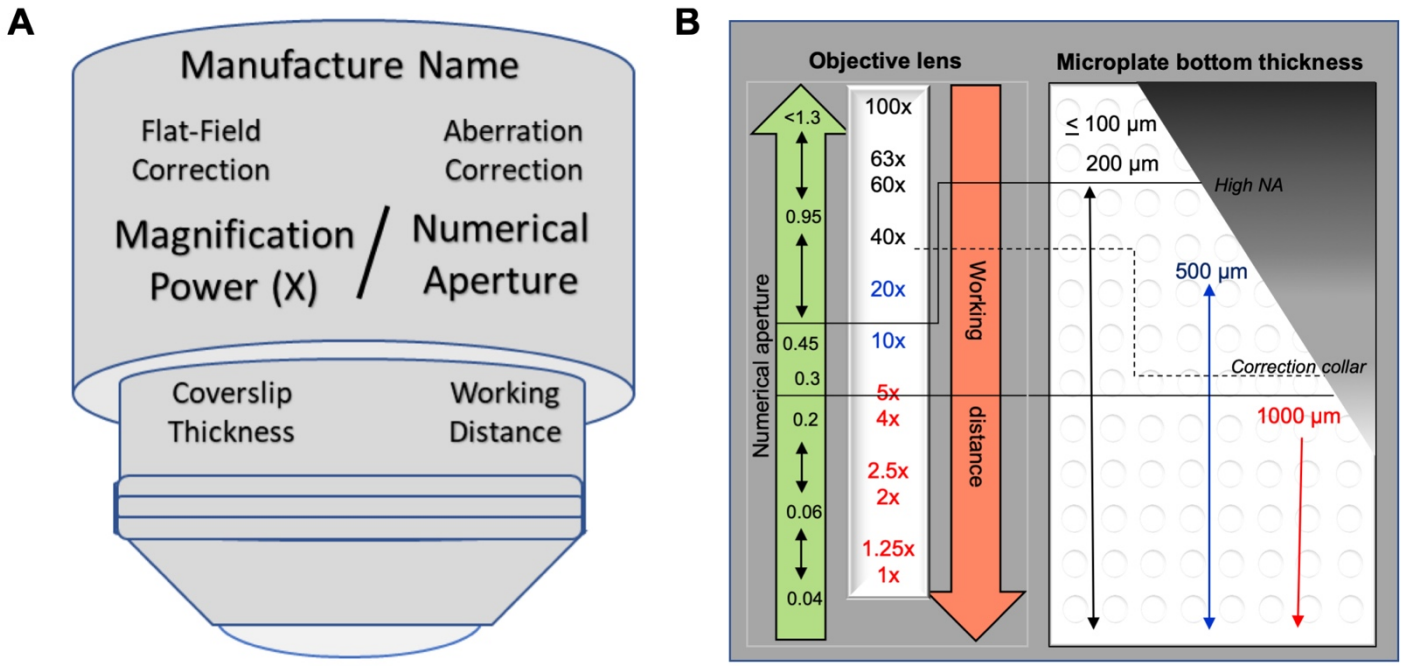


Figure 12. Lens-microplate compatibility considerations for HCS applications. (A) Outside anatomy of microscope objective lens. The information is generic placeholder standard for commercial suppliers. The objective lens is labeled with information about the lens including the magnification power, numerical aperture, and coverslip (microplate bottom) thickness, and working distance. (B) Guidelines to match HCS instrument objective lens with the microplate bottom thickness.

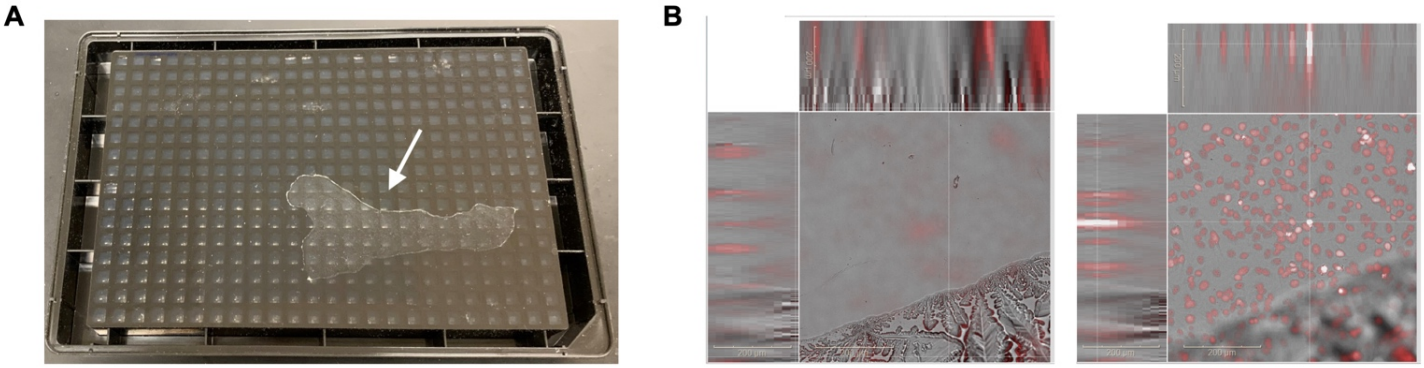


Figure 13. Examples of salt residue from PBS interfering with HCS image quality. (A) Salt residue area from buffer on bottom surface of the 0.19 mm-bottom microplate (B) Brightfield and fluorescent 3D image captured with 10X objective lens. Left: 3D image showing z-position of salt residue in focus at bottom of the microplate well. Right: cells labeled with DRAQ5 (red) showing in-focus z-position.

microplate lots are used, procedures should be in place to identify and account for batch effects. Such batch effects can be detected by using reference compounds in every experiment and comparing assay performance metrics (e.g., Z', signal-to-background ratios, max and min signal), along with specialized data analyses such as control charting (see also "Special Topics and Troubleshooting – Microplate Lots" section for suggested processes to detect batch effects or changes in assay performance over time).

- Certain specialty treatments might require specialized storage conditions, such as refrigeration or protection from light.

- *Assay support*. When performing HTS and structure-activity relationship (SAR) support, consider the length of time the microplate-based assay will need to be performed, and whether it is feasible to maintain the same lot or supply of a given microplate.
- *Inspection*. Carefully inspect microplates from a new case for acceptability, checking for warping (lay microplate on flat surface and check for any wobbling) and cracks (often from shipping and handling).

Mixing

For most assays performed in microplates, several dispensing steps are required to combine the assay reagents and test compounds. These various components must be equilibrated to produce robust data. For example, most compounds are delivered into aqueous solutions from DMSO stocks. DMSO droplets tend to sink to the bottom of microplate wells and slowly diffuse without agitation.

Mixing is *occasionally* performed for microplate-based assays, and this section is included for completeness should mixing be necessary depending on the nature of the bioassay (e.g., simple diffusion might not be an acceptable mixing strategy). Whereas individual microcentrifuge tubes can be inverted or vortexed at the benchtop, such practices are typically avoided with microplates (even sealed microplates) given the number and proximity of samples. The design of the microplate and the desire for throughput necessitates some special considerations with respect to mixing microplate well contents (21).

There are several important considerations with respect to mixing in microplates. The reaction volume is one primary consideration for microplate mixing. Standard 96- and 384-well plates can often be adequately mixed with simple orbital shakers. With 1,536-well plates, the capillary-like dimensions of the wells do not always create a robust vortex. However, the small volumes are more amenable to simple diffusion. For example, sodium ions can diffuse at approximately 0.5 mm in under two minutes (with 100% water and no other ions). Assuming a similar diffusion rate for other assay components, standard pre-incubation times might be sufficient for high-density microplates. Another important consideration is the time and desired throughput. Depending on the protocol, mixing may take several minutes. Users should carefully consider the nature of the assay to determine what lengths of mixing time are acceptable for a given workflow. For instance, kinetic assays with early time-points might place a premium on fast mixing after a reaction is initiated. By contrast, endpoints after several hours of incubation might be more tolerant to longer mixing protocols. *In general, mixing is not done with cell-based assays*, as it may perturb cell function or even disperse adherent cells. For certain cell-free systems, assay components such as detergents and highly proteinaceous solutions have the tendency to form bubbles, which can interfere with some light-based readouts.

There are several technologies available to mix microplate contents, including:

- *Orbital shaking*. Orbital plate shakers or mixers operate by producing hundreds-to-thousands of small-diameter revolutions per minute, effectively creating a vortex within each microplate well. Orbital mixers are available that can fit multiple plates at once, or single plates that can be fitted into automation lines. When using these devices, users should carefully validate mixing protocols to ensure no spillage and adequate mixing.
- *Pipetting*. With this method, a percentage of the well contents can be aspirated and re-dispensed. This approach is straightforward but requires consumables (i.e., each well requires a new pipette tip or thoroughly-washed tip) and certain compounds may preferentially adhere to pipette tips.
- *Diffusion*. In this method, well contents are allowed to diffuse over time unperturbed. This is the slowest but simplest mixing method.
- *Reagent addition*. Additional media/buffer (which may include additional reagents) can be added *after* compound addition to mix compounds. Efficient mixing typically occurs when added volumes are at least 25% of the final assay volume.

Dispensing

There are a variety of approaches for dispensing reagents, cells, and test compounds into microplate wells. The technical details of dispensing assay components into microplates are non-trivial, as imprecise or inaccurate dispensing can reduce the assay performance. In general, care should be taken to minimize the time between dispensing components into the first and last wells.

For reagent and cell dispensing, the main approaches are single- or multi-channel pipetting, single-well dispensers (e.g., BioRAPTR), and multi-well dispensers (e.g., MultiDrop). Each approach has pros and cons:

- *Manual pipetting.* This enables the greatest customization or flexibility, but can be susceptible to user errors and is also the slowest method for dispensing into microplates. Manual pipetting is generally not appropriate for low microliter volumes.
- *Single-well dispensers.* Dispensing into one well at a time allows for more customization of plate maps compared to multi-well dispensers, but at the expense of time. Also, the time delay between dispensing into the first and last well might introduce systematic errors. However, dispensing a reagent from a single tip compared to multiple tips increases the precision and minimizes the variability among dispenses.
- *Multi-well dispensers.* Dispensing into multiple wells at a time (usually column-wise) increases the overall throughput, but can restrict plate layouts such that any given row or column must contain the same reagents. Also, each tip will dispense volumes with a slightly different accuracy, which can cause striping patterns in the data.

For compound dispensing, the main approaches are single- or multi-channel pipetting, noncontact dispensing, and pin tool transfer. The choice of a compound delivery method for microplates can have substantial ramifications on the apparent bioactivity and false-negative rates (22). Each approach has pros and cons:

- *Pin tool transfer.* Utilizes pins to directly transfer a fixed volume (usually 20 - 200 nL) from a compound source plate to an assay plate already containing reagents (23). The advantages of this method include: its speed of dispensing and acceptable precision, and the reduction in consumables (pipette tips) by the reuse of pins. Disadvantages include: [1] fixed-volume dispensing with a given pin type; [2] the destination plate layout is dependent on the source plate layout; [3] the need for washing steps and solvents, as well as the risk of compound carryover or contamination from incompletely washed pins; [4] the need to carefully tune methods based on plate geometry, compound volume, and solvent; and [5] the requirement to deliver compounds into destination wells with solvent ("wet dispense").
- *Non-contact dispensing.* Usually performed by acoustic ejection or piezo sample distribution (24,25). With acoustic droplet ejection (ADE) technology, nanoliter volume droplets are ejected from a source plate to a destination plate, with significant advantages being [1] its ability to deliver compound to multiple destination wells, [2] the elimination of consumables (pipette tips) and contamination sources, and [3] the ability to deliver compounds into destination wells without solvent ("dry dispense") to generate assay-ready plates (ARPs) prior to performing an assay. Disadvantages include: [1] the need for specialized source plates; [2] decreased speed relative to pin tools as only one well is dispensed at a time; [3] introduction of compound-solvent-microplate interactions by virtue of dry-dispensing (n.b., can be avoided with modified protocols utilizing solvent-loaded destination plates). The size and appearance of compound droplets can vary due to the volume transferred, excipients, microplate type, and storage conditions (temperature, humidity, time), which can thereby affect assay performance (25,26).

ADE dispensing requires the assay plate to be inverted to receive the nanoliter acoustic-ejected drop from the source plate. When the source plate contains compounds dissolved in DMSO, several items need to be considered, particularly in cell-based assays. Although cell-based assays have been enabled with ADE technology using assay ready plates, many assays with adherent cell lines require prior seeding of the plates with cells before compound addition (27). The challenges involved with using ADE technology in a direct manner

have been reviewed (28). The main issue is wicking or dripping of cell media from the wells upon inversion which is not an issue for high-density 1536-well plates where the capillary forces are very high (Figure 14). The severity of the liquid loss or wicking upon inversion of lower well-density plates (96-, 384-well) can depend on the well shape, coating, as well as media composition. Direct addition of compounds to 96-well plates has been enabled using a workflow where 70% of the well volume is aspirated before the acoustic dispense to prevent the media from draining out of the wells upon inversion (30 µL was left in the well). In another case, as most of the remaining liquid pooled to the corners of the well, a dispense protocol (using an Labcyte Echo acoustic dispenser) was implemented where the 2.5 nL DMSO droplets were ejected towards the side of the well (less than 1 mm from the side of the well wall), which preserved the cell monolayer (29). Media was then added back following this acoustic dispensing step (29,30). It has also been noted that acoustic addition immediately after media exchanges where the sides of the well walls may still be wet can lead to similar issues as observed in 96-well plates (31). In general, it is important to monitor this during assay development of cell-based assays to ensure the dispensing of compounds by ADE is not adversely affecting the cells.

Compound-solvent-microplate interactions can have substantial impacts on assay performance. Pin tool dispensing into microplates appears to result in more validated hits compared to ARPs, while freezing ARPs (-20 °C) and longer storage times can result in lower hit confirmation rates compared to refrigeration (4 °C) and "just-in-time" use in one study utilizing a biochemical luciferase assay (22). Furthermore, even the choice of microplate material can affect the false-negative rate, as COP results in fewer false-negative hits compared to PS (22). There are likely several important chemical principles explaining these observations. Compounds directly applied to microplate polymers can be potentially sequestered by microplate plastics or precipitated due to a combination of organic solvent evaporation and water uptake from ambient air. We note there may be additional significant relationships between (a) assay performance, (b) compound dispensing methods, (c) assay format (i.e., cell-based versus biochemical, buffer/media composition), and (d) other microplate properties (e.g., other polymers, surface treatments) that have not been reported in the literature.

Some other miscellaneous points with respect to dispensing assay components into microplate wells include:

- *Dispensing speed.* For automated dispensers, note that the dispensing speed can have several effects on assay performance, including mixing. For cell-based assays, excessive flow rates may damage cells by turbulence.
- *Location of dispensing flow.* Contents can be added to microplate wells at either the center of the well or towards a side/corner. The specific location may need to be adjusted to optimize reagent mixing, minimize disruption to cells, or to prevent intra-well effects.
- *Bubbling.* Poorly optimized dispensing can create bubbles, which can adversely affect many light-based readouts. Bubbling most often occurs with detergent-containing solutions and highly proteinaceous solutions, in combination with excessive flow rates and poorly-directed flow directions.
- *Calibration of microplate dispensers*. Any instrument that dispenses a liquid including compounds or reagents (e.g., buffer, media, cells) should be calibrated for accuracy and precision. For the dispensing of low volumes (e.g., pin tool, acoustic dispenser) this is most practically accomplished by dispensing known amounts of fluorescent or colored dyes into each well by the instrument in question and back-calculating the dye concentrations, with the concentration differences being attributable to errors in the dispensing volume (e.g., ARTEL system). For higher volume liquid dispensers commonly used to deliver reagents, this is typically accomplished by a gravimetric analysis. By verifying the accuracy and precision of liquid handlers, scientists can better exclude liquid handling as a source of systematic bias when there are apparent positional effects. In general, such calibration should occur *at least* annually (preferably quarterly with high-use instruments) given the importance of accurate and precise dispensing in microplate-based assays. Users should consult instrument manuals for recommended calibration frequency.

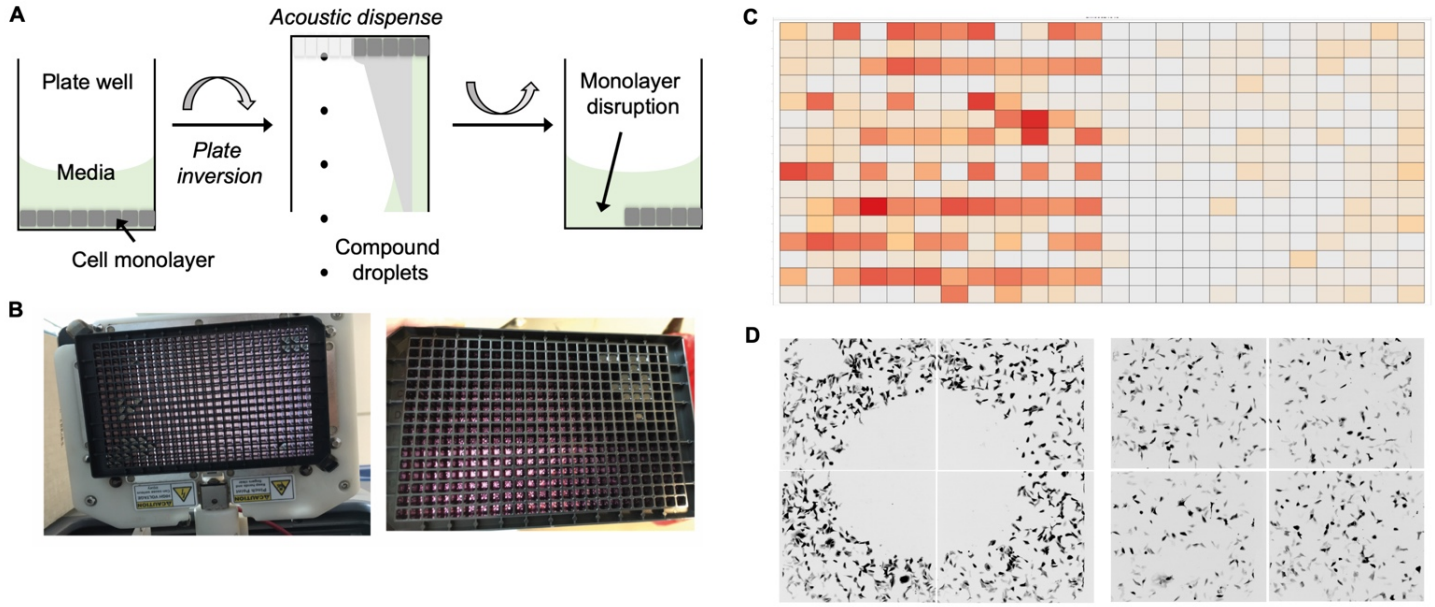


Figure 14. Special considerations for using ADE with cell-based microplate assays. (A) Illustration of the ADE dispense artifact. Plates are inverted for acoustic dispensing which may lead to displacement of the well liquid toward the top of the well. Acoustic dispensing of nanoliter DMSO droplets into the nearly-dry well disrupts the cell monolayer in the dispense area. The plate is reinverted and the cells are lost within the dispense area. (B) Photo of inverted plate showing wicking wells on a Labcyte Echo acoustic dispenser (left) and example of what is often observed once the plate is finished. (C) Heatmap showing wells where holes in the cell monolayer were detected by imaging (analysis of the cell monolayer was performed by Cell Profiler; red wells show loss of cells in wells). The left half of the plate used acoustic dispensing from a DMSO source plate (90% DMSO/10% H_2O, v/v), while the right half was not dispensed by ADE. The plate was imaged immediately after the acoustic dispense. (D) Example of a hole in the cell monolayer caused by the ADE in a wicking well (left) and an undisturbed cell monolayer (right).

Centrifugation

Centrifugation can have a place in various steps of a screening platform. It is a critical component in compound source plate management where a spin before removal of the microplate seal reduces the potential for well-to-well cross contamination. For cell and biochemical microplate assay systems, centrifugation has been used as a surrogate for mixing, especially in the 1536-well format, where well volumes are small and the physics of the well sizes themselves challenge a successful mix with 'traditional' mix strategies such as shaking (linear, orbital, double orbital) or pipetting.

Centrifugation of microplates can be incorporated into experimental protocols at various steps, with some potential advantages (Figure 15). Briefly centrifuging microplates can [1] eliminate dead space at the bottom of the well when the dispensed reagents do not completely settle; [2] eliminate bubbles which can interfere with imaging and light-based readouts (32,33); [3] recover droplets adhering to the side and lids of microplates; [4] pellet suspended particles which may interfere with top-read applications; and [5] enhance settling of adherent or suspension cells. Each of these benefits can increase the overall well-to-well consistency in certain settings.

Centrifugation has also been used as a 'time zero' timestamp for compound/enzyme preincubations in biochemical assay systems as it enables immediate and consistent contact of compound and enzyme solutions across a microplate without loss of reagent. Depending on the detection technology, centrifugation can also play a critical step in reducing variability; for example in radioactive filter binding assays, where scintillant addition is the final step before detection, a centrifugation step before the read ensures contact of the scintillant with the filter and thereby reduces well to well variability due to uneven scintillant dispensing. *However, in some detection technologies, centrifugation will increase variability by creating uneven menisci across the microplate* (MJ Wildey

and colleagues, unpublished observations). This can be difficult to predict *a priori* based on the assay technology and experimental design, but in general it is found in top-read assays. If one canvases the literature, one will find a wide range of recommendations on the value and use of centrifugation as part of *in vitro* microplate-based assay systems. As with many components of our screening toolbox, validation of the impact of centrifugation in your specific automated workflow is critical and highly recommended (e.g., compare performance of two plate groups, one centrifuged and one not centrifuged).

Some technical considerations for centrifuging microplates include:

- Adding centrifugation increases the number of steps in an assay protocol and can decrease the overall throughput. Centrifuging after the final dispensing step (rather than after each dispensing step) might be sufficient, which should be experimentally validated.
- Most larger laboratory centrifuges can accommodate microplates with special holders or buckets. Note that most centrifuges can only accommodate 10 to 20 microplates at a given time, which might necessitate batching and adjustments to optimize the automation and workflow.
- Microplates are generally spun at weaker *g*-forces than tubes, usually less than 400 x *g* for only 1 - 2 min.
- Note the temperature of the centrifuge. Some laboratories may have the default temperature set to 4 °C, which can adversely affect certain reagents or cells. For most applications, the centrifuge should be set at 25 °C.
- Note that the centrifuge chamber is non-sterile and might contain airborne particles. Therefore, utilize lids or seals for microplates to prevent their contamination.

Incubation practices

Depending on the assay design and operation, microplates may need to be incubated at specific temperature and atmospheric conditions. For assays that require incubation at above-ambient temperatures, it must be noted that heating and is hardly instantaneous and not necessarily uniform even in an incubator (Figure 16A). Depending on the amount of thermal mass in the microplate, the heating gradient, and surface contacts between the microplate and temperature sources, it might take several hours to achieve a temperature equilibrium. An example illustrating the importance of surface contact is PCR microplates, which feature separate V-wells that can individually contact the PCR heating block for rapid and uniform heating and cooling. In large oven incubators, there is the potential for a significant heating gradient whereby microplates closest to the heated air flow or higher vertical positions are at higher temperatures than more distal microplates.

The practical implication is heating gradients and incubation practices can contribute to systematic biases (34,35). To mitigate systematic biases related to microplate incubation, there are several recommended strategies and considerations:

- During assay development and operational validation, investigate the effect of plate positioning on the assay readout using uniformity plates positioned at different orientations. The presence of gradients that respond to plate positioning and orientation is suggestive of a bias.
- Minimize the opening and closing of incubators whenever possible to prevent perturbations in heating and atmospheric conditions.
- Avoid simply stacking microplates on top of one another when incubating for prolonged periods of time, as this can prevent airflow along the top and bottom surfaces of the interior microplates and create an uneven heating gradient.
    - A simple and economic solution for smaller labs that do not have industrial-scale plate incubators might be the use of 3D-printed “plate hotels” which can separate microplates but enable easy handling and close proximity within standard incubator ovens (Figure 16B).
- For cell-based experiments, utilize continuous temperature and CO_2 monitoring.

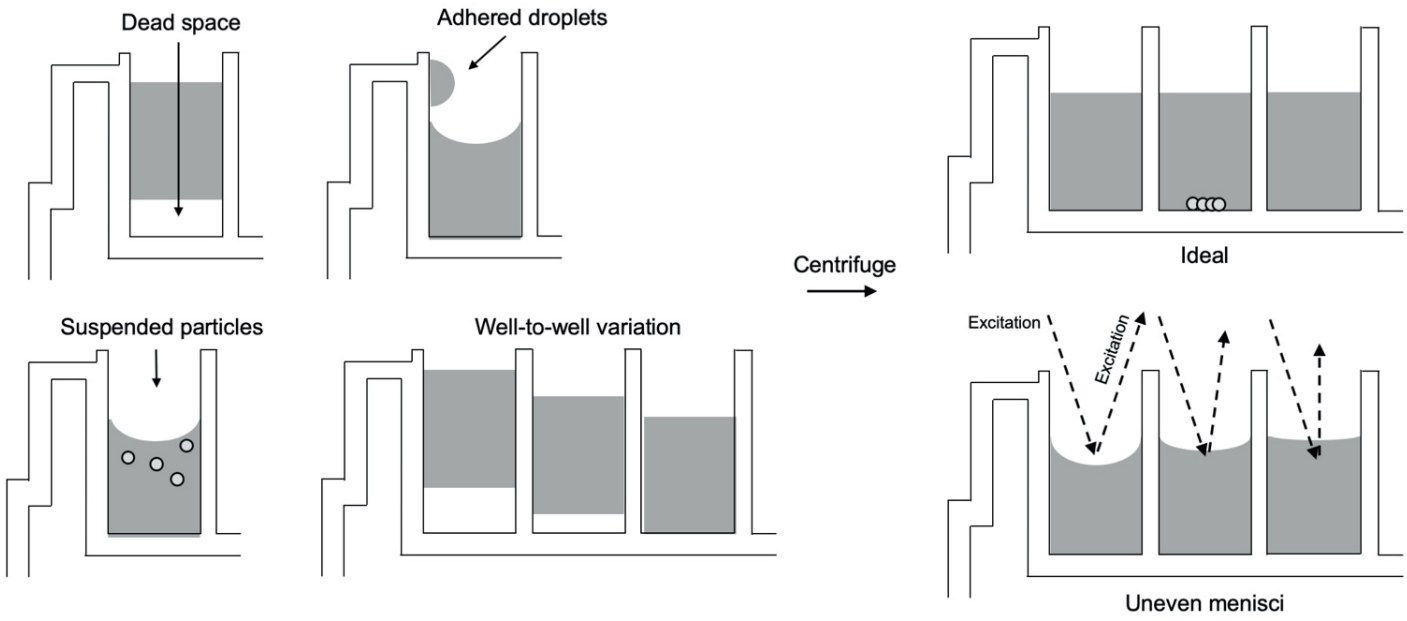


Figure 15. Potential benefits of centrifugation. Briefly centrifuging plates can eliminate dead space, recover droplets from the microplate walls, pellet suspended particles, and create more uniform reactions. Note that in certain cases, centrifugation can produce uneven menisci, which can be counter-productive as it can introduce additional variability (e.g., certain top-read assays).

- Depending on the assay, consider periodically rotating the position and/or orientation of microplates (this is most amenable with prolonged incubations), or randomizing the position of the microplates within incubators with respect to plate order. Regardless, it is prudent to note the location of each plate in case systematic biases need to be investigated after-the-fact.

Section summary

Often overlooked, robust assays depend on the proper use of microplates. During assay design and validation, scientists should consider the use of microplate handling including centrifugation, mixing, dispensing, and incubation steps.

Troubleshooting and Special Topics

This section describes special topics relating to microplates including batch-to-batch variability, well-to-well contamination (e.g., wicking), edge effects, microplate surfaces, and plate washing.

Microplate lots

It is tribal knowledge amongst seasoned screeners that microplates are susceptible to variations among batches that can add variability to assay results. This usually occurs when the manufacturer changes something between batches, such as the raw materials (different composition or even as subtle as a different source of the same material), the microplate mold, or some variable during the manufacturing process. One practical approach to mitigate this batch-to-batch variability is to purchase microplates from the same batch or lot whenever possible (a similar tactic is used for cell culture media, where scientists often purchase bulk animal serum from the same lot). Scientists should calculate the expected number of plates for assay optimization, screening, follow-up assays, and SAR support.

After the assay plate has been selected, the best practices are to secure enough plates to complete the project (such as a screen or SAR support) for at least one year. It is also recommended that a set (case/sleeve) of these

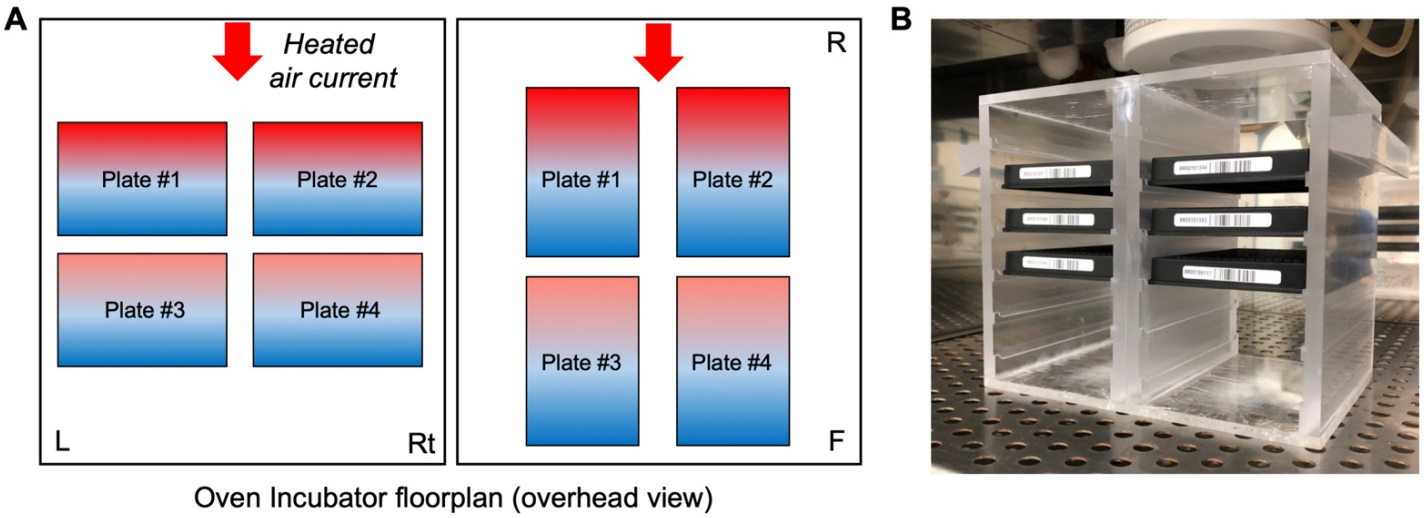


Figure 16. Effects of microplate positioning on systematic biases during incubation. (A) In oven incubators, the positioning and orientation of microplates can introduce systematic biases due to heating gradients. Both individual plates and the wells within these plates, might experience different temperatures depending on their proximity to heating elements. This temperature differential might perturb the assay readout (e.g., alter cell growth, denature or enhance enzymes). A bias that shifts upon microplate rotation (compare left and right panels) or re-orientation is suggestive of an incubation-based systematic error. (B) A microplate hotel for compact storage and uniform microplate incubation. Microplate hotels can be assembled by 3D printing. Within the hotels, microplates can be placed in close-proximity inside standard oven incubators while enabling airflow between the plates.

validated plates be set aside for comparison testing with future plate orders. The Replicate-Experiment format as described in the HTS Assay Validation AGM chapter is one tool that can be used to compare the performance of a new batch of microplates to an existing validated batch when using identical compounds and reagents on each microplate batch tested in parallel. The testing-mode minimum significant ratio (MSR; potency analysis) should be used for SAR support and minimum significant difference (MSD; efficacy analysis) for single-concentration screening (36,37). An Excel template is available to simplify the data analysis. Other approaches to detecting performance drift due to batch effects or degradation include control charting (e.g., Levey-Jennings charts) of assay metrics such as signal strength, signal-to-background ratios, and Z'.

Well-to-well contamination (including wicking)

Due to the proximity of samples within a microplate, well-to-well contamination can be a significant and frustrating source of error. An otherwise perfectly-optimized assay can fail to reliably interrogate bioactivity if well-to-well contamination occurs. The consequences of such contamination are assay-specific, and depend on the amount of contaminants, chemical identity of contaminants, plate layout, and assay technology. For example, a cross-contamination between two adjacent vehicle control wells (DMSO-treated for example) might have minimal consequences to the assay readout. By contrast, the contamination of an otherwise non-bioactive well with even a small amount of highly potent bioactive compound from an adjacent well can produce a false-positive readout.

Cross-contamination can occur by several mechanisms, the most common culprits being [1] simple spillage or overflow from handling or dispensing, and [2] capillary-like effects ("wicking") (Figure 17A,B). Simple spillage can arise from a variety of sources:

- *Non-optimized liquid dispensing to microplates*. Excessive flow rates or poorly-aimed liquid streams can eject contents into adjacent wells.
- *Indelicate microplate handling procedures*. Like any experimental vessel, microplates subjected to course handling procedures (i.e., sudden, jerky movements) can cause contents to spill from well to well.

- *Non-optimized handling of a lid or seal*. Well contents can occasionally adhere to the interior surfaces of microplate lids or seals. Cross-contamination between wells can occur when the contents are not removed from the interior surface prior to opening (e.g., by simple centrifugation) and/or indelicate cover handling.

Wicking occurs by a capillary-like mechanism such that liquid from one well ascends along the vertical well dimension and eventually mixes with the liquid from an adjacent well (Figure 17B). Factors that influence wicking include:

- *Well geometry*. Wells with sharp corners can create a capillary-like conduit for liquids.
- *Laboratory environment*. Static charges can have the effect of accelerating wicking. Non-humid laboratories are often associated with pro-static conditions (e.g., laboratories in northern climates during the winter).
- *Assay buffer components and microplate surfaces*. Buffer components such as organic solvents and detergents can alter the surface tension and adherence to specific microplate surfaces.

For optimizing microplate handling and automation, troubleshooting can be done by mimicking the assay protocol with colored dyes and assay buffer. This approach can often identify cases of gross spillage, such as misaligned liquid dispensers or sloppy lid/seal removal. Non-conspicuous sources of well-to-well contamination can be investigated by utilizing highly potent controls in wells adjacent to inactive controls (Figure 17C). In this straightforward setup, even small amounts of cross-well contamination (e.g., 0.1% well volume) from the bioactive control should produce a measurable change in the negative control well.

If a source of cross-contamination is identified, one should still consider a variety of suggested practices to mitigate well-to-well contamination:

- *Avoid maximal well volumes when possible*. Wells that are filled near the maximal volume have less vertical distance to traverse before spilling into adjacent wells.
- *Utilize microplate wells with rounded corners*. Wells with right angles can act as capillaries that enable fluid to ascend along the microplate well. Wells with rounded corners can mitigate this capillary-like phenomenon (38).
- *Centrifuge microplates*. Incorporating brief centrifugation steps can return well contents to the bottom of wells. See also the "Centrifugation" section.
- *Avoid excessively dry conditions*. Incubator ovens can be humidified with water pans at the bottom. Controlling the humidity of an entire laboratory is more difficult, especially in northern climates during the winter.
- *Utilize microplate wells with ridges or chimney construction*. Some microplates have built-in ridges and valleys between the wells. Other plates feature chimney wells, in which each microplate well exists as a freestanding structure connected to adjacent wells only at the microplate base (Figure 17D).
- *Experiment with different surface treatments and buffer compositions*. Some surface treatments might repel buffer components, possibly accelerating a wicking-like effect.
- *Carefully apply and remove lids or seals*. When removing seals, make sure the removed portion does not re-contact the microplate (i.e., peel "away").
- *Prevent formation and discharge of static electricity.* As is the practice in several industries, labs can incorporate air knifes and de-ionizers, as well as alcohol mists, to mitigate static electricity. Some manufacturers also package microplates in anti-static packaging.

Positional effects ("plate effects")

Microplates can exhibit systematic bias as a function of well position within a given microplate, so-called "plate effects" (positional effects) of which there are several examples in HTS and clinical assays (39-42). Plate effects

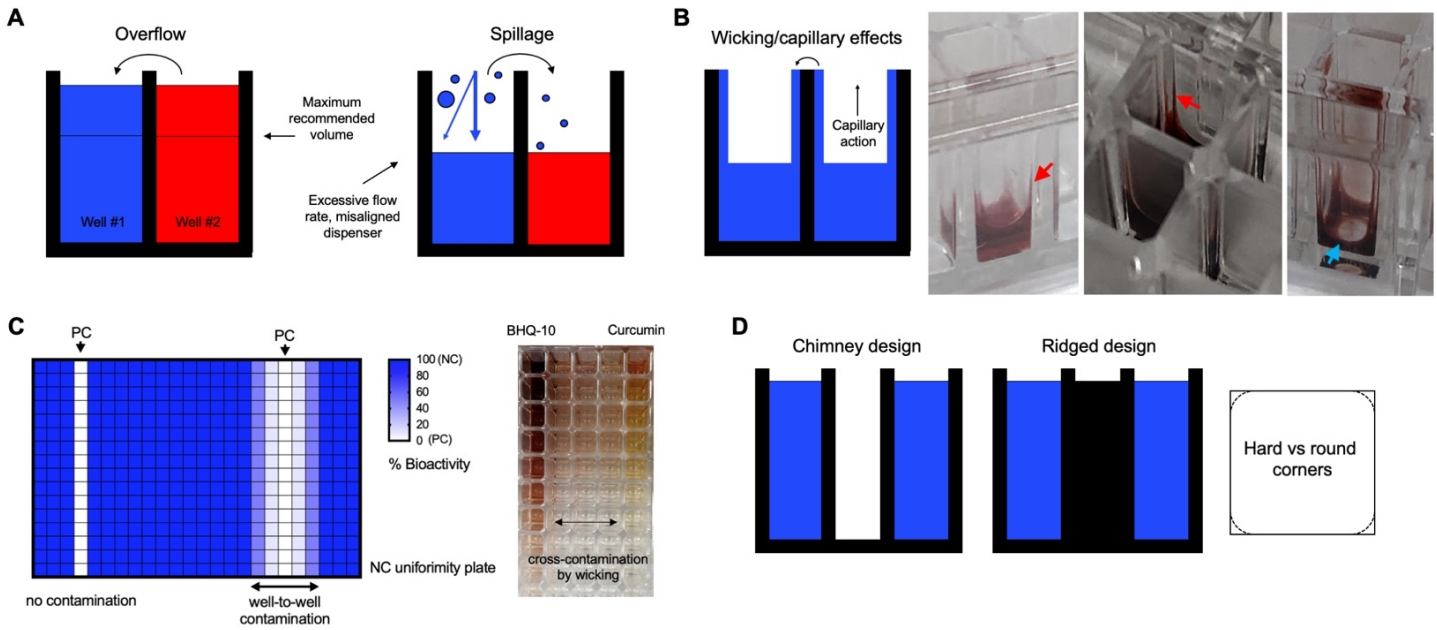


Figure 17 Well-to-well contamination in microplates. (A). Common sources of well-to-well contamination. Simple spillage or overflow is related to over-filled wells and poorly optimized microplate handling (automation and procedures). Wicking can be caused by ambient laboratory conditions (usually dry air), microplate construction (sharp corners), and microplate surface-buffer interactions. (B) Examples of wicking phenomenon in a 384-well PS plate with non-rounded corners. Note the presence of colored compound (BHQ-10 carboxylic acid as 0.5 mM PBS solution) ascending along the corners (red arrows) and adhering to the edges (blue arrows), forming pronounced menisci. (C) Troubleshooting well-to-well contamination using bioactive controls. Sources of contamination can be quantified by assaying highly-active controls adjacent to negative control wells. In this picture DMSO stock solutions of BHQ-10 and curcumin were separated by DMSO wells, and allowed to incubate overnight. Note the colored wells, indicative of well-to-well contamination *despite the use of adhesive seals*. (D) Chimney wells, ridged wells, and rounded corners are specific microplate design features to mitigate well-to-well contamination.

can arise from several sources, including manufacturing (injection mold defects, non-uniform irradiation or surface treatments), incubation effects (evaporation, heating gradients), reagent and compound dispensing, and microplate readers (43).

