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UNITED STATES DISTRICT COURT

CENTRAL DISTRICT OF CALIFORNIA

CHROMACODE, INC. and CALIFORNIA INSTITUTE OF TECHNOLOGY,

Plaintiffs,

v.

BIO-RAD LABORATORIES, INC.,

Defendant.

CASE NO. 2:23-cv-08417-RGK-PD

AMENDED COMPLAINT FOR PATENT INFRINGEMENT OF U.S. PATENT NOS. 10,068,051 AND 10,770,170

DEMAND FOR JURY TRIAL

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And California Institute of Technology

Plaintiffs ChromaCode, Inc. ("ChromaCode") and California Institute of Technology ("Caltech") (collectively, "Plaintiffs"), by and through their counsel, for their Amended Complaint against Defendant Bio-Rad Laboratories, Inc. ("Bio-Rad" or "Defendant"), allege as follows:

NATURE OF THE ACTION

1. This is an action for infringement of U.S. Patent Nos. 10,068,051 (the "'051 Patent") and 10,770,170 (the "'170 Patent").

2. Traditional methods of detecting the presence or absence of multiple targets (called "multiplexing") in a biological sample require tagging every target with a different color—*i.e.*, target A is tagged with a "blue" signal, target B is tagged with a "red" signal, and so on. Although there may be an infinite number of unique colors in a rainbow, there are ***not*** an infinite number of colors that can be unambiguously ***detected*** by modern equipment. Accordingly, traditional assays were limited to only a few acceptable colors, which meant that only a small number of targets could be detected in a single test. ChromaCode's co-founder, Dr. Aditya Rajagopal, together with his colleagues at Caltech, figured out how to break the paradigm and unambiguously identify multiple targets using the same color tag. Plaintiffs bring this action to stop Bio-Rad's infringement of this valuable and groundbreaking intellectual property. Bio-Rad markets and uses ChromaCode's exclusively licensed and patented multiplexing technologies in their digital Polymerase Chain Reaction ("PCR") products without authorization and in violation of ChromaCode's exclusive rights.

3. As a result of Bio-Rad's infringement, and given the threat of its growing infringement, Plaintiffs will be irreparably harmed if such infringement is not halted.

THE PARTIES

4. Plaintiff ChromaCode is a Delaware corporation with its headquarters in Carlsbad, California. ChromaCode is a molecular diagnostics startup company

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with a diagnostic and bioinformatics focus.

5. Plaintiff Caltech is a world-renowned science and engineering institute that marshals some of the world's brightest minds and most innovative tools to address fundamental scientific questions and pressing societal challenges. Caltech is a non-profit private university located in Pasadena, California in this District.

6. Defendant Bio-Rad is a Delaware corporation headquartered in Hercules, California. Bio-Rad is a developer and manufacturer of specialized technological products for the life science research and clinical diagnostics markets. Bio-Rad's Quality Systems Development Division is based in Irvine, California.

JURISDICTION AND VENUE

7. This Court has subject matter jurisdiction over this patent infringement action pursuant to 28 U.S.C. §§ 1331 and 1338(a).

8. This Court has personal jurisdiction over Bio-Rad because of Bio-Rad's purposeful, systematic, and continuous contacts with California, and particularly the Central District of California. Bio-Rad maintains regular and established places of business in this District at least at the following addresses: 9500 Jeronimo Road, Irvine, California 92618; 21 Technology Drive, Irvine, California 92618; and 9 Holland Drive, Irvine, California 92618. Bio-Rad's headquarters are located in Hercules, California. Bio-Rad is therefore physically located in the State of California. Bio-Rad conducts design, development, manufacturing, and distribution of software/informatics products and *in vitro* diagnostic medical devices for clinical laboratory quality control programs in Irvine, California (*i.e.*, in this District). Bio-Rad also sells products, including the Infringing Products (defined below), in Irvine, California, and elsewhere in the Central District of California.

9. Venue is proper in the Central District pursuant to 28 U.S.C. § 1400.

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The facts establishing this are included throughout this Amended Complaint. Bio-Rad maintains regular and established places of business in this District, as identified above, and offers for sale and sells its infringing products in this District.

BACKGROUND

10. The patented technology at issue in this case concerns the ability to detect, unambiguously, multiple targets in a single sample without having to label every target with a different color. This novel approach offers a fast and inexpensive alternative to researchers and clinicians who need unambiguous identification of multiple targets in a single sample.

A. Traditional Methods of Identifying Multiple Targets in One Sample

11. For years, researchers invested substantial resources and time to overcome the single-target limitations of then-existing target identification systems. In an early improvement, researchers were able to modestly increase the number of targets unambiguously identified in a single sample by expanding the variety of colors used to tag targets. Probes designed to bind to particular targets were labeled with fluorophores which, when excited, emit light at different wavelengths (each of which correspond to a color range), and sensors tuned to detect those different wavelengths could then identify the presence or absence of each respective target. The probes were designed such that they would only emit light when a particular target was amplified. In other words, if a blue light probe designed to target A emits blue light, that means A is present in the sample. If no blue light is emitted, that means A is not present in the sample. If, in the same sample, a red light probe designed to target B emits red light, that means B is present in the sample. If no red light is emitted, that means B is not present in the sample. And so on. For every target, a different color could indicate its presence or absence.

12. Theoretically, with a one-target-one-color approach, one could

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unambiguously identify a nearly infinite number of targets in a single sample. For example, consider a target identification approach as indicated in the table below:

Target	Assigned Shades of Blue
A	
B	
C	

13. This table includes just three shades of blue for each target. The average human eye can detect up to around 10 million shades of different colors.[1] Unfortunately, fluorescent probes cannot be relied upon to emit light in any one particular shade with anywhere near this level of precision. Each fluorophore has a wide emission spectra, meaning they emit light in various shades of each color. Accordingly, in our example, rather than unambiguously identifying A from B from C, each shade of blue could correspond to each target, as shown below.

Target	Assigned Shades of Blue
A or B or C	
A or B or C	
A or B or C	

14. For this reason, to avoid what is called spectral overlap (bleeding colors) and preserve spectral resolution (the ability to detect different colors), only a relatively small number of colors (*e.g.*, 4 to 6) are typically used simultaneously in multiplexed fluorescent assays.

15. To overcome this limitation, researchers combined additional labor-intensive processing steps along with fluorescence to identify more targets from a single sample, including aliquoting, spatial arraying, and sequential processing.

[1] *See* https://www.aao.org/eye-health/tips-prevention/how-humans-see-in-color.

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These additional processing steps are labor-intensive and frequently require relatively expensive and complex optical and mechanical systems. Although these tools offered some ability to increase the number of targets detected in a sample, there remained a significant need for inexpensive multiplexed encoding and decoding methods that could identify unambiguously a significantly higher number of targets.

B. ChromaCode Broke the One-Target-One-Color Barrier

16. Researchers continued to investigate a solution to the one-target-one-color approach. Then, in 2011, a team of researchers from Caltech, comprised of Drs. Emil Kartalov, Aditya Rajagopal, and Axel Scherer, conceived of a solution that enabled high definition multiplexing—specifically, the unambiguous identification of multiple targets for each color.

17. Dr. Emil Kartalov earned his B.S. in Physics (1998), M.S. in Applied Physics (2004), and Ph.D. in Applied Physics (2004), all from Caltech. His doctoral thesis focuses on single-molecule fluorescence microscopy, single-molecule DNA sequencing, and microfluidic DNA sequencing by synthesis. The first two developed techniques resulted in the founding of Helicos Biosciences in Boston, MA. For his postdoctoral work, Dr. Kartalov moved to the Biochemistry Dept and later the Pathology Department at the Keck School of Medicine at the University of Southern California ("USC"), where the focus shifted to fundamental microfluidics and biomedical applications of microfluidic devices. Dr. Kartalov invented microfluidic vias and resultant autoregulatory devices, and built multi-analyte microfluidic immunoassay chip for protein diagnostics in fingerprick amounts of human serum and plasma. In 2006, Dr. Kartalov won the NIH K99/R00 Career Award and became tenure-track faculty in Pathology at USC in 2008. His group focuses on microfluidic point-of-care diagnostics and techniques for high-throughput analysis of pathology tissue slices. Dr. Kartalov has over 33

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peer-reviewed publications and 32 issued patents.[2]

18. Dr. Aditya Rajagopal earned his B.S. (2008), M.S. (2010), and Ph.D. (2014) in Electrical Engineering, all from Caltech. He is a Visiting Associate in Electrical Engineering at Caltech. Dr. Rajagopal's research in medical engineering, microfluidics, nanotechnology and bioengineering has resulted in numerous innovative technological developments. He is the inventor of core technologies related to not only multiplexed PCR assay design, but also combinatorics for biochemical labeling and algorithms for data processing. In the aggregate, Dr. Rajagopal has authored over 45 patents and patent publications. He is the recipient of numerous honors including the Caltech Grubstake Award in 2017, the Caltech Innovation Initiative (CI2) in 2014 and 2015, the Demitriadis-Kafka-Kokallis Thesis Prize in January 2013, and various fellowships and scholarships sponsored by the National Science Foundation and Carnation Foundation from 2006 to 2013. In addition to co-founding ChromaCode, Dr. Rajagopal co-founded a healthcare company called Esperto Medical—a venture-backed Caltech spin-out utilizing compressed sensing methods with ultrasound to continuously and non-invasively measure patient blood pressure.

19. Dr. Axel Scherer is the Bernard Neches Professor of Electrical Engineering, Physics, and Applied Physics at Caltech, where his research focuses on the design and microfabrication of optical, magnetic and fluidic devices. He is also a distinguished visiting professor at Thayer School of Engineering at Dartmouth College. He is known for fabricating the world's first semiconducting vertical-cavity surface-emitting laser ("VCSEL") at Bell Labs, now widely used in data communications systems. More recently, his group developed electromagnetic design tools and fabrication techniques for the definition of

[2] *See, e.g.*, https://nps.edu/web/physics/nps-physics-research-kartalov; *see also, e.g.*, https://presentations.akamaized.net/Shows/UBM/Standalone/IVDT/9-7-11/EmilBio.html; https://nps.edu/faculty-profiles/-/cv/epkartal.

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lithographically integrated optical devices. This led to pioneering work in photonic bandgap lasers, silicon photonic circuits, as well as tunable microfluidic dye lasers, leading to new classes of integrated optics. The first demonstration of strong coupling between single quantum dots and optical nanocavities recently emerged from a collaboration between Dr. Scherer and Hyatt Gibbs. Collaborations with Larry Dalton (University of Washington) resulted in some of the world's smallest and fastest light modulators. Dr. Scherer also fabricated some of the first surface plasmon enhanced high brightness light emitting diodes. His group miniaturized fluidic systems and demonstrated the first multi-layer replication molded fluidic chips, with thousands of valves creating microfluidic "laboratories" and single cell analysis systems. He leads a group focused on the miniaturization and integration of fluidic, optical, electronic and magnetic devices for applications in biotechnology. Scherer has co-authored over 300 publications and holds over 100 patents on the area of microfabrication and design of devices.[3]

20. As a result of continued diligent research efforts, the Caltech researchers' high definition multiplexing solution was reduced to practice by at least February 3, 2012, when Caltech filed U.S. Provisional Patent Application No. 61/594,480, to which the '051 and '170 Patents claim priority.

21. The Caltech researchers' innovative approach to high definition multiplexing, referred to as high-definition PCR[4] ("HDPCR"), involves using

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[3] *See, e.g.*, https://www.eas.caltech.edu/people/etcher#profile-d0b3cee6-tab; *see also, e.g.*, https://www.eas.caltech.edu/people/etcher#publications-d0b3cee6-tab; https://feeds.library.caltech.edu/people/Scherer-A/.

[4] "PCR" stands for the Polymerase Chain Reaction, which is a method of exponential amplification of specific target DNA in a reaction mix with a DNA polymerase and primers. Primers are short single-stranded DNA oligonucleotides, which bind to particular regions of a target sequence. The reaction mixture is cycled in heating and cooling steps. The heating cycle denatures or splits the double-stranded DNA target into single-stranded templates. In the cooling cycle, (continued...)

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multiple fluorescent probes in a single reaction, each probe corresponding to a different target but not necessarily a different color, and distinguishing targets unambiguously based on cumulative signal intensities from each fluorophore. HDPCR is the subject of multiple U.S. patents assigned to Caltech, including the '051 and '170 Patents (collectively, the "HDPCR Patents").

C. The Founding and Mission of ChromaCode

22. In 2012, Dr. Aditya Rajagopal teamed up with Dr. Alex Dickinson to found ChromaCode in order to bring the patented HDPCR technology to the market. Drs. Rajagopal and Dickinson understood that the commercialization of multiplexing technologies, like HDPCR, was critical to the creation of widely-available, efficient and affordable PCR assays able to detect various diseases and medical conditions. ChromaCode obtained an exclusive license to the HDPCR Patents from Caltech for the purpose of developing and delivering commercial embodiments of the HDPCR technology to the market. ChromaCode sought to bridge the gap where one-target-one-color multiplexing falls short because it is not comprehensive enough and next generation sequencing ("NGS") falls short because it can be too slow, costly, and require too much tissue. ChromaCode knew that its HDPCR multiplexing technology could deliver results to patients faster, require less painful procedures, and cost significantly less.

23. ChromaCode's market strategy was to be first-to-market with its groundbreaking HDPCR technology, either through its own commercialization efforts or a partnership with an existing multi-color digital PCR ("dPCR") company. Because ChromaCode's first-to-market strategy was protected by its exclusive patent rights, several third parties invested millions of dollars in

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the primers bind to the target. After the template is primed, the DNA polymerase creates a copy of the original template. Repeated cycling exponentially amplifies the target 2 fold with each cycle leading to approximately a billion-fold increase of the target sequence in 30 cycles.

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ChromaCode. Indeed, ChromaCode's value is inextricably tied to its ability to execute on its first-to-market strategy.

24. ChromaCode's exclusive intellectual property rights in the HDPCR Patents provide ChromaCode with a valuable competitive shield against much larger multi-billion-dollar competitors like Bio-Rad. Bio-Rad's infringement of the HDPCR Patents has eroded that shield and severely diminished ChromaCode's economic advantage.

25. The global PCR market is immense. In the United States alone, the PCR market was estimated to be $7.10 billion in 2020.[5] The market only continues to grow. The increase in PCR research and forensic laboratories and increasing demand for advanced diagnostics, which depend on the ability to detect multiple target sequences in single PCR reactions, are expected to drive market growth. Among other things, the increase in the prevalence of chronic and infectious diseases and genetic disorders is expected to boost PCR demand.

26. Thus, ChromaCode's market value will be irreparably harmed if Bio-Rad is not enjoined from selling its infringing products. ChromaCode's primary asset is its intellectual property. If Bio-Rad is allowed to infringe the HDPCR Patents, ChromaCode's market value will be irreparably reduced. Even if ChromaCode recovers money damages from Bio-Rad, its market value will not be repaired.

THE ASSERTED PATENTS

A. The HDPCR Patents

27. On September 4, 2018, the USPTO issued the '051 Patent, entitled "Signal Encoding and Decoding in Multiplexed Biochemical Assays," to Drs. Emil Kartalov, Aditya Rajagopal and Axel Scherer. Caltech is the assignee of the '051

[5] *See* https://www.fortunebusinessinsights.com/polymerase-chain-reaction-pcr-market-102528.

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Patent. ChromaCode is the exclusive licensee of the '051 Patent pursuant to a license agreement with Caltech dated June 8, 2015. A true and correct copy of the '051 Patent is attached hereto as **Exhibit A**.

28. The '051 Patent claims priority to U.S. Provisional Application No. 61/594,480, filed on February 3, 2012. Independent claim 1 of the '051 Patent recites:

> 1. A method of detecting the presence or absence of at least seven polynucleotide analytes, in any combination of presence or absence, in a single sample solution volume, the method comprising:
>
> a) providing a sample solution volume comprising or potentially comprising, at least one of at least seven polynucleotide analytes, and comprising at least seven hybridization probes, each hybridization probe corresponding to one of the at least seven polynucleotide analytes, wherein each hybridization probe comprises at least one fluorophore selected from four fluorophores, and wherein each hybridization probe, when excited and when contacted with its corresponding polynucleotide analyte, generates a cumulative intensity signal, further wherein each generated cumulative intensity signal comprises at least one signal generated by excitement of at least one of the four fluorophores;
>
> b) contacting said sample solution volume with the plurality of hybridization probes and exciting the hybridization probes to generate the cumulative intensity signal(s) if one or more of the at least seven polynucleotide analytes is present in the sample volume; and
>
> c) measuring the cumulative intensity signal(s), thereby generating a cumulative signal measurement, wherein the cumulative signal measurement non-degenerately indicates the presence or absence of the at least seven

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> polynucleotide analytes, in any combination of presence or absence,
>
> wherein said method non-degenerately detects the presence or absence of the at least seven polynucleotide analytes in said single sample solution volume, in any combination of presence or absence, without requiring any step of immobilization of said polynucleotide analytes, physical separation of said polynucleotide analytes, or mass spectrometry.

29. On September 4, 2018, the USPTO issued the '170 Patent, entitled "Signal Encoding and Decoding in Multiplexed Biochemical Assays," to Drs. Emil Kartalov, Aditya Rajagopal and Axel Scherer. Caltech is the assignee of the '170 Patent. ChromaCode is the exclusive licensee of the '170 Patent pursuant to a license agreement with Caltech dated June 8, 2015. A true and correct copy of the '170 Patent is attached hereto as **Exhibit B**.

30. The '170 Patent shares the same specification as the '051 Patent and claims priority to U.S. Provisional Application No. 61/594,480, filed on February 3, 2012. Independent claim 1 of the '170 Patent recites:

> 1. A method of unambiguously detecting any unique combination of presence or absence of at least five polynucleotide analytes in a plurality of droplets, the method comprising:
>
> a) providing a sample comprising, or potentially comprising, at least one of said at least five polynucleotide analytes;
>
> b) forming a mixture of said sample and at least five hybridization probes, wherein each of said at least five hybridization probes further comprises at least one fluorophore and at most four fluorophores;
>
> c) partitioning said mixture into said plurality of droplets;
>
> d) exciting said at least one fluorophore to generate one or more signals if one or more of said at least five

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polynucleotide analytes is present in said plurality of droplets, wherein said one or more signals comprise at least one signal generated by excitement of said at least one fluorophore;

e) measuring said one or more signals to generate a cumulative intensity measurement, wherein said cumulative intensity measurement corresponds to the presence of a unique combination of presence or absence of said at least five polynucleotide analytes in said sample; and

f) determining whether each of said least five polynucleotide analytes is present, in any unique combination of presence or absence, based on said cumulative intensity measurement,

wherein the method does not require any step of immobilization of said at least five polynucleotide analytes or mass spectrometry.

31. The HDPCR Patents describe a method of unambiguously identifying more than one target per color by using a cumulative signal from all fluorescent probes in a reaction. By designing the assay so that each cumulative signal can ***only*** belong to one target—notwithstanding the fact that multiple targets may be assigned the same color—the HDPCR Patents describe a method of doing what others could not: unambiguously identifying multiple targets in a single sample by using less colors than targets.

32. The HDPCR Patents provide solutions to the long-existing problem of how to unambiguously identify multiple targets without expanding the colors used, and in some cases, how to unambiguously identify at least five or more targets in less than the same number of colors (*e.g.*, five targets with four colors or less). ChromaCode's exclusively licensed and patented multiplexing solution dramatically improved on existing dPCR technology and opened the door to providing usable and commercially viable advanced PCR detection assays that

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could aid in the fight against difficult diseases like cancer.

THE INFRINGING PRODUCTS

A. Bio-Rad Launches its Infringing QX600 Droplet Digital PCR System

33. Upon information and belief, Bio-Rad also had been trying to develop an advanced multiplexing technology of its own. In 2011, Bio-Rad researchers allegedly figured out a process of identifying more targets than colors in a single sample and filed for patent protection of their process. *See* U.S. Provisional Patent Application No. 61/454,373, filed on March 18, 2011. Although Bio-Rad received patents on their approach to multiplexing, *see* U.S. Patent Nos. 9,222,128 and 9,921,154, Bio-Rad was unable to unambiguously detect multiple targets per color or unambiguously detect more than four targets with two colors. Despite many years trying to make its own inferior multiplexing technology work, this limitation rendered Bio-Rad's approach commercially unviable.

34. Bio-Rad knew, however, that ChromaCode had been able to develop an elegant, functional multiplexing solution to unambiguously detect multiple targets per color. ChromaCode shared an overview of its patented, proprietary technology with Bio-Rad during fundraising discussions in 2014. These fundraising discussions included an overview of ChromaCode's intellectual property portfolio, including then-pending Caltech patent applications covering the HDPCR multiplexing technology. ChromaCode shared with Bio-Rad that it was the exclusive licensee to these patents.

35. The application that became the '051 Patent published on February 26, 2015. The '051 Patent issued on September 4, 2018. Around this period, discussions with Bio-Rad continued. In those discussions with Bio-Rad, ChromaCode reiterated that it had the exclusive rights to patented HDPCR multiplexing technology. Indeed, in 2017 and 2018, ChromaCode shared slides with Bio-Rad stating that, in its multiplexing assay, "[t]argets are amplitude

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multiplexed using ChromaCode's patented and proprietary methods." The slides ChromaCode shared with Bio-Rad also referred to ChromaCode's "[p]atented multiplex coding methods," and stated that ChromaCode had "[e]xclusive rights to patented HDPCR™ from Caltech." On information and belief, armed with this knowledge, a sophisticated legal department, and significant prior experience with patent disputes and patent litigation, Bio-Rad should have investigated the scope of ChromaCode's patent rights, including the '051 and '170 Patents.

36. Unable to make its own approach commercially viable, Bio-Rad decided instead to copy ChromaCode's multiplexing solution and willfully infringe the HDPCR Patents. In late 2022, Bio-Rad announced its new QX600 Droplet Digital PCR System (the "QX600 System").[6] For example, in an April 5, 2023 press release, Bio-Rad touted that its QX600 System is capable of unambiguously identifying 12 targets in its 6 color instrument:

> Bio-Rad Laboratories, Inc. (NYSE: BIO and BIOb), a global leader in life science research and clinical diagnostic products, is accelerating measurable residual disease (MRD) research through more than a half dozen collaborations with institutions and companies leveraging its new QX600™ Droplet Digital™ PCR System. ***Launched late last year, it boasts six color detection capable of quantifying 12 targets per well***, a simple user workflow, and powerful data analysis. Maintaining Bio-Rad's best-in-class ddPCR™ technology, this platform utilizes the same droplet generation and processing protocols as the QX200™ system, enabling thousands of current customers to easily adopt its advanced multiplexing capabilities.[7]

[6] As used herein, the term "QX600 System" includes Bio-Rad's QX600 Droplet Digital PCR System and related systems, components and assays, such as Bio-Rad's QX600 Auto DG Droplet Digital System.

[7] Emphasis added.

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37. A true and correct copy of Bio-Rad's April 5, 2023 press release is attached hereto as **Exhibit C**.[8]

38. Beginning at least as of December 14, 2022, Bio-Rad's website offered the QX600 System for sale, announcing: "The QX600™ Droplet Digital™ PCR System enables advanced six-color multiplexing, allowing clear discrimination of multiple targets with assays that are cross-compatible with the QX200™ Droplet Digital PCR System. The QX600 System is designed for researchers who need to quantify multiple targets with high accuracy, reproducibility, and sensitivity." A true and correct copy of the product page for the QX600 system is attached hereto as **Exhibit D**.[9] The QX600 System product page continues in the "Description" section:

> The QX600 Droplet Reader offers users:
>
> - Sensitive multiplexing
> - Six-color detection capability (FAM, HEX, Cy5, Cy5.5, ROX, and ATTO 590)
> - Quantification of up to 12 targets in a single well
> - Absolute quantification with 0.1% or better sensitivity
> - Sensitive and precise gene expression multiplexing

39. Bio-Rad also began distributing instructions on how to use the QX600 System and related assays to multiplex PCR processes to customers throughout the United States, including in this District, on its website, including the QX600 Droplet Reader and QX Manager Software Standard Edition User Guide (the "QX600 System User Guide"). A true and correct copy of the QX600 System

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[8] *See also* https://www.bio-rad.com/en-us/life-science-research/news/bio-rads-qx600-droplet-digital-pcr-system-advancing-measurable-residual-disease-research?ID=Bio-Rad-s-QX600-Drop_1680646933.

[9] *See also* https://www.bio-rad.com/en-us/product/qx600-droplet-digital-pcr-system?ID=b07d12ac-0585-fc4c-a586-3ddf20d5c4a0.

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User Guide is attached hereto as **Exhibit E**.[10]

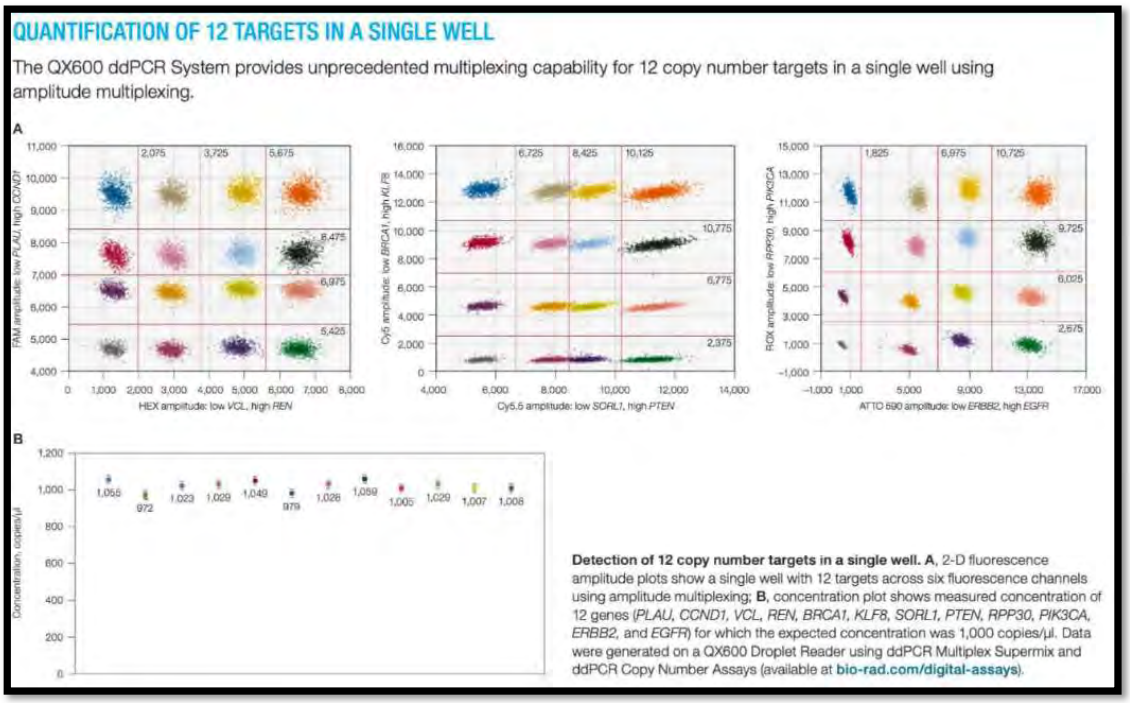
40. The QX600 System User Guide describes multiple assays for the QX600 that include an encoding scheme based on the cumulative signal from each fluorophore information that unambiguously identifies more targets than there are colors. For example, in Table 15 titled "Assay types," Bio-Rad describes assay type "Amplitude multiplex" as "Method to increase multiplexing up to twelve targets per well, with one or two targets detected per [color]." Ex. E at 77. Bio-Rad also describes a "Method assuming up to six probe colors … and up to six targets per [color]." *Id.*; *see also id.* at 79, Table 21 (describing "Fluorophore options" including "Amplitude multiplex, for 1 to 12 targets" and "Probe mix triplex, for 9 targets").

41. In Bio-Rad's "Bulletin 3557," a true and correct copy of which is attached hereto as **Exhibit F**,[11] Bio-Rad provided data demonstrating their new and "unprecedented multiplexing capabilit[ies]," as reflected in the excerpt below labeled "Quantification of 12 Targets in a Single Well."

[10] *See also* https://www.bio-rad.com/sites/default/files/2022-12/10000153877.pdf.

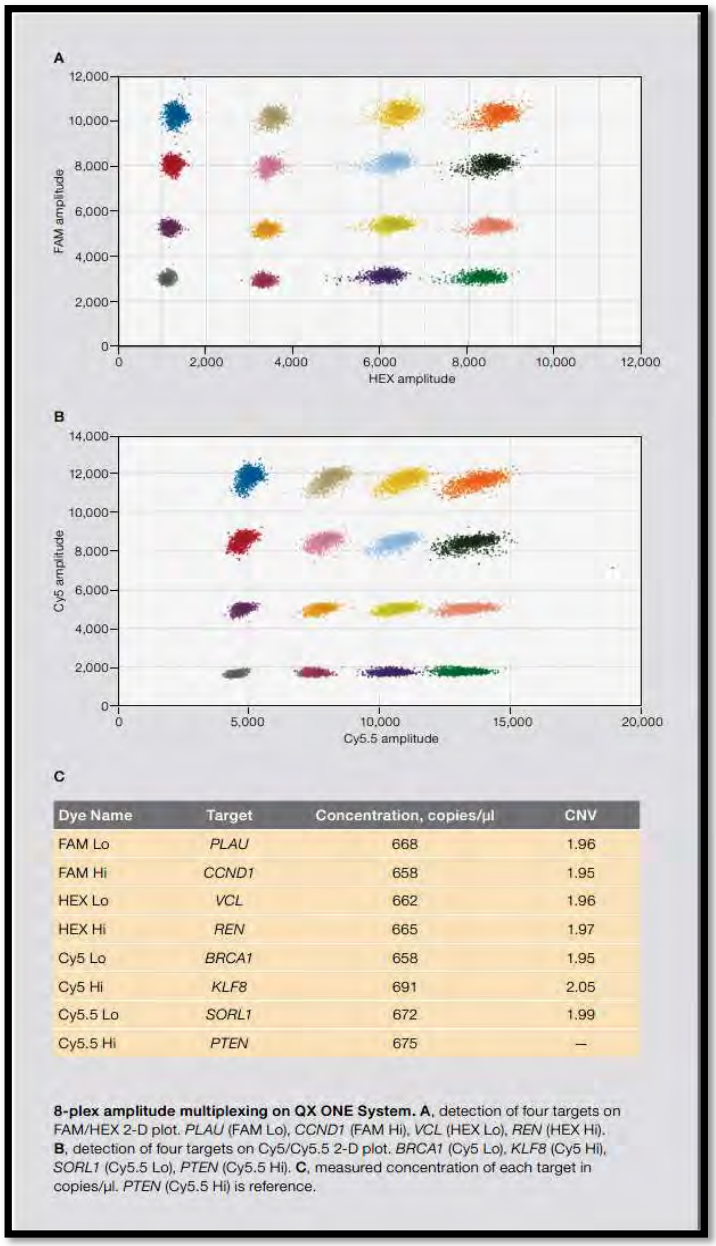
[11] *See also* https://www.bio-rad.com/sites/default/files/2023-08/Bulletin_3557.pdf.

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42. At least the QX600 System practices one or more claims of each of the HDPCR Patents. For example, the QX600 System's ability to detect multiple targets per color using cumulative signals is only possible using ChromaCode's patented approach to multiplexing recited by one or more claims of each of the HDPCR Patents. Thus, Bio-Rad's QX600 System infringes the HDPCR Patents.

43. Bio-Rad's QX ONE Droplet Digital PCR System and ddPCR Multiplex Supermix together also practice one or more claims of each of the



C

Dye Name	Target	Concentration, copies/µl	CNV
FAM Lo	*PLAU*	668	1.96
FAM Hi	*CCND1*	658	1.95
HEX Lo	*VCL*	662	1.96
HEX Hi	*REN*	665	1.97
Cy5 Lo	*BRCA1*	658	1.95
Cy5 Hi	*KLF8*	691	2.05
Cy5.5 Lo	*SORL1*	672	1.99
Cy5.5 Hi	*PTEN*	675	–

8-plex amplitude multiplexing on QX ONE System. A, detection of four targets on FAM/HEX 2-D plot. *PLAU* (FAM Lo), *CCND1* (FAM Hi), *VCL* (HEX Lo), *REN* (HEX Hi). **B**, detection of four targets on Cy5/Cy5.5 2-D plot. *BRCA1* (Cy5 Lo), *KLF8* (Cy5 Hi), *SORL1* (Cy5.5 Lo), *PTEN* (Cy5.5 Hi). **C**, measured concentration of each target in copies/µl. *PTEN* (Cy5.5 Hi) is reference.

HDPCR Patents, as reflected in Bio-Rad's "Bulletin 6512."[12] A true and correct copy of Bulletin 6512 is attached hereto as **Exhibit G** and an excerpt from that

[12] *See* https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6512.pdf.

Bulletin is included above for the Court's convenience.

44. Bulletin 6512 states: "Bio-Rad's QX ONE Droplet Digital PCR System offers four color channels—FAM, HEX, Cy5, and Cy5.5—thereby providing additional multiplexing flexibility. By using Bio-Rad's ddPCR Multiplex Supermix on the QX ONE ddPCR System, as many as eight targets can be detected and measured in a single reaction. Such advanced multiplexing is made possible using strategies such as amplitude multiplexing in conjunction with four color channels. Extract as much information as possible with high sensitivity, using as little sample as possible in a fast, cost-effective manner." Ex. G at 9.

45. Bio-Rad knew or should have known about ChromaCode's exclusive rights to practice the HDPCR Patents. Despite knowledge of ChromaCode's patent rights, Bio-Rad chose not only to infringe, but to induce others to do so as well. Indeed, as described above, Bio-Rad went so far as to provide step-by-step instructions on how to use Bio-Rad's devices to infringe the HDPCR Patents. This egregious misconduct has caused Plaintiffs significant, irreparable harm that will continue unless and until Bio-Rad is enjoined from infringing the HDPCR Patents.

COUNT I

INFRINGEMENT OF THE '051 PATENT

46. Plaintiffs repeat and reallege each and every allegation contained in the preceding paragraphs of this Amended Complaint as if fully set forth herein.

47. Caltech is the assignee and owner of the '051 Patent. ChromaCode is the exclusive licensee of the '051 Patent. ChromaCode's exclusive rights include the right to enforce the '051 Patent.

48. Bio-Rad makes, offers to sell and/or sells its infringing products in the United States, including in this District.

49. For example, Bio-Rad's QX600 System practices each and every limitation, either literally or under the doctrine of equivalents, of at least claim 1 of the '051 Patent in violation of 35 U.S.C. § 271(a). For example, one or more

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assays provided by Bio-Rad with at least the QX600 System perform amplitude-based PCR multiplexing to unambiguously identify the presence or absence of at least seven polynucleotide analytes based on cumulative signal intensity of probes that comprise at least one fluorophore selected from four fluorophores, and perform each of the other elements of claim 1. Bio-Rad's QX600 System, therefore, directly infringes the '051 Patent.

50. Bio-Rad also indirectly infringes the '051 Patent. Bio-Rad has knowingly induced infringement of the '051 Patent by encouraging others to infringe at least claim 1 of the '051 Patent. For example, in Bio-Rad's QX600 System User Guide, Bio-Rad has induced, and will continue to induce, users to infringe the '051 Patent by expressly and intentionally instructing and encouraging users to perform each limitation of at least claim 1 of the '051 Patent in violation of 35 U.S.C. § 271(b). Bio-Rad is, therefore, liable for inducing infringement of the '051 Patent.

51. Bio-Rad's sale of infringing products has contributed, and will continue to contribute, to the infringement of the '051 Patent in violation of 35 U.S.C. § 271(c). For example, users of the QX600 System have infringed, and will continue to infringe, at least claim 1 of the '051 Patent by performing assays in accordance with instructions set forth in the QX600 System User Guide. Bio-Rad is, therefore, liable for contributing to the infringement of the '051 Patent.

52. Upon information and belief, Bio-Rad had and continues to have knowledge that multiple assays it provides for the QX600 System are especially made or especially adapted for a use that infringes the '051 Patent.

53. Upon information and belief, Bio-Rad had actual and constructive notice of the '051 Patent prior to launching the QX600 System and knowingly or intentionally infringed the '051 Patent after acquiring that knowledge. Bio-Rad has no reasonable basis for asserting that the commercial manufacture, use, offer for sale, or sale of the QX600 System will not infringe, induce the infringement of,

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and/or contribute to the infringement of the '051 Patent.

54. Bio-Rad's infringement of the '051 Patent has been, and continues to be, willful, wanton, malicious, bad faith, deliberate, consciously wrong, flagrant, and egregious, entitling Plaintiffs to an award of up to three times their actual damages pursuant to 35 U.S.C. § 284.

55. Bio-Rad's willful infringement of the '051 Patent further renders this an exceptional case under 35 U.S.C. § 285.

56. Plaintiffs have been damaged by Bio-Rad's egregious and willful infringement of the '051 Patent.

57. Plaintiffs will be irreparably harmed if Bio-Rad is not enjoined from infringing, and from actively inducing or contributing to the infringement of, the '051 Patent. Plaintiffs do not have an adequate remedy at law and, considering the balance of hardships between Plaintiffs and Bio-Rad, a remedy in equity is warranted. Further, the public interest would not be disserved by the entry of a permanent injunction.

COUNT II

INFRINGEMENT OF THE '170 PATENT

58. Plaintiffs repeat and reallege each and every allegation contained in the preceding paragraphs of this Amended Complaint as if fully set forth herein.

59. Caltech is the assignee and owner of the '170 Patent. ChromaCode is the exclusive licensee of the '170 Patent. ChromaCode's exclusive rights include the right to enforce the '170 Patent.

60. Bio-Rad makes, offers to sell and/or sells its infringing products in the United States, including in this District.

61. For example, Bio-Rad's QX600 System practices each and every limitation, either literally or under the doctrine of equivalents, of at least claim 1 of the '170 Patent in violation of 35 U.S.C. § 271(a). For example, one or more assays provided by Bio-Rad with at least the QX600 System perform amplitude-

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based PCR multiplexing to unambiguously identify the presence or absence of at least five polynucleotide analytes based on cumulative signal intensity of probes that comprise between one and four fluorophores, and perform each of the other elements of claim 1. Bio-Rad's QX600 System, therefore, directly infringes the '170 Patent.

62. Bio-Rad also indirectly infringes the '170 Patent. Bio-Rad has knowingly induced infringement of the '170 Patent by encouraging others to infringe at least claim 1 of the '170 Patent. For example, in Bio-Rad's QX600 System User Guide, Bio-Rad has induced, and will continue to induce, users to infringe the '170 Patent by expressly and intentionally instructing and encouraging users to perform each limitation of at least claim 1 of the '170 Patent in violation of 35 U.S.C. § 271(b). Bio-Rad is, therefore, liable for inducing infringement of the '170 Patent.

63. Bio-Rad's sale of the infringing products has contributed, and will continue to contribute, to the infringement of the '170 Patent in violation of 35 U.S.C. § 271(c). For example, users of the QX600 System have infringed, and will continue to infringe, at least claim 1 of the '170 Patent by performing assays in accordance with instructions set forth in the QX600 System User Guide. Bio-Rad is, therefore, liable for contributing to the infringement of the '170 Patent.

64. Upon information and belief, Bio-Rad had and continues to have knowledge that multiple assays it provides for the QX600 System are especially made or especially adapted for a use that infringes the '170 Patent.

65. Upon information and belief, Bio-Rad had actual and constructive notice of the '170 Patent prior to launching the QX600 System and knowingly or intentionally infringed the '170 Patent after acquiring that knowledge. Bio-Rad has no reasonable basis for asserting that the commercial manufacture, use, offer for sale, or sale of the QX600 System will not infringe, contribute to the infringement of, and/or induce the infringement of the '170 Patent.

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66. Bio-Rad's infringement of the '170 Patent has been, and continues to be, willful, wanton, malicious, bad faith, deliberate, consciously wrong, flagrant, and egregious, entitling Plaintiffs to an award of up to three times their actual damages pursuant to 35 U.S.C. § 284.

67. Bio-Rad's willful infringement of the '170 Patent further renders this an exceptional case under 35 U.S.C. § 285.

68. Plaintiffs have been damaged by Bio-Rad's egregious and willful infringement of the '170 Patent.

69. Plaintiffs will be irreparably harmed if Bio-Rad is not enjoined from infringing, and from actively inducing or contributing to the infringement of the '170 Patent. Plaintiffs do not have an adequate remedy at law, and, considering the balance of hardships between Plaintiffs and Bio-Rad, a remedy in equity is warranted. Further, the public interest would not be disserved by the entry of a permanent injunction.

PRAYER FOR RELIEF

70. WHEREFORE, Plaintiffs respectfully request the following relief:

a. That the Court enter judgment that Bio-Rad directly infringed (literally and/or under the doctrine of equivalents), contributorily infringed, and induced infringement of the '051 Patent;

b. That the Court enter judgment that Bio-Rad directly infringed (literally and/or under the doctrine of equivalents), contributorily infringed, and induced infringement of the '170 Patent;

c. That, prior to the expiration of the HDPCR Patents, the Court enjoin Bio-Rad from: (1) making, offering for sale and selling its infringing products and related multiplexing assays, and (2) infringing the HDPCR Patents;

d. That the Court award Plaintiffs monetary damages suffered as a result of Bio-Rad's infringement of the '051 and '170 Patents;

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e. That an accounting be performed to determine the monetary damages to be awarded to Plaintiffs as a result of Bio-Rad's infringing activities, including an accounting for infringing conduct not presented at trial and an award of additional damages for any such infringing activities;

f. That the Court find that Bio-Rad's infringement of the HDPCR Patents was egregious and willful and award Plaintiffs three times their actual damages;

g. That the Court declare that this case is exceptional under 35 U.S.C. § 285 and award Plaintiffs their attorneys' fees, costs, and expenses incurred in this action;

h. That the Court award Plaintiffs any further and additional relief as the Court may deem just, equitable, and proper.

JURY DEMAND

Plaintiffs hereby demand a jury trial on all issues and claims so triable.

Dated: October 13, 2023

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Martin R. Bader

Attorneys for Plaintiffs ChromaCode, Inc. and California Institute of Technology

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EXHIBIT A

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US010068051B2

(12) **United States Patent**
Kartalov et al.

(10) **Patent No.: US 10,068,051 B2**
(45) **Date of Patent: Sep. 4, 2018**

(54) **SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS**

(71) Applicant: **California Institute of Technology**, Pasadena, CA (US)

(72) Inventors: **Emil Kartalov**, Pasadena, CA (US); **Aditya Rajagopal**, Irvine, CA (US); **Axel Scherer**, Barnard, VT (US)

(73) Assignee: **California Institute of Technology**, Pasadena, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 90 days.

(21) Appl. No.: **14/451,876**

(22) Filed: **Aug. 5, 2014**

(65) **Prior Publication Data**

US 2015/0057178 A1 Feb. 26, 2015

Related U.S. Application Data

(63) Continuation of application No. 13/756,760, filed on Feb. 1, 2013, now Pat. No. 8,838,394.
(Continued)

(51) **Int. Cl.**
G01N 33/48 (2006.01)
G01N 33/50 (2006.01)
(Continued)

(52) **U.S. Cl.**
CPC ***G06F 19/20*** (2013.01); ***C12Q 1/68*** (2013.01); ***C12Q 1/6825*** (2013.01);
(Continued)

(58) **Field of Classification Search**
None
See application file for complete search history.

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Primary Examiner — Russell S Negin
(74) *Attorney, Agent, or Firm* — Wilson Sonsini Goodrich & Rosati

(57) **ABSTRACT**

This disclosure provides methods, systems, compositions, and kits for the multiplexed detection of a plurality of analytes in a sample. In some examples, this disclosure provides methods, systems, compositions, and kits wherein multiple analytes may be detected in a single sample volume by acquiring a cumulative measurement or measurements of at least one quantifiable component of a signal. In some cases, additional components of a signal, or additional signals (or components thereof) are also quantified. Each signal or component of a signal may be used to construct a coding scheme which can then be used to determine the presence or absence of any analyte.

9 Claims, 8 Drawing Sheets

Specification includes a Sequence Listing.

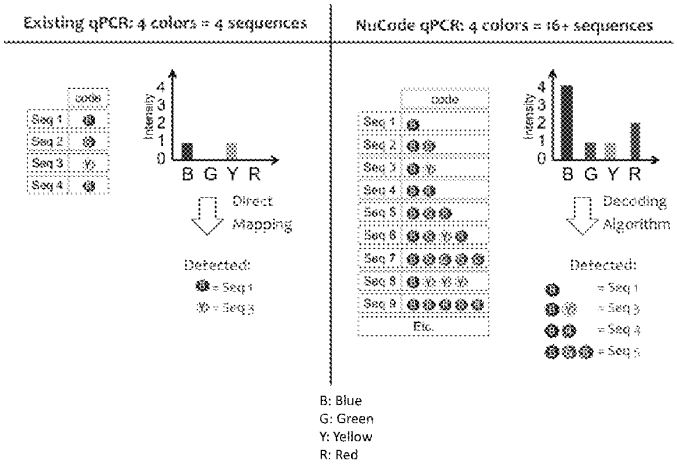


EXHIBIT A

Related U.S. Application Data

(60) Provisional application No. 61/703,093, filed on Sep. 19, 2012, provisional application No. 61/594,480, filed on Feb. 3, 2012.

(51) **Int. Cl.**

G06F 19/20	(2011.01)
G06F 19/12	(2011.01)
C12Q 1/68	(2018.01)
G01N 21/64	(2006.01)
C12Q 1/6825	(2018.01)
C12Q 1/6851	(2018.01)
G06F 19/24	(2011.01)
G06F 17/10	(2006.01)
G06F 17/11	(2006.01)

(52) **U.S. Cl.**

CPC ***C12Q 1/6851*** (2013.01); ***G01N 21/6486*** (2013.01); ***G06F 19/12*** (2013.01); *G06F 17/10* (2013.01); *G06F 17/11* (2013.01); *G06F 19/24* (2013.01); *Y02A 50/53* (2018.01)

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Fig. 1

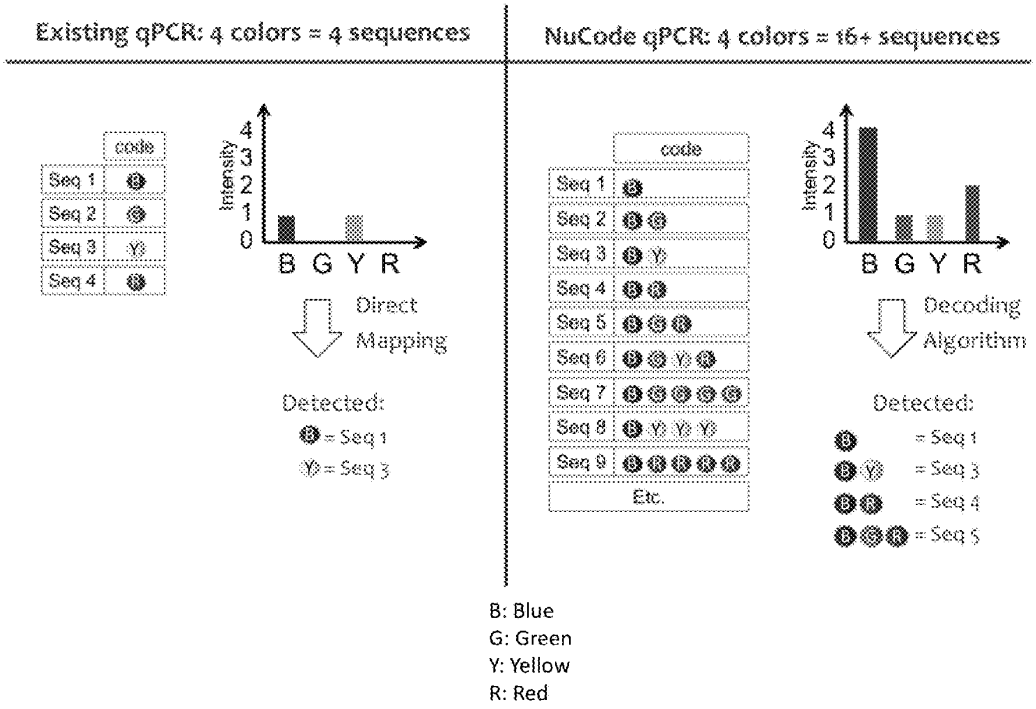
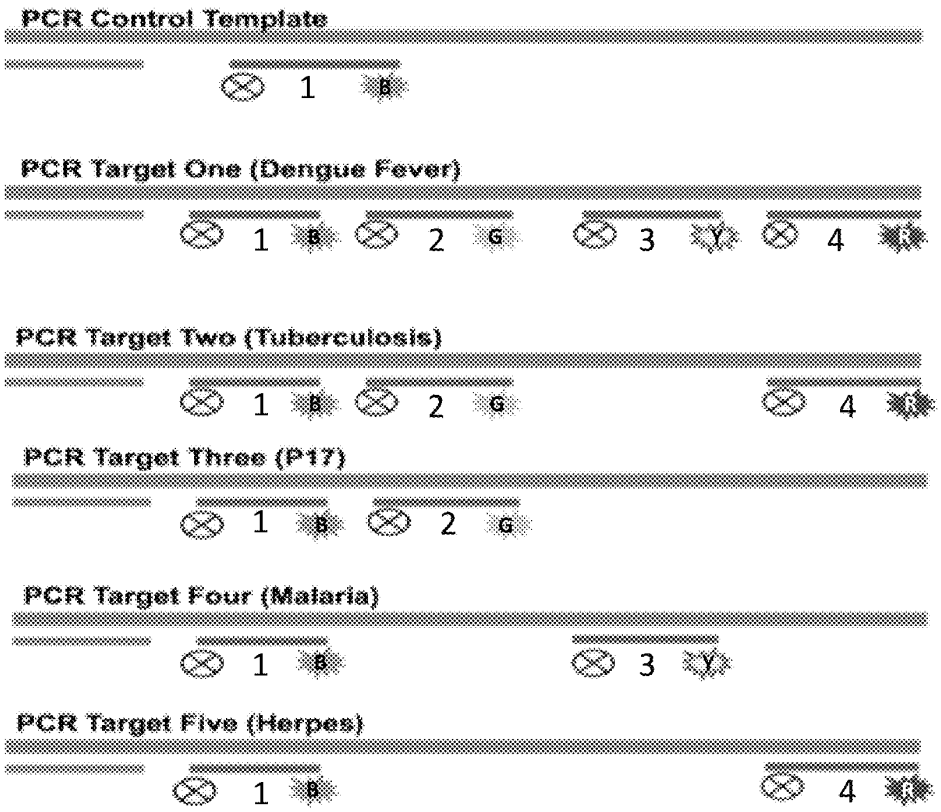
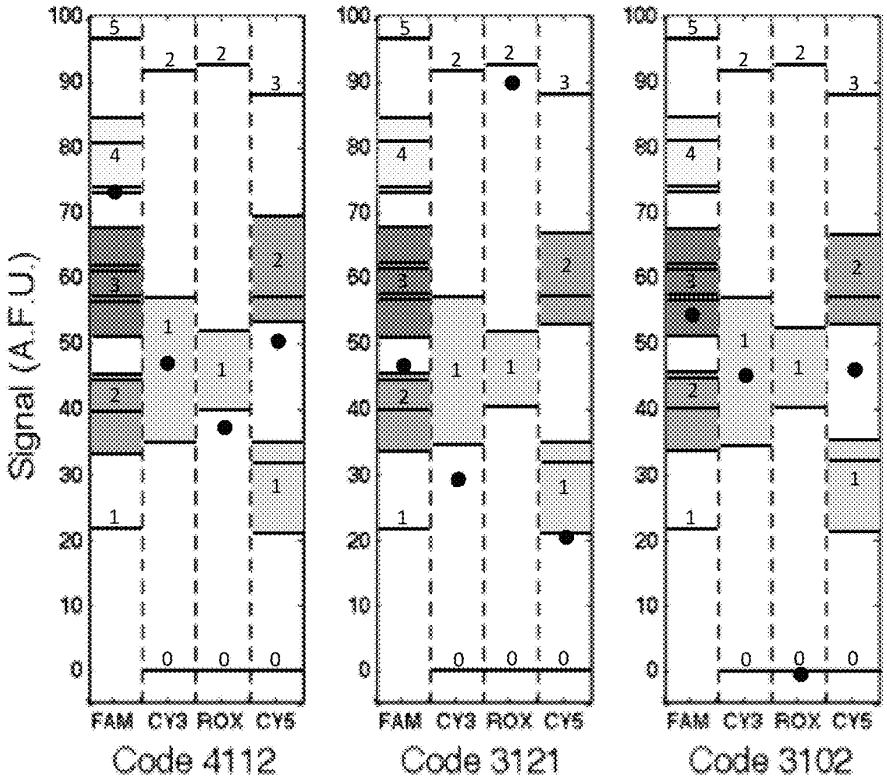

Existing qPCR: 4 colors = 4 sequences
NuCode qPCR: 4 colors = 16+ sequences
code
Seq 1
Seq 2
Seq 3
Seq 4
Intensity
4
3
2
1
0
B G Y R
Direct
Mapping
Detected:
= Seq 1
= Seq 3
code
Seq 1
Seq 2
Seq 3
Seq 4
Seq 5
Seq 6
Seq 7
Seq 8
Seq 9
Etc.
Intensity
4
3
2
1
0
B G Y R
Decoding
Algorithm
Detected:
= Seq 1
= Seq 3
= Seq 4
= Seq 5
B: Blue
G: Green
Y: Yellow
R: Red

Fig. 2



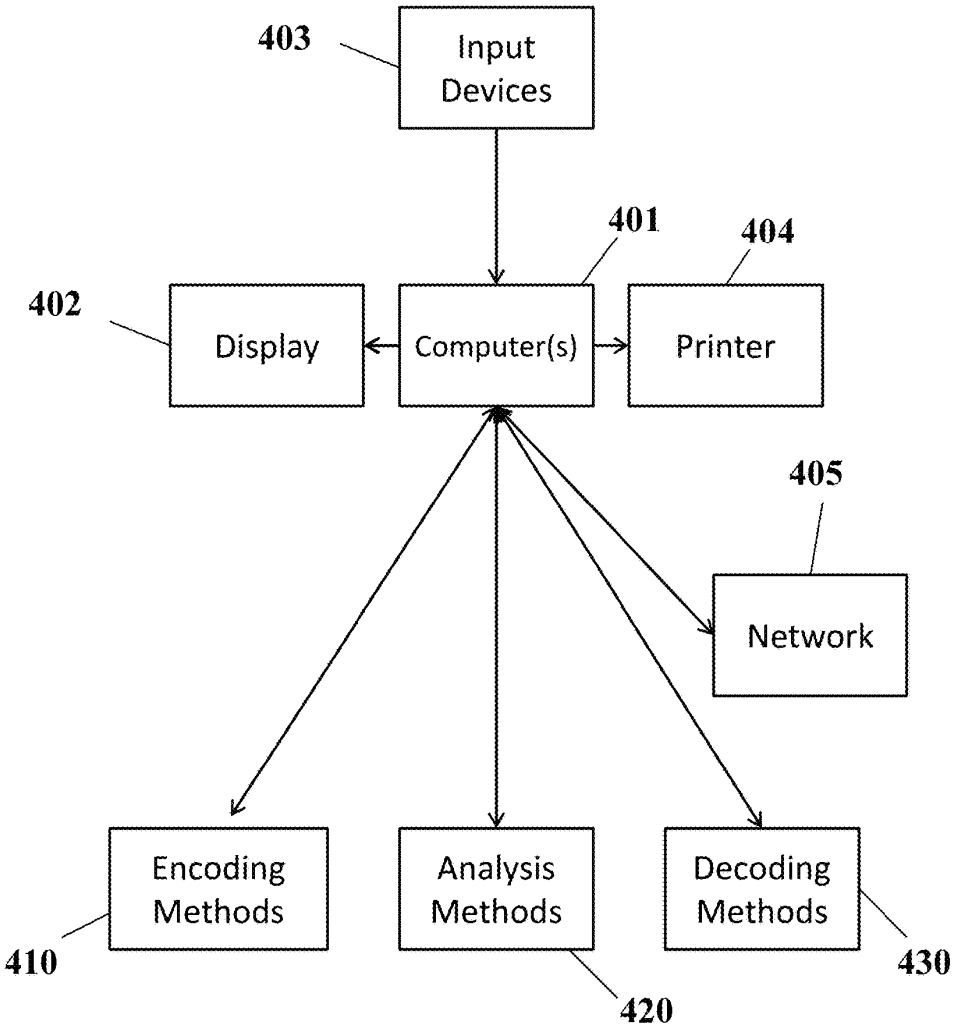
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Fig. 3



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Fig. 4



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Fig. 5

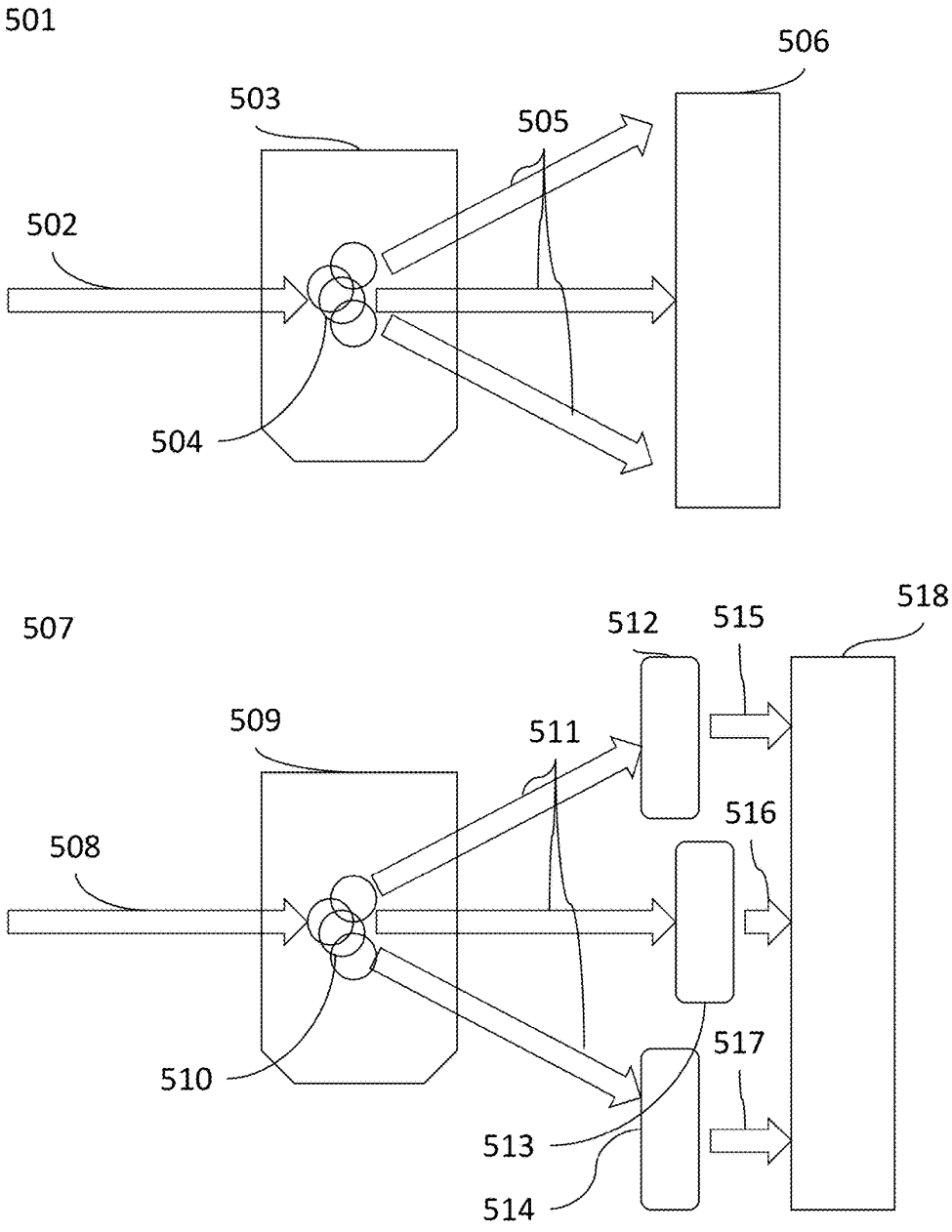
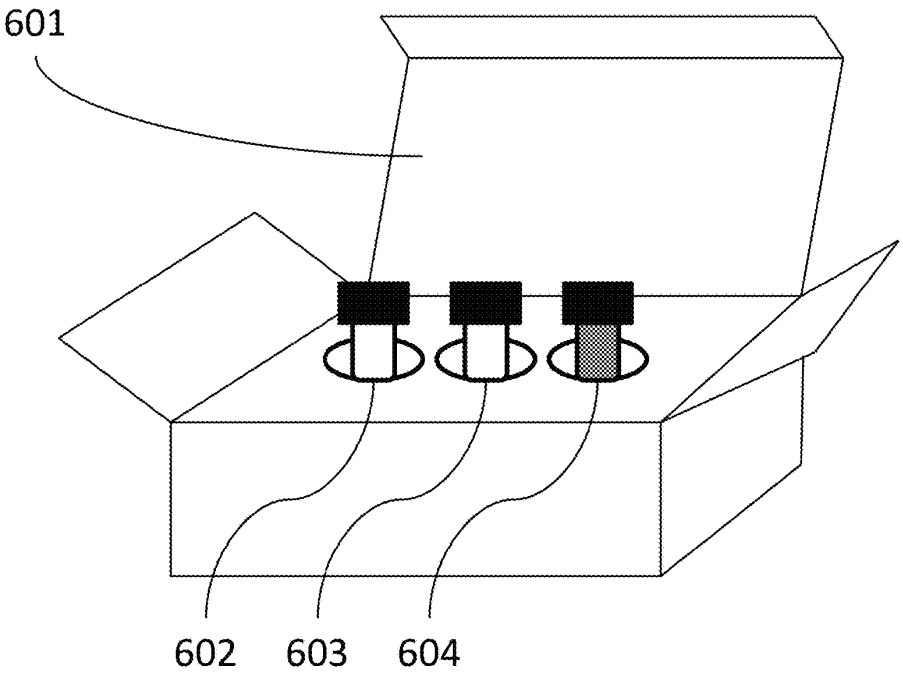
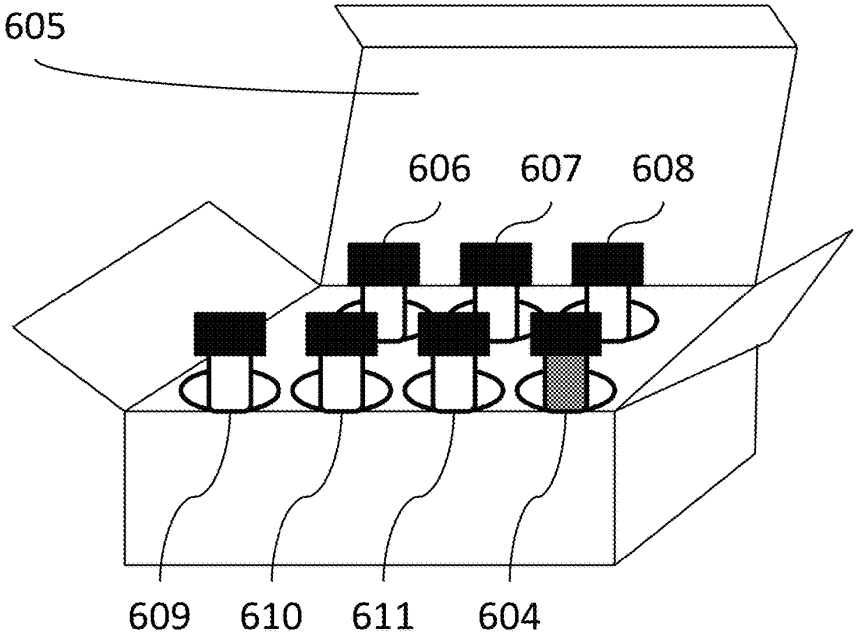

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Fig. 6





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Fig. 7

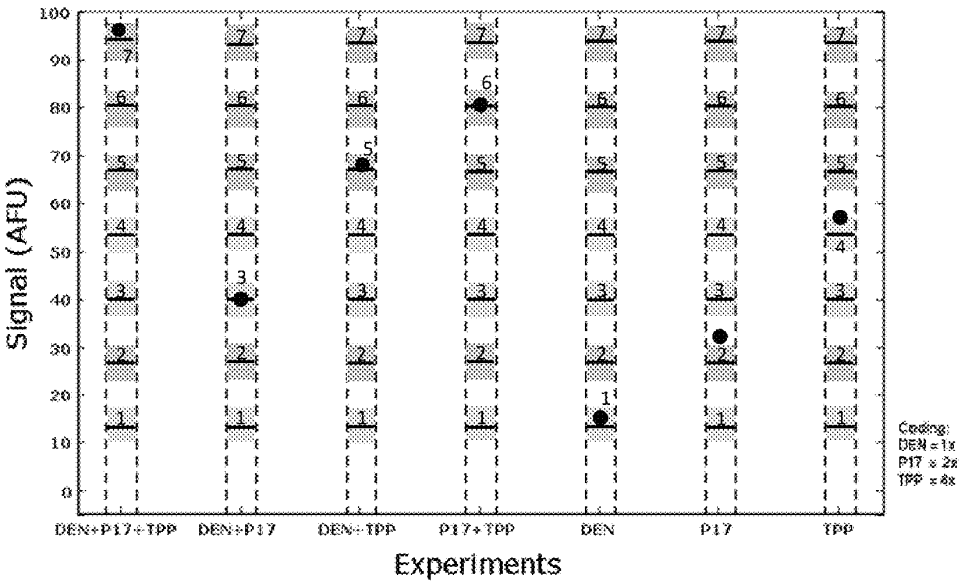
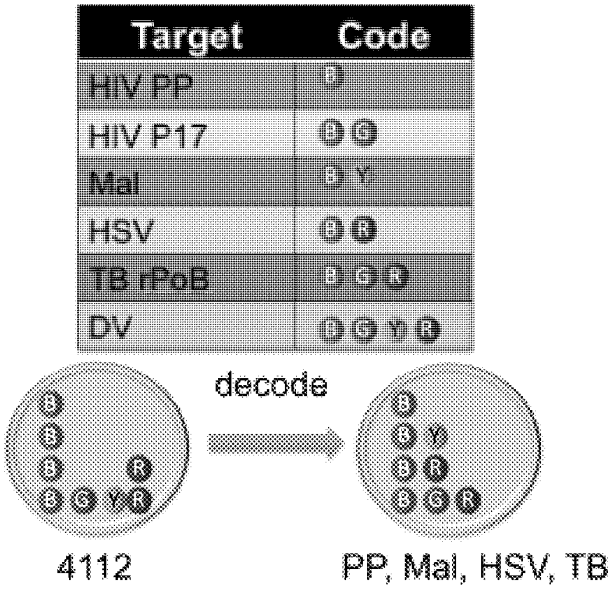
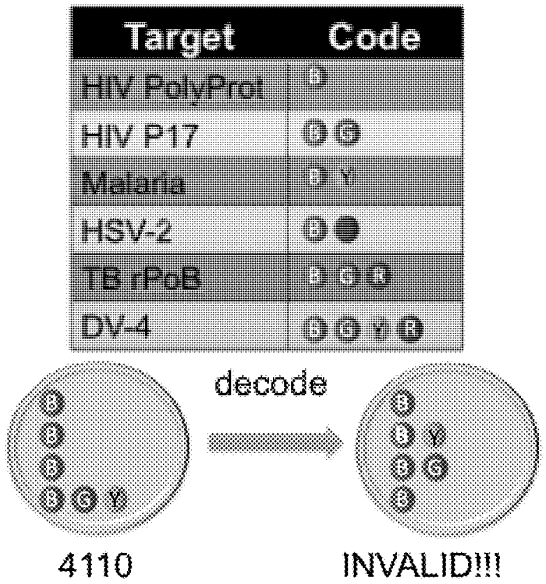

Signal (AFU)
Experiments
DEN+P17+TPP
DEN+P17
DEN+TPP
P17+TPP
DEN
P17
TPP
Coding:
DEN = 1x
P17 = 2x
TPP = 4x

Fig. 8


Target
Code
HIV PP
HIV P17
Mal
HSV
TB rPoB
DV
decode
4112
PP, Mal, HSV, TB


Target
Code
HIV PolyProt
HIV P17
Malaria
HSV-2
TB rPoB
DV-4
decode
4110
INVALID!!!