One of the unique sources of experimental error associated with microplates are edge effects, which is when wells along the perimeter show substantial differences in assay activity relative to more interior wells (Figure 18A) (44). Unlike the interior wells, perimeter wells are not surrounded on all sides by other wells and therefore have at least one side bordering the external environment, which results in different thermal conditions. Edge effects can be significant if they are not mitigated, because they can introduce additional analytical uncertainty. One of the main sources of so-called edge effects is evaporation along the exterior edges of the microplate (45-48). The functional consequence of edge effects (increase or decrease in signal) is assay-dependent. There are several strategies that have been proposed to reduce the impact of edge effects, including:

- Ensure adequate humidity within incubators (by saturating the atmosphere with water so that there is no driving force) (49). In many settings, this is done by placing a water tray at the bottom of the incubator.
- Utilize microplates with lid-lock designs (Figure 18B).
- Utilize microplates with dummy perimeter wells (Figure 18C).
- Fill perimeter wells with assay buffer or media but omit them from analyses.
- Edge effects in cellular assays requiring media changes may also be reduced with centrifugal plate washers (50).

A related source of systematic errors affecting microplates are row and column effects in which there is a systematic bias with respect to the microplate rows and/or columns. Row and column effects are usually caused by instrumentation (dispensers or readers), rather than a specific property of the microplate. Examples include:

- *Dispensing procedures*. Reagents dispensed column-wise (dispense column 1, then column 2, etc.) may have a bias reflecting the difference in dispense timing between the first and last columns. Similarly, reagents dispensed along rows might result in different assay signals that are proportional to the difference in time at which they were dispensed. Cassette-based dispensers, which dispense simultaneously from multiple tips can deliver volumes unevenly or even have a clogged tip, both of which can result in striped patterns. A striped pattern can also result from incomplete priming of the dispenser or adsorption of assay reagents to the dispenser tubes.
- *Detectors*. Microplate readers that read row-wise (i.e., read row 1, then row 2, etc.) may have a bias reflecting the difference in read timing between the first and last rows. Also, the position of a microplate within a reader can cause an apparent signal gradient across the plate if the distance between the detector and plate change during the readout process (51).
- *Temperature-sensitive assays and plate effects*. Occasionally one encounters significant temperature gradients when performing the plate detection step. As an example, there have been assays run in the past that require plates of cells to incubate at 37°C after addition of a detection reagent, followed by a brief incubation at ambient temperature before being placed into a luminescent plate detector. Once read, it became obvious that there was an artifact of some sort as a full plate view showed an oval pattern in the middle of the plate being present, with the values within the oval being higher and those outside of it being lower. This artifact was attributed to the cooling pattern of the plate once it was removed from 37°C and brought to ambient temperature, with the outer edges of the plate cooling faster than the inner portion, leading to a measurable difference due to the nature of the detection reagent being used (S Michael, personal observation). It is important to note that it is very common for an assay biologist to immediately think she has done something wrong when artifacts or inconsistent assay results present themselves, potentially leading to rounds of unnecessary biological troubleshooting where the problem may not be on her side at all.

Positional effects and other systematic errors in microplates can be corrected with appropriate statistical tools (52-56). *This being said, we do not recommended correcting for plate patterns involving greater than 30% change in signal*. One pragmatic strategy is to use uniformity plates (which should in principle produce the same readout in every well). By making the assumption that edge/row/column signals are constant between plates, perimeter wells can be corrected by this strategy. Another strategy involves utilizing plate layouts with controls along an entire row and column, though this comes at a sacrifice to the number of compounds that can be tested on a given plate. For additional discussions on validation and corrections of microplate positional effects, including for HCS data, refer to the AGM chapters, HTS Assay Validation and Advanced Assay Development Guidelines for Image-Based High Content Screening and Analysis.

For cell-based assays, another consideration is the homogeneity of the well contents ("well effects"; Figure 18D). Some practical tips include:

- Incubators can create vortex-like effects that force cells towards the edges of individual microplate wells. For cellular assays, pre-incubation of the newly seeded plates in ambient conditions rather than direct placement in a CO_2 incubator has been proposed (57).
- Briefly centrifuge freshly-seeded cells to facilitate adherence to the bottom of the microplate wells (e.g., 100 *g*, 2 min).
- Limit physical disturbances near incubators, including heavy foot traffic, unnecessary open and closing of incubator, etc.

- Image several fields per well. Compare edges, corners, and center. Determine the optimal number of fields per well that best represents the intra-well variability.

Predictive analytics to reduce plate effects. Statistical analysis of repeated measures designs offers the ability to partition out variability due to repeating individual differences in measurements. In the context of microplates, one can measure the differences between individual wells in a uniformity plate whereby all wells should report identical assay results. Predictive analytics using machine learning can reveal slight differences between the wells that are non-random. Well-to-well differences in optimized assays contribute to about 30% of the overall imprecision (P. Hensley, unpublished results). Incorporating predictive analytics in the post-assay processing step has the potential to correct this sizeable contribution to imprecision, without changing the assay method, lab equipment, or analytical measurement. This can have a substantial impact, as lowering the CV can reduce the statistical threshold for identifying "active" compounds from a HTS, and enhance confidence in ranking compounds. Post-processing with predictive analytics can accommodate other correction factors routinely for parallax, other optical/mechanical repeating biases, pipetting effects, and others. The standard deviation of a given assay can be reduced approximately 30% for both cell-based assays and biochemical assays (Figure 19). Such methods usually require only two or three teaching cycles with uniformity plates (i.e., plates prepared for uniform high signal), with incremental improvement with more teaching cycles.

Well-to-well variance

Well-to-well shapes and volumes can vary within a given microplate, albeit these differences are often relatively very small in magnitude (one non-peer-reviewed study suggests intra-plate well volumes are within 5%) (58). The same study suggests that individual well dimensions are relatively constant between plates of the same plate. This well shape variation can arise because each well is molded from a different feature of the microplate mold. Because molds are machined, wall surfaces are not perfectly smooth and as a result, the surface area of each well can vary and ultimately affect the quality of some assays. Besides gross well shape and volume, wells within a plate can have *different surface properties* that can dramatically alter assay results, and in some instances, this variation can eclipse edge effects in terms of magnitude (41).

Microplate surfaces and associated properties

The uniformity and properties of microplate surfaces are often overlooked, but hardly trivial in that they can dramatically affect assay results. One explanation for differences in microplate performance metrics are microplate surface properties, including surface "roughness", chemical composition, and wettability (i.e., hydrophobicity/hydrophilicity), which can now be analyzed by a variety of sophisticated analytical techniques. Scanning electron micrographs (SEMs) and atomic force microscopy (AFM) have recently been used to assess microplate surface roughness. In one study, SEM identified four distinct problems with some 1,536-well microplates: surface debris, roughness, inclusions (holes into the surface), and exclusions (material extending into the well from the wall) (Figure 20A-F). No brand that was tested seemed better or worse than the other brands (P. Hensley, unpublished observations). Microplate surfaces can vary significantly <u>within</u> a given plate. Contact angle measurements suggest the wettability of individual wells can vary within a given microplate, and have the potential to affect assay results (e.g., higher wettability variability was associated with higher CVs) (39). Even within the same well, there can be patches of hydrophobic contaminants that alter protein binding (59).

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) has also been utilized to analyze microplate surface chemicals (Figure 20G). Of special interest were the chemicals used in production to initialize and control the polymerization rate, control primary and secondary oxidation, improve polymer characteristics (including melting temperature and flow), and mold-release agents. In one analysis, very few ions were unique to a single brand; which is to say almost all were common to every brand tested (P. Hensley, personal observation).

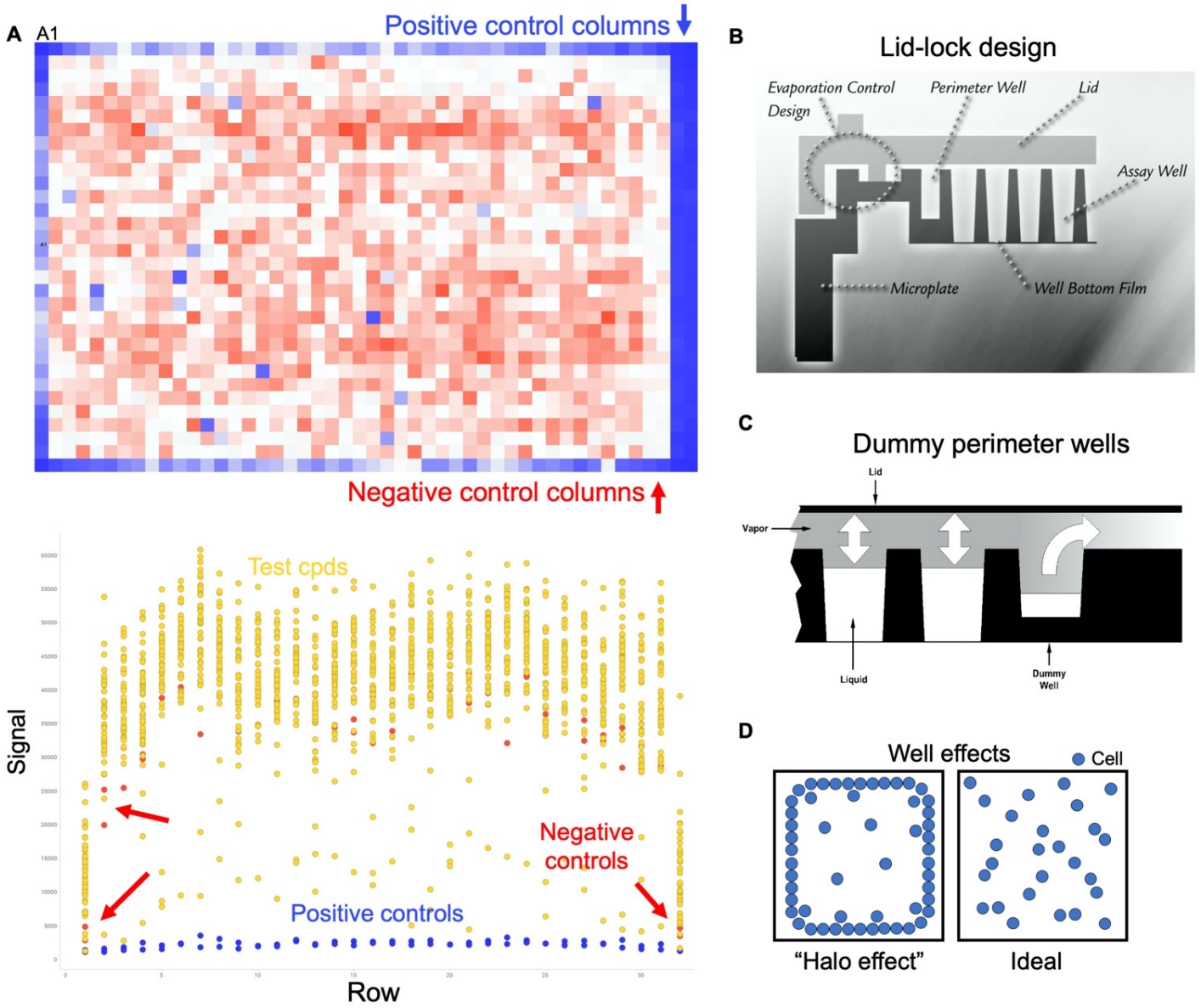


Figure 18. Microplate positional and well effects. (A) Example of microplate edge effects in a 1536-well antagonist assay (red, not active; blue, active). Compared to the interior wells, the perimeter wells show a decreased readout (blue), and note the significant bias of perimeter negative controls (arrows). (B) Microplates and lids can incorporate interlocking designs to mitigate the escape of vapors from within the microplate. (C) Microplates can incorporate dummy perimeter wells that can be filled with media, buffer, or water that will be preferentially evaporated given the proximity to the microplate edge, effectively sparing evaporation from interior wells. (D) Schematic of microplate well effects. In an ideal assay, cells are plated uniformity across the well; well effects can manifest as a "halo"-like effect, whereby cells have a propensity to settle along the microplate edges.

In another study with ELISA plates in a clinical assay for plasma *N*-terminal pro-urocortin 2, SEM revealed particle-like structures on opaque (white) microplate surfaces, which energy-dispersive X-ray (EDX) spectroscopy suggest are TiO_2 remnants from the manufacturing process (39). In the same study, sterilized microplates had higher oxygen content than comparable non-sterilized microplates, thought to be due to γ-irradiation (60,61). Notably, higher oxygen content can confer increased wettability.

Surface area of polymers. Understanding polymer surfaces is importance because they can directly affect assay performance. Determining the surface area of what appears to be a flat polymer surface is a complex process that only results in an approximated value. As there are chemicals on or embedded near the surface of the polymer,

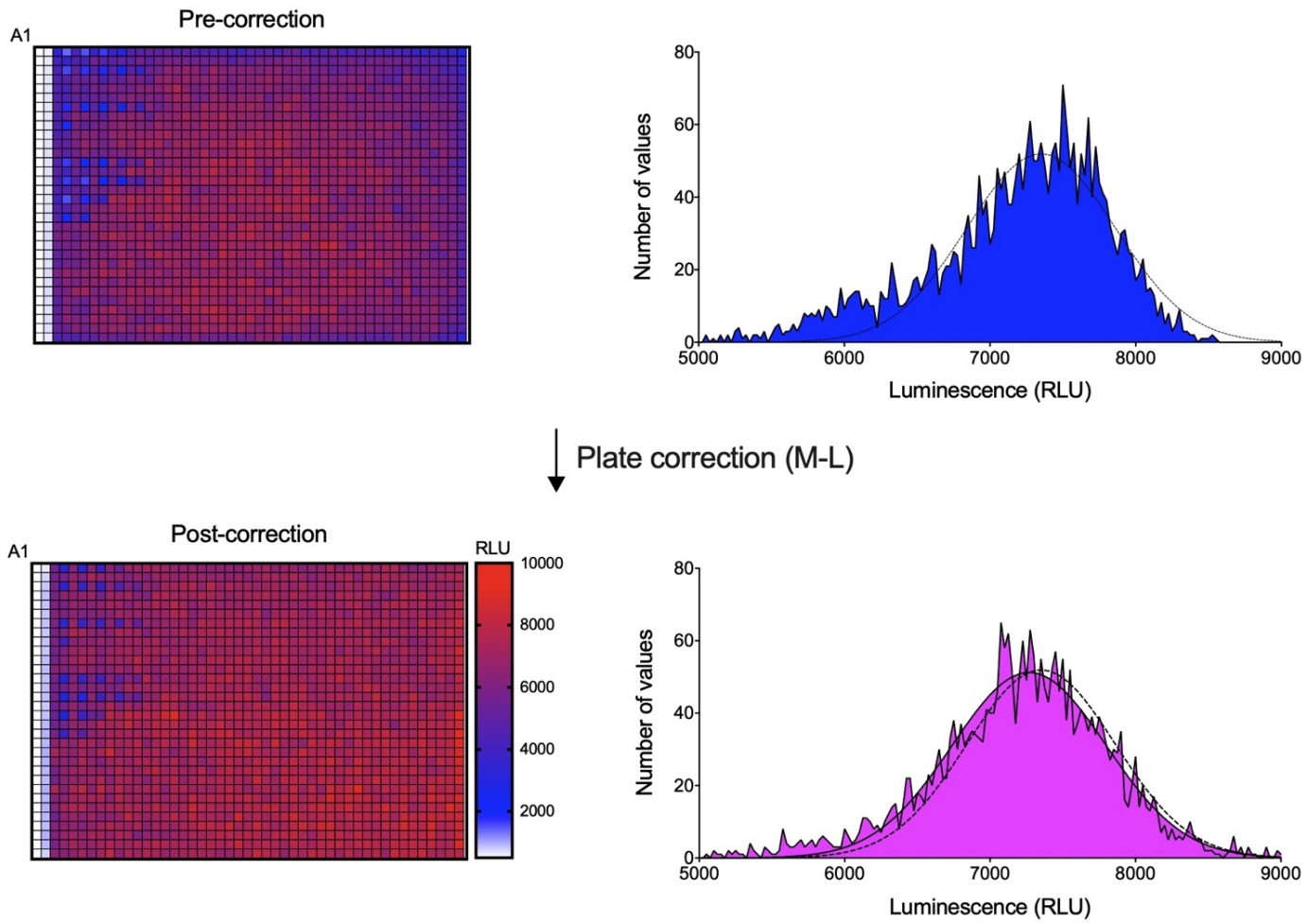


Figure 19. Correction of well-to-well variation using predictive analytics. (Top) Heatmap and corresponding distribution histogram of a uniformity plate read by a ViewLux (PerkinElmer), a microplate reader with a known edge effects. (Bottom) The heatmap and distribution histogram of the same microplate after processing with machine-learning to correct for well-to-well variation. The standard deviation was reduced slightly more than 30% using predictive analytics, as well as a more Gaussian distribution pattern (Assay Analytics, IonField Systems).

understanding the mechanisms of how the chemicals migrate from the polymer to the solvent are critical. The factors related to the polymer surface are: [1] the interface between the surface and the solvent, [2] the solubility of the chemicals into the solvent, and [3] the physical changes to the surface resulting from interfacial forces due to contact with the solvent. A cursory review of the high-magnification SEMs (Figure 20A-F) demonstrates that the surface area of microplate wells is vastly greater than the geometric dimensions. Water comes in direct contact with virtually every polymer molecule on the surface area (Figure 20A-F, note each 'tic' mark on the x-axis is the length of 40,000 water molecules). Using AFM, one study showed pigmented plates had greater roughness compared to non-pigmented plates, and greater roughness in γ-irradiated compared to non-irradiated plates (39). While the increased oxygen content from irradiation can increase wettability, this can be mitigated by increased roughness associated with irradiation.

Physical changes to microplate surfaces/surface dynamics. A logical implication from the ToF-SIMS data is that chemicals added to the polymer mix during production can eventually dissolve into microplate well solutions. Generally, it is the negative ion of the chemical that is the active part of the molecule in starting and controlling the polymerization process. The positive ion is selected for its charge being appropriate or ease of synthesis. The findings from ToF-SIMS is that the positive ion species used are bioactive and as ions all are soluble in water.

Studies of polymer surface properties, specifically the measuring of the contact angle with water, show that the contact angle initially is high (indicating the surface when dry is hydrophobic). If the surface is covered with water and allowed to stay wet and then the contact angle is re-measured, it decreases, indicating that the surface becomes more hydrophilic. These changes in "wettability" are hypothesized to reflect molecular motions in the polymer layer that contacts water (14). Specifically, nonpolar sections of the microplate polymer molecule that are outward facing (to the well contents) when the surface is dry will reverse position with polar sections of the molecule that are inward facing when the surface is in contact with water, resulting in a reduction in contact angle. This phenomenon is commonly seen at the benchtop when one re-pipettes aqueous solutions with the same tip multiple times; with microplates this process is somewhat slower but the contact angle does decrease over time. A practical implication of this molecular movement is that it can expose chemicals in the polymer matrix to the assay solution itself.

Leachable chemicals within microplates can affect assay results

An increasingly acknowledged problem is the ability of chemicals to leach from the microplate material into the microplate well solution. These leachable chemicals and other contaminants can originate from the manufacturing process, such as volatile residues, plastic enhancers, and mold-flow and mold-release agents (59). This can be a significant problem, as some of these chemicals can significantly modulate bioactivity, as shown by a prominent example where the biocide di(2-hydroxyethyl)methyldodecylammonium chloride (DiHEMDA) and the slip agent oleamide inhibited human monamine oxidase-B (Figure 21) (18). Other examples of leachables and assay modulation have also been described (62,63). Metals from polymerization catalysts and impurities (e.g., Zr, Al, V, Cr, Fe, Mg, Ti, Zn, Os, Pd, Ru, Ni, Pb) can also leach and may perturb assay readouts (13).

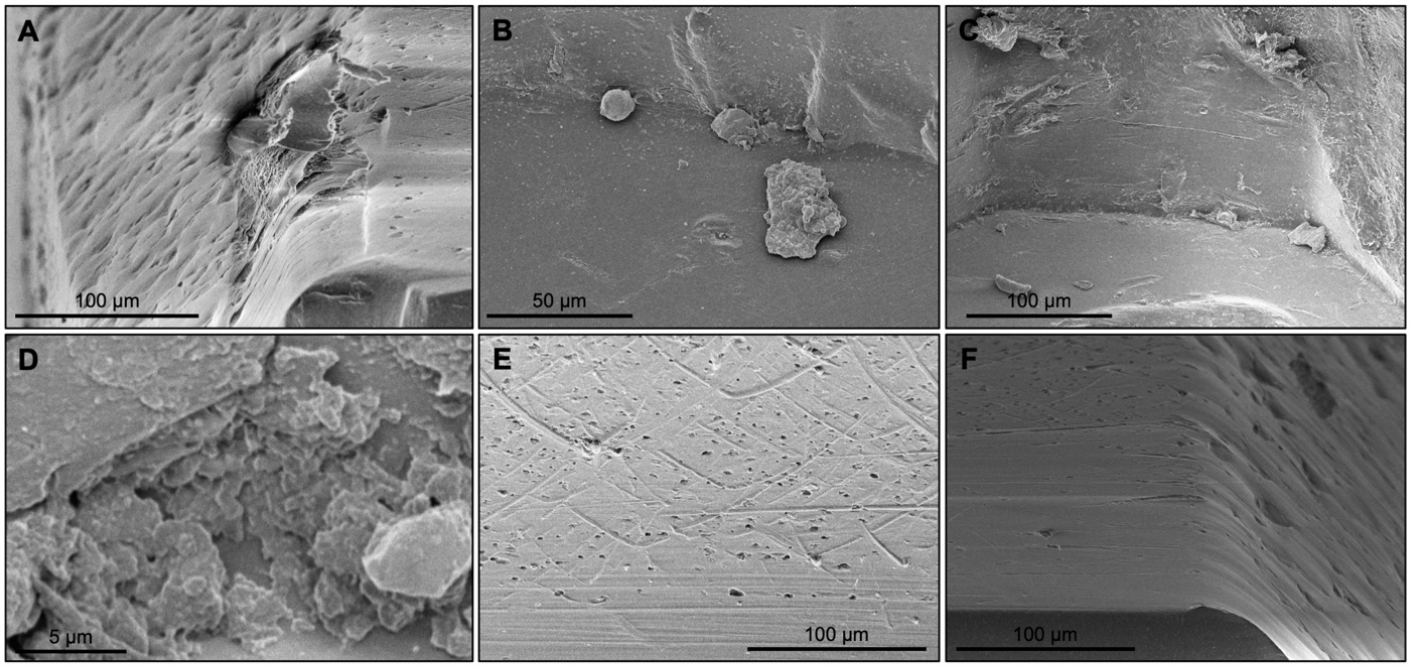
There can also be differences in cell growth rates between comparable microplate brands due to the presence or absence of surface chemicals, as demonstrated by CellTiter-Glo studies with Be2C cells grown in similar microplates with different ToF-SIMS signatures (64). Therefore, assessing cell growth rates and cell health when selecting between microplates might be prudent. Variations in cell growth rates within the same brand can be attributed to well-to-well differences, both chemical and physical, while the variation between brands of cell growth rates is attributed to the effect of chemical and surface differences between brands. Notably, cell growth rates cluster by brand, and all brands in this study showed a skewing toward low values due to edge effect parallax from the ViewLux plate reader used in this testing, which is not microplate related.

Plate washing and reuse

Interest in cleaning microplates to enable their reuse started immediately after the development of microplates as a commercial product. A basic approach involves washing plates with a conventional benchtop plate washer, then centrifuging the microplates with their wells facing "out" and paper towels on the bottom of the microplate holder to adsorb residual assay material and wash solution. The first commercial product intended to clean microplates was the SQUIRT (Matrical), which mechanically inverts a microplate onto a seal and pressure rinses the microplate wells, followed by a blow-drying step.

Through a collaboration between NCATS/NIH and industry (SBIR, HHSN271201400051C), there have been some recent advances in plate washing, including a systematic analysis of washing steps and the application of plasma treatment after plate rinsing. Several critical parameters of an effective microplate wash were identified in this collaboration:

- Fluid pressure: there is a narrow band of pressure for optimal effective cleaning
- Angle of the fluid entering a microplate well: a critical setting as it determines the flow pattern in the well
- Distance of the pressure spray head from the microplate: has a narrow optimized range of maximum effectiveness as the width of the fluid stream changes with time after leaving the spray head



G

(+) ToF-SIMS intensity (relative, per ion)

Individual microplate	Na (23)	Al (27)	Si (28)	Ca (40)	C_3H_5 (41)	C_2H_3O (43)	Ti (48)	C_3H_3O (55)	Zn (64)	C_5H_7 (67)	$C_4H_{12}N$ (74)	C_7H_7 (91)	$C_8H_{20}N$ (130)	$Si_2OC_5H_{15}$ (147)	$C_{11}H_{22}N$ (168)	Pb (208)	$C_{20}H_{40}NO$ (310)	$C_{23}H_{38}N$ (328)	$C_{42}H_{64}PO_3$ (648)
1	16	79	2	12	41	2	100	2	100	9	1	61	1	3	12	9	100	7	100
2	2	4	2	6×10^{-2}	100	100	1	100	1	100	100	100	100	100	100	82	2	25	33
3	100	100	14	4×10^{-1}	44	27	18	21	6	16	16	48	30	37	19	100	3×10^{-1}	11	33
4	7	16	2	3×10^{-1}	11	1	14	1	41	2	1	87	1	7	2	9	0	2	0
5	6	32	3	2×10^{-1}	8	2×10^{-1}	19	1	41	1	2×10^{-1}	23	1×10^{-1}	3	1	7	0	0	33
6	4×10^{-1}	8×10^{-2}	2	4	76	33	1	74	4×10^{-1}	74	39	80	83	54	56	80	2	5	100
7	2×10^{-1}	1×10^{-1}	100	1×10^{-1}	73	24	1	50	1	68	85	68	96	45	5	64	2	7	33
8	3	6	7	100	26	5	2	5	15	8	33	59	29	16	6	80	1	31	67
9	4	2	5	81	50	15	1	13	10	15	89	27	53	31	6	93	1	100	33

Ion (*m/z*)

Figure 20. Microplate surfaces. (A-F) Representative SEM of microplate surfaces. Microplates were frozen with liquid nitrogen, then fractured to expose well walls. (A) Polymer pulled from wall; (B) small loose particles; (c) surface scales; (d) surface tear; (e) surface bubbles; and (f) random non-flat surfaces. (G). ToF-SIMS analyses of microplate surfaces. Using positive ion analysis, ToF-SIMS revealed a large number of positive-ion species either on or within 1 nm of the microplate surfaces. Microplates were tested from three brands. All had at least 27 "non-polymer" positive ions, which one would anticipate based on a review of the literature in this field. This analysis was performed to identify potential differences between the brands. Very few ions were unique to a single brand and almost all were common to every brand tested. However, there were variations in concentration by brand. Examples are that Na^+ varied 498-fold and Ca^{2+} 2860-fold. Typically there were minimal differences for trace ions, such as Pb^{2+} and Ag^+. Labs performing assays known to be sensitive to trace chemicals should check the list of chemicals shown in product attachments and consider testing the microplates they intend to use.

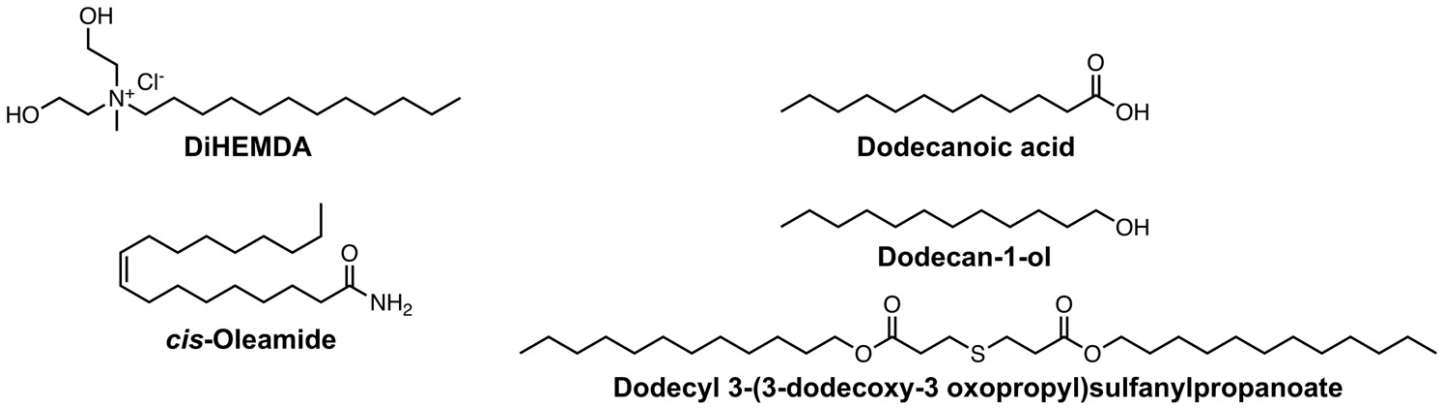


Figure 21. Examples of leachable chemicals from microplates. Chemicals such as the biocide DiHEMDA and the slip agent oleamide from the microplate manufacturing process can leach and dissolve into microplate well solutions, and potentially modulate bioactivity.

- Speed of the microplate as it moves under the pressure spray head: demonstrated a harmonic effect between speed and the width of the fluid

Using conventional plate washers plus centrifugation methods, washing did not consistently reach a 1,000-fold reduction benchmark (the limit of cleaning effectiveness of most serial dilution cleaning processes). Seemingly minor differences in plate handling lead to a variation in cleaning results, even with controlled conditions. With optimized conditions, cleaning effectiveness can be reliably increased to over 10,000-fold.

Considerable research was also done on how to break droplets off a polymer surface, as micro-droplets may contribute to carryover when microplates are reused. Adding a small amount of alcohol to the washing pressure fluid reduced surface tension and improved droplet release, but did not entirely resolve all droplet formation issues. Notably, the biggest improvement in microdroplet removal occurred with the use of a high-performance centrifuge (as opposed to air drying) that are highly precise and allow extremely high rates of rotor acceleration and de-acceleration (0.7 s to reach 1500 rpm, 3.6 s at speed, 0.7 s to stop). The acceleration/de-acceleration rates when centrifuging microplates is a key factor in droplet removal, with extended spin times having minimal additional effects. The "at speed time" is the least amount of time needed for air flow in the centrifuge to clear droplets and aerosol, an important safety consideration.

Another recent advance in microplate washing is the application of atmospheric-pressure plasma (gas excited by electrical current) which is directed into the microplate wells. High-energy plasma causes the breakdown of organic molecules on contact by an oxidative process without the production of liquid or solid waste. Another key technical advance in microplate washing from this public-private collaboration is the importance of a low pressure micro-dispensing process with a trace amount of a solvent mix to enhance residual compound removal. By serendipity, visual inspection of washed clear microplates revealed visible "spots" on the bottom of wells that previously contained high concentrations of certain test compounds (Figure 22A). These spots were presumed to be residual compound, and are significant because they have the potential to perturb bioactivity in subsequent assays if they are not removed. Experimentally it was determined that the solvent mix solubilized both types of residual compound in 3 - 5 s when followed by a high-pressure wash. This approach completely eliminates dried compound as a source of carryover (Figure 22B). In summary, plate washing has advanced from the crude centrifugation of plates to a highly optimized system of solvents, fluid washes, timing, centrifugal force (i.e., precise assay- and microplate-specific application of said force), and plasma treatments.

There are several notable considerations with plasma-based microplate washing (while Microplate Cleaning System powered by PlasmaKnife Technology is specifically discussed, many of these concepts can apply to other systems):

- Applicable to: SBS/ANSI-compliant microplates; multiple microplate heights; 96-, 384-, and 1536-wells; all polymers; glass or polymer clear bottom.
- The plasma cleaning process leaves a trace amount of nitric acid (HNO_3) on the microplate surface. If an assay is pH-sensitive, then post-processing application of a neutralizing buffer is recommended.
- Microplates that have been allowed to completely dry after the assay are less likely to be successfully cleaned for reuse.
- Cell-based assays that include a lysing step with detergents (e.g., Triton X-100) will deposit membrane proteins and lipids on the bottom of the microplate well. In such cases, these microplates must be pre-washed immediately with Tergazyme post-assay while this cellular debris is still wet before commencing plasma-based microplate cleaning.
- Most secondary surface treatments are removed by the plasma cleaning process. Surface treatments can then be reapplied after washing.
- Treatments like the conventional TC process are replaced by the plasma process. In virtually all testing, TC-treated microplates perform better after plasma cleaning (P Hensley, unpublished observations).
- The plasma cleaning does not restore the giga ohm surface properties of patch clamp microplates (e.g., NanIon).

Environmental contaminants

Another factor inherent to microplate-based assays is the effect of environmental contaminations such as airborne particulates ("dust") and even lint from cotton-based laboratory coats on assay performance (65). Microplate-based assays are particularly prone to airborne particle effects because of their relatively large surface compared to vials or microcentrifuge tubes, they are often exposed to air when lids are removed for dispensing and reading steps, and because they may be moved many times during the experimental procedure. Light-based readouts are particularly sensitive to anomalous results from dust.

There are several recommended tactics to mitigate the impact of airborne impurities, including dust:

- Pre-read plates
- Work in clean environments (clean all work articles before placing on systems)
- Keep paper out of screening areas (boxes, packaging)
- Utilize disposable plastic coats to minimize lint contamination (65)

Sterility

For cell-based assays in microplates, it is imperative to maintain sterility to avoid contamination by laboratory microorganisms such bacteria (including *Mycoplasma*) and fungi (yeasts, molds). Most manufactures sterilize microplates by γ-irradiation, which interestingly can have effects on microplate surface properties including wettability, surface roughness, and well uniformity (39). Several best practices are recommended in the context of microplates (in addition to general good laboratory and cell culture processes). These include:

- Refrain from opening plastic sleeves of sterile microplates until prior to experimentation.
- Barcode microplates prior to de-contaminating (autoclaving, etc.); this can often be done by request from microplate manufacturers. This avoids the need to expose opened microplates to barcoding instruments which are often in general laboratory areas and are not sterile.
- Minimize the frequency and duration of lid or seal removal from microplates.
- Whenever possible, dispense reagents (buffer, cells, compounds) into microplates within a tissue culture hood or sterile environment. Some plate dispensers (e.g., NanoDrop, MultiDrop) can fit within standard tissue culture hoods. This minimizes the chances of airborne contaminants from settling into microplate wells when the lids must be inevitably removed for dispensing.