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

CROSS-REFERENCE

This application is a continuation of U.S. patent application Ser. No. 13/756,1760, filed Feb. 1, 2013, which claims the benefit of U.S. provisional applications 61/594,480, filed Feb. 3, 2012 and 61/703,093, filed Sep. 19, 2012. The content of the three applications are incorporated herein by reference in entirety.

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

This invention was made with the support of the United States government under Grant No. 1144469 awarded by NSF. The government has certain rights in the invention.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on Aug. 20, 2014, is named 38075-716-301-Seqlist.txt and is 9,260 bytes in size.

BACKGROUND

Multiplexed reactions offer significant advantages over traditional uniplex reactions, including performance of parallel reactions on the same sample, use of the same chamber to perform multiple reactions, and the ability to extract rich information from a sample in a fast and efficient manner. However, to achieve these benefits, multiplexed assays generally require complex reporting mechanisms, namely spectrally resolved fluorescence or chemiluminescence (e.g., PCR, ELISA), spatially resolved signals (e.g., microarrays, gel electrophoresis), temporally resolved signals (e.g., capillary electrophoresis), or combinations thereof (e.g., Sanger sequencing). There is a need for multiplexed reactions that can be carried out in a single solution.

SUMMARY OF THE INVENTION

This disclosure provides methods, compositions, systems, and kits for the multiplexed detection of analytes. In some cases, this disclosure provides assays that are capable of unambiguously detecting the presence or absence of each of at least 7 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis. In some examples, each of the analytes is encoded as a value of one (or at least one) component of a signal.

In some examples, an assay provided in this disclosure is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M=\log_2 (F+1)$ and F is the maximum cumulative value of one component of a signal (e.g., an intensity) when all the analytes are present.

In some cases, each analyte is encoded as at least one first value in a first component of a signal (e.g., at least one intensity or range of intensities) and at least one second value in a second component of a signal (e.g., at least one wavelength or range of wavelengths). In some examples, each analyte is encoded as a first value in a first component of a signal at each of a plurality of second values in a second component of a signal (e.g., a signal intensity or range of signal intensities at each of a plurality of wavelengths or ranges of wavelengths).

In some examples, an assay provided herein is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M=C*\log_2 (F+1)$, where C is the number of the second values used to encode the analytes and F is the maximum cumulative value of the first component of the signal, for any second value, when all of the analytes are present.

In some cases, an assay provided in this disclosure is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M=(P*T)+1$, where P is the number of codes per tier in a coding scheme and T is the number of tiers. In some cases, the number of tiers $T=\log_4 (F+1)$, and F is the maximum cumulative value of a first component of a signal, for any second value, when all of the analytes are present.

In some examples, the first value is an intensity or range of intensities. In some cases, a coding scheme comprises at least three intensities or ranges of intensities.

In some cases, the second value is a wavelength or range of wavelengths. In some examples, the coding scheme comprises at least five wavelengths or ranges of wavelengths.

In some examples, the signal is an electromagnetic signal. In some cases, the electromagnetic signal is a fluorescence emission signal. In some examples, the intensity of the fluorescence emission signal is measured at at least four wavelengths or ranges of wavelengths.

In some cases, an assay provided herein is performed with reagents that are lyophilized prior to use.

In some cases, a detecting step is performed with reagents comprising hybridization probes. In some examples, the number of the hybridization probes is greater than the number of analytes. In some cases, the set of hybridization probes comprise one or more hybridization probes specific for different analytes and comprising an identical fluorophore or combination of fluorophores. In some examples, a sample is contacted with at least 18 of said hybridization probes. In some examples, a detecting step is performed with reagents comprising at least one pair of primers. In some cases, at least one pair of primers are capable of amplifying a region complementary to at least three of said hybridization probes.

In some examples, the signal that is measured is generated during a polymerase chain reaction. In some cases, the polymerase chain reaction is selected from the group consisting of an end-point polymerase chain reaction, a real-time polymerase chain reaction, a digital polymerase chain reaction, and combinations thereof.

In some examples, at least one cumulative measurement is performed on a solution.

In some examples, at least one analyte is encoded by at least one additional value (i.e., at least two values together), wherein the at least one additional value is selected from the group consisting of a value from at least one additional component of a signal, a value from at least one component of a different signal, and combinations thereof. For example, the at least one additional value may be selected from the group consisting of a fluorescence emission intensity, a fluorescence emission wavelength, a Förster resonance energy transfer (FRET) emission intensity, a FRET emission wavelength, an electrochemical signal, a chemiluminescence wavelength, a chemiluminescence intensity, a fluorescence bleaching rate, a chemiluminescence bleaching rate, and combinations thereof.

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In some cases, a chromatogram is constructed. The chromatogram may be constructed by plotting all possible combinations of first values and second values for positive control samples for each analyte.

In some examples, at least one of the analytes comprises a polynucleotide. In some cases, the polynucleotide is from a source selected from the group consisting of an animal, a plant, a bacteria, a fungus, a parasite, and a virus. In some examples, the polynucleotide is from a source selected from the group consisting of human immunodeficiency virus, herpes simplex virus, human papilloma virus, *Plasmodium, Mycobacterium*, dengue virus, hepatitis virus, and influenza virus. In some cases, the polynucleotide is selected from the group consisting of human immunodeficiency virus polyprotease, human immunodeficiency virus p17, human papilloma virus E6, and human papilloma virus E7.

In some cases, a sample is selected from the group consisting of a clinical sample, a food sample, an environmental sample, a pharmaceutical sample, and a sample from a consumer product.

In some examples, information concerning the presence or absence of an analyte is transmitted through a computer network.

In some cases, information concerning the presence or absence of an analyte is provided to a physician. In some examples, a clinical decision is made based on such information.

In some examples, at least one step of a method provided herein is performed using instructions on a computer readable medium. In some cases, the instructions are located on a remote server. In some examples, the instructions are located on a thermal cycler. In some cases, the instructions are located on a computer in communication with a thermal cycler.

In some cases, at least one of the analytes is a positive control analyte.

In some examples, the coding scheme is non-degenerate. In some cases, the coding scheme is designed to be non-degenerate. In some examples, the coding scheme is made non-degenerate by enumerating every legitimate result that can be obtained from the coding scheme, identifying each legitimate result that is degenerate, and eliminating at least one potential analyte code from the coding scheme to eliminate degeneracy.

In some cases, an assay provided herein is an end-point assay. In some examples an assay provided in this disclosure is ended at a threshold number of cycles set by the limit of detection of an instrument.

In certain examples, an assay provided herein is a liquid phase assay.

In some cases, an assay provided herein is quantitative.

In some cases, this disclosure provides a method of detecting the presence or absence of each analyte of a plurality of analytes, comprising: (a) encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy; (b) providing a sample comprising, or potentially comprising, at least one of said analytes; (c) contacting said sample with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present; (d) cumulatively measuring said first values of said signal within said sample, thereby providing a cumulative measurement; and (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme.

In some examples, the method described in the preceding paragraph is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M=\log_2(F+1)$ and F is the maximum cumulative value of the first values when all of the analytes are present. In some cases, the first value is an intensity or range of intensities. In some examples, the first value has a minimum value in the coding scheme that is selected from the group consisting of at least 1, at least 2, at least 4, at least 8, at least 16, at least 32, and at least 64. In some cases, the first value is incremented in the coding scheme by an amount equal to the cumulative maximum of said preceding first values plus one. In other cases, the first value is incremented in the coding scheme by an amount greater than the cumulative maximum of the preceding first values plus one.

In some cases, this disclosure provides a method of detecting the presence or absence of each analyte of a plurality of analytes, comprising: (a) encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy; (b) providing a sample comprising, or potentially comprising, at least one of said analytes; (c) contacting said sample with analyte-specific reagents that generate said at least one first value and at least one said second value, as represented in said coding scheme, when each of said analytes is present; (d) cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme, wherein, when degeneracy is eliminated, said method is capable of unambiguously detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

In some cases, each of the analytes of the method described in the preceding paragraph is encoded as a first value in a first component of the signal at each of a plurality of second values in a second component of the signal. In some cases, when degeneracy is eliminated, this method is capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single volume using four second values. In some examples, when degeneracy is eliminated, the coding scheme comprises T non-degenerate tiers, wherein $T=\log_4(F+1)$ and F is the maximum cumulative value of the first component of the signal, for any second value, when all of the analytes are present. In some cases, when degeneracy is eliminated, the method is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M=(P*T)+1$ and P is the number of codes per tier. In some examples, when degeneracy is eliminated, the method is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M=C*\log_2(F+1)$, C is the number of second values in the coding scheme, and F is the maximum cumulative value of the first component of the signal, for any second value, when all of the analytes are present.

This disclosure also provides non-degenerate coding schemes capable of unambiguously encoding the presence

or absence of each of at least 7 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis.

In some examples, a non-degenerate coding scheme of this disclosure is generated by a method comprising: (a) generating a code for each potential analyte, wherein each potential analyte is encoded by at least one value of at least one component of a signal; (b) enumerating every legitimate cumulative result for all possible combinations of presence or absence of each analyte; (c) identifying each legitimate result that is degenerate; and (d) eliminating at least one code to eliminate degeneracy. In some cases, the coding scheme may be expanded by adding additional codes that are at least one unit greater than the sum of all previous codes in the at least one component of said signal. In some cases a coding scheme is generated by a method of mathematical iteration that guarantees non-degeneracy by construction.

In some cases, this disclosure provides systems for detecting the presence or absence of each analyte of a plurality of analytes, comprising: (a) encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy; (b) providing a sample comprising, or potentially comprising, at least one of said analytes; (c) contacting said sample with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present; (d) cumulatively measuring said first values of said signal within said sample, thereby providing a cumulative measurement; and (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme.

In some cases, this disclosure provides systems for detecting the presence or absence of each analyte of a plurality of analytes, comprising: (a) encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy; (b) providing a sample comprising, or potentially comprising, at least one of said analytes; (c) contacting said sample with analyte-specific reagents that generate said at least one first value and said at least one second value, as represented in said coding scheme, when each of said analytes is present; (d) cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme, wherein, when degeneracy is eliminated, said system is capable of unambiguously detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

In some cases, this disclosure provides methods of detecting the presence or absence of each analyte of a plurality of analytes for a third party, comprising: (a) obtaining the identity of each of said analytes from a party; (b) encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy; (c) providing said party with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present, said party: (i) contacting a sample comprising, or potentially comprising, at least one of said analytes with said reagents; and (ii) cumulatively measuring said first values within said sample, thereby providing a cumulative measurement; and (d) obtaining said cumulative measurement from said party; (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme; and (f) providing said party with information about the presence or absence of each of said analytes.

In some cases, this disclosure provides methods of detecting the presence or absence of each analyte of a plurality of analytes for a third party, comprising: (a) obtaining the identity of each of said analytes from a party; (b) encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy; (c) providing said party with analyte-specific reagents that generate said at least one first value and said at least one second value, as represented in said coding scheme, when each of said analytes is present, said party: (i) contacting a sample comprising, or potentially comprising, at least one of said analytes with said reagents; and (ii) cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and (d) obtaining said cumulative measurement from said party; (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme; and (f) providing said party with information about the presence or absence of each of said analytes.

In some cases, this disclosure provides compositions for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, each reagent generating a signal comprising a first value, wherein said reagents are capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, mass spectrometry, or melting curve analysis.

In some cases, this disclosure provides compositions for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, each reagent generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said reagents are capable of detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

In some cases, this disclosure provides kits for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, packaging, and instructions, each reagent generating a signal comprising a first value, wherein said kit is capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, mass spectrometry, or melting curve analysis.

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In some examples, this disclosure provides kits for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, packaging, and instructions, each reagent generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said kit is capable of detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

In some cases, this disclosure provides kits for detecting the presence or absence of each analyte of a plurality of analytes, comprising a kit body (**601**), clamping slots arranged in the kit body for placing bottles, a bottle comprising probes for the detection of an analyte (**602**), a bottle comprising primers for amplification (**603**), and a bottle comprising reagents for amplification (**604**), wherein said kit is capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, mass spectrometry, or melting curve analysis.

In some examples, this disclosure provides kits for detecting the presence or absence of each analyte of a plurality of analytes, a kit body (**601**), clamping slots arranged in the kit body for placing bottles, a bottle comprising probes for the detection of an analyte (**602**), a bottle comprising primers for amplification (**603**), and a bottle comprising reagents for amplification (**604**), each probe generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said kit is capable of detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said probes generates only one second value.

INCORPORATION BY REFERENCE

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

FIG. **1** shows a comparison between a traditional encoding method of detecting four analytes with four colors and an encoding method of the invention able to detect 16 or more sequences with four colors. Colors are indicated by B, G, Y, and R, which indicate blue, green, yellow, and red, respectively.

FIG. **2** shows a schematic representation of detection of six analytes (including control) with four colors. The analytes are detected using hybridization probes attached to a fluorophore (B, G, Y, R; blue, green, yellow, and red, respectively) and a quencher (oval with "X"). The control sequence and the five other sequences (Dengue Fever=dengue virus; Tuberculosis=*Mycobacterium tuberculosis*; P17=HIV p17; Malaria=*Plasmodium falciparum*; and Herpes=herpes simplex virus 2) are all detected using a probe labeled with a blue fluorophore. The non-control analytes are each detected using 1-3 additional probes. For example, the dengue virus analyte is detected using three additional probes with green, yellow, and red fluorophores; the herpes simplex virus 2 analyte is detected using one additional probe with a red fluorophore; and so on.

FIG. **3** shows chromatograms of experimental results obtained as described in Example 2.

FIG. **4** shows an exemplary embodiment of the invention in which a computer is used to perform one or more steps of the methods provided herein.

FIG. **5** shows a schematic of two different methods of detecting wavelength and intensity of a light signal, such as a fluorescence emission signal.

FIG. **6** shows a schematic of exemplary kits.

FIG. **7** shows chromatograms of experimental results obtained as described in Example 3.

FIG. **8** shows a schematic of a legitimate result (top) and an illegitimate result (bottom).

DETAILED DESCRIPTION OF THE INVENTION

Fluorescence detection has been a preferred technique for multiplexed assays because of several desirable features, including compatibility with biochemical assays, the relatively small size of fluorescent labels, simple means of conjugation to molecules of interest, affordability, low toxicity, stability, robustness, detectability with inexpensive optics, and an ability to be combined with spatial arrays. However, standard fluorophores have wide emission spectra. Therefore, in order to avoid spectral overlap (i.e., to preserve spectral resolution) only a relatively small number of colors (e.g., 4 to 6) are typically used simultaneously in multiplexed fluorescent assays.

The traditional encoding method for multiplexed fluorescent assays has been to encode each analyte with a single color; i.e., M=N, where M is the number of analytes that can be detected and N is the number of spectrally resolved fluorescent probes. Whenever higher factors of multiplexing are required (i.e., M>N), fluorescence is generally combined with other techniques, such as aliquoting, spatial arraying, or sequential processing. These additional processing steps are labor-intensive and frequently require relatively expensive and complex optical and mechanical systems (e.g., spectrometers, mechanized microscopy stages, microfluidics, droplet generators, scanners, and the like). Such systems are often impractical to deploy in certain settings, particularly point-of care and low-resource settings. Thus, there is a significant need for multiplexed encoding and decoding methods that can provide an inexpensive means of multiplexing while avoiding the use of expensive additional processing steps.

This disclosure provides methods, systems, compositions, and kits for the detection of multiple analytes in a sample. Analytes are detected based on the encoding, analysis, and decoding methods presented herein. In some examples, each analyte to be detected is encoded as a value of a signal (e.g., intensity), where the values are assigned so that the results of the assay unambiguously indicate the presence or absence of the analytes being assayed. In other examples, each analyte to be detected is encoded as a value in each of at least two components of a signal (e.g., intensity and wavelength).

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The at least two components of a signal may be orthogonal. Similarly, as described more fully elsewhere in this disclosure, multiple orthogonal signals may be used, such as a combination of a fluorescent signal and an electrochemical signal. The analyte may be any suitable analyte, such as a polynucleotide, a protein, a small molecule, a lipid, a carbohydrate, or mixtures thereof. The signal may be any suitable signal such as an electromagnetic signal, a light signal, a fluorescence emission signal, an electrochemical signal, a chemiluminescent signal, and combinations thereof. The at least two components of the signal may be any suitable two components, such as an amplitude and a frequency or an intensity and a wavelength.

After encoding of the analytes, a sample is provided wherein the sample comprises or may comprise at least one of the encoded analytes. The sample is contacted with an analyte-specific reagent or reagents that generate a particular signal, as specified for each analyte in the coding scheme, in the presence of an analyte. A reagent may be any suitable reagent that is capable of generating such a signal in the presence of its corresponding analyte, for example, an oligonucleotide probe attached to a fluorophore and a quencher (e.g., a TAQMAN probe). If the reagent is an oligonucleotide probe attached to a fluorophore and a quencher, a nucleic acid amplification may be performed to generate the signal.

After addition of the reagent(s), the signal is quantified. In some cases, this quantification is performed by measuring one component of the signal (e.g., fluorescence intensity) and determining the presence and absence of certain analytes based on the values used to encode the presence of each analyte and the cumulative value of the signal.

In some cases, at least two components of a signal (e.g., intensity and wavelength) are cumulatively measured for the sample. This measurement can be performed, for example, by measuring the intensity at a particular wavelength or the intensity within a particular range of wavelengths. The presence or absence of an analyte may then be determined based on the values of each of the at least two components of the signal and the values used to encode the presence of the analyte (i.e., those values in the coding scheme).

The encoding may be performed in a manner that reduces or eliminates the number of possible degenerate (e.g., ambiguous) results that can be obtained by the method. As described elsewhere in this specification, the full coding capability of a particular coding scheme may be enumerated, and certain potential analyte codes may be eliminated from the coding scheme to reduce or eliminate degeneracy. Similarly, a coding scheme may be designed to be non-degenerate, so that a reduction or elimination of degeneracy is not necessary. A decoding matrix may be constructed to translate cumulative signal measurements (e.g., intensities or intensities at particular wavelengths) into the presence or absence of certain analytes, corresponding to the constituent signals of the cumulative signal measurement.

I. Definitions

The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

As used herein, the singular forms "a," "an," and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise. Furthermore, to the extent that the terms "including," "includes," "having," "has," "with," "such as," or variants thereof, are used in either the specification and/or the claims, such terms are not limiting and are intended to be inclusive in a manner similar to the term "comprising".

The term "about," as used herein, generally refers to a range that is 15% greater than or less than a stated numerical value within the context of the particular usage. For example, "about 10" would include a range from 8.5 to 11.5.

The terms "dimension" and "component," as used herein when referring to a signal, generally refer to an aspect of the signal that may be quantified. For example, if a signal is generated by a fluorescent molecule, it may be quantified in terms of its wavelength (e.g., a first dimension or component) and its intensity (e.g., a second dimension or component).

The term "decoding," as used herein, generally refers to a method of determining which analytes are present based on the cumulative signal and a coding scheme or decoding matrix that enables the conversion of a cumulative signal to information concerning the presence or absence of one or more analytes.

The term "encoding," as used herein, generally refers to the process of representing an analyte using a code comprising values of a signal, such as intensity, or values in each of at least two components of a signal or signals, such as wavelength and intensity.

The terms "oligonucleotide probe attached to a fluorophore and a quencher" and "TAQMAN probe" generally refer to hydrolysis probes used to detect the presence of an analyte in a polynucleotide amplification assay. These probes comprise an oligonucleotide probe attached to a fluorophore and a quencher. So long as the quencher and the fluorophore are in proximity, the quencher quenches the fluorescence emitted by the fluorophore upon excitation by a light source. The sequence of the oligonucleotide probe is designed to be complementary to a polynucleotide sequence present in an analyte, and therefore capable of hybridizing to the polynucleotide sequence present in the analyte. Hybridization of the oligonucleotide probe is performed in a nucleic acid amplification reaction comprising primers (e.g., a polymerase chain reaction). Upon extension of the primers by a DNA polymerase, the 5' to 3' exonuclease activity of the polymerase degrades the probe, releasing the fluorophore and the quencher into the medium. The proximity between the fluorophore and the quencher is broken and the signal from the fluorophore is no longer quenched. Thus, the amount of fluorescence detected is a function of the amount of analyte present. If no analyte is present, the probe will not hybridize to an analyte, and the fluorophore and quencher will remain in close proximity. Little or no signal will be produced.

The term "orthogonal," as used herein, generally refers to at least two components of a signal (e.g., wavelength and intensity), or at least two different signals (e.g., fluorescence emission and electrochemical signal), that can be varied independently or approximately independently. For example, wavelength and intensity are considered orthogonal or approximately orthogonal when fluorescent molecules are used. Among other factors, the wavelength of fluorescence emission will depend on the composition of the fluorescent molecule and the intensity of the fluorescence will depend on the amount of molecule present. Although wavelength and intensity are examples of two components of a signal that can be varied approximately independently, the methods described herein are not limited to components that can be varied independently or approximately independently. Components of a signal, or signals, that vary non-independently may also be used, so long as the components

or signals are characterized well enough to enable encoding, measurement, and decoding. For example, if the variance in one component or signal affects the variance in another component or signal, the two components or signals may still be used so long as the relationship between the variances is understood.

The terms "polynucleotide," "oligonucleotide," or "nucleic acid," as used herein, are used herein to refer to biological molecules comprising a plurality of nucleotides. Exemplary polynucleotides include deoxyribonucleic acids, ribonucleic acids, and synthetic analogues thereof, including peptide nucleic acids.

The term "probe," as used herein, generally refers to a reagent capable of generating a signal in the presence of a particular analyte. A probe generally has at least two portions: a portion capable of specifically recognizing an analyte, or a portion thereof, and a portion capable of generating a signal in the presence of an analyte, or a portion thereof. A probe may be an oligonucleotide probe attached to a fluorophore and a quencher, as described above and elsewhere in this disclosure. A probe may also be any reagent that generates a signal in the presence of an analyte, such as an antibody that detects an analyte, with a fluorescent label that emits or is quenched upon binding of the antibody to an analyte. Any suitable probe may be used with the methods presented in this disclosure, so long as the probe generates a quantifiable signal in the presence of an analyte. For example, the analyte-specific portion of a probe may be coupled to an enzyme that, in the presence of an analyte, converts an uncharged substrate into a charged product, thereby increasing the electrical conductivity in the medium over time. In this case, different analytes may be encoded by coupling the analyte-specific portion of the probe (e.g., hybridization probe or antibody) to an enzyme at different ratios. The resulting rate of increased conductivity in the medium will be cumulative for all analytes present in the medium. Encoding analytes according to the methods provided herein enables conversion of the conductivity measurements into unambiguous (i.e., non-degenerate) results providing information about the presence or absence of particular analytes. Similarly, a probe may comprise an enzyme producing a chemiluminescent product from a substrate. The amount of chemiluminescence may then be used to encode the presence of particular analytes.

II. Encoding and Decoding Methods

A. Traditional Fluorescent Encoding and Decoding Method

A commonly used method of determining the presence of an analyte uses four spectrally resolved fluorescent molecules to indicate the presence or absence of four analytes. An example of this method is presented on the left-hand side of FIG. **1**. The left-hand side of FIG. **1** shows an encoding method where four analytes (Seq 1, Seq 2, Seq 3, and Seq 4) are each encoded by a single color (blue, green, yellow, and red, respectively). The color represents a fluorophore attached to an oligonucleotide probe that also comprises a quencher. In the system shown on the left-hand side of FIG. **1**, there are four different oligonucleotide probes, each comprising a single fluorophore (blue, green, yellow, or red) and a quencher. The presence or absence of an analyte is determined based on the presence or absence of a signal in a particular color.

The chart on the left-hand side of FIG. **1** shows intensity versus color for a hypothetical sample containing two analytes: Seq 1 and Seq 3. The presence of these analytes is determined based on the measurement of a blue signal (corresponding to Seq 1) and a yellow signal (corresponding to Seq 3). The absence of Seq 2 and Seq 4 is indicated by the absence of a blue and red signal.

Table 1 shows a translation of this coding scheme into a binary format. Each analyte is encoded as a value in each of two components of a fluorescent signal: (1) color (also known as wavelength; or range of wavelengths) and (2) intensity (indicated by the numbers within the table). For example, A (e.g., Seq 1) has a color of blue and an intensity of 1; B (e.g., Seq 2) has a color of green and an intensity of 1; and so on. The intensity of the signal within each color range may be quantified as described herein, for example by measuring the intensity of the signal within a particular wavelength range determined by a band pass filter. A result of 1000 indicates that only analyte A is present; a result of 1100 indicates that analytes A and B are present; and so on.

TABLE 1

Traditional encoding of four analytes with four probes, each probe having a single color, and one probe per analyte.

	Blue	Green	Yellow	Red
A	1	0	0	0
B	0	1	0	0
C	0	0	1	0
D	0	0	0	1

B. Encoding Methods Using More than One Color Per Analyte

The traditional method described above suffers from the fact that it is limited by the number of spectrally resolvable fluorophores. More specifically, the number of detectable analytes is equal to the number of spectrally resolvable fluorophores. Therefore, the number of analytes may only be increased by increasing the number of spectrally resolvable fluorophores.

This disclosure provides methods that overcome this limitation. More specifically, in some cases, by utilizing at least two components of a signal during encoding, the methods described herein may be used to detect more than one analyte per fluorophore. For example, using the method provided herein 1.5, 1.6, 1.7, 1.8, 1.9, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes may be detected per fluorophore. In some cases, the methods provided herein may be used to detect at least 1.5, 1.6, 1.7, 1.8, 1.9, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes may be detected per fluorophore. In some cases, the methods provided herein may be used to detect 1.5-2, 2-4, 1.5-4, 4-6, 2-6, 6-10 analytes per fluorophore.

In some cases, the methods provided in this disclosure may include the use of a control color. The control color may be attached to one or more probes binding a positive control analyte, and each analyte to be detected, in a sample. If the same sequence occurs in the positive control analyte and each analyte to be detected, a single control probe may be used. If the same sequence does not occur in the positive control analyte and each analyte to be detected, different probes may be used, but each probe may still be attached to the control color.

For example, building on the traditional methods described above, one color (e.g., blue) may be used to encode the presence of a control analyte that is always

present in the sample. The control analyte may be added to the sample, or may be inherently present in the sample. The remaining colors (e.g., green, yellow, red) may be used to encode the presence of additional analytes. Table 2 shows an example of one such method.

TABLE 2

Encoding of four analytes, including control, with four colors and up to two colors per analyte.

	Blue	Green	Yellow	Red
Ctrl. (p)	1	0	0	0
A	1	0	0	1
B	1	0	1	0
C	1	1	0	0

In Table 2, the presence of the control (p) is indicated by a result of 1000. The presence of the control and analyte A is indicated by a result of 2001. The presence of the control and all three other analytes is indicated by a result of 4111. The control color (blue) provides an indication that the assay is functioning properly. The intensity of the blue color reports the number of analytes that are present in a test sample. Of course, any color may be used as the control color. One of skill in the art will recognize that certain practical considerations might make it preferable to use one color over another as the control color. For example, if one color is better detected in a particular optical system (or system of fluorophores), it might be practically preferable to use that color as a control color.

The coding scheme shown in Table 2 encodes each analyte using one control color and one additional color (up to 2 probes per analyte). However, as shown below, the number of analytes that can be encoded increases when up to 4 colors are used per analyte. Table 3 shows an exemplary coding scheme where each analyte is encoded by up to 4 colors. In the scheme shown in Table 3, the control color (blue) is generated in the presence of the control sequence and each of the other seven analytes (A-G). The other seven analytes are each encoded by the presence of one to three additional colors. The colors may be contained on different probes (e.g., oligonucleotide probes attached to a fluorophore and a quencher) or on the same probe (which can have multiple fluorophores and multiple quenchers).

TABLE 3

Encoding of eight analytes (including control) with four colors and up to four colors per analyte.

	Blue	Green	Yellow	Red
Ctrl. (p)	1	0	0	0
A	1	0	0	1
B	1	0	1	0
C	1	1	0	0
D	1	0	1	1
E	1	1	0	1
F	1	1	1	0
G	1	1	1	1

In Table 3, the presence of the control and all seven other analytes is indicated by a result of 8444. Three analytes (A, B, and C) are encoded by two colors. Three analytes (D, E, and F) are encoded by three colors, and one analyte (G) is encoded by four colors. The presence of the control (p) is indicated by a result of 1000. The presence of the control and analyte A is indicated by a result of 2001. The presence of the control and analyte G is indicated by a result of 2111. These results express the cumulative intensity of the signal in each color. For example, the result 2111 has a 2X signal intensity in the blue channel, while the result 1000 has only a 1 X signal intensity in the blue channel.

Using Table 3, each of the possible cumulative assay results can be enumerated, in terms of color (blue, green, yellow, red) and intensity (0-8). Table 4 shows a "decoding matrix" generated by enumerating each of the possible cumulative assay results based on the encoding method presented in Table 3 and providing the corresponding decoded result of each assay in terms of the analyte(s) present in the sample. A similar decoding matrix may be generated for any coding scheme described herein, by enumerating each of the possible cumulative assay results based on the coding scheme and providing the corresponding decoded result of each assay in terms of the analyte(s) present in (or absent from) the sample. In some cases, as described below, one or more analytes may be removed from the coding scheme in order to reduce or eliminate degeneracy.

TABLE 4

Decoding matrix for encoding method presented in Table 3.

Cumulative Assay Result				
Blue	Green	Yellow	Red	Analyte(s) Present
1	0	0	0	p
2	0	0	1	pA
2	0	1	0	pB
2	1	0	0	pC
2	0	1	1	pD
2	1	0	1	pE
2	1	1	0	pF
2	1	1	1	pG
3	0	1	1	pAB
3	1	0	1	pAC
3	0	1	2	pAD
3	1	0	2	pAE
3	1	1	1	pAF, pCD, pBE
3	1	1	2	pAG, pDE
3	1	1	0	pBC
3	0	2	1	pBD
3	1	2	0	pBF
3	1	2	1	pBG, pDF
3	2	0	1	pCE

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TABLE 4-continued

Decoding matrix for encoding method presented in Table 3.

Cumulative Assay Result				
Blue	Green	Yellow	Red	Analyte(s) Present
3	2	1	0	pCF
3	2	1	1	pCG, pEF
3	1	2	2	pDG
3	2	1	2	pEG
3	2	2	1	pFG
4	1	1	1	pACB
4	0	2	2	pABD
4	2	2	0	pBCF
4	2	0	2	pACE
4	1	1	2	pABE, pACD
4	1	2	1	pABF, pBCD
4	2	1	1	pACF, pBCE
4	1	2	2	pADF, pABG, pBDE
4	2	1	2	pACG, pCDE, pAEF
4	2	2	1	pBCG, pBEF, pCDF
4	2	2	2	pCDG, pAFG, pBEG, pDEF
4	1	1	3	pADE
4	1	3	1	pBDF
4	3	1	1	pCEF
4	1	2	3	pADG
4	3	2	1	pCFG
4	2	1	3	pAEG
4	2	3	1	pBFG
4	1	3	2	pBDG
4	3	1	2	pCEG
4	2	2	3	pDEG
4	3	2	2	pEFG
5	1	2	2	pABCD
5	2	1	2	pABCE
5	2	2	1	pABCF
5	2	2	2	pABCG, pABEF, pBCDE, pACDF
5	1	2	3	pABDE
5	2	1	3	pACDE
5	1	3	2	pABDF
5	2	1	3	pBCDF
5	3	1	2	pACEF
5	3	2	1	pBCEF
5	2	2	3	pABEG, pACDG, pADEF
5	2	3	2	pABFG, pBCDG, pBDEF
5	3	2	2	pACFG, pBCEG, pCDEF
5	1	3	3	pABDG
5	3	1	3	pACEG
5	3	3	1	pBCFG
5	2	3	3	pADFG, pBDEG
5	3	3	2	pBEFG, pDEFG
5	3	2	3	pCDEG, pAEFG
5	2	2	4	pADEG
5	2	4	2	pBDFG
5	4	2	2	pCEFG
5	3	3	3	pDEFG
6	2	2	3	pABCDE
6	2	3	2	pABCDF
6	3	2	2	pABCEF
6	2	3	3	pABCDG, pABDEF
6	3	2	3	pABCEG, pACDEF
6	3	3	2	pABCFG, pBCDEF
6	4	3	2	pBCEFG
6	4	2	3	pACEFG
6	3	3	3	pABEFG, pACDFG, pBCDEG
6	3	4	2	pBCDFG
6	2	4	3	pABDFG
6	3	2	4	pACDEG
6	2	3	4	pABDEG
6	4	3	3	pCDEFG
6	3	4	3	pBDEFG
6	3	3	4	pADEFG
7	4	4	3	pBCDEFG
7	4	3	4	pACDEFG
7	3	4	4	pABDEFG
7	4	3	3	pABCEFG
7	3	4	3	pABCDFG
7	3	3	4	pABCDEG

TABLE 4-continued

Decoding matrix for encoding method presented in Table 3.

Cumulative Assay Result				
Blue	Green	Yellow	Red	Analyte(s) Present
7	3	3	3	pABCDEF
8	4	4	4	pABCDEFG

The decoding matrix provided in Table 4 is constructed using two assumptions. First, the decoding matrix assumes that the positive control (p) always produces a positive outcome. Second, the decoding matrix assumes that, within each color, the intensity is additive and scales in the same way with changing probe concentration, regardless of which probe the signal may come from. This essentially means that the signals are additive and digital. The conditions underlying these assumptions may be met by properly preparing the assay. If a fluorescent signal is used, the intensity need only be approximately additive and digital, as demonstrated in the Examples provided herein.

Table 4 allows the conversion of a cumulative measurement of intensity in four ranges of fluorescent wavelengths (i.e., signal intensity within each color range), into the corresponding analytes present in the sample. For example, a result of 4321 indicates that p, C, F, and G are present and the other analytes are not present. This result is referred to as a "legitimate" result, because it is present in the decoding matrix. By contrast, a result of 4000, while possible to measure, does not occur in the decoding matrix. More specifically, the result of 4000 cannot be achieved by adding any combination of control and analyte codes from Table 3. This result is referred to as an "illegitimate" result. An illegitimate result may indicate that the assay malfunctioned. Thus, the control (p) and the decoding matrix provide a means of verifying that the assay is functioning properly.

Table 4 is exhaustive for any combination of fluorophores generating four resolvable emission spectra (e.g., colors). The term "rank" is used to describe the number of detected analytes, including the control. In the example provided above, if the assay functions properly, the rank is equal to the value of the blue signal. For example, a rank of 8 indicates that the control and all seven other analytes (A-G) are present. A rank of 2 indicates that the control and only one analyte are present. The lowest rank is a rank of 1, which indicates that only the control is present. The number of possibilities at each rank can be enumerated. For example, continuing to refer to the encoding and decoding method described in Tables 3-4, there are 7 possibilities for rank 2. The number of possibilities at rank 3 can be calculated as a combination of 7 take 2, or 7!/(5!*2!)=21 possibilities. More generally, the number of possibilities for a combination of N take K is N!/((N−K)! *K!). Analogously, at ranks 4, 5, 6, 7, and 8, the number of possibilities is 35, 35, 21, 7, and 1, respectively. Referring to Table 4 shows that the table agrees with the theoretical prediction. Thus, Table 4 is an exhaustive decoding matrix for the encoding method provided in Table 3.

C. Reducing or Eliminating Degeneracy

The terms "degenerate" and "degeneracy," as used herein, generally describe a situation where a legitimate result is not definitive, because it can indicate more than one possibility in terms of the presence or absence of an analyte. For example, with reference to Table 4, result 5233 is degenerate because it can be decoded as either pADFG or pBDEG. Similarly, result 4222 is degenerate because it can be decoded as any of the following: pCDG, pAFG, pBEG, pDEF. By contrast, result 3110 can only indicate pBC and thus is not degenerate.

This disclosure provides methods of reducing or eliminating degeneracy, thereby increasing the confidence with which an analyte is detected. In one embodiment, degeneracy is eliminated by a method comprising (i) encoding each potential analyte to be detected as a value of a signal and, optionally, as a value in each of at least two components of a signal; (ii) enumerating every legitimate result that can be obtained from the coding scheme; (iii) identifying each legitimate result that is degenerate; and (iv) eliminating at least one potential analyte (or potential analyte code) from the coding scheme, wherein eliminating the at least one potential analyte reduces or eliminates degeneracy. For example, with reference to the coding scheme described in Table 3 and the decoding matrix described in Table 4 (enumerating every legitimate result), eliminating any two of analytes D, E, and F eliminates the degeneracy. Eliminating any one of analytes D, E, and F would not eliminate the degeneracy, but would reduce it.

With continued reference to the coding scheme described in Table 3, eliminating any two of analytes D, E, and F from the coding scheme results in a scheme where six analytes (including control) can be analyzed, with no degeneracy, using only 4 colors. By contrast, conventional methods of multiplexing would allow for only the reporting of 3 analytes and 1 control using 4 colors. Therefore, the number of analytes that can be analyzed is nearly doubled by using the methods provided herein.

FIG. **2** shows an exemplary embodiment of the invention in which 4 colors are used to detect five analytes and a control. The analytes are nucleic acids from dengue virus ("Dengue Fever"), *Mycobacterium tuberculosis* ("Tuberculosis"), human immunodeficiency virus (HIV) p17 (P17), *Plasmodium* ("Malaria"), and herpes simplex ("Herpes"). The "colors" in this example are fluorophores attached to oligonucleotide probes that also comprise a quencher. The oligonucleotide probes will generally be different for different analytes, whereas the color is the same. For example, all probes designated as Probe 1 have a blue color but generally will have a different oligonucleotide sequence. Of course, probes designated as Probe 1 could also have the same oligonucleotides sequence, if the complementary sequence was present in each of the analytes. The mechanism of detection with these probes is described elsewhere in this disclosure. Probe 1 (blue) hybridizes to all six analytes, including control. Probe 2 (green) hybridizes to analytes from dengue virus, *Mycobacterium*, and HIV p17. Probe 3 (yellow) hybridizes to analytes from dengue fever and *Plasmodium*. Probe 4 hybridizes to analytes from dengue virus, *Mycobacterium*, and herpes simplex. The coding scheme defined by these probes is illustrated in Table 5. With reference to Table 3, potential analytes D (1011) and F (1110) have been eliminated from the coding scheme. There-

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fore, the coding scheme presented in Table 5 is non-degenerate and each legitimate result from the assay corresponds to the presence or absence of a unique combination of analytes in a sample.

TABLE 5

Coding scheme for detection of dengue virus, *Mycobacterium*, HIV, *Plasmodium*, and herpes simplex, as exemplified in FIG. 2.

	Blue	Green	Yellow	Red
Ctrl. (p)	1	0	0	0
Herpes Simplex	1	0	0	1
Plasmodium	1	0	1	0
HIV	1	1	0	0
Mycobacterium	1	1	0	1
Dengue Virus	1	1	1	1

The methods for encoding and decoding presented above, including the methods for eliminating degeneracy, are all equally applicable to encoding methods using additional colors. For example, Table 3 could be extended by including additional colors and additional intensities (described further below). The decoding matrix is then generated as described above, enumerating every legitimate result that can be obtained from the coding scheme. A decoding matrix analogous to the decoding matrix provided in Table 4 may be constructed for any coding scheme described in this disclosure. The legitimate results that are degenerate are then identified and at least one potential analyte code is eliminated from the coding scheme to reduce or eliminate degeneracy. The method of eliminating degeneracy may be carried out using software on a computer readable medium, or hardware configured to carry out the method (e.g., a microchip).

Although degeneracy can be reduced or eliminated by the methods described above, and elsewhere in this disclosure, this disclosure also provides coding schemes that are non-degenerate by design. For example, after elimination of degeneracy in the coding scheme described in Table 3, the coding scheme may be extended indefinitely in a non-degenerate manner where the non-degeneracy is by design (see, e.g., the coding scheme exemplified in Table 6, described more fully below). Similarly, this disclosure provides coding schemes that are completely non-degenerate by design and therefore do not require any reduction or elimination of degeneracy (see, e.g., the coding scheme exemplified in Table 8, described more fully below). Thus, the coding schemes provided in this disclosure may have reduced or eliminated degeneracy, or be non-degenerate by design.

D. Encoding Methods Using More than One Color and More than One Intensity

The encoding method described above (e.g., Tables 3-5) may be further extended by allowing analytes to be encoded by an intensity greater than 1. For example, each of the analytes encoded in the coding scheme provided in Table 5 is encoded by a fluorescence intensity of either 1 or 0. Allowing higher values for the signal intensity in at least one color further increases the number of analytes that can be encoded by any of the methods provided in this disclosure. In some examples, these higher intensity values may be assigned any color except for the control color, in order to maintain the analyte counting capability of the control color.

Table 6 shows the first three tiers of an exemplary coding scheme that utilizes four colors and multiple intensities. Tier 1 of Table 6 is a reproduction of Table 3, showing the encoding of seven analytes and a control with four colors. As described above, any two of potential analytes D, E, and F may be eliminated from the coding scheme in order to produce a non-degenerate coding scheme. Tier 1 of Table 6 indicates that potential analytes D (1011) and F (1110) have been eliminated from the coding scheme to eliminate degeneracy. Therefore, the coding scheme presented in Tier 1 of Table 6 is capable of determining the presence of five analytes and one control using four colors, as described above.

The coding scheme of Tier 1 of Table 6 may be expanded to a second tier (Tier 2) by allowing the intensity in any of the colors to increase. As described above, the intensity of the control color may be maintained at 1, in order to preserve the sequence counting capability of the control. Increasing the intensity of any of the remaining three colors will yield codes 100Y, 10Y0, 1Y00, 10YY, 1Y0Y, 1YY0, and 1YYY, where Y>1. The minimal value of Y for a new tier of encoding is equal to the cumulative maximum value from the prior tier(s) plus 1. As described below, a value greater than 1 could also be used, to maximize the differences between the intensities.

Thus, in this context, the term "tier" is generally used to describe a set of codes that fully utilize the coding capability provided by a particular number of first values (e.g., intensities) and second values (e.g., colors), without degeneracy. For example, Tier 1 of Table 6 fully utilizes the coding capability provided by four colors with up to one intensity in each color, without degeneracy. As shown in Table 6, Tier 1, this results in six encoded analytes, including the control. To introduce a second tier, the minimum value of Y (described above) may be incremented to equal the cumulative maximum result from the prior tier(s) plus one (or more than one). In the example provided in Table 6, the intensity of the blue (control) color is maintained as one, to preserve the sequence counting capability in this color. Thus, Tier 2 consists of five non-degenerate encoding possibilities obtained by incrementing the intensities in the green, yellow, and red channels to equal the cumulative maximum results in each of these channels from Tier 1, plus one. All possibilities of these codes may then be enumerated for Tier 2, and codes resulting in degeneracy may be eliminated, or Tier 2 may be made non-degenerate by design, using the information used to eliminate the corresponding codes from Tier 1. Further coding capacity may then be achieved by adding a third tier, or further tiers, which are constructed according to analogous methods. A coding scheme may have an infinite number of tiers, for example 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100 or more tiers.

More specifically, with reference to Table 6, Tier 2, analyte H is encoded by 1004. The value of the control color is maintained as 1. The value of the red color is equal to the cumulative maximum result from the prior tier (3) plus 1, or 4. Similarly, analytes I and J are encoded by 1030 and 1400, respectively. Combinations of these codes are used to encode the remaining four analytes (K-N), as was done for analytes D-G in Tier 1. This completes Tier 2. In Tier 2, the inclusion of potential analytes K (1034) and M (1430) results in degeneracy. Therefore, these analytes have been eliminated from the coding scheme, to eliminate degeneracy.

Continuing to refer to Table 6, a third tier (Tier 3) is constructed using the same principles described above. Analyte O is encoded by 1-0-0-16. The value of the control color is still maintained as 1. The value of the red color is equal to the cumulative maximum result from the prior tiers (15) plus 1, or 16. Similarly, analytes P and Q are encoded

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by 1-0-9-0 and 1-16-0-0, respectively. Combinations of these codes are used to encode the remaining four analytes (R-U), as was done for analytes D-G in Tier 1 and analytes K-N in Tier 2. In Tier 3, the inclusion of potential analytes R (1-0-9-16) and T (1-16-9-0) results in degeneracy. Therefore, these analytes have been eliminated from the coding scheme, to eliminate degeneracy.

The three-tier coding scheme shown in Table 6 shows the encoding of 15 analytes and one control using four colors. This coding scheme may be indefinitely extended, by adding more intensities to generate additional tiers and/or adding more colors, to generate additional coding capability within the tiers. The methods of reducing or eliminating degeneracy, as described in this disclosure, may be used with the addition of each intensity and/or color, to reduce or eliminate degenerate results.

More generally, the coding scheme depicted in Table 6 is a non-degenerate, infinite extension of the coding scheme depicted in Tables 3-5. The maximum intensity of the cumulative measurement at the first tier is 6. The maximum intensity of the cumulative measurement at the second tier is 15. The maximum intensity of the cumulative measurement at the third tier is 63, and so on. Given a maximum cumulative intensity value (F), the maximum number of tiers (T) available in this coding scheme is $T=\log_4(F+1)$. The coding scheme depicted in Table 6 provides five non-degenerate codes per tier (P). Thus, the maximum number of codes $M=5*\log_4(F+1)$, or $M=P*T$, where P is the number of non-degenerate codes per tier and T is the number of tiers. For example, given F=63, the maximum number of codes (i.e., analytes) is 15 for 5 non-degenerate codes per tier. This formula does not include the 1000 code, which is reserved for a positive control in Table 6. To include the control in the total number of analytes, one would simply add one, to provide the formula M=(P*T)+1. The methods provided in this disclosure may be used to expand this coding scheme infinitely.

For example, by utilizing combinations of different intensities (i.e., first values) and colors (i.e., second values) one can encode any number of analytes (M) by varying the number of non-degenerate codes per tier (P) and the number of tiers (T). For example, the number of non-degenerate codes per tier is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, or more. Similarly, the number of tiers may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, or more.

TABLE 6

Encoding of 15 analytes and one control using four colors and multiple intensities.

Tier	Analyte	B	G	Y	R	Comments
1	Ctrl. (p)	1	0	0	0	
	A	1	0	0	1	
	B	1	0	1	0	
	C	1	1	0	0	
	~~D~~	~~1~~	~~0~~	~~1~~	~~1~~	Eliminated from coding scheme to eliminate degeneracy.
	E	1	1	0	1	
	~~F~~	~~1~~	~~1~~	~~1~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.
	G	1	1	1	1	
Cumulative Maximum Result 2		6	3	2	3	
	H	1	0	0	4	
	I	1	0	3	0	
	J	1	4	0	0	
	~~K~~	~~1~~	~~0~~	~~3~~	~~4~~	Eliminated from coding scheme to eliminate degeneracy.
	L	1	4	0	4	
	~~M~~	~~1~~	~~4~~	~~3~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.
	N	1	4	3	4	
Cumulative Maximum Result 3		11	15	8	15	
	O	1	0	0	16	
	P	1	0	9	0	
	Q	1	16	0	0	
	~~R~~	~~1~~	~~0~~	~~9~~	~~16~~	Eliminated from coding scheme to eliminate degeneracy.
	S	1	16	0	16	
	~~T~~	~~1~~	~~16~~	~~9~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.
	U	1	16	9	16	
Cumulative Maximum Result for Three Non-Degenerate Tiers		16	63	26	63	

The right-hand side of FIG. **1** shows one exemplary embodiment of the method described above. More specifically, the right-hand side of FIG. **1** shows an encoding method where nine or more analytes (Seq 1-Seq 9, etc.) are each encoded by at least two colors, with varying intensities within each color. The color represents a fluorophore attached to an oligonucleotide probe that also comprises a quencher. The system can be designed so that each probe is labeled with single fluorophore, or each probe is labeled with more than one fluorophore. For example, the code for analyte H, in Table 6, is 1004. The intensity of 4 in the red channel may be achieved by either using an H-specific probe comprising 4 red fluorophores, or by using 4 H-specific probes each comprising a single red fluorophore. Of course, any combination of probes and fluorophores producing a result of 4 in the red channel would be equally appropriate, such as 2 probes with 2 red fluorophores each, and 1 probe with 1 red fluorophore and 1 probe with 3 red fluorophores, or simply one probe with one red fluorophore but present at 4× amount in the reaction mixture.

The coding scheme depicted on the right-hand side of FIG. **1** may be represented as in Table 7. The result of the

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analysis shown on the right-hand side of FIG. **1** is **4112**, or an intensity of 4 in the blue channel, 1 in the green channel, 1 in the yellow channel, and 2 in the red channel. Using a decoding matrix constructed as described herein, this result is decoded to indicate the presence of Seq 1, Seq 3, Seq 4, and Seq 5.

TABLE 7

Coding scheme illustrated on the right-hand side of FIG. 1.

Tier	Analyte	B	G	Y	R	Comments
1	Seq 1	1	0	0	0	
	Seq 2	1	1	0	0	
	Seq 3	1	0	1	0	
	Seq 4	1	0	0	1	
	Seq 5	1	1	0	1	
	Seq 6	1	1	1	1	
	~~Eliminated Potential Seq~~	~~1~~	~~0~~	~~1~~	~~1~~	Eliminated from coding scheme to eliminate degeneracy.
	~~Eliminated Potential Seq~~	~~1~~	~~1~~	~~1~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.
Cumulative Maximum Result		6	3	2	3	
2	Seq 7	1	4	0	0	
	Seq 8	1	0	3	0	
	Seq 9	1	0	0	4	

Etc.-i.e. Continue as provided in Table 6.

E. Encoding Methods Using One Color and One Intensity Per Analyte but Different Intensities Among Analytes

In some methods provided herein, each analyte is encoded by a single color and intensity combination. For example, in a four color system, the first four analytes may be encoded by 1000, 2000, 4000, and 8000. The next four analytes may be encoded by 0100, 0200, 0400, and 0800. Analytes 9-12 and 13-16 would be assigned analogously, as shown in Table 8.

Like the encoding method described in Table 6, this coding scheme is theoretically infinite, non-degenerate by construction, and only limited by the bandwidth of the instrument used to measure the signal. However, this coding scheme enables more analytes to be quantified per unit of bandwidth than the encoding method described in Table 6. The reason is that the available multiplicity of signal is used with maximal efficiency, as each level of intensity is utilized in the coding (i.e. there are no gaps in the chromatogram; see below for description of chromatograms). Table 8 shows one embodiment of this method, illustrating four tiers of encoding based on four colors and intensities 1, 2, 4, and 8. The coding scheme is non-degenerate, and the result if all 16 analytes are present is 15-15-15-15. This encoding method is more efficient, in terms of bandwidth utilization, than the encoding methods presented above. However, this method does not have the proofreading capability of the first scheme, as all the results decode to legitimate outcomes in the absence of gaps in the chromatogram.

TABLE 8

Example of encoding method using one color and one intensity per analyte, but different intensities among analytes.

Tier	Analyte	B	G	Y	R
1	A	1	0	0	0
	B	2	0	0	0
	C	4	0	0	0
	D	8	0	0	0
2	E	0	1	0	0
	F	0	2	0	0
	G	0	4	0	0
	H	0	8	0	0
3	I	0	0	1	0
	J	0	0	2	0
	K	0	0	4	0
	L	0	0	8	0
4	M	0	0	0	1
	N	0	0	0	2
	O	0	0	0	4
	P	0	0	0	8
		15	15	15	15

The method presented above, and exemplified in Table 8, may be extended by introducing additional colors and/or intensities. For example, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, or more colors may be used. Additional intensity levels, such as 16, 32, 64, 128, 255, 512, 1024, 2048, 4096, 8192, 16384, 32768, 65536, and so on may also be used. The method is generalizable and may be used to encode an infinite number of analytes. Each analyte is represented by a code in a single color, wherein the value of the code in that single color equal to the sum of all previous values plus one. For example, if the first code contains a 1 in a particular color, the next codes are 2, 4, 8, 16, 32, 64, 128 and so on. Other progressions are possible, but will not be as efficient in terms of usage of the bandwidth. However, one of ordinary skill in the art will recognize that less efficient use of bandwidth may be desirable when one wishes to maximize separation between values. Similarly, the value 1 could be excluded from the coding scheme, for example to maximize the difference in the intensity between the first encoded value and instrumental noise. For example, in some cases a coding scheme may begin with a value of 2, which would provide a progression of 2, 4, 8, 16, 32, 64, 128, and so on for the analyte codes, while the progression of cumulative results would be 2, 4, 6, 8, 10, 12, 14, 16, and so on. This approach generates uniform gaps in the possible cumulative results, allowing for higher tolerance to noise in comparison to an analyte code progression of 1, 2, 4, 8, 16, 32, and so on, and its corresponding cumulative result progression of 1, 2, 3, 4, 5, 6, 7, 8, and so on. In this example, the increased robustness in the measurement comes at a cost of a decrease in the number of available codes within a fixed bandwidth of multiplicity. For example, if only 63 multiplicity states can be measured reliably, the coding scheme starting with 1 offers 7 codes per color whereas the coding scheme starting with 2 offers 6 codes per color. If 4 colors are available, the former will offer 28 codes, while the latter will offer 24 codes. In summary, both the starting signal intensity and the progression (i.e., difference between intensity values) may be scaled in order to maximize the ability to distinguish over instrumental noise and maximize the differences between the intensity values, thereby enhancing the ability to distinguish between distinct experimental outcomes.

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The coding scheme illustrated in Table 8 is non-degenerate by design. Although the coding scheme illustrated in Table 8 uses both intensity and color to encode each of the 16 analytes, each of the analytes could also be encoded by simply utilizing intensity. For example, given the 16 analytes provided in Table 8 (A-P), a single color coding scheme encoding all 16 analytes may assign the values 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048, 4096, 8192, and 16384 to analytes A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, and P, respectively. Such a coding scheme is non-degenerate by design and the cumulative intensity result can be unambiguously decoded to indicate the presence or absence of analytes A-P. This coding scheme is capable of detecting the presence or absence of M analytes, where $M=\log_2(F+1)$ and F is the maximum cumulative value of a signal (e.g., signal intensity). By adding a second component to this signal (e.g., color, as depicted in Table 8), the capacity of this coding scheme can be increased to $M=C*\log_2(F+1)$, where C is the number of colors used in said coding scheme.

As described throughout this specification, any suitable value may be used for C or F. For example, C may be at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, or more. F may be at least about 2, 4, 8, 16, 32, 64, 128, 255, 512, 1024, 2048, 4096, 8192, 16384, 32768, 65536, 131072, 262144, 524288, 1048576, and so on. As described elsewhere in this disclosure, both the initial starting value of F and the steps in its progression may also be varied.

III. Analytes

An analyte may be any suitable analyte that may be analyzed using the methods and compositions of the invention, where the analyte is capable of interacting with a reagent in order to generate a signal with at least two components that can be measured. An analyte may be naturally-occurring or synthetic. An analyte may be present in a sample obtained using any methods known in the art. In some cases, a sample may be processed before analyzing it for an analyte. The methods and compositions presented in this disclosure may be used in solution phase assays, without the need for particles (such as beads) or a solid support.

In some cases, an analyte may be a polynucleotide, such as DNA, RNA, peptide nucleic acids, and any hybrid thereof, where the polynucleotide contains any combination of deoxyribo- and/or ribo-nucleotides. Polynucleotides may be single stranded or double stranded, or contain portions of both double stranded or single stranded sequence. Polynucleotides may contain any combination of nucleotides or bases, including, for example, uracil, adenine, thymine, cytosine, guanine, inosine, xanthine, hypoxanthine, isocytosine, isoguanine and any nucleotide derivative thereof. As used herein, the term "nucleotide" may include nucleotides and nucleosides, as well as nucleoside and nucleotide analogs, and modified nucleotides, including both synthetic and naturally occurring species. Polynucleotides may be any suitable polynucleotide for which one or more reagents (or probes) as described herein may be produced, including but not limited to cDNA, mitochondrial DNA (mtDNA), messenger RNA (mRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), nuclear RNA (nRNA), small interfering RNA (siRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), small Cajal body-specific RNA (scaRNA), microRNA (miRNA), double stranded (dsRNA), ribozyme, riboswitch or viral RNA. Polynucleotides may be contained within any suitable vector, such as a plasmid, cosmid, fragment, chromosome, or genome.

Genomic DNA may be obtained from naturally occurring or genetically modified organisms or from artificially or synthetically created genomes. Analytes comprising genomic DNA may be obtained from any source and using any methods known in the art. For example, genomic DNA may be isolated with or without amplification. Amplification may include PCR amplification, multiple displacement amplification (MDA), rolling circle amplification and other amplification methods. Genomic DNA may also be obtained by cloning or recombinant methods, such as those involving plasmids and artificial chromosomes or other conventional methods (see Sambrook and Russell, *Molecular Cloning: A Laboratory Manual*, cited supra.) Polynucleotides may be isolated using other methods known in the art, for example as disclosed in Genome Analysis: A Laboratory Manual Series (Vols. I-IV) or *Molecular Cloning: A Laboratory Manual*. If the isolated polynucleotide is an mRNA, it may be reverse transcribed into cDNA using conventional techniques, as described in Sambrook and Russell, *Molecular Cloning: A Laboratory Manual*, cited supra.

An analyte may be a protein, polypeptide, lipid, carbohydrate, sugar, small molecule, or any other suitable molecule that can be detected with the methods and compositions provided herein. An analyte may be an enzyme or other protein. An analyte may be a drug or metabolite (e.g., anti-cancer drug, chemotherapeutic drug, anti-viral drug, antibiotic drug, or biologic). An analyte may be any molecule, such as a co-factor, receptor, receptor ligand, hormone, cytokine, blood factor, antigen, steroid, or antibody.

An analyte may be any molecule from any pathogen, such as a virus, bacteria, parasite, fungus, or prion (e.g., PrP^{Sc}). Examples of viruses include those from the families Adenoviridae, Flaviviridae, Hepadnaviridae, Herpesviridae, Orthomyxoviridae, Papovaviridae, Paramyxoviridae, Picornaviridae, Polyomavirus, Retroviridae, Rhabdoviridae, and Togaviridae. Specific examples of viruses include adenovirus, astrovirus, bocavirus, BK virus, coxsackievirus, cytomegalovirus, dengue virus, Ebola virus, enterovirus, Epstein-Barr virus, feline leukemia virus, hepatitis virus, hepatitis A virus, hepatitis B virus, hepatitis C virus, hepatitis D virus, hepatitis E virus, herpes simplex virus (HSV), HSV type 1, HSV type 2, human immunodeficiency virus (HIV), HIV type 1, HIV type 2, human papilloma virus (HPV), HPV type 1, HPV type 2, HPV type 3, HPV type 4, HPV type 6, HPV type 10, HPV type 11, HPV type 16, HPV type 18, HPV type 26, HPV type 27, HPV type 28, HPV type 29, HPV type 30, HPV type 31, HPV type 33, HPV type 34, HPV type 35, HPV type 39, HPV type 40, HPV type 41, HPV type 42, HPV type 43, HPV type 44, HPV type 45, HPV type 49, HPV type 51, HPV type 52, HPV type 54, HPV type 55, HPV type 56, HPV type 57, HPV type 58, HPV type 59, HPV type 68, HPV type 69, influenza virus, JC virus, Marburg virus, measles virus, mumps virus, Norwalk virus, parovirus, polio virus, rabies virus, respiratory syncytial virus, retrovirus, rhinovirus, rotavirus, Rubella virus, smallpox virus, vaccinia virus, West Nile virus, and yellow fever virus.