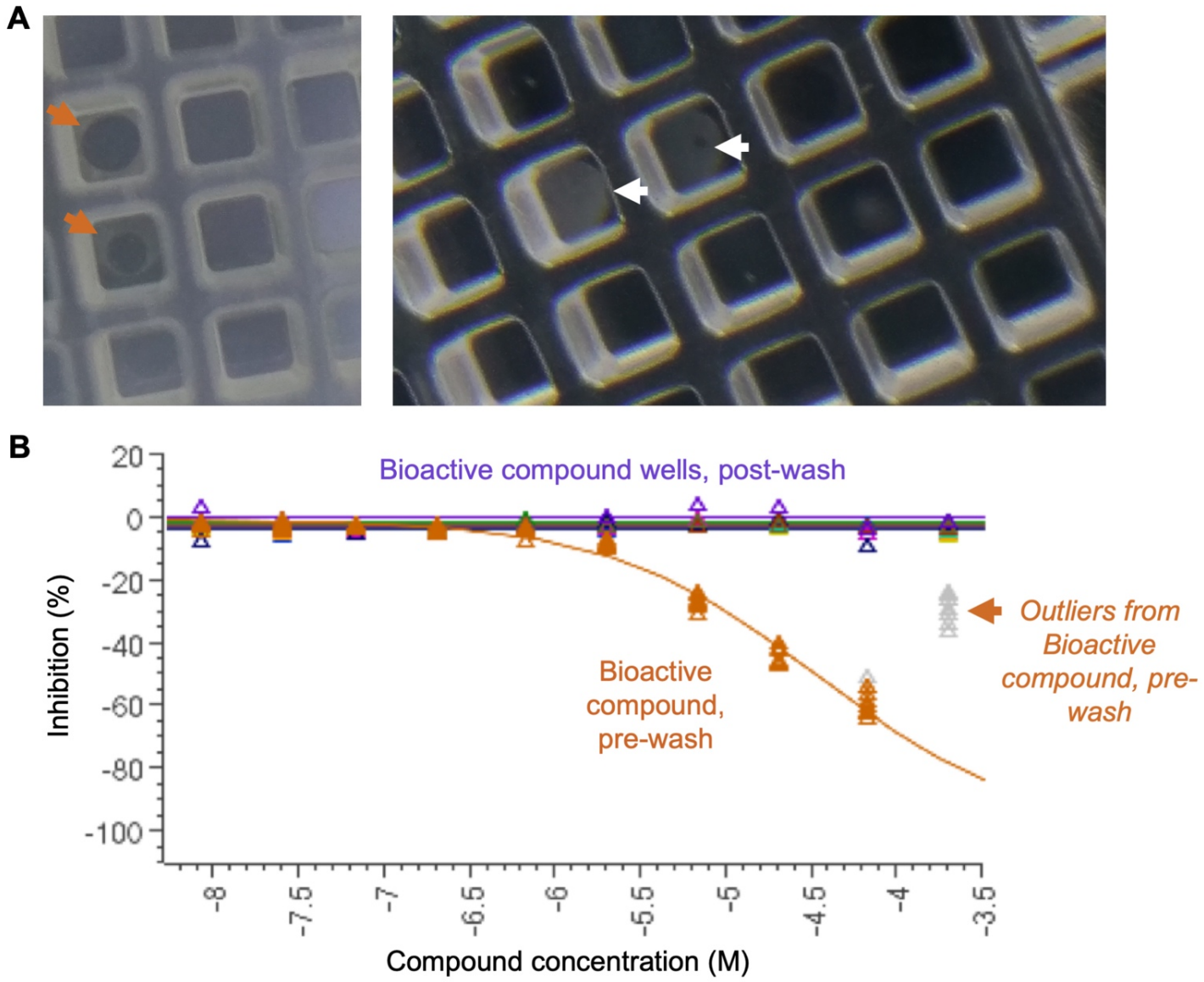


Figure 22. Compound carryover in non-optimized plate washing protocols. (A) Left; circles having a clear area surrounded by a hazy area defining the circle (orange arrows). Right; circles having a hazy area defining the circle with the no haze outside the circle (white arrows). The difference between the two lead to the conclusion that two different phenomena are shown. On the left, compound that "missed" the center droplet dried and once dry would not go into solution when the assay liquid was added. On the right, most of the compound did go into the assay solution but some precipitated and is stuck to the surface of the plate. (B) Effectiveness of microplate washing by incorporation of plasma treatment. A bioactive compound (orange, same as the compound in Figure 22A, left panel) shown was testing using a standard 10-point serial dilution by a homogenous assay. The microplate was then cleaned using a Microplate Cleaning System powered by PlasmaKnife Technology or MCS (ionField Systems). The assay was then repeated in the same wells without test compound. Note that the same wells did not produce significant bioactivity after microplate washing. Data are five technical replicates on a single microplate.

- Sterilize or clean any tissue component that will come into contact with microplates or assay reagents. For example, cassettes for liquid dispensers can be autoclaved and primed with 70% ethanol and then sterile PBS prior to running cells or reagents through.
- Depending on the assay requirements, antibiotics can be included in culture media (e.g., penicillin/streptomycin combination).

- Include control wells or plates without cells and carry it through the assay protocol. Examine these controls for signs of microbial growth. This might be useful for applications not including antimicrobials in culture media.

Chemical compatibility

Some solvents or reagents might not be compatible with a given microplate polymer (Table 5). Perhaps the most important compatibility in the context of biological assays is DMSO, as most test compounds are prepared as DMSO stock solutions, though the total concentration is typically less than 1%. Chemical compatibility is often a more significant issue when certain quenching, detection, or extraction reagents are employed that utilize organic solvents or markedly acidic/basic solutions. Such chemicals can react with the microplate polymers (i.e., oxidization, reaction with functional groups, catalyze de-polymerization), or be absorbed into the bulk microplate material and soften/swell the microplate. It is sound practice to verify the chemical compatibility of assay reagents with microplates in such cases.

Table 5. Generalized compatibility of microplate polymers with select chemicals. Note that compatibility can be concentration- and/or time-dependent. Compatibilities are often assessed using high concentrations of chemicals (e.g., > 10% v/v for most solvents). +, generally compatible; -, generally incompatible; +/-, exercise caution/verify.

		Microplate polymer		
		COP	PP	PS
Alcohols	Ethanol	+	+	+/-
	Isopropanol	+	+	+
	Methanol	+	+	+/-
Other organic solvents	Acetone	+	+	-
	Dimethylformamide (DMF)	+/-	+	-
	Dimethyl sulfoxide (DMSO)	+	+	+/-
Acids	Acetic acid	+	+	+/-
	Hydrochloric acid (HCl)	+	+	+/-
	Sulfuric acid (H_2SO_4)	+/-	-	-
Bases	Sodium hydroxide (NaOH)	+	+	+
Other	Formaldehyde	+	+	-
	Hydrogen peroxide (H_2O_2)	+/-	+	+

Optical isotropy

An optically isotropic material has the same optical properties in all directions. In other words, light rays behave the same in all directions of propagation and polarization when traveling through an optically isotropic material. By contrast, in anisotropic materials, light transmitted through the material is dependent on the ray direction and polarization. Certain microplate plastics (such as polystyrene) can become more anisotropic with mechanical stress from molding procedures. Certain polymers such as COP have more isotropic properties (13).

Optical isotropy is an important consideration for imaging-based assays and those involving polarized light because anisotropic materials can produce certain light-based interferences. Anisotropic materials that affect light polarization can diminish illumination intensity by polarized light sources in fluorescence polarization, FRET, and time-resolved FRET assays. In such cases, the material undesirably depolarizes the polarized incident light, which effectively attenuates the transmitted and emitted light.

Section summary

The optimal use of microplates requires knowledge of special topics including batch-to-batch variability, well-to-well contamination, plate effects, microplate surfaces, and plate washing.

Conclusions

Robust HTS and quantitative biology can be significantly influenced by careful microplate selection and employing best practices. Along with an appreciation that microplate surfaces are non-uniform and can dramatically influence assay readouts, the choice of microplate should not be seen as an afterthought. Particular attention should be made to key parameters such as well number, microplate color, well volume, well shape, and microplate surface coatings. Scientists should carefully optimize experimental procedures including incubation and compound/reagent dispensing to maximize microplate performance. Careful validation processes, plate layouts, and statically tools can help correct for imprecision. Variability in microplates is often be attributed to positional effects, which can be introduced by manufacturing processes and experimental conditions. This information and best practices should therefore enable higher-quality bioassays.

Suggested Readings (alphabetical order)

Knight S, Plant H, McWilliams L, Murray D, Dixon-Steele R, Varghese A, Harper P, Ramne A, McArdle P, Engberg S, Bennett N, Blackett C, Wigglesworth M. Enabling 1536-well high-throughput cell-based screening through the application of novel centrifugal plate washing. SLAS Discov. 2017;22(6):732–742. PubMed PMID: 28027450.

Describes the application of centrifugal plate washing to reduce edge effects in microtiter cellular assays, including those with complex protocols and media changes.

Lilyanna S, Ng EMW, Moriguchi S, Chan SP, Kokawa R, Huynh SH, Chong PCJ, Ng YX, Richards AM, Ng TW, Liew OW. Variability in microplate surface properties and its Impact on ELISA. J Appl Lab Med. 2018;2(5):687–699.

An illustrative case study highlighting the importance of microplate surface properties on assay performance. Includes excellent examples of biophysical and chemical characterization of microplate surfaces including SEM, contact angles, AFM, and elemental analysis.

Smith T, Ho PI, Yue K, Itkin Z, MacDougall D, Paolucci M, Hill A, Auld DS. Comparison of compound administration methods in biochemical assays: effects on apparent compound potency using either assay-ready compound plates or pin tool-delivered compounds. J Biomol Screen. 2013;18(1):14–25. PubMed PMID: 22904199.

An excellent study comparing the performance of compound dispensing technologies and microplate materials in HTS.

Trask OJ. Guidelines for microplate selection in high content imaging. Methods Mol Biol. 1683;2018:75–88. PubMed PMID: 29082488.

A detailed discussion of factors related to microplate selection in high content and phenotypic screening.

Glossary

column/row effects – a type of systematic signal differential in microplates in which rows and/or columns display a bias. Such a bias can be caused by temperature gradients, liquid dispensers and detection instrumentation.

contact angle – the angle where a liquid–vapor interface meets a solid surface; this quantifies the wettability of a solid surface by a liquid

edge effects – a type of systematic signal gradient often observed in microplates in which the perimeter wells have a different readout compared to the interior wells. This is often caused by a temperature gradient and evaporation.

flange – a projecting rim along the base of microplates that serves to maintain plate positioning with various instruments and automation devices

microplate – any type of flat plate containing multiple wells that serve as individual chambers for biological assays; designed for the purpose of increasing throughput

optical isotropy – a material property in which light transmitted through the material behaves the same regardless of its direction and polarization

uniformity plate – a microplate containing the same reagents in every well, usually a positive control achieving maximal assay signal, to estimate systematic biases such as edge, column, and row effects

well effects – a type of systematic signal gradient often observed in microplates in which the signal within a microplate well has a different readout compared to another portion of the same microplate well.

wettability – the ability of a liquid to maintain contact with a microplate surface

wicking – the vertical movement of liquid towards the top of a square microplate well due to capillary-like effects along the right angle edges of the well, often leading to evaporation and well-to-well contamination

Acknowledgements

BKW acknowledges support from the Ono Pharma Foundation, the NIH High-End Instrumentation Program (S10-OD026839), and an NIH NIDDK award (U01-DK123717).

Conflict of Interest Statement

The authors declare no conflicts of interest.

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Basics of Assay Equipment and Instrumentation for High Throughput Screening

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Created: May 1, 2012; Updated: April 2, 2016.

Abstract

This chapter contains a synopsis of general and specialized instrumentation used in screening and lead optimization laboratories. The instrument type is described along with the general principles of operation to familiarize readers considering equipping drug discovery laboratories, principally directed to new investigators. The descriptions are introductory and detailed information on installation and applications should be obtained from instrument vendors and experienced drug discovery scientists and engineers.

Introduction

Instruments employed in quantitative biology laboratories can be broadly categorized into microplates, microplate readers, microplate handling equipment, liquid handling equipment and other miscellaneous instruments such as safety equipment, centrifuges, incubators, electronic balances, microscopes, pH meters, spectrophotometers and other bench-top equipment routinely used in high throughput screening (HTS) assay development laboratories. It is absolutely important to familiarize yourself with the equipment and its proper use.

Common Equipment in HTS Labs

1. **Microplates**
2. **Microplate Readers**
3. **Liquid Handling Equipment**
4. **Microplate Handling Systems**
5. **Misc. Benchtop Equipment**
6. **Hoods, Incubators & Freezers**

Microplates

The microplate is the standard format of miniaturization and automation for bioassays (Biochemical and cell-based assays) associated with drug discovery. Within each microplate is a 2D array of wells with a limited volume for the experimentation to take place. The most common well densities for screening come in 96, 384 and 1536 wells per plate. Numerous manufacturers make microplates in a wide range of materials specific to equipment and customer needs. The footprint of the microplate and well locations has been standardized by ANSI (American National Standards Institute) and SLAS (Society for Laboratory Automation and Screening).

In HTS laboratories, microplates are generally categorized into “Compound Plates” and “Assay Plates”. Compound plates are for storage of the molecular library to be screened against and durable such the same plate can be used for extended periods and across several screens. Assay plates are where the experimentation takes place, selected based on the assay conditions and only used for the extent of an individual screen.

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Available Plate Configurations

- 24-well (Figure 1)
- 96-well & 96-well half-area (Figure 2)
- 384-well (Figure 3)
- 1536-well (Figure 4)

Most commonly used microplates in HTS applications have 96-wells (12 columns × 8 rows) with 250-300 volume µL capacity. Recent introductions include 96-well half area, 384- and 1536-well plates using the same outside plate dimensions (or foot print). The 96-well half area and 384-well plates use less reagents (75-100 µL capacity), and a major advantage is the reduced reagent consumption. However, these plates require special care during reagent delivery. The 1536-well plates are used less commonly, and require special liquid delivery equipment.

Plate Color

Microplates are typically offered in opaque white, opaque black or translucent. Compound plates are typically translucent such that compound volumes and color can be seen. Opaque plates are used to enhance detection technologies. Generally white assay plates are for luminance assays and black assay plates are for florescence assays. The bottom material of an opaque plate may be clear to support bottom reads and colorimetric (absorbance) assays.

Microplate Materials

Microplates are available in numerous materials that have different characteristics and, as such, may be more appropriate for specific applications. The list below provides some examples of microplate materials, their characteristics, and applications for the different plate materials.

- Polystyrene (PS)
  - Typical material for assay plates
  - Low production cost
  - Rigid and brittle
  - Some natural binding properties to biomolecules
- Cyclic Olefin Copolymer (COC)
  - Material for assay and compound plates
  - Works well for acoustic dispensing
  - Less susceptible to breakage during handling
  - DMSO resistant
- Polypropylene (PP)
  - Typical material for compound plates
  - DMSO resistant
  - Thermal Stability
  - More durable than PS

Well Bottom Shape

The shapes of the wells in microplates vary with the application. Flat bottoms are standard and also required for some detection technologies that use transmission of light through the bottom material. Round bottoms can aid in cell washing and V-bottoms reduce dead volumes when transferring volumes between plates.

Figure 1: 24 Well Microplate, Source PerkinElmer http://www.perkinelmer.com

Figure 2: 96 Well Microplate, Source PerkinElmer http://www.perkinelmer.com

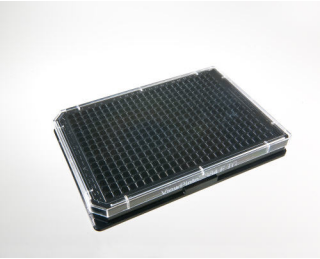


Figure 3: 384-Well Microplate, Source PerkinElmer http://www.perkinelmer.com

Figure 4: 1536-Well Microplate, Source PerkinElmer http://www.perkinelmer.com

Plate Bottom Material

The bottom material of a plate is often specific to the detection technology. Many assays require reading the plate from underneath therefore the plate bottom material must be optically clear. The thickness and quality of clarity varies with the materials used, often a plastic or glass. Microscopy biased detection systems with higher objectives would require more optical clarity then most microplate readers and therefor consideration in comparing materials to costs should be made.

Surface Treatments

To aid in assay performance many surface treatment options are available. Polystyrene inherently binds biomolecules with large hydrophobic regions through passive interactions without treatment. Surface treatment options can enhance or minimize this effect. Tissue culture treated surfaces are also common to aid in cell attachment. These either affect the binding of biomolecules to microplate surfaces or improve the attachment of cell lines.

Microplate Sealing

For the purpose of maintaining specific microplate well conditions there are several options for controlling environmental exposure and contamination including plate lids and plate seals.

Plate Lids

Plate lids rest on the top of a microplate and allow ease of access for biologists and are supported by many automated systems. The material may be machined metal or plastic with a gasket to seal along the parameter of the plate. Automated systems may use a vacuum holding system, specific features built into the lid shape or an empty plate nest to store the lid while the microplate wells are accessed for compound and reagent additions. Assay plate lids may also have a system such as an array of holes for gas exchange.

Plate Seals

The plate seal is an adhesive film that is pressed onto the top of the microplate. The material for the seal and the adhesive can be selected for the application and chemical compatibility required.

Automated Plate Sealing Systems

Automated plate sealing systems can be fed by hand or by robot (Figure 5). Most sealers use a thermal process where the microplate is pressed against a hot plate with the sealing material between. The seal adhesive, and to some extent the top of the microplate, melt making a seal. The seal can be permanent or removed by hand. Besides thermal sealing, there are automated plate sealing systems using press on adhesives. Press-on seals do not have the potential damaging effects of a thermal system such as plate deformation or having a significant heat source in close proximity to the microplate well contents. In addition to sealing plates, there is also instrumentation with the ability to robotically remove seals.

Microplate Readers

The microplate reader is designed to detect and quantify biological, chemical or physical events found within the well of a microplate. There is currently a wide range of detection technologies to suit specific assay requirements many of which can be combined into single multi-purpose instrumentation.

Single & Multi-Mode Readers

Single and multi-mode microplate readers are used for fluorescence, luminescence, absorbance and other light based detection technologies (Figure 6). These detection technologies share many of the same components differing in the light paths through the sample. Single mode microplate readers can be small and economical tailored to a specific technology. A bit larger and costlier then single mode readers, a multi-mode reader can be very advantageous for a lab combining multiple technologies and detection modes into a single more versatile unit. In many cases the use of a detection reagent is required to quantify a specific event.

Figure 5: PlateLoc^{TM} Thermal Microplate Sealer, Source Agilent Technologies http://www.chem.agilent.com

Figure 6: Synergy HT Multi-Mode Microplate Reader, Source Biotek http://www.biotek.com

Common Detection Technologies

- Fluorescence Intensity (FI) - A light source of a specific wavelength illuminates fluorescent molecules within a sample causing the simultaneous light emission from the sample (Figure 7). If the emission light is of a different wavelength it can be filtered from the excitation source light and can then be measured using a light detector such as a photomultiplier tube (PMT). Some variations of the technology.
    - Time-Resolved Fluorescence (TRF) - Uses specific fluorescent molecules called lanthanides that have long light emission times following the removal of an excitation source. The excitation light source is pulsed and the light detector measures the sample after other fluorescent molecule emissions have diminished resulting in lower backgrounds then FI however there is less assay compatibility and higher reagent costs.
    - Fluorescence Polarization - Polarization refers to the orientation waves, in this case waves of light. Similar to FI with the addition of polarizing light filters for the excitation light source and sample emission. Fluorescence polarization measures the mobility of florescent molecules. Fluorescent molecules attached to larger objects will rotate relatively slowly and will emit more polarized light when excited by a polarized light source. Smaller molecules will rotate more rapidly and the light emission will become depolarized. Useful in measuring molecular binding.
- Luminescence - Measurement of light emission caused by a chemical or biochemical reaction (Figure 8). Luminescence does not require an excitation light source. Used in many luciferase-based assays such as gene expression, cytotoxicity and ATP detection.

- Absorbance - Measuring the amount of light absorbed as it passes through the well. A light source of a selected wavelength illuminates the sample while the detector measures the amount of light from the opposing side of the well (Figure 9). The amount of light absorbed can be related to the biology of interest.
- AlphaScreen - Assay technology developed using bead-based chemistry to study bimolecular interactions through homogeneous proximity. Binding of molecules of interest captured on anode and cathode beads leads to an energy transfer from one bead to the other producing a signal when subjected to a specific excitation. Requires the use of a 680nm laser excitation not available on all microplate readers.

Filters and Monochromators

Filters and monochromaters are two competing wavelength selection technologies integral to microplate reader design. Both have their advantages.

- Monochromator - A diffraction grating separates white light into a spectrum such that a slotted material can be positioned isolating the specific wavelength of light. Used for both excitation and emission filtering.
  - Convenient and flexible, does not require an inventory of compatible filters
  - Can perform a spectral scan to characterize unknown fluorophores or spectral shifts
  - Reduction of signal/sensitivity due to the significant loss of light in diffraction gratings
- Filter Based - Optical filters with specific wavelengths and bandwidths are incorporated into the excitation and detection light paths.
  - Less expensive components compared with monochromators
  - Minimal signal loss and effective separation of excitation and emission wavelengths
  - Several filters are typically maintained within the equipment and/or accessed for changing by the equipment operator.
  - Cannot perform spectral scan
  - An initial inventory of commonly used filters is required with the likelihood of purchasing more filters over time to accommodate changing needs

Many microplate readers are modular with numerous upgrade paths.

Common Options to Consider

- Top Read, Bottom Read or Both - Single mode microplate readers often have the detector light path set to read from only the top or bottom of the plate depending on the detection technology while multimode readers are often adapted to read plates from either the top or bottom of the microplate.
  - Bottom reads require a clear bottom microplate such that light can pass through often used for absorbance assays and florescence intensity often performs better for cells adherent to the bottom of the plate.
  - Top reads perform best with solid bottom plates and work well for detection of molecules that are suspended in solution.
- Temperature Control & Incubation
- Gas Purging
- Reagent Injection- Some assays kits signals decay rapidly and require near simultaneous detection that can only be achieved by delivering reagents within the microplate reader.

Major manufacturers of microplate readers include:

- PerkinElmer
- Tecan
- Thermo Scientific
- BMG Labtech
- Molecular Devices

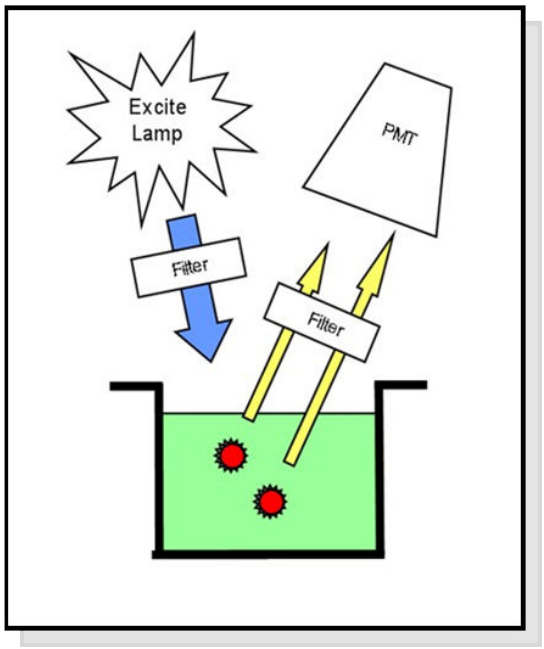


Figure 7: Simplified Fluorescence Detection

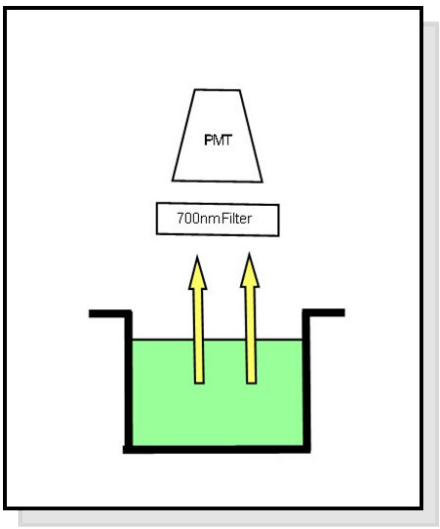


Figure 8: Simplified Luminescence Detection

- Biotek

Ultra High Throughput Screening Microplate Reader

The ViewLux^{TM} uHTS Microplate Imager is a commonly used device in high throughput screening with some unique features of note (Figure 10). The ViewLux^{TM} operates on similar light based detection technologies as other microplate readers however, while most readers provide excitation light and detection on a well to well basis, the ViewLux^{TM} excites the entire microplate at once and using a highly sensitive CCD camera to capture an image of the emission signal. The image is rapidly processed to correct for any parallax error and to provide a numerical representation for each individual well. Though many microplate readers are more sensitive and considerably less costly the time to process an entire microplate on the ViewLux can be reduced from minutes to

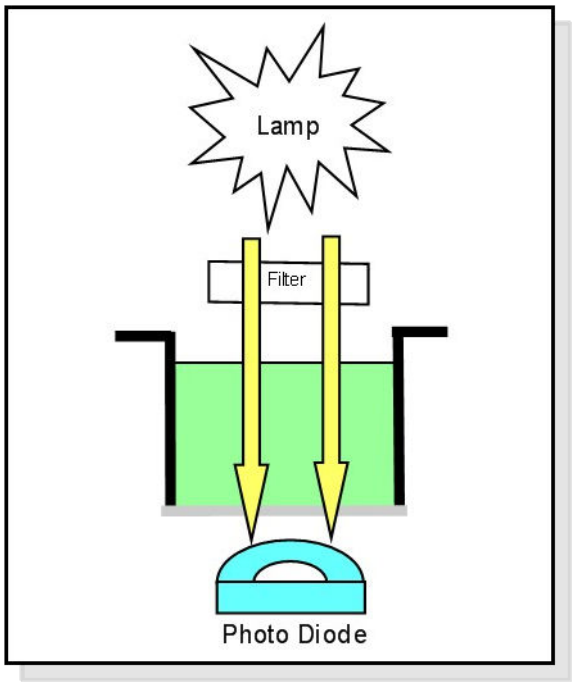


Figure 9: Simplified Absorbance Detection

seconds. Due to its size and cost, the ViewLux^{TM} is typically reserved for assay development and automated screening of a high volume of compounds (manufactured by PerkinElmer).

High Content Imagers

High Content Screening (HCS) an extension of HTS utilizing much of the same processes and equipment for screening except that the microplate read portion of the assay is performed using an automated microscope or high content imager. Much more than a standard microscope, a high content imager incorporates an automated platform for plate handling and image processing software to quantify the data collected under specified parameters. Using one or more fluorescent dyes different excitation light sources can be applied and images saved of the emission. A separate image showing each fluorescent emission is taken in rapid succession and then overlaid onto each other resulting in a high resolution image of the results. High content imagers are able to collect data other microplate readers cannot such as cell morphology or spatially localized proteins.

Excitation light is provided using a lamp, lasers or light emitting diodes (LED). LEDs are currently the optimal choice due to their long life span and stable output. Emission light is collected from the bottom of the plate through microscope objectives that can be changed to different magnifications and the image is captured using a digital camera. Some high content imagers can be equipped with a confocal microscope system. Considerably more complex and expensive, a confocal system can provide further depth resolution and improved contrast by rejecting light from out of focus sources. A confocal system is particular useful in imaging small or 3D cellular systems/structures and samples with strong background fluorescence.

Manufactures include:

- GE
- Thermo Scientific
- PerkinElmer
- Molecular Devices
- Yokogawa

Figure 10: ViewLux^{TM}, Source PerkinElmer http://www.perkinelmer.com

Laser Scanning Cytometers

Laser scanning cytometers in comparison to other microplate readers can be classified as medium content imaging. Excitation is by laser across the surface of a microplate, as a molecule excites and fluoresces it is detected by photo multiplier tube. The technology is effective at detecting cells, colonies and model organisms but not subcellular features or processes. Has large depth of focus allowing differentiation between low and high concentration of signals.

Manufactures include:

- TTP Labtech
- Molecular Devices
- Hamamatsu

Label Free Detection

Label free detection refers to the quantification of biological, chemical or physical events without the use of detection reagents. Detection reagents can be of considerable cost and though they are not used for label free detection the money saved is more than offset by the need of specialized plates. There are currently two methods of label-free detection on the market, monitoring the change in impedance and detecting shifts of the refractive index from the bottom of the plate.

Impedance-based label-free readers use microplates with integrated electrodes molded to bottom of the wells. A voltage is applied to each well and electrical currents flow around, between and through cells. The measurement of impedance or resistances to electrical flow is recorded during the duration of the event to be monitored. The microplate and electrical connections are often routed through and access port of an incubator to maintain environmental conditions while monitoring over long periods. Changes in cell adherence, shape, volume and interactions ultimately affect the recorded impedance logged in real time.

Using a specialized microplate changes near the bottom surface of the well are detected by monitoring the reflected wavelength. A refractive waveguide biosensor grating is imbedded into the bottom surface of the microplate. As a broadband light source illuminates the bottom of the well a reflected wavelength is detected indicating the refractive index near the well bottom. After a cell binding event or intracellular protein movement a shift in the refractive index occurs and is then detected by the change in the reflected wavelength (Figure 11).

Manufactures include:

- Corning
- PerkinElmer
- Molecular Devices
- SRU Biosystems

Spectrophotometry

The electromagnetic spectrum stretches from radio waves to gamma rays (Figure 12).

Spectrometric Techniques

- Absorption
- Emission
- Scattering
- Ultraviolet and Visible Absorption Spectroscopy
- Dual-beam uv-vis spectrophotometer
- Fluorescence Spectroscopy

Electromagnetic Spectrum

Spectroscopy is the use of the absorption, emission, or scattering of electromagnetic radiation by atoms or molecules (or atomic or molecular ions) to qualitatively or quantitatively study the atoms or molecules, or to study physical processes. The interaction of radiation with matter can cause redirection of the radiation and/or transitions between the energy levels of the atoms or molecules. A transition from a lower level to a higher level with transfer of energy from the radiation field to the atom or molecule is called **absorption**. A transition from a higher level to a lower level is called **emission** if energy is transferred to the radiation field or non-radiative decay if no radiation is emitted. Redirection of light due to its interaction with matter is called **scattering**, and may or may not occur with transfer of energy, i.e., the scattered radiation has a slightly different or the same wavelength.

Absorption

When atoms or molecules absorb light, the incoming energy excites a quantized structure to a higher energy level. The type of excitation depends on the wavelength of the light. Electrons are promoted to higher orbitals by ultraviolet or visible light, vibrations are excited by infrared light, and microwaves excite rotations. An absorption spectrum is the absorption of light as a function of wavelength. The spectrum of an atom or molecule depends on its energy level structure, and absorption spectra are useful for identification of compounds. Measuring the concentration of an absorbing species in a sample is accomplished by applying the *Beer-Lambert Law*.

The Beer-Lambert law (or Beer's law) is the linear relationship between absorbance and concentration of an absorbing species. The general *Beer-Lambert law* is usually written as:

$A=a(\lambda)*b*c$

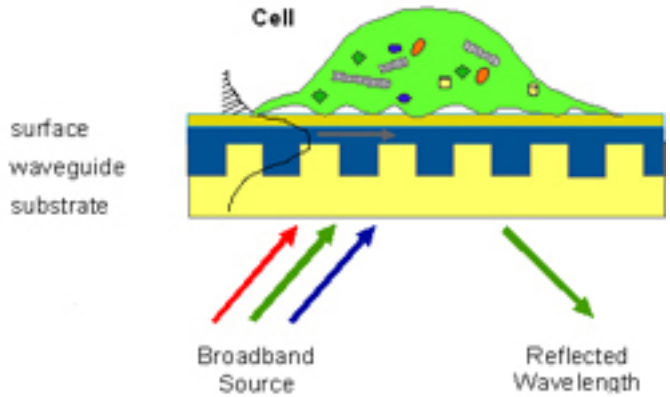


Figure 11: Epic Label Free Detection System, Source Corning http://www.corning.com/lifesciences/epic/en/products/epic_system.aspx

where A is the measured absorbance, a(λ) is a wavelength-dependent absorptivity coefficient, b is the path length, and c is the analyte concentration. When working in concentration units of molarity, the *Beer-Lambert law* is written as:

$A=\varepsilon^{*}b^{*}c$

where ε is the wavelength-dependent molar absorptivity coefficient with units of M^{-1} cm^{-1}. Experimental measurements are usually made in terms of transmittance (T), which is defined as $T = I / I_o$, where I is the light intensity after it passes through the sample and Io is the initial light intensity (Figure 13). The relation between A and T is:

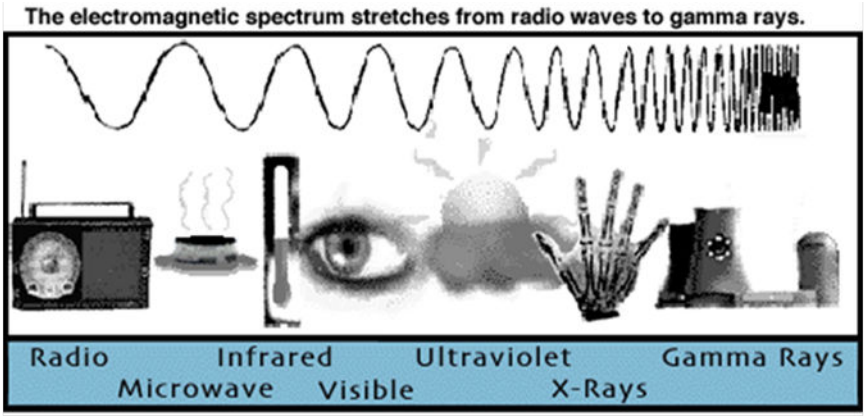
$A=-\log T=-\log(I/I_o)$.

The linearity of the Beer-Lambert law is limited by chemical and instrumental factors. Causes of nonlinearity include:

- Deviations in absorptivity coefficients at high concentrations (>0.01 M) due to electrostatic interactions between molecules in close proximity.
- Scattering of light due to particulates in the sample.
- Fluorescence or phosphorescence of the sample.
- Changes in refractive index at high analyte concentration.
- Shifts in chemical equilibria as a function of concentration.
- Non-monochromatic radiation. (Deviations can be minimized by using a relatively flat part of the absorption spectrum such as the maximum of an absorption band).
- Stray light leaking into the sample compartment.

Emission

Atoms or molecules that are excited to high energy levels can decay to lower levels by emitting radiation (emission or luminescence). For atoms excited by a high-temperature energy source this light emission is commonly called atomic or optical emission (see atomic-emission spectroscopy), and for atoms excited with light it is called atomic fluorescence (see atomic-fluorescence spectroscopy). For molecules it is called molecular fluorescence if the transition is between states of the same spin and phosphorescence if the transition occurs between states of different spin. The emission intensity of an emitting substance is linearly proportional to analyte concentration at low concentrations, and is useful for quantifying emitting species (Figure 14).



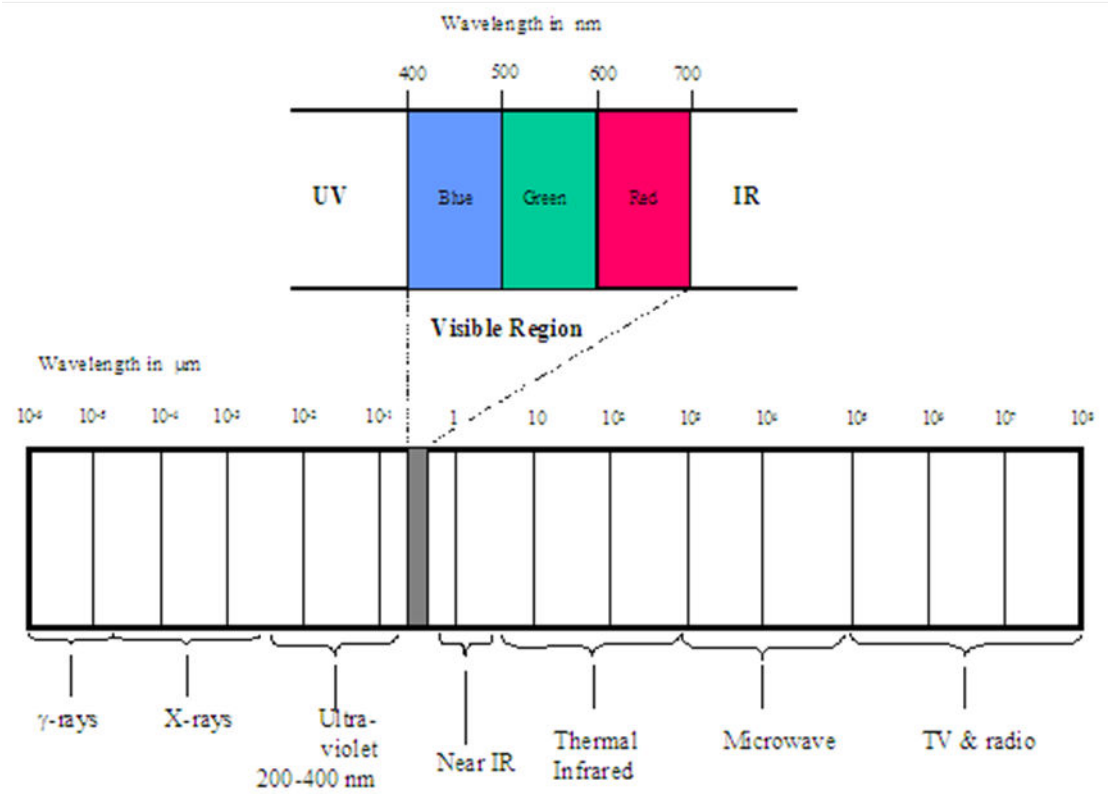


Figure 12: Schematic depictions of the electromagnetic spectrum.

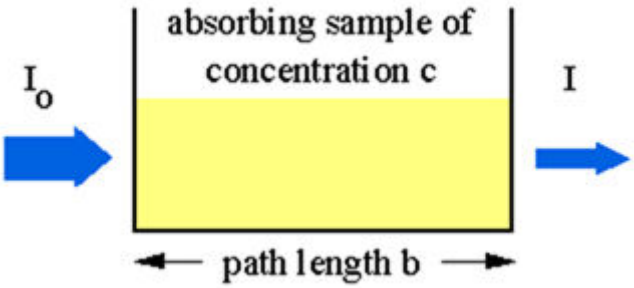


Figure 13: Schematic of Beer-Lambert law

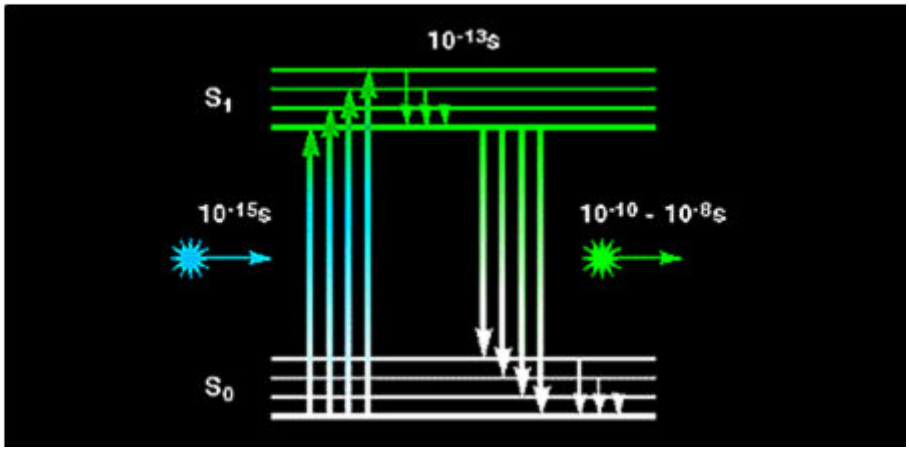


Figure 14: Jablonski Diagram

Scattering

When electromagnetic radiation passes through matter, most of the radiation continues in its original direction but a small fraction is scattered in other directions. Light scattered at the same wavelength as the incoming light is called Rayleigh scattering. Light that is scattered in transparent solids due to vibrations (phonons) is called Brillouin scattering. Brillouin scattering is typically shifted by 0.1 to 1 cm^{-1} from the incident light. Light that is scattered due to vibrations in molecules or optical phonons in solids is called Raman scattering. Raman scattered light is shifted by as much as 4000 cm^{-1} from the incident light.

Ultraviolet and Visible Absorption Spectroscopy

UV-vis spectroscopy is the measurement of the wavelength and intensity of absorption of near-ultraviolet and visible light by a sample. Ultraviolet and visible light are energetic enough to promote outer electrons to higher energy levels. UV-vis spectroscopy is usually applied to molecules and inorganic ions or complexes in solution. The UV-vis spectra have broad features that are of limited use for sample identification but are very useful for quantitative measurements. Measuring the absorbance at some wavelength and applying the Beer-Lambert Law can determine the concentration of an analyte in solution. The light source is usually a hydrogen or deuterium lamp for UV measurements and a tungsten lamp for visible measurements. The wavelengths of these continuous light sources are selected with a wavelength separator such as a prism or grating monochromator. Spectra are obtained by scanning the wavelength separator and quantitative measurements can be made from a spectrum or at a single wavelength (Figure 15).

Dual-beam UV-VIS spectrophotometer

In single-beam UV-vis absorption spectroscopy, obtaining a spectrum requires manually measuring the transmittance (see the Beer-Lambert Law) of the sample and solvent at each wavelength. The double-beam design greatly simplifies this process by measuring the transmittance of the sample and solvent simultaneously (Figure 16). The detection electronics can then manipulate the measurements to give the absorbance. Table 1 provides specifications for a typical spectrophotometer.

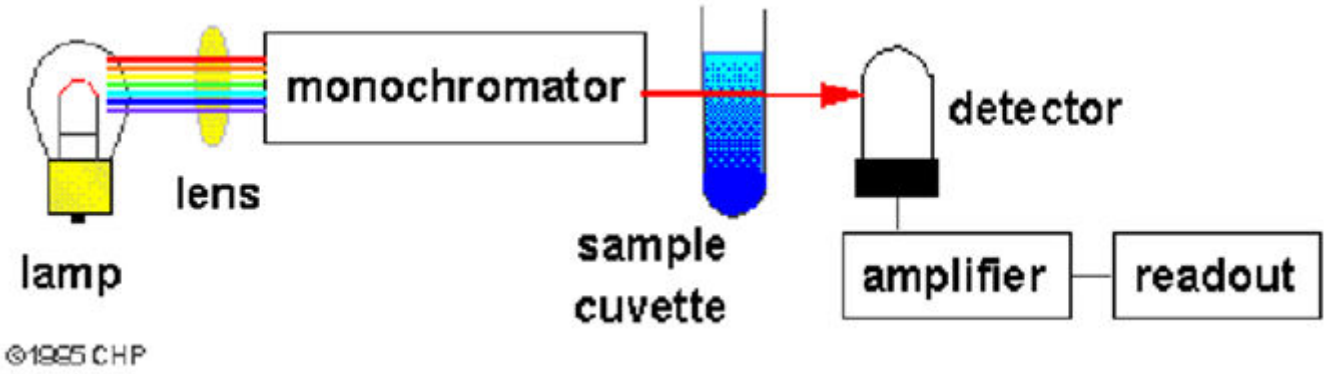


Figure 15: Schematic of a single beam UV-vis spectrophotometer

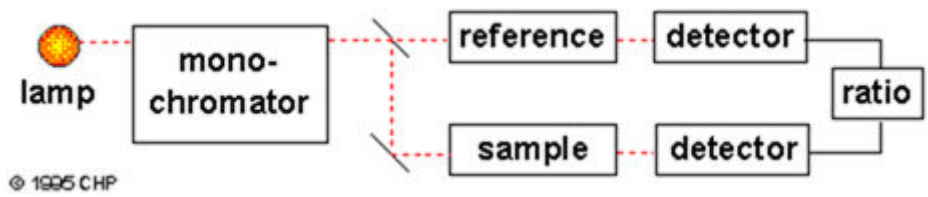


Figure 16: Schematic of a dual-beam UV-VIS spectrophotometer.

Table 1: Specifications of a typical spectrophotometer:

Wavelength range:	200~800 nm
Spectral Band width:	2 nm
Wavelength Accuracy:	1 nm
Stray Light:	Less than 0.002%(300 nm, 630 nm)
Photometric Range:	Absorbance-1~3 Abs
Transmittance:	0~200%T
Photometric Accuracy:	0.5%T
Photometric Repeatability:	0.3%T
Light Source:	Tungsten Lamp, Deuterium Lamp
Monochromator:	Diffraction grating single monochromator system
Cell:	Max. 4 turrets
Dimension:	500mm(L) x 380 mm(W) x 230 mm(H)
Power Requirements:	110/220V, 3A, 50/60 Amps.

Fluorescence Spectroscopy

Light emission from atoms or molecules can be used to quantify the amount of the emitting substance in a sample. The relationship between fluorescence intensity and analyte concentration is:

$F=k*QE*P_o*(1-10[-*b*c])$

where F is the measured fluorescence intensity, k is a geometric instrumental factor, QE is the quantum efficiency (photons emitted/photons absorbed), P_o is the radiant power of the excitation source, is the

wavelength-dependent molar absorptivity coefficient, b is the path length, and c is the analyte concentration (, b, and c are the same as used in the Beer-Lambert law). Expanding the above equation in a series and dropping higher terms gives:

$F=k*QE*P_o*(2.303*b*c)$

This relationship is valid at low concentrations ($<10^{-5}$ M) and shows that fluorescence intensity is linearly proportional to analyte concentration. Determining unknown concentrations from the amount of fluorescence emitted from a sample requires calibration of a fluorimeter with a standard (to determine k and QE) or by using a working curve.

Many of the limitations of the Beer-Lambert law also affect quantitative fluorimetry. Fluorescence measurements are also susceptible to inner-filter effects. These effects include excessive absorption of the excitation radiation (pre-filter effect) and self-absorption of atomic resonance fluorescence (post-filter effect).

pH Meters

The pH meter measures the pH of a solution using an ion-selective electrode (ISE) that responds to the H^+ concentration of the solution. The pH electrode produces a voltage that is proportional to the concentration of the H^+ concentration, and making measurements with a pH meter is therefore a form of potentiometry. The pH electrode is attached to control electronics that convert the voltage to a pH reading and display it on a meter. A pH meter consists of a H^+-selective membrane (ISE), an internal reference electrode, an external reference electrode, and a meter with control electronics and display (Figure 17). Commercial pH electrodes usually combine all electrodes into one unit that are then attached to the pH meter.

An ion-selective electrode (ISE) produces a potential that is proportional to the concentration of an analyte. Making measurements with an ISE is therefore a form of potentiometry. The most common ISE is the pH electrode, which contains a thin glass membrane that responds to the H^+ concentration in a solution. The potential difference across an ion-sensitive membrane is

$$E=K-(2.303RT/nF)\log(a)$$

where K is a constant to account for all other potentials, R is the gas constant, T is temperature, n is the number of electrons transferred, F is Faraday's constant, and a is the activity of the analyte ion. A plot of measured potential versus log(a) will therefore give a straight line. ISEs are susceptible to several interferences. Samples and standards are therefore diluted 1:1 with total ionic strength adjuster and buffer (TISAB). The TISAB consists of 1 M NaCl to adjust the ionic strength, acetic acid/acetate buffer to control pH, and a metal complexing agent. ISEs consist of the ion-selective membrane, an internal reference electrode, an external reference electrode, and a voltmeter. A typical meter is shown in the document on the pH meter. Commercial ISEs often combine the two electrodes into one unit that are then attached to a pH meter.

***NOTE** : All pH meters should be calibrated daily, and preferably, before each use. It is important to know that the ISE potential can drift due to drying of the LaF3 membrane and the evaporation of internal reference solutions.*

Electronic Balances

Analytical balances are accurate and precise instruments to measure weights (Figure 18). They require a draft-free location on a solid bench that is free of vibrations. Modern balances have built-in calibration weights to maintain accuracy. Older balances should be calibrated periodically with a standard weight. A few weighing tips follow:

- Do not bump or place objects on the bench after zeroing the balance.

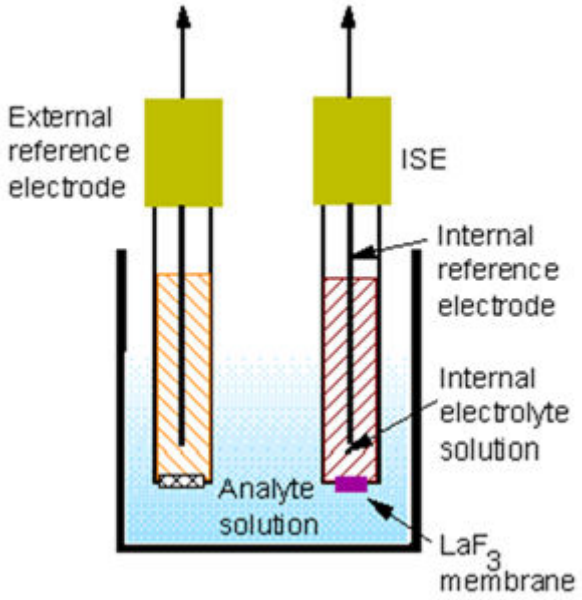


Figure 17: Schematic of an ISE measurement.

- Weigh powders on weighing paper or in weighing dishes. Handle objects with tongs, gloves, or weighing paper to prevent fingerprints.
- Let hot objects cool before weighing.
- Weigh hygroscopic materials rapidly since they will absorb water during weighing.
- When making repetitive weighings always use the same procedure.
- Ensure that the balances are calibrated routinely.
- It is absolutely important to keep the balance pans and the area around precision balances clean. Clean up after each use for safety and convenience of others.
- In dry cold weather, weighing fine powders can be a problem due to static charges that develop on particles. Devices are now available for neutralizing electric charges of the material while it is still in the container. Use of these devices is highly recommended for accurate weighings.

Microscopes

- Microscopes
- Microscope Parts
- Objectives
- Condensers
- Iris Diaphragm
- Eyepiece
- 2.5 Field Limiting Aperture

A microscope is not designed to magnify small objects. For example, you can find in any hobby or toy store a $49.95 instrument capable of magnifying objects to 1200 times. And that includes a zoom lens and light source. Most student and research microscopes magnify no more than 1000 times with costs starting at around $1500.00, with research microscopes going into the tens of thousands of dollars. Is the academic community being taken for a ride? No. The $49.95 microscope only gives you an image that is a soft blur at 1000x magnification, whereas the research microscope's image is crystal sharp. This is called resolution, the ability to see fine details. Once you can resolve fine details then you can magnify them. Every optical system has a finite

Figure 18: Representative image of a balance.

resolution; if you magnify objects beyond the resolution the result will be empty magnification. **So, the actual purpose of a microscope is to see small things clearly.**

A desirable attribute of a microscope is depth of field, which is the range of depth that a specimen is in acceptable focus. A microscope that has a thin depth of field will have to be continuously focused up and down to view a thick specimen. A third feature that a microscope has is its mechanism for contrast formation. Contrast is the ratio between the dark and the light. Typically, most microscopes use absorption contrast; that is, the specimen is subjected to stains in order to be seen. This is called bright field microscopy. There are other types of microscope that use more exotic means to generate contrast, such as phase contrast, dark field, and differential interference contrast. The fourth desirable feature is a strong illumination source. The higher a microscope magnifies the more light will be required. Also, there will be more optical trade off leeway when more light is present. The illumination source should also be at a wavelength (color) that will facilitate the interaction with the specimen. All microscopes fall into either of two categories based on how the specimen is illuminated. In the typical compound microscope the light passes through the specimen and is collected by the image forming optics. This is called diascopic illumination. Dissecting (stereo) microscopes generally use episcopic illumination for use with opaque specimen. The light is reflected onto the specimen and then into the objective lens. The four attributes of an optical system may have trade-offs with each other. For example, resolution and brightness is antagonistic towards contrast and depth of field. One cannot have maximum resolution and maximum contrast simultaneously. Theoretically speaking, if you had an infinite resolving system there would be no contrast to discern the image. It is up to the microscopist to decide which attribute is needed to view a particular specimen. All of which are controlled by the iris diaphragm.

Microscope Parts

Objectives

The objective lens is the lens that is closest to the object or specimen (Figure 19). It is essentially the information-gathering lens of an optical system. Therefore, it is regarded as the most important lens of the microscope. There are many different types of objective lenses. The most common and inexpensive is the achromat. This lens is usually found on student microscopes. It is corrected for spherical aberration for only green light. Chromatic aberration is corrected in only two colors. The apochromat objective is far superior and generally very expensive. Chromatic aberration is corrected for all three colors and it is spherically corrected for two colors. These objectives quite often will require a special compensating eyepiece. Semiapochromat objectives have correction in between the apochromat and achromat. Flat field or plano objectives compensate for curvature of field and are excellent for histology work. The flat field objectives can be optically constructed to be also an achromat, semiapochromat or apochromat. In the latter case the lens would be called a plano apochromat which is generally regarded as the finest lens available. The price of a single plano apochromat will run into the many thousands of dollars. Figure 20 provides a comparison of the resolution using different types of objectives.

Each objective has information critical for the maximum resolution possible written on the side of the barrel. Generally the magnification is printed in the largest text with the manufacturer type designation. The second value is the numerical aperture. Beneath that, in a smaller font the tube length and the cover glass thickness is given. Any special information will also be added such as if it is an oil lens, infinity etc. The tube length usually 160 refers to the distance between the objective and the eyepiece in millimeters. It must be maintained if the aberrations are to be corrected. You can recognize a superior microscope if when adjusting the interpupillary distance you can see the eyepiece extend which happens to maintain the proper tube length. The cover slip thickness usually around 0.17mm is also critical. This corresponds to a cover glass of No. 1.5. The more sophisticated objectives even have a cover glass compensation control that you dial in the thickness of the cover glass.

Condensers

The sub-stage condenser of a microscope is designed to focus the light onto the specimen. In addition it must also fill the numerical aperture of the objective (Figure 21). Like objective lenses there are several different types. The most common being the Abbe condenser. This type is not corrected for optical aberrations. The achromatic condenser is corrected for both spherical and chromatic aberrations. Both types of condenser have their numerical aperture printed on the side. This needs to be of equal or greater value than that of the objective N.A., otherwise, the full resolution of the objective will not be utilized. Most substage condensers can use immersion oil like that of the objectives to achieve their full N. A. This is not recommended unless you are doing very demanding photomicroscopy work.

Iris Diaphragm

The iris diaphragm is the most important single control on the microscope (Figure 22). There is a misconception that it is used to regulate the amount of light. The light intensity control is the sole means to adjust the brightness. The iris diaphragm is the resolution verses contrast control. It does this by varying the size of the numerical aperture of the objective lens. Usually, lenses such as those found on cameras have the iris diaphragm built in the objective lens. In a microscope objective the iris diaphragm would have to be very small, which would be difficult to manufacture. So the optical engineers put the iris diaphragm at the optical equivalent of being in the objective lens, in the condenser assembly. This is one of the reasons why the condenser lens has to be set at the correct distance to the objective. In addition the iris diaphragm controls the depth of field.

Figure 19: Image of objective lens

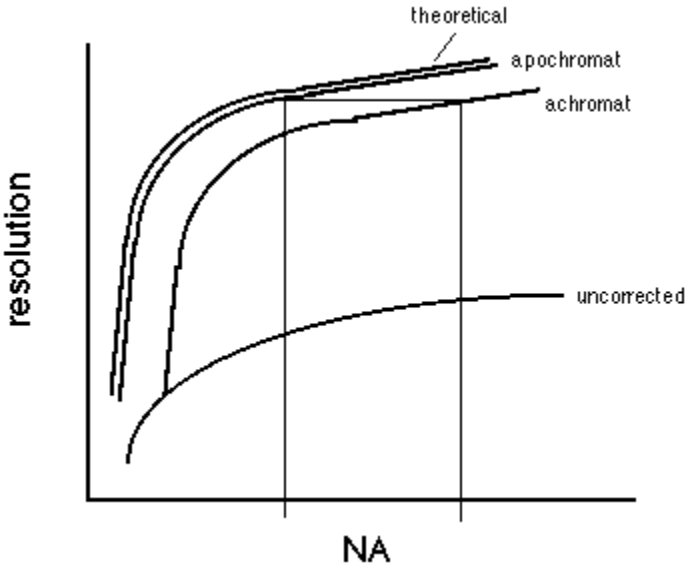


Figure 20: Comparison of the resolution using different types of objectives.

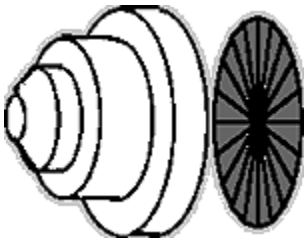


Figure 21: Example of a substage microscope condenser.

Eyepiece

The eyepiece is basically a projection lens system (Figure 23). There are three types generally used in light microscopy. The most common is the Huygenian type. This eyepiece is used with low and medium magnification and is designed to project the image into a human eye. Some of these eyepieces will have a long eyepoint, the spot where your eye should be, so you can focus with your glasses on. If you suffer from astigmatism you should wear your glasses while using the microscope. If you are near or far sighted then you can adjust the eyepiece for your personal correction using the diopter corrector and leave your glasses off. The second type of eyepiece is the compensating eyepiece and is generally used with apochromate or flat field objectives. These provide superior image quality. The third type is the photo eyepiece. These are designed to project a corrected image onto film

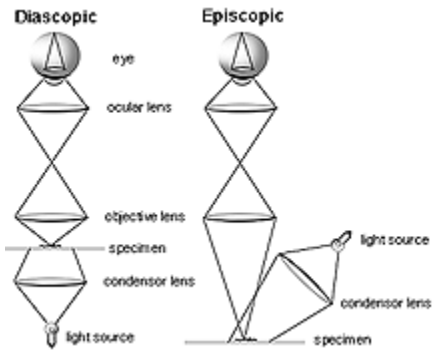


Figure 22: Schematic of iris diaphgram control.

plane in a camera. These are generally considered the finest of eyepieces. All eyepieces will have a relative magnification written on the side of the barrel. They range in magnification from 2.5X to 15X with the lower magnifications used with the photo eyepiece.

Field Limiting Aperture

The field-limiting aperture is used to determine the correct position and center of the condenser lens. It is used in conjunction with the condenser centering knobs to place the illumination in the center. It also helps in reducing the amount of optical flare.

Liquid Handling Devices

The high throughput screening lab has several liquid handling applications and device technologies are chosen to best suit the specific task.

Hand-held Pipette

The pipette transfers precise volumes of liquids through movement of a piston and the displacement of air (Figure 24). Pipettes have specified volume ranges and the user selects the increment. The usable range of volume is 100nl to 1ml. Pipette tips are disposable and available in numerous shapes, sizes and treatments. Accurate manual pipetting is a lab skill acquired with practice. Common variations of pipettes are as follows.

- Single-channel
- Multi-channel – Typically 8 or 12 simultaneous channels
- Electronic
- Repeaters – Allows for multiple dispenses following a single aspiration

Automated Pipetting Devices

Automated pipetting platforms are scalable to the application and level of automation desired (Figure 25). Microplates are positioned in specific locations on the instrument deck or into a plate stacker. The number of available positions can range from 2 - >16 positions and further plate capacity can be added when using stackers. The head, consisting of the liquid handling apparatus, may be outfitted with a single tip, single column of tips or 96/384 2D array of tips for accommodating whole plate transfers referred to as plate stamping. Tips used may be disposable or reusable with washing applications.

Creating methods on these devices can be very involved and uses elements of programming logic such as variable parameters, database access and conditional looping. Once a method is optimized it is then very

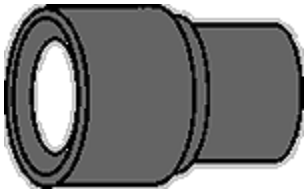


Figure 23: Example of a microscope eyepiece.

Figure 24: Example pipettes

reproducible. These devices are often used as the main integrating platform for compact systems with options to include plate transferring capabilities and auxiliary equipment.

Major manufacturers of automated pipetting systems include:

- Beckman Coulter
- Hamilton
- Perkin Elmer
- Cybio
- Agilant
- Tecan

Solenoid Valve Based Dispensers

Using a pressurized bottle of fluid, a fast acting valve and robotic positioning, these systems can deliver precise volumes (>0.1 µl) into the microplate well at very high rates of speed. Assuming a constant air pressure in the bottle, the volume to be dispensed is controlled by adjusting valve timing.

Most systems offer the ability to dispense multiple fluids simultaneously using separate valves and fluid paths. Dispensing is controlled by a spreadsheet correlating the specific valve and volume to the microplate well. Common fluids for this equipment include cell media, buffers and detection reagents. Dispensing of viscous fluids or cells prone to clumping can be problematic and interfere with valve operation. Dispensing DMSO is possible though may cause rapid valve degeneration.

Most components of the equipment fluid path are reused and therefore cleaning operations are a must. In most cases a combination of 70% ethanol, cleaning detergents and high purity water are sufficient.

These systems are used heavily in high throughput screening due to their dispense accuracy and speed of operation. Dispensing a nominal volume across an entire microplate can take 1 to 3 minutes.

Manufacturers of Solenoid Valve Based Dispensers:

- Beckman Coulter
- Thermo Fisher Scientific

Figure 25: 96 Channel Automated Pipette, Source Tecan http://www.tecan.com

Peristaltic Pump Based Dispensers

Peristaltic pumps are found in numerous applications in the laboratory and medical fields (Figure 25). Peristaltic pumps move fluids using positive displacement by pinching flexible tubing with rotating sets of rollers. The pumping liquid is maintained within the tubing and no external contact is made between pump components and fluid. This lack of contact allows for a large range of chemical capabilities based solely on the tubing material. Tubing is intended to be remove or replaced. Direction and speed are easily varied.

For microplate dispensing the peristaltic pump has some specific features. The fluid path is part of a cassette assembly consisting of tubing, tips, tension adjustment screws and plastic housing. The cassettes are consumable with lifespans varied by manufacturer and operating volume ranges. The equipment has a plate positioning system, motor controlled rotating rollers and the user interface.

The main disadvantage of the peristaltic pump microplate dispenser is the cost of consumable cassettes. Each cassette costs $500-$1000 and is considered accurate for dispensing a few hundred plates. The lifespan may be increased by proper cleaning, storage and recalibration techniques. The advantage is that cassettes are accurate out of the box.

Manufacturers of Peristaltic Pump Based Dispensers:

- Thermo Fisher Scientific
- Biotek

Pintools

The pintool is used for fixed low volume liquid transfers between microplates. For HTS this equipment is used for the transfer of compounds from the library into the assay plate. The pins are stainless steel with precision machined features to set the volume of liquid transfer (Figure 26). As the pin enters the source, pin surfaces

make liquid contact. When the pin is withdrawn, small amounts of liquid adhere to the surfaces until the pin is submerged again and surface tension of the adherent liquid can be broken. Following any liquid transfer, pintool pins must be washed and dried prior to their next use. Washing steps typically include solvent baths, blotting and air drying.

The pintool head is the fixture to hold the pins the proper positions (Figure 27). The head is made to match the diameter of the pins used and plate density, typically 96, 384 or 1536 pins to be used with 96-, 384-, or 1536-well plates, respectively. Though fixtures can be made for hand held operations, higher density plates require higher levels of precision for accurate transfers thus necessitating the need for robotic control. Most liquid handling robotic systems can be adapted to use pintools, while dedicated systems are also available.

The pintool is advantageous for HTS due to its high speed and direct correlation of compound plate well position to assay plate well position. The principal provider of pintool pins and fixtures is V&P Scientific.

Acoustic Dispensers

Acoustic dispensers use focused bursts of sound energy to propel 1-10 nanoliter (nL) sized droplets between source and destination microplates without direct liquid contact (Figure 28). Droplet dispenses are very rapid and the final volume transfer is achieved using increments of droplet size. Contamination is minimized since there is no contact carryover between operations. Contrasting the pintool, the acoustic dispensers is exceedingly flexible using a user-created dispense map that specifically defines source well, destination well and volume for each transfer.

The acoustic dispenser brings flexibility into the screening workflow. In addition to 1-1 plate stamping complex liquid handling operations can easily be achieved at low volumes such as dispensing into dry wells, serial dilutions, matrix/poly-pharmaceutical screening and cherry picking. Dispensing compound into dry plates then storing for later use is known as making “Assay Ready” plates. An “Assay Ready” plate can be created in advance of a screen as resources are available and on a separate system. If screening multiple compound concentrations, the number of concentrations multiplies the number of compound plates needed. Using an acoustic dispenser, different concentrations can be achieved from a single stock greatly reducing the number of compound plates needed. Follow up assays often require the creation compound plates with compounds of specific interest at different concentrations. The creation of a single follow up plate can take a considerable amount of resources. Using the acoustic dispenser screening of only the specific follow up compounds is possible without the need of new plates and potentially done on the fly.

The advantages of acoustic dispensing have some contrasting limitations. The cost for equipment varies with model and options but is typically >$300k per unit. The speed of operation is significantly slower when compared to the pintool requiring multiple units to maintain throughput. A 384-well transfer can take about 4min while 1536 wells can take > 10min. The compound plate must have 384 or 1536 wells and be acoustically compatible, meaning that the well shape and material must effectively transfer sound energy. Many microplate venders are now offering microplates for this specific purpose. The droplet destination can be any labware that fits into an SLAS/SBS microplate footprint though it must inverted for operation. For 384 and 1536 plates, liquid tension maintains fluids in the well during inversion though other labware may have to be initially dry.

Manufacturers of Acoustic Dispensers:

- Labcyte
- EDC Biosystems

The quality of assay data depends critically on the ability of individual scientists to use the liquid delivery devices appropriately. It is highly recommended that you make yourself familiar with all pipetting equipment in your lab. Also note that these liquid dispensers will have to be calibrated on a regular basis. For automated liquid

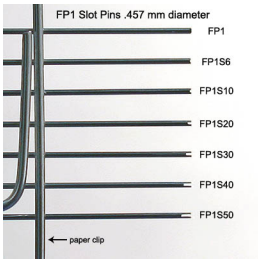


Figure 26: Example Slotted Pin Selection, Source V&P Scientific http://www.vp-scientific.com

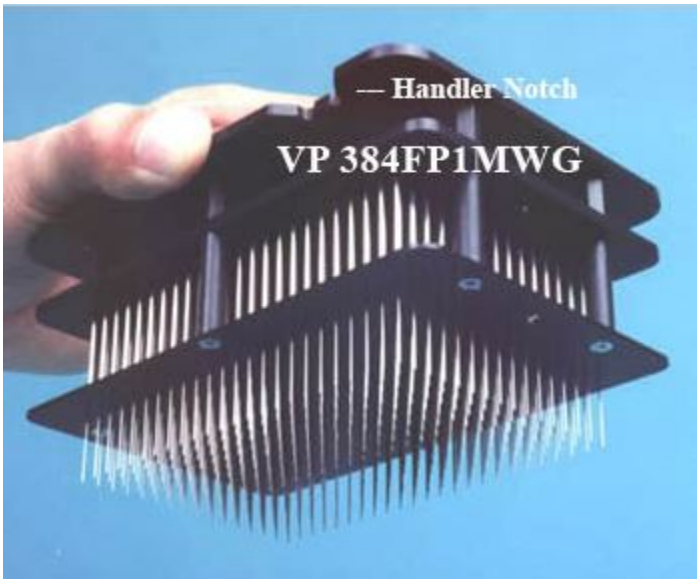


Figure 27: Example Pintool Head, Source V&P Scientific http://www.vp-scientific.com

handlers, special procedures recommended by the manufacturers are employed. These procedures usually involve serial dilutions of dyes from stock solutions and the determination of the accuracy and precision of the dilutions.

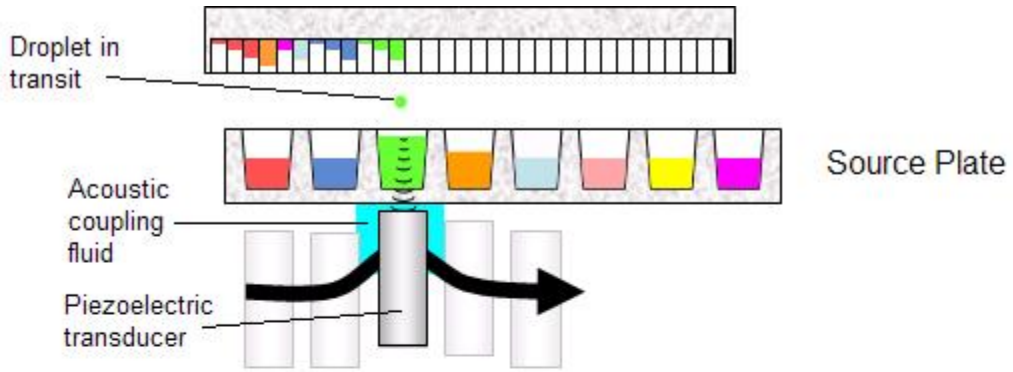


Figure 28: Simplified Acoustic Dispense, Source http://en.wikipedia.org/wiki/File:Acoustic_transfer1.jpg

Suggested Websites and Resources

1. Analytical Instrumentation Review. (Available at: http://www.uam.es/docencia/quimcursos/Scimedia/chem-ed/analytic/ac-meths.htm).
2. Corning Microplate Selection Guide. (Available at: http://catalog2.corning.com/lifesciences/media/pdf/productselectionguide_microplates11_02_cls_mp_014.pdf)
3. Corning Life Sciences | Corning® Epic® Technology | Corning Epic System (Available at: http://www.corning.com/lifesciences/epic/en/products/epic_system.aspx)
4. Agilent PlateLoc Thermal Microplate Sealer Consumables Selection Guide. (Available at: http://www.chem.agilent.com/Library/selectionguide/Public/5990-3659en_lo%20CMS.pdf)
5. Everything You Need To Know About Pin Tools But Were Afraid To Ask. (Available at: http://www.vp-scientific.com/pin_tools.htm)

Suggested Readings (alphabetical order)

1. Buchser W, Collins M, Garyantes T, Guha R, Haney S, Lemmon V, Li Z, Trask OJ., Assay Development Guidelines for Image-Based High Content Screening, High Content Analysis and High Content Imaging. In: Sittampalam GS, Coussens NP, Nelson H, Arkin M, Auld D, Austin C, Bejcek B, Glicksman M, Inglese J, Iversen PW, Li Z, McGee J, McManus O, Minor L, Napper A, Peltier JM, Riss T, Trask OJ Jr., Weidner J, editors. Assay Guidance Manual [Internet]. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2004-. 2012 Oct 1 [updated 2014 Sep22].

2. Eglen RM, Reisine T, Roby P, Rouleau N, Illy C, Bossé R, et al. The use of AlphaScreen technology in HTS: current status. Current chemical genomics. 2008;1(1) PubMed PMID: 20161822.
3. Garbison KE, Heinz BA, Lajiness ME, Weidner JR, Sittampalam GS., Impedance-Based Technologies. In: Sittampalam GS, Coussens NP, Nelson H, Arkin M, Auld D, Austin C, Bejcek B, Glicksman M, Inglese J, Iversen PW, Li Z, McGee J, McManus O, Minor L, Napper A, Peltier JM, Riss T, Trask OJ Jr., Weidner J, editors. Assay Guidance Manual [Internet]. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2004-. 2012 May 1.
4. Millar DP. Time-resolved fluorescence spectroscopy. Curr Opin Struct Biol. 1996;6(5):637–42. PubMed PMID: 8913686.
5. Phillips D. Luminescence lifetimes in biological systems. Analyst. 1994;119(4):543–50. PubMed PMID: 8024118.

Calculations and Instrumentation used for Radioligand Binding Assays

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Created: May 1, 2012; Updated: October 1, 2012.

Abstract

Radioligand binding assays are a "work horse" in biological laboratories and have been adapted for HTS and lead optimization support in drug discovery. The instrumentation is highly specialized to measure radioactivity of the labels on binding ligands and requires specialized calculation procedures. In this chapter, the author thoroughly and systematically describes the instrumentation and calculation principles used in data analysis. Sample calculations are shown along with definitions of terms and important steps in setting up the instrumentation. This is a very useful chapter for beginners, as well as a refresher for experienced investigators.

Introduction

The purpose of this chapter is to 1) describe common calculations used in radioligand binding assays and 2) outline steps for setting up and using microplate scintillation counters (Microbeta Trilux and TopCount).

When performing calculations such as those described on the following pages, it is advised to use unit dimension equations. This ensures that values have the appropriate units for the designated purpose. Unit dimension equations are used through this chapter.

Radioactive Calculations

Determination of Counting Efficiency

Microplate scintillation counters, used for reading Scintillation Proximity Assay (SPA) and filtration assays, detect flashes of light (photons) that occur when a released radioactive particle interacts with and excites a fluor molecule. Not all of the radioactive particles emitted will be detected as photons by the counting instrument. The output from the scintillation counter is the number of photons detected per unit time, typically expressed in counts per minute (CPM). The ratio between CPM detected by the instrument and actual disintegrations per minute (DPM) of the isotope is termed ***efficiency***. The efficiency of counting depends on the geometry of the detector, scintillation properties of the fluor and the energy of the particular isotope. Determination of DPM is important for making conversions to calculate molar concentrations of radioligands, and it is also important if a comparison between different instruments is required. For data that will be normalized (e.g. % Inhibition), CPM can be used as a direct readout from the instrument.

The efficiency for each isotope counting condition should be independently determined for an instrument. Steps to determine average instrument efficiency are shown in the example below for an SPA assay using a [^{3}H]-labeled radioligand and YSi SPA beads:

Example Determination of Efficiency

[^{3}H]-labeled SPA Beads can be prepared by incubating [^{3}H]-labeled biotin with YSi streptavidin beads and washing them (using centrifugation) to remove any unbound radioactivity. Alternatively, a reaction associated with an assay (e.g. WGA beads, membranes, radioligand) can be used.

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1. Remove a 300 µl aliquot of [^{3}H]-SPA beads to a 1.5 ml polypropylene tube.
2. Centrifuge the tube for 5 seconds in a microfuge to pellet the [^{3}H]-SPA beads.
3. Remove the supernatant. Dispose of it properly, treating it as potential radioactive waste.
4. Add 300 µl of PBS and mix beads. Repeat centrifugation and remove supernatant.
5. Resuspend in a final volume of 300 µl PBS.
6. For a Microbeta, pipette 25 µl of beads into three different wells of a microplate. Add 175 µl of PBS. Allow the beads to settle overnight.
7. Count the microplate and determine the average CPM for the three replicates (example: 52,800 CPM).
8. Add 25 µl of beads to three different scintillation vials containing scintillation cocktail. Count the vials on a liquid scintillation counter, which is capable of returning results in DPM, and determine the average for the three replicates (example: 140,582 DPM).
9. Determine efficiency using the following equation:

$$\text{Efficiency} = \frac{\text{CPM}}{\text{DPM}} = \frac{52{,}800}{140{,}582} = 0.38$$

For [^{125}I], a gamma counter with a known efficiency can be used for the determination of the total DPM.

Some typical instrument efficiencies for common isotope configurations on a Trilux Microbeta are shown in Table 1.

These are approximate efficiencies for comparison. Actual efficiencies for your instrument should be determined independently. In addition, some counting conditions require special "window settings" that can impact the apparent efficiency. Alterations or repairs to an instrument (e.g. adjustment of photomultiplier tubes (PMT's)) may also require determination of an updated efficiency value.

Table 1: Typical instrument efficiencies for common isotope configurations on a Trilux Microbeta. These are approximate efficiencies for comparison and actual efficiencies for your instrument should be determined independently.

Isotope	Scintillation Mode	Efficiency
^{3}H	Filtration	0.32
^{3}H	SPA (PVT)	0.23
^{3}H	SPA (YSi)	0.34
^{125}I	Filtration	0.45
^{125}I	SPA (PVT)	0.38
^{125}I	SPA (YSi)	0.56

Conversion from CPM to DPM

DPM are calculated from the equation shown below, where efficiency is expressed as a decimal percent. Determination of instrument efficiency (Eff) is described above.

$$\text{DPM} = \frac{\text{CPM}}{\text{Efficiency}}$$

Example:

1000 CPM detected in an assay using Polyvinyltoluene (PVT) SPA beads and ^{3}H.

The instrument efficiency was determined to be 22%.

DPM	=	CPM/Efficiency
	=	1000/0.22
	=	4545

Specific Activity (SA)

The amount of radioactivity per unit mole for a radioligand is referred to as the ***specific activity*** (often abbreviated as SA) and is typically given in units of Ci/mmol by the manufacturer. Since raw data from assays using radioactivity are in CPM or DPM, conversion of the specific activity from Ci/mmol to CPM/fmol or DPM/fmol is usually more convenient for further data analysis.

Conversion Factors: 1 Ci = 2.22 x 10^{12} DPM

10^{12} fmol = 1 mmol

Equation to convert Ci/mmol to DPM/fmol:

DPM/fmol = [Specific activity (~~Ci~~/~~mmol~~) x [2.22 x 10^{12} DPM/~~Ci~~] x [~~mmol~~/10^{12} fmol] = **SA x 2.22**

Example: SA = 2000 Ci/mmol

DPM/fmol = SA x 2.22 = 2000 x 2.22 = <u>4440 DPM/fmol</u>

Equation to Convert Ci/mmol to CPM/fmol:

CPM/fmol = [SA (~~Ci~~/~~mmol~~) x [2.22 x 10^{12} ~~DPM~~/~~Ci~~] x [~~mmol~~/10^{12} fmol] x Efficiency (CPM/~~DPM~~) = **SA x 2.22 x Eff**

Example: Instrument efficiency = 40%, SA = 2000 Ci/mmol

CPM/fmol = SA x 2.22 x Eff = 2000 x 2.22. x 0.4 = <u>1776 CPM/fmol</u>

Nominal Concentration of a Radioligand

The theoretical or nominal concentration of a radioligand stock solution can be calculated from the stated radioactive concentration (RAC, in µCi/ml) and the specific activity (SA, in Ci/mmol) using the equation shown below:

[Radioligand] = RAC/SA

Example: Radioactive concentration (RAC): 50 µCi/ml

Specific Activity (SA): 2000 Ci/mmol

Conversion factor: 1 Ci = 10^6 µCi

[Radioligand] = RAC/SA = (50 ~~µCi~~/ml ÷ 2000 ~~Ci~~/mmol) x 1 ~~Ci~~/10^6 ~~µCi~~ = 2.5 x 10^{-8} mmol/ml

= 2.5 x 10^{-8} M

= <u>25 nM</u>

This is the nominal concentration of the stock on the reference date. To estimate the concentration on any other day, see the ***Radioactive Decay*** section to determine the fraction remaining and the resulting concentration. See also the ***Dilution of Stock*** section to prepare a dilution of a stock radioligand.

Actual Concentration of a Radioligand

When performing radioligand binding assays, a rough estimate for the concentration of radioligand used in the assay can be computed using the information supplied with the material. This is called the theoretical or nominal concentration (shown above). In order to calculate the ***actual*** concentration of the radioligand used in an assay more accurately, one should count an aliquot of the stock mix and obtain the CPM or DPM for that aliquot, then use the equation below.