Examples of bacteria include those from the genuses *Bordetella, Borrelia, Brucella, Campylobacter, Chlamydia, Clostridium, Corynebacterium, Enterococcus, Escherichia, Francisella, Haemophilus, Helicobacter, Legionella, Leptospira, Listeria, Mycobacterium, Mycoplasma, Neisseria, Pseudomonas, Rickettsia, Salmonella, Shigella, Staphylococcus, Streptococcus, Treponema, Vibrio*, and *Yersinia*. Specific examples of bacteria include *Bordetella par aper-*

tussis, Bordetella pertussis, Borrelia burgdorferi, Brucella abortus, Brucella canis, Brucella melitensis. Brucella suis, Campylobacter jejuni, Chlamydia pneumoniae, Chlamydia psittaci, Chlamydia trachomatix, Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Clostridium tetani, Corynebacterium diphtheriae, Enterococcus faecalis, Enterococcus faecium, Escherichia coli, Francisella tularensis, Haemophilus influenzae, Helicobacter pylori, Legionella pneumophila, Leptospira interrogans, Listeria monocytogenes, Mycobacterium leprae, Mycobacterium tuberculosis, Mycobacterium ulcerans, Mycoplasma pneumoniae, Neisseria gonorrhoeae, Neisseria meningitidis, Pseudomonas aeruginosa, Rickettsia rickettsii, Salmonella choleraesuis, Salmonella dublin, Salmonella enteritidis, Salmonella typhi, Salmonella typhimurium, Shigella sonnei, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus saprophyticus, Streptococcus agalactiae, Streptococcus pneumoniae, Streptococcus pyogenes, Treponema pallidum, Vibrio cholerae, Yersinia pestis, and *Yersinia enterocolitica.*

Examples of parasites include those from the genuses *Acanthamoeba, Babesia, Balamuthia, Balantidium, Blasocystis, Cryptosporidium, Dientamoeba, Entamoeba, Giardia, Isospora, Leishmania, Naegleria, Pediculus, Plasmodium, Rhinosporidium, Sarcocystis, Schistosoma, Toxoplasma, Trichomonas*, and *Trypanosoma*. Specific examples of parasites include *Babesia divergens, Babesia bigemina, Babesia equi, Babesia microfli, Babesia duncani, Balamuthia mandrillaris, Balantidium coli, Dientamoeba fragilis, Entamoeba histolytica, Giardia lamblia, Isospora belli, Naegleria fowleri, Pediculus humanus, Plasmodium falciparum, Plasmodium knowlesi, Plasmodium malariae, Plasmodium ovale, Plasmodium vivax, Rhinosporidium seeberi, Sarcocystis bovihominis, Sarcocystis suihominis, Schistosoma mansoni, Toxoplasma gondii, Trichomonas vaginalis, Trypanosoma brucei*, and *Trypansoma cruzi.*

Examples of fungi include those from the genuses *Apophysomyces, Aspergillus, Blastomyces, Candida, Cladosporium, Coddidioides, Cryptococcos, Exserohilum, Fusarium, Histoplasma, Pichia, Pneumocystis, Saccharomyces, Sporothrix, Stachybotrys*, and *Trichophyton. Specific examples of fungi include Aspergillus fumigatus, Aspergillus flavus, Aspergillus clavatus, Blastomyces dermatitidis, Candida albicans, Coccidioides immitis, Crytptococcus neoformans, Exserohilum rostratum, Fusarium verticillioides, Histoplasma capsulatum, Pneumocystis jirovecii, Sporothrix schenckii, Stachybotrys chartarum*, and *Trichophyton mentagrophytes.*

In some cases, the methods provided in this disclosure may be used to detect any one of the analytes described above, or elsewhere in the specification. In some cases the methods provided in this disclosure may be used to detect panels of the analytes described above, or elsewhere in the specification. For example, a panel may comprise an analyte selected from the group consisting of any 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100 or more analytes described above or elsewhere in the specification.

An analyte may be obtained from any suitable location, including from organisms, whole cells, cell preparations and cell-free compositions from any organism, tissue, cell, or environment. Analytes may be obtained from environmental samples, biopsies, aspirates, formalin fixed embedded tissues, air, agricultural samples, soil samples, petroleum samples, water samples, or dust samples. In some instances, an analyte may be obtained from bodily fluids which may include blood, urine, feces, serum, lymph, saliva, mucosal secretions, perspiration, central nervous system fluid, vaginal fluid, or semen. Analytes may also be obtained from manufactured products, such as cosmetics, foods, personal care products, and the like. Analytes may be the products of experimental manipulation including, recombinant cloning, polynucleotide amplification, polymerase chain reaction (PCR) amplification, purification methods (such as purification of genomic DNA or RNA), and synthesis reactions.

More than one type of analyte may be detected in each multiplexed assay. For example, a polynucleotide, a protein, a polypeptide, a lipid, a carbohydrate, a sugar, a small molecule, or any other suitable molecule may be detected simultaneously in the same multiplexed assay with the use of suitable reagents. Any combination of analytes may be detected at the same time.

Detection of an analyte may be useful for any suitable application, including research, clinical, diagnostic, prognostic, forensic, and monitoring applications. Exemplary applications include detection of hereditary diseases, identification of genetic fingerprints, diagnosis of infectious diseases, cloning of genes, paternity testing, criminal identification, phylogeny, anti-bioterrorism, environmental surveillance, and DNA computing. For example, an analyte may be indicative of a disease or condition. An analyte may be used to make a treatment decision, or to assess the state of a disease. The presence of an analyte may indicate an infection with a particular pathogen, or any other disease, such as cancer, autoimmune disease, cardiorespiratory disease, liver disease, digestive disease, and so on. The methods provided herein may thus be used to make a diagnosis and to make a clinical decision based on that diagnosis. For example, a result that indicates the presence of a bacterial polynucleotide in a sample taken from a subject may lead to the treatment of the subject with an antibiotic.

In some cases the methods and compositions of the invention may be used to detect at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes. In some cases the methods and compositions of the invention may be used to detect 7-50, 8-40, 9-30, 10-20, 10-15, 8-12, or 7-12 analytes.

In some cases, this disclosure provides assays that are capable of unambiguously detecting the presence or absence of each of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis. In some cases, this disclosure provides assays that are capable of unambiguously detecting the presence or absence of each of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 111, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis. In some cases,

this disclosure provides assays that are capable of unambiguously detecting the presence or absence of less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis.

IV. Signals

The methods presented in this disclosure may be used with any quantifiable signal. In some cases, this disclosure provides coding schemes and methods to encode an infinite number of targets without degeneracy, using a single component of a signal (e.g., intensity). For example, as described above and in Example 3, a coding scheme may rely on a multiplicity of signal intensity without consideration of color. Although fluorescent probes have been used to illustrate this principle, the coding scheme is equally applicable to any other method providing a quantifiable signal, including an electrochemical signal and a chemiluminescent signal, as described elsewhere in this disclosure.

The methods presented in this disclosure may also utilize the measurement of a signal in at least two dimensions, also referred to as the measurement of at least two components of a signal. In comparison to the coding scheme described in the paragraph above, which relies on, for example, signal intensity to differentiate between analytes, utilization of at least two components of a signal (e.g., color and intensity) allows the generation of more unique codes per unit of signal intensity bandwidth. When at least two components of a signal are utilized, a cumulative measurement of the at least two components may be obtained for a single sample volume. For example in the coding scheme described in Table 5, the presence of each analyte results in a particular intensity (one component of the signal) in each of the four colors (a second component of a signal). The combination of these constituent signals leads to a cumulative signal that may be measured by measuring an intensity at each wavelength or range of wavelengths. The corresponding coding scheme or decoding matrix may then be used to convert the cumulative measurement into a determination of the presence or absence of an analyte.

In some cases, a quantifiable signal comprises a waveform that has both a frequency (wavelength) and an amplitude (intensity). A signal may be an electromagnetic signal. An electromagnetic signal may be a sound, a radio signal, a microwave signal, an infrared signal, a visible light signal, an ultraviolet light signal, an x-ray signal, or a gamma-ray signal. In some cases, an electromagnetic signal may be a fluorescent signal, for example a fluorescence emission spectrum that may be characterized in terms of wavelength and intensity.

In certain portions of this disclosure, the signal is described and exemplified in terms of a fluorescent signal. This is not meant to be limiting, and one of ordinary skill in the art will readily recognize that the principles applicable to the measurement of a fluorescent signal are also applicable to other signals. For example, like fluorescent signals, any of the electromagnetic signals described above may also be characterized in terms of a wavelength and an intensity. The wavelength of a fluorescent signal may also be described in terms of color. The color may be determined based on measuring intensity at a particular wavelength or range of wavelengths, for example by determining a distribution of fluorescent intensity at different wavelengths and/or by utilizing a band pass filter to determine the fluorescence intensity within a particular range of wavelengths. Such band pass filters are commonly employed in a variety of laboratory instrumentation, including quantitative PCR machines. Intensity may be measured with a photodetector. A range of wavelengths may be referred to as a "channel."

In some cases, the methods provided in this disclosure may be used with any signal where the cumulative signal scales with the constituent signals of the same color, frequency, absorption band, and so on. However, the cumulative signal need not be digital or scale linearly with the number and intensity of the constituent signals. For example, if the physical principle of measurement is absorption, the cumulative attenuation is a product of constituent attenuations while the constituent concentrations are additive, due to the exponential nature of the Beer-Lambert law. The logarithm of the cumulative attenuation will then scale linearly with constituent concentrations in each absorption band (the equivalent of color, if fluorescent detection is used). The methods of the invention are therefore applicable. The methods of the invention may also be used with chemiluminescent signals and electrochemical signals.

The number of signals, and the number of dimensions or components measured, may also be expanded beyond the numbers shown in the exemplary embodiments of the invention, leading to an expansion in multiplexing capability. The exemplary embodiments provided in this disclosure utilize coding schemes constructed utilizing a fluorescent signal with one or two components measured: wavelength and/or intensity. The number of analytes that can be encoded can be increased by increasing the number of wavelengths and/or intensities. The number of analytes that can be encoded can also be increased by increasing the number of signals, for example by combining a fluorescent signal with an electrochemical signal or a FRET signal (fluorescence resonance energy transfer).

In some cases, more than two components of a signal may be measured. For example, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 18, 20 or more components of a signal may be measured. At least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 18, or 20 components of a signal may be measured. At least 2, but fewer than 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 18, or 20 components of a signal may be measured. In some cases, 2-3, 2-4, 2-5, 2-6, 3-5, 3-6, 3-8, or 5-10 components of a signal may be measured. These additional components may include kinetic components, such as a rate of signal decay and rate of photobleaching.

If a fluorescent signal is employed, the number of analytes that can be encoded may be further expanded by utilizing additional fluorophores. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more fluorophores may be used. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 fluorophores may be used. In some cases, fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 fluorophores may be used. In some cases, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 fluorophores may be used.

Generally, the number of analytes that can be encoded may be further expanded by utilizing additional first values (e.g., intensities) that are values or ranges of values from a first component of a signal. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more first values that are values or ranges of values from a first component of a signal may be used. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more first values that are values or ranges of values from a first component of a signal may be used. In some cases, fewer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 first values that are values or ranges of values from a first component of a signal may be used. In some cases, 4-20, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 first values that are values or ranges of values from a first component of a signal may be used.

In instances in which intensity is a component of a signal that is quantified, such as when a fluorescent signal is utilized, the presence of an analyte may be encoded using a variety of intensities or ranges of intensities. For example, a coding scheme may utilize 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more intensities or ranges of intensities. A coding scheme may utilize at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more intensities or ranges of intensities. A coding scheme may utilize fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 intensities or ranges of intensities. In some cases, a coding scheme may utilize 2-20, 2-3, 2-4, 2-5, 2-6, 2-7, 2-8, 2-9, 2-10, 3-4, 3-5, 3-6, 3-7, 3-8, 3-9, 3-10, 4-5, 4-6, 4-7, 4- 8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, or 5-10 intensities or ranges of intensities.

The number of analytes that can be encoded may be further expanded by utilizing additional second values (e.g., wavelengths) that are values or ranges of values from a second component of a signal. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 1, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more second values that are values or ranges of values from a second component of a signal may be used. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more second values that are values or ranges of values from a second component of a signal may be used. In some cases, fewer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 second values that are values or ranges of values from a second component of a signal may be used. In some cases, 4-20, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 second values that are values or ranges of values from a second component of a signal may be used.

In instances in which wavelength is a component of a signal that is quantified, such as when a fluorescent signal is utilized, the presence of an analyte may be encoded using a variety of wavelengths or ranges of wavelengths. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more wavelengths or ranges of wavelengths may be used. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more wavelengths or ranges of wavelengths may be used. In some cases, fewer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 wavelengths or ranges of wavelengths may be used. In some cases, 4-20, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 wavelengths or ranges of wavelengths may be used.

In some cases, when degeneracy is eliminated, the methods of the invention are capable of detecting the presence or absence of at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more analytes in a single volume when each reagent used to generate a signal in the volume generates only one second value (e.g., each reagent, emits light at only one wavelength).

In other cases, when degeneracy is eliminated, the methods of the invention are capable of detecting the presence or absence of at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more analytes in a single volume using a total of four second values in that volume (e.g., a total of four wavelengths or ranges or wavelengths, which might be implemented by using four spectrally resolvable fluorophores).

As described throughout the specification, the assay provided herein utilizes cumulative measurements on a sample. A cumulative measurement may be, for example, a single measurement of intensity values, or a measurement of intensity values at one or more wavelengths or ranges of wavelengths. A plurality of cumulative measurements may be obtained. For example, an intensity may be measured at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more wavelengths or ranges of wavelengths. An intensity may be measured at at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more wavelengths or ranges of wavelengths. An intensity may be measured at less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 wavelengths or ranges of wavelengths.

More generally a cumulative measurement may be obtained for any quantifiable component of a signal, and for any quantifiable component of a signal at another quantifiable component of a signal. For example, at least a first component of a signal may be measured at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more second components of a signal. At least a first component of a signal may be measured at at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27,

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28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more second components of a signal. At least a first component of a signal may be measured at less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 second components of a signal.

As is apparent from this disclosure, each analyte to be detected can be encoded as a code utilizing any number of suitable components of a signal or any number of signals. For example, each analyte to be detected can be encoded in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more components of a signal or signals. In some cases, each analyte to be detected can be encoded in at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more components of a signal or signals. In some cases, each analyte to be detected can be encoded in fewer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 components of a signal or signals. In some cases, 1-4, 4-20, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 components of signal or signals may be used to encode each analyte. Each analyte in a coding scheme may be encoded by the same number of components of a signal, or signals, or different numbers of components of a signal, or signals.

A. Probes, Primers, Fluorophores, and Quenchers

Some of the methods provided in this disclosure utilize a reagent that generates a signal in the presence of an analyte. Any suitable reagent may be used with the invention. Generally, a reagent will have an analyte-specific component and a component that generates a signal in the presence of the analyte. In some cases, these reagents are referred to as probes. The probes may be hybridization probes. The hybridization probes may be an oligonucleotide probe attached to a fluorophore and a quencher (e.g., a TAQMAN probe).

The methods of the invention may use one or more reagents or probes to detect the presence or absence of each analyte. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more or probes may be used to detect the presence or absence of each analyte. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 probes may be used to detect the presence or absence of each analyte. In some cases, fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 probes may be used to detect the presence or absence of each analyte. In some cases, the number of probes used to detect the presence or absence of each analyte is 1-10, 2-9, 2-8, 2-7, 2-6, 2-5, 2-4, 1-3, or 1-4.

In some cases, a sample is contacted with 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more probes to detect the presence or absence of all analytes. In some cases, a sample is contacted with at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more probes to detect the presence or absence of all analytes. In some cases, a sample is contacted with fewer than 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 probes to detect the presence or absence of all analytes. In some cases, the number of probes that a sample is contacted with to detect the presence or absence of all analytes is 4-50, 4-40, 4-30, 4-20, 5-15, 5-10, or 3-10.

As described above, oligonucleotide probes attached to a fluorophore and a quencher may be used to detect the presence of an analyte in a polynucleotide amplification assay. So long as the quencher and the fluorophore are in proximity, the quencher quenches the fluorescence emitted by the fluorophore upon excitation by a light source. The sequence of the oligonucleotide probe is designed to be complementary to a polynucleotide sequence present in an analyte, and therefore capable of hybridizing to the polynucleotide sequence present in the analyte. Hybridization of the oligonucleotide probe may be performed in a nucleic acid amplification reaction comprising primers (e.g., a polymerase chain reaction). Upon extension of the primers by a DNA polymerase, the 5' to 3' exonuclease activity of the polymerase degrades the probe, releasing the fluorophore and the quencher into the medium. The proximity between the fluorophore and the quencher is broken and the signal from the fluorophore is no longer quenched. Thus, the amount of fluorescence detected is a function of the amount of analyte present. If no analyte is present, the probe will not hybridize to an analyte, and the fluorophore and quencher will remain in close proximity. Little or no signal will be produced.

Oligonucleotide probes may have one or a plurality of fluorophores and quenchers per probe. For example, in some embodiments an oligonucleotide probe may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more fluorophores. An oligonucleotide probe may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 fluorophores. An oligonucleotide probe may comprise fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 fluorophores.

An oligonucleotide probe may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more quenchers. An oligonucleotide probe may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 quenchers. An oligonucleotide probe may comprise fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 quenchers.

Attachment of probes and quenchers to a probe may be performed in the same reaction or in serial reactions. A series of reactions may be performed to label probes with at least one fluorophore and the reaction products may be mixed to generate a mixture of probes with different fluorophores.

If an oligonucleotide probe comprises two or more fluorophores, these fluorophores may be arranged to allow Förster (Fluorescence) resonance energy transfer (FRET) to occur between the fluorophores. Briefly, FRET is a mechanism of energy transfer between fluorophores. Using FRET-based probes allows one excitation source to generate two different fluorescence emission signals by excitation of a single fluorophore. For example, the methods described herein may used paired probes, where one probe in the pair is attached to a fluorophore and a quencher and a second probe is attached to two fluorophores (in close enough proximity for FRET to occur) and a quencher. Any combination of fluorophores and quenchers that provide for FRET may be used. Such an approach doubles the number of fluorescent probes that may be used with an excitation source, because a single excitation source can be used to produce two signals: one from the probe with one fluorophore and one from the probe with two fluorophores in close enough proximity to provide for FRET. For example, using the encoding method described in Table 8, the number of unambiguously detectable analytes could be increased from 16 to 32 by pairing each of the probes in Table 8 with a corresponding FRET probe.

The primers may be specific for a particular analyte and capable of amplifying a region complementary to a probe. In some cases, the number of pairs of primers used is equivalent to the number of probes. In other cases, the number of probes used may exceed the number of primer pairs used. In still other cases, the number of primer pairs used may exceed the number of probes used. In some cases a sample to be analyzed is contacted with 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more pairs of primers. In some cases a sample to be analyzed is contacted with at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more pairs of primers. In some cases a sample to be analyzed is contacted with fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 pairs of primers. In some cases, the number of pairs of primers is 2-10, 3-15, 4-20, 3-10, 4-10, 5-10, 6-8, or 6-10.

The primers may amplify regions of a polynucleotide in which different numbers of hybridization probes hybridize. For example, at least one pair of primers may amplify a region complementary to at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 hybridization probes. In some cases, all of said pairs of primers may amplify a region complementary to at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 hybridization probes.

In one example, a first probe may be labeled with the fluorophore 5'-fluorescein amidite (5'-FAM) and the quencher black hole quencher 1 (BHQ-1). A second probe may be labeled with 5'-FAM in close proximity (e.g., attached to) to cyanine 5.5 (Cy5.5) and the quencher black hole quencher 3 (BHQ-3). Upon digestion of the probe, via the nuclease activity of the polymerase, two fluorescent signals are generated from a single excitation wavelength (e.g., 470 nm). The fluorophore from the first probe will fluoresce at about 520 nm. The fluorophores on the second probe undergo FRET. The donor fluorophore, FAM is excited by the excitation light source (e.g., 470 nm) and transfers its energy to the acceptor fluorophore, Cy5.5. The acceptor fluorophore emits at about 705 nm. These fluorophores and quenchers are merely exemplary. Any fluorophores that can undergo FRET and any quenchers that can quench fluorescence are suitable for use with the invention. Methods for producing FRET-based probes are described in Jothikumar et al., BioTechniques, 2009, 46(7):519-524.

In some examples, a single hybridization probe may be used for each analyte. In order to utilize multi-color encoding (e.g., as shown in Table 3), each probe may be labeled with a plurality of fluorophores at pre-determined ratios. The ratio may be determined so that a positive control signal provides the same intensity as other positive control signals of the same intensity, in the same color. This approach reduces the number of probes that must be synthesized and may be less expensive to deploy than methods utilizing multiple probes. Moreover, in the case of hybridization probes, it is easier to fit one hybridization probe to an analyte sequence than multiple probes.

Although many aspects of the invention are exemplified using nucleic acid-based probes, one of ordinary skill in the art will readily recognize that other forms of probes would work equally well with the invention described in this disclosure. For example, a binding molecule specific to an analyte could be used as a probe. Non-limiting exemplary binding molecules include an antibody recognizing an analyte, and generating a signal in the presence of an analyte.

In embodiments of the invention that utilize fluorescent labels, any suitable fluorescent label may be used. Exemplary fluorescent labels suitable for use with the invention include rhodamine, rhodol, fluorescein, thiofluorescein, aminofluorescein, carboxyfluorescein, chlorofluorescein, methylfluorescein, sulfofluorescein, aminorhodol, carboxyrhodol, chlororhodol, methylrhodol, sulforhodol; aminorhodamine, carboxyrhodamine, chlororhodamine, methylrhodamine, sulforhodamine, and thiorhodamine, cyanine, indocarbocyanine, oxacarbocyanine, thiacarbocyanine, merocyanine, cyanine 2, cyanine 3, cyanine 3.5, cyanine 5, cyanine 5.5, cyanine 7, oxadiazole derivatives, pyridyloxazole, nitrobenzoxadiazole, benzoxadiazole, pyrene derivatives, cascade blue, oxazine derivatives, Nile red, Nile blue, cresyl violet, oxazine 170, acridine derivatives, proflavin, acridine orange, acridine yellow, arylmethine derivatives, auramine, crystal violet, malachite green, tetrapyrrole derivatives, porphin, phtalocyanine, and bilirubin. Exemplary quenchers include black hole quenchers, such as BHQ-0, BHQ-1, BHQ-2, BHQ-3; ATTO quenchers, such as ATTO 540Q, ATTO580Q, and ATTO612Q; and the like.

The fluorophores that may be used with the invention are not limited to any of the fluorophores described herein. For example, fluorophores with improved properties are continually developed, and these fluorophores could readily be used with the methods provided in this disclosure. Such improved fluorophores include quantum dots, which may emit energy at different wavelengths after being excited at a single wavelength. The advantage of using such fluorophores is that only a single excitation source is needed, but many different signals may be quantified, for example in terms of color and intensity. Moreover, fluorophores with narrow emission spectra would be particularly useful with the method described herein, as such fluorophores could be included in multiplex assays with minimal or no overlap between their emission spectra, thereby offering many more "colors" and boosting significantly the overall number of coded analytes.

In some cases, one or more reagents are lyophilized. Any suitable reagent may be lyophilized. For example, probes, primers, enzymes, antibodies, or any other reagent used for detection may be lyophilized. In some cases, a sample comprising an analyte may also be lyophilized. Lyophilization may be useful, for example, when distributing reagents and/or samples in developing regions where access to cold storage is expensive, not readily available, or not reliably available. In one example, lyophilized reagents comprise any probe or analyte-specific reagent described in this disclosure or otherwise suitable for use with the invention. In another example, lyophilized reagents comprise PCR primers. In yet another example, lyophilized reagents comprise reagents suitable for performing a PCR reaction.

V. Analytical Techniques and Instrumentation

The methods provided herein are suitable for use with a variety of detection methods. For example, the methods may be applied using an analytical technique that measures the wavelength and intensity of a fluorescent signal. This may be accomplished by measuring the intensity of a signal across a spectrum of wavelengths, or by using band pass filters that restrict the passage of certain wavelengths of light, thereby allowing only light of certain wavelengths to reach a photodetector. Many real-time PCR and quantitative PCR instruments comprise an excitation light source and band pass filters that enable the detection of fluorescent signals in four colors (e.g., blue, green, yellow, and red). Therefore, the methods of the invention can be readily applied using instruments widely used in the art. Importantly, the methods and compositions provided herein may be used to detect multiple analytes by obtaining a cumulative measurement on a single solution. No separation is necessary. The invention does not require the use of beads or a solid phase. Of course, one of ordinary skill in the art would understand that the invention could be used with separation, beads, or a solid phase, if desired.

FIG. **5** shows two examples of instrumental configurations that may be used to obtain cumulative measurements useful for the methods of the invention. With reference to FIG. **5**, an instrument with a detector configured to detect both wavelength and intensity (e.g., a fluorometer) is shown in **501**. With reference to **501**, at least one excitation light source **502** is directed into a chamber **503** containing analytes and reagents that generate a signal in the presence of the analytes **504**. As described elsewhere, the analyte may be a nucleic acid and the reagents generating the signal may be hybridization probes comprising a fluorophore and a quencher. If the analyte is present, an emission signal **505** is generated. In the configuration shown in **501**, the wavelength and intensity of this emission signal are measured across a spectrum by a detector capable of generating a fluorescence emission spectrum **506**.

The wavelength and intensity may also be determined using a combination of a photodetector and band pass filters. This configuration is used in several thermal cyclers known in the art. With reference to FIG. **5**, an instrument with band pass filters and a photodetector is depicted in **507**. With reference to **507**, at least one excitation light source **508** is directed into a chamber **509** containing analytes and reagents that generate a signal in the presence of the analytes **510**. As described elsewhere, the analyte may be a nucleic acid and the reagents generating the signal may be hybridization probes comprising a fluorophore and a quencher. If the analyte is present, an emission signal **511** is generated. In the configuration shown in **507**, band pass filters **512**, **513**, and **514** are used to restrict the passage of light to light within certain ranges of wavelengths. For example band pass filter **512** may restrict the passage of light to light within wavelength range 1. Band pass filter **513** may restrict the passage of light to light within wavelength range 2. Band pass filter **514** may restrict the passage of light to light within wavelength range 3. Any number of band pass filters (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, or more) may be used with the invention. In addition, the positions of the band pass filters in **507** are not meant to be limiting. In some cases, emitted light may be passed through more than one band pass filter at any time. In other cases, the band pass filters may be configured so that all emitted light passes through only one band pass filter at a time. After passage through a band pass filter, filtered light **515**, **516**, and **517** is detected by a photodetector. For example, filtered light **515** may be light within wavelength range 1, filtered light **516** may be light within wavelength range 2, and filtered light **517** may be light within wavelength range 3. The intensity of each of the filtered lights **515**, **516**, and **517** may then be quantified by a photodetector **518**.

The methods described in this disclosure are compatible with a variety of amplification methods, including quantitative PCR (qPCR) methods, end point PCR methods, reverse transcriptase PCR, and digital PCR methods. Digital PCR methods (e.g., DROPLET DIGITAL (BIORAD) and DYNAMIC ARRAY (FLUIDIGM)) produce highly sensitive quantification of polynucleotide copy numbers. The methods provided herein can be easily integrated into these systems to significantly expand their throughput by allowing multiplexing in a droplet or a dynamic array.

Similarly, the methods described in this disclosure may be applied in a real-time PCR assay. For example, real-time data may be fully recorded as the PCR runs to completion. The end-point values may then be decoded to indicate the presence or absence of analytes. The individual cycle thresholds (Ct) values may be analyzed for each color by detecting the maxima of the second derivative of the real-time curve in that color. As a particular analyte is amplified, fluorophores are released from its corresponding probes in the same ratios as the color multiplicities in the coding of that sequence. Hence, e.g. a code of 1100 will have the same Ct in blue and green, while a code of 1400 will have its green Ct precede its blue Ct by 2 cycles. This additional data enables determination of the identity and starting quantity of each analyte.

The methods described herein may also be used directly on a tissue sample. For example, a tissue sample may be obtained and photolithographic methods may be used to build wells directly onto the tissue sample. The tissue sample may be fixed prior to building the wells. The tissue sample may then be analyzed by dispensing appropriate reagents into the wells in the tissue sample. The encoding and decoding methods described in this disclosure may be used for the multiplexed detection of analytes within the wells etched into the tissue. Each well may correspond to a single cell or a few cells, providing excellent spatial resolution when analyzing different portions of a tissue for an analyte. This method may be used to detect analytes in different areas of the same tissue.

In some cases, instruments may be modified or constructed, for example, to provide additional excitation light sources, at multiple wavelength, and/or to provide additional band pass filters or a capability of determining a complete spectrum. Including additional excitation sources would allow for the excitation of a larger variety of fluorophores.

Including additional band pass filters, or modifying or constructing an instrument capable of determining an entire spectrum allows detection of a wider variety of emissions from fluorophores. These techniques can be used to increase the number of fluorophores that can be used with the methods described in this disclosure and, accordingly, to increase the number of analytes that be simultaneously detected.

In some cases, the methods described in this disclosure utilize end-point PCR methods. However, in some cases a result may be obtained before the end-point. This may be advantageous when, for example, results are needed as soon as possible. For example, in one case calibration experiments are performed in which different starting concentrations of an analyte are analyzed to determine the cycle number at which the signal becomes saturated, as a function of the starting amount of the analyte. Using this data, a computer monitoring a PCR reaction in real-time can be programmed to search for saturation up to the maximal cycle number according to the limit of detection (LOD) of the particular system. If there is no amplification of an analyte (e.g., other than a positive control, if present), by the maximal cycle number, the result for that analyte is negative. If there is amplification of the analyte by the maximal cycle number, the result for that analyte is positive and the saturation intensity in each color may be used to decode the result using the coding scheme. In both cases, there would be no need to run the PCR reaction beyond the number of cycles set by the LOD for that instrument. Although PCR has been used to illustrate this quantitative method, one of ordinary skill in the art will readily recognize that similar principles could be applied in any catalytic system where the starting amount of an analyte limits the rate of reaction, and the rate of reaction can be measured and reported by an evolution in a signal intensity over time.

VI. Compositions and Kits

This disclosure also provides compositions and kits for use with the methods described herein. The compositions may comprise any component, reaction mixture and/or intermediate described herein, as well as any combination. For example, the disclosure provides detection reagents for use with the methods provided herein. Any suitable detection reagents may be provided, including hybridization probes labeled with a fluorophore and a quencher and primers, as described elsewhere in the specification.

In some cases, compositions comprise reagents for the detection of at least seven analytes using four fluorophores. In some cases, compositions comprise reagents for the detection of at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes using four fluorophores. In some cases, compositions comprise reagents for the detection of at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes using five fluorophores. In some cases, compositions comprise reagents for the detection of at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes using six fluorophores. In some cases, compositions comprise reagents for the detection of at least 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes using seven fluorophores.

In some cases the compositions comprise primers. The compositions may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 pairs of primers.

The invention also provides kits for carrying out the methods of the invention. Accordingly, a variety of kits are provided in suitable packaging. The kits may be used for any one or more of the uses described herein, and, accordingly, may contain instructions for detecting the presence or absence of each analyte of a plurality of analytes. A kit may comprise a coding scheme, or a decoding matrix, to assist the user in converting a cumulative measurement to a result indicating the presence or absence of each of a plurality of analytes. A kit may be a diagnostic kit, for example, a diagnostic kit suitable for the detection of any analyte, including the analytes recited herein. A kit may contain any of the compositions provided in this disclosure, including those recited above.

FIG. **6** shows schematics of exemplary kits of the invention. With reference to FIG. **6**, a kit comprising a kit body **601** is shown that contains reagents for the detection of analytes (e.g., polynucleotide probes comprising a fluorophore and a quencher) **602** and primers for the amplification of a nucleic acid **603**. The kit may also contain any other reagents, such as reagents suitable for performing an amplification reaction **604**. In the embodiment depicted in **601**, the reagents for the detection of analytes and the primers are each contained in a single volume (e.g., tube or a bottle). However, these reagents may also be provided in separate volumes, as depicted the kit body shown in **605**. The kit body **605** contains seven separate volumes. Three volumes (**606**, **607**, and **608**) contain reagents for the detection of analytes (or mixtures of such reagents) and three volumes (**609**, **610**, and **611**) contain primers (or mixtures of primers). The kit may also contain reagents suitable for performing an amplification reaction **604**.

FIG. **6** is provided for illustrative purposes only. Any number of reagents for the detection of analytes (e.g., probes) and any number of primers may be included in a single tube or bottle, as appropriate for the application. For example, in some embodiments, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more reagents for the detection of analytes may be included in a single bottle. In other cases, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more primers for amplification may be included in a single bottle or tube. In some cases, primers and probes may be provided in the same bottle or tube. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more probes may be provided per pair of primers. In other cases, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more primers may be provided per pair of probes. The kits may contain 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more bottles or tubes. Control analytes, and

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their associated detection reagents and primers, may also be included in the kit. These may be included in a separate bottle or tube or, for example, included within the bottle or tube providing reagents for an amplification reaction **604**. As described elsewhere in the specification, any reagent in the kits may be lyophilized.

VII. Systems and Software

The methods of this disclosure may also be performed as part of a system. For example, one or more steps of the methods described herein may be performed by software contained on a computer readable medium. The software may be used to encode analytes, check for degeneracy, reduce or eliminate degeneracy, and/or produce a decoding matrix. The software may also be used to convert cumulative measurements made on an instrument (e.g., a PCR machine) into information concerning the presence or absence of an analyte. For example, with reference to FIG. **4**, a computer **401** may be connected to an input device **403**, a display **402**, and a printer **404**. The computer may execute one or more of the steps of any of the methods described in this disclosure, including, for example, encoding methods **410**, analysis methods **420**, and decoding methods **430**. A computer may reduce or eliminate degeneracy, for example as part of the encoding methods **410**. Similarly, a computer may generate a coding schme that is non-degenerate by design using, for example, mathematical iteration. A computer may also be connected to a network **405**. The network may be a local network and/or the Internet. The network may be used to perform the methods of the invention on multiple computers in different locations. For example, one computer may perform the encoding, another computer may perform the measuring, and yet another computer may perform the decoding. A computer may perform any or all of these methods. The network may be used to transmit information obtained using the methods described in this disclosure throughout the world, for example across a border.

In one example, the software is embedded in the instrument making the measurement, such as a thermal cycler. In another example, the software is installed on a computer attached to the instrument making the measurement, such as a computer attached to a thermal cycler. In another example, the software is in the "cloud"—i.e., on a computer that another computer may communicate with through a network.

Each of the elements described above may be connected to a controller that communicates with each of the elements and coordinates their actions. For example, a controller may initiate the encoding of a series of analytes into values of color and intensity. As described throughout this disclosure, the controller can execute software to reduce or eliminate degeneracy by removing one or more potential analyte codes from the encoding matrix. The controller may then automatically order probes corresponding to this code from a manufacturer of probes. Alternatively, the controller may operate an oligonucleotide synthesis instrument that can synthesize the appropriate probes. After analysis of the sample on the instrument (which may also be automated), the controller may process the results of the analysis using the decoding matrix. The controller may then provide these results to a user.

VIII. Services

The methods provided herein may also be performed as a service. For example, a service provider may obtain the identity of a plurality of analytes that a customer wishes to analyze. The service provider may then encode each analyte to be detected by any of the methods described herein and provide appropriate reagents to the customer for the assay. The customer may perform the assay and provide the results to the service provider for decoding. The service provider may then provide the decoded results to the customer. The customer may also encode analytes, generate probes, and/or decode results by interacting with software installed locally (at the customer's location) or remotely (e.g., on a server reachable through a network). Exemplary customers include clinical laboratories, physicians, manufacturers of food and consumer products, industrial manufacturers (e.g., petroleum companies) and the like. A customer or party may be any suitable customer or party with a need or desire to use the methods, systems, compositions, and kits of the invention.

EXAMPLES

Example 1: General Materials & Methods

1. General Reagents

TE Buffer, pH7 (LIFE TECHNOLOGIES, Carlsbad, Calif.); UltraPure RNAse-free Water (LIFE TECHNOLOGIES, Carlsbad, Calif.); Taq 5× Master Mix (FISHER SCIENTIFIC COMPANY, Tustin, Calif.).

2. DNA Sequences, Primers, and Probes

Five nucleic acids from organisms of clinical relevance were chosen for the exemplary study: (1) human immunodeficiency virus 1 (HIV-1); (2) *Plasmodium falciparum* (Malaria); (3) herpes simplex virus-2 (HSV-2); (4) *Mycobacterium tuberculosis* (TB); (5) and dengue virus type 3 (dengue fever).

Two regions of diagnostic relevance from the HIV-1 genome, p17 and poly-protease, were selected from the Los Alamos National Laboratory HIV-1 reference sequence. A diagnostic sequence from the *Plasmodium falciparum* ChR7 gene was obtained from the UCSC *Plasmodium falciparum* Genome Browser. A sequence for Herpes Simplex Virus-2 was synthesized from the sequence obtained from the European Molecular Biology Library. Similarly a diagnostic sequence for the rpoB gene in *Mycobacterium tuberculosis* was synthesized from a sequence obtained from the European Molecular Biology Library. A PCR diagnostic sequence for Dengue Virus Type 3 was obtained from the National Institute of Health genetic sequence database.

Oligonucleotides were synthesized by INTEGRATED DNA TECHNOLOGIES (IDT). Probes and primer pairs were chosen for each analyte using OLIGOANALYZER, from IDT. Sense probes containing a fluorophore at the 5' end and a quencher at the 3' end were synthesized for all analytes. All oligonucleotides were lyophilized and reconstituted in TE buffer before use. Tables 9-14 show sequence information, target sequence, primers, and probes for each of the six analytes.

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TABLE 9

HIV-1 polyprotease sequence information.

Sequence Information	HIV-1 Poly protease 198mer synthesized from bases 2253-2550
Source	HIV-1 Reference Sequence, Los Alamos National Laboratory
Target sequence (3' to 5')	GGAAGCTCTATTAGATACAGGAGCAGATGATACAGTATTAGAAGAAATGA GTTTGCCAGGAAGATGGAAACCAAAAATGATAGGGGGAATTGGAGGTTTT ATCAAAGTAAGACAGTATGATCAGATACTCATAGAAATCTGTGGACATAA AGCTATAGGTACAGTATTAGTAGGACCTACACCTGTCAACATAATTGG (SEQ ID NO: 1)
Forward Prime (3' to 5')	GGAAGCTCTATTAGATACAGGAGCAG (SEQ ID NO: 2)
Reverse Primer (3' to 5')	CCAATTATGTTGACAGGTGTAGGTCC (SEQ ID NO: 3)
Probe 1 (3' to 5')	/56-FAM/TGAGTTTGCCAGGAAGATGGAAACCA/3BHQ_1/ (SEQ ID NO: 4)

TABLE 10

HIV p17 sequence information.

Sequence Name	HIV-1 P17 199mer synthesized from bases 790-1186
Source	HIV-1 Reference Sequence, Los Alamos National Laboratory
Target sequence (3' to 5')	CAGCTACAACCATCCCTTCAGACAGGATCAGAAGAACTTAGATCATTATA TAATACAGTAGCAACCCTCTATTGTGTGCATCAAAGGATAGAGATAAAAG ACACCAAGGAAGCTTTAGACAAGATAGAGGAAGAGCAAAACAAAAGTAAG AAAAAAGCACAGCAAGCAGCAGCTGACACAGGACACAGCAATCAGGTCA (SEQ ID NO: 5)
Forward Primer (3' to 5')	CAGCTACAACCATCCCTTCAGACA (SEQ ID NO: 6)
Reverse Primer (3' to 5')	TGACCTGATTGCTGTGTCCTGTGT (SEQ ID NO: 7)
Probe 1 (3' to 5')	/56-FAM/AGCAACCCTCTATTGTGTGCATCAAAGG/3BHQ_1 (SEQ ID NO: 8)
Probe 2 (3' to 5')	/5Cy3/AAAGCACAGCAAGCAGCAGCTGA/3BHQ_2/ (SEQ ID NO: 9)

TABLE 11

Plasmodium ChR7 sequence information.

Sequence Name	*Plasmodium* ChR7 199mer synthesized from bases 1139138-1141223
Source	*Plasmodium falciparum* Genome Browser Gateway
Target sequence (3' to 5')	GCCTAACATGGCTATGACGGGTAACGGGGAATTAGAGTTCGATTCCGGAG AGGGAGCCTGAGAAATAGCTACCACATCTAAGGAAGGCAGCAGGCGCGTA AATTACCCAATTCTAAAGAAGAGAGGTAGTGACAAGAAATAACAATGCAA GGCCAATTTAAAACCTTCCCAGAGTAACAATTGGAGGGCAAGTCTGGTG (SEQ ID NO: 10)
Forward Primer (3' to 5')	GCCTAACATGGCTATGACGGGTAA (SEQ ID NO: 11)
Reverse Primer (3' to 5')	CACCAGACTTGCCCTCCAATTGTT (SEQ ID NO: 12)

TABLE 11-continued

Plasmodium ChR7 sequence information.

Probe 1 (3' to 5')	/56-FAM/ATTCCGGAGAGGGAGCCTGAGAAATA/3BHQ_1/ (SEQ ID NO: 13)
Probe 2 (3' to 5')	/56-ROXN/AAGGAAGGCAGCAGGCGCGTAAATTA/3BHQ_2/ (SEQ ID NO: 14)

TABLE 12

Herpes simplex virus 2 sequence information.

Sequence Name	HSV-2 193mer synthesized from HSV-2 genome
Source	EMBL Bank AJ303204
Target sequence (3' to 5')	TCAGCCCATCCTCCTTCGGCAGTATGGAGGGTGTCGCGGCGGCGAGCCGC CGTCCCCAAAGACGTGCGGGTCGTACACGTACACGTACCAGGGCGGCGGG CCTCCGACCCGGTACGCTCTCGTAAATGCTTCCCTGCTGGTGCCGATCTG GGACCGCGCCGCGGAGACATTCGAGTACCAGATCGAACTCGG (SEQ ID NO: 15)
Forward Primer (3' to 5')	TCAGCCCATCCTCCTTCGGCAGTAT (SEQ ID NO: 16)
Reverse Primer (3' to 5')	CCGAGTTCGATCTGGTACTCGAATGT (SEQ ID NO: 17)
Probe 1 (3' to 5')	/56-FAM/AAAGACGTGCGGGTCGTACACGTACA/3BHQ_1/ (SEQ ID NO: 18)
Probe 2 (3' to 5')	/5Cy5/TAAATGCTTCCCTGCTGGTGCCGAT/31AbRQSp/ (SEQ ID NO: 19)

TABLE 13

Mycobacterium tuberculosis rpoB sequence information

Sequence Name	*Mycobacterium Tuberculosis* rpoB 200mer synthesized from Mycobacterium tuberculosis rpoB genome
Source	EMBL Bank GQ395623
Target sequence (3' to 5')	GAGTGCAAAGACAAGGACATGACGTACGCGGCCCCGCTGTTCGTCACGGC CGAGTTCATCAACAACAACACCGGCGAGATCAAGAGCCAGACGGTGTTCA TGGGTGACTTCCCGATGATGACCGAGAAGGGCACCTTCATCATCAACGGC ACCGAGCGCGTCGTGGTCAGCCAGCTGGTCCGCTCGCCCGGTGTGTACTT (SEQ ID NO: 20)
Forward Primer (3' to 5')	GAGTGCAAAGACAAGGACATGACG (SEQ ID NO: 21)
Reverse Primer (3' to 5')	AAGTACACACCGGGCGAGC (SEQ ID NO: 22)
Probe 1 (3' to 5')	/56-FAM/CGCTGTTCGTCACGGCCGAGTTCAT/3BHQ_1/ (SEQ ID NO: 23)
Probe 2 (3' to 5')	/5Cy3/AGATCAAGAGCCAGACGGTGTTCATG/3BHQ_2/ (SEQ ID NO: 24)
Probe 3 (3' to 5')	/5Cy5/AAGGGCACCTTCATCATCAACGGCA/3IAbRQSp/ (SEQ ID NO: 25)

TABLE 14

Dengue virus type 3 sequence information.

Sequence Name	Dengue Virus Type 3 200mer synthesized from Dengue Virus Type 3 genome
Source	GenBank M93130
Target sequence (3' to 5')	ATGCCAACTGTGATTGAGCACTTAGAAAGACTACAAAGGAAACATGGAGG AATGCTTGTGAGAAATCCACTCTCACGAAACTCCACGCACGAAATGTATT GGATATCCAATGGTACAGGCAACATCGTCTCATTCACAATGACACACAGG AGACCCACCATAGAGAAAGATGTGGATTTAGGAGCAGGAACCCGACATGT (SEQ ID NO: 26)
Forward Primer (3' to 5')	ATGCCAACTGTGATTGAGCACT (SEQ ID NO: 27)
Reverse Primer (3' to 5')	ACATGTCGGGTTCCTGCTCCTAAA (SEQ ID NO: 28)
Probe 1 (3' to 5')	/56-FAM/ACAAAGGAAACATGGAGGAATGCTTGTGA/3BHQ_1/ (SEQ ID NO: 29)
Probe 2 (3' to 5')	/5Cy3/ACTCTCACGAAACTCCACGCACGAAA/3BHQ_2/ (SEQ ID NO: 30)
Probe 3 (3' to 5')	/56-ROXN/ACAATGACACACAGGAGACCCACCAT/3BHQ_2/ (SEQ ID NO: 31)
Probe 4 (3' to 5')	/5Cy5/GGATATCCAATGGTACAGGCAACATCGT/3IAbRQSp/ (SEQ ID NO: 32)

3. Polymerase Chain Reactions

All PCR reactions were performed in a ROCHE 480 LIGHTCYCLER instrument. Forty-five thermal cycles were performed, with a 60 second hot-start at 95° C. The cycling conditions for denaturation, annealing, and extension were 45 seconds, 50 seconds, and 60 seconds at 95° C., 65° C., and 70° C., respectively. Each experiment was run in quintuplicate, with a reaction volume of 15 μL. Fluorescence measurements were obtained in the following channels after every annealing step: 483 nm to 533 nm (FAM), 523 nm to 568 nm (Cy3), 558 nm to 610 nm (ROX), and 615 nm to 670 nm (Cy5). Fluorescence measurements were also obtained after the hot-start and the end of the 45 thermal cycles. The change in the fluorescence intensity between these two measurements (after hot-start and at the end of the 45 cycles) determined the end-point fluorescent signal.

Example 2: Simultaneous Detection of Polynucleotide Markers from Pathogens Based on an Encoding Method Using More than One Color Per Analyte

This example describes the simultaneous detection of up to four polynucleotides selected from HIV (2 polynucleotides), HSV-2, *Mycobacterium, Plasmodium*, and dengue virus. Fourteen PCR reactions were assembled, using the reagents described in Table 15.

TABLE 15

Reagents for 14 PCR reactions.

Reaction No.	Type of Reagent	Reagent	Concentration	Volume
1		UltraPure Water	—	34 μL
		Taq 5x Master Mix	—	42 μL
	Template	Poly Protease Template	17 nM	2 μL

TABLE 15-continued

Reagents for 14 PCR reactions.

Reaction No.	Type of Reagent	Reagent	Concentration	Volume
	Primers	Poly Protease FWD Primer	1 nM	2 μL
		Protease RVS Primer	1 nM	2 μL
	Probe	Poly Protease Probe 1	1 nM	2 μL
2		UltraPure Water	—	28 μL
		Taq 5x Master Mix	—	42 μL
	Templates	Malaria Template	15 nM	2 μL
		Herpes Template	17 nM	2 μL
		TB Template	16 nM	2 μL
		Dengue Virus Template	17 nM	2 μL
	Primers	Poly Protease FWD Primer	1 nM	2 μL
		Protease RVS Primer	1 nM	2 μL
	Probe	Poly Protease Probe 1	1 nM	2 μL
3		UltraPure Water	—	32 μL
		Taq 5x Master Mix	—	42 μL
	Template	Malaria Template	15 nM	2 μL
	Primers	Malaria FWD Primer	1 μM	2 μL
		Malaria RVS Primer	1 μM	2 μL
	Probes	Malaria Probe 1	1 μM	2 μL
		Malaria Probe 2	1 μM	2 μL
4		UltraPure Water	—	26 μL
		Taq 5x Master Mix	—	42 μL
	Templates	Poly Protease Template	17 nM	2 μL
		Herpes Template	17 nM	2 μL
		TB Template	16 nM	2 μL
		Dengue Virus Template	17 nM	2 μL
	Primers	Malaria FWD Primer	1 μM	2 μL
		Malaria RVS Primer	1 μM	2 μL
	Probes	Malaria Probe 1	1 μM	2 μL
		Malaria Probe 2	1 μM	2 μL
5		UltraPure Water	—	32 μL
		Taq 5x Master Mix	—	42 μL
	Template	Herpes Template	17 nM	2 μL
	Primers	Herpes FWD Primer	1 μM	2 μL
		Herpes RVS Primer	1 μM	2 μL
	Probes	Herpes Probe 1	1 μM	2 μL
		Herpes Probe 2	1 μM	2 μL

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TABLE 15-continued

Reagents for 14 PCR reactions.

Reaction No.	Type of Reagent	Reagent	Concentration	Volume
6		UltraPure Water	—	26 µL
		Taq 5x Master Mix	—	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		TB Template	16 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
	Probes	Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
7		UltraPure Water	—	30 µL
		Taq 5x Master Mix	—	42 µL
	Template	TB Template	16 nM	2 µL
	Primers	TB FWD Primer	1 µM	2 µL
		TB RVS Primer	1 µM	2 µL
	Probes	TB Probe 1	1 µM	2 µL
		TB Probe 2	1 µM	2 µL
		TB Probe 3	1 µM	2 µL
8		UltraPure Water	—	30 µL
		Taq 5x Master Mix	—	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	TB FWD Primer	1 µM	2 µL
		TB RVS Primer	1 µM	2 µL
	Probes	TB Probe 1	1 µM	2 µL
		TB Probe 2	1 µM	2 µL
		TB Probe 3	1 µM	2 µL
9		UltraPure Water	—	28 µL
		Taq 5x Master Mix	—	42 µL
	Template	Dengue Virus Template	17 nM	2 µL
	Primers	Dengue Virus FWD Primer	1 µM	2 µL
		Dengue Virus RVS Primer	1 µM	2 µL
	Probes	Dengue Virus Probe 1	1 µM	2 µL
		Dengue Virus Probe 2	1 µM	2 µL
		Dengue Virus Probe 3	1 µM	2 µL
		Dengue Virus Probe 4	1 µM	2 µL
10		UltraPure Water	—	22 µL
		Taq 5x Master Mix	—	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
	Primers	Dengue Virus FWD Primer	1 µM	2 µL
		Dengue Virus RVS Primer	1 µM	2 µL
	Probes	Dengue Virus Probe 1	1 µM	2 µL
		Dengue Virus Probe 2	1 µM	2 µL
		Dengue Virus Probe 3	1 µM	2 µL
		Dengue Virus Probe 4	1 µM	2 µL
11		Taq 5x Master Mix	—	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Poly Protease FWD Primer	1 µM	2 µL
		Poly Protease RVS Primer	1 µM	2 µL
		Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
		Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
		Dengue Virus FWD Primer	1 µM	2 µL
		Dengue VirusRVS Primer	1 µM	2 µL
	Probes	Poly Protease Probe 1	1 µM	2 µL
		Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
		Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
		Dengue Virus Probe 1	1 µM	2 µL
		Dengue Virus Probe 2	1 µM	2 µL
		Dengue Virus Probe 3	1 µM	2 µL
		Dengue Virus Probe 4	1 µM	2 µL
12		UltraPure Water	—	2 µL
		Taq 5x Master Mix	—	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
	Primers	Poly Protease FWD Primer	1 µM	2 µL
		Poly Protease FWD Primer	1 µM	2 µL
		Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
		Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
		TB FWD Primer	1 µM	2 µL
		TB RVS Primer	1 µM	2 µL
	Probes	Poly Protease Probe 1	1 µM	2 µL
		Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
		Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
		TB Probe 1	1 µM	2 µL
		TB Probe 2	1 µM	2 µL
		TB Probe 3	1 µM	2 µL
13		UltraPure Water	—	12 µL
		Taq 5x Master Mix	—	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
	Primers	Poly Protease FWD Primer	1 µM	2 µL
		Poly Protease FWD Primer	1 µM	2 µL
		Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
		TB FWD Primer	1 µM	2 µL
		TB RVS Primer	1 µM	2 µL
	Probes	Poly Protease Probe 1	1 µM	2 µL
		Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
		TB Probe 1	1 µM	2 µL
		TB Probe 2	1 µM	2 µL
		TB Probe 3	1 µM	2 µL
14		UltraPure Water	—	10 µL
		Taq 5x Master Mix	—	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Poly Protease FWD Primer	1 µM	2 µL
		Poly Protease FWD Primer	1 µM	2 µL
		Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
		Dengue Virus FWD Primer	1 µM	2 µL
		Dengue Virus RVS Primer	1 µM	2 µL
	Probes	Poly Protease Probe 1	1 µM	2 µL
		Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
		Dengue Virus Probe 1	1 µM	2 µL
		Dengue Virus Probe 2	1 µM	2 µL
		Dengue Virus Probe 3	1 µM	2 µL
		Dengue Virus Probe 4	1 µM	2 µL

Referring to Table 15, reactions 1, 3, 5, 7, and 9 (containing HIV polyprotease, *Plasmodium*, HSV, *Mycobacterium*, and dengue analytes, respectively) were positive controls that provided baseline fluorescence intensity for each polynucleotide analytes alone, in the absence of other analytes. The change in fluorescence intensity in each color was used to provide the expected cumulative intensity level for each color in the chromatogram (FIG. **3**), as described more fully below. Reactions 2, 4, 6, 8, and 10 were negative controls used to determine the extent of non-specific amplification. In these reactions, the primers provided were not specific for the analytes provided.

Experiments 11, 12, 13, and 14 were multiplex detection experiments. Encoding was performed as indicated in Table

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16. The measured fluorescence intensity for each assay, in each color, was plotted as black circles in the chromatogram of FIG. **3**.

TABLE 16

Coding scheme for detection of pathogens.

	FAM	Cy3	ROX	Cy5
HIV poly-protease	1	0	0	0
HIV p17	1	1	0	0
Plasmodium	1	0	1	0
HSV	1	0	0	1
Mycobacterium	1	1	0	1
Dengue Virus	1	1	1	1

To construct the chromatogram, the signals of the positive controls (reactions 1, 3, 5, 7, and 9) were used to assemble every possible combination of present sequences, in each color. The cumulative signals were plotted in a diagram, called a "chromatogram" (FIG. **3**). Cumulative signals corresponding to all possible combinations of the same rank in the same color were organized into their own "band." Doing this for all four colors produced a level and band structure, against which the experimental results of each combination (reactions 1, 12, 13, and 14) could be evaluated. FIG. **3** shows three replicates of a chromatogram constructed using the positive control samples, with experimental results 4112, 3121, and 3102 superimposed on the chromatogram. The experimental results are indicated by black dots.

The intensity value for each color in reactions 11-14 was measure in arbitrary fluorescence units (AFU), plotted on the chromatogram, and then assigned to a band based on two criteria. First, if an experimental result was within a band, then the result was assigned to the multiplicity of the band (e.g., the Cy3 value of 1 in the left-most chromatogram of FIG. **3**). Second, if an experimental result was between two bands, a two-step analysis was employed. If the experimental result was between two legitimate results, the experimental result was assigned to the closest band. (e.g., the Cy5 value of 2 in the right-most chromatogram of FIG. **3**). If the experimental result was between a legitimate result and an illegitimate result, the result was assigned to the band with the legitimate result (e.g., the FAM value of 3 in the middle chromatogram of FIG. **3**). FIG. **8** schematically shows a legitimate result of **4112** (top) indicating the presence of HIV polyprotease, *Plasmodium*, herpes simplex, and *Mycobacterium*; and an illegitimate result of **4110** (bottom), which cannot be decoded and indicates a malfunction in the assay.

Using these criteria, the output of Experiment 12 was designated **4112**, which was the correct answer. The output of Experiment 14 could have been designated as **2121** or **3121**, but **2121** is illegitimate, so the result must be 3121, which is the correct answer. The output of Experiment 13 could be designated as **3102** or **3101**, both of which are legitimate, but the result is clearly closer to 2 than 1 in the Cy5 channel, so **3102**, the correct answer, was designated as the result. These results provide experimental validation of the methods of the provided herein.

The results indicate a trend of slightly lower combined signal than what would be expected from a simple summation of positive-control signals. This may be because the combination of signals is not perfectly linear. This may be a result of additional effects for which the chromatogram construction does not account for at present. These effects may be related to the absolute concentration of quenchers. The higher the absolute concentration of quenchers, the larger the percentage of reaction volume they quench, so that the same percentage of released fluorophores fails to contribute as much as expected to the cumulative signal. One solution to this problem is to use low concentrations of probes, so that the percentile loss in signal is negligible, regardless of how much quencher is released.

FIG. **3** shows that each rank containing multiple combinations has a relatively wide band. The width of the band is ultimately the difference between the highest and lowest cumulative signals for the positive control samples. If all positive controls in the particular color produced exactly the same fluorescence signal, then the width of each band would be zero. Since instead those signals are somewhat different, the resulting bands are relatively wide. However, there is a simple means to tighten the bands. Instead of loading all probes at the same concentration, as was done for the experiment above, the concentration of each probe can be adjusted so that each of the resulting positive-control signals is the same as the others in the same color. This approach would significantly tighten the bands, making it easier to determine the multiplicity of a signal for a sample containing analytes.

Example 3: Simultaneous Detection of Polynucleotide Markers from Pathogens Based on an Encoding Method Using One Color Per Analyte and Different Intensities

This example describes the simultaneous detection of up to three polynucleotides selected from dengue virus, HIV p17, and HIV polyprotease. The coding scheme was as depicted in Table 11. As shown in Table 17, each analyte was encoded as a single value of fluorescence intensity in a single color. In this case, as described elsewhere in this disclosure, the coding scheme is non-degenerate by design. This is achieved by assigning an intensity value to each analyte that is equal to the cumulative intensity values for the prior analytes, plus one. Of course, as described elsewhere in the specification, the initial assigned value can be greater than one, for example to better distinguish over noise. Similarly, the values assigned to each analyte can be greater than the cumulative intensity values for the prior analytes plus one, for example, to increase the separation between the intensities and enable more simple assignment of intensities.

TABLE 17

Coding scheme for detection of dengue virus, HIV polyprotease, and HIV p17.

	FAM
Dengue Virus	1
HIV p17	2
HIV polyprotease	4

The same reagents (e.g., templates, primers, and probes) used in Example 2 were used here. The fluorescence signals generated by the respective probes in positive-control endpoint PCR reactions were measured and used to calculate the probe concentrations that would produce 1×, 2×, and 4× signal intensities. Experiments were then set up to detect the seven non-null combinations of analyte occurrences. Tables 18-24 provide the PCR reaction components for each of these mixtures.

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TABLE 18

Binary FAM Experiment 1 Cocktail: HIV Polyprotease, HIV p17, and dengue virus

	Concentration	Volume Added
Reagents		
UltraPure Water	—	50 µL
Tag 5x Master Mix	—	25 µL
Templates		
Poly Protease Template	17 nM	4 µL
P17 Template	15 nM	4 µL
Dengue Virus Template	17 nM	4 µL
Primers		
Poly Protease FWD Primer	1 µM	4 µL
Poly Protease RVS Primer	1 µM	4 µL
P17 FWD Primer	1 µM	4 µL
P17 RVS Primer	1 µM	4 µL
Dengue Virus FWD Primer	1 µM	4 µL
Dengue Virus RVS Primer	1 µM	4 µL
Probes		
Poly Protease Probe 1	800 nM	4 µL
P17 Probe 1	400 nM	4 µL
Dengue Virus Probe 1	200 nM	4 µL

TABLE 19

Binary FAM Experiment 2 Cocktail: dengue virus and HIV p17

	Concentration	Volume Added
Reagents		
UltraPure Water	—	54 µL
Tag 5x Master Mix	—	25 µL
Templates		
Poly Protease Template	17 nM	—
P17 Template	15 nM	4 µL
Dengue Virus Template	17 nM	4 µL
Primers		
Poly Protease FWD Primer	1 µM	4 µL
Poly Protease RVS Primer	1 µM	4 µL
P17 FWD Primer	1 µM	4 µL
P17 RVS Primer	1 µM	4 µL
Dengue Virus FWD Primer	1 µM	4 µL
Dengue Virus RVS Primer	1 µM	4 µL
Probes		
Poly Protease Probe 1	800 nM	4 µL
P17 Probe 1	400 nM	4 µL
Dengue Virus Probe 1	200 nM	4 µL

TABLE 20

Binary FAM Experiment 3 Cocktail: dengue virus and HIV polyprotease

	Concentration	Volume Added
Reagents		
UltraPure Water	—	54 µL
Tag 5x Master Mix	—	25 µL

TABLE 20-continued

Binary FAM Experiment 3 Cocktail: dengue virus and HIV polyprotease

	Concentration	Volume Added
Templates		
Poly Protease Template	17 nM	4 µL
P17 Template	15 nM	—
Dengue Virus Template	17 nM	4 µL
Primers		
Poly Protease FWD Primer	1 µM	4 µL
Poly Protease RVS Primer	1 µM	4 µL
P17 FWD Primer	1 µM	4 µL
P17 RVS Primer	1 µM	4 µL
Dengue Virus FWD Primer	1 µM	4 µL
Dengue Virus RVS Primer	1 µM	4 µL
Probes		
Poly Protease Probe 1	800 nM	4 µL
P17 Probe 1	400 nM	4 µL
Dengue Virus Probe 1	200 nM	4 µL

TABLE 21

Binary FAM Experiment 4 Cocktail: HIV polyprotease and HIV p17

	Concentration	Volume Added
Reagents		
UltraPure Water	—	54 µL
Tag 5x Master Mix	—	25 µL
Templates		
Poly Protease Template	17 nM	4 µL
P17 Template	15 nM	4 µL
Dengue Virus Template	17 nM	—
Primers		
Poly Protease FWD Primer	1 µM	4 µL
Poly Protease RVS Primer	1 µM	4 µL
P17 FWD Primer	1 µM	4 µL
P17 RVS Primer	1 µM	4 µL
Dengue Virus FWD Primer	1 µM	4 µL
Dengue Virus RVS Primer	1 µM	4 µL
Probes		
Poly Protease Probe 1	800 nM	4 µL
P17 Probe 1	400 nM	4 µL
Dengue Virus Probe 1	200 nM	4 µL

TABLE 22

Binary FAM Experiment 5 Cocktail: *dengue virus*

	Concentration	Volume Added
Reagents		
UltraPure Water	—	58 µL
Tag 5x Master Mix	—	25 µL
Templates		
Poly Protease Template	17 nM	—
P17 Template	15 nM	—
Dengue Virus Template	17 nM	4 µL
Primers		
Poly Protease FWD Primer	1 µM	4 µL
Poly Protease RVS Primer	1 µM	4 µL
P17 FWD Primer	1 µM	4 µL
P17 RVS Primer	1 µM	4 µL

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TABLE 22-continued

Binary FAM Experiment 5 Cocktail: *dengue virus*

	Concentration	Volume Added
Dengue Virus FWD Primer	1 μM	4 μL
Dengue Virus RVS Primer	1 μM	4 μL
Probes		
Poly Protease Probe 1	800 nM	4 μL
P17 Probe 1	400 nM	4 μL
Dengue Virus Probe 1	200 nM	4 μL

TABLE 23

Binary FAM Experiment 6 Cocktail: HIV p17

	Concentration	Volume Added
Reagents		
UltraPure Water	—	58 μL
Tag 5x Master Mix	—	25 μL
Templates		
Poly Protease Template	17 nM	—
P17 Template	15 nM	4 μL
Dengue Virus Template	17 nM	—
Primers		
Poly Protease FWD Primer	1 μM	4 μL
Poly Protease RVS Primer	1 μM	4 μL
P17 FWD Primer	1 μM	4 μL
P17 RVS Primer	1 μM	4 μL
Dengue Virus FWD Primer	1 μM	4 μL
Dengue Virus RVS Primer	1 μM	4 μL
Probes		
Poly Protease Probe 1	800 nM	4 μL
P17 Probe 1	400 nM	4 μL
Dengue Virus Probe 1	200 nM	4 μL

TABLE 24

Binary FAM Experiment 7 Cocktail: HIV polyprotease

	Concentration	Volume Added
Reagents		
UltraPure Water	—	58 μL
Tag 5x Master Mix	—	25 μL
Templates		
Poly Protease Template	17 nM	4 μL
P17 Template	15 nM	—
Dengue Virus Template	17 nM	—

TABLE 24-continued

Binary FAM Experiment 7 Cocktail: HIV polyprotease

	Concentration	Volume Added
Primers		
Poly Protease FWD Primer	1 μM	4 μL
Poly Protease RVS Primer	1 μM	4 μL
P17 FWD Primer	1 μM	4 μL
P17 RVS Primer	1 μM	4 μL
Dengue Virus FWD Primer	1 μM	4 μL
Dengue Virus RVS Primer	1 μM	4 μL
Probes		
Poly Protease Probe 1	800 nM	4 μL
P17 Probe 1	400 nM	4 μL
Dengue Virus Probe 1	200 nM	4 μL

The total fluorescence signal of each experiment was plotted on a chromatogram (FIG. **7**) that was constructed as described above. The width of the bands around each positive control measurement is the propagated uncertainty of the 1X measurement. That uncertainty was equated to the standard deviation of the fluorescence signals of the last five PCR cycles in saturation.

FIG. **7** shows the results of this experiment. The three right-most results show the measured intensities for dengue virus alone (DEN), HIV p17 alone (P17), and HIV polyprotease (TPP). The assigned intensities for each of these analytes were as indicated in Table 1, and FIG. **7** confirms that the three analytes yielded the expected intensity results when alone (see DEN, P17, and TPP). At the end of the assay, essentially all of the probes have been hydrolyzed, releasing essentially all of the fluorophores, which emit a signal. A fixed amount of a fluorophore produces a fixed and predictable amount of signal. Therefore a target that is present should generally produce a fixed amount of signal, as determined by the amount of probe added to the reaction volume.

As expected, the intensity values for the combinations of analytes are equivalent to the sum of the assigned intensities. Since this coding scheme is designed to be non-degenerate, each result can be unambiguously decoded into the presence or absence of a particular analyte. For example, starting from the left side of FIG. **7**, a result of 7 indicates the presence of all three analytes. A result of 3 indicates the presence of dengue virus and HIV p17 analytes, and the absence of HIV polyprotease. A result of 5 indicates the presence of dengue virus and HIV polyprotease, and the absence of HIV p17. Finally a result of 6 indicates the presence of HIV p17 and HIV polyprotease, and the absence of dengue virus. This coding scheme can be extended to encode additional analytes, as described elsewhere in this disclosure.

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gagaggtagt gacaagaaat aacaatgcaa ggccaattta aaaccttccc agagtaacaa 180

ttggagggca agtctggtg 199

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<220> FEATURE:
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<400> SEQUENCE: 13

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cgctcgcccg gtgtgtactt 200

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aacatcgtct cattcacaat gacacacagg agacccacca tagagaaaga tgtggattta 180

ggagcaggaa cccgacatgt 200

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<400> SEQUENCE: 31

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What is claimed is:

1. A method of detecting the presence or absence of at least seven polynucleotide analytes, in any combination of presence or absence, in a single sample solution volume, the method comprising:

a) providing a sample solution volume comprising or potentially comprising, at least one of at least seven polynucleotide analytes, and comprising at least seven hybridization probes, each hybridization probe corresponding to one of the at least seven polynucleotide analytes, wherein each hybridization probe comprises at least one fluorophore selected from four fluorophores, and wherein each hybridization probe, when excited and when contacted with its corresponding polynucleotide analyte, generates a cumulative intensity signal, further wherein each generated cumulative intensity signal comprises at least one signal generated by excitement of at least one of the four fluorophores;

b) contacting said sample solution volume with the plurality of hybridization probes and exciting the hybridization probes to generate the cumulative intensity signal(s) if one or more of the at least seven polynucleotide analytes is present in the sample volume; and

c) measuring the cumulative intensity signal(s), thereby generating a cumulative signal measurement, wherein the cumulative signal measurement non-degenerately indicates the presence or absence of the at least seven polynucleotide analytes, in any combination of presence or absence,

wherein said method non-degenerately detects the presence or absence of the at least seven polynucleotide analytes in said single sample solution volume, in any combination of presence or absence, without requiring any step of immobilization of said polynucleotide analytes, physical separation of said polynucleotide analytes, or mass spectrometry.

2. The method of claim **1**, wherein said hybridization probes comprise an identical fluorophore, and wherein said plurality of hybridization probes are specific for different polynucleotide analytes.

3. The method of claim **1**, wherein said detecting is performed with a plurality of hybridization probes comprising different fluorophores, wherein said plurality of hybridization probes are specific for different analytes.

4. The method of claim **1**, wherein said cumulative intensity signal(s) are generated during a polymerase chain reaction.

5. The method of claim **1**, further comprising transmitting information concerning the presence or absence of said polynucleotide analytes through a computer network.

6. The method of claim **1**, further comprising providing information concerning the presence or absence of said polynucleotide analytes to a health care professional, wherein the health care professional makes a clinical decision based on said information.

7. The method of claim **1**, wherein at least one step of said method is performed using a computer.

8. The method of claim **7**, wherein said computer is located on a remote server.

9. The method of claim **7**, wherein said computer is located on a thermal cycler.

* * * * *

EXHIBIT B

US010770170B2

(12) **United States Patent**
Kartalov et al.

(10) **Patent No.: US 10,770,170 B2**
(45) **Date of Patent: *Sep. 8, 2020**

(54) **SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS**

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G01N 33/50 (2006.01)
(Continued)

(52) **U.S. Cl.**
CPC ***G16B 25/00*** (2019.02); ***C12Q 1/68*** (2013.01); ***C12Q 1/6825*** (2013.01);
(Continued)

(58) **Field of Classification Search**
None
See application file for complete search history.

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(57) **ABSTRACT**

This disclosure provides methods, systems, compositions, and kits for the multiplexed detection of a plurality of analytes in a sample. In some examples, this disclosure provides methods, systems, compositions, and kits wherein multiple analytes may be detected in a single sample volume by acquiring a cumulative measurement or measurements of at least one quantifiable component of a signal. In some cases, additional components of a signal, or additional signals (or components thereof) are also quantified. Each signal or component of a signal may be used to construct a coding scheme which can then be used to determine the presence or absence of any analyte.

20 Claims, 8 Drawing Sheets

Specification includes a Sequence Listing.

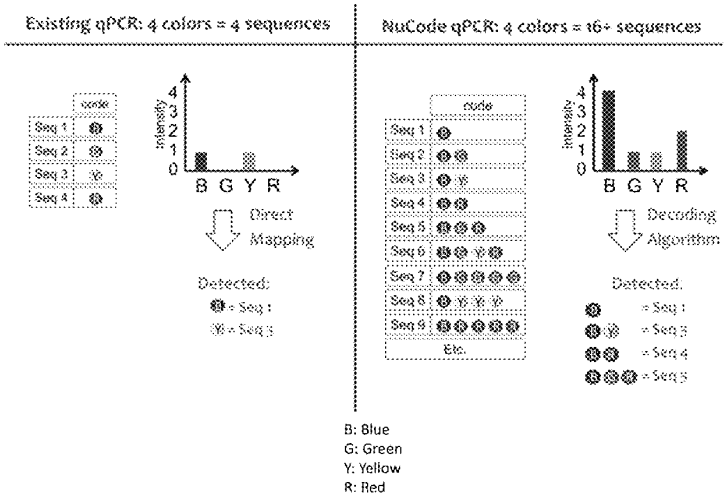


EXHIBIT B

Related U.S. Application Data

continuation of application No. 13/756,760, filed on Feb. 1, 2013, now Pat. No. 8,838,394.

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Fig. 1

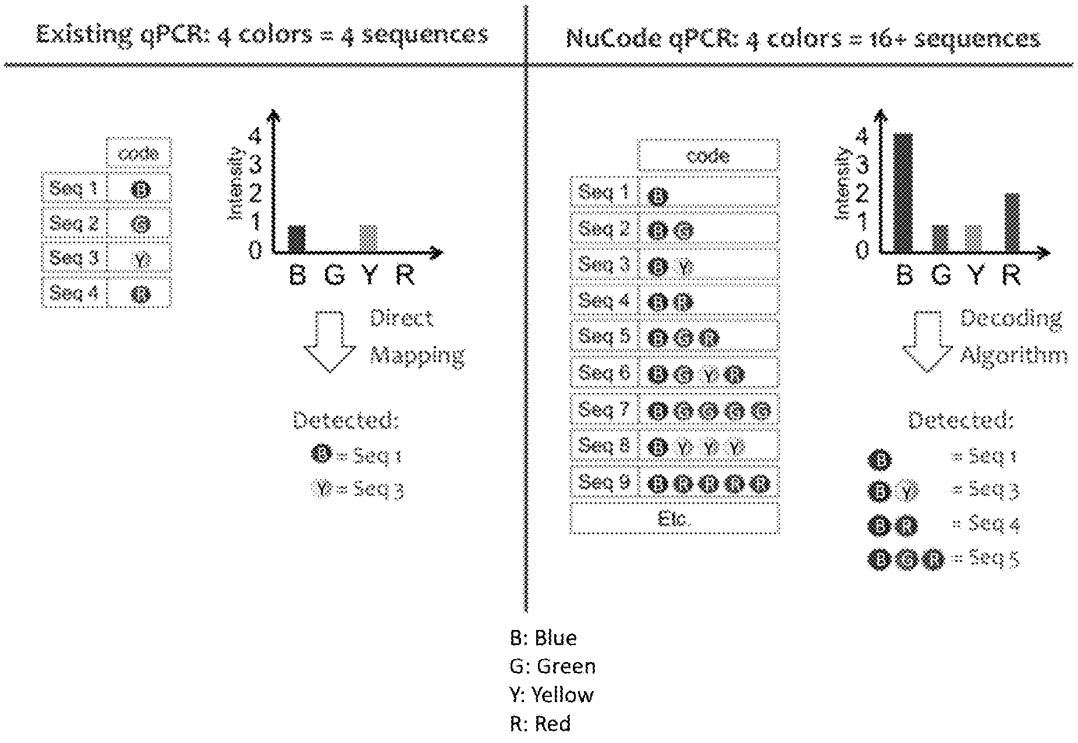
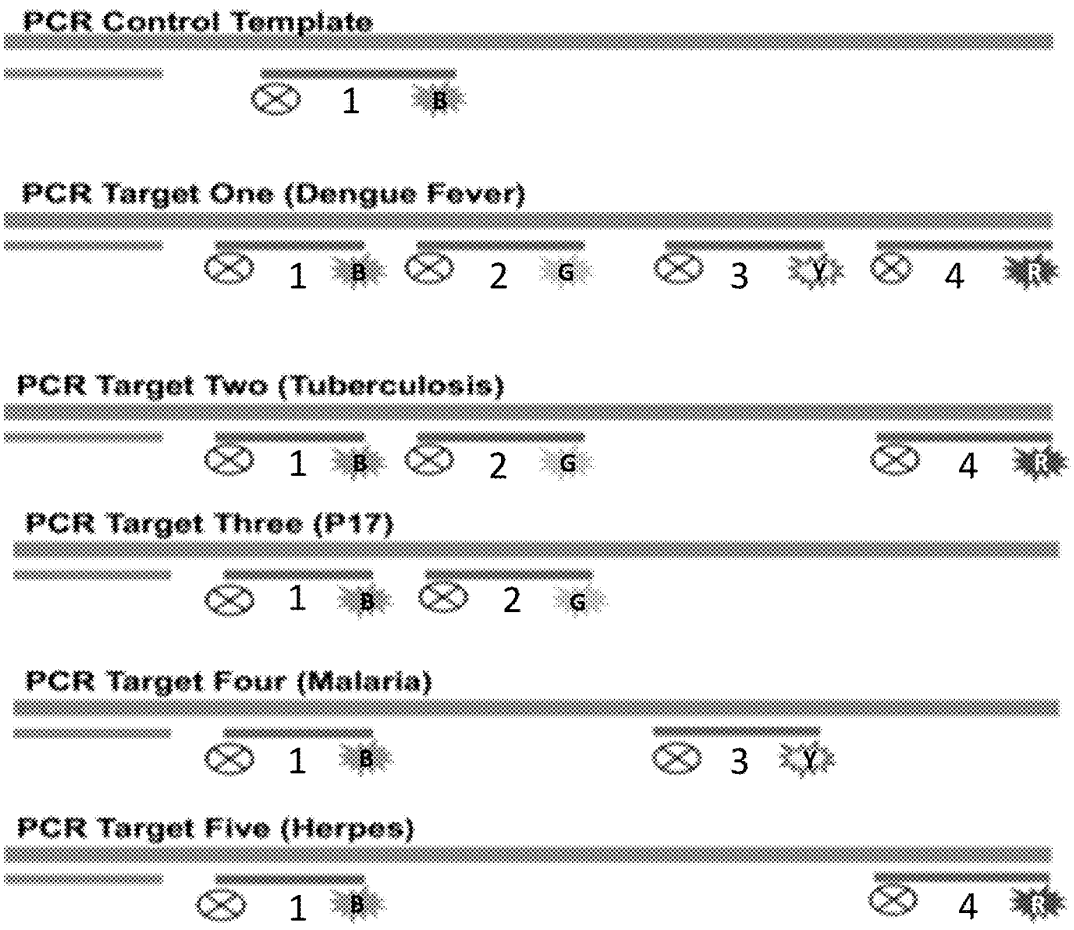
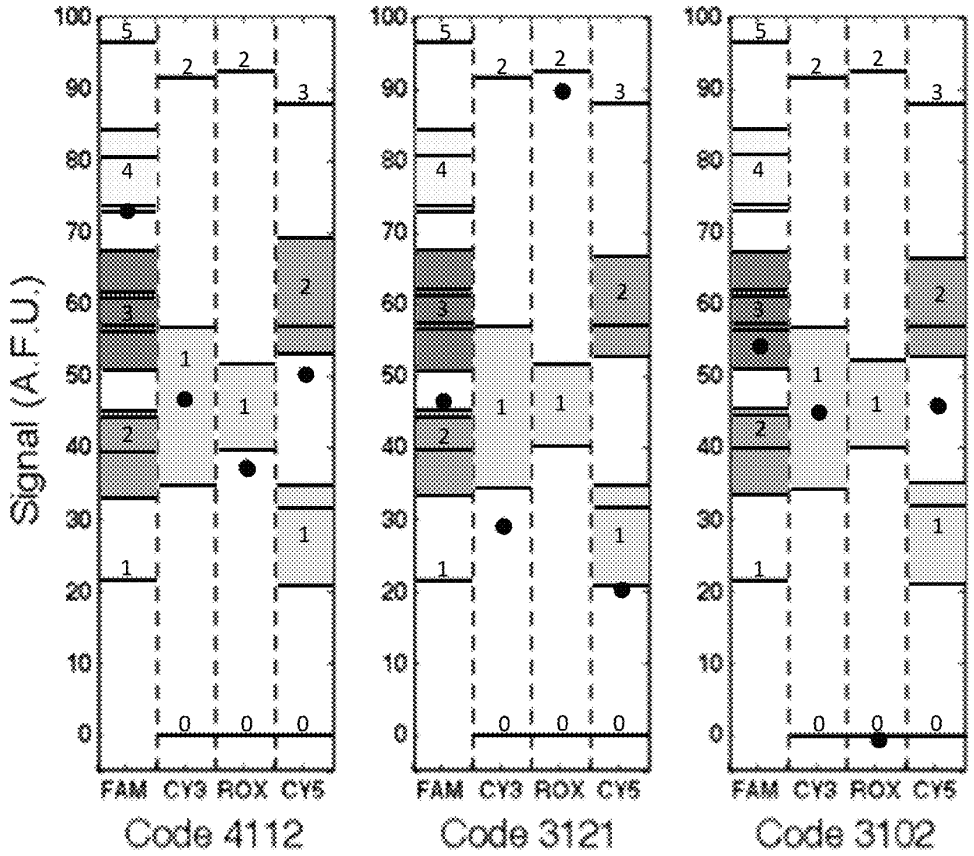

Existing qPCR: 4 colors = 4 sequences
NuCode qPCR: 4 colors = 16+ sequences
code
Seq 1
Seq 2
Seq 3
Seq 4
Intensity
B G Y R
Direct Mapping
Detected:
= Seq 1
= Seq 3
Seq 5
Seq 6
Seq 7
Seq 8
Seq 9
Etc.
Decoding Algorithm
= Seq 4
= Seq 5
B: Blue
G: Green
Y: Yellow
R: Red

Fig. 2



B: Blue
G: Green
Y: Yellow
R: Red

Fig. 3



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Fig. 4

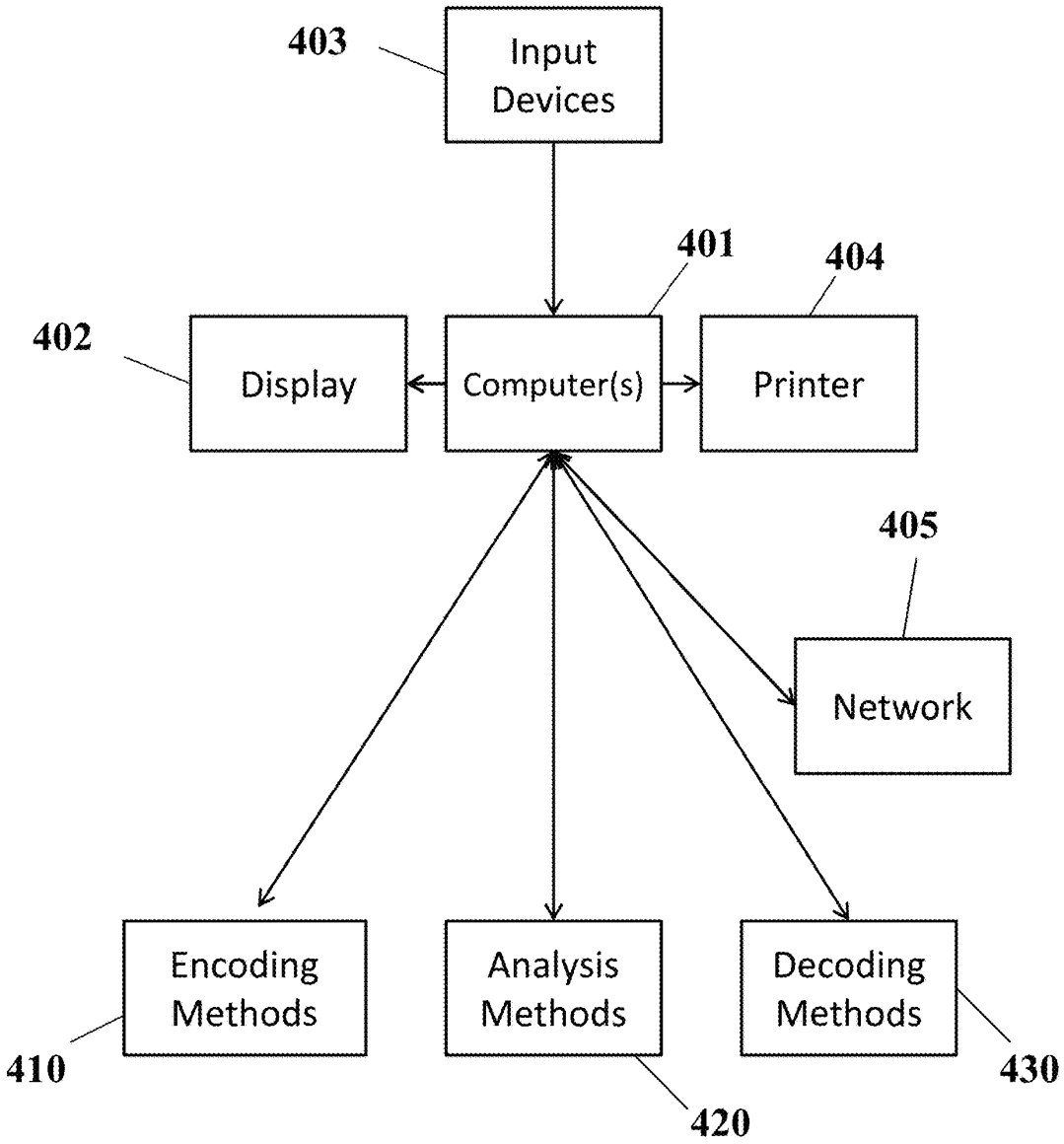
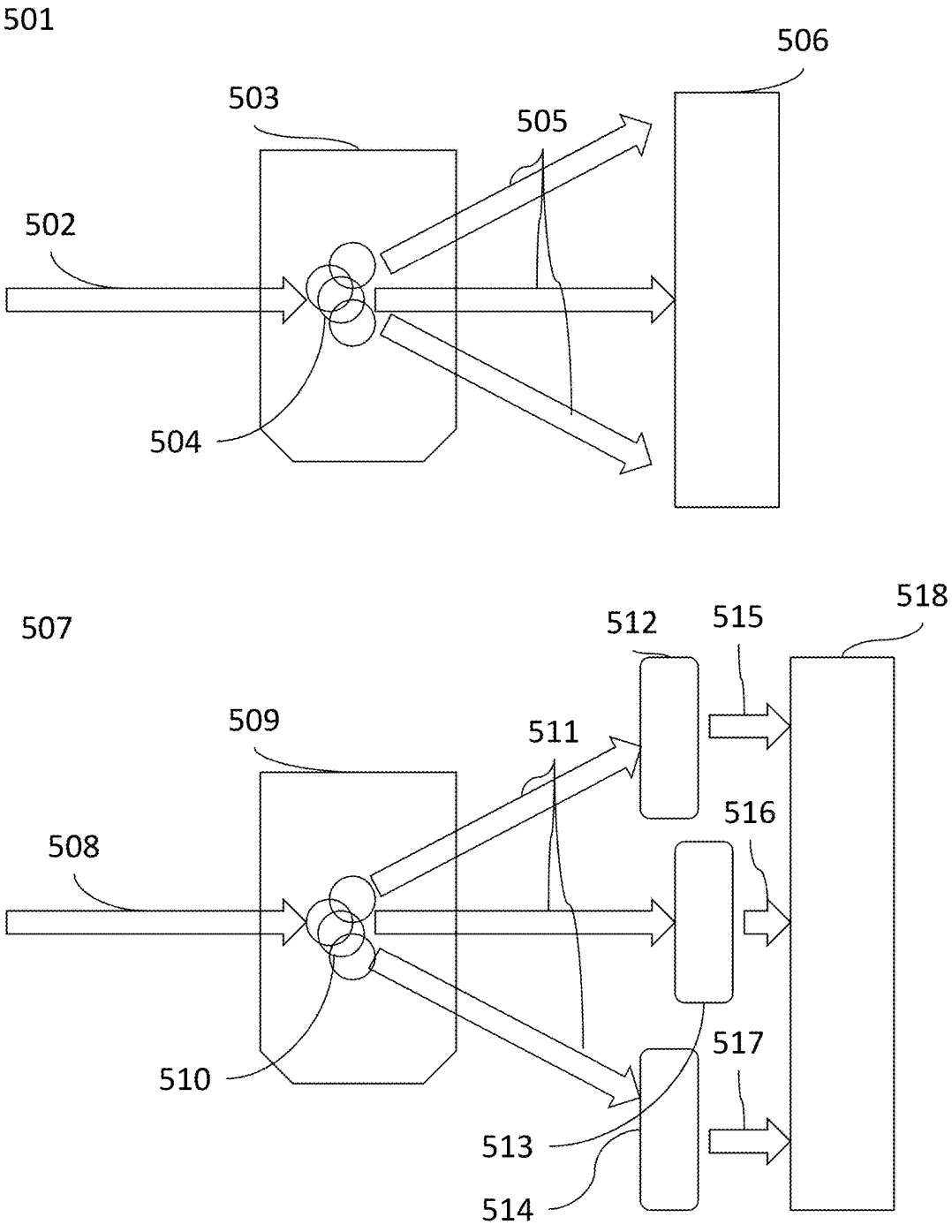
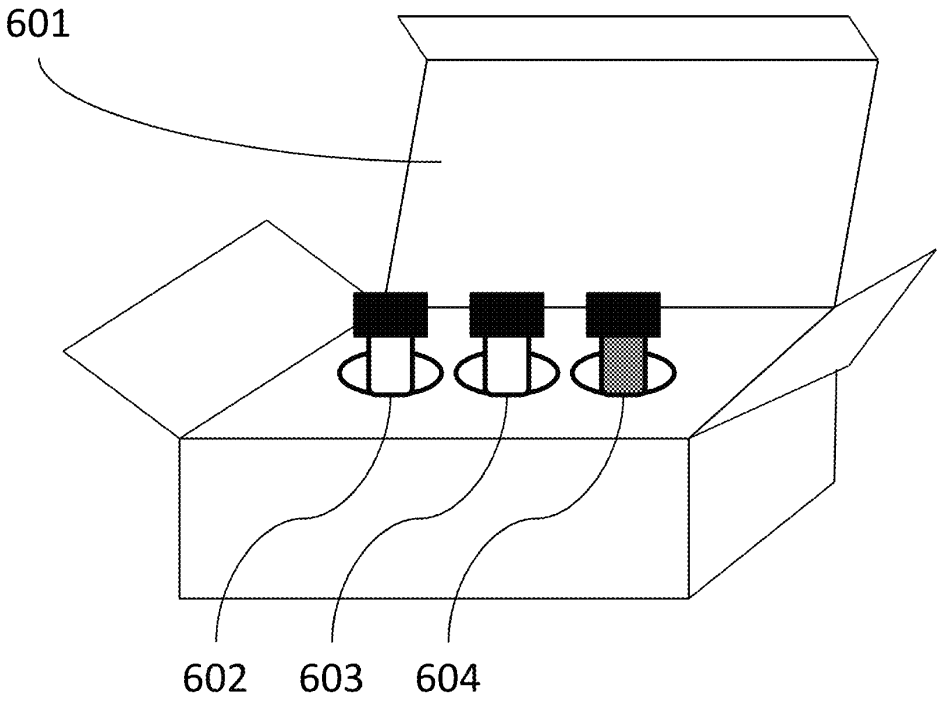

403
Input Devices
401
404
402
Display
Computer(s)
Printer
405
Network
Encoding Methods
Analysis Methods
Decoding Methods
410
420
430

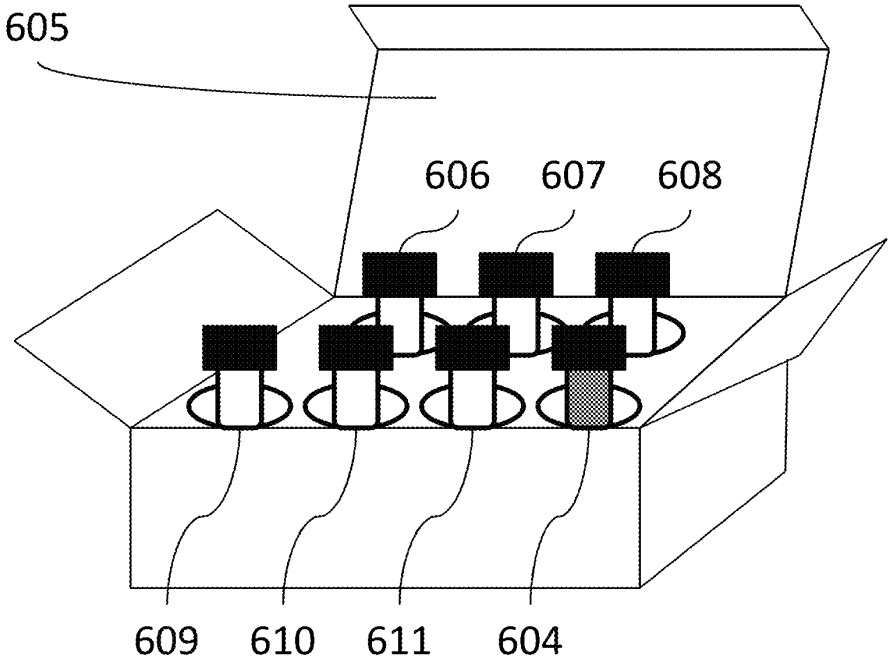
Fig. 5



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Fig. 6





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Fig. 7

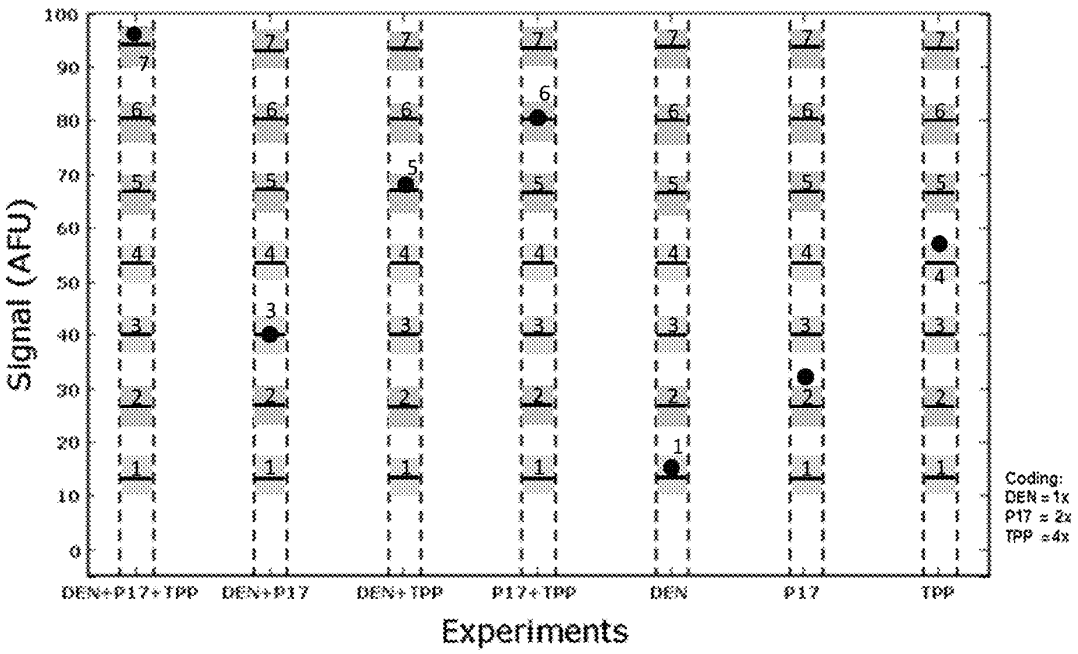
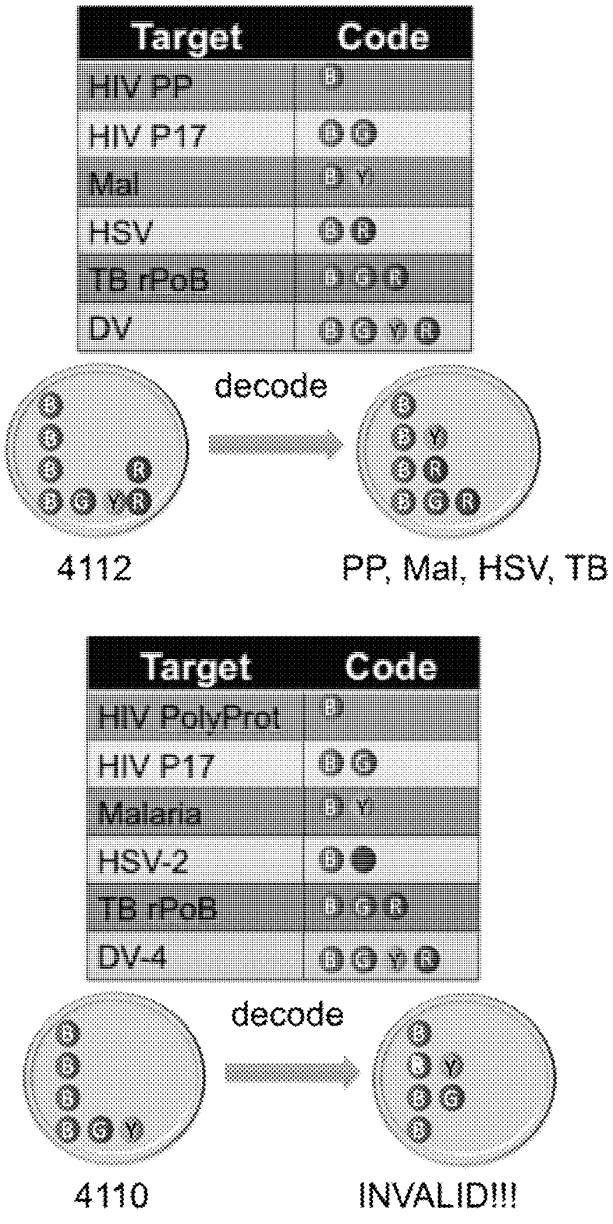

Signal (AFU)
Experiments
DEN+P17+TPP
DEN+P17
DEN+TPP
P17+TPP
DEN
P17
TPP
Coding:
DEN = 1x
P17 = 2x
TPP = 4x

Fig. 8



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SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

CROSS-REFERENCE

This application is a continuation of U.S. patent application Ser. No. 14/451,876, filed Aug. 5, 2014, which is a continuation of U.S. patent application Ser. No. 13/756,760, filed Feb. 1, 2013, which claims the benefit of U.S. provisional applications 61/594,480, filed Feb. 3, 2012 and 61/703,093, filed Sep. 19, 2012. The content of these applications is incorporated herein by reference in its entirety.

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

This invention was made with the support of the United States government under Grant No. 1144469 awarded by NSF. The government has certain rights in the invention.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on Aug. 20, 2014, is named 38075-716-301-Seqlist.txt and is 9,260 bytes in size.

BACKGROUND

Multiplexed reactions offer significant advantages over traditional uniplex reactions, including performance of parallel reactions on the same sample, use of the same chamber to perform multiple reactions, and the ability to extract rich information from a sample in a fast and efficient manner. However, to achieve these benefits, multiplexed assays generally require complex reporting mechanisms, namely spectrally resolved fluorescence or chemiluminescence (e.g., PCR, ELISA), spatially resolved signals (e.g., microarrays, gel electrophoresis), temporally resolved signals (e.g., capillary electrophoresis), or combinations thereof (e.g., Sanger sequencing). There is a need for multiplexed reactions that can be carried out in a single solution.

SUMMARY OF THE INVENTION

This disclosure provides methods, compositions, systems, and kits for the multiplexed detection of analytes. In some cases, this disclosure provides assays that are capable of unambiguously detecting the presence or absence of each of at least 7 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis. In some examples, each of the analytes is encoded as a value of one (or at least one) component of a signal.

In some examples, an assay provided in this disclosure is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M=\log_2 (F+1)$ and F is the maximum cumulative value of one component of a signal (e.g., an intensity) when all the analytes are present.

In some cases, each analyte is encoded as at least one first value in a first component of a signal (e.g., at least one intensity or range of intensities) and at least one second value in a second component of a signal (e.g., at least one wavelength or range of wavelengths). In some examples, each analyte is encoded as a first value in a first component of a signal at each of a plurality of second values in a second component of a signal (e.g., a signal intensity or range of signal intensities at each of a plurality of wavelengths or ranges of wavelengths).

In some examples, an assay provided herein is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M=C*\log_2 (F+1)$, where C is the number of the second values used to encode the analytes and F is the maximum cumulative value of the first component of the signal, for any second value, when all of the analytes are present.

In some cases, an assay provided in this disclosure is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M=(P*T)+1$, where P is the number of codes per tier in a coding scheme and T is the number of tiers. In some cases, the number of tiers $T=\log_4 (F+1)$, and F is the maximum cumulative value of a first component of a signal, for any second value, when all of the analytes are present.

In some examples, the first value is an intensity or range of intensities. In some cases, a coding scheme comprises at least three intensities or ranges of intensities.

In some cases, the second value is a wavelength or range of wavelengths. In some examples, the coding scheme comprises at least five wavelengths or ranges of wavelengths.

In some examples, the signal is an electromagnetic signal. In some cases, the electromagnetic signal is a fluorescence emission signal. In some examples, the intensity of the fluorescence emission signal is measured at at least four wavelengths or ranges of wavelengths.

In some cases, an assay provided herein is performed with reagents that are lyophilized prior to use.

In some cases, a detecting step is performed with reagents comprising hybridization probes. In some examples, the number of the hybridization probes is greater than the number of analytes. In some cases, the set of hybridization probes comprise one or more hybridization probes specific for different analytes and comprising an identical fluorophore or combination of fluorophores. In some examples, a sample is contacted with at least 18 of said hybridization probes. In some examples, a detecting step is performed with reagents comprising at least one pair of primers. In some cases, at least one pair of primers are capable of amplifying a region complementary to at least three of said hybridization probes.

In some examples, the signal that is measured is generated during a polymerase chain reaction. In some cases, the polymerase chain reaction is selected from the group consisting of an end-point polymerase chain reaction, a real-time polymerase chain reaction, a digital polymerase chain reaction, and combinations thereof.

In some examples, at least one cumulative measurement is performed on a solution.

In some examples, at least one analyte is encoded by at least one additional value (i.e., at least two values together), wherein the at least one additional value is selected from the group consisting of a value from at least one additional component of a signal, a value from at least one component of a different signal, and combinations thereof. For example, the at least one additional value may be selected from the group consisting of a fluorescence emission intensity, a fluorescence emission wavelength, a Förster resonance energy transfer (FRET) emission intensity, a FRET emission wavelength, an electrochemical signal, a chemiluminescence wavelength, a chemiluminescence intensity, a fluo-

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rescence bleaching rate, a chemiluminescence bleaching rate, and combinations thereof.

In some cases, a chromatogram is constructed. The chromatogram may be constructed by plotting all possible combinations of first values and second values for positive control samples for each analyte.

In some examples, at least one of the analytes comprises a polynucleotide. In some cases, the polynucleotide is from a source selected from the group consisting of an animal, a plant, a bacteria, a fungus, a parasite, and a virus. In some examples, the polynucleotide is from a source selected from the group consisting of human immunodeficiency virus, herpes simplex virus, human papilloma virus, *Plasmodium, Mycobacterium,* dengue virus, hepatitis virus, and influenza virus. In some cases, the polynucleotide is selected from the group consisting of human immunodeficiency virus polyprotease, human immunodeficiency virus p17, human papilloma virus E6, and human papilloma virus E7.

In some cases, a sample is selected from the group consisting of a clinical sample, a food sample, an environmental sample, a pharmaceutical sample, and a sample from a consumer product.

In some examples, information concerning the presence or absence of an analyte is transmitted through a computer network.

In some cases, information concerning the presence or absence of an analyte is provided to a physician. In some examples, a clinical decision is made based on such information.

In some examples, at least one step of a method provided herein is performed using instructions on a computer readable medium. In some cases, the instructions are located on a remote server. In some examples, the instructions are located on a thermal cycler. In some cases, the instructions are located on a computer in communication with a thermal cycler.

In some cases, at least one of the analytes is a positive control analyte.

In some examples, the coding scheme is non-degenerate. In some cases, the coding scheme is designed to be non-degenerate. In some examples, the coding scheme is made non-degenerate by enumerating every legitimate result that can be obtained from the coding scheme, identifying each legitimate result that is degenerate, and eliminating at least one potential analyte code from the coding scheme to eliminate degeneracy.

In some cases, an assay provided herein is an end-point assay. In some examples an assay provided in this disclosure is ended at a threshold number of cycles set by the limit of detection of an instrument.

In certain examples, an assay provided herein is a liquid phase assay.

In some cases, an assay provided herein is quantitative.

In some cases, this disclosure provides a method of detecting the presence or absence of each analyte of a plurality of analytes, comprising: (a) encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy; (b) providing a sample comprising, or potentially comprising, at least one of said analytes; (c) contacting said sample with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present; (d) cumulatively measuring said first values of said signal within said sample, thereby providing a cumulative measurement; and (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme.

In some examples, the method described in the preceding paragraph is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M=\log_2 (F+1)$ and F is the maximum cumulative value of the first values when all of the analytes are present. In some cases, the first value is an intensity or range of intensities. In some examples, the first value has a minimum value in the coding scheme that is selected from the group consisting of at least 1, at least 2, at least 4, at least 8, at least 16, at least 32, and at least 64. In some cases, the first value is incremented in the coding scheme by an amount equal to the cumulative maximum of said preceding first values plus one. In other cases, the first value is incremented in the coding scheme by an amount greater than the cumulative maximum of the preceding first values plus one.

In some cases, this disclosure provides a method of detecting the presence or absence of each analyte of a plurality of analytes, comprising: (a) encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy; (b) providing a sample comprising, or potentially comprising, at least one of said analytes; (c) contacting said sample with analyte-specific reagents that generate said at least one first value and at least one said second value, as represented in said coding scheme, when each of said analytes is present; (d) cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme, wherein, when degeneracy is eliminated, said method is capable of unambiguously detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

In some cases, each of the analytes of the method described in the preceding paragraph is encoded as a first value in a first component of the signal at each of a plurality of second values in a second component of the signal. In some cases, when degeneracy is eliminated, this method is capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single volume using four second values. In some examples, when degeneracy is eliminated, the coding scheme comprises T non-degenerate tiers, wherein $T=\log_4 (F+1)$ and F is the maximum cumulative value of the first component of the signal, for any second value, when all of the analytes are present. In some cases, when degeneracy is eliminated, the method is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M=(P*T)+1$ and P is the number of codes per tier. In some examples, when degeneracy is eliminated, the method is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M=C*\log_2 (F+1)$, C is the number of second values in the coding scheme, and F is the maximum cumulative value of the first component of the signal, for any second value, when all of the analytes are present.

This disclosure also provides non-degenerate coding schemes capable of unambiguously encoding the presence or absence of each of at least 7 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis.

In some examples, a non-degenerate coding scheme of this disclosure is generated by a method comprising: (a) generating a code for each potential analyte, wherein each potential analyte is encoded by at least one value of at least one component of a signal; (b) enumerating every legitimate cumulative result for all possible combinations of presence or absence of each analyte; (c) identifying each legitimate result that is degenerate; and (d) eliminating at least one code to eliminate degeneracy. In some cases, the coding scheme may be expanded by adding additional codes that are at least one unit greater than the sum of all previous codes in the at least one component of said signal. In some cases a coding scheme is generated by a method of mathematical iteration that guarantees non-degeneracy by construction.

In some cases, this disclosure provides systems for detecting the presence or absence of each analyte of a plurality of analytes, comprising: (a) encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy; (b) providing a sample comprising, or potentially comprising, at least one of said analytes; (c) contacting said sample with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present; (d) cumulatively measuring said first values of said signal within said sample, thereby providing a cumulative measurement; and (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme.

In some cases, this disclosure provides systems for detecting the presence or absence of each analyte of a plurality of analytes, comprising: (a) encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy; (b) providing a sample comprising, or potentially comprising, at least one of said analytes; (c) contacting said sample with analyte-specific reagents that generate said at least one first value and said at least one second value, as represented in said coding scheme, when each of said analytes is present; (d) cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme, wherein, when degeneracy is eliminated, said system is capable of unambiguously detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

In some cases, this disclosure provides methods of detecting the presence or absence of each analyte of a plurality of analytes for a third party, comprising: (a) obtaining the identity of each of said analytes from a party; (b) encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy; (c) providing said party with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present, said party: (i) contacting a sample comprising, or potentially comprising, at least one of said analytes with said reagents; and (ii) cumulatively measuring said first values within said sample, thereby providing a cumulative measurement; and (d) obtaining said cumulative measurement from said party; (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme; and (f) providing said party with information about the presence or absence of each of said analytes.

In some cases, this disclosure provides methods of detecting the presence or absence of each analyte of a plurality of analytes for a third party, comprising: (a) obtaining the identity of each of said analytes from a party; (b) encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy; (c) providing said party with analyte-specific reagents that generate said at least one first value and said at least one second value, as represented in said coding scheme, when each of said analytes is present, said party: (i) contacting a sample comprising, or potentially comprising, at least one of said analytes with said reagents; and (ii) cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and (d) obtaining said cumulative measurement from said party; (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme; and (f) providing said party with information about the presence or absence of each of said analytes.

In some cases, this disclosure provides compositions for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, each reagent generating a signal comprising a first value, wherein said reagents are capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, mass spectrometry, or melting curve analysis.

In some cases, this disclosure provides compositions for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, each reagent generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said reagents are capable of detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

In some cases, this disclosure provides kits for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, packaging, and instructions, each reagent generating a signal comprising a first value, wherein said kit is capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single sample volume, in any combina-

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tion of presence or absence, without immobilization, separation, mass spectrometry, or melting curve analysis.

In some examples, this disclosure provides kits for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, packaging, and instructions, each reagent generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said kit is capable of detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

In some cases, this disclosure provides kits for detecting the presence or absence of each analyte of a plurality of analytes, comprising a kit body (**601**), clamping slots arranged in the kit body for placing bottles, a bottle comprising probes for the detection of an analyte (**602**), a bottle comprising primers for amplification (**603**), and a bottle comprising reagents for amplification (**604**), wherein said kit is capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, mass spectrometry, or melting curve analysis.

In some examples, this disclosure provides kits for detecting the presence or absence of each analyte of a plurality of analytes, a kit body (**601**), clamping slots arranged in the kit body for placing bottles, a bottle comprising probes for the detection of an analyte (**602**), a bottle comprising primers for amplification (**603**), and a bottle comprising reagents for amplification (**604**), each probe generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said kit is capable of detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said probes generates only one second value.

INCORPORATION BY REFERENCE

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

FIG. **1** shows a comparison between a traditional encoding method of detecting four analytes with four colors and an encoding method of the invention able to detect 16 or more sequences with four colors. Colors are indicated by B, G, Y, and R, which indicate blue, green, yellow, and red, respectively.

FIG. **2** shows a schematic representation of detection of six analytes (including control) with four colors. The analytes are detected using hybridization probes attached to a fluorophore (B, G, Y, R; blue, green, yellow, and red, respectively) and a quencher (oval with "X"). The control sequence and the five other sequences (Dengue Fever=dengue virus; Tuberculosis=*Mycobacterium tuberculosis;* P17=HIV p17; Malaria=*Plasmodium falciparum;* and Herpes=herpes simplex virus 2) are all detected using a probe labeled with a blue fluorophore. The non-control analytes are each detected using 1-3 additional probes. For example, the dengue virus analyte is detected using three additional probes with green, yellow, and red fluorophores; the herpes simplex virus 2 analyte is detected using one additional probe with a red fluorophore; and so on.

FIG. **3** shows chromatograms of experimental results obtained as described in Example 2.

FIG. **4** shows an exemplary embodiment of the invention in which a computer is used to perform one or more steps of the methods provided herein.

FIG. **5** shows a schematic of two different methods of detecting wavelength and intensity of a light signal, such as a fluorescence emission signal.

FIG. **6** shows a schematic of exemplary kits.

FIG. **7** shows chromatograms of experimental results obtained as described in Example 3.

FIG. **8** shows a schematic of a legitimate result (top) and an illegitimate result (bottom).

DETAILED DESCRIPTION OF THE INVENTION

Fluorescence detection has been a preferred technique for multiplexed assays because of several desirable features, including compatibility with biochemical assays, the relatively small size of fluorescent labels, simple means of conjugation to molecules of interest, affordability, low toxicity, stability, robustness, detectability with inexpensive optics, and an ability to be combined with spatial arrays. However, standard fluorophores have wide emission spectra. Therefore, in order to avoid spectral overlap (i.e., to preserve spectral resolution) only a relatively small number of colors (e.g., 4 to 6) are typically used simultaneously in multiplexed fluorescent assays.

The traditional encoding method for multiplexed fluorescent assays has been to encode each analyte with a single color; i.e., M=N, where M is the number of analytes that can be detected and N is the number of spectrally resolved fluorescent probes. Whenever higher factors of multiplexing are required (i.e., M>N), fluorescence is generally combined with other techniques, such as aliquoting, spatial arraying, or sequential processing. These additional processing steps are labor-intensive and frequently require relatively expensive and complex optical and mechanical systems (e.g., spectrometers, mechanized microscopy stages, microfluidics, droplet generators, scanners, and the like). Such systems are often impractical to deploy in certain settings, particularly point-of care and low-resource settings. Thus, there is a significant need for multiplexed encoding and decoding methods that can provide an inexpensive means of multiplexing while avoiding the use of expensive additional processing steps.

This disclosure provides methods, systems, compositions, and kits for the detection of multiple analytes in a sample. Analytes are detected based on the encoding, analysis, and decoding methods presented herein. In some examples, each analyte to be detected is encoded as a value of a signal (e.g., intensity), where the values are assigned so that the results of the assay unambiguously indicate the presence or absence of the analytes being assayed. In other examples, each analyte to be detected is encoded as a value in each of at least

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two components of a signal (e.g., intensity and wavelength). The at least two components of a signal may be orthogonal. Similarly, as described more fully elsewhere in this disclosure, multiple orthogonal signals may be used, such as a combination of a fluorescent signal and an electrochemical signal. The analyte may be any suitable analyte, such as a polynucleotide, a protein, a small molecule, a lipid, a carbohydrate, or mixtures thereof. The signal may be any suitable signal such as an electromagnetic signal, a light signal, a fluorescence emission signal, an electrochemical signal, a chemiluminescent signal, and combinations thereof. The at least two components of the signal may be any suitable two components, such as an amplitude and a frequency or an intensity and a wavelength.

After encoding of the analytes, a sample is provided wherein the sample comprises or may comprise at least one of the encoded analytes. The sample is contacted with an analyte-specific reagent or reagents that generate a particular signal, as specified for each analyte in the coding scheme, in the presence of an analyte. A reagent may be any suitable reagent that is capable of generating such a signal in the presence of its corresponding analyte, for example, an oligonucleotide probe attached to a fluorophore and a quencher (e.g., a TAQMAN probe). If the reagent is an oligonucleotide probe attached to a fluorophore and a quencher, a nucleic acid amplification may be performed to generate the signal.

After addition of the reagent(s), the signal is quantified. In some cases, this quantification is performed by measuring one component of the signal (e.g., fluorescence intensity) and determining the presence and absence of certain analytes based on the values used to encode the presence of each analyte and the cumulative value of the signal.

In some cases, at least two components of a signal (e.g., intensity and wavelength) are cumulatively measured for the sample. This measurement can be performed, for example, by measuring the intensity at a particular wavelength or the intensity within a particular range of wavelengths. The presence or absence of an analyte may then be determined based on the values of each of the at least two components of the signal and the values used to encode the presence of the analyte (i.e., those values in the coding scheme).

The encoding may be performed in a manner that reduces or eliminates the number of possible degenerate (e.g., ambiguous) results that can be obtained by the method. As described elsewhere in this specification, the full coding capability of a particular coding scheme may be enumerated, and certain potential analyte codes may be eliminated from the coding scheme to reduce or eliminate degeneracy. Similarly, a coding scheme may be designed to be non-degenerate, so that a reduction or elimination of degeneracy is not necessary. A decoding matrix may be constructed to translate cumulative signal measurements (e.g., intensities or intensities at particular wavelengths) into the presence or absence of certain analytes, corresponding to the constituent signals of the cumulative signal measurement.

I. Definitions

The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

As used herein, the singular forms "a," "an," and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise. Furthermore, to the extent that the terms "including," "includes," "having," "has," "with," "such as," or variants thereof, are used in either the specification and/or the claims, such terms are not limiting and are intended to be inclusive in a manner similar to the term "comprising".

The term "about," as used herein, generally refers to a range that is 15% greater than or less than a stated numerical value within the context of the particular usage. For example, "about 10" would include a range from 8.5 to 11.5.

The terms "dimension" and "component," as used herein when referring to a signal, generally refer to an aspect of the signal that may be quantified. For example, if a signal is generated by a fluorescent molecule, it may be quantified in terms of its wavelength (e.g., a first dimension or component) and its intensity (e.g., a second dimension or component).

The term "decoding," as used herein, generally refers to a method of determining which analytes are present based on the cumulative signal and a coding scheme or decoding matrix that enables the conversion of a cumulative signal to information concerning the presence or absence of one or more analytes.

The term "encoding," as used herein, generally refers to the process of representing an analyte using a code comprising values of a signal, such as intensity, or values in each of at least two components of a signal or signals, such as wavelength and intensity.

The terms "oligonucleotide probe attached to a fluorophore and a quencher" and "TAQMAN probe" generally refer to hydrolysis probes used to detect the presence of an analyte in a polynucleotide amplification assay. These probes comprise an oligonucleotide probe attached to a fluorophore and a quencher. So long as the quencher and the fluorophore are in proximity, the quencher quenches the fluorescence emitted by the fluorophore upon excitation by a light source. The sequence of the oligonucleotide probe is designed to be complementary to a polynucleotide sequence present in an analyte, and therefore capable of hybridizing to the polynucleotide sequence present in the analyte. Hybridization of the oligonucleotide probe is performed in a nucleic acid amplification reaction comprising primers (e.g., a polymerase chain reaction). Upon extension of the primers by a DNA polymerase, the 5' to 3' exonuclease activity of the polymerase degrades the probe, releasing the fluorophore and the quencher into the medium. The proximity between the fluorophore and the quencher is broken and the signal from the fluorophore is no longer quenched. Thus, the amount of fluorescence detected is a function of the amount of analyte present. If no analyte is present, the probe will not hybridize to an analyte, and the fluorophore and quencher will remain in close proximity. Little or no signal will be produced.

The term "orthogonal," as used herein, generally refers to at least two components of a signal (e.g., wavelength and intensity), or at least two different signals (e.g., fluorescence emission and electrochemical signal), that can be varied independently or approximately independently. For example, wavelength and intensity are considered orthogonal or approximately orthogonal when fluorescent molecules are used. Among other factors, the wavelength of fluorescence emission will depend on the composition of the fluorescent molecule and the intensity of the fluorescence will depend on the amount of molecule present. Although wavelength and intensity are examples of two components of a signal that can be varied approximately independently, the methods described herein are not limited to components that can be varied independently or approximately independently. Components of a signal, or signals, that vary non-independently may also be used, so long as the components

or signals are characterized well enough to enable encoding, measurement, and decoding. For example, if the variance in one component or signal affects the variance in another component or signal, the two components or signals may still be used so long as the relationship between the variances is understood.

The terms "polynucleotide," "oligonucleotide," or "nucleic acid," as used herein, are used herein to refer to biological molecules comprising a plurality of nucleotides. Exemplary polynucleotides include deoxyribonucleic acids, ribonucleic acids, and synthetic analogues thereof, including peptide nucleic acids.

The term "probe," as used herein, generally refers to a reagent capable of generating a signal in the presence of a particular analyte. A probe generally has at least two portions: a portion capable of specifically recognizing an analyte, or a portion thereof, and a portion capable of generating a signal in the presence of an analyte, or a portion thereof. A probe may be an oligonucleotide probe attached to a fluorophore and a quencher, as described above and elsewhere in this disclosure. A probe may also be any reagent that generates a signal in the presence of an analyte, such as an antibody that detects an analyte, with a fluorescent label that emits or is quenched upon binding of the antibody to an analyte. Any suitable probe may be used with the methods presented in this disclosure, so long as the probe generates a quantifiable signal in the presence of an analyte. For example, the analyte-specific portion of a probe may be coupled to an enzyme that, in the presence of an analyte, converts an uncharged substrate into a charged product, thereby increasing the electrical conductivity in the medium over time. In this case, different analytes may be encoded by coupling the analyte-specific portion of the probe (e.g., hybridization probe or antibody) to an enzyme at different ratios. The resulting rate of increased conductivity in the medium will be cumulative for all analytes present in the medium. Encoding analytes according to the methods provided herein enables conversion of the conductivity measurements into unambiguous (i.e., non-degenerate) results providing information about the presence or absence of particular analytes. Similarly, a probe may comprise an enzyme producing a chemiluminescent product from a substrate. The amount of chemiluminescence may then be used to encode the presence of particular analytes.

II. Encoding and Decoding Methods

A. Traditional Fluorescent Encoding and Decoding Method

A commonly used method of determining the presence of an analyte uses four spectrally resolved fluorescent molecules to indicate the presence or absence of four analytes. An example of this method is presented on the left-hand side of FIG. **1**. The left-hand side of FIG. **1** shows an encoding method where four analytes (Seq 1, Seq 2, Seq 3, and Seq 4) are each encoded by a single color (blue, green, yellow, and red, respectively). The color represents a fluorophore attached to an oligonucleotide probe that also comprises a quencher. In the system shown on the left-hand side of FIG. **1**, there are four different oligonucleotide probes, each comprising a single fluorophore (blue, green, yellow, or red) and a quencher. The presence or absence of an analyte is determined based on the presence or absence of a signal in a particular color.

The chart on the left-hand side of FIG. **1** shows intensity versus color for a hypothetical sample containing two analytes: Seq 1 and Seq 3. The presence of these analytes is determined based on the measurement of a blue signal (corresponding to Seq 1) and a yellow signal (corresponding to Seq 3). The absence of Seq 2 and Seq 4 is indicated by the absence of a blue and red signal.

Table 1 shows a translation of this coding scheme into a binary format. Each analyte is encoded as a value in each of two components of a fluorescent signal: (1) color (also known as wavelength; or range of wavelengths) and (2) intensity (indicated by the numbers within the table). For example, A (e.g., Seq 1) has a color of blue and an intensity of 1; B (e.g., Seq 2) has a color of green and an intensity of 1; and so on. The intensity of the signal within each color range may be quantified as described herein, for example by measuring the intensity of the signal within a particular wavelength range determined by a band pass filter. A result of 1000 indicates that only analyte A is present; a result of 1100 indicates that analytes A and B are present; and so on.

TABLE 1

Traditional encoding of four analytes with four probes, each probe having a single color, and one probe per analyte.

	Blue	Green	Yellow	Red
A	1	0	0	0
B	0	1	0	0
C	0	0	1	0
D	0	0	0	1

B. Encoding Methods Using More Than One Color Per Analyte

The traditional method described above suffers from the fact that it is limited by the number of spectrally resolvable fluorophores. More specifically, the number of detectable analytes is equal to the number of spectrally resolvable fluorophores. Therefore, the number of analytes may only be increased by increasing the number of spectrally resolvable fluorophores.

This disclosure provides methods that overcome this limitation. More specifically, in some cases, by utilizing at least two components of a signal during encoding, the methods described herein may be used to detect more than one analyte per fluorophore. For example, using the method provided herein 1.5, 1.6, 1.7, 1.8, 1.9, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes may be detected per fluorophore. In some cases, the methods provided herein may be used to detect at least 1.5, 1.6, 1.7, 1.8, 1.9, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes may be detected per fluorophore. In some cases, the methods provided herein may be used to detect 1.5-2, 2-4, 1.5-4, 4-6, 2-6, 6-10 analytes per fluorophore.

In some cases, the methods provided in this disclosure may include the use of a control color. The control color may be attached to one or more probes binding a positive control analyte, and each analyte to be detected, in a sample. If the same sequence occurs in the positive control analyte and each analyte to be detected, a single control probe may be used. If the same sequence does not occur in the positive control analyte and each analyte to be detected, different probes may be used, but each probe may still be attached to the control color.

For example, building on the traditional methods described above, one color (e.g., blue) may be used to encode the presence of a control analyte that is always present in the sample. The control analyte may be added to

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the sample, or may be inherently present in the sample. The remaining colors (e.g., green, yellow, red) may be used to encode the presence of additional analytes. Table 2 shows an example of one such method.

TABLE 2

Encoding of four analytes, including control, with four colors and up to two colors per analyte.

	Blue	Green	Yellow	Red
Ctrl. (p)	1	0	0	0
A	1	0	0	1
B	1	0	1	0
C	1	1	0	0

In Table 2, the presence of the control (p) is indicated by a result of 1000. The presence of the control and analyte A is indicated by a result of 2001. The presence of the control and all three other analytes is indicated by a result of 4111. The control color (blue) provides an indication that the assay is functioning properly. The intensity of the blue color reports the number of analytes that are present in a test sample. Of course, any color may be used as the control color. One of skill in the art will recognize that certain practical considerations might make it preferable to use one color over another as the control color. For example, if one color is better detected in a particular optical system (or system of fluorophores), it might be practically preferable to use that color as a control color.

The coding scheme shown in Table 2 encodes each analyte using one control color and one additional color (up to 2 probes per analyte). However, as shown below, the number of analytes that can be encoded increases when up to 4 colors are used per analyte. Table 3 shows an exemplary coding scheme where each analyte is encoded by up to 4 colors. In the scheme shown in Table 3, the control color (blue) is generated in the presence of the control sequence and each of the other seven analytes (A-G). The other seven analytes are each encoded by the presence of one to three additional colors. The colors may be contained on different probes (e.g., oligonucleotide probes attached to a fluorophore and a quencher) or on the same probe (which can have multiple fluorophores and multiple quenchers).

TABLE 3

Encoding of eight analytes (including control) with four colors and up to four colors per analyte.

	Blue	Green	Yellow	Red
Ctrl. (p)	1	0	0	0
A	1	0	0	1
B	1	0	1	0
C	1	1	0	0
D	1	0	1	1
E	1	1	0	1
F	1	1	1	0
G	1	1	1	1

In Table 3, the presence of the control and all seven other analytes is indicated by a result of 8444. Three analytes (A, B, and C) are encoded by two colors. Three analytes (D, E, and F) are encoded by three colors, and one analyte (G) is encoded by four colors. The presence of the control (p) is indicated by a result of 1000. The presence of the control and analyte A is indicated by a result of 2001. The presence of the control and analyte G is indicated by a result of 2111. These results express the cumulative intensity of the signal in each color. For example, the result 2111 has a 2× signal intensity in the blue channel, while the result 1000 has only a 1× signal intensity in the blue channel.

Using Table 3, each of the possible cumulative assay results can be enumerated, in terms of color (blue, green, yellow, red) and intensity (0-8). Table 4 shows a "decoding matrix" generated by enumerating each of the possible cumulative assay results based on the encoding method presented in Table 3 and providing the corresponding decoded result of each assay in terms of the analyte(s) present in the sample. A similar decoding matrix may be generated for any coding scheme described herein, by enumerating each of the possible cumulative assay results based on the coding scheme and providing the corresponding decoded result of each assay in terms of the analyte(s) present in (or absent from) the sample. In some cases, as described below, one or more analytes may be removed from the coding scheme in order to reduce or eliminate degeneracy.

TABLE 4

Decoding matrix for encoding method presented in Table 3.

Cumulative Assay Result				
Blue	Green	Yellow	Red	Analyte(s) Present
1	0	0	0	p
2	0	0	1	pA
2	0	1	0	pB
2	1	0	0	pC
2	0	1	1	pD
2	1	0	1	pE
2	1	1	0	pF
2	1	1	1	pG
3	0	1	1	pAB
3	1	0	1	pAC
3	0	1	2	pAD
3	1	0	2	pAE
3	1	1	1	pAF, pCD, pBE
3	1	1	2	pAG, pDE
3	1	1	0	pBC
3	0	2	1	pBD
3	1	2	0	pBF
3	1	2	1	pBG, pDF
3	2	0	1	pCE
3	2	1	0	pCF
3	2	1	1	pCG, pEF
3	1	2	2	pDG
3	2	1	2	pEG
3	2	2	1	pFG
4	1	1	1	pACB
4	0	2	2	pABD
4	2	2	0	pBCF
4	2	0	2	pACE
4	1	1	2	pABE, pACD
4	1	2	1	pABF, pBCD
4	2	1	1	pACF, pBCE
4	1	2	2	pADF, pABG, pBDE
4	2	1	2	pACG, pCDE, pAEF
4	2	2	1	pBCG, pBEF, pCDF
4	2	2	2	pCDG, pAFG, pBEG, pDEF
4	1	1	3	pADE
4	1	3	1	pBDF
4	3	1	1	pCEF
4	1	2	3	pADG
4	3	2	1	pCFG
4	2	1	3	pAEG
4	2	3	1	pBFG
4	1	3	2	pBDG
4	3	1	2	pCEG
4	2	2	3	pDEG
4	3	2	2	pEFG
5	1	2	2	pABCD
5	2	1	2	pABCE
5	2	2	1	pABCF

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TABLE 4-continued

Decoding matrix for encoding method presented in Table 3.

Cumulative Assay Result				
Blue	Green	Yellow	Red	Analyte(s) Present
5	2	2	2	pABCG, pABEF, pBCDE, pACDF
5	1	2	3	pABDE
5	2	1	3	pACDE
5	1	3	2	pABDF
5	2	1	3	pBCDF
5	3	1	2	pACEF
5	3	2	1	pBCEF
5	2	2	3	pABEG, pACDG, pADEF
5	2	3	2	pABFG, pBCDG, pBDEF
5	3	2	2	pACFG, pBCEG, pCDEF
5	1	3	3	pABDG
5	3	1	3	pACEG
5	3	3	1	pBCFG
5	2	3	3	pADFG, pBDEG
5	3	3	2	pBEFG, pDEFG
5	3	2	3	pCDEG, pAEFG
5	2	2	4	pADEG
5	2	4	2	pBDFG
5	4	2	2	pCEFG
5	3	3	3	pDEFG
6	2	2	3	pABCDE
6	2	3	2	pABCDF
6	3	2	2	pABCEF
6	2	3	3	pABCDG, pABDEF
6	3	2	3	pABCEG, pACDEF
6	3	3	2	pABCFG, pBCDEF
6	4	3	2	pBCEFG
6	4	2	3	pACEFG
6	3	3	3	pABEFG, pACDFG, pBCDEG
6	3	4	2	pBCDFG
6	2	4	3	pABDFG
6	3	2	4	pACDEG
6	2	3	4	pABDEG
6	4	3	3	pCDEFG
6	3	4	3	pBDEFG
6	3	3	4	pADEFG
7	4	4	3	pBCDEFG
7	4	3	4	pACDEFG
7	3	4	4	pABDEFG
7	4	3	3	pABCEFG
7	3	4	3	pABCDFG
7	3	3	4	pABCDEG
7	3	3	3	pABCDEF
8	4	4	4	pABCDEFG

The decoding matrix provided in Table 4 is constructed using two assumptions. First, the decoding matrix assumes that the positive control (p) always produces a positive outcome. Second, the decoding matrix assumes that, within each color, the intensity is additive and scales in the same way with changing probe concentration, regardless of which probe the signal may come from. This essentially means that the signals are additive and digital. The conditions underlying these assumptions may be met by properly preparing the assay. If a fluorescent signal is used, the intensity need only be approximately additive and digital, as demonstrated in the Examples provided herein.

Table 4 allows the conversion of a cumulative measurement of intensity in four ranges of fluorescent wavelengths (i.e., signal intensity within each color range), into the corresponding analytes present in the sample. For example, a result of 4321 indicates that p, C, F, and G are present and the other analytes are not present. This result is referred to as a "legitimate" result, because it is present in the decoding matrix. By contrast, a result of 4000, while possible to measure, does not occur in the decoding matrix. More specifically, the result of 4000 cannot be achieved by adding any combination of control and analyte codes from Table 3. This result is referred to as an "illegitimate" result. An illegitimate result may indicate that the assay malfunctioned. Thus, the control (p) and the decoding matrix provide a means of verifying that the assay is functioning properly.