Equation to convert CPM to pM:

$$\text{pM} = \frac{\text{CPM}/\text{SA (CPM/fmol)}}{\text{Volume (ml)}} \times \frac{0.001\ \text{pmol/fmol}}{0.001\ \text{liter/ml}} = \frac{\text{CPM}/\text{SA}}{\text{Vol}}$$

Example: Counted a 50 µl aliquot of a stock mix, which yielded 50,000 CPM; SA = 1776 CPM/fmol (see above for calculation).

$$\text{pM} = \frac{50{,}000\ \text{CPM}/1776\ \text{CPM/fmol}}{0.05\ \text{ml}} = \underline{\underline{563\ \text{pM}}}$$

If values are in DPM, one should use specific activity (SA) expressed in DPM/fmol. Use appropriate unit conversions to determine the concentration in nM, µM, etc.

It is best practice to use the ***actual*** concentration of radioligand determined for each assay in calculations such as K_i, rather than the theoretical or nominal concentration.

Radioactive Decay

Radioactive decay is a random event and follows an exponential decay trend. You can calculate the fraction remaining in a radioactive sample if you know the date (reference date) when the specific activity or radioactive concentration was known using the following equation:

$$\text{Fraction Remaining} = e^{(-0.693/t_{1/2})\bullet \text{time}}$$

where $t_{1/2}$ is the half-life of the isotope (time it takes for half the isotope to decay), and time is the number of days before or after the known reference date. The term ($-0.693/t_{1/2}$) is also referred to as the decay rate constant, K_{decay}.

Example: [^{125}I] radioligand with a known specific activity on 10/1/07.

Half-life for [^{125}I] = 60 days.

Fraction remaining on 10/20/07 (20 days):

$$\text{Fraction Remaining} = e^{(-0.693/60)\bullet 20} = 0.794 \text{ or } 79.4\% \text{ remaining}$$

The fraction remaining following radioactive decay can also be determined from tables. Note that for the activity on a day prior to the stated reference date, the fraction remaining will be greater than 1.

An assumption typically made is that radioactive decay results in unlabeled decay product(s), which no longer bind to the target or receptor of interest. This implies that the specific activity remains constant over time and that the concentration of ligand changes with time. This assumption may not be valid with all radioligands used.

Half-Life

Table 2 shows half-lives (time for half of the isotope to decay) for common isotopes, along with typical values for specific activity of a single-labeled molecule. One should always consult the manufacturer's information for the exact specific activity of a radioligand.

Note that half lives (even for the same isotope) can vary from one manufacturer to another. In addition, if software is used for tracking of decay of isotope inventories, one must make sure that the half life value used is consistent throughout.

Table 2: Half-lives for common isotopes and typical values for specific activity of a single-labeled molecule. One should always consult the manufacturer's information for the exact specific activity of a radioligand.

Isotope	Half-life	Specific Activity
^{3}H	12.43 years	85.0 Ci/mmol
^{125}I	60 days	2000 Ci/mmol
^{32}P	14.3 days	9128 Ci/mmol
^{35}S	87.4 days	1493 Ci/mmol
^{14}C	5730 years	0.064 Ci/mmol

Dilution of Stock

To calculate the amount of a radioligand stock solution required to prepare a specific volume of a dilution, the parameters listed below will be needed. The values listed for each parameter are for use in the example calculations.

Radioactive Concentration (RAC): 50 μCi/ml

Specific Activity (SA): 2000 Ci/mmol

Half-life for isotope: 60 days (I-125)

Reference date: 10/1/07

Date of preparation: 10/20/07

Volume of final diluted mix: 50 ml

Desired concentration of final diluted mix: 0.1 nM

1 Determine nominal stock concentration – described above in ***Stock Concentration*** section:

[Radioligand] = RAC/SA = (50 ~~μCi~~/ml ÷ 2000 ~~Ci~~/mmol) x 1 ~~Ci~~/10^6 ~~μCi~~ = 2.5 x 10^{-8} mmol/ml

= 2.5 x 10^{-8} M

= <u>25 nM</u>

2) Determine stock concentration on day of use – described in ***Radioactive Decay*** section above:

Date of use – Reference Date = 20 days

$$\text{Fraction Remaining} = e^{(-0.693/60)\bullet 20} = 0.794 \text{ or } 79.4\% \text{ remaining}$$

Therefore, stock concentration on day of use = 0.794 x 25 nM = <u>19.85 nM</u>

3) Determine amount of stock required:

$C_1V_1 = C_2V_2$ solving for V_1, yields $V_1 = C_2V_2/C_1$ = (50 ml x 0.1 ~~nM~~)/19.85 ~~nM~~ = <u>0.252 ml</u>

This is the theoretical or nominal concentration. To determine actual concentration, count an aliquot of the diluted mix and calculate as shown in the ***Actual Concentration of Radioligand*** section above.

Instrumentation

Microbeta Trilux

General Concepts

A Microbeta Trilux comes with either 6 or 12 detectors. Each detector is comprised of two photomultiplier tubes (PMT's), one on top of the sample and one on the bottom. The PMT's operate using conventional coincidence circuitry, as shown in Figure 1.

Each detector counts only a portion of a 96-well microplate (16 wells per detector on the 6-detector Microbeta model, ~9 wells on the 12-detector model). The area of the plate counted by each detector of a 6- or 12-detector model is shown in Figure 2.

Although the use of multiple detectors can increase throughput, since the performance of PMT's are not identical, a calibration procedure (Normalization) is required. An identical sample is counted by all of the detectors, and a relative efficiency (fractional value) is determined. If an activity (DPM) for the sample is known, this can be inputted into the software, and the detectors are normalized to this activity. This will result in the efficiency factors being lower than if the detectors are normalized against each other. As an example, the typical efficiency relative to activity for [3H] with SPA beads is 0.20 – 0.30. When the detectors are normalized against each other, the relative efficiencies should be 0.9 – 1.0.

Modes of Normalization

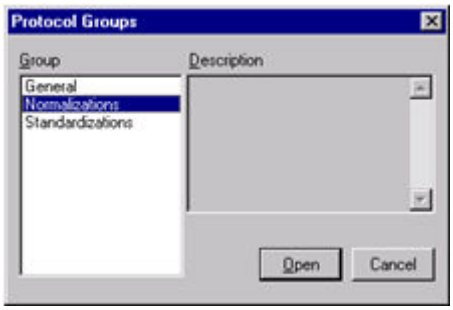
There are two ways to normalize the Trilux with a single sample in well G11 (for 96 well plate):

1. Relative to the detector with the highest reading (CCPM = CPM)
2. Relative to the activity inputted in well G11 (CCPM = DPM)

The basic principle for each of these modes is shown at the end of this section. The sample to be used for normalization must be in well G11. Both normalization protocols are set up the same way, with one additional step for mode 2, when results in DPM are desired. There are other features for Standardization (e.g. using quench curves) or Easy DPM, Paralux, etc. that are not discussed in this document.

Setup of a Normalization Protocol

1. Click on the Protocols button at the top of the Microbeta software toolbar.
2. Select ***Normalizations*** followed by the ***Open*** button.



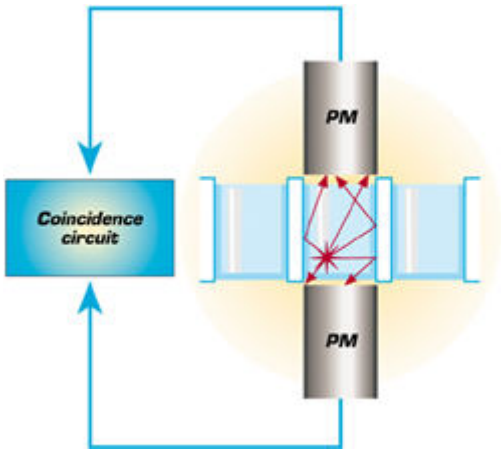


Figure 1: Diagram of a Microbeta Detector. Each detector includes two photomultiplier tubes that operate in coincidence counting mode. In this mode, background photons not related to the sample are eliminated because they do not possess the energy required for both PMT's to distinguish it in a discrete amount of time. The lower PMT can be disabled to count opaque-bottomed microplates. (Diagram from Perkin Elmer Life and Anlaytical Sciences Document 1450-1017-07).

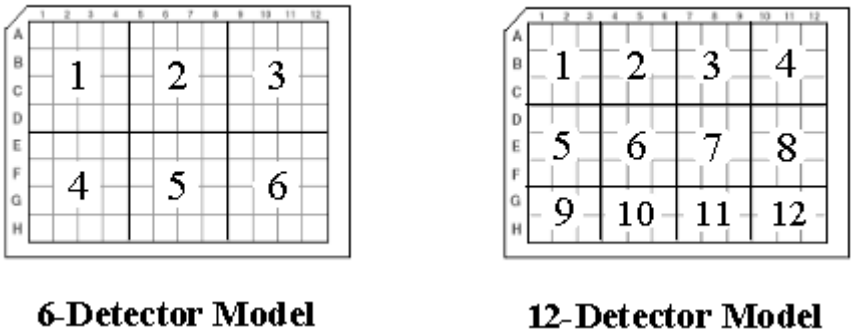
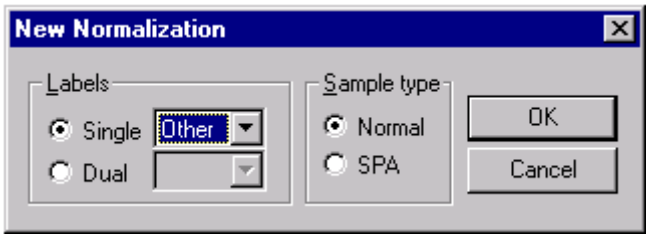


Figure 2: The area of the plate counted by each detector of a 6- or 12-detector model of a Microbeta Detector.

3. Click on the ***New*** button to create a new normalization protocol.

4. Select the appropriate label from the pull-down menu in the pop-up dialog box and click ***OK***. Do not check SPA unless you want to use Paralux counting mode (consult instrument manual).

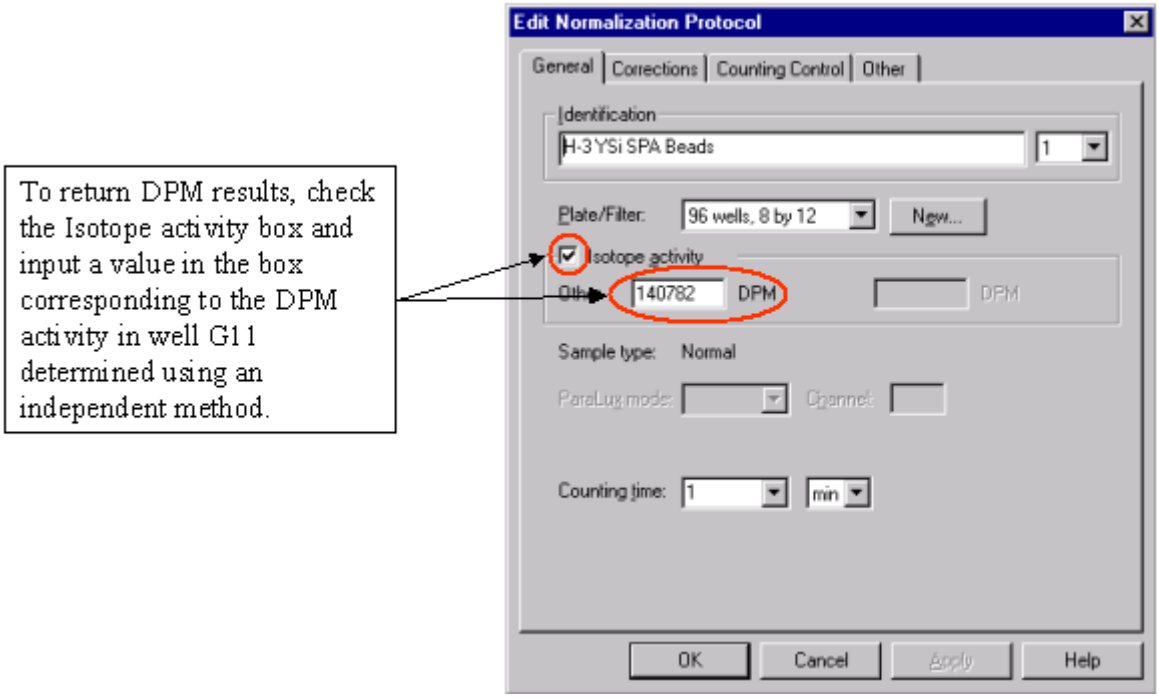


In many cases, particularly with YSi SPA beads, you should select ***Other*** and use the manual energy spectrum window settings shown in the table below in Step 6. The default settings were designed for PVT SPA beads.

5. Under the ***General*** tab, type in a name for the protocol and select a number for the protocol from the pull-down list (only unused, available protocol numbers are listed).

If it is desired to express results in DPM: Check the ***Isotope activity*** box and input a number for the activity (in DPM) that is in well G11. This activity should be determined by counting an identical aliquot in a liquid

scintillation counter (for 3H) or a gamma counter (for 125I) that has a known efficiency (DPM = CPM/ Efficiency). In this example, replicate aliquots of YSi SPA beads were counted in a liquid scintillation counter with an average of 140,782 DPM. An identical aliquot was placed in well G11 of a microplate for normalization. The value 140,782 is entered into the area on the General tab, as shown below.

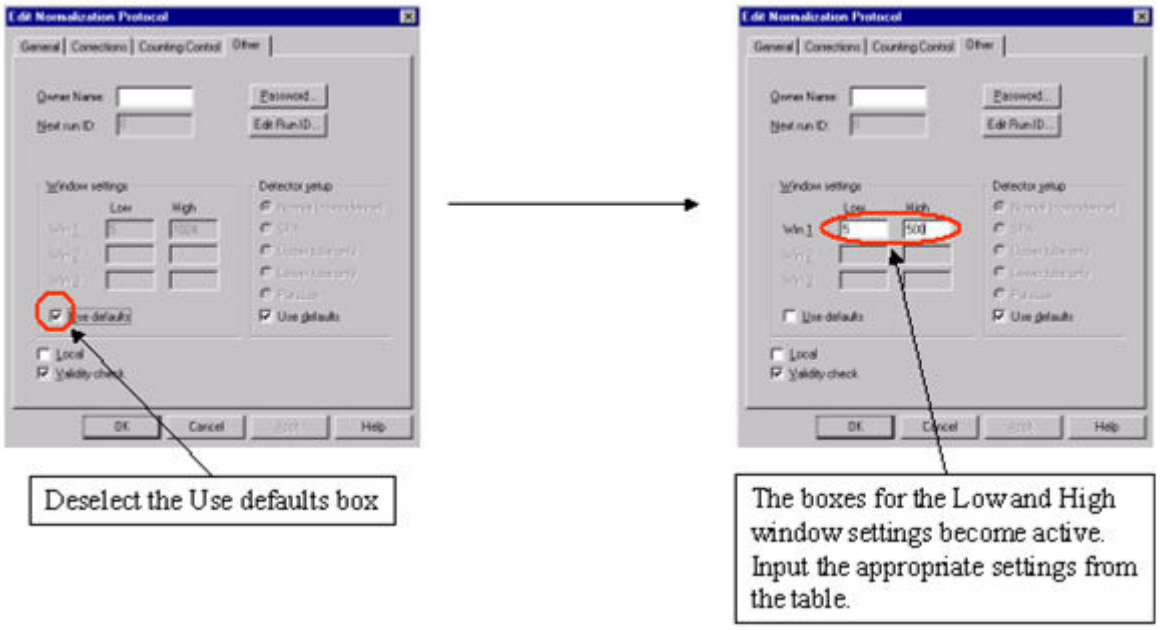


6. If ***Other*** was selected as the label, the energy spectrum window settings may need to be manually defined. By default it will appear under the Other tab as a window from 5 to 1024, an open energy spectrum window.

Uncheck the box next to ***Use defaults***. The window settings for Low and High can now be changed.

Table 3 indicates the suggested settings for several isotopes and types of SPA beads and Cytostar-T plates. The screen capture below shows the ***Other*** tab, after new window settings have been inputted for tritium YSi SPA beads and the Microbeta (Table from www.perkinelmer.com).

Under the ***Other*** tab:



7. Click OK to save the normalization protocol.

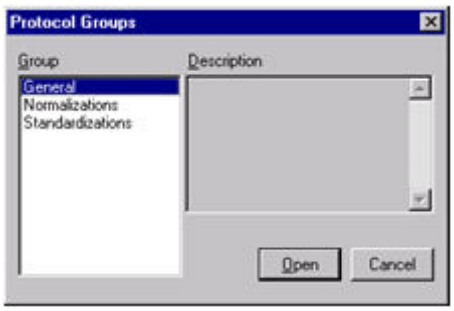
Table 3: suggested settings for several isotopes and types of SPA beads and Cytostar-T plates

ISOTOPE	SPA PVT		SPA Yttrium silicate		Cytostar-T microplates	
	MicroBeta	TopCount	MicroBeta	TopCount	MicroBeta	TopCount
Tritium	5 - 360	1.5 - 35	5 - 500	0 - 50	5 - 250	1.5 - 45
Iodine-125	5 -530	1.5 - 100	5 - 650	0 - 100	5 - 350	1.5 - 50
Phosphorus-33	5 - 650	2.9 - 256	5 - 750	2.9 - 256	5 - 700	1.5 - 100
Sulphur-35	5 - 600	2.9 - 256	5 - 700	2.9 - 256	5 - 500	1.5 - 60
Carbon-14	5-650	2.9 - 256	5 - 750	2.9 - 256	5 - 500	1.5 - 60
Calcium-45					5 - 550	1.5 - 60
Rubidium-86 [energy range HIGH]						2.9 - 256

Setup of a General Counting Protocol

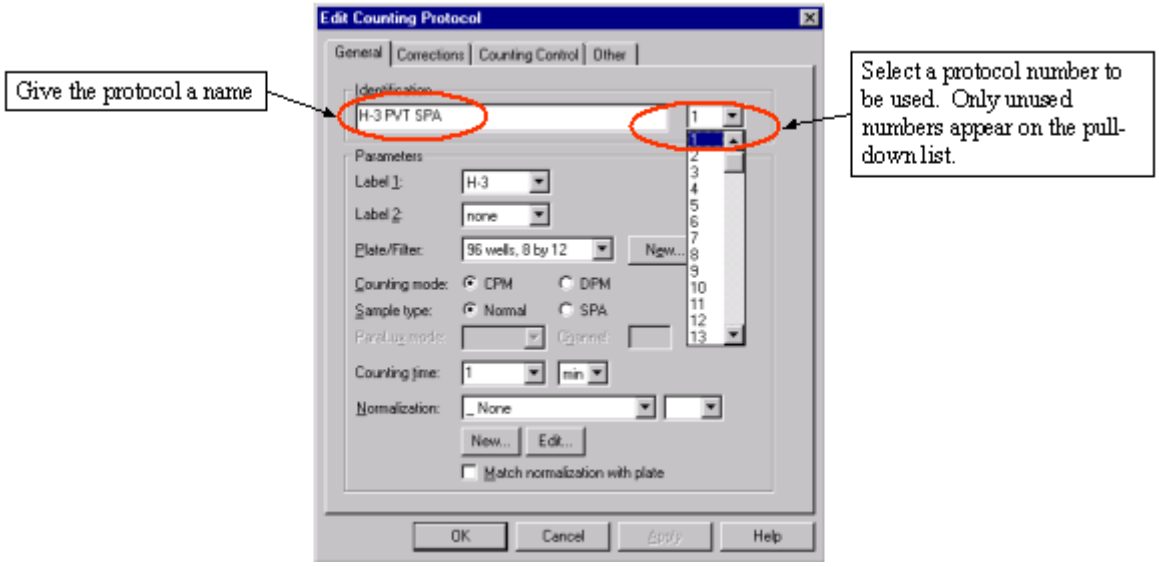
A Normalization protocol is linked to a General Counting protocol, in order to define the counting parameters (i.e. isotope, window settings, etc.) and the detector efficiencies (relative to the highest detector reading or relative to DPM activity) needed to correct raw counting data.

1. Click on the Protocols button at the top of the Microbeta software toolbar.
2. Select ***General*** followed by the ***Open*** button.

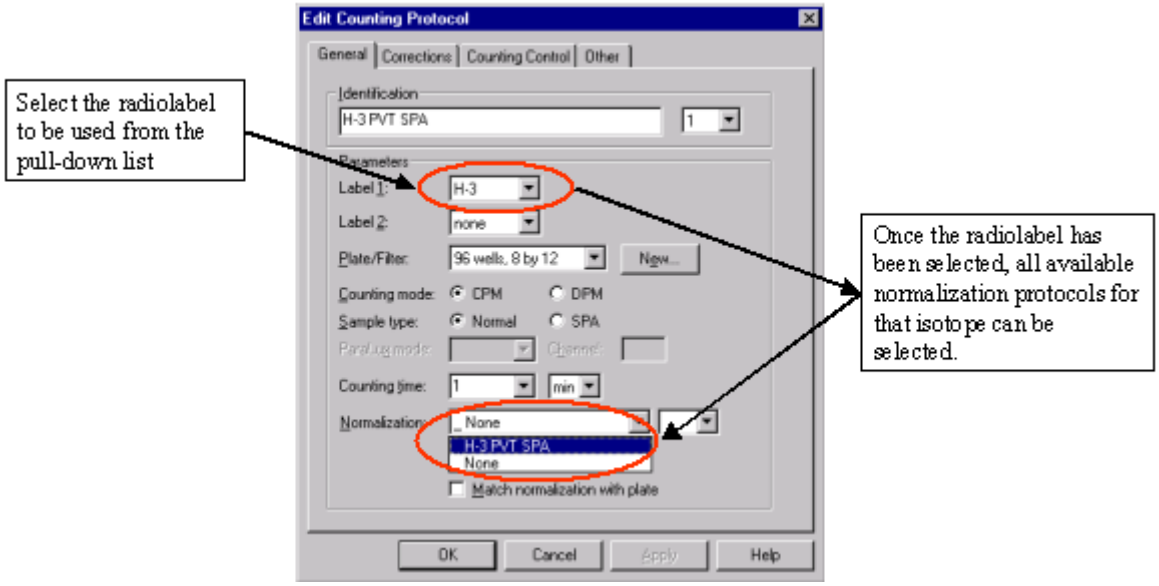


3. Click on the ***New*** button to create a new General counting protocol.

4. In the Edit Counting Protocol window, type in a name for the protocol in the ***Identification*** space. Select a protocol number from the pull-down list to the right of the ***Identification*** name. Only unused protocol numbers will appear in this pull-down list.



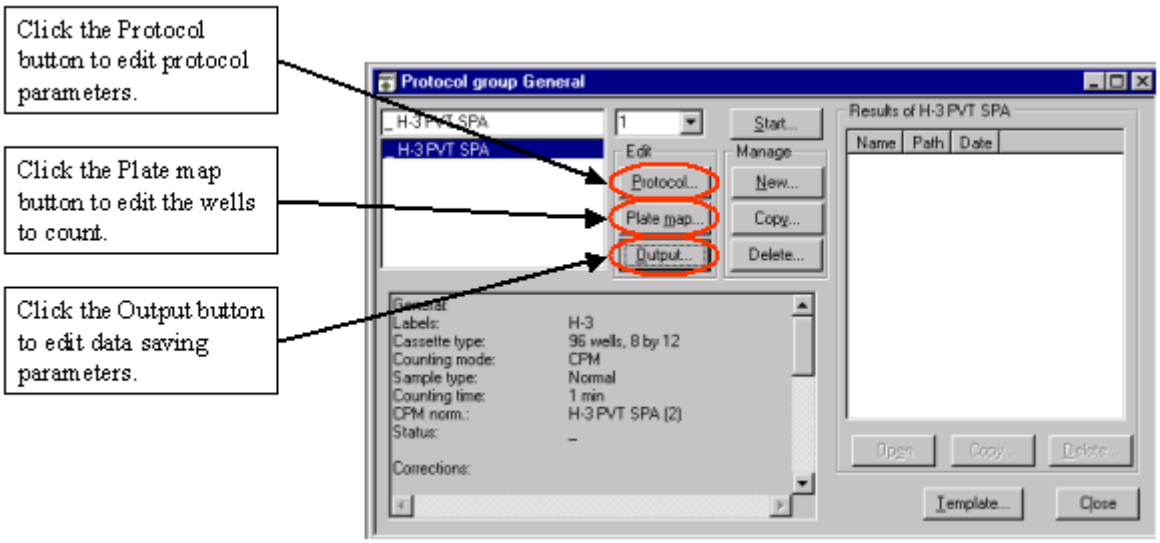
5. Select the isotope from the pull-down list. Once the isotope is selected, Normalization protocols that have been created using that isotope will appear in the Normalization pull-down area. Select the appropriate Normalization protocol to link to the General counting protocol. Note that an underscore (_) before the name of a Normalization protocol indicates that the Normalization plate has not been counted yet. Once the Normalization data has been stored, an (n) will appear before the name of the Normalization protocol.



6. Change the Counting time if desired (default is 1 min). The other tabs in the Edit Counting Protocol window (***Corrections, Counting Control, Other***) usually do not need to be modified unless special counting circumstances are being used.

7. Click OK to save the General counting protocol. Click Yes on the dialog box that pops up.

8. From the ***Protocol group General*** window, the General counting protocols can be edited.



9. The Protocol button allows editing of the protocol parameters (i.e. counting time).

10. The Plate map button allows selection of microplate wells to count (default set to entire plate).

11. The Output button allows selection of file and printing options. There are a couple of changes that should be made in the output as outlined below:

If the instrument is connected to a network and does not have a dedicated printer attached to the PC controller, it may be desirable to deselect the printing option. Quality printouts of the data directly from the instrument to a network laser printer are difficult. Deselect Generate print output in the ***Print*** tab.

Under the ***File 1*** tab, it is advisable to change the path where data files are electronically stored. By default, they

are stored in the Results subdirectory where the Microbeta software is stored. This can be dangerous, as the Normalization parameters are also stored in that subdirectory. Accidental deletion or moving of Normalization protocol results files will render the Normalization protocols useless. To prevent this, direct General counting output to a different subdirectory.

Under the ***File 1*** Items tab, if you do not want the electronic data file to have the data expressed as 96 numbers in a column (for a 96-well plate), deselect the Column section box. The data file will have results in plate format only (8 x 12 array for 96-well microplates).

The suggested outline shown above is for general counting conditions. One should consult instrument owners or the manufacturer for advanced counting options such as cross-talk correction, background correction or manual setting of count windows.

Figure 3 demonstrates the linking of a Normalization protocol to a General counting protocol for a 12-detector Trilux using either a relative detector efficiency set up or anefficiency relative to a known activity. Similar linking occurs for a 6 detector instrument.

TopCount

General Concepts

The TopCount is different than the Microbeta because it uses a single photomultiplier tube (PMT) counting from the top of the microplate instead of one PMT on top and one on bottom. Consequently, the TopCount determines background from true photon events using a time-resolved discrimination method of scintillation counting. This means that appropriate scintillators (known as slow scintillators) must be used for proper signal detection (Figure 4). The TopCount is available in 6- and 12-detector models.

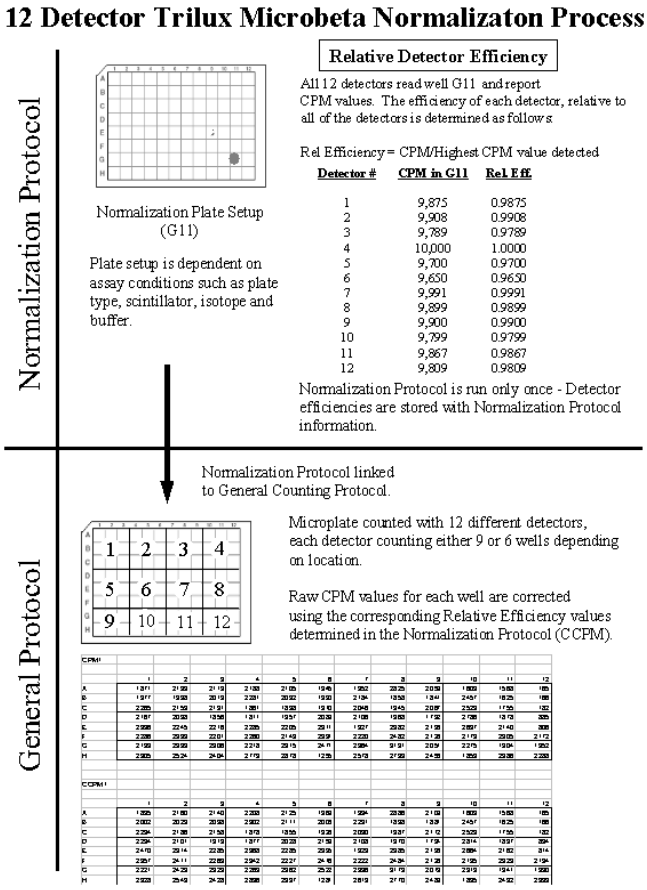
Normalization of the TopCount is similar to the Microbeta, except that ***Well A10*** is used by the detectors as the common read well. In addition, the TopCount NXT software does not have a provision to enter in an activity (in DPM) for the normalization amount on the plate. Therefore, results are always reported in corrected CPM, with the detectors normalized relative to each other. Efficiency of the TopCount must be determined manually, and the correction factor must be applied to determine DPM activity. Further information about normalization procedures and applications for the TopCount can be obtained from the manufacturer.

A stepwise procedure for setting up a counting protocol on a TopCount NXT is shown below.

Setup of Counting Assay

1. Click on the Assay Wizard icon located in the tool bar at the top of the software window (hold the mouse over a button to obtain a description of each icon).
2. Select Create a New Assay.
3. Define the assay name and number; select CPM as the Assay Type; select the desired plate type if requested.
4. Accept the default selection of Unknowns, unless you need to add Totals and Blanks for additional calculations.
5. Select Counting Options including delays and repeats, and select the Radionuclide from the drop-down list. Table 4 lists preset window settings on the TopCount NXT.

6. Define printed and ASCII file outputs, as well as post-processing user application programs.
7. Select Instrument Correction Factors.
8. Establish Instrument Correction Factors.
9. Define Sample Map and finish Setup.



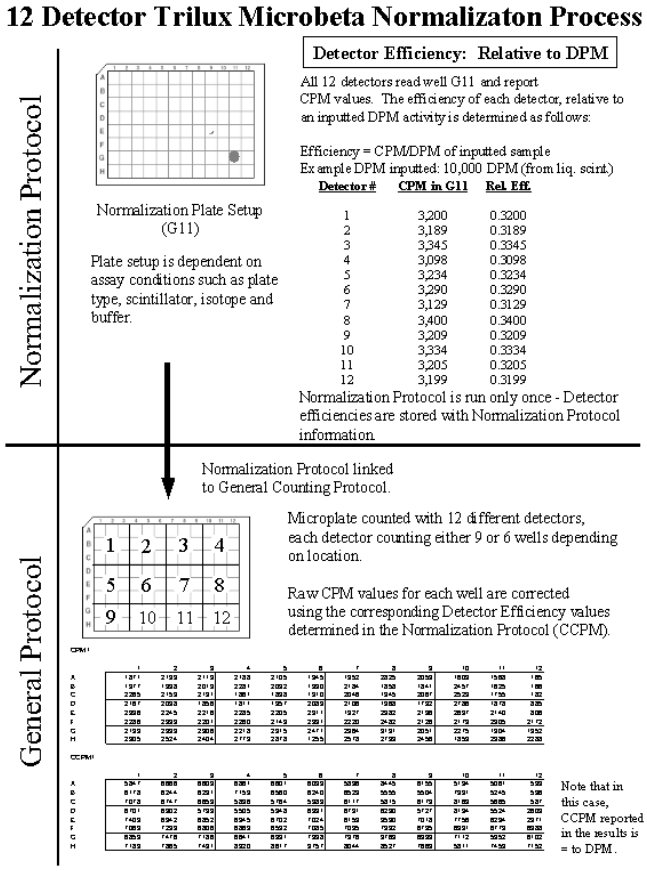


Figure 3: Linking of a Normalization protocol to a General counting protocol for a 12-detector Trilux using either a relative detector

efficiency set up or an efficiency relative to a known activity.

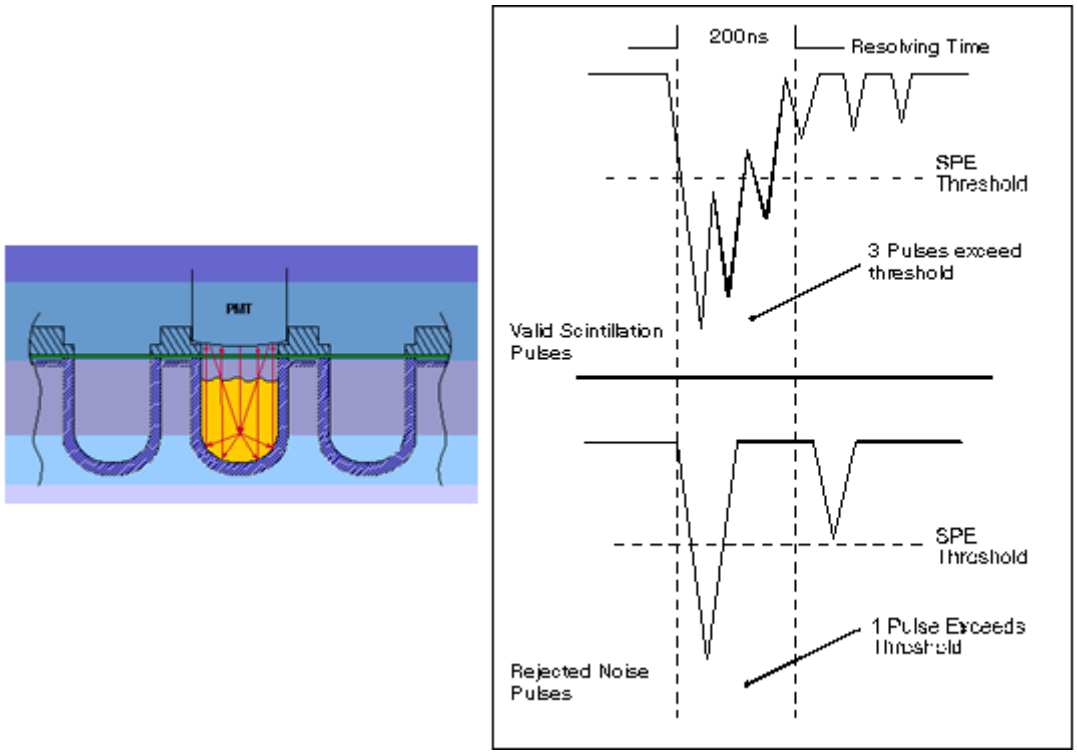


Figure 4: Diagram of TopCount Pulse Discrimination. Appropriate slow scintillators must be used to allow the photon energy to dissipate in a time resolved manner (multiple pulses detected during resolving time). Single pulses detected by the PMT during the resolving time would be eliminated as background noise. The TopCount uses a single PMT positioned on top. Diagrams from Perkin Elmer Life and Analytical Sciences (Document TCA-003).

10. The first time the Assay Protocol is selected, a normalization plate with a sample of activity in well A10 will be expected. Future runs will count plates using the stored normalization parameters.

Table 4: **Preset window settings on the TopCount NXT.**

		Energy	Efficiency	Channels	
Name	Scintillator	Range	Mode	Region A	Region B
Polyvinyltoluene (PVT) SPA					
3H-PVT-SPA		Liq/Plastic	Low	Normal	1.5-35.0	1.5-256.0
125I-PVT-SPA		Liq/Plastic	Low	Normal	1.5-100.0	1.5-256.0
33P-PVT-SPA		Liq/Plastic	Low	Normal	2.9-256.0	2.9-256.0
35S-PVT-SPA		Liq/Plastic	Low	Normal	2.9-256.0	2.9-256.0
Yttrium Silicate (YS) SPA					
3H-YS-SPA		Glass	Low	High Sens.	0.0-50.0	0.0-256.0
125I-YS-SPA		Glass	Low	High Sens.	0.0-100.0	0.0-256.0

Uniformity Plate

To test instrument detector variation, a uniformity plate with the same level of radioactivity in all wells is generated. The counting results are analyzed for each detector, as well as across the plate by columns and rows, to determine if any detectors require adjustment. Periodic counting of a uniformity plate (called a Performance Check) can identify detector drift or other instrument problems. This procedure can be performed regardless of the instrument type or the number of detectors.

Since many assays are performed in a concentration response mode, a gradient signal across the plate is an expected result. An example of how a Performance Check using a uniformity plate can assist in reducing instrument variability is shown in Figure 5.

To generate a 96-well microplate for [^{125}I] SPA beads, labeled beads are prepared using WGA beads, [^{125}I]-ligand and receptor membranes. A brief procedure is described below:

Add receptor membranes, [^{125}I]-ligand and WGA SPA beads in an appropriate buffer in a single tube. After a incubation time (consistent with the biological system), add 200 µl of diluted bead mixture per well using a 12-channel pipette. Change tips for each row. Allow beads to settle overnight (stable counting conditions) or centrifuge if the receptor/ligand interaction is not stable. Count radioactivity in Microbeta (use clear bottom plate) or TopCount (use opaque bottom plate

Results for a typical read using a clear bottom plate and a 12-detector Microbeta Trilux are shown in Figure 6. The relative efficiency between all 12 detectors is >95%.

Color Quench Correction

If colored compounds are to be tested and are present during the counting step (as in a non-separation technique such as SPA) color quenching may be present. This occurs when the photons emitted by the fluor are absorbed by the colored compound resulting in attenuation of signal. The emission spectra (~420 nm max) of SPA beads detectable in the Trilux and TopCount and the absorption spectra for common colors are shown in Figure 7.

For the Trilux and TopCount, compounds that are red, yellow or orange (absorption max ~400 nm) have the biggest impact on signal attenuation (with PVT or YSi SPA beads) if they are present while reading the plates.

Correction of color quenching can be performed within the software of the Trilux or TopCount using a prepared quench curve. Typically, this is performed with a yellow dye, such as tartrazine.

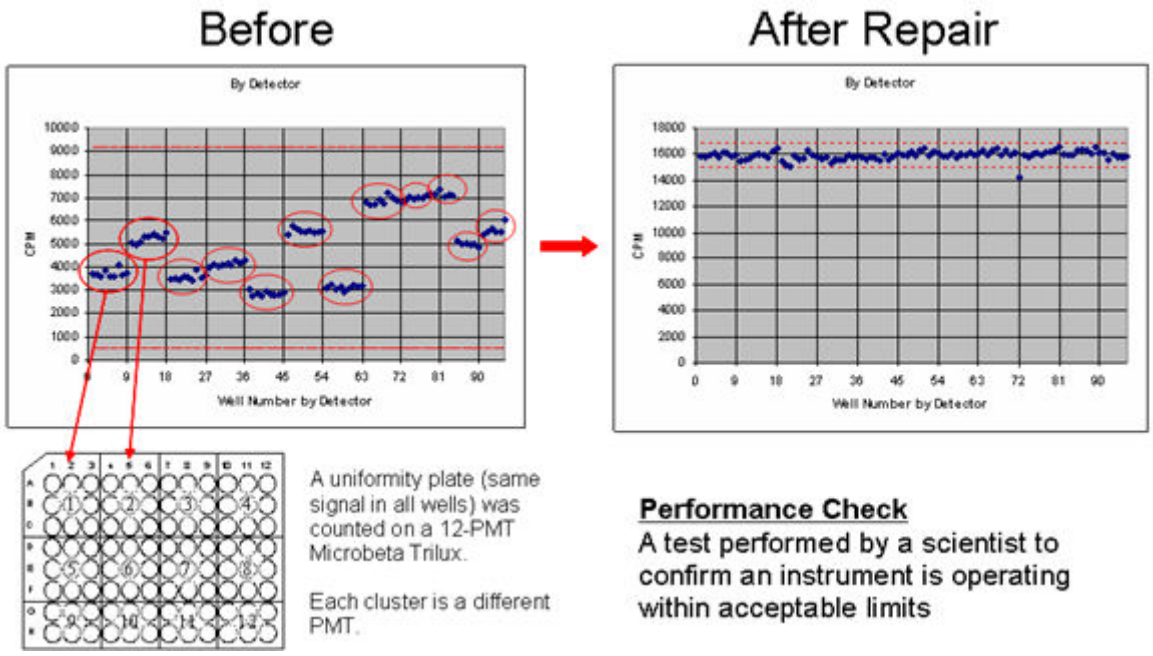


Figure 5: Example a performance check, which reduces instrument variability.

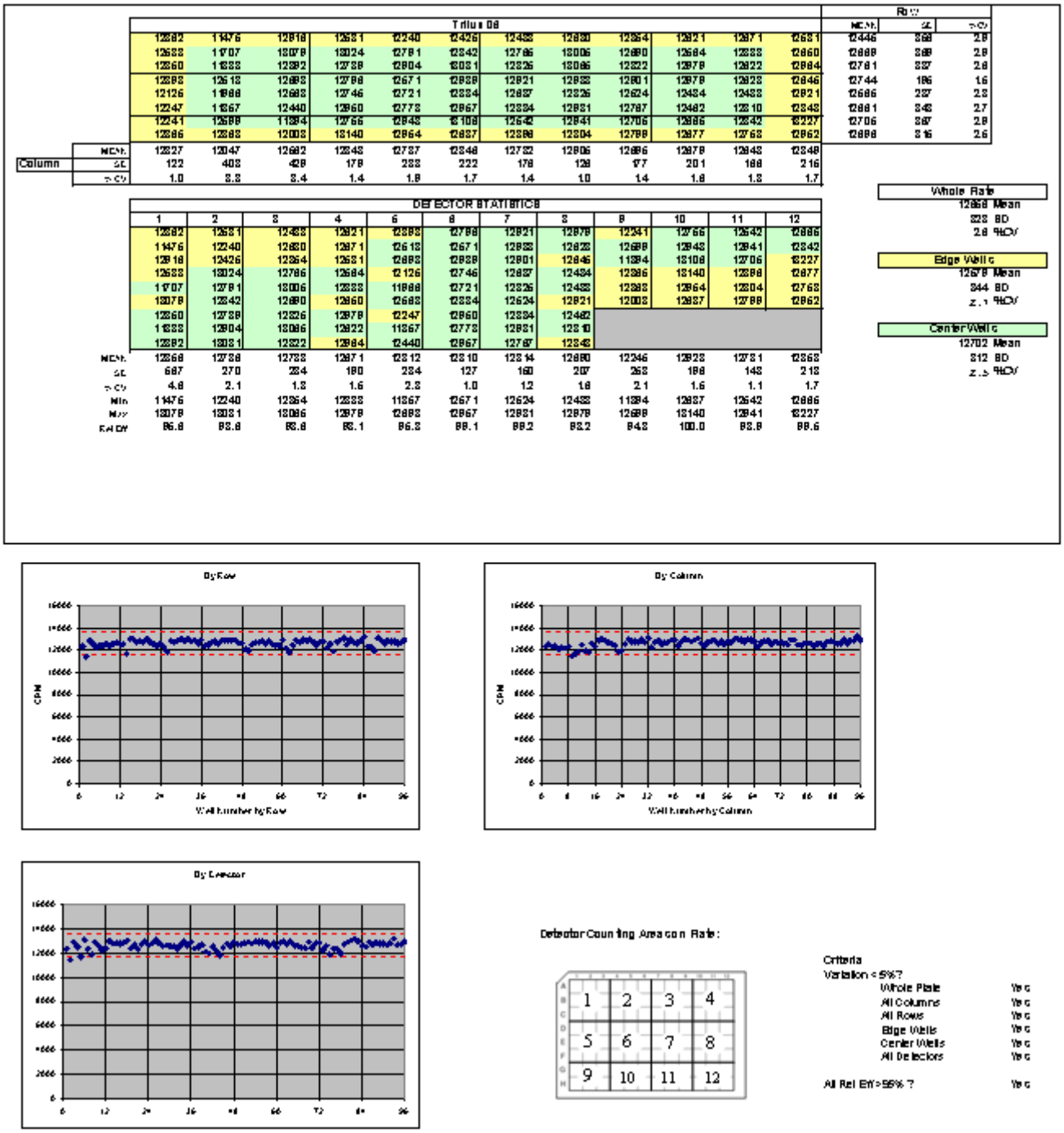


Figure 6: Results for a typical read using a clear bottom plate and a 12-detector Microbeta Trilux.

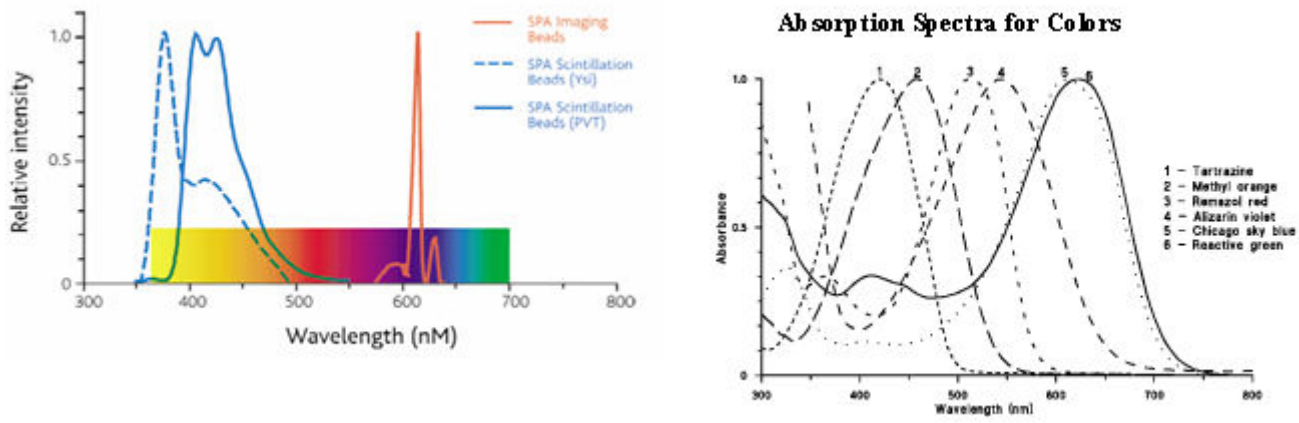


Figure 7: The emission spectra (~420 nm max) of SPA beads detectable in the Trilux and TopCount and the absorption spectra for common colors. Diagrams from PE LAS.

Abbreviations

CPM = Counts per minute

DPM = Disintegrations per minute

SA = Specific activity, example: Ci/mmol

RAC = Radioactive concentration, example: µCi/ml or mCi/ml

Eff = Efficiency, defined as CPM/DPM

PMT = Photomultiplier tube, detects photons of light emitted by a source (e.g. fluor)

SPA = Scintillation Proximity Assay

YSi = Yttrium silicate, a rare earth metal based SPA bead

PVT = Polyvinyltoluene, a plastic-based SPA bead

PE LAS = Perkin Elmer Life and Analytical Sciences

Pharmacokinetics and Drug Metabolism

Thomas D.Y. Chung and Xin Xu

In Vitro and *In Vivo* Assessment of ADME and PK Properties During Lead Selection and Lead Optimization – Guidelines, Benchmarks and Rules of Thumb

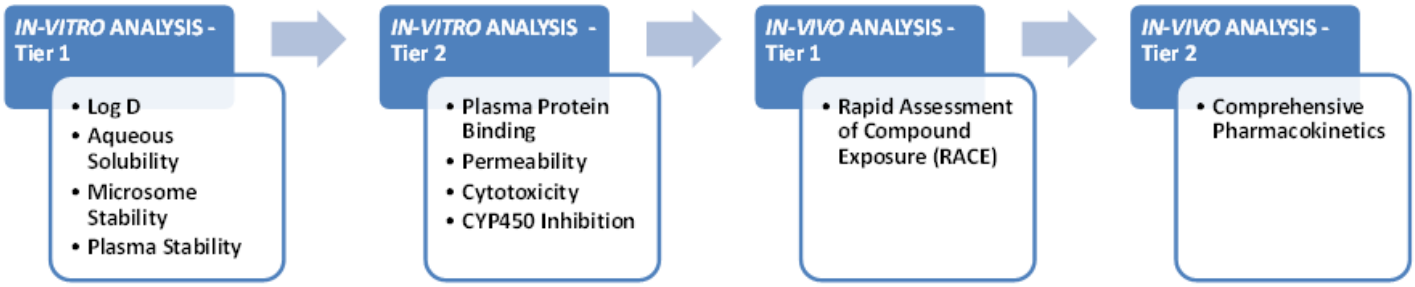
Thomas D.Y. Chung,[1] David B. Terry,[2] and Layton H. Smith[3]

Created: September 9, 2015.

Abstract

Assessment of the pharmacological properties of small molecule chemical compounds is critical to the initial selection or identification of a chemical lead, and during the further lead optimization to elucidate the Structure-Activity Relationships (SAR) and Structure Property Relationships (SPR), and ultimately to select the compound(s) that will enter Investigational New Drug (IND)-enabling studies. While extensive discussion of how Absorption, Distribution, Metabolism, and Excretion (ADME) of compounds affects their ultimate pharmacokinetics (PK) is beyond the scope of this chapter, herein, we provide guidelines for ADME and PK assessments, benchmarks and practical "rules of thumb" for selecting compounds with sufficient PK to be viable efficacious drugs.

Flow Chart of a Two-tier Approach for *In Vitro* and *In Vivo* Analysis



Background

As well-reviewed in the Assay Guidance Manual (AGM) chapter on Early Drug Discovery and Development Guidelines, there is an evolving paradigm for drug discovery and early development with the academic and non-profit enterprises focused on delivering innovative, novel, new chemical entities (NCE) through collaboration, partnering and licensing optimized leads for final clinical development to pharmaceutical companies. That chapter outlined the overall process, critical steps and key decision points at each step. Each of these steps and associated technologies, protocols, techniques, and case examples are covered elsewhere in the AGM. Ultimately, an exemplary compound(s) emerges from systematic elucidation of the Structure Activity Relationship (SAR) through examining the potency, specificity and selectivity of analogs around a chemical scaffold. This comprises identification of a chemical lead, where the most potent, specific and selective compound(s) are chosen.

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Assessments of the pharmacological properties of Absorption, Distribution, Metabolism, and Excretion (ADME) of a candidate chemical lead(s) are critical to their initial selection, and establishes benchmarks against which compounds synthesized during lead optimization can be evaluated. Further improvements in ADME properties during lead optimization are sought, while preserving the potency and selectivity of the chemical lead(s), though sometimes more efficacious compounds have lower *in vitro* potencies, but better ADME properties.

These activities often reside in an exploratory pharmacology group that provides *in vitro* and *in vivo* pharmacologic and physicochemical property analysis of biologically active small molecules in support of small molecule probe/drug discovery projects. Early pharmacological assessment has been adopted within the pharmaceutical industry as a critical feature of a robust drug/probe discovery process. This is because the development and optimization of useful molecules is a multi-parameter process. Simply designing new analogs and developing a SAR for increased potency against the biological target is inadequate for the development of small molecule probes or drugs suitable for cellular, tissue, or whole animal disease model(s). The assessment and optimization of Structure-Pharmacologic/Property-Relationships (SPR) is a further critical step for efficacy evaluation. In addition to assessing compound characteristics such as solubility, protein binding, and serum stability, the data allows the chemistry team to prioritize different structural classes and rank order them not only based on potency but also in relation to potential downstream absorption or metabolism liabilities. An excellent additional overview of pharmacokinetics (PK) can be found on the online version of the Merck Manual (http://www.merckmanuals.com/professional/clinical-pharmacology/pharmacokinetics/overview-of-pharmacokinetics)

Prior to actual dosing in animals, a number of relatively rapid and cost effective *in vitro* assays can serve as surrogates and indicators of the ADME fate of compounds *in vivo*. Improvements in ADME properties of compounds translate to their improved PK properties. Simply stated, if a compound is rapidly absorbed, well distributed, minimally metabolically degraded and not rapidly eliminated, while not being toxic, then it more likely will rapidly achieve peak levels in the blood, maintain the desired levels (n-fold above the IC_{50}) for a longer duration, before falling to low trough levels, and ultimately being cleared by the body.

In the sections below, we describe the basic component and provide high level protocols for a two-tiered approach for these key *in vitro* ADME assays (see Flow Chart). We provide an example of an ADME table, and benchmarks for a series of probe compounds developed through the NIH Molecular Libraries Program (MLP) by our group when we were part of the Molecular Libraries Probe Production Centers Network (MLPCN). We then summarize a two-tiered approach to doing pharmacokinetic studies (see Flow Chart). First an abbreviated "rapid" assessment for compound exposure (R.A.C.E.) developed by us (LHS), then the more typical "comprehensive" pharmacokinetic analysis used during late lead optimization toward candidate selection for final preclinical IND.

Ultimately, the *in vivo* efficacy of an optimized lead will be better served by having good pharmacological properties, so that the compound administered at a given dose actually achieves the required concentration, for sufficient duration in the target tissue to achieve the desired biological effect, while minimizing any undesired off target effects. Improvement in these ADME properties is sought prior to actual dosing in animals to assess PK, and certainly for larger compound efficacy studies, since animals are expensive and the ethics of sacrificing animals in poorly designed studies uninformed by pharmacological guidance are indefensible.

In Vitro Analysis - Low Compound Requirements and Relative Moderate Capacity

Lipophilicity

Pharmacologic question addressed: "*Will my parent compound be stored in lipid compartments or how well will my parent compound bind to a target protein?*"

Lipophilicity is an important physicochemical property of a potential drug. It plays a role in solubility, absorption, membrane penetration, plasma protein binding, distribution, CNS penetration and partitioning into other tissues or organs such as the liver and has an impact on the routes of clearance. It is important in ligand recognition, not only to the target protein but also CYP450 interactions, HERG binding, and PXR mediated enzyme induction.

Lipophilicity is typically measured as the neutral (non-ionized) compound distribution between non-aqueous (octanol) and aqueous (water) phase and the result is expressed as a 10-base logarithm of the concentration ratios between these phases (partition coefficient), log P.

Another common measure for lipophilicity is the distribution coefficient, log D, which takes into account the compound's ionized and non-ionized forms, and therefore the measurement is done at different pH values. Typically the most interesting is pH 7.4, since the majority of known drugs contain ionizable groups and are likely to be charged at physiological pH.

Assay Design:

- Test articles are assayed in triplicate
- One concentration of test article (typically 10 μM)
- n-Octanol is the partition solvent
- Ratio of buffer: Octanol is 1:1 (other ratios available)
- Positive control: Testosterone (high log $D_{7.4}$ value)
- Negative control: Tolbutamide (low log $D_{7.4}$ value)

Analysis: LC/MS/MS measurement of parent compound

Report: Log $D_{7.4}$ value

Quantity of test article required: 1.0 - 2.0 mg

Summary of Assay:

Lipophilicity of compounds is assessed using the golden standard "shake-flask" method. The compound is dissolved in a solution with equal amounts of octanol and water, shaken for 3 hours, and then measured for the amount of compound in each phase. Log D values are calculated by the log ($[compound]_{octanol}$ / $[compound]_{buffer}$).

Solubility

Pharmacologic question addressed: "*What is the bioavailability of my compound?*"

Aqueous solubility, another common physicochemical parameter for drug discovery compounds, is an important analysis as it reflects the bioavailability of the compound. The ability of a compound to dissolve in a solvent to give a homogenous system is one of the important parameters to achieve a desired concentration of drug in systemic circulation for the desired (anticipated) pharmacological response. Formulation and routes of administration, especially oral dosing, are challenging for poorly soluble drugs, as it limits the absorption of compound from the gastrointestinal tract. Also, poor solubility will affect other AMDE/DMPK analyses, if some fraction of the compound precipitates and is unavailable (e.g. in assays for metabolite stability and various CYP identification/inhibition/induction assays). Also, since the majority of known drugs contain ionizable groups, the aqueous solubility is assessed over a range of pH values.

Assay Design:

- Test articles are assayed in duplicate
- One concentration of test article (typically 1 μM)

- Phosphate buffered solution (other buffers available)
- Three point pH range (5.0, 6.2, 7.4)
- Positive control: Diclofenac (high solubility)
- Negative control: Dipyridamole (low solubility)
- Background control: DMSO only

Analysis: UV spectrophotometry measurement of parent compound

Report: Amount of compound dissolved (μM)

Quantity of test article required: 1.0 - 2.0 mg

Summary of Assay:

The compound is dissolved in buffer solutions at the indicated pH values. The compound is allowed to reach thermodynamic equilibrium by incubating for 18 hours. Compound UV absorption is compared to fully saturated solution in 1-propanol.

Hepatic Microsome Stability

Pharmacologic question addressed: "*How long will my parent compound remain circulating in plasma within the body?*"

The assay uses subcellular fractions of liver, microsomes, to investigate the metabolic fate of compounds. Liver microsomes consist mainly of endoplasmatic reticulum and contain many drug-metabolizing enzymes, including cytochrome P450s (CYPs), flavin monooxygenases, carboxylesterases, and epoxide hydrolase (1). Liver microsomes are available commercially (example, Xenotech, LifeTechnologies and DB Biosciences) as frozen preparations that are usually prepared in bulk with pooled livers from sacrificed mice, rat or human cadavers. As a result, hepatic microsomal metabolic activity can vary significantly from batch to batch. Therefore, in critical studies, it is recommended that planning to obtain the same lot of microsomes be considered in the experimental plans. If lots of microsomes do run out, a few bridging comparisons to establish comparable values for microsomal stability of reference compounds, should be done.

Assay Design:

- Test articles are assayed in triplicate
- Human liver microsomes (or other species as needed) (0.5 mg/mL)
- One concentration of test article (typically 10 μM)*
- Two time points $t = 0$ and $t = 60$ min*
- Positive control: Substrates with known activity
- Negative control: NADPH deficient

Analysis: LC/MS/MS measurement of parent compound at specific time points

Report: % metabolism of the test article (single time point); also, intrinsic clearance and half-life (multiple time points)*

Quantity of test article required: 1.0 - 2.0 mg

Summary of Assay:

Metabolic stability of compounds are assessed at a single concentration (typically 10 μM) at $t = 0$ and at $t = 60$ min. Stability of compounds are tested in human (other species available) liver microsomes. Compounds are tested in triplicate with or without NADP wells as a negative control for P450 metabolism. Each assay will include a substrate with known activity (such as the CYP3A4 substrate testosterone) as a positive control (2).

*Note: the number of concentrations/time points in the assay can be expanded for drug development SAR efforts.

Plasma Stability

Pharmacologic question addressed: "*Is my compound degraded in plasma?*"

In addition to hepatic metabolism, compounds are also subjected to degradation/modification by enzymes in plasma, particularly hydrolysis and esterases. Thus, the stability of test compounds in plasma is an important parameter, which not only affects *in vivo* results, but also the bioanalytical assay strategy and design. Investigation of plasma stability should be performed early in the discovery process in order to assess potential degradation and/or protein binding issues.

Assay Design:

- Test articles are assayed in triplicate
- Two concentrations (10 μM and 100 μM or if known C_{max} and 10x C_{max})
- Two time points $t = 0$ and $t = 180$ min
- Positive control: Procaine (50 μM)
- Negative control: Procainamide (50 μM)

Analysis: LC/MS/MS detection of the remaining test article

Report: % parent compound remaining

Quantity of test article required: 1.0 - 2.0 mg

Summary of Assay:

A solution of test compound in plasma is prepared and incubated for a predetermined time period. Aliquots are removed at pre-defined time points and analyzed by LC/MS/MS. The peak area for the parent compound is compared to the time zero sample in order to assess the amount of compound still available.

Plasma Protein Binding

Pharmacologic question addressed: "*What percent of the compound plasma protein is bound, to which component (sub-fraction), and what is the free fraction available to cover the target?*"

The binding of test compounds to plasma proteins is an important factor affecting drug efficacy, metabolism and pharmacokinetic properties. In many cases, drug efficacy is determined by the concentration of free drug (unbound), rather than the total concentration in plasma. If the drug is highly bound to plasma proteins, the amount of drug available to reach the target is reduced. Subsequently, the efficacy of that compound may be significantly reduced. Therefore, information on the free drug fraction is essential for drug development and may be helpful in correlating with *in vivo* efficacy.

Rapid equilibrium dialysis (RED) is an accurate and reliable method for determining the degree to which a compound binds to plasma proteins. Plasma spiked with test compound is added to the center chamber of a commercial plate based RED device. Blank, isotonic sodium phosphate buffer is added to the peripheral chamber of the RED device and the plate is incubated at 37°C for 4 hours. Equilibrium of free compound is achieved by the diffusion of the unbound compound across the dialysis membrane. Several manufacturers provide RED devices (Thermo Scientific). Aliquots of the buffer and the plasma are taken at pre-determined time points and the concentration of free and bound test compound is determined by LC/MS/MS analysis.

Assay Design:

- Test articles are assayed in duplicate
- Test articles are mixed with human plasma (other species available)
- One concentration of test article (10 µM, different concentrations available)
- One time point (t = 4 hours at 37°C)
- Positive control: Propranolol (high binding) and Metoprolol (low binding)
- Negative control: No plasma (PBS only)

Analysis: LC/MS/MS detection of the test compound in plasma and in buffer

Report: % compound bound

Quantity of test article required: 1.0 - 2.0 mg

Summary of Assay:

Human or specific species of interest plasma in the sample chamber are spiked with test compounds at 100x dilution of stock solution (typically 10 mM in DMSO). The chamber is sealed, and the compound is dialyzed against PBS, pH 7.4 at 37°C for 4 hours. Aliquots from each chamber (plasma and PBS) are collected and the concentrations of compound in each sample are determined by LC/MS/MS. Adjustments are made for non-specific binding.

Screening Cytotoxicity / Hepatotoxicity Test

Pharmacologic question addressed: "*Is my compound too toxic to be therapeutic?*"

Cytotoxicity is a well-established and easily accessible endpoint to gather early information about the general / acute toxic potential of a test article. The *in vitro* cytotoxicity test with primary hepatocytes is used to identify the cytotoxic potential of a test substance. The relative cell viability upon incubation with test article compared to the solvent control is determined (single point).

The ATP-lite 1step Cytotoxicity Assay (PerkinElmer) is a single reagent addition, homogeneous, luminescence ATP detection assay that measures the number of live cells in culture wells.

Assay Design:

- Primary hepatocytes (other cells available)
- 12-dose concentration response curve (CRC) of the test article (100x IC_{50} or 50 µM maximum concentration)
- Two replicates of CRC
- One incubation time 24 hours
- Positive control: Compounds with known toxicity
- Negative control: Compound with known non-toxicity
- Background control: Vehicle only

Analysis: Luminescence is measured from 550 - 620 nm.

Report: IC_{50}

Quantity of test article required: 1.0 - 2.0 mg

Summary of Assay:

Hepatocyte cells are incubated for 24 hours with known toxic and non-toxic compounds at a range of different concentrations. At the end of the incubation period the cells are loaded with the ATP-lite™ 1step ATP

monitoring reagent and scanned using an automated plate reader with luminescence detection (Tecan Infinite M200 reader) to determine the number of active cells.

CYP450 Inhibition Profiling

This assay extends the findings of the microsomal stability assay. Pharmacologic question addressed: "*Does my compound inhibit a key oxidative metabolic enzyme that would lead to subsequent drug-drug interactions?*"

Cytochrome P450s (CYPs) are a superfamily of heme-containing enzymes that mediate the inactivation and metabolism of many drugs as well as endogenous substances. Compounds that inhibit P450s may cause the toxic accumulation of other substrates. CYP inhibition profiling examines the effects of a test compound on the metabolism of other known enzyme substrates of the five primary drug human metabolizing CYP: 1A2, 2B6, 2C9, 2D6, 3A4. The levels of the CYP isoform marker substrate and metabolites are measured in the presence and absence of a test compound by LC/MS/MS.

Assay Design:

- Five CYP isoenzymes: 1A2, 2B6, 2C9, 2D6, 3A4
- Test articles are run in triplicate
- One concentration of human liver microsomes (0.5 mg/mL)
- One concentration of test item (10 μM)
- One time point 0.5 hours
- Positive control: CYP marker reaction (Table 1)
- Negative control: NADPH deficient reaction control

Analysis: LC/MS/MS detection (appearance of metabolite)

Report: Data are expressed as % inhibition of selected metabolites formation for each CYP450 enzyme (1A2, 2B6, 2C9, 2D6, 3A4).

(See FDA guideline for CYP substrates:

http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/DrugInteractionsLabeling/ucm093664.htm)

Quantity of test article required: 1.0 - 2.0 mg

Summary of Assay:

In an assay similar to the metabolic stability assay, liver microsomes are used to determine the CYP450 inhibition profile of test compounds by measuring the % metabolism of a known substrate. Microsomes (and NADPH regenerating system) are dispensed into a 96-well plate containing a substrate and test compound (10 μM), and the reaction is allowed to proceed for 0.5 hours at 37°C with shaking. The reaction is quenched by the addition of MeOH, centrifuged and the amount of product is measured by LC/MS/MS. Each plate will contain a known inhibitor of each CYP450 profiled as positive control and NADP-/- negative controls.

Table 1: Inhibition profiling for the five primary drug human metabolizing cytochrome P450s.

CYP Enzyme	Substrate	Metabolite	Known Inhibitor
1A2	Phenacetin	Acetaminophen	Furafylline
2B6	Bupropion	Hydroxybupropion	Ticlopidine
2C9	Diclofenac	4-hydroxydiclofenac	Tienilic Acid
2D6	Dextromethorphan	Dextrorphan	Paroxetine

Table 1 continued from previous page.

CYP Enzyme	Substrate	Metabolite	Known Inhibitor
3A4	Testosterone	6β-hydroxytestosterone	Azamulin

Permeability

Pharmacologic question addressed: "*How well is my drug absorbed in the gastrointestinal tract?*"

Evaluating compound permeability through a cell monolayer is a good indication of intestinal permeability and oral bioavailability. The Parallel Artificial Membrane Permeability Assay (PAMPA) provides a high throughput, non-cell based method for predicting passive, transcellular intestinal absorption, the process by which the majority of small molecule drugs enter circulation. In the PAMPA method, an artificial membrane immobilized on a filter is placed between a donor and acceptor compartment. The compound is introduced in the donor compartment. Following the permeation period, the amount of compound in the donor and acceptor compartments are quantified using scanning UV spectrophotometry.

The gastrointestinal tract (GT) has a pH range from pH 1 – 8. The pH of the blood is constant at pH 7.4; therefore it is possible for a pH gradient to exist between the GT and the plasma that can affect the transport of ionizable molecules. In an effort to mimic this pH gradient *in vitro*, alternative assays with pH 7.4 for the acceptor compartment and pH values 5.0, 6.2, and 7.4 in the donor compartment are used.

PAMPA is a well-established and predictive assay that models the absorption of drugs in the gut. However, PAMPA is an artificial system that may provide inaccurate and potentially misleading results. Despite these limitations, PAMPA can be a useful tool to prioritize lead compounds in early stages of development. The colon carcinoma (Caco-2) cell permeability assay is the industry standard for *in vitro* prediction of intestinal absorption of drugs, but it too has limitations. Caco-2 cells require extensive culturing (>20 days), and often fail to form the cohesive monolayer necessary for uniform transport of compounds across the cell layer. The assay requires a significant amount of compound to perform the assay (typically ~20 mg). Together, the limitations of time and compound consumption decrease the value of the results obtained by Caco-2 at the early stages of drug discovery. One variant of PAMPA is the Blood Brain Barrier (BBB) PAMPA in where the artificial monolayer contains brain specific membrane components, such as sphingolipids.

Assay Design:

- Test articles are run in triplicate
- One concentration (25 µM)
- One time point (18 hours)
- One pH (7.4) or three point pH range (5.0, 6.2, 7.4) for acceptor compartment
- Single polar membrane lipid (phosphatidylcholine in dodecane)
- Multiscreen PVDF membrane (0.45 µm)
- Positive control: Verapamil (high permeability)
- Negative control: Theophylline (low permeability)

Analysis: The concentration of the compound remaining in the donor well, diffused through the membrane and into the acceptor well, and reference compounds are measured by UV spectrophotometry

Report: Bin the results as high, medium, or low predicted absorption and report direct permeability units (10-6 cm/s)

Quantity of test article required: 5.0 - 7.0 mg

Summary of Assay:

A lipid bilayer is established on a membrane filter and a test compound solution is added to the top of the membrane-lipid interface. The ability of compounds to passively diffuse through the lipid treated membrane is an indication of the overall compound permeability. This approach is helpful in compound profiling and supporting the relative rank ordering of compounds.

Example of *In Vitro* ADME Profiling Assays

Table 2 below shows an example tabulation of the ADME properties assessed and evaluated as a requirement for nomination of a chemical probe in the Molecular Libraries Program (MLP) for probes ML301, ML314 and a few key related analogs, as described in the NCBI MLP probe report: http://www.ncbi.nlm.nih.gov/books/NBK184496/ that describes a novel small molecule agonist for the Neurotensin 1 Receptor (NTR1).

ADME profile of ML301 (Probe 1) and related compounds: ML301, its intriguing naphthyl analog (MLS-0446079), and the prior art, pyrazole, were evaluated in a detailed *in vitro* ADME screen as shown in Table 2. Despite its structural similarity (imidazole vs. pyrazole) to the prior art compound, ML301 exhibited substantial advantages in this testing, especially with regard to plasma and microsomal stability. These three compounds all exhibited good solubility due to the presence of the carboxylic acid moiety.

The PAMPA assay is used as an *in vitro* model of passive, transcellular permeability. The compounds exhibited good overall permeability, inversely related to the pH of the donor compartment. Because these NTR1 agonists are envisioned as predecessors of psychoactive drugs, a preliminary assessment of their potential to cross the BBB was performed. When incubated with an artificial membrane that models the BBB, much lower permeability was observed. These observations are also consistent with the carboxylic acid function in the compounds, and may present an opportunity for future enhancements.

Plasma protein binding is a measure of a drug's efficiency of binding proteins within blood plasma. The less bound a drug is, the more efficiently it can traverse cell membranes or diffuse. Drugs that are highly bound to plasma proteins are confined to the vascular space, thereby having a relatively low volume of distribution. In contrast, drugs that remain largely unbound in plasma are generally available for distribution to other organs and tissues. The imidazole scaffold compounds (ML301 and its MLS-0446079) exhibited substantial protein binding, but significantly lower than that of the prior art, pyrazole.

The stability of small molecules and peptides in plasma may strongly influence *in vivo* efficacy. Drug candidates are susceptible to enzymatic processes, such as those mediated by proteases or esterases in plasma. They may also undergo intramolecular rearrangement or bind irreversibly (covalently) to proteins. ML301 showed excellent stability in plasma, significantly better than that of either analog.

The microsomal stability assay is commonly used to rank compounds according to their metabolic stability, which influences how long the candidate may remain intact while circulating in plasma. ML301 showed excellent stability in human and modest stability in mouse liver homogenates, which was much better than that observed for the prior art analog, MLS-0437103. None of the compounds showed toxicity (>50 μM) toward human hepatocytes.

ADME profile of ML314 (Probe 2): As described above for ML301, *in vitro* ADME screening was also conducted for ML314. Consistent with its aqueous solubility data, ML314 exhibited high permeability in the PAMPA assay with increasing pH of the donor compartment. When incubated with an artificial membrane that models the BBB, ML314 was found to be highly permeable. ML314 was highly bound to plasma protein and exhibited very high plasma stability. ML314 was metabolized rapidly when incubated *in vitro* with human and mouse liver microsomes. This result is not completely surprising because of the presence of several unsubstituted aryl and alkyl positions and Ar-OMe ethers, which are prone to oxidation, hydrolysis, conjugation and other metabolic reactions. ML314 showed a >15-fold window for toxicity (LC_{50} = 30 μM) towards human hepatocytes.

Improving the metabolic stability and toxicity profile of ML314 represents a challenge as well as an avenue for further optimization studies in the future.

Table 2: Summary of *in vitro* ADME/T properties of NTR1 agonists ML301 (& analogs) and ML314.

ADME/T Assay Panel Component		MLS-0437103 *(prior art pyrazole)*	MLS-0446079 *(ML301-napthyl analog)*	ML301 (Probe 1)	ML314 (Probe 2) *β-arrestin biased*
Aqueous Solubility in pION's buffer (μg/mL) *[μM]*[a] pH 5.0/6.2/7.4		52.9 / >155 / >155 *[113 / >296 / >296]*	102.6 / >145 / >145 *[247 / >297 / >297]*	>52 / >52 / >52 *[>99 / >99 / >99]*	>125 / 9.0 / 0.52 *[>297 / 21.4 / 1.2]*
Aqueous Solubility in 1x PBS, pH 7.4 (μg/mL) *[μM]*[a]		ND	ND	ND	0.45 *[1.1]*
PAMPA Permeability, P_e ($x10^{-6}$ cm/s) Donor pH: 5.0 / 6.2 / 7.4 Acceptor pH: 7.4		1267 / 725 / 70	953 / 145 / 12	363 / 17 / 6	1163 / 2145 / 2093
BBB-PAMPA Permeability, Pe ($x10^{-6}$ cm/s) Donor pH: 7.4 Acceptor pH: 7.4		4.8	1.1	1.2	399
Plasma Protein Binding (% Bound)	Human 1 μM / 10 μM	99.60 / 99.71	97.93 / 97.96	98.69 / 98.70	99.45 / 99.22
	Mouse 1 μM / 10 μM	96.63 / 97.10	96.43 / 95.41	92.15 / 91.36	99.67 / 98.84
Plasma Stability (%Remaining at 3 hrs) Human/ Mouse		88.94 / 70.74	76.00 / 75.97	100 / 100	100 / 99.55
Hepatic Microsome Stability (% Remaining at 1hr) Human/Mouse		75.50 / 45.67	100 / 75.70	100 / 59.48	1.36 / 0.16
Toxicity Towards Fa2N-4 Immortalized Human Hepatocytes LC_{50} (μM)		>50	>50	>50	29.6

[a] Solubility also expressed in molar units (μM) as indicated in *italicized [bracketed values],* in addition to more traditional μg/mL units.
ND = Not Determined

In Vivo Analysis - High Compound Requirements and Low Capacity

Rapid Assessment of Compound Exposure (R.A.C.E.)

This experiment is a rapid and efficient compressed *in vivo* PK screening method to determine the pharmacokinetic attributes of novel chemical probes (3). A small cohort of animals (4 mice or 2 rats/ experiment), is administered compound orally (*p.o.*) or by intraperitoneal injection (*i.p.*) at a single dose (5 - 50 mg/kg). Blood samples are collected at 20 and 120 minutes. The plasma samples are analyzed with a mini-standard curve (Figure 1). This experiment provides a snapshot of compound exposure sufficient to estimate total compound exposure as the area under the curve ($AUC_{(20\text{-}120\ min)}$), providing a rank order of estimated AUC values to prioritize compounds for further investigation.

Another utilization for R.A.C.E. is to evaluate varying formulation excipients for improving solubility and absorption. Generally, formulation excipients approved by the FDA are available for this assay. Please refer to reference (4) for FDA approved excipients and amounts.

Assay Design:

- Test articles are formulated for *p.o.* or *i.p.* dosing
- One dose of test article (5 – 50 mg/kg)

- Two time points (t = 20 min and 120 min)

Analysis: LC/MS/MS detection of the test compound(s) in plasma samples

Report: Estimated $AUC_{(20\text{-}120\ min)}$ and a rank order of compound exposure

Quantity of test article required: 5.0 - 50.0 mg

Comprehensive Pharmacokinetic Analysis

Compounds that show promising PK profiles or that are further developed, can be subjected to a comprehensive pharmacokinetic analysis and metabolite identification study. An example set of graphs representing typical time course drug plasma concentrations following oral dosing can be found in Wikipedia http://en.wikipedia.org/wiki/Pharmacokinetics and are reproduced to illustrate the key related parameters: time to reach (t_{max}) maximal concentration (C_{max}) of compound, time to reduce concentration by half of the initial value ($t_{1/2}$), dosing interval (τ), and area-under-curve (AUC) or total compound exposure (Figure 2).

A minimum of six 300 gram rats are required, per compound tested, for appropriate sampling. For each drug, the compound will be formulated in suitable vehicle to typically 1.0 mg/mL (final concentration) and administered to 3 rats at 1 mg/kg *i.v.* into a femoral vein catheter. The same substance is administered to an additional 3 rats at 2 mg/kg by oral gavage (*direct dosing of compound into the stomach through a feeding tube*). Blood is drawn (0.25 mL) via the femoral artery catheter at 5, 15, and 30 minutes and at 1, 2, 4, 6, 8 and 24 hours post dose for a total maximum volume of 2.25 mL. Plasma samples are analyzed by LC/MS/MS. A comprehensive PK analysis requires 6-12 animals and blood collections at 8 time points.