Table 4 is exhaustive for any combination of fluorophores generating four resolvable emission spectra (e.g., colors). The term "rank" is used to describe the number of detected analytes, including the control. In the example provided above, if the assay functions properly, the rank is equal to the value of the blue signal. For example, a rank of 8 indicates that the control and all seven other analytes (A-G) are present. A rank of 2 indicates that the control and only one analyte are present. The lowest rank is a rank of 1, which indicates that only the control is present. The number of possibilities at each rank can be enumerated. For example, continuing to refer to the encoding and decoding method described in Tables 3-4, there are 7 possibilities for rank 2. The number of possibilities at rank 3 can be calculated as a combination of 7 take 2, or 7!/(5!*2!)=21 possibilities. More generally, the number of possibilities for a combination of N take K is N!/((N-K)!*K!). Analogously, at ranks 4, 5, 6, 7, and 8, the number of possibilities is 35, 35, 21, 7, and 1, respectively. Referring to Table 4 shows that the table agrees with the theoretical prediction. Thus, Table 4 is an exhaustive decoding matrix for the encoding method provided in Table 3.

C. Reducing or Eliminating Degeneracy

The terms "degenerate" and "degeneracy," as used herein, generally describe a situation where a legitimate result is not definitive, because it can indicate more than one possibility in terms of the presence or absence of an analyte. For example, with reference to Table 4, result 5233 is degenerate because it can be decoded as either pADFG or pBDEG. Similarly, result 4222 is degenerate because it can be decoded as any of the following: pCDG, pAFG, pBEG, pDEF. By contrast, result 3110 can only indicate pBC and thus is not degenerate.

This disclosure provides methods of reducing or eliminating degeneracy, thereby increasing the confidence with which an analyte is detected. In one embodiment, degeneracy is eliminated by a method comprising (i) encoding each potential analyte to be detected as a value of a signal and, optionally, as a value in each of at least two components of a signal; (ii) enumerating every legitimate result that can be obtained from the coding scheme; (iii) identifying each legitimate result that is degenerate; and (iv) eliminating at least one potential analyte (or potential analyte code) from the coding scheme, wherein eliminating the at least one potential analyte reduces or eliminates degeneracy. For example, with reference to the coding scheme described in Table 3 and the decoding matrix described in Table 4 (enumerating every legitimate result), eliminating any two of analytes D, E, and F eliminates the degeneracy. Eliminating any one of analytes D, E, and F would not eliminate the degeneracy, but would reduce it.

With continued reference to the coding scheme described in Table 3, eliminating any two of analytes D, E, and F from the coding scheme results in a scheme where six analytes (including control) can be analyzed, with no degeneracy, using only 4 colors. By contrast, conventional methods of multiplexing would allow for only the reporting of 3 analytes and 1 control using 4 colors. Therefore, the number of analytes that can be analyzed is nearly doubled by using the methods provided herein.

FIG. **2** shows an exemplary embodiment of the invention in which 4 colors are used to detect five analytes and a control. The analytes are nucleic acids from dengue virus

("Dengue Fever"), *Mycobacterium tuberculosis* ("Tuberculosis"), human immunodeficiency virus (HIV) p17 (P17), *Plasmodium* ("Malaria"), and herpes simplex ("Herpes"). The "colors" in this example are fluorophores attached to oligonucleotide probes that also comprise a quencher. The oligonucleotide probes will generally be different for different analytes, whereas the color is the same. For example, all probes designated as Probe 1 have a blue color but generally will have a different oligonucleotide sequence. Of course, probes designated as Probe 1 could also have the same oligonucleotides sequence, if the complementary sequence was present in each of the analytes. The mechanism of detection with these probes is described elsewhere in this disclosure. Probe 1 (blue) hybridizes to all six analytes, including control. Probe 2 (green) hybridizes to analytes from dengue virus, *Mycobacterium,* and HIV p17. Probe 3 (yellow) hybridizes to analytes from dengue fever and *Plasmodium.* Probe 4 hybridizes to analytes from dengue virus, *Mycobacterium,* and herpes simplex. The coding scheme defined by these probes is illustrated in Table 5. With reference to Table 3, potential analytes D (1011) and F (1110) have been eliminated from the coding scheme. Therefore, the coding scheme presented in Table 5 is non-degenerate and each legitimate result from the assay corresponds to the presence or absence of a unique combination of analytes in a sample.

TABLE 5

Coding scheme for detection of dengue virus, Mycobacterium, HIV, Plasmodium, and herpes simplex, as exemplified in FIG. 2.

	Blue	Green	Yellow	Red
Ctrl. (p)	1	0	0	0
Herpes Simplex	1	0	0	1
Plasmodium	1	0	1	0
HIV	1	1	0	0
Mycobacterium	1	1	0	1
Dengue Virus	1	1	1	1

The methods for encoding and decoding presented above, including the methods for eliminating degeneracy, are all equally applicable to encoding methods using additional colors. For example, Table 3 could be extended by including additional colors and additional intensities (described further below). The decoding matrix is then generated as described above, enumerating every legitimate result that can be obtained from the coding scheme. A decoding matrix analogous to the decoding matrix provided in Table 4 may be constructed for any coding scheme described in this disclosure. The legitimate results that are degenerate are then identified and at least one potential analyte code is eliminated from the coding scheme to reduce or eliminate degeneracy. The method of eliminating degeneracy may be carried out using software on a computer readable medium, or hardware configured to carry out the method (e.g., a microchip).

Although degeneracy can be reduced or eliminated by the methods described above, and elsewhere in this disclosure, this disclosure also provides coding schemes that are non-degenerate by design. For example, after elimination of degeneracy in the coding scheme described in Table 3, the coding scheme may be extended indefinitely in a non-degenerate manner where the non-degeneracy is by design (see, e.g., the coding scheme exemplified in Table 6, described more fully below). Similarly, this disclosure provides coding schemes that are completely non-degenerate by design and therefore do not require any reduction or elimination of degeneracy (see, e.g., the coding scheme exemplified in Table 8, described more fully below). Thus, the coding schemes provided in this disclosure may have reduced or eliminated degeneracy, or be non-degenerate by design.

D. Encoding Methods Using More Than One Color and More Than One Intensity

The encoding method described above (e.g., Tables 3-5) may be further extended by allowing analytes to be encoded by an intensity greater than 1. For example, each of the analytes encoded in the coding scheme provided in Table 5 is encoded by a fluorescence intensity of either 1 or 0. Allowing higher values for the signal intensity in at least one color further increases the number of analytes that can be encoded by any of the methods provided in this disclosure. In some examples, these higher intensity values may be assigned any color except for the control color, in order to maintain the analyte counting capability of the control color.

Table 6 shows the first three tiers of an exemplary coding scheme that utilizes four colors and multiple intensities. Tier 1 of Table 6 is a reproduction of Table 3, showing the encoding of seven analytes and a control with four colors. As described above, any two of potential analytes D, E, and F may be eliminated from the coding scheme in order to produce a non-degenerate coding scheme. Tier 1 of Table 6 indicates that potential analytes D (1011) and F (1110) have been eliminated from the coding scheme to eliminate degeneracy. Therefore, the coding scheme presented in Tier 1 of Table 6 is capable of determining the presence of five analytes and one control using four colors, as described above.

The coding scheme of Tier 1 of Table 6 may be expanded to a second tier (Tier 2) by allowing the intensity in any of the colors to increase. As described above, the intensity of the control color may be maintained at 1, in order to preserve the sequence counting capability of the control. Increasing the intensity of any of the remaining three colors will yield codes 100Y, 10Y0, 1Y00, 10YY, 1Y0Y, 1YY0, and 1YYY, where Y>1. The minimal value of Y for a new tier of encoding is equal to the cumulative maximum value from the prior tier(s) plus 1. As described below, a value greater than 1 could also be used, to maximize the differences between the intensities.

Thus, in this context, the term "tier" is generally used to describe a set of codes that fully utilize the coding capability provided by a particular number of first values (e.g., intensities) and second values (e.g., colors), without degeneracy. For example, Tier 1 of Table 6 fully utilizes the coding capability provided by four colors with up to one intensity in each color, without degeneracy. As shown in Table 6, Tier 1, this results in six encoded analytes, including the control. To introduce a second tier, the minimum value of Y (described above) may be incremented to equal the cumulative maximum result from the prior tier(s) plus one (or more than one). In the example provided in Table 6, the intensity of the blue (control) color is maintained as one, to preserve the sequence counting capability in this color. Thus, Tier 2 consists of five non-degenerate encoding possibilities obtained by incrementing the intensities in the green, yellow, and red channels to equal the cumulative maximum results in each of these channels from Tier 1, plus one. All possibilities of these codes may then be enumerated for Tier 2, and codes resulting in degeneracy may be eliminated, or Tier 2 may be made non-degenerate by design, using the information used to eliminate the corresponding codes from Tier 1. Further coding capacity may then be achieved by adding a third tier, or further tiers, which are constructed according

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to analogous methods. A coding scheme may have an infinite number of tiers, for example 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100 or more tiers.

More specifically, with reference to Table 6, Tier 2, analyte H is encoded by 1004. The value of the control color is maintained as 1. The value of the red color is equal to the cumulative maximum result from the prior tier (3) plus 1, or 4. Similarly, analytes I and J are encoded by 1030 and 1400, respectively. Combinations of these codes are used to encode the remaining four analytes (K-N), as was done for analytes D-G in Tier 1. This completes Tier 2. In Tier 2, the inclusion of potential analytes K (1034) and M (1430) results in degeneracy. Therefore, these analytes have been eliminated from the coding scheme, to eliminate degeneracy.

Continuing to refer to Table 6, a third tier (Tier 3) is constructed using the same principles described above. Analyte O is encoded by 1-0-0-16. The value of the control color is still maintained as 1. The value of the red color is equal to the cumulative maximum result from the prior tiers (15) plus 1, or 16. Similarly, analytes P and Q are encoded by 1-0-9-0 and 1-16-0-0, respectively. Combinations of these codes are used to encode the remaining four analytes (R-U), as was done for analytes D-G in Tier 1 and analytes K-N in Tier 2. In Tier 3, the inclusion of potential analytes R (1-0-9-16) and T (1-16-9-0) results in degeneracy. Therefore, these analytes have been eliminated from the coding scheme, to eliminate degeneracy.

The three-tier coding scheme shown in Table 6 shows the encoding of 15 analytes and one control using four colors. This coding scheme may be indefinitely extended, by adding more intensities to generate additional tiers and/or adding more colors, to generate additional coding capability within the tiers. The methods of reducing or eliminating degeneracy, as described in this disclosure, may be used with the addition of each intensity and/or color, to reduce or eliminate degenerate results.

More generally, the coding scheme depicted in Table 6 is a non-degenerate, infinite extension of the coding scheme depicted in Tables 3-5. The maximum intensity of the cumulative measurement at the first tier is 6. The maximum intensity of the cumulative measurement at the second tier is 15. The maximum intensity of the cumulative measurement at the third tier is 63, and so on. Given a maximum cumulative intensity value (F), the maximum number of tiers (T) available in this coding scheme is $T=\log_4(F+1)$. The coding scheme depicted in Table 6 provides five non-degenerate codes per tier (P). Thus, the maximum number of codes $M=5*\log_4(F+1)$, or $M=P*T$, where P is the number of non-degenerate codes per tier and Tis the number of tiers. For example, given F=63, the maximum number of codes (i.e., analytes) is 15 for 5 non-degenerate codes per tier. This formula does not include the 1000 code, which is reserved for a positive control in Table 6. To include the control in the total number of analytes, one would simply add one, to provide the formula $M=(P*T)+1$.

The methods provided in this disclosure may be used to expand this coding scheme infinitely. For example, by utilizing combinations of different intensities (i.e., first values) and colors (i.e., second values) one can encode any number of analytes (M) by varying the number of non-degenerate codes per tier (P) and the number of tiers (7). For example, the number of non-degenerate codes per tier is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, or more. Similarly, the number of tiers may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, or more.

TABLE 6

Encoding of 15 analytes and one control using four colors and multiple intensities.

Tier	Analyte	B	G	Y	R	Comments
1	Ctrl. (p)	1	0	0	0	
	A	1	0	0	1	
	B	1	0	1	0	
	C	1	1	0	0	
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	Eliminated from coding scheme to eliminate degeneracy.
	E	1	1	0	1	
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	Eliminated from coding scheme to eliminate degeneracy.
	G	1	1	1	1	
Cumulative Maximum Result		6	3	2	3	
2	H	1	0	0	4	
	I	1	0	3	0	
	J	1	4	0	0	
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	Eliminated from coding scheme to eliminate degeneracy.
	L	1	4	0	4	
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	Eliminated from coding scheme to eliminate degeneracy.
	N	1	4	3	4	
Cumulative Maximum Result		11	15	8	15	
3	O	1	0	0	16	
	P	1	0	9	0	
	Q	1	16	0	0	
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	Eliminated from coding scheme to eliminate degeneracy.
	S	1	16	0	16	
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	Eliminated from coding scheme to eliminate degeneracy.
	U	1	16	9	16	
Cumulative Maximum Result						
Cumulative Maximum Result for Three Non-Degenerate Tiers		16	63	26	63	

The right-hand side of FIG. **1** shows one exemplary embodiment of the method described above. More specifically, the right-hand side of FIG. **1** shows an encoding method where nine or more analytes (Seq 1-Seq 9, etc.) are each encoded by at least two colors, with varying intensities within each color. The color represents a fluorophore attached to an oligonucleotide probe that also comprises a quencher. The system can be designed so that each probe is labeled with single fluorophore, or each probe is labeled with more than one fluorophore. For example, the code for analyte H, in Table 6, is 1004. The intensity of 4 in the red channel may be achieved by either using an H-specific probe comprising 4 red fluorophores, or by using 4 H-specific

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probes each comprising a single red fluorophore. Of course, any combination of probes and fluorophores producing a result of 4 in the red channel would be equally appropriate, such as 2 probes with 2 red fluorophores each, and 1 probe with 1 red fluorophore and 1 probe with 3 red fluorophores, or simply one probe with one red fluorophore but present at 4× amount in the reaction mixture.

The coding scheme depicted on the right-hand side of FIG. **1** may be represented as in Table 7. The result of the analysis shown on the right-hand side of FIG. **1** is 4112, or an intensity of 4 in the blue channel, 1 in the green channel, 1 in the yellow channel, and 2 in the red channel. Using a decoding matrix constructed as described herein, this result is decoded to indicate the presence of Seq 1, Seq 3, Seq 4, and Seq 5.

TABLE 7

Coding scheme illustrated on the right-hand side of FIG. 1.

Tier	Analyte	B	G	Y	R	Comments
1	Seq 1	1	0	0	0	
	Seq 2	1	1	0	0	
	Seq 3	1	0	1	0	
	Seq 4	1	0	0	1	
	Seq 5	1	1	0	1	
	Seq 6	1	1	1	1	
	~~Eliminated Potential Seq~~	~~1~~	~~0~~	~~1~~	~~1~~	Eliminated from coding scheme to eliminate degeneracy.
	~~Eliminated Potential Seq~~	~~1~~	~~1~~	~~1~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.
Cumulative Maximum Result		6	3	2	3	
2	Seq 7	1	4	0	0	
	Seq 8	1	0	3	0	
	Seq 9	1	0	0	4	

Etc. - i.e. Continue as provided in Table 6.

E. Encoding Methods Using One Color and One Intensity Per Analyte but Different Intensities Among Analytes

In some methods provided herein, each analyte is encoded by a single color and intensity combination. For example, in a four color system, the first four analytes may be encoded by 1000, 2000, 4000, and 8000. The next four analytes may be encoded by 0100, 0200, 0400, and 0800. Analytes 9-12 and 13-16 would be assigned analogously, as shown in Table 8.

Like the encoding method described in Table 6, this coding scheme is theoretically infinite, non-degenerate by construction, and only limited by the bandwidth of the instrument used to measure the signal. However, this coding scheme enables more analytes to be quantified per unit of bandwidth than the encoding method described in Table 6. The reason is that the available multiplicity of signal is used with maximal efficiency, as each level of intensity is utilized in the coding (i.e. there are no gaps in the chromatogram; see below for description of chromatograms). Table 8 shows one embodiment of this method, illustrating four tiers of encoding based on four colors and intensities 1, 2, 4, and 8. The coding scheme is non-degenerate, and the result if all 16 analytes are present is 15-15-15-15. This encoding method is more efficient, in terms of bandwidth utilization, than the encoding methods presented above. However, this method does not have the proofreading capability of the first scheme, as all the results decode to legitimate outcomes in the absence of gaps in the chromatogram.

TABLE 8

Example of encoding method using one color and one intensity per analyte, but different intensities among analytes.

Tier	Analyte	B	G	Y	R
1	A	1	0	0	0
	B	2	0	0	0
	C	4	0	0	0
	D	8	0	0	0
2	E	0	1	0	0
	F	0	2	0	0
	G	0	4	0	0
	H	0	8	0	0
3	I	0	0	1	0
	J	0	0	2	0
	K	0	0	4	0
	L	0	0	8	0
4	M	0	0	0	1
	N	0	0	0	2
	O	0	0	0	4
	P	0	0	0	8
		15	15	15	15

The method presented above, and exemplified in Table 8, may be extended by introducing additional colors and/or intensities. For example, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, or more colors may be used. Additional intensity levels, such as 16, 32, 64, 128, 255, 512, 1024, 2048, 4096, 8192, 16384, 32768, 65536, and so on may also be used. The method is generalizable and may be used to encode an infinite number of analytes. Each analyte is represented by a code in a single color, wherein the value of the code in that single color equal to the sum of all previous values plus one. For example, if the first code contains a 1 in a particular color, the next codes are 2, 4, 8, 16, 32, 64, 128 and so on. Other progressions are possible, but will not be as efficient in terms of usage of the bandwidth. However, one of ordinary skill in the art will recognize that less efficient use of bandwidth may be desirable when one wishes to maximize separation between values. Similarly, the value 1 could be excluded from the coding scheme, for example to maximize the difference in the intensity between the first encoded value and instrumental noise. For example, in some cases a coding scheme may begin with a value of 2, which would provide a progression of 2, 4, 8, 16, 32, 64, 128, and so on for the analyte codes, while the progression of cumulative results would be 2, 4, 6, 8, 10, 12, 14, 16, and so on. This approach generates uniform gaps in the possible cumulative results, allowing for higher tolerance to noise in comparison to an analyte code progression of 1, 2, 4, 8, 16, 32, and so on, and its corresponding cumulative result progression of 1, 2, 3, 4, 5, 6, 7, 8, and so on. In this example, the increased robustness in the measurement comes at a cost of a decrease in the number of available codes within a fixed bandwidth of multiplicity. For example, if only 63 multiplicity states can be measured reliably, the coding scheme starting with 1 offers 7 codes per color whereas the coding scheme starting with 2 offers 6 codes per color. If 4 colors are available, the former will offer 28 codes, while the latter will offer 24 codes. In summary, both the starting signal intensity and the progression (i.e., difference between intensity values) may be scaled in order to maximize the ability to distinguish over instrumental noise and maximize the differences between the intensity values, thereby enhancing the ability to distinguish between distinct experimental outcomes.

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The coding scheme illustrated in Table 8 is non-degenerate by design. Although the coding scheme illustrated in Table 8 uses both intensity and color to encode each of the 16 analytes, each of the analytes could also be encoded by simply utilizing intensity. For example, given the 16 analytes provided in Table 8 (A-P), a single color coding scheme encoding all 16 analytes may assign the values 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048, 4096, 8192, and 16384 to analytes A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, and P, respectively. Such a coding scheme is non-degenerate by design and the cumulative intensity result can be unambiguously decoded to indicate the presence or absence of analytes A-P. This coding scheme is capable of detecting the presence or absence of M analytes, where $M=\log_2(F+1)$ and F is the maximum cumulative value of a signal (e.g., signal intensity). By adding a second component to this signal (e.g., color, as depicted in Table 8), the capacity of this coding scheme can be increased to $M=C*\log_2(F+1)$, where C is the number of colors used in said coding scheme.

As described throughout this specification, any suitable value may be used for C or F. For example, C may be at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, or more. F may be at least about 2, 4, 8, 16, 32, 64, 128, 255, 512, 1024, 2048, 4096, 8192, 16384, 32768, 65536, 131072, 262144, 524288, 1048576, and so on. As described elsewhere in this disclosure, both the initial starting value of F and the steps in its progression may also be varied.

III. Analytes

An analyte may be any suitable analyte that may be analyzed using the methods and compositions of the invention, where the analyte is capable of interacting with a reagent in order to generate a signal with at least two components that can be measured. An analyte may be naturally-occurring or synthetic. An analyte may be present in a sample obtained using any methods known in the art. In some cases, a sample may be processed before analyzing it for an analyte. The methods and compositions presented in this disclosure may be used in solution phase assays, without the need for particles (such as beads) or a solid support.

In some cases, an analyte may be a polynucleotide, such as DNA, RNA, peptide nucleic acids, and any hybrid thereof, where the polynucleotide contains any combination of deoxyribo- and/or ribo-nucleotides. Polynucleotides may be single stranded or double stranded, or contain portions of both double stranded or single stranded sequence. Polynucleotides may contain any combination of nucleotides or bases, including, for example, uracil, adenine, thymine, cytosine, guanine, inosine, xanthine, hypoxanthine, isocytosine, isoguanine and any nucleotide derivative thereof. As used herein, the term "nucleotide" may include nucleotides and nucleosides, as well as nucleoside and nucleotide analogs, and modified nucleotides, including both synthetic and naturally occurring species. Polynucleotides may be any suitable polynucleotide for which one or more reagents (or probes) as described herein may be produced, including but not limited to cDNA, mitochondrial DNA (mtDNA), messenger RNA (mRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), nuclear RNA (nRNA), small interfering RNA (siRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), small Cajal body-specific RNA (scaRNA), microRNA (miRNA), double stranded (dsRNA), ribozyme, riboswitch or viral RNA. Polynucleotides may be contained within any suitable vector, such as a plasmid, cosmid, fragment, chromosome, or genome.

Genomic DNA may be obtained from naturally occurring or genetically modified organisms or from artificially or synthetically created genomes. Analytes comprising genomic DNA may be obtained from any source and using any methods known in the art. For example, genomic DNA may be isolated with or without amplification. Amplification may include PCR amplification, multiple displacement amplification (MDA), rolling circle amplification and other amplification methods. Genomic DNA may also be obtained by cloning or recombinant methods, such as those involving plasmids and artificial chromosomes or other conventional methods (see Sambrook and Russell, *Molecular Cloning: A Laboratory Manual.*, cited supra.) Polynucleotides may be isolated using other methods known in the art, for example as disclosed in *Genome Analysis: A Laboratory Manual Series* (Vols. I-IV) or *Molecular Cloning: A Laboratory Manual.* If the isolated polynucleotide is an mRNA, it may be reverse transcribed into cDNA using conventional techniques, as described in Sambrook and Russell, *Molecular Cloning: A Laboratory Manual.*, cited supra.

An analyte may be a protein, polypeptide, lipid, carbohydrate, sugar, small molecule, or any other suitable molecule that can be detected with the methods and compositions provided herein. An analyte may be an enzyme or other protein. An analyte may be a drug or metabolite (e.g., anti-cancer drug, chemotherapeutic drug, anti-viral drug, antibiotic drug, or biologic). An analyte may be any molecule, such as a co-factor, receptor, receptor ligand, hormone, cytokine, blood factor, antigen, steroid, or antibody.

An analyte may be any molecule from any pathogen, such as a virus, bacteria, parasite, fungus, or prion (e.g., PrP^{Sc}). Examples of viruses include those from the families Adenoviridae, Flaviviridae, Hepadnaviridae, Herpesviridae, Orthomyxoviridae, Papovaviridae, Paramyxoviridae, Picornaviridae, Polyomavirus, Retroviridae, Rhabdoviridae, and Togaviridae. Specific examples of viruses include adenovirus, astrovirus, bocavirus, BK virus, coxsackievirus, cytomegalovirus, dengue virus, Ebola virus, enterovirus, Epstein-Barr virus, feline leukemia virus, hepatitis virus, hepatitis A virus, hepatitis B virus, hepatitis C virus, hepatitis D virus, hepatitis E virus, herpes simplex virus (HSV), HSV type 1, HSV type 2, human immunodeficiency virus (HIV), HIV type 1, HIV type 2, human papilloma virus (HPV), HPV type 1, HPV type 2, HPV type 3, HPV type 4, HPV type 6, HPV type 10, HPV type 11, HPV type 16, HPV type 18, HPV type 26, HPV type 27, HPV type 28, HPV type 29, HPV type 30, HPV type 31, HPV type 33, HPV type 34, HPV type 35, HPV type 39, HPV type 40, HPV type 41, HPV type 42, HPV type 43, HPV type 44, HPV type 45, HPV type 49, HPV type 51, HPV type 52, HPV type 54, HPV type 55, HPV type 56, HPV type 57, HPV type 58, HPV type 59, HPV type 68, HPV type 69, influenza virus, JC virus, Marburg virus, measles virus, mumps virus, Norwalk virus, parovirus, polio virus, rabies virus, respiratory syncytial virus, retrovirus, rhinovirus, rotavirus, Rubella virus, smallpox virus, vaccinia virus, West Nile virus, and yellow fever virus.

Examples of bacteria include those from the genuses *Bordetella, Borrelia, Brucella, Campylobacter, Chlamydia, Clostridium, Corynebacterium, Enterococcus, Escherichia, Francisella, Haemophilus, Helicobacter, Legionella, Leptospira, Listeria, Mycobacterium, Mycoplasma, Neisseria, Pseudomonas, Rickettsia, Salmonella, Shigella, Staphylococcus, Streptococcus, Treponema, Vibrio,* and *Yersinia.* Specific examples of bacteria include *Bordetella par apertussis, Bordetella pertussis, Borrelia burgdorferi, Brucella abortus, Brucella canis, Brucella melitensis, Brucella suis,*

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Campylobacter jejuni, Chlamydia pneumoniae, Chlamydia psittaci, Chlamydia trachomatix, Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Clostridium tetani, Corynebacterium diphtherias, Enterococcus faecalis, Enterococcus faecium, Escherichia coli, Francisella tularensis, Haemophilus influenzae, Helicobacter pylori, Legionella pneumophila, Leptospira interrogans, Listeria monocytogenes, Mycobacterium leprae, Mycobacterium tuberculosis, Mycobacterium ulcerans, Mycoplasma pneumoniae, Neisseria gonorrhoeae, Neisseria meningitidis, Pseudomonas aeruginosa, Rickettsia rickettsii, Salmonella choleraesuis, Salmonella dublin, Salmonella enteritidis, Salmonella typhi, Salmonella typhimurium, Shigella sonnei, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus saprophyticus, Streptococcus agalactiae, Streptococcus pneumoniae, Streptococcus pyogenes, Treponema pallidum, Vibrio cholerae, Yersinia pestis, and *Yersinia enterocolitica.*

Examples of parasites include those from the genuses *Acanthamoeba, Babesia, Balamuthia, Balantidium, Blasocystis, Cryptosporidium, Dientamoeba, Entamoeba, Giardia, Isospora, Leishmania, Naegleria, Pediculus, Plasmodium, Rhinosporidium, Sarcocystis, Schistosoma, Toxoplasma, Trichomonas,* and *Trypanosoma.* Specific examples of parasites include *Babesia divergens, Babesia bigemina, Babesia equi, Babesia microfti, Babesia duncani, Balamuthia mandrillaris, Balantidium coli, Dientamoeba fragilis, Entamoeba histolytica, Giardia lamblia, Isospora belli, Naegleria fowleri, Pediculus humanus, Plasmodium falciparum, Plasmodium knowlesi, Plasmodium malariae, Plasmodium ovale, Plasmodium vivax, Rhinosporidium seeberi, Sarcocystis bovihominis, Sarcocystis suihominis, Schistosoma mansoni, Toxoplasma gondii, Trichomonas vaginalis, Trypanosoma brucei,* and *Trypansoma cruzi.*

Examples of fungi include those from the genuses *Apophysomyces, Aspergillus, Blastomyces, Candida, Cladosporium, Coddidioides, Cryptococcos, Exserohilum, Fusarium, Histoplasma, Pichia, Pneumocystis, Saccharomyces, Sporothrix, Stachybotrys,* and *Trichophyton.* Specific examples of fungi include *Aspergillus fumigatus, Aspergillus flavus, Aspergillus clavatus, Blastomyces dermatitidis, Candida albicans, Coccidioides immitis, Crytptococcus neoformans, Exserohilum rostratum, Fusarium verticillioides, Histoplasma capsulatum, Pneumocystis jirovecii, Sporothrix schenckii, Stachybotrys chartarum,* and *Trichophyton mentagrophytes.*

In some cases, the methods provided in this disclosure may be used to detect any one of the analytes described above, or elsewhere in the specification. In some cases the methods provided in this disclosure may be used to detect panels of the analytes described above, or elsewhere in the specification. For example, a panel may comprise an analyte selected from the group consisting of any 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100 or more analytes described above or elsewhere in the specification.

An analyte may be obtained from any suitable location, including from organisms, whole cells, cell preparations and cell-free compositions from any organism, tissue, cell, or environment. Analytes may be obtained from environmental samples, biopsies, aspirates, formalin fixed embedded tissues, air, agricultural samples, soil samples, petroleum samples, water samples, or dust samples. In some instances, an analyte may be obtained from bodily fluids which may include blood, urine, feces, serum, lymph, saliva, mucosal secretions, perspiration, central nervous system fluid, vaginal fluid, or semen. Analytes may also be obtained from manufactured products, such as cosmetics, foods, personal care products, and the like. Analytes may be the products of experimental manipulation including, recombinant cloning, polynucleotide amplification, polymerase chain reaction (PCR) amplification, purification methods (such as purification of genomic DNA or RNA), and synthesis reactions.

More than one type of analyte may be detected in each multiplexed assay. For example, a polynucleotide, a protein, a polypeptide, a lipid, a carbohydrate, a sugar, a small molecule, or any other suitable molecule may be detected simultaneously in the same multiplexed assay with the use of suitable reagents. Any combination of analytes may be detected at the same time.

Detection of an analyte may be useful for any suitable application, including research, clinical, diagnostic, prognostic, forensic, and monitoring applications. Exemplary applications include detection of hereditary diseases, identification of genetic fingerprints, diagnosis of infectious diseases, cloning of genes, paternity testing, criminal identification, phylogeny, anti-bioterrorism, environmental surveillance, and DNA computing. For example, an analyte may be indicative of a disease or condition. An analyte may be used to make a treatment decision, or to assess the state of a disease. The presence of an analyte may indicate an infection with a particular pathogen, or any other disease, such as cancer, autoimmune disease, cardiorespiratory disease, liver disease, digestive disease, and so on. The methods provided herein may thus be used to make a diagnosis and to make a clinical decision based on that diagnosis. For example, a result that indicates the presence of a bacterial polynucleotide in a sample taken from a subject may lead to the treatment of the subject with an antibiotic.

In some cases the methods and compositions of the invention may be used to detect at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes. In some cases the methods and compositions of the invention may be used to detect 7-50, 8-40, 9-30, 10-20, 10-15, 8-12, or 7-12 analytes.

In some cases, this disclosure provides assays that are capable of unambiguously detecting the presence or absence of each of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis. In some cases, this disclosure provides assays that are capable of unambiguously detecting the presence or absence of each of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis. In some cases, this disclosure provides assays that are capable of unambiguously detecting the presence or absence of less than 1,

2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis.

IV. Signals

The methods presented in this disclosure may be used with any quantifiable signal. In some cases, this disclosure provides coding schemes and methods to encode an infinite number of targets without degeneracy, using a single component of a signal (e.g., intensity). For example, as described above and in Example 3, a coding scheme may rely on a multiplicity of signal intensity without consideration of color. Although fluorescent probes have been used to illustrate this principle, the coding scheme is equally applicable to any other method providing a quantifiable signal, including an electrochemical signal and a chemiluminescent signal, as described elsewhere in this disclosure.

The methods presented in this disclosure may also utilize the measurement of a signal in at least two dimensions, also referred to as the measurement of at least two components of a signal. In comparison to the coding scheme described in the paragraph above, which relies on, for example, signal intensity to differentiate between analytes, utilization of at least two components of a signal (e.g., color and intensity) allows the generation of more unique codes per unit of signal intensity bandwidth. When at least two components of a signal are utilized, a cumulative measurement of the at least two components may be obtained for a single sample volume. For example in the coding scheme described in Table 5, the presence of each analyte results in a particular intensity (one component of the signal) in each of the four colors (a second component of a signal). The combination of these constituent signals leads to a cumulative signal that may be measured by measuring an intensity at each wavelength or range of wavelengths. The corresponding coding scheme or decoding matrix may then be used to convert the cumulative measurement into a determination of the presence or absence of an analyte.

In some cases, a quantifiable signal comprises a waveform that has both a frequency (wavelength) and an amplitude (intensity). A signal may be an electromagnetic signal. An electromagnetic signal may be a sound, a radio signal, a microwave signal, an infrared signal, a visible light signal, an ultraviolet light signal, an x-ray signal, or a gamma-ray signal. In some cases, an electromagnetic signal may be a fluorescent signal, for example a fluorescence emission spectrum that may be characterized in terms of wavelength and intensity.

In certain portions of this disclosure, the signal is described and exemplified in terms of a fluorescent signal. This is not meant to be limiting, and one of ordinary skill in the art will readily recognize that the principles applicable to the measurement of a fluorescent signal are also applicable to other signals. For example, like fluorescent signals, any of the electromagnetic signals described above may also be characterized in terms of a wavelength and an intensity. The wavelength of a fluorescent signal may also be described in terms of color. The color may be determined based on measuring intensity at a particular wavelength or range of wavelengths, for example by determining a distribution of fluorescent intensity at different wavelengths and/or by utilizing a band pass filter to determine the fluorescence intensity within a particular range of wavelengths. Such band pass filters are commonly employed in a variety of laboratory instrumentation, including quantitative PCR machines. Intensity may be measured with a photodetector. A range of wavelengths may be referred to as a "channel."

In some cases, the methods provided in this disclosure may be used with any signal where the cumulative signal scales with the constituent signals of the same color, frequency, absorption band, and so on. However, the cumulative signal need not be digital or scale linearly with the number and intensity of the constituent signals. For example, if the physical principle of measurement is absorption, the cumulative attenuation is a product of constituent attenuations while the constituent concentrations are additive, due to the exponential nature of the Beer-Lambert law. The logarithm of the cumulative attenuation will then scale linearly with constituent concentrations in each absorption band (the equivalent of color, if fluorescent detection is used). The methods of the invention are therefore applicable. The methods of the invention may also be used with chemiluminescent signals and electrochemical signals.

The number of signals, and the number of dimensions or components measured, may also be expanded beyond the numbers shown in the exemplary embodiments of the invention, leading to an expansion in multiplexing capability. The exemplary embodiments provided in this disclosure utilize coding schemes constructed utilizing a fluorescent signal with one or two components measured: wavelength and/or intensity. The number of analytes that can be encoded can be increased by increasing the number of wavelengths and/or intensities. The number of analytes that can be encoded can also be increased by increasing the number of signals, for example by combining a fluorescent signal with an electrochemical signal or a FRET signal (fluorescence resonance energy transfer).

In some cases, more than two components of a signal may be measured. For example, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 18, 20 or more components of a signal may be measured. At least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 18, or 20 components of a signal may be measured. At least 2, but fewer than 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 18, or 20 components of a signal may be measured. In some cases, 2-3, 2-4, 2-5, 2-6, 3-5, 3-6, 3-8, or 5-10 components of a signal may be measured. These additional components may include kinetic components, such as a rate of signal decay and rate of photobleaching.

If a fluorescent signal is employed, the number of analytes that can be encoded may be further expanded by utilizing additional fluorophores. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more fluorophores may be used. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 fluorophores may be used. In some cases, fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 fluorophores may be used. In some cases, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 fluorophores may be used.

Generally, the number of analytes that can be encoded may be further expanded by utilizing additional first values (e.g., intensities) that are values or ranges of values from a first component of a signal. For example, 1, 2, 3, 4, 5, 6, 7,

8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more first values that are values or ranges of values from a first component of a signal may be used. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more first values that are values or ranges of values from a first component of a signal may be used. In some cases, fewer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 first values that are values or ranges of values from a first component of a signal may be used. In some cases, 4-20, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 first values that are values or ranges of values from a first component of a signal may be used.

In instances in which intensity is a component of a signal that is quantified, such as when a fluorescent signal is utilized, the presence of an analyte may be encoded using a variety of intensities or ranges of intensities. For example, a coding scheme may utilize 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more intensities or ranges of intensities. A coding scheme may utilize at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more intensities or ranges of intensities. A coding scheme may utilize fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 intensities or ranges of intensities. In some cases, a coding scheme may utilize 2-20, 2-3, 2-4, 2-5, 2-6, 2-7, 2-8, 2-9, 2-10, 3-4, 3-5, 3-6, 3-7, 3-8, 3-9, 3-10, 4- 5, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, or 5-10 intensities or ranges of intensities.

The number of analytes that can be encoded may be further expanded by utilizing additional second values (e.g., wavelengths) that are values or ranges of values from a second component of a signal. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more second values that are values or ranges of values from a second component of a signal may be used. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more second values that are values or ranges of values from a second component of a signal may be used. In some cases, fewer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 second values that are values or ranges of values from a second component of a signal may be used. In some cases, 4-20, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 second values that are values or ranges of values from a second component of a signal may be used.

In instances in which wavelength is a component of a signal that is quantified, such as when a fluorescent signal is utilized, the presence of an analyte may be encoded using a variety of wavelengths or ranges of wavelengths. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more wavelengths or ranges of wavelengths may be used. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more wavelengths or ranges of wavelengths may be used. In some cases, fewer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 wavelengths or ranges of wavelengths may be used. In some cases, 4-20, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 wavelengths or ranges of wavelengths may be used.

In some cases, when degeneracy is eliminated, the methods of the invention are capable of detecting the presence or absence of at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more analytes in a single volume when each reagent used to generate a signal in the volume generates only one second value (e.g., each reagent, emits light at only one wavelength).

In other cases, when degeneracy is eliminated, the methods of the invention are capable of detecting the presence or absence of at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more analytes in a single volume using a total of four second values in that volume (e.g., a total of four wavelengths or ranges or wavelengths, which might be implemented by using four spectrally resolvable fluorophores).

As described throughout the specification, the assay provided herein utilizes cumulative measurements on a sample. A cumulative measurement may be, for example, a single measurement of intensity values, or a measurement of intensity values at one or more wavelengths or ranges of wavelengths. A plurality of cumulative measurements may be obtained. For example, an intensity may be measured at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more wavelengths or ranges of wavelengths. An intensity may be measured at at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more wavelengths or ranges of wavelengths. An intensity may be measured at less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 wavelengths or ranges of wavelengths.

More generally a cumulative measurement may be obtained for any quantifiable component of a signal, and for any quantifiable component of a signal at another quantifiable component of a signal. For example, at least a first component of a signal may be measured at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more second components of a signal. At lest a first component of a signal may be measured at at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more second components of a signal. At least a first component of a signal may be measured at less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13,

14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 second components of a signal.

As is apparent from this disclosure, each analyte to be detected can be encoded as a code utilizing any number of suitable components of a signal or any number of signals. For example, each analyte to be detected can be encoded in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more components of a signal or signals. In some cases, each analyte to be detected can be encoded in at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more components of a signal or signals. In some cases, each analyte to be detected can be encoded in fewer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 components of a signal or signals. In some cases, 1-4, 4-20, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 components of signal or signals may be used to encode each analyte. Each analyte in a coding scheme may be encoded by the same number of components of a signal, or signals, or different numbers of components of a signal, or signals.

A. Probes, Primers, Fluorophores, and Quenchers

Some of the methods provided in this disclosure utilize a reagent that generates a signal in the presence of an analyte. Any suitable reagent may be used with the invention. Generally, a reagent will have an analyte-specific component and a component that generates a signal in the presence of the analyte. In some cases, these reagents are referred to as probes. The probes may be hybridization probes. The hybridization probes may be an oligonucleotide probe attached to a fluorophore and a quencher (e.g., a TAQMAN probe).

The methods of the invention may use one or more reagents or probes to detect the presence or absence of each analyte. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more or probes may be used to detect the presence or absence of each analyte. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 probes may be used to detect the presence or absence of each analyte. In some cases, fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 probes may be used to detect the presence or absence of each analyte. In some cases, the number of probes used to detect the presence or absence of each analyte is 1-10, 2-9, 2-8, 2-7, 2-6, 2-5, 2-4, 1-3, or 1-4.

In some cases, a sample is contacted with 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more probes to detect the presence or absence of all analytes. In some cases, a sample is contacted with at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more probes to detect the presence or absence of all analytes. In some cases, a sample is contacted with fewer than 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 probes to detect the presence or absence of all analytes. In some cases, the number of probes that a sample is contacted with to detect the presence or absence of all analytes is 4-50, 4-40, 4-30, 4-20, 5-15, 5-10, or 3-10.

As described above, oligonucleotide probes attached to a fluorophore and a quencher may be used to detect the presence of an analyte in a polynucleotide amplification assay. So long as the quencher and the fluorophore are in proximity, the quencher quenches the fluorescence emitted by the fluorophore upon excitation by a light source. The sequence of the oligonucleotide probe is designed to be complementary to a polynucleotide sequence present in an analyte, and therefore capable of hybridizing to the polynucleotide sequence present in the analyte. Hybridization of the oligonucleotide probe may be performed in a nucleic acid amplification reaction comprising primers (e.g., a polymerase chain reaction). Upon extension of the primers by a DNA polymerase, the 5' to 3' exonuclease activity of the polymerase degrades the probe, releasing the fluorophore and the quencher into the medium. The proximity between the fluorophore and the quencher is broken and the signal from the fluorophore is no longer quenched. Thus, the amount of fluorescence detected is a function of the amount of analyte present. If no analyte is present, the probe will not hybridize to an analyte, and the fluorophore and quencher will remain in close proximity. Little or no signal will be produced.

Oligonucleotide probes may have one or a plurality of fluorophores and quenchers per probe. For example, in some embodiments an oligonucleotide probe may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more fluorophores. An oligonucleotide probe may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 fluorophores. An oligonucleotide probe may comprise fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 fluorophores.

An oligonucleotide probe may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more quenchers. An oligonucleotide probe may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 quenchers. An oligonucleotide probe may comprise fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 quenchers.

Attachment of probes and quenchers to a probe may be performed in the same reaction or in serial reactions. A series of reactions may be performed to label probes with at least one fluorophore and the reaction products may be mixed to generate a mixture of probes with different fluorophores.

If an oligonucleotide probe comprises two or more fluorophores, these fluorophores may be arranged to allow Förster (Fluorescence) resonance energy transfer (FRET) to occur between the fluorophores. Briefly, FRET is a mechanism of energy transfer between fluorophores. Using FRET-

based probes allows one excitation source to generate two different fluorescence emission signals by excitation of a single fluorophore. For example, the methods described herein may used paired probes, where one probe in the pair is attached to a fluorophore and a quencher and a second probe is attached to two fluorophores (in close enough proximity for FRET to occur) and a quencher. Any combination of fluorophores and quenchers that provide for FRET may be used. Such an approach doubles the number of fluorescent probes that may be used with an excitation source, because a single excitation source can be used to produce two signals: one from the probe with one fluorophore and one from the probe with two fluorophores in close enough proximity to provide for FRET. For example, using the encoding method described in Table 8, the number of unambiguously detectable analytes could be increased from 16 to 32 by pairing each of the probes in Table 8 with a corresponding FRET probe.

The primers may be specific for a particular analyte and capable of amplifying a region complementary to a probe. In some cases, the number of pairs of primers used is equivalent to the number of probes. In other cases, the number of probes used may exceed the number of primer pairs used. In still other cases, the number of primer pairs used may exceed the number of probes used. In some cases a sample to be analyzed is contacted with 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more pairs of primers. In some cases a sample to be analyzed is contacted with at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more pairs of primers. In some cases a sample to be analyzed is contacted with fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 pairs of primers. In some cases, the number of pairs of primers is 2-10, 3-15, 4-20, 3-10, 4-10, 5-10, 6-8, or 6-10.

The primers may amplify regions of a polynucleotide in which different numbers of hybridization probes hybridize. For example, at least one pair of primers may amplify a region complementary to at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 hybridization probes. In some cases, all of said pairs of primers may amplify a region complementary to at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 hybridization probes.

In one example, a first probe may be labeled with the fluorophore 5'-fluorescein amidite (5'-FAM) and the quencher black hole quencher 1 (BHQ-1). A second probe may be labeled with 5'-FAM in close proximity (e.g., attached to) to cyanine 5.5 (Cy5.5) and the quencher black hole quencher 3 (BHQ-3). Upon digestion of the probe, via the nuclease activity of the polymerase, two fluorescent signals are generated from a single excitation wavelength (e.g., 470 nm). The fluorophore from the first probe will fluoresce at about 520 nm. The fluorophores on the second probe undergo FRET. The donor fluorophore, FAM is excited by the excitation light source (e.g., 470 nm) and transfers its energy to the acceptor fluorophore, Cy5.5. The acceptor fluorophore emits at about 705 nm. These fluorophores and quenchers are merely exemplary. Any fluorophores that can undergo FRET and any quenchers that can quench fluorescence are suitable for use with the invention. Methods for producing FRET-based probes are described in Jothikumar et al., BioTechniques, 2009, 46(7):519-524.

In some examples, a single hybridization probe may be used for each analyte. In order to utilize multi-color encoding (e.g., as shown in Table 3), each probe may be labeled with a plurality of fluorophores at pre-determined ratios. The ratio may be determined so that a positive control signal provides the same intensity as other positive control signals of the same intensity, in the same color. This approach reduces the number of probes that must be synthesized and may be less expensive to deploy than methods utilizing multiple probes. Moreover, in the case of hybridization probes, it is easier to fit one hybridization probe to an analyte sequence than multiple probes.

Although many aspects of the invention are exemplified using nucleic acid-based probes, one of ordinary skill in the art will readily recognize that other forms of probes would work equally well with the invention described in this disclosure. For example, a binding molecule specific to an analyte could be used as a probe. Non-limiting exemplary binding molecules include an antibody recognizing an analyte, and generating a signal in the presence of an analyte.

In embodiments of the invention that utilize fluorescent labels, any suitable fluorescent label may be used. Exemplary fluorescent labels suitable for use with the invention include rhodamine, rhodol, fluorescein, thiofluorescein, aminofluorescein, carboxyfluorescein, chlorofluorescein, methylfluorescein, sulfofluore cein, aminorhodol, carboxyrhodol, chlororhodol, methylrhoda suiforhodol; aminorhodarnine, carboxyrhodamine, chiororhodamine, methylrhodamine, suiforhodamine, and thiorhodatnine, cyanine, indocarhocyanine, oxacarbocyanine, thiacarbocyanine, tnerocyanine, cyanine 2, cyanine 3, cyanine 3.5, cyanine 5, cyanine 5.5, cyanine 7, oxadiazole derivatives, pyridyloxazole, nitrobenzoxadiazole, benzoxadiazole, pyrene derivatives, cascade blue, oxazine derivatives, Nile red, Nile blue, cresyl violet, oxazine 170, acridine derivatives, proflavin, acridine orange, acridine yellow, arylmethine derivatives, auramine, crystal violet, malachite green, tetrapyrrole derivatives, porphin, phtalocyanine, and bilirubin. Exemplary quenchers include black hole quenchers, such as BHQ-0, BHQ-1, BHQ-2, BHQ-3; ATTO quenchers, such as ATTO 540Q, ATT0580Q, and ATTO612Q; and the like.

The fluorophores that may be used with the invention are not limited to any of the fluorophores described herein. For example, fluorophores with improved properties are continually developed, and these fluorophores could readily be used with the methods provided in this disclosure. Such improved fluorophores include quantum dots, which may emit energy at different wavelengths after being excited at a single wavelength. The advantage of using such fluorophores is that only a single excitation source is needed, but many different signals may be quantified, for example in terms of color and intensity. Moreover, fluorophores with narrow emission spectra would be particularly useful with the method described herein, as such fluorophores could be included in multiplex assays with minimal or no overlap between their emission spectra, thereby offering many more "colors" and boosting significantly the overall number of coded analytes.

In some cases, one or more reagents are lyophilized. Any suitable reagent may be lyophilized. For example, probes, primers, enzymes, antibodies, or any other reagent used for detection may be lyophilized. In some cases, a sample comprising an analyte may also be lyophilized. Lyophiliza-

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tion may be useful, for example, when distributing reagents and/or samples in developing regions where access to cold storage is expensive, not readily available, or not reliably available. In one example, lyophilized reagents comprise any probe or analyte-specific reagent described in this disclosure or otherwise suitable for use with the invention. In another example, lyophilized reagents comprise PCR primers. In yet another example, lyophilized reagents comprise reagents suitable for performing a PCR reaction.

V. Analytical Techniques and Instrumentation

The methods provided herein are suitable for use with a variety of detection methods. For example, the methods may be applied using an analytical technique that measures the wavelength and intensity of a fluorescent signal. This may be accomplished by measuring the intensity of a signal across a spectrum of wavelengths, or by using band pass filters that restrict the passage of certain wavelengths of light, thereby allowing only light of certain wavelengths to reach a photodetector. Many real-time PCR and quantitative PCR instruments comprise an excitation light source and band pass filters that enable the detection of fluorescent signals in four colors (e.g., blue, green, yellow, and red). Therefore, the methods of the invention can be readily applied using instruments widely used in the art. Importantly, the methods and compositions provided herein may be used to detect multiple analytes by obtaining a cumulative measurement on a single solution. No separation is necessary. The invention does not require the use of beads or a solid phase. Of course, one of ordinary skill in the art would understand that the invention could be used with separation, beads, or a solid phase, if desired.

FIG. **5** shows two examples of instrumental configurations that may be used to obtain cumulative measurements useful for the methods of the invention. With reference to FIG. **5**, an instrument with a detector configured to detect both wavelength and intensity (e.g., a fluorometer) is shown in **501**. With reference to **501**, at least one excitation light source **502** is directed into a chamber **503** containing analytes and reagents that generate a signal in the presence of the analytes **504**. As described elsewhere, the analyte may be a nucleic acid and the reagents generating the signal may be hybridization probes comprising a fluorophore and a quencher. If the analyte is present, an emission signal **505** is generated. In the configuration shown in **501**, the wavelength and intensity of this emission signal are measured across a spectrum by a detector capable of generating a fluorescence emission spectrum **506**.

The wavelength and intensity may also be determined using a combination of a photodetector and band pass filters. This configuration is used in several thermal cyclers known in the art. With reference to FIG. **5**, an instrument with band pass filters and a photodetector is depicted in **507**. With reference to **507**, at least one excitation light source **508** is directed into a chamber **509** containing analytes and reagents that generate a signal in the presence of the analytes **510**. As described elsewhere, the analyte may be a nucleic acid and the reagents generating the signal may be hybridization probes comprising a fluorophore and a quencher. If the analyte is present, an emission signal **511** is generated. In the configuration shown in **507**, band pass filters **512**, **513**, and **514** are used to restrict the passage of light to light within certain ranges of wavelengths. For example band pass filter **512** may restrict the passage of light to light within wavelength range 1. Band pass filter **513** may restrict the passage of light to light within wavelength range 2. Band pass filter **514** may restrict the passage of light to light within wavelength range 3. Any number of band pass filters (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, or more) may be used with the invention. In addition, the positions of the band pass filters in **507** are not meant to be limiting. In some cases, emitted light may be passed through more than one band pass filter at any time. In other cases, the band pass filters may be configured so that all emitted light passes through only one band pass filter at a time. After passage through a band pass filter, filtered light **515**, **516**, and **517** is detected by a photodetector. For example, filtered light **515** may be light within wavelength range 1, filtered light **516** may be light within wavelength range 2, and filtered light **517** may be light within wavelength range 3. The intensity of each of the filtered lights **515**, **516**, and **517** may then be quantified by a photodetector **518**.

The methods described in this disclosure are compatible with a variety of amplification methods, including quantitative PCR (qPCR) methods, end point PCR methods, reverse transcriptase PCR, and digital PCR methods. Digital PCR methods (e.g., DROPLET DIGITAL (BIORAD) and DYNAMIC ARRAY (FLUIDIGM)) produce highly sensitive quantification of polynucleotide copy numbers. The methods provided herein can be easily integrated into these systems to significantly expand their throughput by allowing multiplexing in a droplet or a dynamic array.

Similarly, the methods described in this disclosure may be applied in a real-time PCR assay. For example, real-time data may be fully recorded as the PCR runs to completion. The end-point values may then be decoded to indicate the presence or absence of analytes. The individual cycle thresholds (Ct) values may be analyzed for each color by detecting the maxima of the second derivative of the real-time curve in that color. As a particular analyte is amplified, fluorophores are released from its corresponding probes in the same ratios as the color multiplicities in the coding of that sequence. Hence, e.g. a code of 1100 will have the same Ct in blue and green, while a code of 1400 will have its green Ct precede its blue Ct by 2 cycles. This additional data enables determination of the identity and starting quantity of each analyte.

The methods described herein may also be used directly on a tissue sample. For example, a tissue sample may be obtained and photolithographic methods may be used to build wells directly onto the tissue sample. The tissue sample may be fixed prior to building the wells. The tissue sample may then be analyzed by dispensing appropriate reagents into the wells in the tissue sample. The encoding and decoding methods described in this disclosure may be used for the multiplexed detection of analytes within the wells etched into the tissue. Each well may correspond to a single cell or a few cells, providing excellent spatial resolution when analyzing different portions of a tissue for an analyte. This method may be used to detect analytes in different areas of the same tissue.

In some cases, instruments may be modified or constructed, for example, to provide additional excitation light sources, at multiple wavelength, and/or to provide additional band pass filters or a capability of determining a complete spectrum. Including additional excitation sources would allow for the excitation of a larger variety of fluorophores. Including additional band pass filters, or modifying or constructing an instrument capable of determining an entire spectrum allows detection of a wider variety of emissions from fluorophores. These techniques can be used to increase the number of fluorophores that can be used with the

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methods described in this disclosure and, accordingly, to increase the number of analytes that be simultaneously detected.

In some cases, the methods described in this disclosure utilize end-point PCR methods. However, in some cases a result may be obtained before the end-point. This may be advantageous when, for example, results are needed as soon as possible. For example, in one case calibration experiments are performed in which different starting concentrations of an analyte are analyzed to determine the cycle number at which the signal becomes saturated, as a function of the starting amount of the analyte. Using this data, a computer monitoring a PCR reaction in real-time can be programmed to search for saturation up to the maximal cycle number according to the limit of detection (LOD) of the particular system. If there is no amplification of an analyte (e.g., other than a positive control, if present), by the maximal cycle number, the result for that analyte is negative. If there is amplification of the analyte by the maximal cycle number, the result for that analyte is positive and the saturation intensity in each color may be used to decode the result using the coding scheme. In both cases, there would be no need to run the PCR reaction beyond the number of cycles set by the LOD for that instrument. Although PCR has been used to illustrate this quantitative method, one of ordinary skill in the art will readily recognize that similar principles could be applied in any catalytic system where the starting amount of an analyte limits the rate of reaction, and the rate of reaction can be measured and reported by an evolution in a signal intensity over time.

VI. Compositions and Kits

This disclosure also provides compositions and kits for use with the methods described herein. The compositions may comprise any component, reaction mixture and/or intermediate described herein, as well as any combination. For example, the disclosure provides detection reagents for use with the methods provided herein. Any suitable detection reagents may be provided, including hybridization probes labeled with a fluorophore and a quencher and primers, as described elsewhere in the specification.

In some cases, compositions comprise reagents for the detection of at least seven analytes using four fluorophores. In some cases, compositions comprise reagents for the detection of at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes using four fluorophores. In some cases, compositions comprise reagents for the detection of at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes using five fluorophores. In some cases, compositions comprise reagents for the detection of at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes using six fluorophores. In some cases, compositions comprise reagents for the detection of at least 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes using seven fluorophores.

In some cases the compositions comprise primers. The compositions may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 pairs of primers.

The invention also provides kits for carrying out the methods of the invention. Accordingly, a variety of kits are provided in suitable packaging. The kits may be used for any one or more of the uses described herein, and, accordingly, may contain instructions for detecting the presence or absence of each analyte of a plurality of analytes. A kit may comprise a coding scheme, or a decoding matrix, to assist the user in converting a cumulative measurement to a result indicating the presence or absence of each of a plurality of analytes. A kit may be a diagnostic kit, for example, a diagnostic kit suitable for the detection of any analyte, including the analytes recited herein. A kit may contain any of the compositions provided in this disclosure, including those recited above.

FIG. **6** shows schematics of exemplary kits of the invention. With reference to FIG. **6**, a kit comprising a kit body **601** is shown that contains reagents for the detection of analytes (e.g., polynucleotide probes comprising a fluorophore and a quencher) **602** and primers for the amplification of a nucleic acid **603**. The kit may also contain any other reagents, such as reagents suitable for performing an amplification reaction **604**. In the embodiment depicted in **601**, the reagents for the detection of analytes and the primers are each contained in a single volume (e.g., tube or a bottle). However, these reagents may also be provided in separate volumes, as depicted the kit body shown in **605**. The kit body **605** contains seven separate volumes. Three volumes (**606**, **607**, and **608**) contain reagents for the detection of analytes (or mixtures of such reagents) and three volumes (**609**, **610**, and **611**) contain primers (or mixtures of primers). The kit may also contain reagents suitable for performing an amplification reaction **604**.

FIG. **6** is provided for illustrative purposes only. Any number of reagents for the detection of analytes (e.g., probes) and any number of primers may be included in a single tube or bottle, as appropriate for the application. For example, in some embodiments, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more reagents for the detection of analytes may be included in a single bottle. In other cases, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more primers for amplification may be included in a single bottle or tube. In some cases, primers and probes may be provided in the same bottle or tube. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more probes may be provided per pair of primers. In other cases, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more primers may be provided per pair of probes. The kits may contain 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more bottles or tubes. Control analytes, and their associated detection reagents and primers, may also be included in the kit. These may be included in a separate bottle or tube or, for example, included within the bottle or tube providing reagents for an amplification reaction **604**. As described elsewhere in the specification, any reagent in the kits may be lyophilized.

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VII. Systems and Software

The methods of this disclosure may also be performed as part of a system. For example, one or more steps of the methods described herein may be performed by software contained on a computer readable medium. The software may be used to encode analytes, check for degeneracy, reduce or eliminate degeneracy, and/or produce a decoding matrix. The software may also be used to convert cumulative measurements made on an instrument (e.g., a PCR machine) into information concerning the presence or absence of an analyte. For example, with reference to FIG. **4**, a computer **401** may be connected to an input device **403**, a display **402**, and a printer **404**. The computer may execute one or more of the steps of any of the methods described in this disclosure, including, for example, encoding methods **410**, analysis methods **420**, and decoding methods **430**. A computer may reduce or eliminate degeneracy, for example as part of the encoding methods **410**. Similarly, a computer may generate a coding schme that is non-degenerate by design using, for example, mathematical iteration. A computer may also be connected to a network **405**. The network may be a local network and/or the Internet. The network may be used to perform the methods of the invention on multiple computers in different locations. For example, one computer may perform the encoding, another computer may perform the measuring, and yet another computer may perform the decoding. A computer may perform any or all of these methods. The network may be used to transmit information obtained using the methods described in this disclosure throughout the world, for example across a border.

In one example, the software is embedded in the instrument making the measurement, such as a thermal cycler. In another example, the software is installed on a computer attached to the instrument making the measurement, such as a computer attached to a thermal cycler. In another example, the software is in the "cloud"—i.e., on a computer that another computer may communicate with through a network.

Each of the elements described above may be connected to a controller that communicates with each of the elements and coordinates their actions. For example, a controller may initiate the encoding of a series of analytes into values of color and intensity. As described throughout this disclosure, the controller can execute software to reduce or eliminate degeneracy by removing one or more potential analyte codes from the encoding matrix. The controller may then automatically order probes corresponding to this code from a manufacturer of probes. Alternatively, the controller may operate an oligonucleotide synthesis instrument that can synthesize the appropriate probes. After analysis of the sample on the instrument (which may also be automated), the controller may process the results of the analysis using the decoding matrix. The controller may then provide these results to a user.

VIII. Services

The methods provided herein may also be performed as a service. For example, a service provider may obtain the identity of a plurality of analytes that a customer wishes to analyze. The service provider may then encode each analyte to be detected by any of the methods described herein and provide appropriate reagents to the customer for the assay. The customer may perform the assay and provide the results to the service provider for decoding. The service provider may then provide the decoded results to the customer. The customer may also encode analytes, generate probes, and/or decode results by interacting with software installed locally (at the customer's location) or remotely (e.g., on a server reachable through a network). Exemplary customers include clinical laboratories, physicians, manufacturers of food and consumer products, industrial manufacturers (e.g., petroleum companies) and the like. A customer or party may be any suitable customer or party with a need or desire to use the methods, systems, compositions, and kits of the invention.

EXAMPLES

Example 1

General Materials & Methods

1. General Reagents

TE Buffer, pH7 (LIFE TECHNOLOGIES, Carlsbad, Calif.); UltraPure RNAse-free Water (LIFE TECHNOLOGIES, Carlsbad, Calif.); Taq 5× Master Mix (FISHER SCIENTIFIC COMPANY, Tustin, Calif.).

2. DNA Sequences, Primers, and Probes

Five nucleic acids from organisms of clinical relevance were chosen for the exemplary study: (1) human immunodeficiency virus 1 (HIV-1); (2) *Plasmodium falciparum* (Malaria); (3) herpes simplex virus-2 (HSV-2); (4) *Mycobacterium tuberculosis* (TB); (5) and dengue virus type 3 (dengue fever).

Two regions of diagnostic relevance from the HIV-1 genome, p17 and poly-protease, were selected from the Los Alamos National Laboratory HIV-1 reference sequence. A diagnostic sequence from the *Plasmodium falciparum* ChR7 gene was obtained from the UCSC *Plasmodium falciparum* Genome Browser. A sequence for Herpes Simplex Virus-2 was synthesized from the sequence obtained from the European Molecular Biology Library. Similarly a diagnostic sequence for the rpoB gene in *Mycobacterium tuberculosis* was synthesized from a sequence obtained from the European Molecular Biology Library. A PCR diagnostic sequence for Dengue Virus Type 3 was obtained from the National Institute of Health genetic sequence database.

Oligonucleotides were synthesized by INTEGRATED DNA TECHNOLOGIES (IDT). Probes and primer pairs were chosen for each analyte using OLIGOANALYZER, from IDT. Sense probes containing a fluorophore at the 5' end and a quencher at the 3' end were synthesized for all analytes. All oligonucleotides were lyophilized and reconstituted in TE buffer before use. Tables 9-14 show sequence information, target sequence, primers, and probes for each of the six analytes.

TABLE 9

HIV-1 polyprotease sequence information.

Sequence Information	HIV-1 Poly protease 198mer synthesized from bases 2253-2550
Source	HIV-1 Reference Sequence, Los Alamos National Laboratory
Target sequence (3' to 5')	GGAAGCTCTATTAGATACAGGAGCAGATGATACAGTATTAGAAGAAATGAGTTTGCC AGGAAGATGGAAACCAAAAATGATAGGGGGAATTGGAGGTTTTATCAAAGTAAGACA

TABLE 9-continued

HIV-1 polyprotease sequence information.

	GTATGATCAGATACTCATAGAAATCTGTGGACATAAAGCTATAGGTACAGTATTAGT AGGACCTACACCTGTCAACATAATTGG (SEQ ID NO: 1)
Forward Primer (3' to 5')	GGAAGCTCTATTAGATACAGGAGCAG (SEQ ID NO: 2)
Reverse Primer (3' to 5')	CCAATTATGTTGACAGGTGTAGGTCC (SEQ ID NO: 3)
Probe 1 (3' to 5')	/**56-FAM**/TGAGTTTGCCAGGAAGATGGAAACCA/3BHQ_1/ (SEQ ID NO: 4)

TABLE 10

HIV p17 sequence information.