Assay Design:

- Single test article is formulated for both *p.o.* and *i.v.* dosing
- *I.v* and *p.o.* doses of test article (e.g., 1.0 mg/kg and 2.0 mg/kg)
- Nine time points (5, 15, and 30 min and at 1, 2, 4, 6, 8 and 24 hours).

Analysis: LC/MS/MS detection of the test compound(s) in individual plasma samples

Report: Time course of plasma drug concentration versus time, PK parameters (for example, AUC, $t_{1/2}$, oral bioavailability) and metabolite identification

Quantity of test article required: 10 - 100 mg

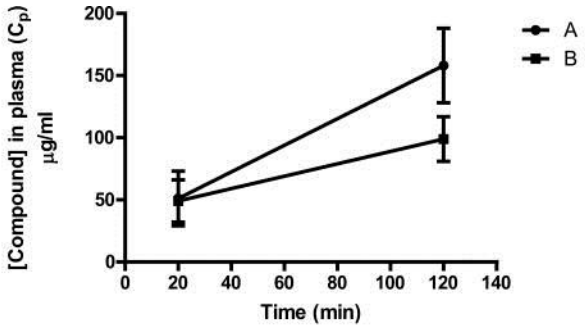
Suggested Equipment and Resources

Automated workstation: A flexible versatile high-throughput/high-capacity workstation system for automated liquid handling is a useful work horse of the pharmacology lab. The system should be capable of processing compound solutions for analysis and executing the various *in vitro* profiling assays. A variable spanning 8 channel pipette arm, a 96-well multichannel pipetting head, and plate gripper arm are useful for reformatting samples between vials, tubes, and 96-well plates. Specialized devices include vacuum filtration and magnetic separation modules, as well as integrated devices for heating, cooling, and shaking samples.

Multimodal Plate Reader: A multi-mode plate reader capable of UV/Vis, top/bottom fluorescence, and flash/glow luminescence read modes accessible to the plate gripper arm of a workstation is useful for unattended reading for a batch of samples.

Liquid Chromatography/Mass Spectrometric/Mass Spectrometry (LC/MS/MS): This technology is the workhorse of all ADME/T and PK analyses of samples from both the *in vitro* and *in vivo* assay. After extraction of biomatrix from small molecules, the small molecule sample and any related compounds (metabolized,

A



B

Compound	First X	Last X	Peak X	Peak Y	Total Area	Calculated eAUC**
A	20.0	120.0	120.0	158.00	10483	10.483 µg*hr/mL
B	20.0	120.0	120.0	98.87	7828	7.828 µg*hr/mL

Figure 1: Example of data from a previously performed RACE study. The purpose of this study was to select which compound from a series exhibited the highest estimated exposure. (A) Representative exposure data from two compounds in a series obtained by sequential RACE studies (n=3 mice/time point; vehicle mice showed no compound in the plasma, data not shown). (B) Results from the GraphPad Prism5 AUC analysis. **eAUC (estimated exposure ($AUC_{20\text{-}120\ min}$) using a "trapezoidal" estimation of AUC (Copyright © 2012 John Wiley & Sons, Inc., republished with permission from (3)).

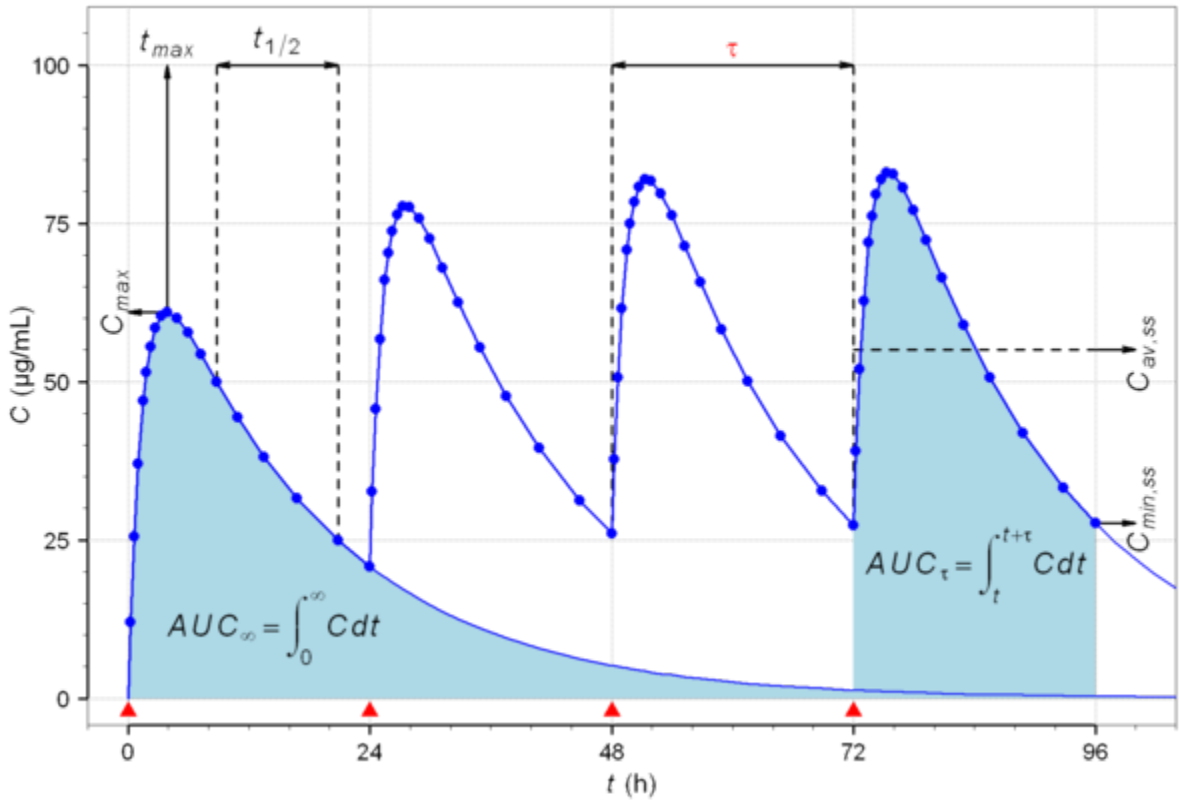


Figure 2: Time course of drug plasma concentrations over 96 hours following oral administrations every 24 hours (τ). Absorption half-life is 1 hour and elimination half-life is 12 hours. Note that in steady state and in linear pharmacokinetics $AUC_\tau = AUC_\infty$. Steady state is reached after about 5 × 12 = 60 hours (courtesy of Helmut Schütz).

hydrolyzed, or broken down compounds) are first separated through high-performance liquid chromatography (HPLC). The effluent from the HLPC are monitored in real-time for ultraviolet absorbance at 254 and 280 nm, and absorbing samples are diverted into an on-line electrospray unit to introduce samples into the mass spectrometer, followed by sequential quadrapole selection and detection of mass/charge (m/z) distributions of samples that are characteristic of parent and derived molecules. For example, we use a Shimadzu UPLC coupled with an automated AB Sciex API 4000 MS/MS system with QTRAP detection. This is a high performance hybrid triple quadrupole/linear ion trap system with excellent dynamic range and sensitivity. However, this is a very mature sector of the MS market and several vendors with comparable instruments exist.

Microplate Scintillation and Luminescence Counter: For studies with radiolabeled compounds, scintillation devices are still required. Currently a few manufacturers still make multi-wall, microtiter plate based scintillation counters (e.g. PerkinElmer TopCount NXT™), these devices can also plate luminescence readers.

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Glossary

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Glossary of Quantitative Biology Terms

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Created: April 15, 2012; Updated: September 22, 2014.

Abstract

In this chapter, the authors have provided standardized definitions of cell culture and quantitative biology terms generally employed in assays for HTS and lead optimization. The quantitative biology terms include definitions both biological and statistical concepts used assay design, development and validation. These definitions are intended to facilitate streamlined communications across multiple disciplines of scientists involved in drug discovery and development research.

Cell Culture Terms

Acclimatization — Adaption of a culture to different environments such as temperature, humidity or CO2.

Anaerobic Growth — Growth of an organism in the absence of ambient O2.

Anchorage Dependent (Attached) Cells — Cells that require a substrate to survive in culture.

Apoptosis — A process in cells leading to programmed cell death.

Batch Culture — Cells or microbes cultured in a bioreactor that contains all nutrients needed until all is harvested as a unit.

Bioreactor — Environmentally controlled vessel for creation of biological products.

Carry — To maintain a cell line by subculturing in tissue culture medium containing nutrients that will maintain the phenotype and genotype.

Cell Clone — Cell line derived from a single cell and therefore all cells are identical

Cell Culture — Establishment and maintenance of cells derived from dispersed tissue taken from original tissues, primary culture, or from a cell line or cell strain.

Cell Line — Immortalized cell that has undergone transformation and can be passed indefinitely in culture.

Cell Passage — The splitting (dilution) and subsequent redistribution of a monolayer or cell suspension into culture vessels containing fresh media, also known as subculture.

Cell Strain — Cells which can be passed repeatedly but only for a limited number of passages.

Cell Transformation — Permanent alteration in cell genotype that results in phenotypic change from the original cell. It may result in a primary cell becoming immortalized.

Chemically Defined — Solution such as culture medium in which the ingredients are completely known.

Cloning Efficiency — Percent of cells that can become a clone when plated at single cell densities.

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Confluency — Based on the amount of space between the cells on the growth surface. A culture is confluent if no plate can be seen between cells. Degrees of confluency is based on percent of culture dish that is covered. Thus 100% to lower percent exists. The confluency of the culture may influence cell division and gene expression.

Cytopathic — Causing cell pathology.

Cytotoxic — Causing cell death.

Density-Dependent Growth Inhibition — Reduced proliferation of cells upon reaching a threshold density, i.e. reaching confluence.

Differentiation — Property of cells to exhibit tissue-specific properties in culture.

Endotoxin — a material that is part of the cell wall of some bacteria. It can be released upon bacterial death and cause cell sepsis. Medias and media components are routinely tested for its presence.

Generation Time — Time it takes for a cell to complete a cycle of division from same stage to same stage.

Growth Curve — A plot of cell number vs. time. Should demonstrate exponential log phase growth up to stationary phase.

Hatch — To bring cells out of the freezer; to start a culture from a freezer stock.

Heat Inactivation — heating serum at 56C for 30 minutes to inactivate complement proteins that can affect cell growth of some cell lines.

Micoplasma — Prokaryotic organism. Smallest form of bacteria with no cell walls. It can cause infections in plants and animals. It can infect cultured cells without obvious change in cellular morphology but can result in alterations in cellular behaviors. Its presence is routinely monitored.

Monolayer — A layer of cells one cell thick, grown in a culture.

Primary Cells — Cells resulting from the seeding of dissociated tissues, i.e. HUVEC cells. Primary cultures will eventually die or lose their original phenotype and genotype with passaging.

Pass — See cell passage or subculture

Selective Medium — culture medium containing a factor to limit the growth of particular organisms. It could be used in transfection (contain antibiotic) to eliminate cells that are not transfected and do not receive the antibiotic resistance factor from transfection.

Serum Free Medium (SFM) — culture growth solution that does not contain serum but may contain other components as supplements.

Split — To subculture/passage cells; see cell passage.

Stable Cell Line — Cell line that stably expresses a transfected product. Typical systems that exist include resistance to antibotics such as neomycin phosphotransferase, conferring resistance to G418, etc. The culturing of the cells can be done as a mixed population or by single cell culture to obtain cell clones from one single integration event.

Sterile Techniques — methods to ensure lack of contamination with microorganisms.

Suspension Culture — Cells which do not require attachment to a substrate to proliferate, i.e. anchorage independent. Cell cultures derived from blood are typically grown in suspension. Cells can grow as single cells or

as clumps. To subculture, the cultures that grow as single cell suspension can simply be diluted. However, cultures containing clumps must have the clumps disasociated prior to subculturing.

Surfactant — A substance used to minimize the shear stress of water or medium. It can be used in media designed for suspension culture. A common one is Pluronic.

Transient Transfection — The introduction of foreign DNA into a cell to allow the expression of DNA and protein. The DNA does not incorporate into the genome therefore, with cell division, the effect is lost (diluted out) making the event transient (transient transfection). Cells capable of being transfected are referred to as "competent". Protocols are available for opening transient "holes" in the cell membranes allowing introduction of plasmids or siRNA.

Thaw — Same as hatch.

Quantitative Biology Terms

Absolute EC50

The molar concentration of a substance that increases the measured activity in an agonist assay to 50% of the range of activity expressed relative to maximum and minimum controls.

Absolute IC50

The molar concentration of an inhibitor required to block a given response by 50%, half way between the maximum and minimum controls if the response is expressed relative to the maximum and minimum controls.

Accuracy

A measure of the closeness of agreement of a measured test result obtained by the analytical method to its theoretical true (or accepted reference) value. This is relevant only for calibration-curve based applications where a purified or relative reference standard material is available to quantify the analyte levels in test samples.

Accuracy Profile

A plot of the mean percent recovery (or percent relative error) and its confidence interval versus the concentration of the spiked standards (validation or QC samples). It is used to judge the quality of a calibration-curve based assay in terms of total error (bias plus variance).

Assays/Methods

- **Assay-Biochemical:** Biological measurements performed with purified or crude biochemical reagents.
- **Assay-Cell-based:** Biological measurements performed in which at least one of the reagents consists of a population of live cells.
- **Assay Design:** see Multivariate (Factorial Experiments), aka, Experimental Design.
- **Assay-In vitro:** From the Latin meaning 'in glass'. Any assay (biochemical or cell-based) conducted in a synthetic container (e.g. microtiter plate, microfluidic cell).
- **Assay-In vivo:** From the Latin meaning 'in life'. Typically used for assays conducted in living animals (e.g. mice, rats, etc.) with the exception of microorganisms (e.g. yeast, bacteria or C. elegans).
- **Assay Platform:** Technology used to measure response or output. (e.g. Fluorescence polarization or Radiometric counting).
- **Assay-Phenotypic:** An assay where the measured signal corresponds to a complex response such as cell survival, proliferation, localization of a protein, nuclear translocation etc. The molecular target is not assumed.

- **Assay-Primary:** The first assay performed in a testing scheme to identify biologically active chemical entities in a screening mode.
- **Assay-Secondary:** Assays that follow the primary assays to confirm the biological activity of chemical entities identified in the primary assays. This can also include selectivity and specificity assays.
- **Assay-Selectivity/Specificity:** Assays employed to elucidate the specificity of biologically active chemical entities towards a set of closely related disease targets.
- **Assay Validation:** see Validation
- **Assay-Separation:** Prior to detection a physical separation of at least one component from the assay is performed. (Standard ELISA, filtration, HPLC etc.)
- **Assay-non-separation:** Any assay where a physical separation is not required prior to detection.
- **Assay-Target based:** An assay where the measured response can be linked to a known set of biological reagents such as a purified enzyme, domain or a reporter gene.

Biological Target

A macromolecule or a set of macromolecules in a biochemical pathway that is responsible for the disease pathology.

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.

Calibration Curve

Also called "standard curve", a calibration curve is a regression of the assay response on the known concentrations in "standard" samples. It is a model that fits data from standards and is used for calculating (calibrating) values of unknown test samples. For example, measurement of protein/biomarker expression levels of various compounds from in-vitro and in-vivo samples.

Central Composite Designs

A type of multi-factor experiment that is used to optimize the most important factors in an assay (usually 3 to 5 factors).

Classification & Regression Tree Models

A set of statistical methods in which observations are classified into groups based on a set of predictor variables and their relationship with a response variable. These models can be used for multivariate correlation analysis.

Cluster Analysis

A set of statistical methods in which objects (e.g., compounds) are divided into groups such that objects within a group are similar across a set of 2 or more variables.

Concordance Correlation Coefficient

A measure of agreement between two variables, i.e., how closely the paired values match. That is, it measures the degree of closeness of the data to the agreement line (Y=X line). Since the Pearson's correlation measures the degree of departure of the data from the best straight line, which can be considerably different from the agreement line, the concordance correlation coefficient is more stringent than the Pearson's correlation.

Control Compound

A compound that is routinely run in the same manner as the test compounds in every run of the assay. This term does not refer to the plate controls used to define the maximum and minimum responses, and they may or may not be a “literature standard” or “reference” compound.

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.

Dynamic Range

It is the interval between the upper and lower concentration of the analyte in the sample for which the assay has been demonstrated to have acceptable level of accuracy, precision, linearity, etc.

EC50

The effective concentration of an agonist, which produces 50% of the maximum possible response for that agonist.

Emin

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.

Factor

An assay variable that can be changed by the user. Examples include the amount of a reagent, incubation time, buffer type, etc.

False Positive

A hit where the signal modulation is not related to the targeted activity. The sources of false positives include, random or systematic errors in liquid handling, spectrophotometric or fluorescence interference of the assay signal by chemical compounds, reagent instability etc. It is important to note that false positives can be reproducible when they are not related to random errors (as in the case of compound interference).

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 among the concentrations included in a concentration response curve. 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.

Four Parameter Logistic Model

A non-linear regression model commonly used for fitting dose-response and concentration-response data. The four parameters are Minimum (response at zero dose), Maximum (response at infinite dose), Relative EC50 (or IC50, ED50, etc.) and Slope. The 4PL model can be written in several mathematically equivalent versions. Two popular versions are given below.

$$Y = bottom + \frac{top - bottom}{1 + \left(\frac{x}{EC50}\right)^{slope}}$$

$$Y = bottom + \frac{top - bottom}{1 + 10^{(\log(x) - \log(EC50)) * slope}}$$

Fractional Factorial Experiments

A type of multi-factor experiment in which only a subset of factor level combinations is tested. These experiments are very efficient for screening a large number of factors prior to optimizing the most important factors.

Generalized Additive Models

Statistical models in which more general (e.g., nonlinear) relationships between variables can be examined. These models can be used for multivariate correlation analysis.

High Throughput Screening (HTS)

Greater than 100,000 compounds screened per screen.

Homogeneous Assay

All assay components exist in solution phase at the time of detection (e.g. none of the components are in beads or cells). Technically no component scatters light.

Heterogeneous Assay

One or more assay components are present in solid phase at time detection. (e.g.: SPA, cells or IMAP).

Hill Coefficient

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.

Inhibition

Reduction of a predefined stimulus. Unit of Measure is always % when normalized to the dynamic range of the assay.

Inhibition at Max Included Concentration

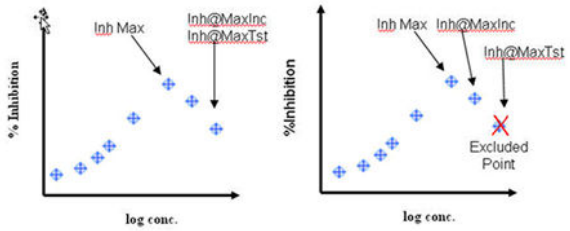
Inhibition observed at the highest included (i.e. not excluded) concentration of a substance tested in a concentration response mode method version regardless of whether it was included in the parametric fit to produce derived results. (see Illustration below)

Inhibition at Max Tested Concentration

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)

Inhibition Max

Maximum inhibition produced by any concentration that was included for the application of a curve fit algorithm (see Illustration below)



Inverse Agonist

When an inverse agonist binds to a receptor, it stabilizes the inactive form of the receptor, shifts the equilibrium toward that state and produces a response opposite to that produced by an agonist in the biological system under investigation. These substances possess negative intrinsic activity.

Least Squares (Pearson's) Correlation Coefficient

A measure of linear correlation between two variables.

LsA (Limits of Agreement)

These are statistical limits that define the region that contains 95% of all potency ratios.

Mean Ratio (MR)

The average ratio of potencies between the two runs.

Multivariate (Factorial) Experiments (Experimental Design)

A system of experimentation for optimizing assays in which multiple factors are varied simultaneously in such a way that the effect of each factor can still is determined. In addition, one can also measure interactions between factors and use this information to more efficiently optimize an assay.

Multiple Linear Regression

A statistical method where the response variable is a linear function of several predictor variables. This can be used for multivariate correlation analysis.

Multivariate Correlation Analysis

A statistical analysis method where correlative relationships between 3 or more variables are examined.

Nonlinear Regression

Statistical methodology for fitting models that is nonlinear in their parameters, for example, the four-parameter logistic model.

One Factor at a Time Experiments

A series of experiments in which one factor is changed at a time. Once the "best" condition for one factor is found, it is fixed at that setting for subsequent experiments. This approach to assay optimization will not find the optimum conditions if at least one factor interacts with another, i.e., the best level of one factor depends on the levels of another factor.

Overall MSD (Minimum Significant Difference)

The minimum difference in efficacies of two compounds evaluated in different runs that is statistically significant, i.e. that should be considered a real change in efficacy. The Overall MSD is defined for a single run of each compound.

Overall MSR (Minimum Significant Ratio)

The minimum ratio in potencies of two compounds evaluated in different runs that is statistically significant, i.e. that should be considered real change in potency. The Overall MSR is defined for a single run of each compound.

Optimization

The process of developing an assay (prior to validation) wherein the variables affecting the assay are elucidated (e.g., Antibody concentration, incubation time, wash cycles, etc.). This process is ideally carried out using a multi-variate factorial approach where the inter-dependence between multiple variables/parameters can be taken into account.

Orphan Receptor

A biological target 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 created.

Percent Recovery

The calibrated value of a standard or validation sample divided by its expected value (known concentration), expressed as a percentage.

Plate Format

Microtiter plate well density (e.g., 96-, 384- or 1536-well) and plate composition (e.g., clear bottom black or clear bottom polystyrene, etc.)

Potentiation

Many 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, if an increased stimulus is observed the test compound is deemed a potentiator. Potentiation is the response produced by the combination of substances minus the response produced by the specific concentration of Reference Agonist alone.

Precision

A quantitative measure (usually expressed as standard deviation, coefficient of variation, MSR) of the random variation between a series of measurements from multiple sampling of the same homogenous sample under the prescribed conditions.

Precision Profile

A plot of the variability of calibrated values (expressed as a CV) versus concentration of standard. It is used to judge the quality of a calibration curve in terms of the variability in the calibrated values. It also determines the working range of a calibration curve.

Production MSD (Minimum Significant Difference)

The minimum difference in efficacies of two compounds evaluated in different runs that is statistically significant, taking into account the number of runs routinely applied to all compounds in the assay. For example, if all compounds are routinely tested twice on separate days then the average of both runs will have greater precision than each individual run, and the Production MSD reflects this increased precision.

Production MSR (Minimum Significant Ratio)

The minimum ratio in potencies of two compounds evaluated in different runs that is statistically significant, taking into account the number of runs routinely applied to all compounds in the assay. For example, if all compounds are routinely tested twice on separate days then the average of both runs will have greater precision than each individual run, and the Production MSR reflects this increased precision.

Quantitative Biology

A set of skills that is essential for the design, optimization and validation of reproducible and robust assays/methods to establish the pharmacological profiles of biologically active chemical entities. The practice of quantitative biology requires the understanding of how cellular, biochemical and pharmacological principles can be integrated with analytical and automation technologies, employing appropriate statistical data analysis and information technology tools.

Relative 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.

Relative EC50

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.

Relative EC50 Inv

The Relative EC50 of an inverse agonist.

Relative Efficacy

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.

Relative Efficacy 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.

Relative IC50

Relative IC50; 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.

Relative Potentiator Efficacy

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.

Response Surface Analysis

A statistical analysis method that is used for central composite designs. A quadratic polynomial model is fit to the data in order to find the optimum conditions for an assay.

Repeatability

Repeatability is the precision of repeated measurements within the same analytical run under the same operating conditions over a short interval of time. It is also termed intra-assay or intra-batch precision.

Reproducibility (Run to Run)

A general term to describe the precision of results generated from multiple runs of a compound (or any homogenous test sample) in an assay. An assay may lack reproducibility because of either high within-run or across-run variability, or because of systematic trend (drift) over time in the response. An assay that is reproducible across runs is one that has variation within acceptable limits and has no material systematic trends.

Reproducibility (Lab to Lab)

Reproducibility across labs expresses the precision between laboratories. It is useful for assessing the "transferability" of an assay and/or the validity of comparing results from samples that are run in two or more laboratories.

Robustness/Ruggedness of the Assay

Robustness is a measure of the capacity of the assay to remain unaffected by small, but deliberate changes in method parameters and provides an indication of its reliability during normal run conditions.

Schild Kb

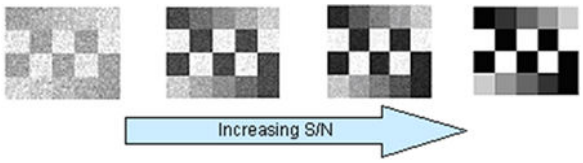
A measure of affinity for a competitive antagonist that is calculated using the ratios of equi-active concentrations of a full agonist (most typically EC50 concentrations are used) in the absence and presence of one or more concentrations of the antagonist. See pp. 335-339, Pharmacologic Analysis of Drug-Receptor Intercation, 3rd Ed. by Terry Kenakin.

Signal-to-background (S:B or S/B)

The ratio between the mean max signal and the mean minimum or background signal. This definition has been useful for describing the dynamic range of the assay.

Signal to Noise Ratio (S:N or S/N)

Defined as the mean signal (eg. Max or Min signal) divided by the standard deviation of this signal. This definition has been useful for describing the "signal strength" of an assay. The figure below illustrates how a two signals (black= background or Min and white=max) may appear as the "noise" in the system varies.



Signal Window

A measure of separation between max. and min. controls in an assay that accounts for the amount of variability in the assay. The formula is:

$$SW = \frac{mean_{\max} - mean_{\min} - 3(SD_{\max} + SD_{\min})}{SD_{\max}}$$

Simple Linear Regression

A statistical method for fitting a straight line to paired (X, Y) data.

Spearman's Correlation Coefficient

A nonparametric measure of correlation between two variables. It is applied to ranked values of the data and is therefore robust to outliers in the data.

Specificity

The ability of the assay to determine unequivocally the analyte in the presence of components that may be expected to be present in the sample.

Spike

Addition of a known quantity of a specific reference material or positive control to a sample matrix for recovery studies.

Stephenson's Kp

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 Intercation, 3rd Ed. by Terry Kenakin.

Stimulation

Increase of a measured output. Unit of Measure is always % when normalized to the dynamic range of the assay (Min and Max control wells). Note that this calculation can generate percents much higher than 100.

Stimulation at Max Included

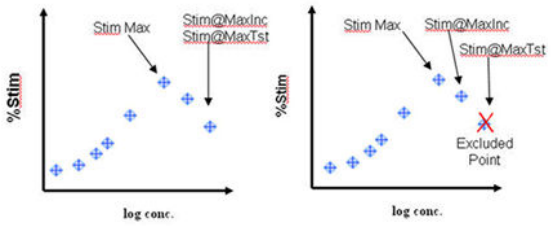
Stimulation observed at the highest included (i.e. not excluded) concentration of a substance tested in a concentration response mode method version regardless of whether it was included in the parametric fit to produce derived results. (See illustration below)

Stimulation at Max Tested

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)

Stimulation Max

Maximum stimulation produced by any concentration that was included for the application of a curve fit algorithm (See illustration below)



Target Platform

A set of biochemically and biologically related targets implicated in disease pathologies. Examples include G-protein coupled receptors (GPCRs), nuclear hormone receptors (NHRs), Kinases, Proteases, Transporters, Ion channels and Chemokines.

Testing Flow Scheme

Stages of testing NCEs as it progresses from active to hit to lead to a clinical candidate. Tests include in-vitro assays, animal model tests, ADME assays biopharmaceutical and toxicological tests.

Test-retest experiment

An experiment in which a set of (usually) 20-30 compounds is tested in two independent runs of an assay. Its purpose is to estimate the MSR (for dose-response assays) or the MSD (for single-point assays or an efficacy measure in dose-response assays). This experiment will provide a reliable estimate of only the within-run MSR.

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. The Unit of Measure is always %.

Ultra High Throughput Screening (uHTS)

Greater than 500,000 compounds screened per screen.

Validation

Validation includes all the laboratory investigations that demonstrate that the performance characteristics of an assay are suitable and reliable for its intended analytical use. It describes in mathematical and quantifiable terms the performance characteristics of an assay.

Validation/QC Samples

Samples of standard material that are prepared independently of the standards used for a calibration curve. They are not used to fit the calibration curve, but they are calibrated against it to determine percent recovery.

Within-Run MSD (Minimum Significant Difference)

The minimum difference in efficacies of two compounds evaluated in the same run that is statistically significant, i.e. that should be considered a real change in efficacy.

Within-Run MSR (Minimum Significant Ratio)

The minimum ratio in potencies of two compounds evaluated in the same run that is statistically significant, i.e. that should be considered real change in potency.

Z′-Factor

Another measure of separation between maximum and minimum controls in an assay that accounts for the amount of variability in the assay. The formula is:

$$\text{Z'-Factor} = 1 - \frac{(3\sigma_+ + 3\sigma_-)}{|\mu_+ - \mu_-|}$$

Genetics Terms

Allelic drop-out — Failure to detect an allele in a sample or failure to amplify an allele during PCR.

Allelic ladder — Comprised of DNA fragments that represent common alleles at a locus.

Allele — A different form of a gene at a particular locus. The characteristics of a single copy of a specific gene or of a single copy of a specific location on a chromosome. For example, one copy of a specific STR region might have 10 repeats, while the other copy might have 11 repeats. These would represent two alleles of that STR region.

Heterozygous — Having two different alleles at the same locus.

Homozygous — Having two of the same alleles at the same locus.

Off Ladder (OL) Alleles — Alleles that size outside allele categories represented in the ladder.

Phenotype — The detectable outward manifestations of a specific genotype; the physical characteristics of a living object.

Polymerase Chain Reaction (PCR) — A technique for enzymatically replicating DNA without using a living organism.

Polymorphism — Variations in DNA sequences in a population that are detected in human DNA identification testing.

Short Tandem Repeats (STR) — Multiple copies of a short identical DNA sequence arranged in direct succession in particular regions of chromosomes.

Authentication of Human Cell Lines by STR DNA Profiling Analysis

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Created: May 1, 2013.

ABSTRACT

Since 1951 when the first human cell line, HeLa, was established there has been an increase in the use of human cell lines as models for human diseases such as cancer, substrates for the production of viruses for vaccine production and as tools for the production of recombinant proteins for therapeutics. Unfortunately this accelerated use of human cell lines and the lack of best practices in tissue culture have led to increase in cellular cross-contamination which has resulted in spurious results. Now a simple molecular technique, Short Tandem Repeat (STR) DNA profiling of human cell lines, is available and if applied routinely in cell culture management can greatly improve the detection of cellular cross-contamination resulting in more accurate assays.

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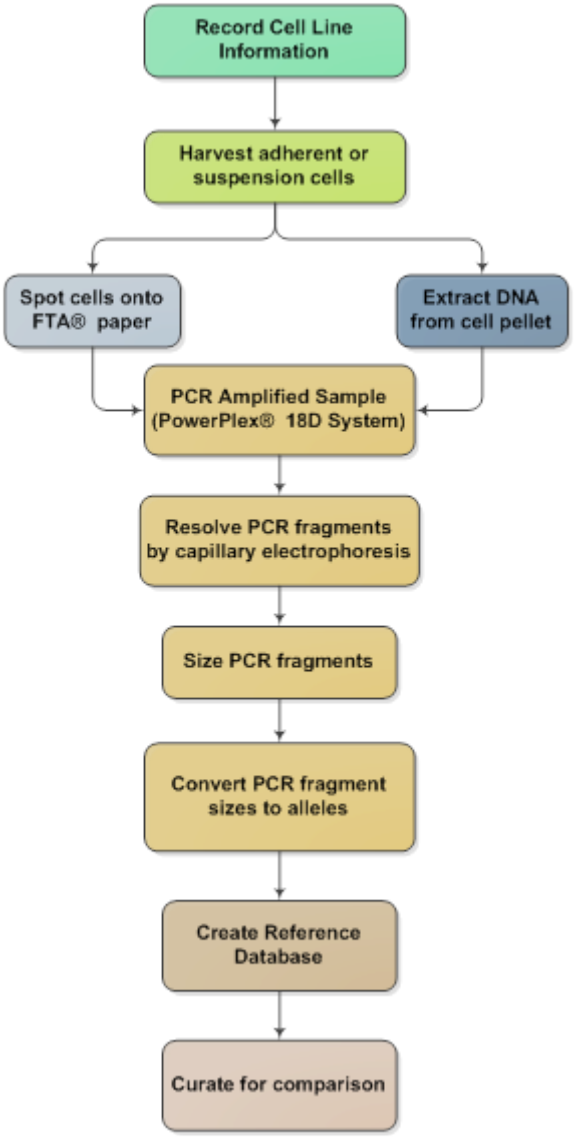
* Editor

† Editor

FLOWCHART: STR DNA ANALYSIS FOR HUMAN CELL LINE AUTHENTICATION



INTRODUCTION

Over the past 50 years there has been a progressive increase in the use of cells as models, substrates and tools for both basic research and industrial applications. The rapid growth in areas such as cell biology, genomics and proteomics has triggered a remarkable increase in cell culture activities, consequently resulting in an increase in the potential risk of cross-contamination of cell cultures. Cross-contamination among cultured cell lines is a persistent problem and has occurred at frequencies ranging from 16 to 35% (1, 2). Detection is particularly difficult if co-cultivated cells express similar phenotypes. Indeed, HeLa, a cell line derived from an invasive cervical carcinoma in 1951, was shown to contaminate more than 90 cell lines (3, 4). At one point, the number of cell lines contaminated with HeLa represented one-third of all human tumor cell lines developed for research in cancer and cell biology. In 1999 Drexler et al. found that 15% of 117 hematopoietic cell lines received from original investigators or a secondary source were cross-contaminated with other cell lines (5). More recent reports have shown cross-contaminated cell lines from breast and prostate cancers (6), thyroid cancer (7), adenoid cystic carcinoma (8), and esophagus (9). Cross-contamination of cell lines has persisted as a result of

mishandling and a lack of attention to best practices in tissue culture. Advances in molecular biology have now made it possible, using Short Tandem Repeat (STR) DNA profiling, to uniquely identify human cell lines derived from the tissue of a single individual allowing researchers to ascertain if their cultures were misidentified or cross-contaminated.

The discovery of DNA hypervariable regions within genomes has made it possible to identify each human cell line derived from a single donor. In 1985 Alec Jeffreys and others (10, 11) demonstrated that hypervariable regions, which consist of variable number tandem repeat (VNTR) units from minisatellite DNA, are capable of hybridizing to many loci distributed throughout the genome to produce a DNA 'fingerprint". The DNA fingerprints are very complex and not easily interpretable. However, subsequent advances in the technology have given rise to the use of STR of microsatellite regions which consist of core sequences of 1-6 bp. The core sequences of these human microsatellite DNAs can serve as hotspots for homologous recombination events which are believed to maintain the variability of these loci (12). The polymorphism or informativeness of these STR markers display many variations in the number of the repeating units between alleles and among loci in unrelated cell lines and shown in detail in Figure 1. There are two copies of an allele at a given locus on the chromosome obtained from a diploid cell line; one allele derived from the mother (material chromosome - M) and the other allele is derived from the father (paternal chromosome - P). If the two copies of the alleles (maternal and paternal) have the same number of repeating units, the fragments, which have the same size, will co-migrate during electrophoresis. For example, in Figure 1 the alleles 9, 10 (designated allele 8) is considered homozygous and a single peak is observed on the electropherogram. If the number of repeating units on the paternal chromosome is different from that of the maternal chromosome, at the same locus, this is considered heterozygous (for example alleles 8 and 9) and two peaks are observed in the electropherogram.

ASSAY CONCEPT: STR DNA PROFILING FOR HUMAN CELL LINE AUTHENTICATION (INTRASPEFICIC IDENTIFICATION AND DETECTION OF CROSS-CONTAMINATING CELLS)

STR analysis can be performed in most laboratories that have the capabilities to execute molecular techniques. It is an easy, low cost and reliable method for the authentication of human cell lines.

When a cell line is first received into the laboratory it is essential to capture as much information as possible on its history, growth and functional characteristics. This information is important for tracking the behavior of the cells during culturing, characterization and in the event there is misidentification, see Flow chart.

Such information may include:

- Name of cell line
- Name of donor of cell line
- Name of the originator of cell line
- Date cell line was established
- Reference describing the establishment of cell line
- Tissue of origin
- Species
- Population doubling levels
- Unique characteristics and function
- Complete growth medium
- Doubling time

A plan should be developed for managing the cells during expansion and the use of cells for experiments such as the creation of a Seed Stock (Master Cell Bank) and a Distribution stock (Working Cell Bank). Upon thaw, spot about 20 µL of cell suspension (200,000 cells) from the donor vial directly onto a FTA® paper for subsequent STR

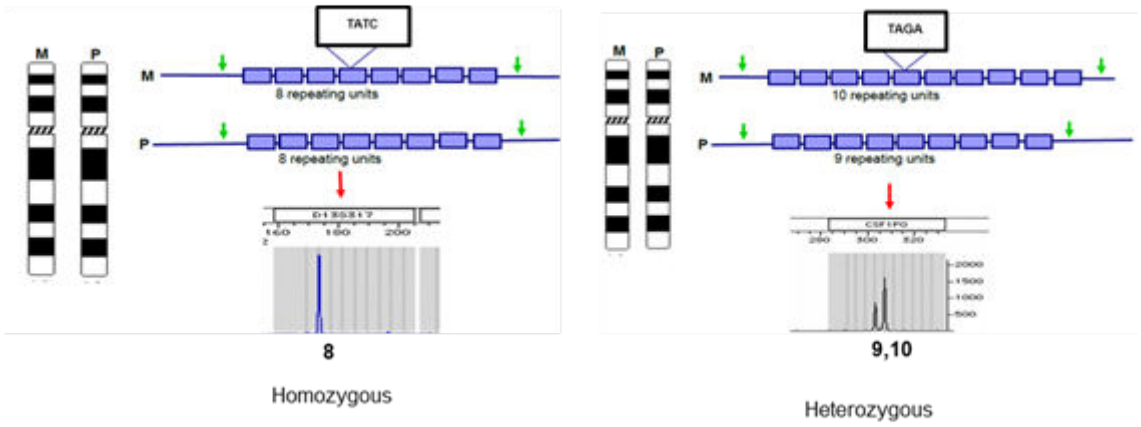


Figure 1: Schematic Diagram of STR Profiling Polymorphism

analysis to establish the baseline STR profile. The baseline STR profile from the original donor material is then used to compare against all subsequent STR DNA profiles performed on the various cell banks. The remaining cells are expanded to create a Seed stock from which a Distribution stock is prepared. Representative vials from both the Seed and Distribution stocks are subjected to another round of STR DNA profile analysis in addition to other quality control procedures. The STR DNA profiles for both Seed and Distribution stocks are compared to the baseline profile of the original donor material to determine if the results from these quality control tests meet the acceptance criteria (≥ 80% match between the cell line and its original tissue or its derivatives), consistent STR profile, for a given line.

STR DNA profile analysis involves the simultaneous amplification of 17 STR markers plus amelogenin for gender determination and is capable of discriminating among human cell lines at about 1×10^{-18}. The amplicons from the PCR are resolved by capillary electrophoresis and sized using internal size standards (ISS). The sized fragments are then converted into alleles with comparison to the allelic ladders and the assigned alleles are converted to numeric values which are used to create a baseline profile. The baseline DNA profiles are used to create a reference database. All subsequent STR DNA profile analyses performed on the various cell banks are compared to the baseline profile of that cell line in the reference database. The STR DNA profile should also be compared to profiles of other cell lines in the reference database (Figure 2).

ASSAY DEVELOPMENT

The preparation of cell suspension obtained from adherent or suspension cultures can be used as a source of DNA. The cell suspension is either spotted directly onto FTA® paper or may be used for liquid DNA extraction. Both forms of DNA are suitable for STR DNA Profile analysis.

Harvest of Adherent Culture

1. Remove and discard culture medium
2. Rinse cell surface by adding PBS (without calcium and magnesium) to the side of the flask (away from the cells) and rinse cells with a gentle rocking motion.
3. Remove and discard PBS.
4. Add dissociation solution, enough to cover the cells. Incubate at 37°C and rock gently until all cells detach.
5. Add medium and gently resuspend cells.
6. Centifuge at 200 × g and for 10 minutes to pellet cells.
7. Discard supernatant and wash cell pellet twice in PBS.

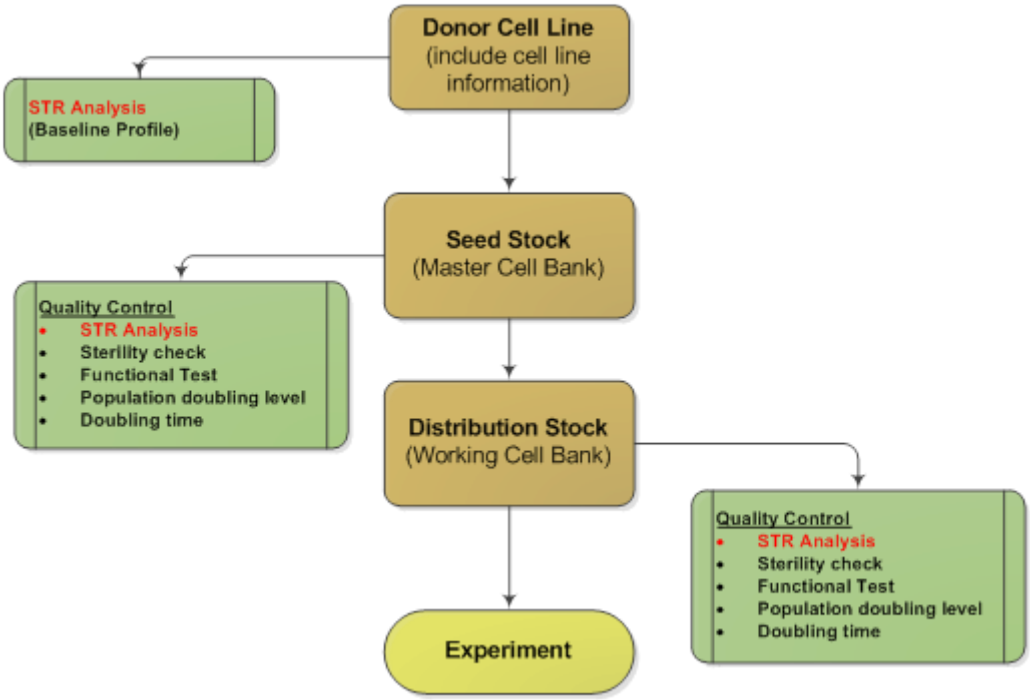


Figure 2: Accessioning Scheme for New Cell Line

8. Resuspend cell pellet in a small volume of PBS and determine cell count and viability.
9. Use cell suspension for spotting onto FTA® paper or for the isolation of DNA.

Harvest of Suspension Culture

1. Transfer cell suspension to a centrifuge tube.
2. Centrifuge at 200 × g for 10 minutes to pellet cells.
3. Discard supernatant and wash cell pellet twice in PBS.
4. Resuspend cell pellet in a small volume of PBS and determine cell count and viability.
5. Use cell suspension for spotting onto FTA® paper or for the isolation of DNA.

Note: Isolate or prepare FTA® paper from cells harvested in log-phase of growth cycle; cells are in the exponential phase of growth and are the most viable. Use best practices in tissue culture to prevent cross-contamination of cells.

PRODUCT COMPONENTS AND STORAGE CONDITIONS FOR STR ANALYSIS

PowerPlex® 18D System (Cat. No DC1802, Promega Corporation)

The PowerPlex® 18D System allows co-amplification and four-color fluorescent detection of eighteen loci, D3S1358, TH01, D21S11, D18S51, Penta E, D5S818, D13S317, D7S820, D16S539, CSF1PO, Penta D, Amelogenin (gender determination), vWA, D8S1179, TPOX, FGA, D19S433 and D2S1338. The system is optimized for analysis of common database samples, such as unwashed FTA® card punches (i.e., direct amplification).

The PowerPlex® 18D System is compatible with the ABI PRISM® 3100 and 3100-Avant and Applied Biosystems 3130, 3130xl, 3500 and 3500xL Genetic Analyzers. Refer to manufacturer manual for detailed protocol.

Tables 1A and 1B provide a listing of the pre- and post-amplification components and long-term storage instruction for the PowerPlex® 18D system.

Table 1A: PowerPlex® 18D System pre-amplification components and storage

Pre-amplification components*		Long-term storage temperature
PowerPlex® D 5X master mix	1 mL	Store at -30 °C to -10 °C
PowerPlex® 18D 5X primer pair mix	1 mL	Store at -30 °C to -10 °C; light sensitive, store in dark
2800M control DNA, 10 ng/µL	25 µL	2 °C to 10 °C
Water, amplification grade	5 x 1,250 µL	Room temperature (18 °C to 22 °C)

* Recommend that the pre-amplification and post-amplification reagents are stored at different locations; use different pipette, tips and racks.

Table 1B: PowerPlex® 18D System post-amplification components and storage

Post-amplification components*		Long-term storage temperature
PowerPlex® 18D allelic ladder mix	100 µL	Store at -30 °C to -10 °C; light sensitive, store in dark
CC5 internal lane standard 500	2 x 300 µL	Store at -30 °C to -10 °C; light sensitive, store in dark
Note: Matrix standard required for initial setup of the color separation matrix (not a component of kit above)		
PowerPlex® 5-Dye Matrix standard 3100/3130		Suitable for genetic analyzers: ABI PRISM® 3100, 3100-Avant Genetic Analyzers, Applied Biosystems 3130, 3130xl, 3500, 3500xL

* **Recommend that the pre-amplification and post-amplification reagents are stored at different locations; use different pipette, tips and racks.**
Available separately the proper panel and bins text files for the use with GeneMapper ID software is available for download at: www.promega.com/geneticidtools/panels_bins/

Materials

- GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems) or equivalent
- MicroAmp® optical 96-well reaction plate (Applied Biosystems) or equivalent
- Microcentrifuge
- Aerosol-resistant pipet tips
- 1.2 mm Harris Micro-Punch or equivalent
- Cutting mat

SPOTTING OF CELLS ONTO FTA® CARD

The prodcedure is a modification of the protocol available at http://www.promega.com/resources/articles/profiles-in-dna/direct-amplification-of-saliva-and-blood-samples-using-powerplex-esx-16/ (13).

1. Spot about 200,000 cells, suspended in about 20 µL of PBS onto FTA® card, or equivalent and allow to dry. **Note**: for long-term storage, cards may be stored in a cool, dry environment for further use.
2. Manually punch a 1.2 mm disk in the center of the sample spot.
3. Use plunger to eject the disk into the appropriate well of a reaction plate.

AMPLIFICATION SETUP

1. Completely thaw the PowerPlex® D 5X master Mix and PowerPlex® 18D 5X Primer Pair mix.
2. Determine the number of reactions needed. Note: Include negative and positive controls and add an additional 2 reactions to compensate for pipetting errors.
3. For reaction assembly, label a clean 0.2 mL MicroAmp® plate.
4. Add reagents in the order listed in Table 2 to each sterile tube.
5. Vortex the PCR amplification mix for about 10 seconds.
6. Add 25 µL PCR amplification mix to each of the reaction tubes containing FTA® disks.
7. For the positive amplification control:
   a. Vortex the tube of 2800M Control DNA
   b. Dilute 2800 M Control DNA to a concentration of 5 ng/µL.
   c. Pipet 1 µL of diluted Control DNA into reaction well containing 25 µL of PCR amplification mix.
   d. For the negative amplification control use either a reaction well containing 25 µL amplification mix without DNA or a reaction well containing 25 µL amplification mix with FTA® disk without DNA.
8. Seal the plate and briefly centrifuge the plate to bring the disks to the bottom of the wells.

Note: Amount of cells spotted onto the FTA® card and the amount of Control DNA used to obtain positive results must be predetermined.

Table 2: Amplification Setup PCR Reaction Volumes

PCR Amplification Mix Components	Volume per reaction	×	Number of reactions	×	Final volume
Water, amplification grade	15 µL	×		×	
PowerPlex® D 5X Master Mix	5 µL	×		×	
PowerPlex® 18D 5X Primer Pair mix	5 µL	×		×	
Total Reaction Volume	**25 µL**	×		×	

THERMAL CYCLING

1. Place the MicroAmp® plate in the thermal cycler.
2. Select and run the recommended protocol provided in Table 3.
3. Store amplified Samples at 4°C after completion of the thermal cycling protocol.

Note: Optimize protocol pertaining to cycle number and injection conditions based on instrumentation used.

Table 3: Thermal Cycling Protocol

Steps	Temperature	Duration	Cycles
1	96 °C	2 minutes	
2	94 °C	10 seconds	27 cycles
3	60 °C	1 minute	
4	60 °C	20 minutes	
4	60 °C	soak	

DETECTION OF AMPLIFIED FRAGMENTS USING THE APPLIED BIOSYSTEMS 3500 OR 3500XL GENETIC ANALYZER

The PowerPlex® 18D primers are covalently linked to fluorescent molecules. Multiple sets of primers with different "color" fluorescent labels are used to analyze numerous different loci in a single PCR reaction. Following the PCR reaction, internal size standards are added to the reaction mixture and the DNAs are separated by size via capillary gel electrophoresis. Once the data are collected, the GeneMapper ID-X software is used to size the amplicons based on the internal size standard. STR genotyping of each cell line is performed by converting amplicons size to alleles by comparing to allelic ladders (14). See Table 4 for a listing of the reagents that can be used in this assay.

Table 4: Detection of Amplified Fragment

Material	Manufacturer	Catalog Number
Dry heating block	N/A	N/A
Water bath	N/A	N/A
Ice-water bath or crushed iced	N/A	N/A
Centrifuge compatible with 96-well plates	N/A	N/A
Aerosol-resistant pipet tips	N/A	N/A
36 cm 3500/3500*xL* capillary array	Life Technologies	4404687
96-well retainer and base set (standard)	Applied Biosystems	4410228
POP-4™ polymer in a pouch for the Applied Biosystems 3500 or 3500*xL* genetic Analyzer	Life Technologies	4393710
Anode buffer container	Life Technologies	4393927
Cathode buffer container	Life Technologies	4408256

Table 4 continued from previous page.

Material	Manufacturer	Catalog Number
Conditioning reagent pouch for the Applied Biosystems 3500 or 3500xL genetic Analyzer	Life Technologies	4393718
Hi-Di formamide	Applied Biosystems	4311320
PowerPlex® 5-Dye Matrix Standards, 3100/3130	Promega Corporation	DG4700

SAMPLE PREPARATION

1. Preparation of loading cocktail:
   Combine and mix CC5 Internal Lane Standard 500 and Hi-Di™ formamide as follows:

   (1 µL CC5 ILS 500) × (number of injections) + (10 µL Hi-Di™ formamide) × (number of injections) = amount of loading cocktail

   Note: the amount of internal lane standard may be adjusted based on the intensity of the size standard peak.
2. Vortex loading cocktail (formamide/lane standard mix)
3. Pipet 11 µL of loading cocktail into each well.
4. Add 1 µL of amplified sample or 1µl PowerPlex® 18D Allelic Ladder Mix.
5. Cover wells with appropriate septa.
6. Centrifuge plate briefly to remove bubbles from the wells.
7. Denature samples at 95°C for 3 minutes just prior to loading instrument.
8. Immediately chill plate on crushed ice or an ice-water bath for 3 minutes.

INSTRUMENT PREPARATION AND USE

Detect amplified fragments using the Applied Biosystems 3500 or 3500 xL Genetic Analyzer. Refer to the User Guide for use and care of the instrument and Promega for a detailed protocol for data analysis.

DATA ANALYSIS

The software for data analysis is PowerPlex® 18D Panel, Bins, Stutter Text Files and GeneMapper ID-X Software, version 1.2. To facilitate the analysis of data generated with the PowerPlex 18D System, go to www.promega.com/geneticidtools/panels_bins/ for panel and bins text files for automatic assignment of genotypes using the GeneMapper ID-X software.

DATA INTERPRETATION

A cell line is considered authentic when the STR profile shows a ≥80% match between the cell line and its original tissue or its derivatives. A STR profile match of ≤56% is considered unrelated. However, there are some cell lines that have a match profile between 56 and 80% match. In these cases, additional studies should be performed to confirm identity (15).

A minimum of eight core STR loci is needed to uniquely identify a human cell line. The eight loci are D5S818, D13S317, D7S820, D16S539, vWA, Th01, TPOX, CSF1PO. A unique cell line has a STR profile that is different

from another unique cell line (Figure 3). Cell lines derived from various tissues from the same individual have the same STR profile (Figure 4).

Data interpretations of STR profiles on human cell lines, especially those derived from tumor tissue, present certain nuances such as:

- Loss of heterozygosity (LOH), also known as allele drop-out (ADO)
- Peak imbalance
- Multiple peaks at several loci

Loss of Heterozygosity

The incidence of genetic instability in STR markers used to evaluate human cancers is not uncommon (16, 17). Most human cell lines are derived from a cancer, which differ genetically from normal tissue. Moreover, cell lines are capable of acquiring additional genetic changes while in culture. In a study of 24 lung samples, there were complete deletions of alleles at multiple loci when compared to normal tissue (18). Great care should be taken when evaluating biopsy tissue (which may contain normal tissue) for baseline STR profile to be compared to STR profile of cell line derived from tumor of that same tissue.

Peak Imbalance

Peak imbalance is also a feature of cancer cells. The favored amplification of one allele over the over another may be due to gene duplication, aneuploidy or chimeric cell population (18). This characteristic brings a unique feature to cell line identification, see Figure 5.

Multiple Peaks at Many Loci

Another relatively common occurrence of STR typing of human cancer cell lines is multiple peaks at several loci. Three or more peaks at one or two loci may be due to somatic mutation, trisomy or gene duplications. Events with more than three peaks at more than three loci may be due to cellular contamination. In either event, an independent method should be used to eliminate cellular cross-contamination, see Figure 6 as an example for cellular cross contamination.

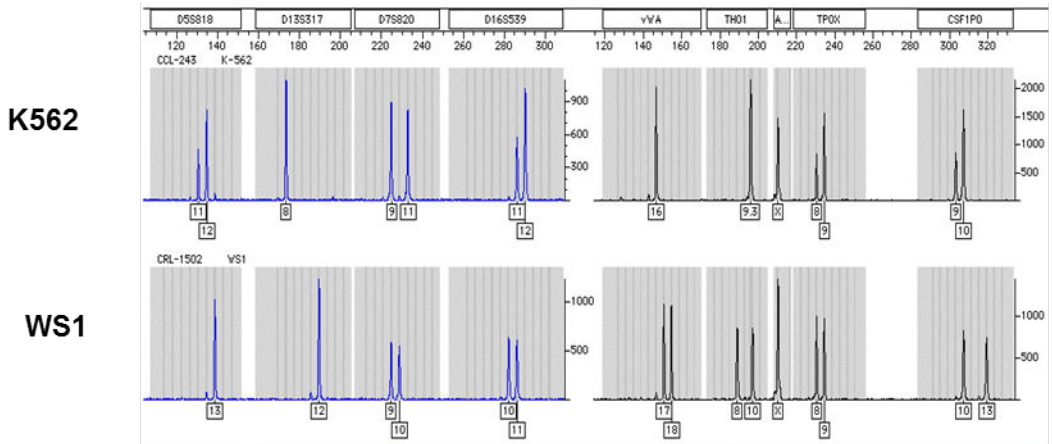
CRITERIA FOR DETERMINING QUALITY STR PROFILE ANALYSIS FOR RELIABLE AND INTERPRETABLE RESULTS

Validation of Procedure

Method validation is an important element of quality control and provides the assurance that data will be reliable. Method validation is the process of demonstrating that a laboratory procedure is robust and reliable and reproducible in the hands of the laboratory personnel performing the test. The factors for method validation include: precision, accuracy, limit detection, specificity, linearity, range, robustness and system suitability. For more information on method validation of STR Systems, refer to http://www.cstl.nist.gov/strbase/validation.htm and Promega's Validation of STR Systems Reference Manual at http://www.promega.com/resources/articles/pubhub/applications-notes/ge053-validation-of-str-systems-reference-manual/.

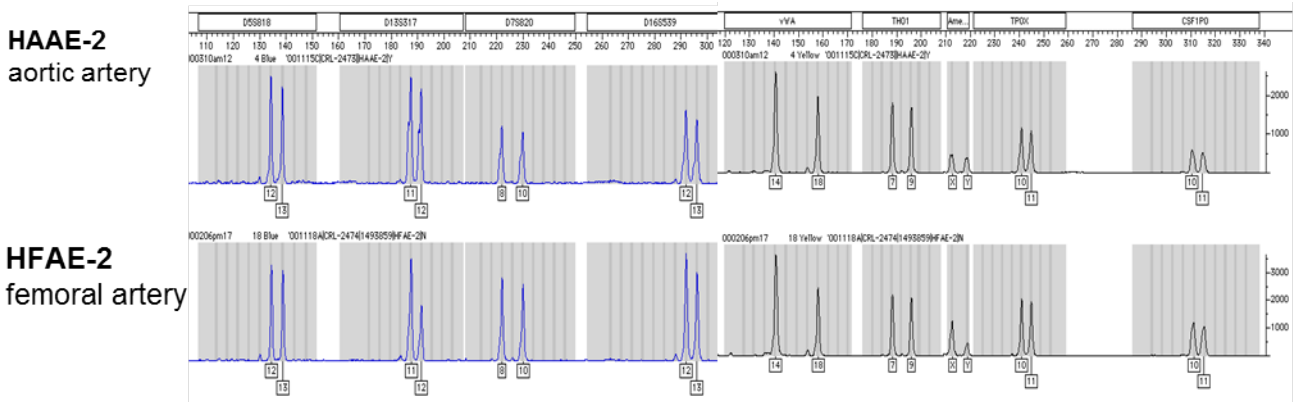
Setting of Analytical Thresholds and Peak Height Ratios

Each laboratory should determine the analytical threshold or the level at which detected signals are above baseline noise. Data falling below this threshold may not be suitable for allele calls; however, data at or above the threshold is of sufficient quality to determine an allele call.



	D5S818	D13S317	D7S820	D16S539	vWA	THO1	Amel.	TPOX	CSF1PO
K562	11, 12	8	9, 11	11, 12	16	9.3	X	8, 9	9, 10
WS1	13	12	9, 10	10, 11	17, 18	8, 10	X	8, 9	10, 13

Figure 3: Electropherogram of two unrelated human cell lines, K562 (chronic myelogenous leukemia) and WS1 (skin fibroblast) obtained from two individuals. STR profile is different between the two cell lines. STR analysis performed with PowerPlex® 1.2. Allele numerical values are used to create database.



	D5S818	D13S317	D7S820	D16S539	vWA	THO1	Amel.	TPOX	CSF1PO
HAAE-2	12,13	11,12	8,10	12,13	14,18	7,9	X,Y	10,11	10,11
HFAE-2	12,13	11,12	8,10	12,13	14,18	7,9	X,Y	10,11	10,11

Figure 4: Electropheragram of two related human cell lines, HAAE-2 (human aortic artery) and HFAE-2 (femoral artery) obtained from the same individuals. STR profiles are identical between the two cell lines. STR analysis performed with PowerPlex® 1.2. Allele numerical values are used to create database.

Setting the appropriate peak height or peak threshold

The genetic material for some tumor cell lines is very complex and this could lead to difficulty in distinguishing true low-level peaks from technical artifacts, including noise. There are no set rules for establishing threshold values; consequently, each laboratory must establish peak-height thresholds for "calling" alleles as part of its validation process. Only peak-height, expressed in relative fluorescent units (RU) that exceed the accepted value will be accepted.

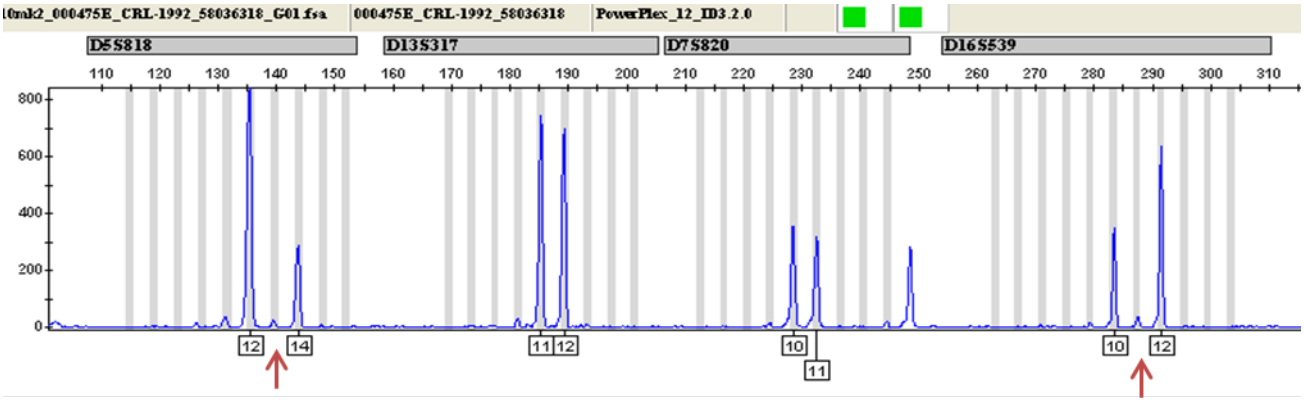


Figure 5: Peak imbalance (arrow) in STR profile of a tumor cell line. Most cell lines are aneuploidy with multiple copies of a chromosome and the total chromosome numbers exceeding 46.

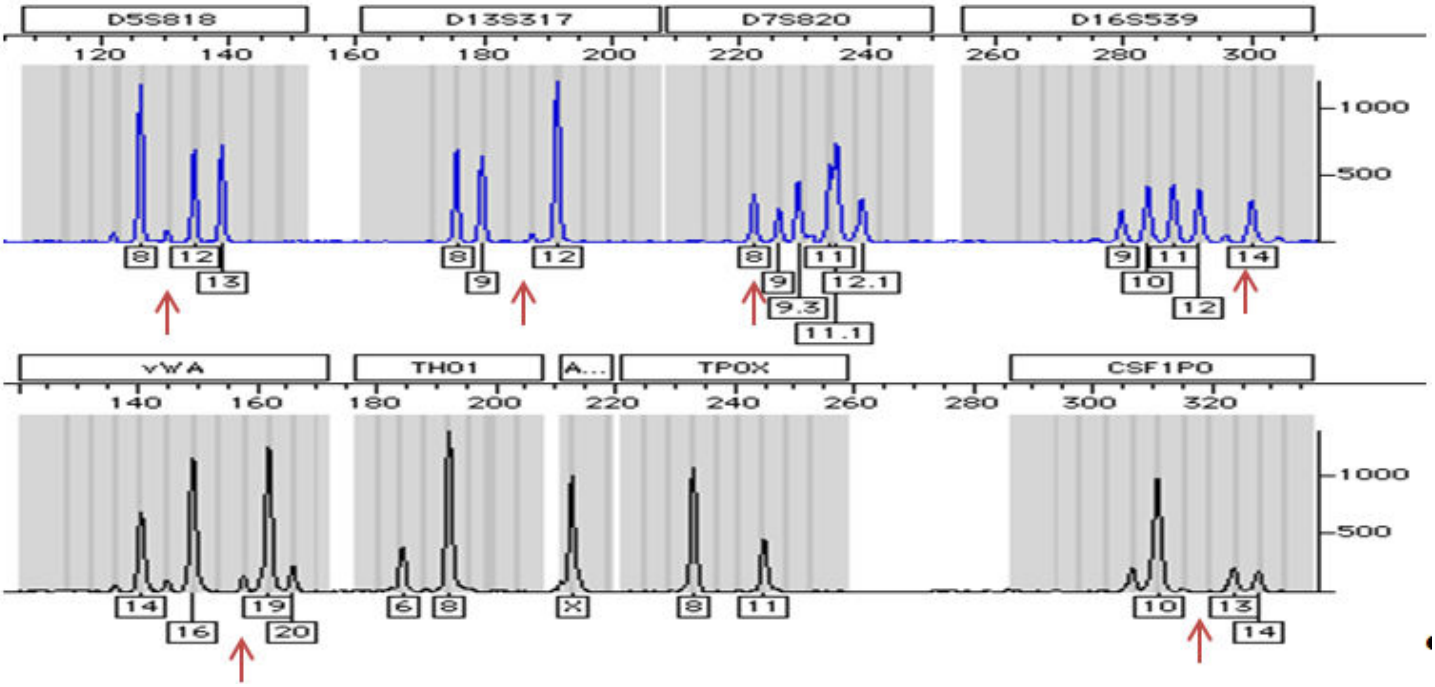


Figure 6: Electropherogram of cellular cross-contamination; multiple peaks at D5S818, D13S317, D7S820, D16S539, vWA, CSF1PO loci. STR typing of human cell lines with PowerPlex® 1.2.

The threshold may be determined experimentally on the basis of observed signal-to-noise ratios or be an arbitrarily set value established by manufacturers (150 RFU) or published data. The lower threshold is a measure of the procedure sensitivity. The upper threshold is essential when reviewing data from samples with high quantity DNA. These samples have high RFU values that can oversaturate the instrument's ability to detect the sample This can lead to difficulty in interpretation, especially when working with early stages of cellular contaminated samples.

Use of appropriate controls (positive and negative):

Including controls during the STR profile analysis is very important as it allows the technician to identify and troubleshoot possible problems and ensure that the data is accurate and reliable.

During the amplification process, positive and negative controls are used. A positive control is a DNA sample with a known STR profile that is added to the sample set. The positive control confirms that the analysis processes are working accurately. (Positive controls are provided in the manufacturer's STR kits). A negative control which could be a reagent blank that is substituted for DNA is included and treated exactly the same manner as other samples. This allows the technician to determining if the reagents and/or techniques used may have introduced contamination.

Ability to evaluate internal lane size standards (ISS) and allelic ladders.

All commercial STR kits include allelic ladders and internal size standards (ISS). Each sample during a run is assessed at completion for the correct calling of the ISS peaks. In general, the peaks from an ISS are uniform in size or intensity. Lack of uniformity or miscalled peaks can indicate problems with the sample, injection, and/or run.

The ISS is valuable in determining the accuracy of a capillary electrophoresis run. Temperature variations during electrophoresis can cause the in-run precision to exceed 1 base pair and evaluation of the ISS can assist analysts in identifying this issue.

Allelic ladders should be assessed to ensure that all peaks have been called correctly. Ladder peaks that have not been called or have been miscalled can indicate a problem with the ladder sample, injection, and/or run.

Ability to evaluate and detect artifacts such as:

There are several artifacts during STR profile analysis that can lead to misinterpretation of the data. Identification and resolution of these artifacts are explained in great detail in the ANSI/ATCC ASN-0002-2011 (19).

- Stutter peaks
- Dye blobs
- Dye pull-ups
- Off-ladder alleles; microvariants

Stutter peaks (products)

Stutter products are a common amplification artifact associated with STR analysis. Stutter products are often observed as one repeat unit below the true allele peak and, occasionally, two repeat units smaller or one repeat unit larger than the true allele peak. Frequently, alleles with a greater number of repeat units will exhibit a higher percent stutter. The pattern and intensity of stutter may differ slightly between primer sets for the same loci (see Figure 7 and Promega's manual on troubleshooting PowerPlex® 18D System).

Dye Blobs

Dye blobs, are fairly common in STR analysis. Evidence suggests that the fluorescent dye tags attached to the primers begin to break down over time. Disassociated primer dyes can show up in the sample analysis range and can mask true data. Dye blobs are usually wider than real peaks and are typically only seen in one color. Follow the manufacturer's specifications for storage of amplification kits to avoid problems associated with free dyes. If problems persist, clean-up or re-amplify sample. See example in Figure 8 below of dye blob.

Dye Pull-up or Bleed-through

Dye pull-up, sometimes referred to as bleed-through, represents a failure of the analysis software to discriminate between the different dye colors used during the generation of the data. Oversaturated data can also cause the dyes to "bleed" over or pull-up into another color. If pull-up occurs, inject less of the sample or re-amplify the sample with less DNA. Reoccurring pull-up (due to too much DNA) may indicate that the quantitation method

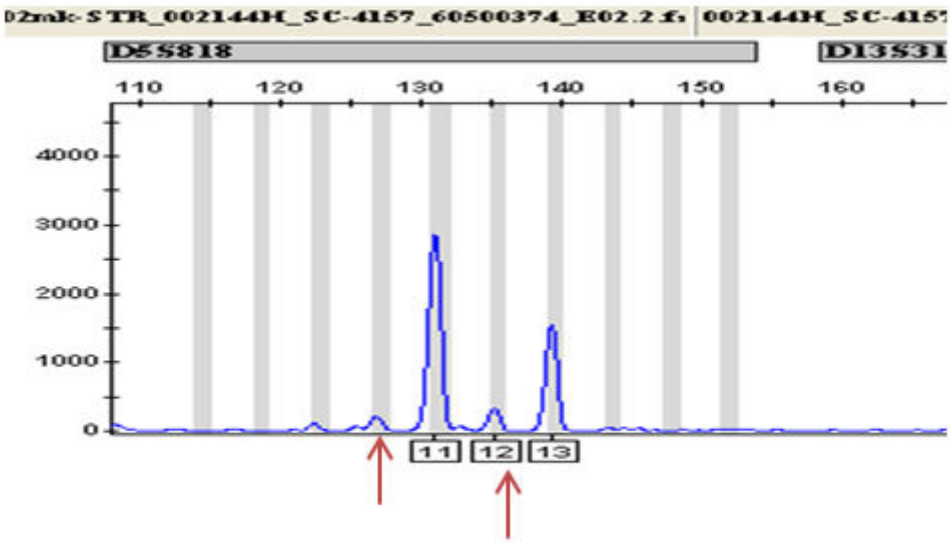


Figure 7: Stutter peaks.

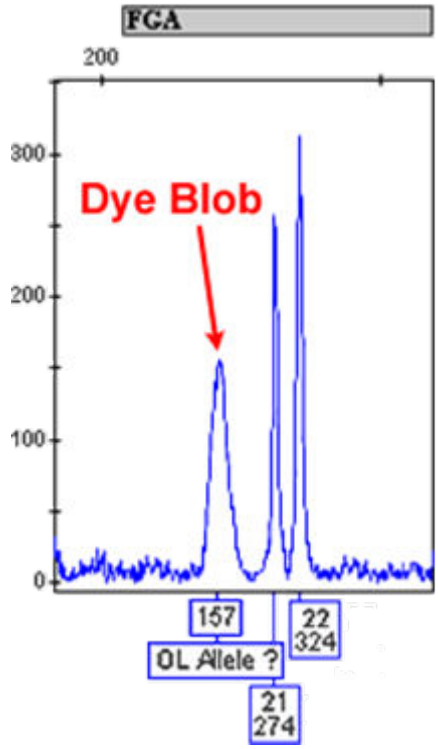


Figure 8: Dye blob

or the amount of DNA used for amplification should be reevaluated. If this problem is not due to too much DNA, it may be necessary to run a new matrix and apply it to the sample.

Off-ladder alleles or microvariants

Allelic ladders represent the most common alleles at each locus and were established through the evaluation of data from several hundred individuals. Alleles within the STR loci are known to differ significantly between

individuals and the manufacturers' kit ladders do not represent all possible types of known alleles. Alleles that size outside allele categories represented in the ladder are known as off ladder (OL) alleles.

The sizing software is designed to assign allele size within one of the allele categories defined by the ladder. The software designates an allele that falls outside of these allele categories as off ladder alleles.

It is not unusual for STR profiling of cells from tumor tissue to see microvariants. A microvariant represents an incomplete repeat for a given allele. For instance, at D18S51 a 15 allele designation means that there are 15 AGAA's along the fragment. However, a 14.2 allele designation means that there are 14 AGAA repeats along the fragment plus an additional 2 bases: AG. Microvariants are reported as the number of complete repeat units and are designated as an integer (e.g. 14). Any partial repeat is designated as a decimal, followed by the number of bases in the partial repeat, see example in Figure 9.

Many microvariants are represented in allelic ladders or have virtual allele categories within the software program; those that do not have established categories are designated as off ladder alleles.

While off ladder alleles have been well documented with forensic STR testing, some may not have been previously characterized. The National Institute of Standards and Technology (NIST) website (http://www.cstl.nist.gov/div831/strbase/) has a listing of off ladder alleles and can be used as a reference in these instances. If it is determined that an allele has not been characterized, it may be advisable to rerun the sample to confirm the type.

Characteristics of STR

STRs selected for cell line authentication are chosen because they display the highest possible variation for discriminating among human cell lines and can detect cross-contaminating cells. The criteria for selecting STR loci for human cell line authentication include the following characteristics.

- High discriminating power
- High observed heterozygosity >70%
- Robust and reproducible results
- Low stutter characteristics
- Low mutation rate
- Alleles fall in the range of 90-500 bp – smaller fragments better to allow for degraded DNA

Advantages of STR analysis

STR analysis is a universally accepted method for human cell line authentication. This method is robust in its ability to identify unique human cell lines; easy to perform; accessible to scientists and affordable. Some of the advantages of STR analysis are listed below.

- Target sequence consists of microsatellite DNA
- Typically use 1-2 ng DNA
- 1 to 2 fragments; discrete alleles allow digital record of data
- Highly variable within populations; highly informative
- Banding pattern is reproducible
- PCR amplifiable, high throughput
- Small size range allows multiplexing
- Allelic ladders simplify interpretation
- Small product size compatible with degraded DNA
- Rapid processing is attainable

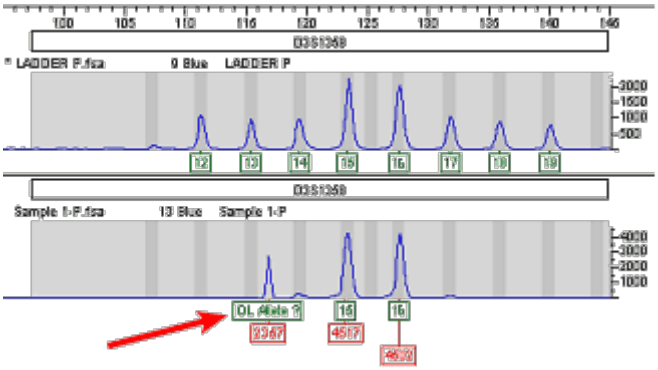


Figure 9: Off-ladder allele or microvariant.

SERVICES FOR STR TYPING OF CELL LINES

Over the past few years, several institutions are offering service for STR typing of human cell lines. When choosing a testing laboratory, considerations should be made based on experience of testing laboratory personnel to propagate human cell lines and to perform and interpret the data from STR analysis. The following are some institutions who are currently offering STR typing services.

- Cell Banks
- Paternity testing labs
- Universities
- Core labs

TROUBLESHOOTING

Reducing Cellular Misidentification of Cell Lines

The following list provides some suggestions to assist in reducing cellular misidentification of cell lines.

- Good documentation
- Highly trained technicians
- Good aseptic techniques
- Use one reservoir of medium per cell line
- Aliquot stock solutions/reagents
- Label flasks (name of cell line, passage number, date of transfer (use barcoded flasks when available)
- Work with one cell line at a time in biological safety cabinet
- Clean biological safety cabinet between each cell line
- Allow a minimum of 5 minutes between each cell line
- Quarantine “dirty” cell line from “clean” cell line
- Manageable work load (reduce accidents)
- Clean laboratory (reduce bioburden)
- Legible handwriting (printed labels)
- Monitor for cell line identity and characteristics contamination, routinely
- Use seed stock (create master stocks)
- Create “good” working environment
- Review and approve laboratory notebook

Preventing contamination during PCR

Preventing contamination during PCR is of critical importance to ensure that you are getting useful results. The following list provides some suggestions of how to reduce and/or prevent contamination during PCR.

- Separate pre-amplification space (low copy) from post-amplification space (high copy)
- Use separate lab coat, gloves, tubes, pipette tip in pre-amplification room from post-amplification room
- Use aerosol-resistant pipette tips
- Keep pre-amplification and post-amplification reagents in separate rooms
- Prepare amplification reactions in a room dedicated for reaction setup
- Use a separate aliquot of DEPC water stock for each round of PCR
- Prepare your PCR mix in a hood with laminar flow. Decontaminate it with bleach, alcohol, RNAse, DNase, etc...
- Use a different pipette tip when pipetting all your reagents, even the same master mix to each tube
- Keep your tubes closed during the procedure, even your master mix tube.
- Be sure that your tubes are closed when discarding the pipette tip!!! Aerosols are dangerous!!!
- Open the tubes only when necessary

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ADDITIONAL REFERENCES

ICLAC List of misidentified cell lines: http://standards.atcc.org/kwspub/home/the_international_cell_line_authentication_committee-iclac_/Database_of_Cross_Contaminated_or_Misidentified_Cell_Lines.pdf

Commercially available STR Multiplex Kits: http://www.cstl.nist.gov/strbase/multiplx.htm

GLOSSARY OF TERMS

Please refer to the “Genetics Terms” section of the “Glossary of Quantitative Biology Terms” for a list of terms and definitions used in this chapter.