Sequence Name	HIV-1 P17 199mer synthesized from bases 790-1186
Source	HIV-1 Reference Sequence, Los Alamos National Laboratory
Target sequence (3' to 5')	CAGCTACAACCATCCCTTCAGACAGGATCAGAAGAACTTAGATCATTATATAATACAGT AGCAACCCTCTATTGTGTGCATCAAAGGATAGAGATAAAAGACACCAAGGAAGCTTTAG ACAAGATAGAGGAAGAGCAAAACAAAAGTAAGAAAAAAGCACAGCAAGCAGCAGCTGAC ACAGGACACAGCAATCAGGTCA (SEQ ID NO: 5)
Forward Primer (3' to 5')	CAGCTACAACCATCCCTTCAGACA (SEQ ID NO: 6)
Reverse Primer (3' to 5')	TGACCTGATTGCTGTGTCCTGTGT (SEQ ID NO: 7)
Probe 1 (3' to 5')	/**56-FAM**/AGCAACCCTCTATTGTGTGCATCAAAGG/3BHQ_1 (SEQ ID NO: 8)
Probe 2 (3' to 5')	/**5Cy3**/AAAGCACAGCAAGCAGCAGCTGA/3BHQ_2/ (SEQ ID NO: 9)

TABLE 11

Plasmodium ChR7 sequence information.

Sequence Name	Plasmodium ChR7 199mer synthesized from bases 1139138-1141223
Source	USCS Plasmodium falciparum Genome Browser Gateway
Target sequence (3' to 5')	GCCTAACATGGCTATGACGGGTAACGGGGAATTAGAGTTCGATTCCGGAGAGGGAGCCTGA GAAATAGCTACCACATCTAAGGAAGGCAGCAGGCGCGTAAATTACCCAATTCTAAAGAAGA GAGGTAGTGACAAGAAATAACAATGCAAGGCCAATTTAAAACCTTCCCAGAGTAACAATTG GAGGGCAAGTCTGGTG (SEQ ID NO: 10)
Forward Primer (3' to 5')	GCCTAACATGGCTATGACGGGTAA (SEQ ID NO: 11)
Reverse Primer (3' to 5')	CACCAGACTTGCCCTCCAATTGTT (SEQ ID NO: 12)
Probe 1 (3' to 5')	/**56-FAM**/ATTCCGGAGAGGGAGCCTGAGAAATA/3BHQ_1/ (SEQ ID NO: 13)
Probe 2 (3' to 5')	/**56-ROXN**/AAGGAAGGCAGCAGGCGCGTAAATTA/3BHQ_2/ (SEQ ID NO: 14)

TABLE 12

Herpes simplex virus 2 sequence information.

Sequence Name	HSV-2 193mer synthesized from HSV-2 genome
Source	EMBL Bank AJ303204
Target sequence (3' to 5')	TCAGCCCATCCTCCTTCGGCAGTATGGAGGGTGTCGCGGCGGCGAGCCGCCGTCCCCA AAGACGTGCGGGTCGTACACGTACACGTACCAGGGCGGCGGGCCTCCGACCCGGTACG CTCTCGTAAATGCTTCCCTGCTGGTGCCGATCTGGGACCGCGCCGCGGAGACATTCGA GTACCAGATCGAACTCGG (SEQ ID NO: 15)

EXHIBIT B

TABLE 12-continued

Herpes simplex virus 2 sequence information.

Forward Primer (3' to 5')	TCAGCCCATCCTCCTTCGGCAGTAT (SEQ ID NO: 16)
Reverse Primer (3' to 5')	CCGAGTTCGATCTGGTACTCGAATGT (SEQ ID NO: 17)
Probe 1 (3' to 5')	/**56-FAM**/AAAGACGTGCGGGTCGTACACGTACA/3BHQ_1/ (SEQ ID NO: 18)
Probe 2 (3' to 5')	/**5Cy5**/TAAATGCTTCCCTGCTGGTGCCGAT/3IAbRQSp/ (SEQ ID NO: 19)

TABLE 13

Mycobacterium tuberculosis rpoB sequence information

Sequence Name	Mycobacterium Tuberculosis rpoB 200mer synthesized from Mycobacterium tuberculosis rpoB genome
Source	ENBL Bank GQ395623
Target sequence (3' to 5')	GAGTGCAAAGACAAGGACATGACGTACGCGGCCCCGCTGTTCGTCACGGCCGAGTTC ATCAACAACAACACCGGCGAGATCAAGAGCCAGACGGTGTTCATGGGTGACTTCCCG ATGATGACCGAGAAGGGCACCTTCATCATCAACGGCACCGAGCGCGTCGTGGTCAGC CAGCTGGTCCGCTCGCCCGGTGTGTACTT (SEQ ID NO: 20)
Forward Primer (3' to 5')	GAGTGCAAAGACAAGGACATGACG (SEQ ID NO: 21)
Reverse Primer (3' to 5')	AAGTACACACCGGGCGAGC (SEQ ID NO: 22)
Probe 1 (3' to 5')	/**56-FAM**/CGCTGTTCGTCACGGCCGAGTTCAT/3BHQ_1/ (SEQ ID NO: 23)
Probe 2 (3' to 5')	/**5Cy3**/AGATCAAGAGCCAGACGGTGTTCATG/3BHQ_2/ (SEQ ID NO: 24)
Probe 3 (3' to 5')	/**5Cy5**/AAGGGCACCTTCATCATCAACGGCA/3IAbRQSp/ (SEQ ID NO: 25)

TABLE 14

Dengue virus type 3 sequence information.

Sequenc Name	Dengue Virus Type 3 200mer synthesized from Dengue Virus Type 3 genome
Source	GenBank M93130
Target sequence (3' to 5')	ATGCCAACTGTGATTGAGCACTTAGAAAGACTACAAAGGAAACATGGAGGAATGCTTGTGA GAAATCCACTCTCACGAAACTCCACGCACGAAATGTATTGGATATCCAATGGTACAGGCAA CATCGTCTCATTCACAATGACACACAGGAGACCCACCATAGAGAAAGATGTGGATTTAGGA GCAGGAACCCGACATGT (SEQ ID NO: 26)
Forward Primer (3' to 5')	ATGCCAACTGTGATTGAGCACT (SEQ ID NO: 27)
Reverse Primer (3' to 5')	ACATGTCGGGTTCCTGCTCCTAAA (SEQ ID NO: 28)
Probe 1 (3' to 5')	/**56-FAM**/ACAAAGGAAACATGGAGGAATGCTTGTGA/3BHQ_1/ (SEQ ID NO: 29)
Probe 2 (3' to 5')	/**5Cy3**/ACTCTCACGAAACTCCACGCACGAAA/3BHQ_2/ (SEQ ID NO: 30)
Probe 3 (3' to 5')	/**56-ROXN**/ACAATGCACACAGGAGACCCACCAT/3BHQ_2/ (SEQ ID NO: 31)
Probe 4 (3' to 5')	/**5Cy5**/GGATATCCAATGGTACAGGCAACATCGT/3IAbRQSp/ (SEQ ID NO: 32)

3. Polymerase Chain Reactions

All PCR reactions were performed in a ROCHE 480 LIGHTCYCLER instrument. Forty-five thermal cycles were performed, with a 60 second hot-start at 95° C. The cycling conditions for denaturation, annealing, and extension were 45 seconds, 50 seconds, and 60 seconds at 95° C., 65° C., and 70° C., respectively. Each experiment was run in quintuplicate, with a reaction volume of 15 μL. Fluorescence measurements were obtained in the following channels after every annealing step: 483 nm to 533 nm (FAM), 523 nm to 568 nm (Cy3), 558 nm to 610 nm (ROX), and 615 nm to 670 nm (Cy5). Fluorescence measurements were also obtained after the hot-start and the end of the 45 thermal cycles. The change in the fluorescence intensity between these two measurements (after hot-start and at the end of the 45 cycles) determined the end-point fluorescent signal.

Example 2

Simultaneous Detection of Polynucleotide Markers from Pathogens Based on an Encoding Method Using More Than One Color Per Analyte

This example describes the simultaneous detection of up to four polynucleotides selected from HIV (2 polynucleotides), HSV-2, *Mycobacterium, Plasmodium,* and dengue virus. Fourteen PCR reactions were assembled, using the reagents described in Table 15.

TABLE 15

Reagents for 14 PCR reactions.

Reaction No.	Type of Reagent	Reagent	Concentration	Volume
1		UltraPure Water	—	34 µL
		Taq 5x Master Mix	—	42 µL
	Template	Poly Protease Template	17 nM	2 µL
	Primers	Poly Protease FWD Primer	1 nM	2 µL
		Protease RVS Primer	1 nM	2 µL
	Probe	Poly Protease Probe 1	1 nM	2 µL
2		UltraPure Water	—	28 µL
		Taq 5x Master Mix	—	42 µL
	Templates	Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Poly Protease FWD Primer	1 nM	2 µL
		Protease RVS Primer	1 nM	2 µL
	Probe	Poly Protease Probe 1	1 nM	2 µL
3		UltraPure Water	—	32 µL
		Taq 5x Master Mix	—	42 µL
	Template	Malaria Template	15 nM	2 µL
	Primers	Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
	Probes	Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
4		UltraPure Water	—	26 µL
		Taq 5x Master Mix	—	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
	Probes	Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
5		UltraPure Water	—	32 µL
		Taq 5x Master Mix	—	42 µL
	Template	Herpes Template	17 nM	2 µL
	Primers	Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
	Probes	Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
6		UltraPure Water	—	26 µL
		Taq 5x Master Mix	—	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		TB Template	16 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
	Probes	Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
7		UltraPure Water	—	30 µL
		Taq 5x Master Mix	—	42 µL
	Template	TB Template	16 nM	2 µL
	Primers	TB FWD Primer	1 µM	2 µL
		TB RVS Primer	1 µM	2 µL
	Probes	TB Probe 1	1 µM	2 µL
		TB Probe 2	1 µM	2 µL
		TB Probe 3	1 µM	2 µL
8		UltraPure Water	—	30 µL
		Taq 5x Master Mix	—	42 µL

TABLE 15-continued

Reagents for 14 PCR reactions.

Reaction No.	Type of Reagent	Reagent	Concentration	Volume
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	TB FWD Primer	1 µM	2 µL
		TB RVS Primer	1 µM	2 µL
	Probes	TB Probe 1	1 µM	2 µL
		TB Probe 2	1 µM	2 µL
		TB Probe 3	1 µM	2 µL
9		UltraPure Water	—	28 µL
		Taq 5x Master Mix	—	42 µL
	Template	Dengue Virus Template	17 nM	2 µL
	Primers	Dengue Virus FWD Primer	1 µM	2 µL
		Dengue Virus RVS Primer	1 µM	2 µL
	Probes	Dengue Virus Probe 1	1 µM	2 µL
		Dengue Virus Probe 2	1 µM	2 µL
		Dengue Virus Probe 3	1 µM	2 µL
		Dengue Virus Probe 4	1 µM	2 µL
10		UltraPure Water	—	22 µL
		Taq 5x Master Mix	—	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
	Primers	Dengue Virus FWD Primer	1 µM	2 µL
		Dengue Virus RVS Primer	1 µM	2 µL
	Probes	Dengue Virus Probe 1	1 µM	2 µL
		Dengue Virus Probe 2	1 µM	2 µL
		Dengue Virus Probe 3	1 µM	2 µL
		Dengue Virus Probe 4	1 µM	2 µL
11		Taq 5x Master Mix	—	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Poly Protease FWD Primer	1 µM	2 µL
		Poly Protease RVS Primer	1 µM	2 µL
		Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
		Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
		Dengue Virus FWD Primer	1 µM	2 µL
		Dengue Virus RVS Primer	1 µM	2 µL
	Probes	Poly Protease Probe 1	1 µM	2 µL
		Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
		Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
		Dengue Virus Probe 1	1 µM	2 µL
		Dengue Virus Probe 2	1 µM	2 µL
		Dengue Virus Probe 3	1 µM	2 µL
		Dengue Virus Probe 4	1 µM	2 µL
12		UltraPure Water	—	2 µL
		Taq 5x Master Mix	—	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
	Primers	Poly Protease FWD Primer	1 µM	2 µL
		Poly Protease FWD Primer	1 µM	2 µL
		Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
		Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
		TB FWD Primer	1 µM	2 µL
		TB RVS Primer	1 µM	2 µL
	Probes	Poly Protease Probe 1	1 µM	2 µL
		Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
		Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
		TB Probe 1	1 µM	2 µL
		TB Probe 2	1 µM	2 µL
		TB Probe 3	1 µM	2 µL

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TABLE 15-continued

Reagents for 14 PCR reactions.

Reaction No.	Type of Reagent	Reagent	Concentration	Volume
13		UltraPure Water	—	12 µL
		Taq 5x Master Mix	—	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
	Primers	Poly Protease FWD Primer	1 µM	2 µL
		Poly Protease FWD Primer	1 µM	2 µL
		Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
		TB FWD Primer	1 µM	2 µL
		TB RVS Primer	1 µM	2 µL
	Probes	Poly Protease Probe 1	1 µM	2 µL
		Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
		TB Probe 1	1 µM	2 µL
		TB Probe 2	1 µM	2 µL
		TB Probe 3	1 µM	2 µL
14		UltraPure Water	—	10 µL
		Taq 5x Master Mix	—	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Poly Protease FWD Primer	1 µM	2 µL
		Poly Protease FWD Primer	1 µM	2 µL
		Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
		Dengue Virus FWD Primer	1 µM	2 µL
		Dengue Virus RVS Primer	1 µM	2 µL
	Probes	Poly Protease Probe 1	1 µM	2 µL
		Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
		Dengue Virus Probe 1	1 µM	2 µL
		Dengue Virus Probe 2	1 µM	2 µL
		Dengue Virus Probe 3	1 µM	2 µL
		Dengue Virus Probe 4	1 µM	2 µL

Referring to Table 15, reactions 1, 3, 5, 7, and 9 (containing HIV polyprotease, *Plasmodium,* HSV, *Mycobacterium,* and dengue analytes, respectively) were positive controls that provided baseline fluorescence intensity for each polynucleotide analytes alone, in the absence of other analytes. The change in fluorescence intensity in each color was used to provide the expected cumulative intensity level for each color in the chromatogram (FIG. **3**), as described more fully below. Reactions 2, 4, 6, 8, and 10 were negative controls used to determine the extent of non-specific amplification. In these reactions, the primers provided were not specific for the analytes provided.

Experiments 11, 12, 13, and 14 were multiplex detection experiments. Encoding was performed as indicated in Table 16. The measured fluorescence intensity for each assay, in each color, was plotted as black circles in the chromatogram of FIG. **3**.

TABLE 16

Coding scheme for detection of pathogens.

	FAM	Cy3	ROX	Cy5
HIV poly-protease	1	0	0	0
HIV p17	1	1	0	0
Plasmodium	1	0	1	0
HSV	1	0	0	1
Mycobacterium	1	1	0	1
Dengue Virus	1	1	1	1

To construct the chromatogram, the signals of the positive controls (reactions 1, 3, 5, 7, and 9) were used to assemble every possible combination of present sequences, in each color. The cumulative signals were plotted in a diagram, called a "chromatogram" (FIG. **3**). Cumulative signals corresponding to all possible combinations of the same rank in the same color were organized into their own "band." Doing this for all four colors produced a level and band structure, against which the experimental results of each combination (reactions 11, 12, 13, and 14) could be evaluated. FIG. **3** shows three replicates of a chromatogram constructed using the positive control samples, with experimental results 4112, 3121, and 3102 superimposed on the chromatogram. The experimental results are indicated by black dots.

The intensity value for each color in reactions 11-14 was measure in arbitrary fluorescence units (AFU), plotted on the chromatogram, and then assigned to a band based on two criteria. First, if an experimental result was within a band, then the result was assigned to the multiplicity of the band (e.g., the Cy3 value of 1 in the left-most chromatogram of FIG. **3**). Second, if an experimental result was between two bands, a two-step analysis was employed. If the experimental result was between two legitimate results, the experimental result was assigned to the closest band. (e.g., the Cy5 value of 2 in the right-most chromatogram of FIG. **3**). If the experimental result was between a legitimate result and an illegitimate result, the result was assigned to the band with the legitimate result (e.g., the FAM value of 3 in the middle chromatogram of FIG. **3**). FIG. **8** schematically shows a legitimate result of 4112 (top) indicating the presence of HIV polyprotease, *Plasmodium,* herpes simplex, and *Mycobacterium;* and an illegitimate result of 4110 (bottom), which cannot be decoded and indicates a malfunction in the assay.

Using these criteria, the output of Experiment 12 was designated 4112, which was the correct answer. The output of Experiment 14 could have been designated as 2121 or 3121, but 2121 is illegitimate, so the result must be 3121, which is the correct answer. The output of Experiment 13 could be designated as 3102 or 3101, both of which are legitimate, but the result is clearly closer to 2 than 1 in the Cy5 channel, so 3102, the correct answer, was designated as the result. These results provide experimental validation of the methods of the provided herein.

The results indicate a trend of slightly lower combined signal than what would be expected from a simple summation of positive-control signals. This may be because the combination of signals is not perfectly linear. This may be a result of additional effects for which the chromatogram construction does not account for at present. These effects may be related to the absolute concentration of quenchers. The higher the absolute concentration of quenchers, the larger the percentage of reaction volume they quench, so that the same percentage of released fluorophores fails to contribute as much as expected to the cumulative signal. One solution to this problem is to use low concentrations of probes, so that the percentile loss in signal is negligible, regardless of how much quencher is released.

FIG. **3** shows that each rank containing multiple combinations has a relatively wide band. The width of the band is ultimately the difference between the highest and lowest cumulative signals for the positive control samples. If all positive controls in the particular color produced exactly the same fluorescence signal, then the width of each band would be zero. Since instead those signals are somewhat different, the resulting bands are relatively wide. However, there is a simple means to tighten the bands. Instead of loading all probes at the same concentration, as was done for the experiment above, the concentration of each probe can be

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adjusted so that each of the resulting positive-control signals is the same as the others in the same color. This approach would significantly tighten the bands, making it easier to determine the multiplicity of a signal for a sample containing analytes.

Example 3

Simultaneous Detection of Polynucleotide Markers from Pathogens Based on an Encoding Method Using One Color Per Analyte and Different Intensities

This example describes the simultaneous detection of up to three polynucleotides selected from dengue virus, HIV p17, and HIV polyprotease. The coding scheme was as depicted in Table 11. As shown in Table 17, each analyte was encoded as a single value of fluorescence intensity in a single color. In this case, as described elsewhere in this disclosure, the coding scheme is non-degenerate by design. This is achieved by assigning an intensity value to each analyte that is equal to the cumulative intensity values for the prior analytes, plus one. Of course, as described elsewhere in the specification, the initial assigned value can be greater than one, for example to better distinguish over noise. Similarly, the values assigned to each analyte can be greater than the cumulative intensity values for the prior analytes plus one, for example, to increase the separation between the intensities and enable more simple assignment of intensities.

TABLE 17

Coding scheme for detection of dengue virus, HIV polyprotease, and HIV p17.

	FAM
Dengue Virus	1
HIV p17	2
HIV polyprotease	4

The same reagents (e.g., templates, primers, and probes) used in Example 2 were used here. The fluorescence signals generated by the respective probes in positive-control end-point PCR reactions were measured and used to calculate the probe concentrations that would produce 1×, 2×, and 4× signal intensities. Experiments were then set up to detect the seven non-null combinations of analyte occurrences. Tables 18-24 provide the PCR reaction components for each of these mixtures.

TABLE 18

Binary FAM Experiment 1 Cocktail: HIV Polyprotease, HIV p17, and dengue virus

	Concentration	Volume Added
Reagents		
UltraPure Water	—	50 µL
Taq 5x Master Mix	—	25 µL
Templates		
Poly Protease Template	17 nM	4 µL
P17 Template	15 nM	4 µL
Dengue Virus Template	17 nM	4 µL

TABLE 18-continued

Binary FAM Experiment 1 Cocktail: HIV Polyprotease, HIV p17, and dengue virus

	Concentration	Volume Added
Primers		
Poly Protease FWD Primer	1 µM	4 µL
Poly Protease RVS Primer	1 µM	4 µL
P17 FWD Primer	1 µM	4 µL
P17 RVS Primer	1 µM	4 µL
Dengue Virus FWD Primer	1 µM	4 µL
Dengue Virus RVS Primer	1 µM	4 µL
Probes		
Poly Protease Probe 1	800 nM	4 µL
P17 Probe 1	400 nM	4 µL
Dengue Virus Probe 1	200 nM	4 µL

TABLE 19

Binary FAM Experiment 2 Cocktail: dengue virus and HIV p17

	Concentration	Volume Added
Reagents		
UltraPure Water	—	54 µL
Taq 5x Master Mix	—	25 µL
Templates		
Poly Protease Template	17 nM	—
P17 Template	15 nM	4 µL
Dengue Virus Template	17 nM	4 µL
Primers		
Poly Protease FWD Primer	1 µM	4 µL
Poly Protease RVS Primer	1 µM	4 µL
P17 FWD Primer	1 µM	4 µL
P17 RVS Primer	1 µM	4 µL
Dengue Virus FWD Primer	1 µM	4 µL
Dengue Virus RVS Primer	1 µM	4 µL
Probes		
Poly Protease Probe 1	800 nM	4 µL
P17 Probe 1	400 nM	4 µL
Dengue Virus Probe 1	200 nM	4 µL

TABLE 20

Binary FAM Experiment 3 Cocktail: dengue virus and HIV polyprotease

	Concentration	Volume Added
Reagents		
UltraPure Water	—	54 µL
Taq 5x Master Mix	—	25 µL
Templates		
Poly Protease Template	17 nM	4 µL
P17 Template	15 nM	—
Dengue Virus Template	17 nM	4 µL
Primers		
Poly Protease FWD Primer	1 µM	4 µL
Poly Protease RVS Primer	1 µM	4 µL
P17 FWD Primer	1 µM	4 µL
P17 RVS Primer	1 µM	4 µL
Dengue Virus FWD Primer	1 µM	4 µL
Dengue Virus RVS Primer	1 µM	4 µL

TABLE 20-continued

Binary FAM Experiment 3 Cocktail: dengue virus and HIV polyprotease		
	Concentration	Volume Added
Probes		
Poly Protease Probe 1	800 nM	4 µL
P17 Probe 1	400 nM	4 µL
Dengue Virus Probe 1	200 nM	4 µL

TABLE 21

Binary FAM Experiment 4 Cocktail: HIV polyprotease and HIV p17		
	Concentration	Volume Added
Reagents		
UltraPure Water	—	54 µL
Taq 5x Master Mix	—	25 µL
Templates		
Poly Protease Template	17 nM	4 µL
P17 Template	15 nM	4 µL
Dengue Virus Template	17 nM	—
Primers		
Poly Protease FWD Primer	1 µM	4 µL
Poly Protease RVS Primer	1 µM	4 µL
P17 FWD Primer	1 µM	4 µL
P17 RVS Primer	1 µM	4 µL
Dengue Virus FWD Primer	1 µM	4 µL
Dengue Virus RVS Primer	1 µM	4 µL
Probes		
Poly Protease Probe 1	800 nM	4 µL
P17 Probe 1	400 nM	4 µL
Dengue Virus Probe 1	200 nM	4 µL

TABLE 22

Binary FAM Experiment 5 Cocktail: dengue virus		
	Concentration	Volume Added
Reagents		
UltraPure Water	—	58 µL
Taq 5x Master Mix	—	25 µL
Templates		
Poly Protease Template	17 nM	—
P17 Template	15 nM	—
Dengue Virus Template	17 nM	4 µL
Primers		
Poly Protease FWD Primer	1 µM	4 µL
Poly Protease RVS Primer	1 µM	4 µL
P17 FWD Primer	1 µM	4 µL
P17 RVS Primer	1 µM	4 µL
Dengue Virus FWD Primer	1 µM	4 µL
Dengue Virus RVS Primer	1 µM	4 µL
Probes		
Poly Protease Probe 1	800 nM	4 µL
P17 Probe 1	400 nM	4 µL
Dengue Virus Probe 1	200 nM	4 µL

TABLE 23

Binary FAM Experiment 6 Cocktail: HIV p17		
	Concentration	Volume Added
Reagents		
UltraPure Water	—	58 µL
Taq 5x Master Mix	—	25 µL
Templates		
Poly Protease Template	17 nM	—
P17 Template	15 nM	4 µL
Dengue Virus Template	17 nM	—
Primers		
Poly Protease FWD Primer	1 µM	4 µL
Poly Protease RVS Primer	1 µM	4 µL
P17 FWD Primer	1 µM	4 µL
P17 RVS Primer	1 µM	4 µL
Dengue Virus FWD Primer	1 µM	4 µL
Dengue Virus RVS Primer	1 µM	4 µL
Probes		
Poly Protease Probe 1	800 nM	4 µL
P17 Probe 1	400 nM	4 µL
Dengue Virus Probe 1	200 nM	4 µL

TABLE 24

Binary FAM Experiment 7 Cocktail: HIV polyprotease		
	Concentration	Volume Added
Reagents		
UltraPure Water	—	58 µL
Taq 5x Master Mix	—	25 µL
Templates		
Poly Protease Template	17 nM	4 µL
P17 Template	15 nM	—
Dengue Virus Template	17 nM	—
Primers		
Poly Protease FWD Primer	1 µM	4 µL
Poly Protease RVS Primer	1 µM	4 µL
P17 FWD Primer	1 µM	4 µL
P17 RVS Primer	1 µM	4 µL
Dengue Virus FWD Primer	1 µM	4 µL
Dengue Virus RVS Primer	1 µM	4 µL
Probes		
Poly Protease Probe 1	800 nM	4 µL
P17 Probe 1	400 nM	4 µL
Dengue Virus Probe 1	200 nM	4 µL

The total fluorescence signal of each experiment was plotted on a chromatogram (FIG. **7**) that was constructed as described above. The width of the bands around each positive control measurement is the propagated uncertainty of the 1× measurement. That uncertainty was equated to the standard deviation of the fluorescence signals of the last five PCR cycles in saturation.

FIG. **7** shows the results of this experiment. The three right-most results show the measured intensities for dengue virus alone (DEN), HIV p17 alone (P17), and HIV poly-protease (TPP). The assigned intensities for each of these analytes were as indicated in Table 11, and FIG. **7** confirms that the three analytes yielded the expected intensity results when alone (see DEN, P17, and TPP). At the end of the assay, essentially all of the probes have been hydrolyzed, releasing essentially all of the fluorophores, which emit a signal. A fixed amount of a fluorophore produces a fixed and predictable amount of signal. Therefore a target that is

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present should generally produce a fixed amount of signal, as determined by the amount of probe added to the reaction volume.

As expected, the intensity values for the combinations of analytes are equivalent to the sum of the assigned intensities. Since this coding scheme is designed to be non-degenerate, each result can be unambiguously decoded into the presence or absence of a particular analyte. For example, starting from the left side of FIG. **7**, a result of 7 indicates the presence of all three analytes. A result of 3 indicates the presence of dengue virus and HIV p17 analytes, and the absence of HIV polyprotease. A result of 5 indicates the presence of dengue virus and HIV polyprotease, and the absence of HIV p17. Finally a result of 6 indicates the presence of HIV p17 and HIV polyprotease, and the absence of dengue virus. This coding scheme can be extended to encode additional analytes, as described elsewhere in this disclosure.

SEQUENCE LISTING

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What is claimed is:

1. A method of unambiguously detecting any unique combination of presence or absence of at least five polynucleotide analytes in a plurality of droplets, the method comprising:

(a) providing a sample comprising, or potentially comprising, at least one of said at least five polynucleotide analytes;

(b) forming a mixture of said sample and at least five hybridization probes, wherein each of said at least five hybridization probes further comprises at least one fluorophore and at most four fluorophores;

(c) partitioning said mixture into said plurality of droplets;

(d) exciting said at least one fluorophore to generate one or more signals if one or more of said at least five polynucleotide analytes is present in said plurality of droplets, wherein said one or more signals comprise at least one signal generated by excitement of said at least one fluorophore;

(e) measuring said one or more signals to generate a cumulative intensity measurement, wherein said cumulative intensity measurement corresponds to the presence of a unique combination of presence or absence of said at least five polynucleotide analytes in said sample; and

(f) determining whether each of said least five polynucleotide analytes is present, in any unique combination of presence or absence, based on said cumulative intensity measurement,

wherein the method does not require any step of immobilization of said at least five polynucleotide analytes or mass spectrometry.

2. The method of claim **1**, wherein each of said at least five hybridization probes is complementary to one of said at least five polynucleotide analytes.

3. The method of claim **1**, wherein said at least five hybridization probes comprise one or more fluorophores selected from one, two, three, or four fluorophores.

4. The method of claim **1**, wherein, in (b), a droplet of said plurality of droplets comprises said at least five hybridization probes.

5. The method of claim **1**, wherein, in (b), a droplet of said plurality of droplets comprises one or more of said at least five polynucleotide analytes.

6. The method of claim **1**, wherein, in (b), a droplet of said plurality of droplets comprises at most one of said at least five polynucleotide analytes.

7. The method of claim **1**, wherein said one or more signals is generated during a polymerase chain reaction.

8. The method of claim **1**, wherein said at least five polynucleotide analytes are at least seven polynucleotide analytes.

9. The method of claim **1**, wherein said at least five hybridization probes are at least seven hybridization probes.

10. The method of claim **1**, wherein said one or more of said at least five polynucleotide analytes comprise cell-free nucleic acid, tumor nucleic acid, fetal nucleic acid, viral nucleic acid, or any combination thereof.

11. The method of claim **1**, wherein said sample is a biological sample derived from a subject.

12. The method of claim **11**, wherein detecting the presence or absence of said at least five polynucleotide analytes indicates the presence of cancer or an infection with a pathogen in said subject.

13. The method of claim **1**, further comprising transmitting information concerning the presence or absence of said at least five polynucleotide analytes through a computer network.

14. The method of claim **1**, further comprising providing information concerning the presence or absence of said at least five polynucleotide analytes to a health care professional, wherein said health care professional makes a clinical decision based on said information.

15. The method of claim **14**, wherein said clinical decision is a treatment decision.

16. The method of claim **15**, wherein said treatment decision comprises treatment with an antibiotic or an anticancer therapeutic.

17. The method of claim **1**, wherein at least part of said method is performed using a computer.

18. The method of claim **17**, wherein said computer is located on a remote server.

19. The method of claim **17**, wherein said computer is located on a droplet reader.

20. The method of claim **1**, wherein each of said at least five hybridization probes are provided at a given concentration such that said cumulative intensity measurement corresponds to said presence of said unique combination.

* * * * *

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EXHIBIT C

Bio-Rad's QX600 Droplet Digital PCR System Advancing Measurable Residual Disease Research

Date: 2023-04-05

HERCULES, Calif.—Bio-Rad Laboratories, Inc. (NYSE: BIO and BIOb), a global leader in life science research and clinical diagnostic products, is accelerating measurable residual disease (MRD) research through more than a half dozen collaborations with institutions and companies leveraging its new QX600™ Droplet Digital™ PCR System. Launched late last year, it boasts six color detection capable of quantifying 12 targets per well, a simple user workflow, and powerful data analysis. Maintaining Bio-Rad's best-in-class ddPCR™ technology, this platform utilizes the same droplet generation and processing protocols as the QX200™ system, enabling thousands of current customers to easily adopt its advanced multiplexing capabilities.

One such collaborator is already planning ways to apply the new QX600 ddPCR System. "The QX600 is an incredibly powerful instrument," said Gary Pestano, PhD, Chief Development Officer at Biodesix, Inc., who also served as a beta tester. "We anticipate leveraging this tool for rare variant detection as a method of analyzing multiple mutations for MRD and therapeutic response testing for our blood-based oncology offerings. We also expect that this will drive the development of novel, multiplexed assays that were not possible before."

The introduction of the QX600 ddPCR System expands the company's family of QX platforms, which currently includes the CE Marked IVDR compliant QX200 system, the FDA-cleared QXDx™ system; and the high-throughput, fully automated QX ONE™ system.

"Combining high multiplexing power with the proven sensitivity and absolute quantification of ddPCR technology will allow us to obtain greater volumes of high-quality clinical research data without burdening patients, ultimately driving precision medicine forward," said Boris Wong, MSc, a translational researcher at the University of Melbourne who participated in beta testing for the QX600 ddPCR System earlier this year.

"The QX200 system has been an outstanding workhorse, cited in over 6,300 publications with nearly 1,000 of those focused on oncology," said Stephen Kulisch, Vice President of Marketing for Bio-Rad's Digital Biology Group. "With the launch of the QX600 system, we're excited to bring advanced multiplexing and unmatched sensitivity to molecular oncology investigators engaged in everything from biomarker discovery to clinical trials and patient monitoring of MRD."

BIO-RAD, ddPCR, Droplet Digital , QXDx, QX200 and QX600 are trademarks of Bio-Rad Laboratories, Inc. in certain jurisdictions.

About Bio-Rad

Bio-Rad Laboratories, Inc. (NYSE: BIO and BIOb) is a global leader in developing, manufacturing, and marketing a broad range of innovative products for the life science research and clinical diagnostic markets. With over 70 years of focus on quality and customer service, our products advance the scientific discovery process and improve healthcare. Our customers are universities, research institutions, hospitals, biotechnology and pharmaceutical companies, as well as public health and commercial laboratories, including food safety and environmental quality testing

facilities. Based in Hercules, California, Bio-Rad has a global network of operations with approximately 8,300 employees worldwide and $2.8 billion in revenues in 2022. For more information, please visit bio-rad.com.

Forward-Looking Statements

This release may be deemed to contain certain forward-looking statements within the meaning of the Private Securities Litigation Reform Act of 1995. These forward-looking statements include, without limitation, statements we make regarding our products and our expectations about our products. Forward-looking statements generally can be identified by the use of forward-looking terminology such as "plan", "believe," "expect," "anticipate," "may," "will," "intend," "estimate," "continue," or similar expressions or the negative of those terms or expressions, although not all forward-looking statements contain these words. Such statements involve risks and uncertainties, which could cause actual results to vary materially from those expressed in or indicated by the forward-looking statements. These risks and uncertainties include the duration and severity of the COVID-19 pandemic, supply chain risks, our ability to develop and market new or improved products, our ability to compete effectively, international legal and regulatory risks, and product quality and liability issues. For further information regarding our risks and uncertainties, please refer to the "Risk Factors" and "Management's Discussion and Analysis of Financial Condition and Results of Operation" in Bio-Rad's public reports filed with the Securities and Exchange Commission, including our most recent Annual Report on Form 10-K and our Quarterly Reports on Form 10-Q. Bio-Rad cautions you not to place undue reliance on forward-looking statements, which reflect an analysis only and speak only as of the date hereof. We disclaim any obligation to update these forward-looking statements.

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EXHIBIT D

BIO-RAD

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QX600 Droplet Digital PCR System



Overview

The QX600™ Droplet Digital™ PCR System enables advanced six-color multiplexing, allowing clear discrimination of multiple targets with assays that are cross-compatible with the QX200™ Droplet Digital PCR System. The QX600 System is designed for researchers who need to quantify multiple targets with high accuracy, reproducibility, and sensitivity.

Read full description

Ordering Information

Contact a Specialist

Description Specifications Ordering Accessories Kits & Reagents Consumables Documents

Description

The QX600 Droplet Digital PCR System (ddPCR™) offers advanced six-color multiplexing and absolute quantification of nucleic acid, while delivering the best-in-class performance you have come to expect from Bio-Rad's ddPCR systems.

Benefits of the QX600 Droplet Digital PCR System

The QX600 Droplet Reader offers users:

- **Sensitive multiplexing**
  - Six-color detection capability (FAM, HEX, Cy5, Cy5.5, ROX, and ATTO 590)
  - Quantification of up to 12 targets in a single well
  - Absolute quantification with 0.1% or better sensitivity
  - Sensitive and precise gene expression multiplexing
- **Accurate and reproducible results**

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- Confidence in your results bolstered by a system qualified by standards from National Measurement Institute (NMI), Australia
- Sets the standard for reproducibility as demonstrated in numerous studies

- **Powerful software**
  - User-friendly analysis
  - Audit trails and tools for U.S. FDA 21 CFR Part 11 compliance with premium edition
  - Assay protocol files for automated data analysis and reporting with premium edition
- **Portfolio compatibility**
  - Cross-compatibility with QX200 reagents, assays, and consumables
  - Extensive menu of pre-existing ddPCR assays
  - System capable of running both TaqMan® and EvaGreen chemistries

Applications of the QX600 Droplet Digital PCR System

The QX600 System enhances a wide variety of applications, including: oncology research, gene expression, next generation sequencing (NGS) orthogonal testing, cell and gene therapy, food and wastewater testing.

- **Oncology research** — The QX600 System collects more information per well, preserving precious circulating tumor DNA (ctDNA) and tumor samples.
  - Structural variant analysis
  - Rare variant detection
  - Copy number variation
  - Methylation detection
  - Loss of heterozygosity
- **Gene Expression** — Employ the extreme precision of the QX600 System to quantify small-fold changes in low abundance RNA molecules.
- **Next generation sequencing (NGS) orthogonal testing** — With the QX600 System perform accurate quantification and qualification of NGS libraries without the use of a standard curves.
- **Cell and gene therapy** — Accelerate therapeutics discovery with highly-advanced multiplexing afforded by the QX600 System.
  - Residual DNA detection in biologics
  - Genome edit confirmation
- **Food testing** — Use the QX600 System to perform routine evaluation of genetically modified organisms (GMO) and pathogen contamination detection.
- **Wastewater testing** — Leverage the QX600 System to perform population-wide monitoring of established and emerging pathogens.

Additional Instruments

The QX600 Droplet Digital PCR System requires the use of the PTC Tempo Deepwell Thermal Cycler or C1000 Touch Thermal Cycler with 96–Deep Well Reaction Module and the PX1 PCR Plate Sealer.

Required Accessories

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- DG8 Cartridges
- DG8 Gaskets

ddPCR Reagents for Hydrolysis Probe-Based Applications

- PrimePCR ddPCR Assays
- ddPCR Multiplex Supermix
- ddPCR Supermixes for Probes (No dUTP)
- ddPCR Supermix for Probes
- One-Step RT-ddPCR Advanced Kit for Probes
- Residual DNA Quantification Supermixes
- Droplet Generation Oil for Probes
- Droplet Reader Oil

ddPCR Reagents for EvaGreen Applications

- QX200 ddPCR EvaGreen Supermix
- QX200 Droplet Generation Oil for EvaGreen
- Droplet Reader Oil

Related Products

- PTC Tempo Thermal Cycler
- PrimePCR™ PCR Primers, Assays, and Arrays
- PX1 PCR Plate Sealer
- PTC Tempo Thermal Cycler

Specifications

Starting sample size, µl	20
QX200 Droplet Generator capacity	1–8 samples/cartridge
Droplets per 20 µl sample	20,000
QX600 Droplet Reader capacity	1–96 samples
Sample illumination	Light-emitting diodes
Sample detection	Avalanche photodiodes
Detection channels	FAM (EvaGreen), HEX (VIC), Cy5, Cy5.5, ROX, and ATTO 590
QX200 Droplet Generator dimensions (W x D x H)	28 x 36 x 13 cm (11 x 14 x 5")

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QX600 Droplet Reader dimensions (W x D x H) 66 x 52 x 29 cm (26 x 20 x 11")

Ordering



QX600 Droplet Digital PCR System

17007769

Discover how the QX600 Droplet Digital PCR System enables 6-color multiplexing, allowing clear discrimination of multiple targets within samples.

List Price: **Inquire**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

Add to Quote

Add to Hot List



QX600 Droplet Reader

12013328

The QX600™ Droplet Reader enables advanced six-color multiplexing, allowing clear discrimination of multiple targets with assays that are cross-compatible with the QX200™ Droplet Digital™ PCR (ddPCR™) System. The QX600 Droplet Reader is designed for investigators who need to quantify multiple targets with high accuracy, reproducibility, and sensitivity.

List Price: **Inquire**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

Add to Quote

Add to Hot List



QX200™ Droplet Generator

1864002

Droplet generator for EvaGreen or probe-based digital PCR applications, for use with QX200™ Droplet Digital™ PCR System

List Price: **Inquire**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

Add to Quote

Add to Hot List

QX Manager Software, Premium Edition

12018108

QX600 and QX200 System control and analysis software, with

List Price: **$10,000.00**

Your Price: Log in to see your price

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traceability for secure environments



Quantity: 1

Add to Cart

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Add to Hot List

Accessories



PX1 PCR Plate Sealer

1814000

PCR plate sealer, includes heat sealing instrument, plate support block that holds 96-well and 384-well plates, sealing frame, power cord

List Price: **$4,928.00**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

Add to Quote

Add to Hot List



PTC Tempo Deepwell Thermal Cycler

12015392

96-well, thermal cycler for PCR with cables; for research use only.

List Price: **$11,500.00**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

Add to Quote

Add to Hot List



C1000 Touch™ Thermal Cycler with 96–Deep Well Reaction Module

1851197

Modular thermal cycler platform, includes C1000 Touch Thermal Cycler chassis, 96–deep well reaction module, USB flash drive

List Price: **$9,578.00**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

Add to Quote

Add to Hot List

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Kits & Reagents



ddPCR Multiplex Supermix, 1.2 ml

12005909

1.2 ml (2 x 0.6 ml), 4x supermix, for use in sample preparation for droplet generation in the QX600/QX200 Droplet Digital PCR Systems

List Price: **$368.00**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

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ddPCR Multiplex Supermix, 3 ml

12005910

3 ml (5 x 0.6 ml), 4x supermix, for use in sample preparation for droplet generation in the QX600/QX200 Droplet Digital PCR Systems

List Price: **$920.00**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

Add to Quote

Add to Hot List



ddPCR Multiplex Supermix, 12.5 ml

12005911

12.5 ml (5 x 2.5 ml), 4x supermix, for use in sample preparation for droplet generation in the QX600/QX200 Droplet Digital PCR Systems

List Price: **$4,601.00**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

Add to Quote

Add to Hot List



ddPCR™ Supermix for Probes (No dUTP)

1863023

2 ml (2 x 1 ml), 2x supermix, for use in sample preparation for droplet generation in the QX600/QX200 Droplet Digital PCR Systems

List Price: **$245.00**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

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Add to Quote

Add to Hot List



ddPCR Supermix for Probes (No dUTP)

1863024

5 ml (5 x 1 ml), 2x supermix, for use in sample preparation for droplet generation in the QX600/QX200 Droplet Digital PCR Systems

List Price: **$613.00**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

Add to Quote

Add to Hot List



ddPCR Supermix for Probes (No dUTP)

1863025

25 ml (5 x 5 ml), 2x supermix, for use in sample preparation for droplet generation in the QX600/QX200 Droplet Digital PCR Systems

List Price: **$3,068.00**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

Add to Quote

Add to Hot List



ddPCR™ Supermix for Probes

1863026

2 ml (2 x 1 ml), 2x supermix, for use in sample preparation for droplet generation in the QX600/QX200 Droplet Digital™ PCR Systems

List Price: **$249.00**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

Add to Quote

Add to Hot List



ddPCR™ Supermix for Probes

1863010

5 ml (5 x 1 ml), 2x supermix, for use in sample preparation for droplet generation in the QX600/QX200 Droplet Digital™ PCR Systems

List Price: **$625.00**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

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Add to Quote

Add to Hot List



ddPCR™ Supermix for Probes

1863027

25 ml (5 x 5 ml), 2x supermix, for use in sample preparation for droplet generation in the QX600/QX200 Droplet Digital™ PCR Systems

List Price: **$3,125.00**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

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ddPCR™ Supermix for Probes

1863028

50 ml (10 x 5 ml), 2x supermix, for use in sample preparation for droplet generation in the QX600/QX200 Droplet Digital™ PCR Systems

List Price: **$6,251.00**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

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One-Step RT-ddPCR Advanced Kit for Probes

1864021

200 x 20 µl reaction kit, for absolute quantification of target RNA in the QX600/QX200 Droplet Digital™ PCR Systems

List Price: **$1,329.00**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

Add to Quote

Add to Hot List

One-Step RT-ddPCR Advanced Kit for Probes

1864022

500 x 20 µl reaction kit, for absolute quantification of target RNA in the

List Price: **$3,321.00**

Your Price: Log in to see your price

Quantity: 1

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QX600/QX200 Droplet Digital™ PCR Systems

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ddPCR™ Buffer Control for Probes

1863052

9 ml (2 x 4.5 ml), 2x buffer, for use as blank control with Droplet Generation Oil for Probes

List Price: **$308.00**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

Add to Quote

Add to Hot List



QX200™ ddPCR™ EvaGreen Supermix

1864033

200 x 20 µl reactions, 2 ml (2 x 1 ml), 2x supermix, for use in sample preparation with the droplet generator in the QX600/QX200 Droplet Digital™ PCR System

List Price: **$233.00**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

Add to Quote

Add to Hot List



QX200™ ddPCR™ EvaGreen Supermix

1864034

500 x 20 µl reactions, 5 ml (5 x 1 ml), 2x supermix, for use in sample preparation with the droplet generator in the QX600/QX200 Droplet Digital™ PCR System

List Price: **$584.00**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

Add to Quote

Add to Hot List

QX200™ ddPCR™ EvaGreen Supermix

1864035

List Price: **$2,921.00**

Your Price: Log in to see your price

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2,500 x 20 µl reactions, 25 ml (5 x 5 ml), 2x supermix, for use in sample preparation with the droplet generator in the QX600/QX200 Droplet Digital™ PCR System

Quantity: 1

Add to Cart

Add to Quote

Add to Hot List



QX200™ ddPCR™ EvaGreen Supermix

1864036

5,000 x 20 µl reactions, 50 ml (10 x 5 ml), 2x supermix, for use in sample preparation with the droplet generator in the QX600/QX200 Droplet Digital™ PCR System

List Price: **$5,842.00**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

Add to Quote

Add to Hot List



QX200™ Buffer Control for EvaGreen

1864052

9 ml (2 x 4.5 ml), 2x buffer, for use as a blank control with Droplet Generation Oil for EvaGreen

List Price: **$341.00**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

Add to Quote

Add to Hot List



ddPCR™ Library Quantification Kit for Illumina TruSeq

1863040

200 x 20 µl reactions, includes ddPCR Library Quantification Assay and ddPCR Supermix for Probes (No dUTP), for quantification of Illumina TruSeq libraries using the QX200™/QX100™ ddPCR™ Systems

List Price: **$1,221.00**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

Add to Quote

Add to Hot List

Consumables

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ddPCR™ 96-Well Plates

12001925

Pkg of 25, clear well/clear shell semi-skirted plates, for use with QX600/QX200 Droplet Digital™ PCR Systems

List Price: **$195.00**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

Add to Quote

Add to Hot List



Droplet Generation Oil for Probes

1863005

70 ml (10 x 7 ml), oil for use with droplet generator in the QX600/QX200 Droplet Digital™ PCR Systems

List Price: **$375.00**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

Add to Quote

Add to Hot List



QX200™ Droplet Generation Oil for EvaGreen

1864005

14 ml (2 x 7 ml), oil for use with the droplet generator in the QX200™ Droplet Digital™ PCR System

List Price: **$82.00**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

Add to Quote

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QX200™ Droplet Generation Oil for EvaGreen

1864006

70 ml (10 x 7 ml), oil for use with the droplet generator in the QX200™ Droplet Digital™ PCR System

List Price: **$364.00**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

Add to Quote

Add to Hot List



ddPCR™ Droplet Reader Oil

1863004

2 L (2 x 1 L), oil for use with droplet reader in the QX600/QX200 Droplet Digital™ PCR Systems

List Price: **$1,748.00**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

Add to Quote

Add to Hot List



DG8™ Cartridges for QX200™/QX100™ Droplet Generator

1864008

Pkg of 24, cartridges for use with the QX200 Droplet Generator

List Price: **$316.00**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

Add to Quote

Add to Hot List

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DG8™ Gaskets for QX200™/QX100™ Droplet Generator

1863009

Pkg of 24, gaskets for use with QX200 Droplet Generator

List Price: **$78.00**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

Add to Quote

Add to Hot List



DG8™ Cartridges and Gaskets

1864007

5 pkg of 24 DG8 Cartridges, 5 pkg of 24 DG8 Gaskets, for use with the QX200 Droplet Generator

List Price: **$1,902.00**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

Add to Quote

Add to Hot List



DG8 Cartridge Holder

1863051

Pkg of 1, cartridge holder, positions and holds the cartridge in the QX200 Droplet Generator

List Price: **$748.00**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

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PCR Plate Heat Seal, foil, pierceable
1814040
Pkg of 100, foil seals for PCR and QX200/QX100 Droplet Digital PCR System applications, for use with PX1 PCR Plate Sealer

List Price: **$116.00**

Your Price: Log in to see your price

Quantity: 1

Add to Cart

Add to Quote

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Documents

Login Required

Number	Description	Options
10000153877	QX600 and QX Manager Software Standard Edition User Guide	
3266	QX600 Droplet Reader	
3557	QX600™ Droplet Digital™ PCR System Brochure	

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QX600™ Droplet Reader and QX Manager Software Standard Edition

User Guide



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QX600 Droplet Reader and QX Manager Software Standard Edition

User Guide

Version 2.1

BIO-RAD

Bio-Rad Technical Support Department

The Bio-Rad Technical Support department is open Monday through Friday, 5:00 AM to 5:00 PM, Pacific time.

Phone: 1-800-424-6723, option 2

Email: Support@bio-rad.com (U.S./Canada Only)

For technical assistance outside the U.S. and Canada, contact your local technical support office.

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Revision History

Document	Date	Description of Change
QX600 Droplet Reader and QX Manager Software Standard Edition User Guide Software version 2.1 DIR No. 10000153877 Ver C	June 2023	Change DIR version to C
QX600 Droplet Reader and QX Manager Software Standard Edition User Guide Software version 2.1 DIR No. 10000153877 Ver C	May 2023	Update the user guide with new and improved features for Version 2.1; add new legal statement and Managing Waste section
QX600 Droplet Reader and QX Manager Software Standard Edition User Guide Software version 2.0 DIR No. 10000153877 Ver A	August 2022	Create new document for QX Manager with QX600 instrument functionality

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Safety and Regulatory Compliance

This section cites regulatory requirements for laboratory and electrical equipment, as well as requirements for working with chemicals and hazardous substances, and also explains safety precautions and recommendations.

Important: Only trained personnel should use this instrument.

Regulatory Compliance

The instrument has been tested and found to be in compliance with all applicable requirements of the following safety and electromagnetic standards:

- IEC 61010-1:2010 (3rd:2016, EN61010-1:2010 (3rd ed.). Electrical equipment for measurement, control, and laboratory use - Part 1: General requirements
- IEC 61326-1:2012 (Class A), EN 61326-1:2013 (Class A). Electrical equipment for measurement, control, and laboratory use. EMC requirements, Part 1: General requirements
- IEC 61010-2-081:2015, 3.0 edition, UL 61010-2-081:2015, CAN/CSA C22.2 No. 61010-2-081:19. Safety requirements for electrical equipment for measurement, control, and laboratory use. Part 2-081: Particular requirements for automatic and semi-automatic laboratory equipment for analysis and other purposes (includes Amendment 1)
- CAN/CSA 22.2 No 61010-1-04, Safety requirements for electrical, equipment for measurement, control, and laboratory use, Part 1: General requirements
- CAN/CSA C22.2 No. 61010-2-101:2019 IEC 61010-2-101: 2018 (3rd ed.)
- Restriction of hazardous substances (ROHS) directive (European Union)
- Registration, evaluation, authorization and restriction of chemicals (REACH). European Chemicals Agency (ECHA) June 1, 2007
- Waste electrical and electronic equipment (WEEE) directive

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This equipment has been tested and found to comply with the limits for a Class A digital device pursuant to Part 15 of FCC rules. These limits are designed to provide reasonable protection against harmful interference when the equipment is operated in a commercial environment.

Table 1. Regulatory symbols

Symbol	Definition
CE	The CE symbol indicates that the manufacturer ensures the product conforms with the essential requirements of the applicable EN directives.
C SA US	The CSA symbol indicates that a project has been tested to Canadian and U.S. standards, and it meets the requirements of those applicable standards. This equipment has been tested and found to comply with the limits for a Class A digital device pursuant to Part 15 of the FCC Rules. These limits are designed to provide reasonable protection against harmful interference when the equipment is operated in a commercial environment.
2012/19/EU www.bio-rad.com	The Waste Electrical and Electronic Equipment (WEEE) Directive symbol indicates that when the end-user wishes to discard this product, it must be sent to separate collection facilities for recovery and recycling.

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Safety Warning Labels

Warning labels alert you about sources of injury or harm. Table 2 defines each safety warning label.

Table 2. Meaning of safety warning labels

Icon	Meaning
	**Warning about risk of harm to body or equipment** Operating the systerm before reading this manual can constitute a personal injury hazard. For safe use, do not operate this instrument in any manner unspecified in this manual. Only qualified laboratory personnel trained in the safe use of electrical equipment should operate this instrument. Always handle all components of the system with care and with clean, dry hands.
	**Warning about handling biohazardous materials** When handling biohazardous samples, adhere to the recommended precautions and guidelines and comply with any local guidelines specific to your laboratory and location.
	**Warning about risk of electric shock** In order to prevent electric shock, use caution when plugging and unplugging the instrument. Always turn off and unplug the instrument when performing maintenance procedures.

Safe Use Specifications

For safe operation of the instrument, Bio-Rad strongly recommends that you comply with instructions listed in this section and in Appendix B, Instrument Maintenance.

This instrument is intended for laboratory use only. Bio-Rad is not responsible for any injury or damage caused by use of this instrument for purposes other than those for which it is intended, or by modifications to the instrument not performed by Bio-Rad or an authorized agent.

- This instrument is for use only by trained personnel.
- Use only the power cord, power switch, and USB port supplied with the instrument, and the plug adapter corresponding to the electrical outlets in your region.
- Position the instrument on a solid, stable surface, with adequate room at the back and on each side so that users can easily reach the power cord and USB port.
- This equipment generates, uses, and can radiate radio frequency energy and, if not installed and used in accordance with the provided instructional documentation, may cause harmful interference to radio communications. Operation of the systems in a residential area is likely to cause harmful interference, in which case users will be required to correct the interference at their own expense.

Note: Bio-Rad recommends maintaining a backup power source in case of power outages. A universal power supply (UPS) can protect from brown outs and power surges, while a regular backup generator does not.

For information on environmental requirements, see Environmental Requirements on page 26.

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General Precautions

- Always wear laboratory coat, laboratory gloves, and safety glasses with side shields or goggles.
- Keep your hands away from your mouth, nose, and eyes.
- Completely protect any cut or abrasion before working with potentially infectious materials.
- Wash your hands thoroughly with soap and water after working with any potentially infectious material before leaving the laboratory.
- Store all infectious or potentially infectious material in unbreakable leak-proof containers.
- Before leaving the laboratory, remove protective clothing.
- Change gloves frequently. Do not use a gloved hand to write, answer the telephone, turn on a light switch, or touch anything that other people may touch without gloves. Remove gloves immediately when they are visibly contaminated.
- Do not expose materials that cannot be properly decontaminated to potentially infectious material.
- Upon completion of an operation involving biohazardous material, decontaminate the work area with an appropriate disinfectant (for example, a 1:10 dilution of household bleach).

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Personal Protective Equipment

Proper use of gloves is recommended with use of oils and sample plates. OSHA requirements for PPE are set forth in the Code of Federal Regulations (CFR) at 29 CFR 1910.132 (General requirements); 29 CFR 1910.138 (Hand protection); 29 CFR 1926.95 (Criteria for standard personal protective equipment). Any gloves with impaired protective ability should be discarded and replaced. Consider the toxicity of the chemicals and factors such as duration of exposure, storage, and temperature when deciding to reuse chemically exposed gloves.

Listed below are features to aid glove selection for handling of machines, assays, oils, and cleaning solvents:

- Butyl gloves are made of a synthetic rubber and protect against peroxide, hydrofluoric acid, strong bases, alcohols, aldehydes, and ketones
- Natural (latex) rubber gloves are comfortable to wear and feature outstanding tensile strength, elasticity, and temperature resistance
- Neoprene gloves are made of synthetic rubber and offer good pliability, finger dexterity, high density, and tear resistance; they protect against alcohols, organic acids, and alkalis
- Nitrile gloves are made of copolymer and provide protection from chlorinated solvents such as trichloroethylene and tetrachloroethene; they offer protection when working with oils, greases, acids, and caustic substances

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Hazards

The instrument is designed to operate safely when used in the manner prescribed by the manufacturer. If the instrument or any of its associated components is used in a manner not specified by the manufacturer, the inherent protection provided by the instrument may be impaired.

Bio-Rad Laboratories, Inc. is not liable for any injury or damage caused by the use of this equipment in any unspecified manner, or by modifications to the instrument not performed by Bio-Rad or an authorized agent. Only trained Bio-Rad personnel should perform service on the system.

Biohazards

The instrument is a laboratory product. However, if biohazardous samples are present, adhere to the following guidelines and comply with any local guidelines specific to your laboratory and location.

Note: No biohazardous substances are expended during normal operations of this instrument.

Chemical Hazards

The instrument contains no potentially hazardous chemical materials.

Explosive or Flammability Hazards

The instrument poses no uncommon hazard related to flammability or explosion when used in a proper manner as specified by Bio-Rad.

Electrical Hazards

The instrument poses no uncommon electrical hazard to operators if installed and operated properly without physical modification and connected to a power source of proper specification.

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Surface Decontamination



WARNING! To prevent electrical shock, always turn off and unplug the instrument before performing decontamination procedures.

Important: Do not use abrasive or corrosive detergents or strong alkaline solutions. These agents can scratch surfaces and damage the system.

The following areas can be cleaned with 10% bleach solution:

- Outer area and chassis
- Inner plate holders
- Droplet reading surfaces

To prepare and apply the disinfectant, refer to the instructions provided by the product manufacturer. For questions regarding the use of other cleaning agents, contact Bio-Rad Technical Support.

For more information on surface cleaning, see Appendix B, Instrument Maintenance.

Important: Do not clean the handler Y-axis rail when the front door is open. This is a lubricated surface, and failures will occur if the lubrication is removed.

Decommissioning and Disposal

The instrument contains electrical materials that should be disposed of as unsorted waste and must be collected separately, according to European Union Directive 2012/19/EU on waste electrical and electronic equipment — WEEE Directive. The purpose of decommissioning is to make sure that the equipment is electrically and environmentally safe for disposal. Before disposal, contact your local Bio-Rad representative for country-specific instructions.

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Transport

You must perform the specified decontamination procedures before moving or shipping the system. Always move or ship the instrument with the supplied packaging materials, which will protect the instrument from damage. If appropriate containers cannot be found, contact your local Bio-Rad office.

Warranty

Important: The instrument is for research use only, and not for use in diagnostic procedures.

The instrument and its associated accessories are covered by a standard Bio-Rad warranty. Contact your local Bio-Rad office for the details of the warranty. Follow the safety specifications listed in this chapter and throughout this guide.

The instrument is intended for laboratory use only. Bio-Rad Laboratories, Inc. is not responsible for any injury or damage caused by use of this instrument for purposes other than those for which it is intended, or by modifications of the instrument not performed by Bio-Rad Laboratories or an authorized agent. Alteration of this instrument voids the warranty and safety certification, as it creates a potential safety hazard.

Use of unapproved supermixes or additives may harm the instrument and voids the warranty.

Use only the power cord supplied with the instrument, using only the plug adaptor that corresponds to the electrical outlets in your region.

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Chapter 1 Introduction to Droplet Digital PCR

Droplet Digital™ PCR (ddPCR™) is a digital polymerase chain reaction method based on water-oil emulsion droplet technology. ddPCR uses a combination of microfluidics and proprietary surfactant chemistries to divide each sample into water-in-oil droplets. The technology uses reagents and workflows similar to those used for most standard TaqMan probe-based assays, and provides absolute quantification of nucleic acid target sequences by counting nucleic acid molecules encapsulated in discrete, volumetrically defined water-in-oil droplet partitions.

ddPCR is highly effective in the following areas:

- **Absolute quantification** — ddPCR provides a concentration of target DNA copies per input sample without the need for running standard curves, making this technique ideal for target DNA measurements, viral load analysis, and microbial quantification.
- **Genomic alterations such as gene copy number variation (CNV)** — CNVs result in too few or too many dosage-sensitive genes responsible for phenotypic variability, complex behavioral traits, and disease. ddPCR enables measurement of 1.2x differences in gene copy number.
- **Detection of rare sequences** — researchers must amplify single genes in a complex sample, such as a few tumor cells in a wild-type background. ddPCR is sensitive enough to detect rare mutations or sequences.
- **Gene expression and microRNA analysis** — ddPCR provides stand-alone absolute quantification of expression levels, especially low-abundance microRNAs, with sensitivity and precision.
- **Next-generation sequencing (NGS)** — ddPCR quantifies NGS sample library preparations to increase sequencing accuracy and reduce run repeats. Validate sequencing results such as single nucleotide polymorphisms or copy number variations with absolute quantification.
- **Single cell analysis** — the high degree (10-fold to 100-fold) of cell-cell variation in gene expression and genomic content among homogeneous post-mitotic, progenitor, and stem cell populations drives a need for analysis from single cells. ddPCR enables low copy number quantification and gene expression of individual cells.
- **Genome edit detection** — ddPCR enables fast, precise, and cost-effective assessment of HDR (Homology directed repair) and NHEJ (non-homologous end joining) generated by CRISPR-Cas9 or other genome editing tools.

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ddPCR has the following benefits for nucleic acid quantification:

- **Unparalleled precision** — The massive sample partitioning afforded by ddPCR enables small fold differences in target DNA sequence between samples to be reliably measured.
- **Increased signal-to-noise ratio** — High-copy templates and background are diluted, effectively enriching template concentration in target-positive partitions. This allows for the sensitive detection of rare targets and enables a ±10% precision in quantification.
- **Removal of PCR efficiency bias** — Error rates are reduced by removing the amplification efficiency reliance of qPCR, enabling accurate quantification of targets to near zero.
- **Simplified quantification** — There is no requirement for a standard curve for absolute quantification.

ddPCR Workflow

The ddPCR process adheres to the following workflow:

- You prepare your samples for PCR by combining DNA or RNA with primers, probes dye, and Bio-Rad ddPCR supermix.
- A droplet generator fractionates a sample into~20,000 nanoliter-sized droplets with target and background DNA distributed randomly into the droplets during the partitioning process.
- Following droplet generation, the droplets are run through a thermal cycler, which performs PCR amplification of the nucleic acid target in each individual droplet.
- A droplet reader reads each droplet to determine the fraction of positive droplets in the original sample and uses Poisson statistical formulas to determine the absolute quantity.

  Note: Positive droplets containing at least one copy of the target DNA molecule exhibit increased fluorescence compared to negative droplets.

Finding Out More

Click the Help tab ( ) on the left, and then click the Bio-Rad Website to access links to technical notes, manuals, videos, product information, and technical support. The website also provides many technical resources on a wide variety of methods and applications related to PCR, Droplet Digital PCR, and gene expression.

Chapter 2 Using the QX600 Droplet Reader

Using the QX600™ Droplet Reader, you can detect rare DNA target copies, determine copy number variation, and precisely measure gene expression levels. Applications and uses include:

- Copy number variation
- Rare sequence detection
- Next generation sequencing (NGS) library quantification
- Viral load determination, including SARS-CoV-2 assays
- mRNA analysis
- Gene expression analysis and single cell gene expression analysis
- Absolute quantification
- Rare mutant detection
- NGS sample preparation
- GMO detection
- Contaminant testing
- High-order multiplexing

Following the droplet generation and thermal cycling processes, the QX600 Droplet Reader collects binary data for analysis as it singulates the ~20,000 nanoliter-sized droplets in each sample, and then streams them in single-file past a six-color detector to determine which droplets contain target molecules (positive reading) and which do not (negative reading).

The QX600 Droplet Reader detects multiple targets in six fluorescent dyes using three interrogation points across defined spectrums. The first interrogation point detects targets in FAM or EvaGreen® and HEX or VIC, the second in Cy5 and Cy5.5, and the third in ROX and ATTO 590.

Important: Use only the dyes listed above (FAM or EvaGreen®, HEX/VIC, Cy5, Cy5.5, ROX, and ATTO 590).

Important: Your QX600 Droplet Reader performs the post-PCR droplet reading function only. A droplet generator and a thermal cycler are required to perform a full ddPCR cycle. You can purchase a droplet generator and thermal cycler, as well as associated accessories and consumables, from Bio-Rad. For information, see Appendix D, Ordering Information.

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The following graphic and legend illustrate the functional areas on the instrument exterior:



LEGEND

1	Button to open and close lid
2	Status indicator lights (see Status Indicator Lights on page 31)
3	On the back of the instrument, power and USB outlets
4	Oil and waste compartment (see Fluid Maintenance and Waste Management on page 195)

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Instrument Specifications

This section contains information on the instrument size and weight of the instrument. Safety information is cited in Safe Use Specifications on page 14 and the proper environmental maintenance is cited in Environmental Requirements on page 26.

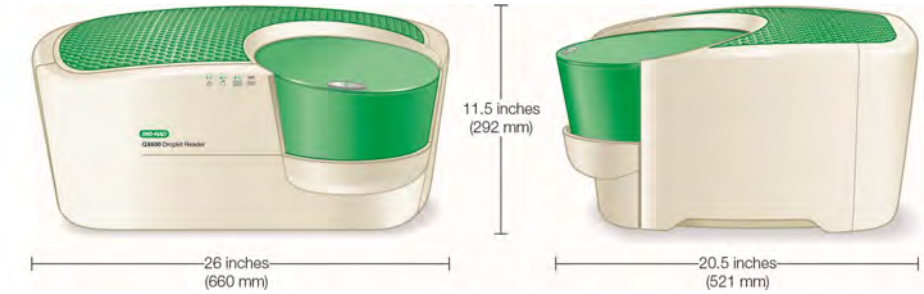


Table 3. Instrument specifications

Element	Specifications
Weight	56.6 lbs (26 kg)
Size (W x D x H)	26 x 20.5 x 11.5” (660 x 521 x 292 cm)

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Environmental Requirements

Table 4 lists the environmental requirements for the QX600 Droplet Reader.

Important: You must use the supplied shielded cables with your instrument to ensure compliance with the Class A FCC limits.

Table 4. Conditions for safe use

Usage aspect	Requirements
Rated input power	100–240 V, 50–60 Hz, 2A (plugs into standard AC receptacle)
Fuses	T5AH, 250 V
Voltage fluctuations	± 10% for the included external power supply **Note:** Use only the power cord supplied with the equipment.
Pollution degree	2
Usage temperature	18–30° C
Relative humidity	85% max non-condensing
Altitude	0 to 8,200 ft (0 to 2,500 m) above sea level
Installation category	II (external power supply plugs into a standard AC receptacle) Indoor use only
Ventilation requirements	The following distances should be unobstructed for proper ventilation: ■ 5 in (12 cm) on the left and right sides of the instrument ■ 10 in (25 cm) behind the instrument

Included Items

This section identifies the functional items included with your droplet reader purchase, as well as additional components and consumables. For information on items you may need for a full ddPCR cycle, see Appendix D, Ordering Information.

Table 5 explains the components that ship with the QX600 Droplet Reader.

Table 5. QX600 Droplet Reader and Included Components

Component	Description	Catalog Number
QX600 Droplet Reader	Droplet reading instrument	12013328
USB cable and power cord	USB cable that connects the computer to the instrument and power cord that connects the instrument to the power source **Note:** To obtain cables or power cords, contact Bio-Rad technical support.	Included
Droplet reader plate holders (2)	Positions and secures the 96-well plate in the droplet reader plate compartment	12006834

Additional Components

The following tables identify additional components you can purchase for use with your system or instrument.

Table 6. Instrument components

Component	Description	Catalog Number
Computer	Computer that connects to the QX600 Droplet Reader for data collection and analysis	12017458
Software purchase	Bio-RadQX Manager Software, Premium Edition **Note:** Standard Edition is included with your droplet reader purchase.	12012172
ddPCR 96-Well Plates	Clear well/clear shell semi-skirted plates	12001925 (pkg of 25)

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Component	Description	Catalog Number
Droplet Reader Oil	ddPCR droplet reader oil for QX600 and QX200 instruments and systems	1863004
Rainin Pipets	■ 20 µl pipet for sample loading ■ 50 µl pipet for droplet transfer ■ 8-channel 200 µl pipet for oil	Rainin L-20 Rainin L-50, L8-50 Rainin L8-200
Rainin Pipet Tips	Filtered	Rainin GP-L10F Rainin GP-L200F

Table 7. Thermal cycler and plate sealer

Product	Description	Catalog Number
C1000™ Touch Thermal Cycler with 96–Deepwell Reaction Module	Includes the C1000 Touch Thermal Cycler chassis, 96–deepwell reaction module, USB flash drive	1851197
PTC Tempo Deepwell Thermal Cycler	Includes the thermal cycler, power cord, USB cable, and Ethernet cable	12015392
PX1 PCR Plate Sealer	PCR plate sealer, includes heat sealing instrument, plate support block that holds 96-well and 384-well plates, sealing frame, power cord	1814000
Piercable Foil Heat Seal	Heat seal for the PX1 PCR Plate Sealer	1814040

Table 8. ddPCR supermixes

Supermix	Description	Catalog number	
ddPCR EvaGreen® Supermix	2x supermix, for use in nucleic acid sample preparation with the QX600 and QX200 ddPCR systems	1864033 1864034 1864035 1864036	2ml (2 x 1ml) 5ml (5 x 1ml) 25ml (25 x 1ml) 50ml (50 x 1ml)
ddPCR Supermix for Probes (no dUTP)	2x supermix, for use in nucleic acid sample preparation with the QX600 and QX200 ddPCR systems	1863023 1863024 1863025	2ml (2 x 1ml) 5ml (5 x 1ml) 25ml (25 x 1)

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Table 8. ddPCR supermixes, continued

Supermix	Description	Catalog number	
ddPCR Supermix for Probes	2x supermix, for use in sample preparation with the QX600 and QX200 ddPCR systems	1863026 1863010 1863027 1863028	2ml (2 x 1ml) 5ml (5 x 1ml) 25ml (25 x 1ml) 50ml (50 x 1ml)
ddPCR Multiplex Supermix	4x supermix especially suited for probe-based detection of multiple targets in DNA samples using the QX600 and QX200 ddPCR systems	12005909 12005910 12005911	1.2ml (2 x 0.6ml) 3 ml (5 x 0.6 ml) 12.5 ml (5 x 2.5 ml),
ddPCR Supermix for Residual DNA Quantification	2x supermix, for use in residual DNA detection with the QX600 and QX200 ddPCR systems	1864037 1864038 1864039 1864040	2ml (2 x 1ml) 5ml (5 x 1ml) 25ml (25 x 1ml) 50ml (50 x 1ml)
One-step RT-ddPCR Advanced Kit for Probes	200 or 500x 20 µl reaction kit, for absolute quantification of target RNA in a one-step format with the QX600 and QX200 ddPCR systems	1864021 1864022	2ml (2 x 1ml) 5ml (5 x 1ml)

Table 9. Buffer controls

Buffer	Catalog Number
ddPCR Buffer Control Kit for Probes	1863052
QX200 Buffer Control Kit for EvaGreen®	1864052

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Compatible ddPCR Process Instruments

Along with the QX600 Droplet Reader, you can purchase the droplet generators shown below from Bio-Rad.

QX200 Droplet Generator



Automated Droplet Generator (left)



QX600 Droplet Reader



Important: A thermal cycler, which can be purchased separately, is required to perform a full ddPCR cycle. For information on additional instruments, accessories, and consumables, see Appendix D, Ordering Information.

Connecting and Starting the Instrument

To execute runs on the QX600 Droplet Reader, you must connect the instrument to a computer on which the QX Manager Software is installed and configured. Bio-Rad recommends purchasing at least one configured computer that can remain connected to the QX600 Droplet Reader and be used for adding and running plates in QX Manager Software. For information on additional items you can purchase for your instrument, see Additional Components on page 27.

To connect and start the instrument

1. Connect the USB cable provided by Bio-Rad to the USB port on the back of the QX600 Droplet Reader, and then to the computer.
2. Press the Power button on the instrument.

   The Power indicator light turns solid green to indicate that power is on.
3. Turn on the connected computer and log in with a Windows account. For information, see your system administrator.
4. Open and log into QX Manager Software.

Important: The first time you log into the software, the Instrument window appears and you must select the instrument you are using. For information on selecting the correct instrument in QX Manager Software, see Selecting the Instrument on page 40.

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Status Indicator Lights

This section describes the indicator lights on the front of the QX600 Droplet Reader.



Before you start a run, check the indicator lights to ensure the instrument is ready. For information, see Table 10.

Table 10. Droplet Reader Status Indicator Lights

Indicator	(Power)	(Fluids)	(Plate)	(Run Status)
Solid Green	Power is on	Fluid levels OK	Plate loaded properly	Complete
Flashing Green	N/A	■ Less than 40% oil is available ■ More than 70% waste in container	N/A	In progress
Flashing Amber	N/A	■ Less than 10% oil available ■ More than 90% waste in container	N/A	Error during run
No color	Power is off	N/A	No plate loaded	Instrument is idle

If the bottle indicator light is solid green or flashing green, there is enough oil in the bottle to complete a run on 96 wells. You can configure the full plate in QX Manager Software for your run. Once the low level sensor is triggered, the Add Plate button is disabled and you cannot continue to run plates (less than 10%). You must replace the oil and waste bottles with new ones before the instrument can start the run.

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Chapter 3 About QX Manager Software

QX Manager Software, when connected to your QX600 Droplet Reader, provides the necessary functionality to create, run, and analyze droplet reading experiments on your samples.

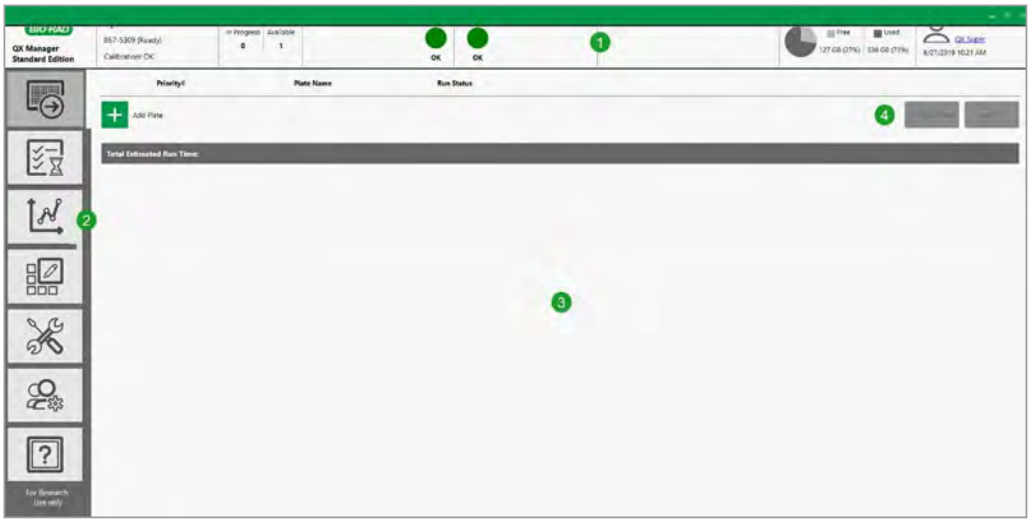
During the installation of Standard Edition, you can enable the User Management module for all user management functions and preferences. If you do not enable User Management, you can use the software with a single generic user.

Using the instrument and software, you can

- Set up customized, real-time ddPCR experiments for plate runs
- Read standard droplets
- Choose between two droplet volumes
- See the available disk space and number of plates you can process
- Use the live analysis function during the droplet reading process
- Analyze your data files in a variety of charts and tables in the Analysis module
- Reprocess data with updated color calibration values
- Create and store plate and report templates, and produce reports on your data

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The following image highlights functional areas.



LEGEND

1	The status bar displays information about the instrument and user. For information on specific functionality, see Instrument Status Bar on page 46.
2	Tabs provide access to the main functional windows.
3	The main pane displays the details of the selected tab.
4	The buttons allow you to configure the plate and start the run.

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Enabling User Management

During the installation of QX Manager Software, Standard Edition, you can choose to enable or bypass the User Management module:

- If the Enable User Management checkbox is selected, all user functions are included in the installation.
- If the checkbox is not selected, User Management is not enabled and the corresponding tab is not included in the side pane. A single generic user, with a small set of user preferences, is always logged in. For information, see Partial Preferences on page 44.

Important: Before you can set up users in the software, all users must be set up on the computer as local Windows users or in Active Directory on your network.



To bypass the User Management module

▶ Ensure the checkbox is cleared and click Continue in the User Management dialog box to complete the installation.

To enable the User Management module

1. Ensure the Enable User Management checkbox is selected.
2. Enter the user name and click Check Name to validate the user.
3. Click Continue to add the user and complete the installation.

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Chapter 4 Getting Started

Use the information in this chapter to

- Sign into the software or change users
- Select the instrument
- Become familiar with the instrument status bar and main functional windows
- Understand the compatible file types for your software edition

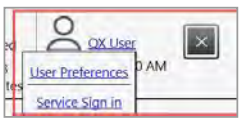
Tip: If QX Manager Software goes into standby mode after being idle, click in the lockout screen to log into the software again.

Signing In, Viewing Privileges, and Changing Preferences

Typically, a system or software administrator sets up users and privileges for QX Manager Software, and communicates the information to you.

Note the following:

- When you sign in the first time after the software is installed, you must select the instrument to which the computer is connected. See Selecting the Instrument on page 40 for information.
- You cannot change your user privileges unless you are assigned the Add/Manage Users privilege. For information, see Viewing User Privileges on page 41.
- You can change your user preferences, which are set to defaults. For information, see Managing Your Preferences on page 43. If User Management was not enabled during the software installation, QX Manager Software creates a single generic user, which is already logged in and is available to all users for setting up and running plates, and analyzing the results. The user can access a set of partial preferences using the generic user link, as shown in the following graphic. See Partial Preferences on page 44 for more information.



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Signing into the Software

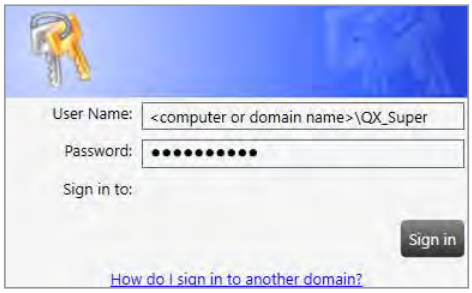
Important: If User Management was not enabled during the installation, the generic user is automatically signed in when you open the software.

To sign in

1. Right-click the software icon on the desktop and select Open to display the Sign in dialog box.
2. Do one of the following to enter your user name:
   - If the Sign in to: label shows the correct domain name, enter only the user name and password.



   - If the Sign in to: label is blank or shows a different domain, then enter it as **<computer or domain name\user name>**, followed by the password.



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For more information, click the How do I sign into another domain? link.



Note: If you are logging into a network domain, but don't know the name, contact your system administrator.

3. Ensure the Do not show EULA on sign in checkbox is selected and tap I Agree.



Each user must agree to the end user license agreement on initial login in order to user the software. If you clear the checkbox, the End User License Agreement appears the next time you log into the software, and you must agree again to the terms before the software opens. If you do not agree to the EULA, the application closes immediately.

When you agree to the EULA, the dialog box closes and the application opens. Your user name appears in the Instrument Status bar.

If you are connected to the QX600 Droplet Reader, the Add Plate window opens.

If you are opening the software on a computer not connected to an instrument, the Data Analysis window opens.

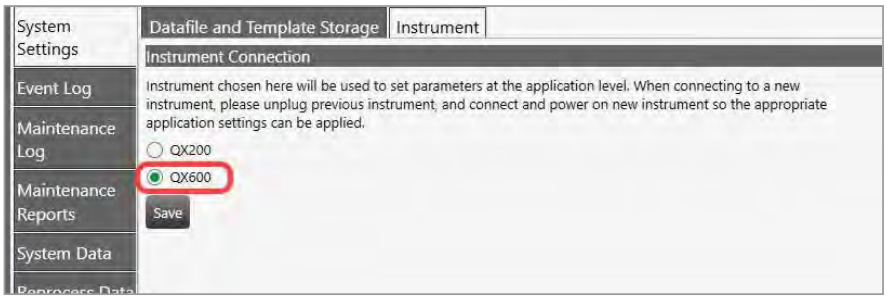
Selecting the Instrument

When you open the software and sign in for the first time, the instrument connection window appears and you must select the connected instrument and a droplet volume.

Note: This procedure assumes the instrument is connected to the computer and QX Manager Software is installed. You can use QX Manager Software with the QX200 Droplet Reader and the QX600 Droplet Reader. If you are using both instruments, you can change instruments at any time.

To select the instrument

1. Select QX600, and then click Save.



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Viewing User Privileges

Important: If you did not enable the User Management module during the installation, the related functions are not available.

In the User Management window, your privileges are identified by the selected checkboxes.

To view your assigned privileges

▶ Select the User Management tab.



Unless you are assigned the Add/Manage Users privilege, checkboxes for your assigned privileges are selected but not enabled, as shown in the following graphic. To request changes to your privileges, contact your software administrator.



Table 11 lists the available privileges, which can be assigned in any combination.

Table 11. User privileges

Privilege	Purpose
Add and manage users	Add or remove users, set privileges, and change preferences. **Important:** Users who are assigned the Add/Manage Users privilege can create users and update privileges and preferences for any user. Only the superuser can remove this privilege from user accounts.
Create new templates	Save a design for a plate or analysis report as a template.
View data files (created by other users)	See run files that were created from runs executed by other users.
Overwrite existing data file name	Save the file with a new file name. Files are always saved with the extension for the application you are using. For information, see Compatible File Types on page 48. **Note:** Users without this permission can open files and perform analysis but cannot create a new file.
System settings	View logs, reprocess data with new calibration values, and change file locations. All users can view the file locations. **Important:** If your administrator selected the Preferred Locations checkbox in System Settings, individual user location preferences are overridden.
Maintenance	View the event log and the system data settings. **Note:** All users can reprocess data, view system folder settings, as well as the maintenance log and maintenance reports.
System Data	Move raw data from the computer to free up disk space. Access troubleshooting data.
Manage Plate	Cancel a run while it is in process on the QX600 Droplet Reader.

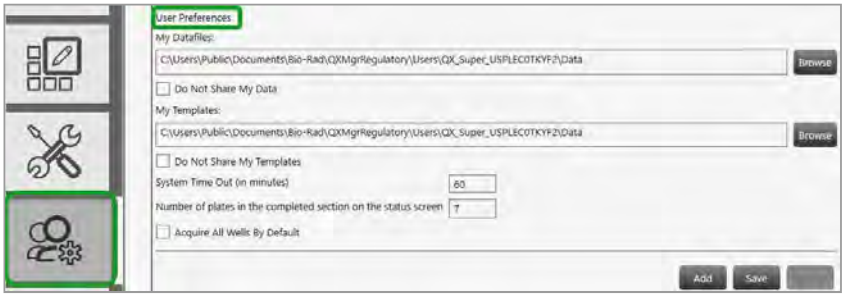
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Managing Your Preferences

Use the User Management window to modify your personal user preferences. Default folder locations appear in your preferences, but you can change them. You can also choose to keep all data files and templates private. The software prompts you to choose either your personal folder or the shared folder each time you save a file or template.

Important: If you did not enable User Management during the software installation, the User Management module is not included and a truncated set of preferences is available. See Partial Preferences on page 44 for information.



To change your preferences

1. Change any of the following preferences:
   - Enter a different file path for your data files and templates.
   - Select or clear the check boxes to change your data file and template privacy settings.

     Important: The paths and checkboxes are disabled if your administrator has set Preferred Locations in System Settings.
   - Enter a different system timeout period.
   - Enter a different total of completed plates to show in the Run Status window, up to a maximum of 100.
   - Select the Acquire all Wells By Default checkbox. If you do not select the checkbox, all wells are excluded by default and you must manually include the wells for each droplet reading run.
2. Do one of the following:
   - If you are setting preferences for a new user, click Add.
   - If you are updating preferences, click Save.
3. When the confirmation message appears, click Yes, and then click OK.

Partial Preferences

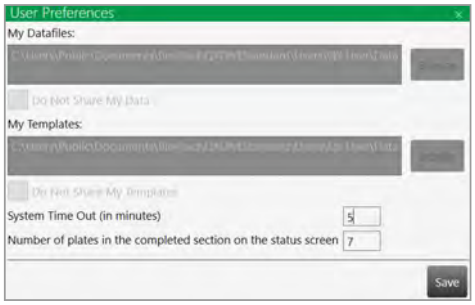
If User Management was not enabled during the software installation, you can still access partial functionality.

To open the menu

1. Click the user name link in the upper-right corner to display the pop-up.

   Important: The Service Sign in option is used by Bio-Rad service engineers only.

2. Click User Preferences to display the dialog box.



3. You can change the following:

   - System time out in minutes (default is 5 minutes)
   - Number of plates in the completed section of the status screen (default is 7)

4. After you have made your changes, click Save.

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Signing Out or Changing Users

While the instrument is idle, one user can log out and another user can log in.

Important: If User Management was not enabled during the software installation, the sign in, sign out, and change users functions are not available.

Note the following:

- Do not change users while a run is in progress or data might be affected.
- If the screen is locked and the initial user left unsaved changes, an advisory prompt appears. Click Save before proceeding or the previous user's work is lost.

To sign out

1. Click the user name link in the upper-right corner and select Sign Out.
2. Click Yes to confirm.

To change users

1. Click anywhere in the lockout screen to display the Sign in window.
2. Enter a user name.
   - The domain name appears below the user login fields. If you are on the same domain as the previous user, you can enter your user name only.



   - If the user is on a different domain, enter the domain name followed by a backslash and the user name.

     <domain name>\<user name> (for example, global\john_smith)

3. Enter the password and click Sign in.

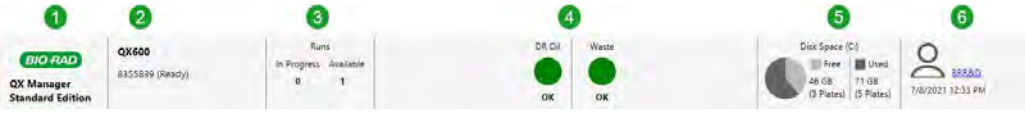
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Instrument Status Bar

An instrument status bar appears above all windows except the analysis displays, which open in a separate module.

When a computer is connected to the instrument, the status bar appears as shown in the following graphics. If a computer is not connected to the instrument, the status bar shows only the software, disk space, and user information.



LEGEND

1	Software name and edition
2	Information and status of the connected instrument
3	Instrument availability
4	Oil and waste levels
5	Used and free disk space Available storage is displayed in GB, and also in number of plates that you can run before running out of space on the computer. **Note:** When QX Manager Software detects low disk space, an advisory prompt to archive data appears.
6	Name of the current user, along with the current date and time.

Important: If User Management was not enabled during the installation, the generic user name appears on the far right.

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Functional Windows

Table 12 describes the functional areas and primary windows in QX Manager Software.

Table 12. Window Tabs

Tab	Name	Purpose
	Add Plate	■ Add and configure a plate for droplet reading
	Run Status	■ In the top pane, view the run in progress ■ In the bottom pane, view up to 100 completed runs
	Data Analysis	■ Access the Data Analysis and Gene Study modules
	Templates	■ Set up templates for plates and reports, and search for existing template files
	System	■ View storage locations, logs, and maintenance reports ■ Archive data, reprocess data with new calibration values, access troubleshooting information **Important:** Available functionality depends on your assigned user privileges. Instrument calibration is available only to the Bio-Rad service engineer.
	Users	■ View the user privileges you have been assigned, and view and modify your personal preferences ■ Create, edit, or remove QX Manager Software users (if you are assigned the Add/Manage Users privilege) **Important:** In Standard Edition, user functionality is available only if the User Management was enabled during software installation.
	Help	■ Access software version information, current calibration values, the End User License Agreement, the companywebsite, and open source software license information **Tip:** You can also link the user and instrument guides to the labeled buttons.

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Compatible File Types

This section describes the file types you can open in QX Manager Software.

The following applies if your organization is using more than one edition of the software:

- You can open files created using Standard Edition only in Standard Edition.
- If you open a .qlp file in QX Manager, Standard Edition, the file opens and is saved as a .ddpcr file.

Table 13. Compatible file types

File Type	Extension	Details
Plate template	.ddplt	Plate template file containing setup details to perform experiments; this file type opens in the Plate Editor.
Plate template	.csv	Importable plate template file containing setup details in CSV format; this file type opens in the Plate Editor.
Data	.ddpcr	Contains the results of an experiment run performed on the droplet reading instrument and opened in QX Manager Software, Standard Edition.
Data	.qlp	File type from earlier QuantaSoft™ versions, for two-channel experiment runs and data analysis; this file type opens in the Analysis module.

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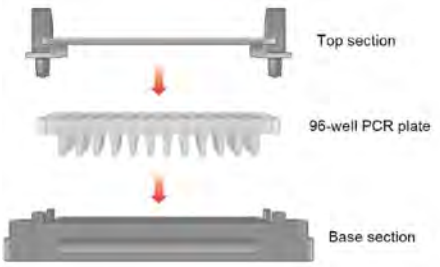
Chapter 5 Running Experiments

When the Start Run button is enabled, you can start the run. You can also cancel a run after it has started if you are assigned the Manage Plate user privilege. For information, see Canceling a Run on page 65.

Assembling a Plate

The QX600 Droplet Reader accepts a solid 96-well plate that fits into a secure holder. You must assemble the plate components before you can insert the plate into the QX600 Droplet Reader.Three separate components comprise the plate:

- A base section to hold the plate
- The plate itself
- A top section to secure the plate



To assemble the plate

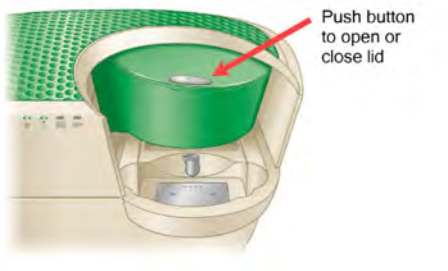
1. Place the plate into the base section, and then raise the release tabs on the top section.
2. Place the top section over the plate, and then lower the release tabs to secure the plate in the holder.



Inserting an Assembled Plate into the Instrument

To insert a plate into the QX600 Droplet Reader

1. Press the button to open the compartment.



2. Fit the assembled plate into the compartment. Well A1 must be in the top-left position.



Important: Make sure the tabs on the plate holder are securely clamped and the plate lays flat. Otherwise, the sampling needle can strike the lid and damage the instrument.



3. Press the button again to close the lid.

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Adding the Plate in QX Manager Software

This section explains how to add the plate in QX Manager Software, Standard Edition when using the QX600 Droplet Reader for your droplet reading run. You can insert an assembled plate into the instrument before or after you add the plate in the software.

To add a plate in QX Manager Software

1. Click the Add Plate tab.

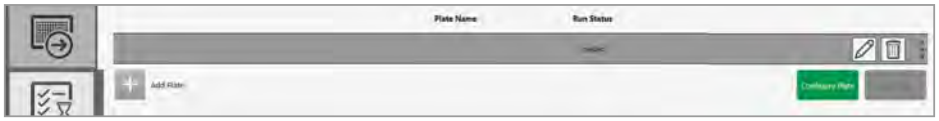


If the Runs Available number in the Instrument Status Bar is 1, the Add Plate button is enabled.

2. Click + next to Add Plate.



When QX Manager Software recognizes the plate holder in the compartment, the software enables the Configure Plate button.



Note: If the software does not recognize the plate holder, the software displays an advisory prompt. Remove and reinsert the plate. If necessary, contact Bio-Rad Technical Support.

3. Click Configure Plate. The Plate Configuration window opens.

Configuring the Plate

After you click Configure Plate, the Plate Configuration window opens to the Plate Information display. The window features the following tabs:

- Plate Information
- Well Selection
- Well Information

All users can start runs on selected plates using the initial configuration, an altered configuration, or just the plate and data file names and the supermix. Users with the Create New Template user privilege can save the configuration as a template before the droplet reading run.

Plate Information Tab

The Plate Information tab is selected by default when the Plate Configuration window opens, and provides functionality allowing you to quickly set up a plate for a run.

You can

- Select, modify (optional), and run an existing template

  Note: CSV template files appear in the dropdown list on the Plate Information tab, but not in the Templates window. For information, see Creating and Storing Plate Templates from CSV Files on page 83.

- Create and run a plate design using a manual configuration or a template
- With the Create New Templates user privilege, save plate designs as templates for reuse
- For an expedited run, identify a plate name and a supermix, and then start the run

  Note: The data file name field is automatically filled with the plate name, but you can to change it. You can identify the remainder of your experiment parameters either in the Plate Editor before you start the run, or in the plate editor layout in the Analysis module after the run has concluded.

- Select to acquire wells by columns or rows

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Identifying Plate Information

When you click Configure Plate, the following window appears.



To identify the plate

1. Do one of the following:
   - **Use an existing template** — click the dropdown arrow under Choose Existing and select a template file. The software automatically populates the fields under Create New Plate.
   - **Create a new template** — click Create New, and see Creating or Editing Templates on page 67.
   - **Create a new plate** — enter a plate name and select a supermix. When you enter the plate name, the Save data file as field is automatically populated, but you have the option to change it.
2. (Optional) Under Acquire Wells By, select the alternative well acquisition method. The default is Column.

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Well Selection Tab

The wells in the plate layout are automatically excluded and you must manually include the wells to be processed. Use this window to select wells to include wells for droplet reading.

Tip: If you typically run full plates, you can change the setting in User Preferences to acquire all wells by default. For information, see Managing Your Preferences on page 43.

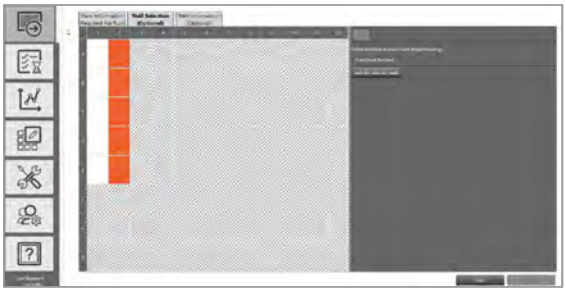
Note: The Well Selection window is identical to the Exclude window in Template Setup.

Including or Excluding Wells in the Plate Layout

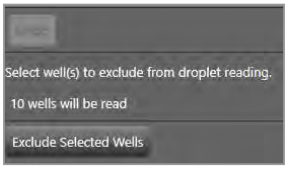
All wells are excluded by default when the plate editor appears.

To include wells in the run

1. Select the Well Selection tab.
2. In the Plate Editor layout, select the wells containing sample and then click the Include Selected Wells toggle button.



3. On the right, QX Manager Software specifies the number of wells that will be included in the droplet reading run and the button text changes to Exclude Selected Wells.



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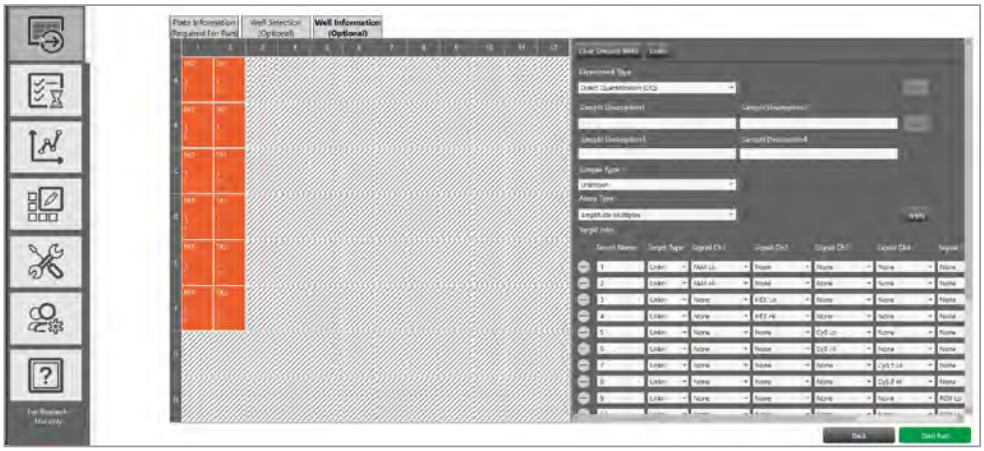
Well Information Tab

When you select the Well Information tab, the Plate Editor opens. You can configure your experiment parameters for selected wells before or after the run.

Note: QX Manager Software can calculate automatic thresholds for positive control wells, but you must select Pos Ctrl from the Sample Type drop-down list. You can make the selection before or after the run. For information, see Sample Types on page 75.

Defining or Editing Well Information

You can assign different experiment parameters to each well or groups of wells.



You can apply your entries at any time. You must click Apply for the software to recognize your new or edited information. If you enter information in a field and need to apply earlier changes first, QX Manager Software displays an advisory message.

To define the configuration for the wells

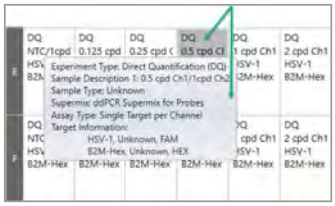
1. Select a well or group of wells.
2. Select an experiment type; see Experiment Types on page 73.
3. In the Sample Description fields, enter up to four words or phrases that describe the sample; see Sample Descriptions on page 75.
4. Select a sample type; see Sample Types on page 75.

   Note: For automatic thresholding of positive control wells, you must select Pos Ctrl.
5. Select a supermix; see Supermixes on page 76.

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6. Select an assay type. Available assay types vary, depending on the selected experiment type; see the fluorophore options in Assay Types and Fluorophores on page 77.
7. (Optional) Under Target Info, change the fluorophore assigned to a channel. The fluorophore information populates automatically for each target, with the maximum number of rows allowed for the assay method. However, you can modify the default fluorophore assignments, if applicable.
   a. Click the dropdown arrow and select an alternative.
   b. To delete a row, click the minus (-) icon next to the target.
   c. To add a row, click the plus (+) icon.
8. (Optional) To clear one or more wells at any time, select the wells and click Clear Selected Wells.
9. Click Apply. After you have applied your entries and selections, you can pause the cursor on a well to see the well information.



10. When you finish configuring the plate, click Save.

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Starting the Run

When all required elements to set up a plate for a run are satisfied, QX Manager Software enables the Start Run button. You can configure the elements of the plate before you start the run, or you can run the plate with just the plate name and supermix, and define the configuration from the Analysis module.

To start the droplet reading run

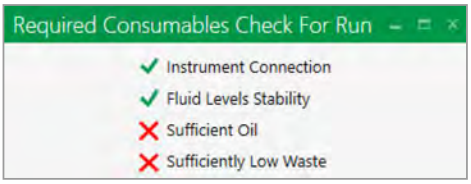
1. Tap Start Run.



The software performs a mandatory prerequisites check to ensure that the instrument is ready.

One of the following occurs:

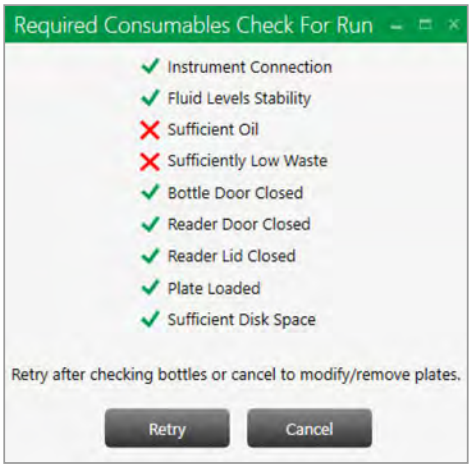
- If the instrument passes the check, the run starts normally. Continue to Running the Experiment on page 61.
- If the check finds issues with the instrument, the software displays a dialog box in which the issues are identified. The instrument cannot start the run until all issues are resolved. For information, see Run Prerequisites Checklist on page 58.



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Run Prerequisites Checklist

If the prerequisites check on the instrument status finds one or more problems with instrument readiness, QX Manager Software identifies them with an ✗ in the Check for Run dialog box. You must resolve all issues before the instrument can start the run.



Note: If you encounter a problem with the instrument or software that is not described here, see Appendix C, Troubleshooting.

For information on each issue that can prevent the instrument from starting the run, see Table 14 on page 59.

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Table 14. Pre-Run Error Indicators

Status Check Item	Issue	Suggested Resolution
Instrument Connection	Power connection not recognized.	Ensure the power outlet is working. Disengage the power cord from the outlet, and then reinsert it into the outlet.
	USB cable connection not recognized.	Disconnect, and then reconnect, the USB cable between the instrument and the computer.
	Instrument connection not recognized.	Ensure the correct instrument is selected in the System Settings tab > Instrument tab.
Fluid Levels Stability	The oil or waste is moving within the bottle so the level cannot be accurately determined.	Wait until the liquid is settled, then try again. Ensure the instrument rests on a solid, stable surface.
Sufficient Oil	Oil level is below the acceptable level for a run.	Remove the oil bottle and replace it with a full one.
Sufficiently Low Waste	Waste level is above the acceptable level for a run.	1. Empty any remaining oil from the bottle and then place a Waste label (provided by Bio-Rad) over the Oil label. 2. Insert the bottle into the Waste slot.
Bottle Door Closed	Bottle door is not closed properly.	Open the bottle door and ensure there are no blockages, and then close the door again.
Reader Door Closed	Reader door is not closed properly.	Open the reader door and ensure there are no blockages, and then close the door again.
Reader Lid Closed	Reader lid is not closed properly.	Open the reader lid and ensure there are no blockages, and then close the lid again.

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Table 14. Pre-Run Error Indicators, continued

Status Check Item	Issue	Suggested Resolution
Plate Loaded	Plate is not loaded properly.	1. Open the reader door and remove the plate. 2. Ensure the tabs of the plate cover are securely clamped. 3. Insert the plate again and ensure it lays flat.
Sufficient Disk Space	There is not enough storage space available for the run data.	Archive older data files to clear space.

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Running the Experiment

The instrument allows you to do the following:

- During the run, perform real-time analysis on the droplets in each well after the well has been read
- Cancel a run (with the Manage Plate permission).
- When the run concludes, QX Manager Software moves the run to the Completed list in the Run Status window, and saves the analysis data file.

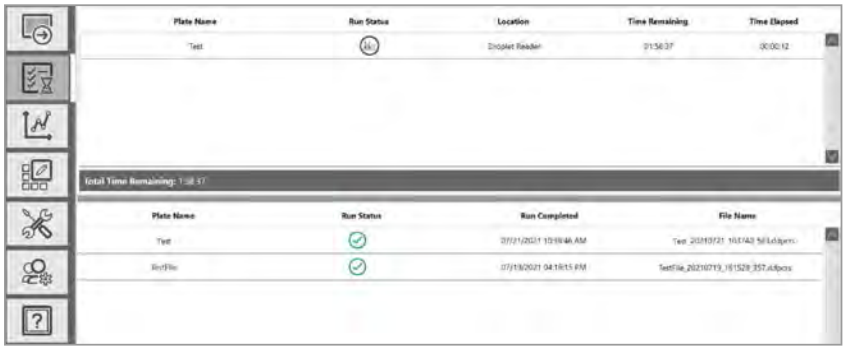
  Data files are always saved to your personal file location or a preferred location, and can also be saved to a shared location.

  Important: if your system administrator has designated preferred locations in System Settings, they override any file paths indicated in your preferences.
- During and after the run, QX Manager Software displays run information in the Run Status window.

During and after the run, the software displays run information in the Run Status window.

- During the run, you can perform real-time analysis on the droplets in each well after the well has been read.
- You can cancel the run at any time.

  Note: Canceling a run also shuts down the instrument.

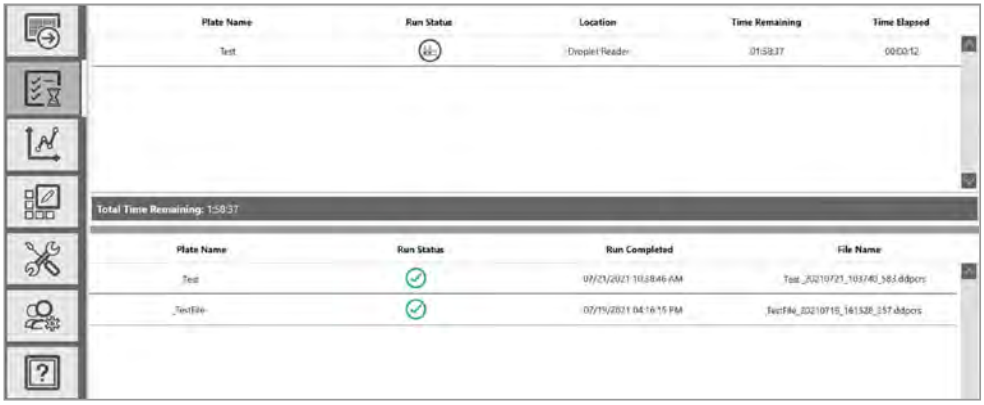


- When the run concludes, the software moves the run to the Completed list in the Run Status window, and saves the analysis data file either to your personal file location or to the shared location.

  Note: The default location is the shared folder shown in the System Settings window, but you can opt to keep your files private in your personal preferences. For information, see Managing Your Preferences on page 43.

Tracking the Run Status

You can track each run from the Run Status window.



The top pane shows the run in progress. The run remains in the top pane for the duration of the processing.

Icons displayed in the Run Status column indicate each process point, as shown in Table 15.

Table 15. Run status indicators

Status Icon	Description
	Ready to begin
	Droplet reading run
	Run completed
	Run completed, plate removed
	Run stopped or canceled by the system, or canceled by the user before it began

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When the run is finished, the software moves the run to the bottom pane, where up to 100 completed runs are visible.

Tip: You set the number of completed runs to display in your user preferences. See Managing Your Preferences on page 43.

The content of each column is explained in Table 16.

Table 16. Run Information Columns

Column Name	Location	Description
Plate name	Top and bottom panes	Name entered under Create New Plate **Note:** If you selected an existing template, the template name appears.
Run status	Top and bottom panes	Icons for each process step (see Table 15 on page 62)
Time remaining	Top pane only	Hours and minutes until the run will be finished
Time elapsed	Top pane only	Time, in hours and minutes the run has been in progress
Total time remaining	Below the top pane	Total hours and minutes remaining for all in-progress runs
Run completed	Bottom pane only	Date and time stamp showing when the instrument completed the run
File name	Bottom pane only	Plate name and system-generated identifier

After run completion, the software saves the analysis data file in a location designated by you or a system administrator.

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Using Live Analysis

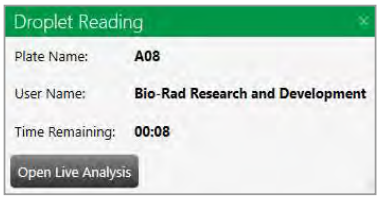
When the QX600 Droplet Reader initiates droplet reading, QX Manager Software provides an option for real-time analysis of the wells that have been read, while the process as a whole is still active.

QX Manager Software initially shows each well in the Plate Editor as disabled. As the software confirms that the instrument has acquired a well and read the droplets, the software enables the well for live analysis in read-only mode. The process continues until all wells are enabled and the process is finished.

To use live analysis

1. Tap the droplet reading icon ( ) for the run in progress.

   The Droplet Reading dialog box opens.



2. Click Open Live Analysis.
3. Select an analysis window.
4. In the well selector, select one or more wells that are enabled.

   Important: QX Manager Software displays read-only data in the selected analysis window. You can view the data but to make changes, you must wait for the run to complete, and then change the data in the Analysis module.

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Canceling a Run

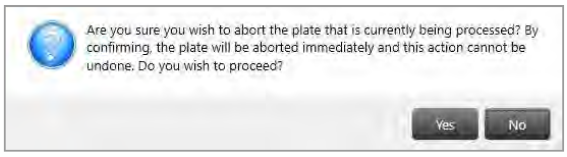
You can cancel a run if you are assigned the Manage Plate user privilege.

1. In the Run Status window, click the X on the right to cancel the run.



   You can also click the icon under Run Status before the run begins. The run cannot be canceled after droplet reading has commenced.

2. When the Are you sure message appears, click Yes.



   The instrument finishes reading the current well, stops the process and reverts to its pre-run state.

   Note: If you click No, or if you do not respond to the confirmation message within 30 sec, the process continues without interruption.

   The icon appears in the Run Status column.

   QX Manager Software creates the data file for the wells that were processed on the canceled plate and the status in the Event Log is specified as “run aborted by user.”

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Chapter 6 Creating or Editing Templates

If you have been assigned the Create New Templates user privilege, you can create, edit, and save plate and report templates for your common experiments. Templates that are available to you appear in the dropdown list in the Plate Information window, and also in the default view of the Template Setup tab. If the list is long, you can use the Search field to narrow the results.

For information on report templates, see Creating or Editing Report Templates on page 80.

All users can configure a plate template in a CSV file, and then import the template into QX Manager Software. For information, see Creating and Storing Plate Templates from CSV Files on page 83.



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Templates you created are saved to one of the following folders:

- The path specified in your user preferences; these appear under My Templates.
- The paths specified by your administrator in the shared or preferred location fields in System Settings.

  Important: If a shared location is specified in System Settings, you can save your templates there or in your personal storage location. Preferred locations override personal folder settings so that templates from all users are saved there.

Template Plate Editor Window

Use the Template Plate Editor to

- Create new or edit existing plate template files, and save new or modified templates for reuse
- Add up to four sample descriptions per well
- Exclude wells from droplet reading

  Important: When you create an experiment from a template with wells excluded in the plate layout, the instrument pierces the foil for all wells in the physical plate, but reads droplets for active wells only.

To open the template plate editor

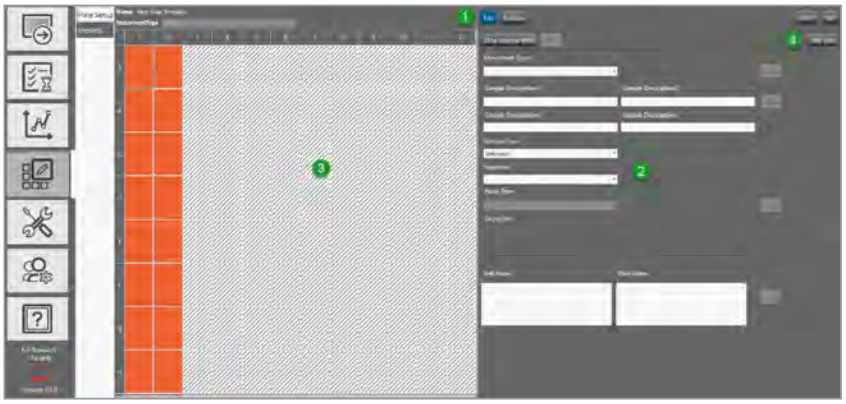
1. Click the Templates tab on the left.



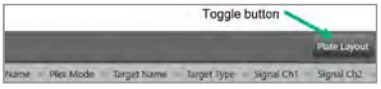
2. In the Templates window, click Edit Plate Layout



You can also open the template plate editor from the Add Plate window.



LEGEND

1	The Edit and Exclude buttons are accessible from the top of the screen.
2	The right pane contains the interface for defining your experiment parameters.
3	The left pane displays the plate grid and configuration information in each well.
4	The Well Data button toggles to the Well Data table, where you can see your plate setup in a tabular format, and then back to the Plate Editor. When the Well Data table appears, the button name changes to Plate Layout. 

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Plate Editor Tools

Use the buttons described in Table 17 when you are creating a template or defining an experiment in the Plate Template Editor.

Table 17. Plate Editor Buttons

Button	Description
Edit	Allows you to ■ Set the experiment type, sample name and type, supermix, and assay type ■ Set the target name, type, and fluorophore for each channel ■ Add well or plate notes
Exclude	Toggle button that allows you to include or exclude wells from droplet reading **Note:** Wells in the plate editor are initially excluded by default.
Cancel	Allows you to cancel and return to the Template Setup window
Save	Opens a dialog box where you can enter a file name and storage location
Clear Selected Wells	Allows you to delete information from selected wells
Undo	Allows you to reverse your last action **Note:** This button is enabled only after you have applied at least one experiment parameter (for example, the selection for Experiment Type).
Well Data or Plate Layout	Toggle button that allows you to access the Well Data table and return to Plate Layout view
Apply	Applies your entries and choices for selected wells

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Creating or Editing Plate Templates

Important: You must be assigned the Create New Template user privilege to create or edit, and then save, a template.

To create or edit a template

1. Do one of the following:
   - Click Create New under Plate Template in the Plate information window, or you can select the Templates tab.
   - Click Edit Plate Layout in the main Templates window.
   - Search for and select a template under Plate Template in the Plate Information window.
   - Select the Templates tab, and then double-click an existing template under My Templates.

     Note: Templates imported from a CSV file are available from the dropdown list only.

   The Plate Editor opens in Edit mode. The Instrument Type field is grayed out.
2. Click the Exclude button, and select the wells to include in the plate layout, and then click Include selected wells.

   By default, all wells in the Plate Editor are automatically excluded. You must select and include new wells in the template. If you typically run full plates, you can change the setting to Acquire all wells by default in your user preferences. For information, see Managing Your Preferences on page 43.
3. Click the Edit button to display the layout for experiment and sample identification.

   The fields are enabled when you include wells.
4. For one or more wells at a time, complete any or all of the following substeps to include the selections and entries that should be static. Leave blank any field that should be completed at the time of the experiment run.
   a. Select an experiment type, sample type, supermix, and assay type, and enter sample descriptions. For information, see
      - Experiment Types on page 73
      - Sample Types on page 75
      - Sample Descriptions on page 75
      - Supermixes on page 76
      - Assay Types and Fluorophores on page 77

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Note: For automatic thresholding of positive control wells, you must select the Pos Ctrl sample type.

b. If you selected Residual DNA Quantification as the experiment type, enter a conversion factor.

c. Enter up to four sample descriptions, and any relevant well or plate notes.

d. Edit the Target Name and Target Type fields.

5. Click Apply.

6. Click Save and select one of the following:

- Shared Templates. The template is saved in the folder specified in the System Settings.
- My Templates (saves the file to the path in your user preferences) or System Templates (saves the file to the preferred location specified by your system administrator).
- If your system administrator has specified preferred locations for all users, templates are automatically saved to that location.

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Experiment Types

QX Manager Software offers the experiment types described in Table 18, each enabling different assay options and analysis tools.

Tip: You can assign multiple experiment types across the plate, but you can assign only one experiment type to each well.

Table 18. Experiment types

Type	Description
Direct quantification (DQ)	DQ uses absolute quantification to determine the concentration (copies/µl) of target DNA copies in a sample. DQ measures the number of positive and negative droplets for each target in a well based on their fluorescence amplitude, and uses a Poisson algorithm to calculate the starting concentration of each target DNA molecule. **Note:** The ABS experiment type created in QuantaSoft Software versions 1.4 through 1.7 is automatically mapped into the software as a DQ experiment type.
Copy number variation (CNV)	CNV determines concentration and calculates the copy number of an unknown (CNV) target relative to a known reference or references within the same well.
Mutation detection (MUT)	MUT determines concentration and calculates the fractional abundance of an unknown mutant present at a low frequency in a wild-type background.
Rare event detection (RED)	RED determines the concentration of a known mutant or a rare target species relative to a given reference species within a large pool of background DNA.
Drop-off (DOF)	DOF determines absolute quantification of targets for assays designed to detect non-wild type sequences, such as indels and genome edits; DOF is designed to support an assay strategy where one probe counts all alleles and one “drop-off” probe sits on top of a predicted cut site or mutation site.
Gene expression (GEX)	GEX determines concentration (as with DQ) and calculates relative expression levels of an unknown target relative to a known reference or references within the same well.

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Table 18. Experiment types, continued

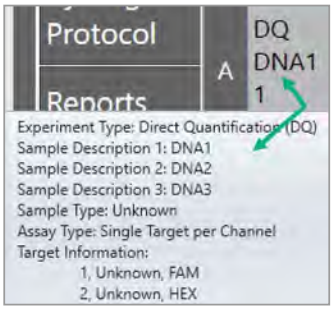
Type	Description
Residual DNA quantification (RDQ)	RDQ provides a precise method for residual host cell DNA quantification. **Note:** Users can enter a conversion factor (1/C-value) for a given genome (human, mouse, etc.) to calculate the mass for a well based on the number of copies measured, up to three decimals. The software displays the result in pg/ul of the ddPCR well in the Molecular Weight column of the Data Table. Starting sample concentration can then be back-calculated based on dilution factor.

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Sample Descriptions

QX Manager Software allows you to enter descriptive words or phrases for your sample in up to four fields per well. Descriptors can include information such as research type, dilution factors, and so forth.

Tip: Only the information from the first field appears in the Plate Editor layout. However, you can see entries in the second, third, and fourth fields in the Well Data table, or you can pause on a well to display a tool tip, as shown in the following graphic.



Sample Types

You can assign multiple sample types within the plate, but you can assign only one sample type to each well.

QX Manager Software provides four sample types for selection:

- Unknown
- NTC (no template control)
- Pos Ctrl (positive control) where one or more targets are expected
- Neg Ctrl (negative control), where no response is expected

Important: Beginning in v1.2, QX Manager Software can automatically calculate thresholds for positive control wells, but you must select Pos Ctrl from the Sample Type dropdown list. You can select Pos Ctrl during plate setup or in the Analysis module after the run is finished.

Supermixes

QX Manager Software offers five PCR supermixes. Each supermix is optimized to deliver maximum PCR efficiency and sensitivity for the amplification and detection of DNA and RNA targets.

Important: Use of unapproved supermixes can harm the instrument and void the warranty.

ddPCR Supermix	Description
Multiplex Supermix	For use in nucleic acid sample preparations to amplify and detect DNA targets in probe-based experiments involving multiple targets. **Note:** Bio-Rad recommends using ddPCR Multiplex Supermix for Cy5 and Cy5.5 probes assays.
Supermix for Probes (No dUTP)	For use in nucleic acid sample preparations, with sensitivity for the amplification and detection of DNA targets using hydrolysis probe-based assays.
Supermix for Probes	For use in sample preparation with uracil N-glucosylase (UNG) decontamination protocols to prevent the reamplification of carryover PCR products between experiments.
Supermix for Residual DNA Quantification	For use in residual DNA detection.
One-Step RT Advanced Kit for Probe	For use in absolute quantification of target RNA molecules.
EvaGreen® Supermix	For use in gene expression.

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Assay Types and Fluorophores

QX Manager Software offers the assay methods explained in Table 19.

Table 19. Assay types

Assay type	Description
Single target per channel	Method assuming up to six probe colors (FAM/EvaGreen®, HEX/VIC, Cy5, Cy5.5, ROX, and ATTO 590) and up to six targets per channel. This is the default assay type, with the maximum six rows displayed. You can delete up to three rows.
Amplitude multiplex	Method to increase multiplexing up to twelve targets per well, with one or two targets detected per channel. The maximum of eight rows are displayed. You can delete up to seven rows.
Probe mix triplex	Triplex mode that allows three groups of three targets each. Group 1 has one target in FAM, one target in HEX/VIC, and one target in both channels. Group 2 has one target in Cy5, one target in Cy5.5, and one target in both.Group 3 has 1 target in ROX, 1 target in ATTO 590, and one target in both groups. You can delete a group, but not the individual targets within the group. **Note:** The channels within each group are not interchangeable therefore FAM is always paired with HEX/VIC and so forth.
Advanced classification	Method to increase multiplexing rate in any combination of targets detected per channel. This method requires manual classification of droplets.
Basic drop-off	This mode allows two groups of two targets each.The first group appears by default, with one target in FAM and HEX/VIC and one target in FAM or HEX/VIC. This assay type is available only for the DOF experiment type. You can add one more group of two targets, with one target in Cy5 and Cy5.5, one target in Cy5 or Cy5.5. You can delete a group, but not the individual targets in the group. **Note:** The channels within each group are not interchangeable therefore FAM is always paired with HEX/VIC and Cy5 with Cy5.5.

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Table 19. Assay types, continued

Assay type	Description
Double drop-off	This assay type is available only for the DOF experiment type, and allows three groups of three targets each. The first group appears by default, and looks for one target in FAM and in HEX/VIC (target type reference) one target in FAM (target type unknown), and one target in HEX/VIC (target type unknown). You can add the second group to look for one target in Cy5 and Cy5.5 (target type reference, with two reference copies), one target in Cy5 (target type unknown), and one target in Cy5.5 (target type unknown). You can also add a third group to look for one target in ROX and ATTO 590 (target type reference), one target in ROX (target type unknown), and one target in ATTO 590 (target type unknown). By default, all reference copies are set to 2. The channels are static for each group. (Channel 1 FAM is always paired with Channel 2 HEX/VIC,Channel 3 Cy5 is always paired with Channel 4 Cy5.5, and Channel 5 ROX is always paired withChannel 6 ATTO 590.

The experiment type you select determines the available assay methods.Table 20 displays the sample type associated with each assay method.

Table 20. Assay types by experiment type

Assay type	Experiment type						
	DQ	CNV	MUT	DOF	GEX	RDQ	RED
Single target per channel	✓	✓	✓		✓	✓	✓
Amplitude multiplex	✓	✓	✓		✓	✓	✓
Probe mix triplex	✓	✓	✓		✓	✓	✓
Advanced classification	✓						✓
Basic drop-off				✓			
Double drop-off				✓			

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QX Manager Software automatically populates the fluorophore in each channel for each target but you can change the default selections.

Use Table 21 to select from the fluorophore options for each assay method listed.

Table 21. Fluorophore options

Assay method	Signal Ch1	Signal Ch2	Signal Ch3	Signal Ch4	Signal Ch5	Signal Ch6
Single target per channel, for1 to 6 targets	FAM EvaGreen® None	HEX VIC None	Cy5 None	Cy5.5 None	ROX None	ATTIO590 None
Amplitude multiplex, for 1 to 12 targets	FAM Hi FAM Lo EvaGreen® Hi EvaGreen® Lo None	HEX Hi HEX Lo VIC Hi VIC Lo None	Cy5 Hi Cy 5 Lo None	Cy5.5 Hi Cy 5.5 Lo None	ROX Hi ROX Lo None	ATTO 590 Hi ATTO 590 Lo None
Probe mix triplex, for 9 targets	FAM None	HEX VIC None	Cy5 None	Cy5.5 None	ROX None	ATTO 590 None
Advanced classification	FAM +1-10 EvaGreen® 1 to 10 None	HEX 1 to 10 VIC 1 to 10 None	Cy5 1 to 10 None	Cy5.5 1 to 10 None	ROX 1 to 10 None	ATTO 590 1 to 10 None

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Creating or Editing Report Templates

If you are assigned the Create New Templates user privilege, you can create and save a report template by editing the default report structure or by opening and editing an existing report.

To create or edit a report template

1. Select the Template Setup tab.



2. Click Reports and do one of the following:

   - To create a new template, click Add New Template. and click Edit Report Template.
   - To open an existing template, select the template and click Edit Report Template.



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3. Click the arrow to the left of each category to expand the list of reportable items.



4. Select and clear checkboxes to create the report structure.

   Note: Use the buttons at the bottom of the screen to expand or collapse all categories at once, or to select or clear all checkboxes at once. You can also drag and drop the section to rearrange the order of appearance in the report, and drag and drop items in the section to reorder them.



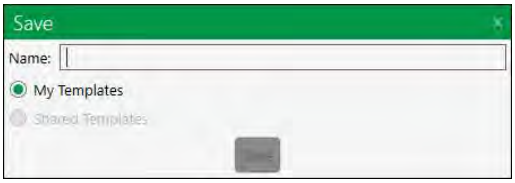


5. Enter a name for your report in the Report Title field and click Save.



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6. When the Save dialog box appears, enter a file name.



7. Select a storage location. Choose from the following:
   - System Templates (preferred location, if specified by the administrator)
   - My Templates (personal storage location specified in user preferences)
   - Shared Templates (files saved to this location are available to all users)
8. Click Save again to save the report format as a template.

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Creating and Storing Plate Templates from CSV Files

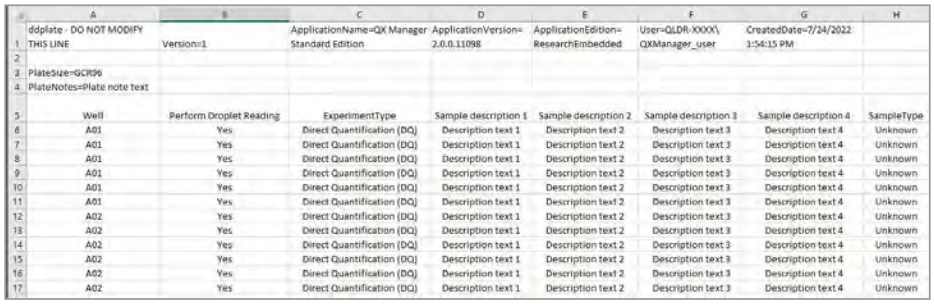
You can create a plate template in a spreadsheet application, save it in CSV format, and then store it in a personal or shared folder for use in your droplet reading runs. Note the following:

- The headings for the columns containing the experiment information match the fields in the Plate Editor.
- Once you specify the information in row 1, the field entries must remain static until you install a new software version or edition.
- CSV template files appear in the Plate Setup dropdown list only.

To set up and save a CSV template

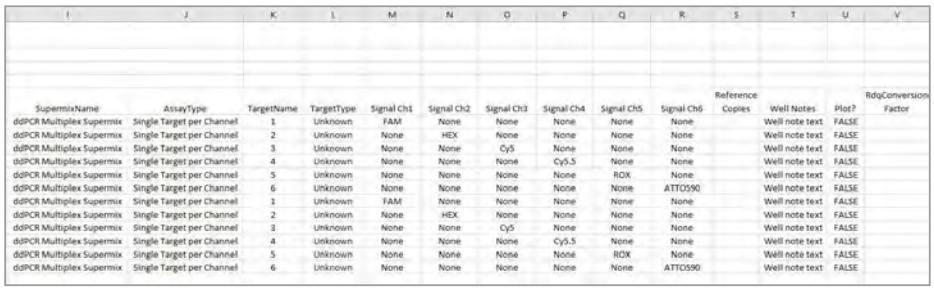
1. Open a spreadsheet program and enter the run settings into the template in the format shown below.

Fig. 1: Template CSV Layout Example, Columns A through H



	A	B	C	D	E	F	G	H
1	ddplate - DO NOT MODIFY THIS LINE	Version=1	ApplicationName=QX Manager Standard Edition	ApplicationVersion= 2.0.0.11098	ApplicationEdition= ResearchEmbedded	User=QLDR-XXXX\ QXManager_user	CreatedDate=7/24/2022 1:54:15 PM	
2								
3	PlateSize=GCR96							
4	PlateNotes=Plate note text							
5	Well	Perform Droplet Reading	ExperimentType	Sample description 1	Sample description 2	Sample description 3	Sample description 4	SampleType
6	A01	Yes	Direct Quantification (DQ)	Description text 1	Description text 2	Description text 3	Description text 4	Unknown
7	A01	Yes	Direct Quantification (DQ)	Description text 1	Description text 2	Description text 3	Description text 4	Unknown
8	A01	Yes	Direct Quantification (DQ)	Description text 1	Description text 2	Description text 3	Description text 4	Unknown
9	A01	Yes	Direct Quantification (DQ)	Description text 1	Description text 2	Description text 3	Description text 4	Unknown
10	A01	Yes	Direct Quantification (DQ)	Description text 1	Description text 2	Description text 3	Description text 4	Unknown
11	A01	Yes	Direct Quantification (DQ)	Description text 1	Description text 2	Description text 3	Description text 4	Unknown
12	A02	Yes	Direct Quantification (DQ)	Description text 1	Description text 2	Description text 3	Description text 4	Unknown
13	A02	Yes	Direct Quantification (DQ)	Description text 1	Description text 2	Description text 3	Description text 4	Unknown
14	A02	Yes	Direct Quantification (DQ)	Description text 1	Description text 2	Description text 3	Description text 4	Unknown
15	A02	Yes	Direct Quantification (DQ)	Description text 1	Description text 2	Description text 3	Description text 4	Unknown
16	A02	Yes	Direct Quantification (DQ)	Description text 1	Description text 2	Description text 3	Description text 4	Unknown
17	A02	Yes	Direct Quantification (DQ)	Description text 1	Description text 2	Description text 3	Description text 4	Unknown

Fig. 2: Template CSV Layout Example, Columns I through R



	I	J	K	L	M	N	O	P	Q	R	S	T	U	V
	SupermixName	AssayType	TargetName	TargetType	Signal Ch1	Signal Ch2	Signal Ch3	Signal Ch4	Signal Ch5	Signal Ch6	Reference Copies	Well Notes	Plot?	RdqConversion Factor
	ddPCR Multiplex Supermix	Single Target per Channel	1	Unknown	FAM	None	None	None	None	None		Well note text	FALSE	
	ddPCR Multiplex Supermix	Single Target per Channel	2	Unknown	None	HEX	None	None	None	None		Well note text	FALSE	
	ddPCR Multiplex Supermix	Single Target per Channel	3	Unknown	None	None	Cy5	None	None	None		Well note text	FALSE	
	ddPCR Multiplex Supermix	Single Target per Channel	4	Unknown	None	None	None	Cy5.5	None	None		Well note text	FALSE	
	ddPCR Multiplex Supermix	Single Target per Channel	5	Unknown	None	None	None	None	ROX	None		Well note text	FALSE	
	ddPCR Multiplex Supermix	Single Target per Channel	6	Unknown	None	None	None	None	None	ATTO590		Well note text	FALSE	
	ddPCR Multiplex Supermix	Single Target per Channel	1	Unknown	FAM	None	None	None	None	None		Well note text	FALSE	
	ddPCR Multiplex Supermix	Single Target per Channel	2	Unknown	None	HEX	None	None	None	None		Well note text	FALSE	
	ddPCR Multiplex Supermix	Single Target per Channel	3	Unknown	None	None	Cy5	None	None	None		Well note text	FALSE	
	ddPCR Multiplex Supermix	Single Target per Channel	4	Unknown	None	None	None	Cy5.5	None	None		Well note text	FALSE	
	ddPCR Multiplex Supermix	Single Target per Channel	5	Unknown	None	None	None	None	ROX	None		Well note text	FALSE	
	ddPCR Multiplex Supermix	Single Target per Channel	6	Unknown	None	None	None	None	None	ATTO590		Well note text	FALSE	

2. When the configuration is finished, navigate to one of the following locations and save the .csv file to ensure it is available for your runs:

   - The Templates folder location specified in your user preferences. For information on your personal storage locations, see Managing Your Preferences on page 43.
   - The shared or preferred Templates location determined by your system administrator. For information on shared folders, including preferred locations, see Shared Settings on page 166.

   Important: The .csv file must be in one of these locations to be available for selection from the dropdown list in the Plate Information window. CSV templates are not listed with standard templates in the Plate Templates window.

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Chapter 7 Data Analysis Module Overview

As the instrument reads droplets in each well, the QX600 Droplet Reader collects raw data. At the end of each run, QX Manager Software uses the raw data to produce a data file, which contains analysis information based on the number of positive and negative droplets identified for each target in each sample. The fraction of positive droplets is fit to a Poisson algorithm to determine the starting concentration of the target DNA molecule in units of copies/µL input.

This section summarizes the analysis functionality, and also describes the general options governing your chart displays. More detailed information, including specific information and uses for each analysis window, as well as information on data calculations, is contained in Chapter 8, Data Analysis Methodology.

When you select the Data Analysis tab, you can access the Analysis module and the Gene Study module.



When you open the data file in the Analysis module, you can view the data with the automatic analysis thresholds and calculations, or you can choose different options to recalculate, replot, or recolor the results.

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The Data Analysis module functionality allows you to

- View your droplet data in 1D, 2D, and 3D plots
- Analyze multiple targets within a well using amplitude multiplexing and probe mixing strategies
- Detect genomic editing events and non-wild type events with a “drop-off assay” analysis option
- Visualize and export data with improved flexibility
- Generate reports for different analysis types
- The Gene Study module allows you to
- Collect data by adding one or more data files
- View gene expression calculations across multiple experiments

Important: The Gene Study module is accessible from a standalone computer only. The maximum number of samples you can analyze in a gene study is limited by the size of the RAM and virtual memory of the standalone computer.

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Opening a Data File for Analysis

You can open the following file types in the Data Analysis module:

- .qlp
- .ddpcr

To open data files, you must be on a computer on which QX Manager Software is installed and have access to the storage locations.

To open a data file for analysis

1. In QX Manager Software, select the Data Analysis tab.



Available data files are displayed under the following headings.

- **Recent Datafiles**

  Displays a list of files you have recently opened.

- **System Datafiles** or **My Datafiles**

  Files are always saved to one of the following locations:

  - If preferred locations are enabled, then files for all users are saved there and they appear under System Datafiles.
  - If preferred locations are not enabled for all users, your files are saved to your personal folder and appear under My Datafiles.

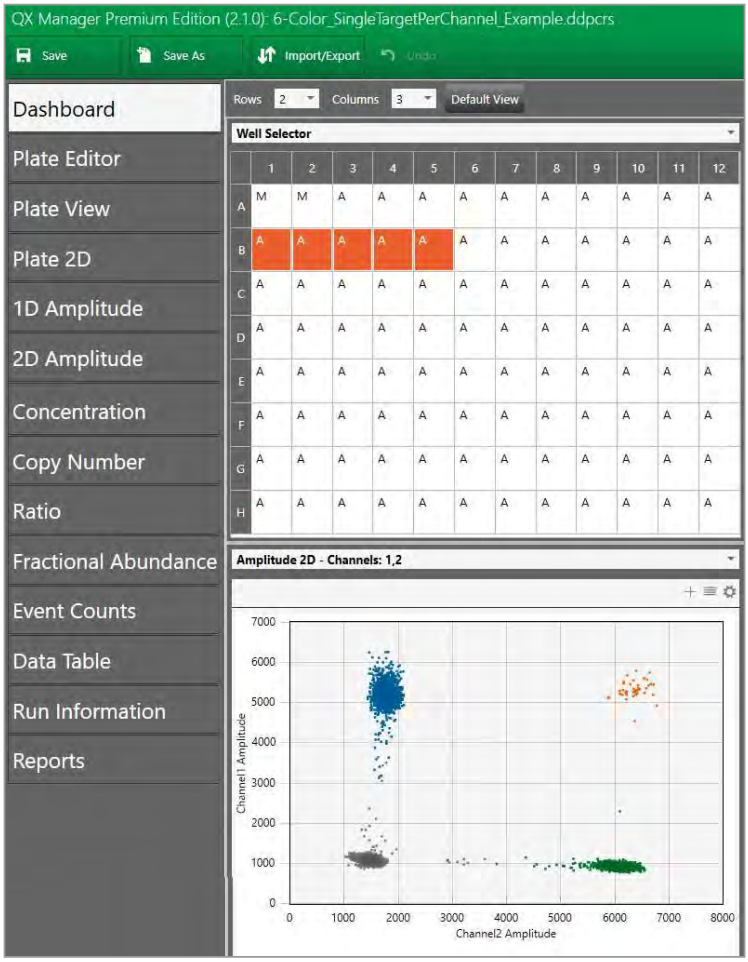
- **Shared Datafiles**

  You can also save your data files to a shared location; these appear under Shared Datafiles.

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2. Click a file to open it in the Analysis module.

   Tabs for each analysis and data window appear on the left of the Analysis window.



Tip: From the computer attached to the QX600 Droplet Reader, you can also open the data file from the Run Status window. In the completed files section, click the completed run icon ( ) for the file, and then click Launch Analysis.

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Saving a Data File

You can save data files with or without modifications, with the same or different file names, and in the same or different locations.

Important: When you save a .qlp.qlps file type in QX Manager Software, the software automatically saves the file as a .ddpcr file. This creates a new file and does not overwrite the original.

To save a data file

1. Do one of the following:
   - To save the file under the same file name and in the same location, click Save.
   - To save the file under a different file name or in a different location, click Save as, and then do one of the following:
     - If you are saving the file under the same name and in the same location, QX Manager Software saves the file immediately.
     - If you are saving the file under a different file name or in a different location, navigate to the new location (if applicable) and enter the new file name. You must be assigned the Overwrite existing file name user privilege to change the file name.
2. Click Save.

Analysis Display and Output Options

QX Manager Software provides the following option that you can choose or set:

- **Universal Options** — available from the primary toolbar to save, import, or export data files
- **Chart Display Preferences** — to change how your chart appears in the analysis window
- **Chart Scale Options** — to change the scale represented on an axis
- **Chart Menu Options** — available in chart views (from the icon) to copy, save, and print chart images. In Amplitude charts showing data points, you can show or hide
- **Table Menu Options** — available in table views (from the icon) to copy data, export data to Excel or CSV files, and show or hide columns

You can also expand the chart display to full-screen size and then return to the original display.

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Universal Options

Universal options appear on the top menu bar, and are available from all windows, although functionality within the main option might differ according to the analysis window that appears.



Universal options are explained in Table 22, and are available in all Analysis module windows.

Table 22. Universal options

Option	Description
Save	Saves changes to the analysis file under its current file name or file extension
Save as	Saves the file with a different name If you open a file in the latest application that was created in an earlier application, the file assumes the file extension of the new application. See Saving a Data File on page 89 for information.
Import/export	■ Imports or exports plate setup data to a CSV file ■ Imports or exports plate setup data to a plate template file ■ Exports visible data to a CSV file ■ Exports amplitude, cluster, and linkage data ■ Exports and prints the visible charts for the 2D plate layout
Undo	Enabled in the Plate Editor only, after you have made a change
User Settings	Allows you to restore your user preferences to the default settings and to show or hide color matrix change messages

To import or export setup or analysis data

▶ Click Import/Export.

Table 23 describes the available import/export options.

Note: If there is unsaved data, the software prompts you to save the file before its contents can be imported or exported.

Table 23. Import/export options

Import/export option	Description
Import plate setup CSV	Import plate layout data from a CSV file to the Plate Editor **Note:** This option is only available in the Plate Editor. The new plate layout overwrites the existing layout.
Export plate setup CSV	Export a configured plate layout to a CSV file
Import plate setup	Import a plate layout from a plate template (.ddplt) file **Note:** This option is only available in the Plate Editor.
Export plate setup	Export the plate layout to a plate template file **Note:** This option is only available in the Plate Editor.
Export visible data to CSV	Export only the data from the wells you selected in the Well Selector to a CSV file
Export amplitude data	Export the data to an Excel spreadsheet, and indicates whether or not the target was found (1 = yes, 0 = no)
Export cluster data	Export the data to an Excel spreadsheet **Note:** Includes (for each well) whether or not the target was found (1 = yes, 0 = no), the droplet count, and the mean and standard deviation for each dye, and the cluster ID.
Export linkage data	Exports to a CSV file the number of copies of a target that are present in overabundance, versus. the expected value in copies per µL
Export visible charts	Exports all charts that are displayed
Print visible charts	Prints all charts that are displayed

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Table 24 shows where the options are available.

Table 24. Option availability

Import/export option	Plate editor	Plate view	Plate 2D	Ampl plots	Data visual charts	Event counts	Data table
Import plate setup from CSV	✓						
Export plate setup to CSV	✓						
Import plate setup	✓						
Export plate setup	✓						
Export visible data to CSV				✓	✓	✓	✓
Export amplitude data	✓	✓	✓	✓	✓	✓	✓
Export cluster data	✓	✓	✓	✓	✓	✓	✓
Export linkage data	✓	✓	✓	✓	✓	✓	✓
Export visible charts			✓	✓	✓	✓	
Print visible charts			✓	✓	✓	✓	

Note: Amplitude plots are available in 1D, 2D and 3D, and Data Visualization charts are Concentration, Copy Number, Ratio, and Fractional Abundance. In the Dashboard view corresponding options are available for each plot or chart displayed.

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Expanding or Reducing the Chart

You can expand or reduce the chart size in the following windows:

- Dashboard
- 1D Amplitude
- 2D Amplitude
- 3D Amplitude
- Concentration
- Copy Number
- Ratio
- Fractional Abundance
- Event Counts

If you expand an amplitude or data visualization chart to full size, the Well Selector and the Well Data table are hidden from view until you collapse the chart again.

To expand or reduce the chart size

1. To increase the chart size to the full window, click the icon in the upper-right corner of the chart.
2. To reduce the window to its original size, click the icon.

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Selecting Chart Display Preferences

The Chart Display preferences primarily control font sizes and positioning. Table 25 explains each preference, as well as the windows in which it is available.

Table 25. Chart display preferences

Preference	Window	Description
Channel or Dye	Plate 2D 1D Amplitude 2D Amplitude 3D Amplitude	Select Channel or Dye to identify your chart accordingly ■ If you select Channel, the channel number appears as the chart title ■ If you select Dye, FAM, HEX, Cy5, C5.5, ROX, or ATTO 590 appears as the chart title
Axis Data Label Size	1D Amplitude 2D Amplitude 3D Amplitude Concentration Copy Number Ratio Fractional Abundance Event Counts	Select a different font size
Chart Data Label Size	Concentration Copy Number Ratio Fractional Abundance Event Counts	Select a different font size
Chart Marker Size	Concentration Copy Number Ratio Fractional Abundance	Select a different marker size
Chart Marker Label Position	Concentration Copy Number Ratio Fractional Abundance	Reposition the marker label

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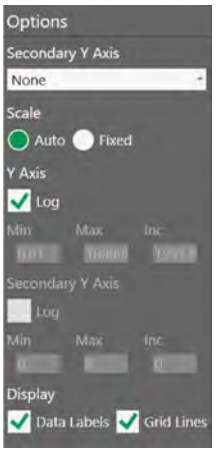
To select chart display preferences

1. Click the Chart Display button.
2. Select your options.

   The display changes immediately to reflect your choices.
3. Do one of the following:
   - To save your choices as your default settings, click Save as my default. When the success message appears, click OK to close the dialog.
   - To close the menu without saving the settings, click anywhere in the chart display.

Selecting Chart Scale and Label Options

Chart scale options primarily control chart scale and label displays.



Each preference, as well as the windows in which it is available, is explained in Table 26 on page 97.

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Table 26. Chart scale options

Option	Description
Scale	Appears in options for all charts The Scale option determines the numerical range for your chart data: Auto — Selected by default; QX Manager Software automatically displays a scale to match the data distribution. Fixed — Selecting Fixed enables the Axis fields, and allows you to manually adjust the range and increment.
Axis	Appears in options for all charts When the Axis fields are enabled, you can manually adjust the minimum (Min) and maximum (Max) points for the data display on each axis, as well as the increments (Incr). X — Horizontal axis Y — Vertical axis **Note:** You can modify the x axis scale in the 2D Amplitude chart only.
Secondary Y Axis	Appears in options for Concentration only From the dropdown list, you can select from Channel 2 concentration, ratio, copy number, or fractional abundance to display the corresponding data in a secondary row on the y axis.
Log	Appears in options for all Data Visualization charts (Concentration, Copy Number, Ratio, and Fractional Abundance) You can select the Log checkbox to display the y axis in base 10 logarithmic format. **Note:** In the Concentration chart, you can also select Log for the secondary y axis.
Inverse	Appears in options for Ratio and Fractional Abundance only The Inverse option equals the opposing number of the Ratio data point to a total of 2, and the opposing number of the Fractional Abundance data point to a total of 100.
Data labels	Appears in options for all Data Visualization charts A data label appears by default for each point in the chart, but you can clear the checkbox to remove the labels.
Grid Lines	Appears in options for all charts Gridlines appear by default. You can clear the check box to remove them.

To select chart scale and label options

1. Click the icon in the upper-left corner of the chart to display the corresponding chart options.
2. To manually define the chart scale, select Fixed.

   Note: Auto is the default setting.
3. In the Min, Max, and Inc fields, enter your lower and upper limits, and the increment, for the y axis. For 2D Amplitude, you can also change the scale on the x axis.
4. For Concentration, Copy Number, Ratio, Fractional Abundance, and Event Counts, select an option for a secondary y axis display. To change to a base 10 logarithmic scale on the y axis, select the Log checkbox.
5. For Ratio and Fractional Abundance, to view an inverse calculation, select the Inverse checkbox.
6. To remove the data labels or grid lines from the display, clear the Data Labels or Grid Lines checkbox.

Using Chart Menu Options

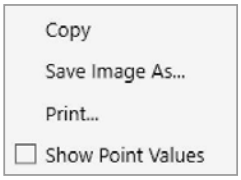


Chart menu options are explained in Table 27.

Table 27. Chart menu options

Option	Description
Copy	Copies the chart to the clipboard, which you can paste into a separate document, such as an MS Word file
Save Image as	Saves the chart as an image file
Print	Prints the image
Show Point Values	Shows corresponding point values when you pause on a data point  **Tip:** Select the Show Point Values checkbox to see the data. Data is available for 1D and 2D amplitude only.

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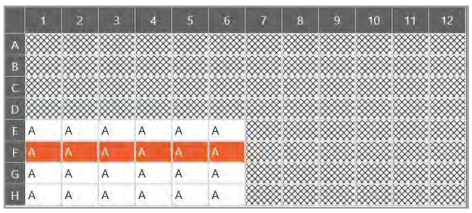
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Chart menu options are available in the following windows:

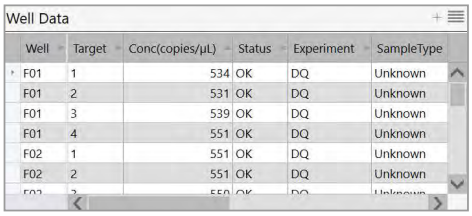
- 1D Amplitude
- 2D Amplitude
- 3D Amplitude
- Concentration
- Copy Number
- Ratio
- Fractional Abundance
- Event Counts

To use the chart menu options

1. Select one or more wells in the Well Selector.



Corresponding data appears in the Well Data table and the chart.



2. In the upper-right corner of the chart, click the menu icon ( ) and select from the dropdown list.
   - Select Copy to copy the chart to the Clipboard. To paste the data, navigate to the location (such as a Word file), right-click and select Paste.
   - Select Save Image As and follow the prompts to save the file.
   - Select Print and follow the prompts to print the image.
3. Click anywhere in the display to close the dialog box.

Using Table Menu Options

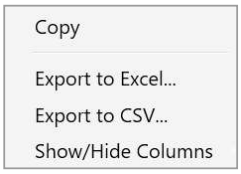


Table menu options are explained in Table 28.

Table 28. Table options

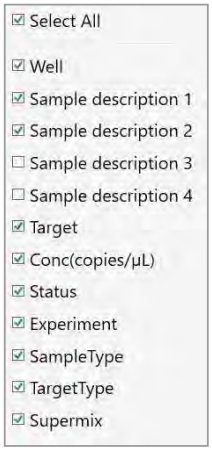
Option	Description
Copy	Copies the information from the selected wells to the clipboard
Export to Excel	Exports the data to an .xlsx file
Export to CSV	Exports the data to a .csv file
Show/Hide Columns	Add or remove columns from a table display When you select Show/Hide Columns, the following dialog box opens. Select or clear check boxes to show or hide columns in the data table. 

Table menu options are available in the following windows:

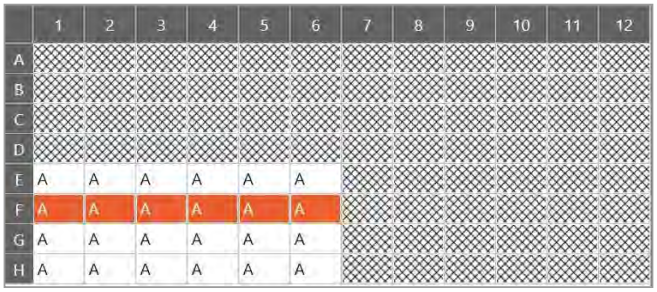
- 1D Amplitude
- 2D Amplitude
- 3D Amplitude
- Concentration
- Copy Number
- Ratio
- Fractional Abundance
- Event Counts

Tip: You can access the menu icon from the individual windows, or from the windows in the Dashboard display.

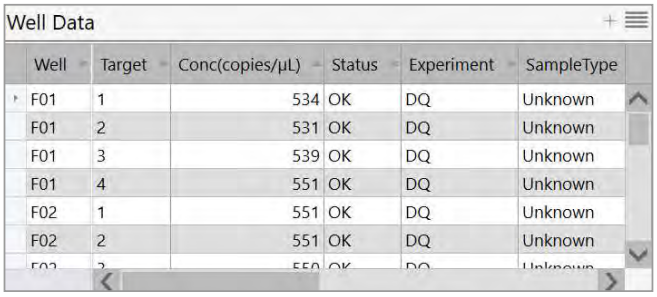
To use the table menu options

If you are using these options in the Data Table, skip to Step 3.

1. Select wells in the Well Selector.



Corresponding data appears in the Well Data table.



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2. In the upper-right corner of the table, click the menu icon ( ) and select from the dropdown list.
   - To copy the data from the selected wells to the Clipboard, select Copy.

     Note: To paste the data, navigate to the location (for example, an Excel file) and then right-click and select Paste.
   - To export your data, select Export to Excel or Export to CSV, and then follow the prompts to save the file.

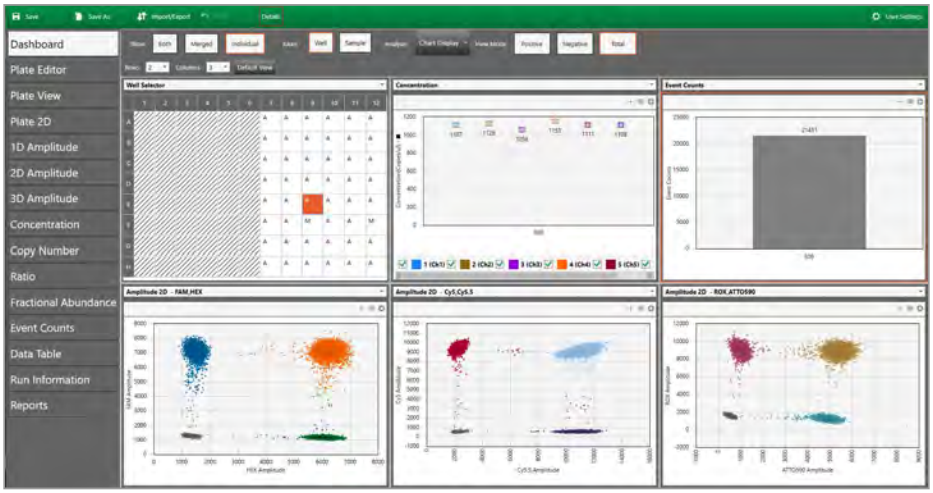
     Note: Exporting to Excel exports the data in spreadsheet format, while Exporting to CSV exports the data in comma-delimited format.
   - To show or hide the corresponding column in the table, select Show/Hide Columns, and then select or clear checkboxes.
3. Click anywhere in the display to close the dialog box.

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Analysis Dashboard

When you open a data file, the Analysis module opens to a Dashboard window containing default analysis and data panes in a summary view. You can customize the view to change panes, or increase or decrease the number of panes.



Note: If you open a .qlp file, the Plate Editor opens as the default view and you must select the Dashboard tab to open it.

From the tabs on the left, you can access plate views, amplitude charts, data visualization distribution charts, droplet counts, and a complete data table. You can also view data specific to the run in the Run Information window, and run reports on any of the analysis charts from the Reports window.

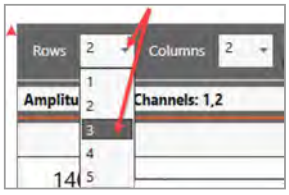
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Modifying the Dashboard Display

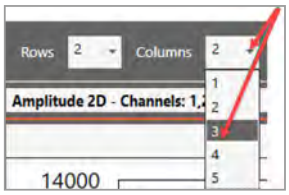
You can modify the column number and row number to display any or all of the analysis windows simultaneously.

To change the number of windows in the display

1. Click the dropdown arrow for Rows and then select a different number.



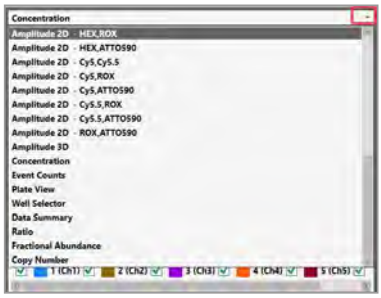
2. Click the dropdown arrow for Columns, and then select a different number.



You can also use the dropdown menus to change the windows that appear in the Dashboard.

To display a different window in a particular Dashboard slot

1. Click the dropdown arrow in the upper-right corner to display the list of available windows, and then select a window. The dropdown list closes and the window changes immediately.

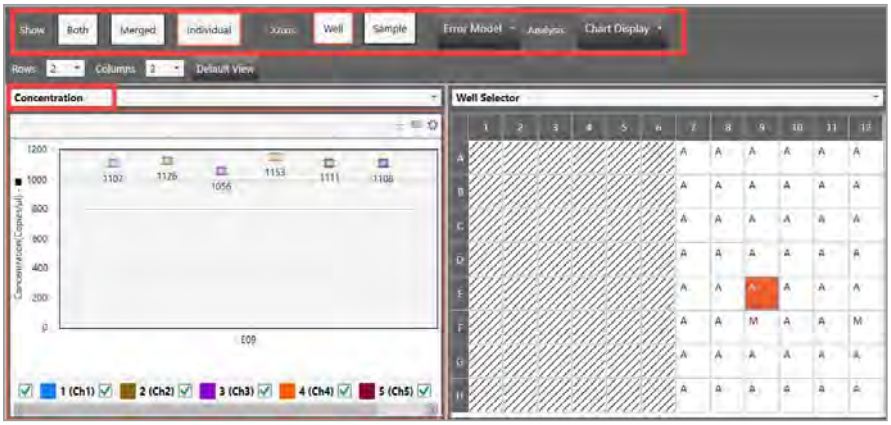


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Dashboard Options

From the Dashboard window, depending on the individual dashboard window you have selected, you can use a range of options as described below:

- When you select a window, the options that appear in the menu bar correspond to the options you see if you open the same window from the side menu. For example, if you select the Concentration chart in the Dashboard, the Concentration menu options appear.



- You can reset the entire dashboard display by adding or removing rows or columns to display more or fewer individual windows. For information, see Modifying the Dashboard Display on page 104.



  Click Default View to return to the default display of two rows and two columns.

- From any window you can
  - Expand or collapse the window
  - Copy, save, or print the image
  - Select display, scale, axis, and logarithmic options
  - Use the dropdown list to replace the current display with a different display
  - Use the menu bar options for the particular chart type

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Table 29 describes the option sets identified in the following graphic.

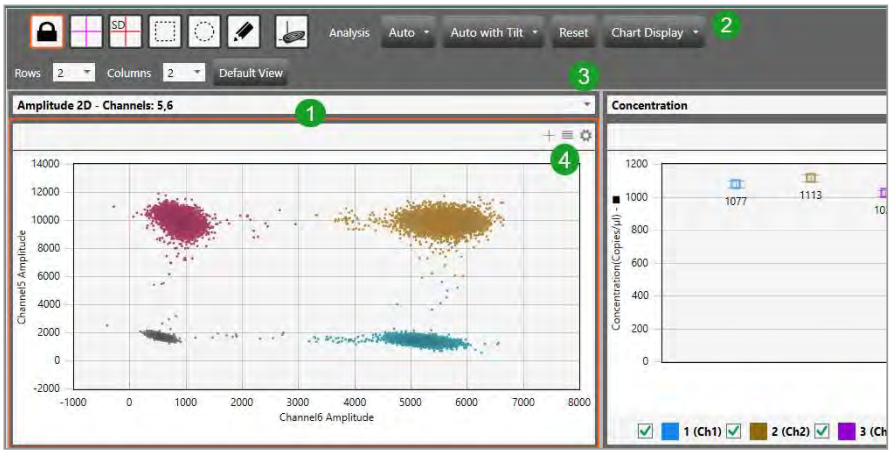


Table 29. Dashboard options

Item	Description
1	Border to identify selected window
2	Toolbar corresponding to the selected window type in the Dashboard
3	Dropdown arrow to replace the current display with a new display
4	Chart options

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Analysis Windows

In its Analysis module, QX Manager Software provides amplitude displays and data visualization charts in the Analysis module. For more information on each chart, see Chapter 8, Data Analysis Methodology.

Amplitude Charts

Amplitude charts illustrate the droplets by degree of dye fluorescence. QX Manager Software displays fluorescence for positive and negative targets identified with FAM/EvaGreen®, HEX/VIC, Cy5, Cy5.5, ROX, and ATTO 590 fluorophores in the following analysis windows:

- 1D Amplitude
- 2D Amplitude
- 3D Amplitude

Data Visualization Charts

Data visualization charts show calculations based on positive droplet concentrations. QX Manager Software counts the number of positive and negative droplets for each fluorophore in each sample, and then fits the fraction of positive droplets to a Poisson algorithm to determine the starting concentration of the target DNA molecule in units of copies/µL input in the following analysis windows:

- Concentration
- Copy Number
- Ratio
- Fractional Abundance

When you open the analysis data file in QX Manager Software and select one or more wells in the grid, the software plots each calculated Poisson distribution average as a data point in the grid. You can pause on the data point to see the high, median, and low numbers.

Note: You can display the data using a confidence level of either 68% or 95% (default setting).

Event Counts

Event counts record the number of droplets in which the target was found (positive) and was not found (negative). The Event Counts tab shows a bar chart view of the number of positive, negative, and total droplets for each of the targets in each of the selected wells.

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Plate View Windows

In the QX Manager Software Analysis module you can open the following windows that contain data on all wells in the run:

- **Plate Editor (analysis view)** — Displays the experiment type, sample names, sample types, supermixes, and assay type for each well in the run (similar to the Template Setup Plate Editor)
- **Plate View** — Displays concentration results in a text format for each processed well
- **2D Plate View** — Displays a two-dimensional amplitude chart for each processed well

Plate Editor — Analysis View

You can use the Plate Editor tab in the Analysis module to select wells, and then view or change the experiment parameters. Similar to other Plate Editor displays, the Analysis Plate Editor displays the experiment information for each well in the run.

Wells you select in the Analysis Plate Editor determine the data initially displayed in other analysis windows.

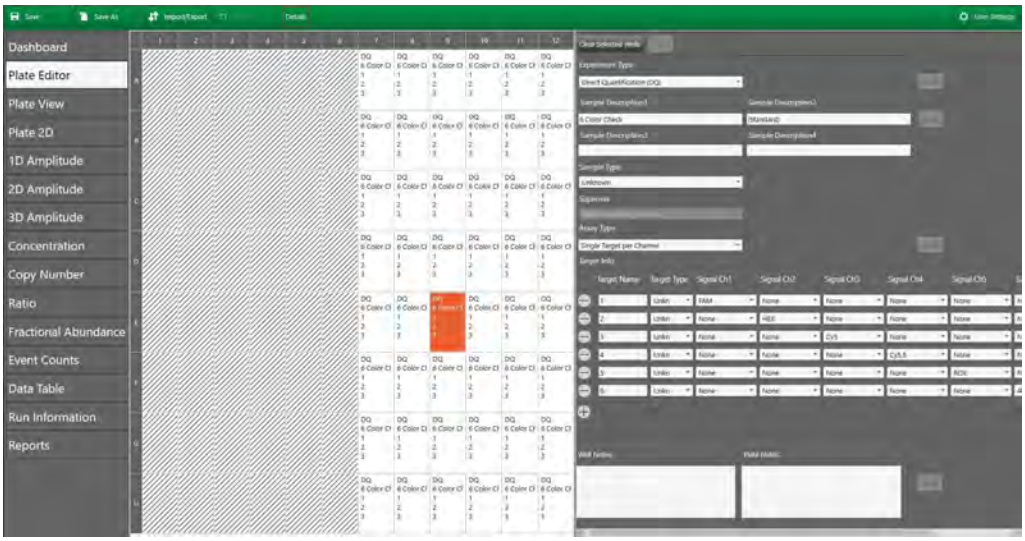
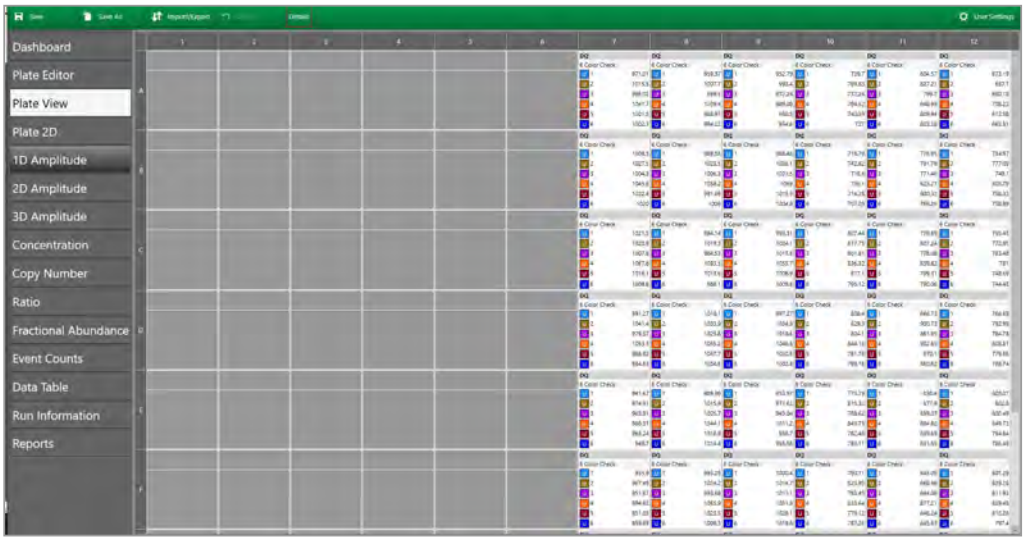


Plate View

The Plate View tab provides, within the plate layout grid, a text view of the calculated concentration for each target in each well processed in the run. For information on the concentration results in chart format, see Concentration on page 138.



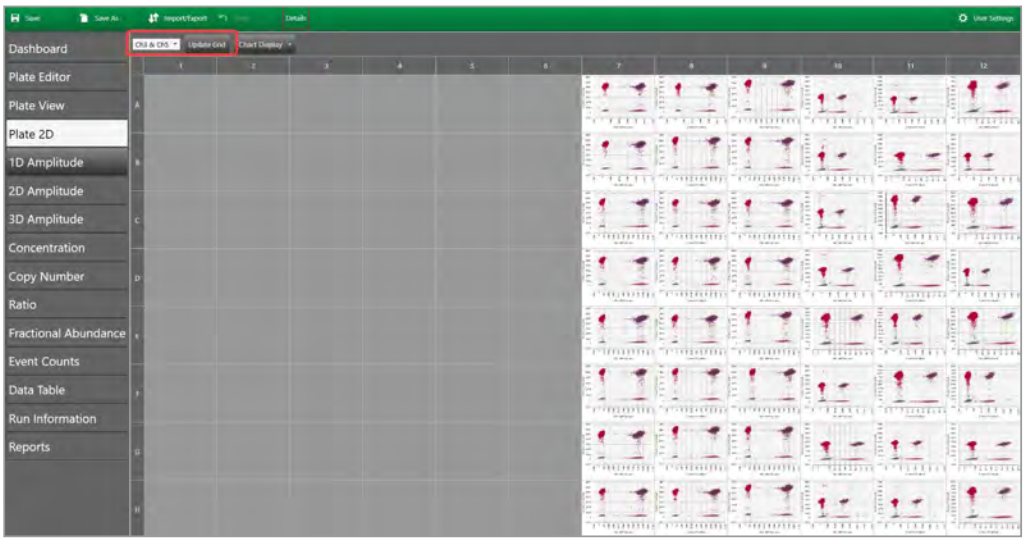
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2D Plate View

To facilitate comparisons of amplitude in the processed wells, the Plate 2D tab provides a two-dimensional amplitude view in a separate chart for each well in a single display. To see a larger representation of a well, select the well in the 2D Amplitude view.

To view different color arrays

▶ Select the channel combination from the dropdown on the left, and then click Update Grid.



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Data Windows

QX Manager Software provides the following table views for your well and run data:

- **Data Table** — available from the main tabs on the left, shows your configured experiment parameters, plus all calculations from the run

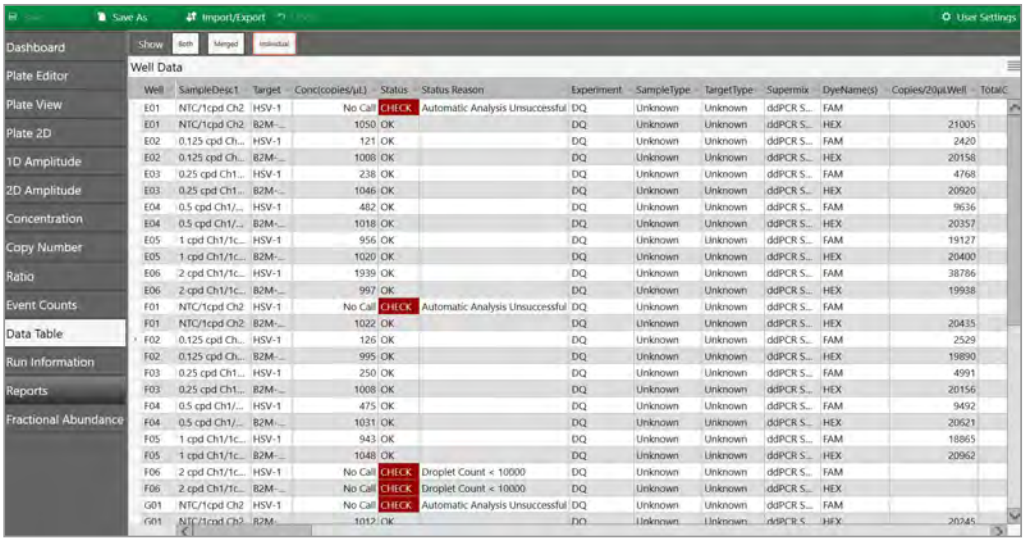
  Note: You can display the data in individual well format, merged format, or both.

- **Plate Well Data Table** — available in plate setup windows (including the Analysis module Plate Editor), shows only your configuration information for each individual well

- **Analysis Well Data Table** — available in the Analysis module, shows a subset of configuration and run data for the wells selected in the Well Selector

Data Table Tab

The Data Table window contains columns of data generated for each target in each well, as droplet clusters are identified through thresholding or manual clustering tools.



Data Table Column Definitions

Table 30 defines each field in the Data Table.

Table 30. Data Table columns

Column name	Content description
Well	The well location in the plate **Note:** The instrument accepts 96-well plates only.
Sample description 1 – 4	Words or phrases to describe the sample in each well **Note:** Only the sample description 1 data appears in plate layout views but you can pause on a well in the Plate Editor to display a tool tip containing the entries in all completed Sample description fields.
Target	Target name from Plate Editor tab
Conc(copies/µL)	Concentration of the target molecules recorded as copies per microliter
pg/µL	Mass concentration of DNA in the well in pg/µL
Status	Contains either Check, OK, Multi, or Manual ■ If Check, either the automatic analysis for the well failed or fewer than 10,000 droplets were counted in the well ■ If OK, enough droplets were counted to trigger automatic analysis ■ If Multi, the data was automatically analyzed as part of a multi-well selection ■ If Manual, the droplets were analyzed manually
Status Reason	Identifies the reason for the status (unsuccessful automatic analysis, not enough droplets counted, or droplets analyzed manually)
Experiment	Experiment type selected for the well in the Plate Editor
SampleType	Sample type selected for the well in the Plate Editor
TargetType	Target type selected for each dye (FAM or EvaGreen®, HEX or VIC, Cy5, Cy5.5, ROX, and ATTO 590) in the Plate Editor

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Table 30. Data Table columns, continued

Column name	Content description
Supermix	Supermix selected for the well in Plate Editor
DyeName(s)	Dye assigned to channels 1, 2, 3, 4, 5, and 6, respectively, for the well in the Plate Editor
Copies/20µLWell	Concentration of the target normalized to a volume of 20 µL
TotalConfMax	For merged wells the high error bar for the target concentration of the combined wells at a 95% confidence interval
TotalConfMin	For merged wells the low error bar for the target concentration of the combined wells at a 95% confidence interval
PoissonConfMax	Maximum target concentration normalized for the high error bar of the droplet Poisson distribution for the 95% confidence interval
PoissonConfMin	Minimum target concentration normalized for the low error bar of the droplet Poisson distribution for the 95% confidence interval
Accepted Droplets	Total number of droplets accepted by quality algorithm
Positives	Number of droplets that contain the target
Negatives	Number of droplets that do not contain the target
Copies/µL linked molecules	Shows number of copies of a target that are present in overabundance versus the expected value in copies per µL
CNV	Copy number calculated for the target relative to the reference
TotalCNVMax	For merged wells, the high error bar for the copy number of the combined wells at a 95% confidence interval
TotalCNVMin	For merged wells, the low error bar for the copy number of the combined wells at a 95% confidence interval
PoissonCNVMax	Maximum copy number normalized for the high error bar of the droplet Poisson distribution for the 95% confidence interval
PoissonCNVMin	Minimum copy number normalized for the low error bar of the droplet Poisson distribution for the 95% confidence interval

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Table 30. Data Table columns, continued

Column name	Content description
ReferenceCopies	Copy number identified for the reference target in the Plate Editor Default is 2, indicating 2 copies per diploid genome.
UnknownCopies	This field is currently unused in QX Manager Software
Threshold1	Threshold value of first threshold line from left to right and bottom to top
Threshold2	Threshold value of second threshold line from left to right and bottom to top
Threshold3	Threshold value of third threshold line from left to right and bottom to top
ThresholdSigmaAbove	Distance of the threshold from the mean of the negative cluster, as a multiple of the negative cluster standard deviation
ThresholdSigmaBelow	Distance of the threshold from the mean of the positive cluster, as a multiple of the positive cluster standard deviation
ReferenceUsed	Identifies which target was used as a reference
Ratio	The ratio of the target against the identified reference
TotalRatioMax	For merged wells the high error bar for the ratio of the unknown against the reference of the combined wells at a 95% confidence interval
TotalRatioMin	For merged wells the low error bar for the ratio of the unknown against the reference of the combined wells at a 95% confidence interval
PoissonRatioMax	Maximum ratio of the unknown against the reference normalized for the high error bar of the droplet Poisson distribution for the 95% confidence interval
PoissonRatioMin	Minimum ratio of the unknown against the reference normalized for the low error bar of the droplet Poisson distribution for the 95% confidence interval

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Table 30. Data Table columns, continued

Column name	Content description
FractionalAbundance	Calculation of fractional abundance of this unknown target vs. the reference target
TotalFractionalAbundanceMax	For merged wells the high error bar for the fractional abundance of the combined wells at a 95% confidence interval
TotalFractionalAbundanceMin	For merged wells the low error bar for the fractional abundance of the combined wells at a 95% confidence interval
PoissonFractionalAbundanceMax	Maximum fractional abundance normalized for the high error bar of the droplet Poisson distribution for the 95% confidence interval
PoissonFractionalAbundanceMin	Minimum fractional abundance normalized for the low error bar of the droplet Poisson distribution for the 95% confidence interval
MeanAmplitudeOfPositives	Mean amplitude value of all droplets that contain the target
MeanAmplitudeOfNegatives	Mean amplitude value of all droplets that contain no target
MeanAmplitudeTotal	Mean amplitude value of all droplets
ExperimentComments	Data populates this field only from files created in QuantaSoft versions 1.4 – 1.7, if comments were added.
MergedWells	Identifies which wells were merged together
TotalConfidenceMax68	For merged wells the high error bar for the target concentration of the combined wells at a 68% confidence interval
TotalConfidenceMin68	For merged wells the low error bar for the target concentration of the combined wells at a 68% confidence interval
PoissonConfidenceMax68	Maximum target concentration normalized for the high error bar of the droplet Poisson distribution for the 68% confidence interval
PoissonConfidenceMin68	Minimum target concentration normalized for the low error bar of the droplet Poisson distribution for the 68% confidence interval

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Table 30. Data Table columns, continued

Column name	Content description
TotalCNVMax68	For merged wells the high error bar for the copy number of the combined wells at a 68% confidence interval
TotalCNVMin68	For merged wells the low error bar for the copy number of the combined wells at a 68% confidence interval
PoissonCNVMax68	Maximum target concentration normalized for the high error bar of the droplet Poisson distribution for the 68% confidence interval
PoissonCNVMin68	Minimum target concentration normalized for the low error bar of the droplet Poisson distribution for the 68% confidence interval
TotalRatioMax68	For merged wells the high error bar for the ratio of the unknown against the reference of the combined wells at a 68% confidence interval
TotalRatioMin68	For merged wells the low error bar for the ratio of the unknown against the reference of the combined wells at a 68% confidence interval
PoissonRatioMax68	Maximum ratio of the unknown against the reference normalized for the high error bar of the droplet Poisson distribution for the 68% confidence interval
PoissonRatioMin68	Minimum ratio of the unknown against the reference normalized for the low error bar of the droplet Poisson distribution for the 68% confidence interval
TotalFractionalAbundanceMax68	For merged wells the high error bar for the fractional abundance of the combined wells at a 68% confidence interval
TotalFractionalAbundanceMin68	For merged wells the low error bar for the fractional abundance of the combined wells at a 68% confidence interval
PoissonFractionalAbundanceMax68	Maximum fractional abundance normalized for the high error bar of the droplet Poisson distribution for the 68% confidence interval
PoissonFractionalAbundanceMin68	Minimum fractional abundance normalized for the low error bar of the droplet Poisson distribution for the 68% confidence interval plate list after correcting the issue

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Table 30. Data Table columns, continued

Column name	Content description
TiltCorrected	Indicates if tilt correction was applied to the well **Note:** Tilt correction rotates the angles from the double-negative cluster centroid to each droplet, such that the two single positive cluster centroids are orthogonal with the double-negative cluster centroid.
Ch1+Ch2+	Number of droplets that contain both channel 1 and channel 2 targets
Ch1+Ch2-	Number of droplets that contain just the channel 1 target
Ch1-Ch2+	Number of droplets that contain just the channel 2 target
Ch1-Ch2-	Number of droplets that contain neither channel 1 nor channel 2 targets
Ch3+Ch4+	Number of droplets that contain both channel 3 and channel 4 targets
Ch3+Ch4-	Number of droplets that contain just the channel 3 target
Ch3-Ch4+	Number of droplets that contain just the channel 4 target
Ch3-Ch4-	Number of droplets that contain neither channel 3 nor channel 4 targets
Ch5+Ch6-	Number of droplets that contain just the channel 5 target
Ch5-Ch6+	Number of droplets that contain just the channel6 target
Ch5-Ch6-	Number of droplets that contain neither channel 5 nor channel 6 targets
Ch5+Ch6+	Number of droplets that contain both channel 5 and channel 6 targets

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Plate Editor Well Data Table

A well data table containing both configuration and run data appears in the analysis windows, next to the Well Selector. This table displays a subset of data columns for only the wells selected in the Plate Editor or Well Selector. Table 31 defines each column in the consolidated table.

Table 31. Fields in the Analysis Well Data table

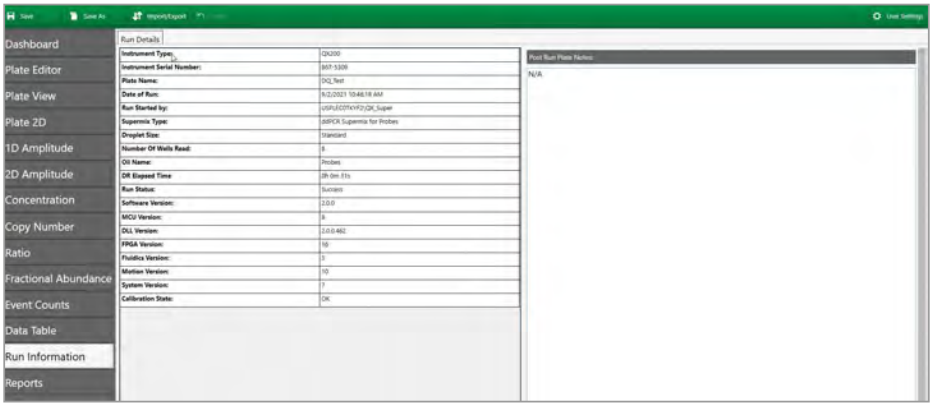
Column name	Content description
Well	The well location in the plate the sample is from
Perform Droplet Reading	Yes or No
Experiment Type	Experiment type from the Plate Editor
Sample description 1 – 4	The sample name used to identify the well in the Plate Editor tab
SampleType	Sample type from the Plate Editor
Supermix Name	Supermix from the Plate Editor
Assay type	Assay type selected in the Plate Editor
Target Name	Target Name from Plate Editor tab
TargetType	Target type assigned to the target in the Plate Editor
Signal Ch1	Selected fluorophore in Channel 1
Signal Ch2	Selected fluorophore in Channel 2
Signal Ch3	Selected fluorophore in Channel 3
Signal Ch4	Selected fluorophore in Channel 4
Signal Ch5	Selected fluorophore in Channel 5
Signal Ch6	Selected fluorophore in Channel 6
Reference Copies	Copy number identified for the reference target in the Plate Editor tab
Well Notes	Entries by the user
Plot?	If selected, target will be used to calculate the copy number for the well
RDQ Conversion Factor	The Residual DNA Quantification conversion factor for the well **Note:** RDQ is only available in Residual DNA Quantification experiments.

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Viewing Run Information

Use the Run Information window to view the information about the plate and run. You can also view or add post-run notes regarding the plate. .



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Chapter 8 Data Analysis Methodology

As summarized in Chapter 7, Data Analysis Module Overview, the software presents analysis data in several different formats. When you open the data file in the Analysis module, you can view the data with the automatic analysis thresholds and calculations or you can choose different options to recalculate, replot, or recolor the results.

Data is shown in the following formats:

- Amplitude, which shows each droplet in each well in a scatter plot, in views
- Data visualizations, which show estimated target molecule concentration, ratio, and fractional abundance of one target against another target based on selected error model and confidence interval

  Note: Copy number variation is also calculated if the user ran a CNV experiment.
- Event counts, which are precise counts of positive and negative droplets for each target in each well

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Amplitude Plot Analysis Options

QX Manager Software provides three amplitude plot displays in which you can view the data with automatic thresholding where applicable, or choose different options to replot or recolor the results.

In the 1D amplitude and 2D amplitude plots, you can

- Show well calculations

  Note: The 1D amplitude plot shows the data for each selected well in its own section, while the 2D amplitude plot shows all selected wells together.

- Modify automatic thresholds calculated by QX Manager Software
- View data in a heat map
- View histograms (1D amplitude tab only)
- Re-apply automatic analysis
- Apply tilt correction to automatic analysis

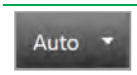
In the 3D amplitude plot, you can

- Rotate the plot to gain a better view of the droplets in 3D orientation
- View thresholds previously set in 1D or 2D amplitude display
- View 2D data behind 3D droplets in clusters or heat maps

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Table 32 explains the analysis options that are available in both 1D amplitude and 2D amplitude plots.

Table 32. 1D and 2D amplitude tool options

Icon	Mode	Description
	View mode	Data appears with the thresholds or clusters assigned by QX Manager Software. When this icon is selected, the view is locked and you cannot modify the thresholds.
	Heat map	Shows the distribution of droplets, with higher density of droplets shown as hot.
	Automatic analysis	Disables tilt correction and automatically reanalyzes the data.
	Automatic analysis with tilt correction	Enables tilt correction and automatically reanalyzes the data. **Note:** Tilt correction rotates the angles from the double-negative cluster centroid to each droplet, such that the two single positive cluster centroids are orthogonal with the double-negative cluster centroid.
	Reset	Resets the data to the original automatically analyzed data.
	Chart display options	Choose whether to display axis with channel number or fluorophore, and change the size of axis markers, data labels, and axis labels.

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Table 33 explains the analysis options available only for 1D amplitude plots.

Table 33. 1D amplitude tool options

Icon	Mode	Description
Automatic thresholding		Individual Wells: Clusters are generated for each selected well separately. Combined Wells: Droplets from all selected wells are combined, and clusters are generated on that combined set. Positive Control Wells: Thresholds are generated for each channel based on any positive controls currently selected, and applies those thresholds to all currently selected wells. **Note:** If you change the thresholds manually, you can click Reset to return to the automatic thresholds.
 	Threshold, single well Threshold, multiple wells	For a single well or multiple wells, the user can manually input thresholding values in the 1D amplitude plot. You can click anywhere in the plot to apply threshold lines to the data. **To adjust the threshold values** ■ Drag the lines horizontally in the plot area ■ Enter a new threshold value in the boxes at the end of each threshold line ■ Click the up and down arrows to incrementally adjust the numbers
 	Threshold, single well SD Threshold, multiple wells SD	For a single well or multiple wells, QX Manager Software automatically calculates and populates thresholding values with standard deviations (SD). **Important:** SD thresholds are not recommended for RMD, since these clusters are not typically located at right angles to each other. **Note:** The SD thresholds are set at a limit that is less than the mean number of positive targets, and more than the mean number of negative targets. Starting from where the user clicks in the plot, SD locates the threshold between the positive and negative clusters such that the classification error is minimized.
	Show histogram	Hides or displays histogram plots for 1D amplitude

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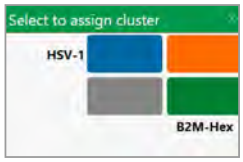
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Table 34 explains the analysis options available only for 2D amplitude plots.

Table 34. 2D amplitude tool options

Icon	Mode	Description
	Automatic thresholding	Individual Wells: Clusters are generated for each selected well separately. Combined Wells: Droplets from all selected wells are combined, and clusters are generated on that combined set. Positive Control Wells: The software generates thresholds for each channel based on any positive controls currently selected, and applies those thresholds to all currently selected wells. **Note:** If you change the thresholds manually, you can click Reset to return to the automatic thresholds.
	Threshold line	Use the Threshold line icons to establish thresholds across a pair of channels, dividing droplets into distinct clusters separated by different colors. You can click anywhere in the plot to apply threshold lines to the data. **To adjust the threshold values, you can** ■ Drag the lines horizontally or vertically in the plot area ■ Enter a new threshold value in the boxes at the end of each threshold line ■ Click the up and down arrows to incrementally adjust the numbers
	Threshold line SD	QX Manager Software automatically calculates and populates threshold line values with standard deviations (SD). **Important:** SD thresholds are not recommended for RMD, since these clusters are not typically located at right angles to each other. **Note:** The SD thresholds are set at a limit that is less than the mean number of positive targets, and more than the mean number of negative targets. Starting from where the user clicks in the plot, SD locates the threshold between the positive and negative clusters such that the classification error is minimized.

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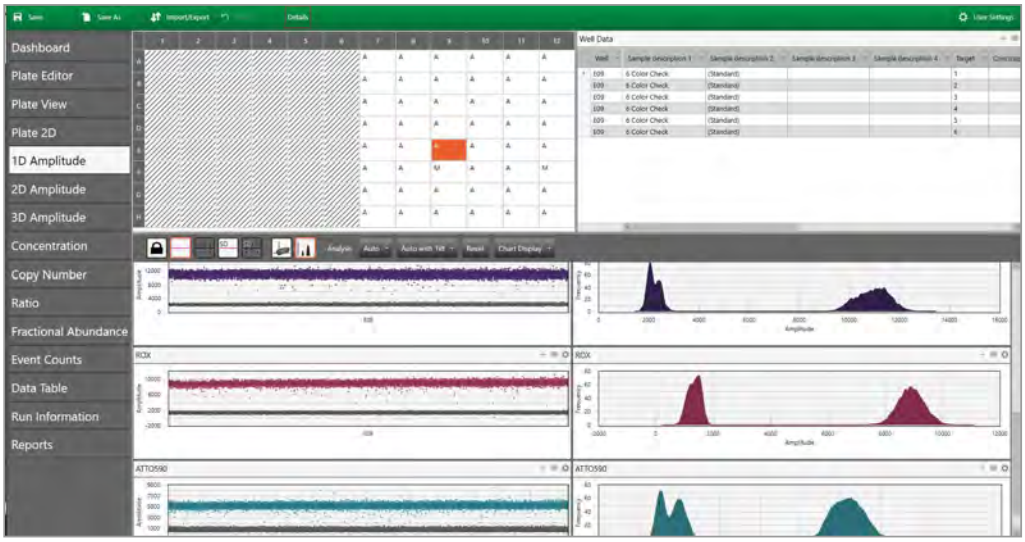
Icon	Mode	Description
	Threshold cluster square	Use the Threshold Cluster icons to establish thresholds across a pair of channels, dividing droplets into distinct clusters separated by different colors. To classify your droplets, you can ■ Click any of the three Threshold Cluster Mode buttons (square, circle, or free form) ■ Draw the chosen shape around the cluster of droplets **Note:** When using the cluster mode tools to draw a shape around a cluster of droplets, a dialog box with color selections by target appears in the window to help you select the correct target combination.  Target names are shown along the two axes based on the signal values assigned during plate setup. Click the colored box that best represents the location of the cluster of droplets selected to automatically classify the droplets as positive or negative for each given target. **Note:** The Cluster dialog box display varies according to the plex mode.
	Threshold cluster circle	
	Threshold cluster freeform	

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1D Amplitude

Use the 1D Amplitude analysis window to view droplet amplitudes for each channel, in selection order, for each well selected in the Well Selector.

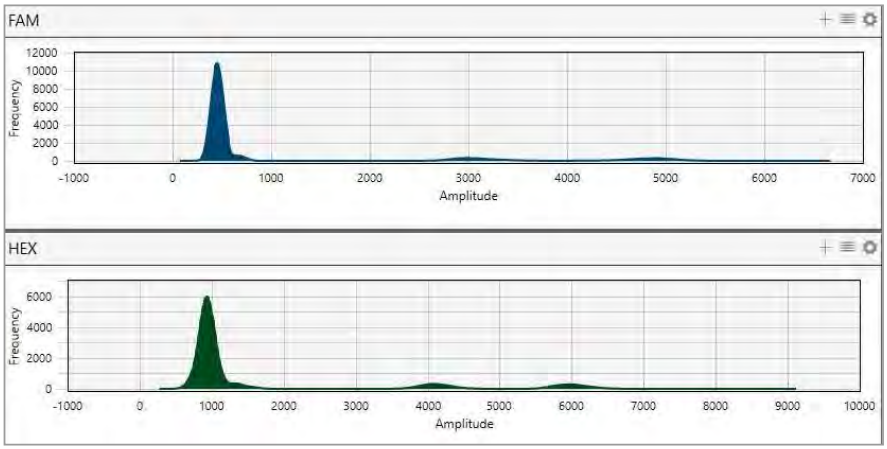
The following graphic shows six colors in standard-size droplets.



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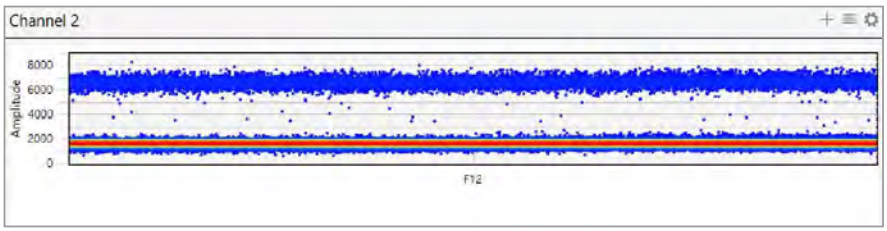
The 1D Amplitude plots illustrate

- All accepted droplets, with a visual representation of the positive and negative droplets in each well
  - Positive droplets appear in color, while negative droplets appear in gray.
  - The Well Selector displays an A in each well where QX Manager Software automatically thresholded the droplet data. An M appears in any well in which you manually changed the threshold.
- Amplitude in a histogram plot



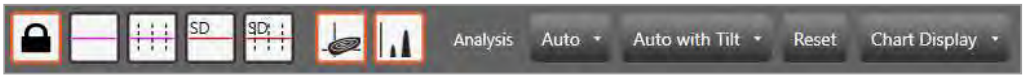
Note: The Histogram icon is enabled by default so a Histogram plot appears automatically for each fluorophore (channel).

- Amplitude in a Heat Map view



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When you select the 1D Amplitude tab, the toolbar options that allow you to change thresholds and show or hide the heat map and histogram appear above the plots.



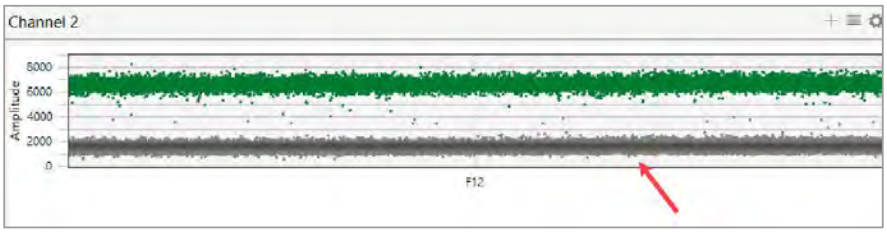
Note: For detailed information on each toolbar option, see Amplitude Plot Analysis Options on page 122.

Default color assignments for 1D amplitude plots are

Positive FAM	=	Blue
Positive HEX	=	Green
Positive Cy5	=	Red
Positive Cy5.5	=	Violet
Positive ROX	=	Maroon
Positive ATTO 590	=	Aqua
Negative	=	Gray

The images in this section display data from a direct quantification experiment involving six targets:

Negative droplets appear in grayscale in each plot.

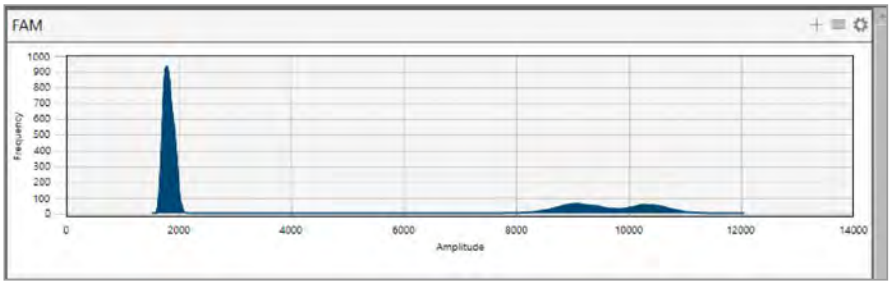


For experiments where enough droplets are recognized, QX Manager Software automatically configures an amplitude threshold, which distinguishes between positive and negative droplets.

Note: Well data must meet certain quality metrics (minimum of 10,000 droplets) before the software will automatically calculate a threshold above which droplets are considered positive. You can use the manual thresholding tools to ensure correct quantification of positive and negative droplets.

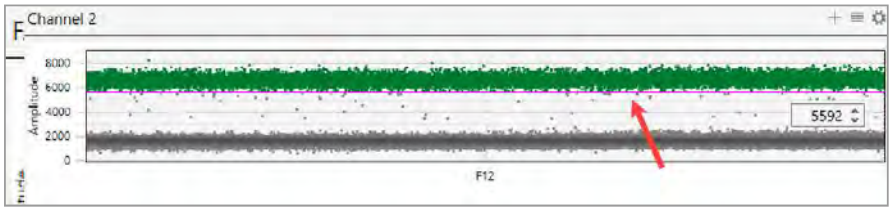
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By default, the Histogram icon ( ) is enabled so the Histogram plot automatically appears with the amplitude plot.



To manually adjust the thresholds

1. Select one or more wells.
2. Click any of the threshold icons ( ) to set manual thresholds:
   - Regular threshold for a single well; when multiple wells are selected, you can set a different threshold for each well
   - Regular threshold for multiple wells, where you can set one threshold for all selected wells
   - Standard deviation threshold for a single well; when multiple wells are selected, you can set a different threshold for each well
   - Standard deviation for multiple wells, where you can set one threshold for all selected wells
3. Click on the location in the plot where you want to draw the new threshold.
4. When the line appears, use the up or down arrows, or click and drag the threshold, to adjust the location.



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QX Manager Software displays the manual threshold and changes the color of the droplets that are now above or below the threshold.

Note: You can also manually enter a threshold number.

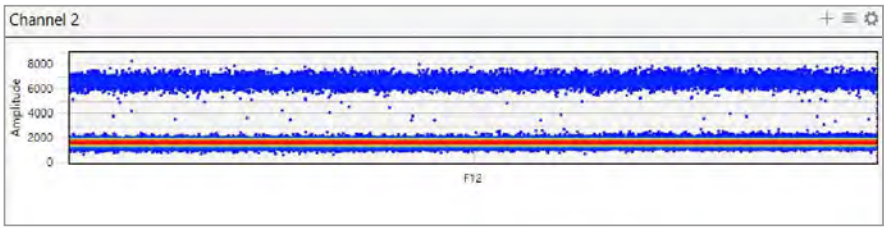
When you manually modify a threshold, the well selector displays an M for manual instead of the A for Automatic.



5. (Optional) To reset to the automatic threshold, click Auto.
6. (Optional) To adjust the automatic threshold to consider tilt correction, click Auto with Tilt.
7. (Optional) To reset everything to the original display, click Reset.

To view a heat map representation of the plot data

▶ Click the heat map ( ) icon.

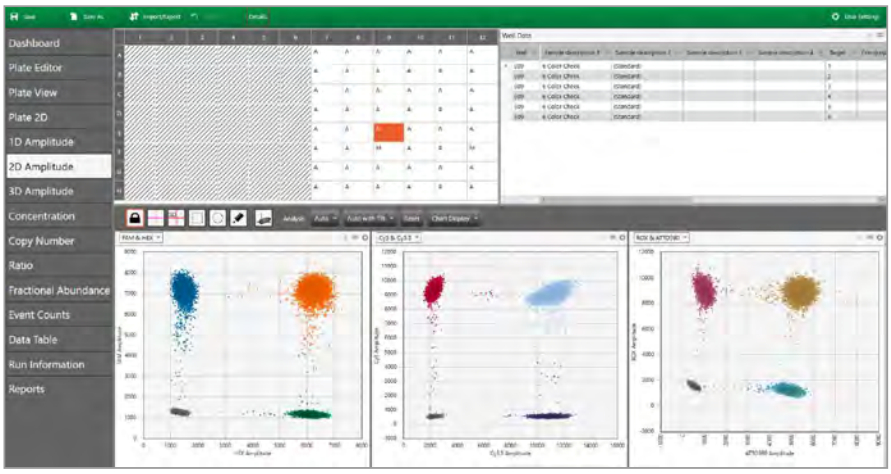


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2D Amplitude

For the wells you select in the analysis view of the Plate Editor, the 2D Amplitude window illustrates fluorescence for six targets. The images in this section display data from an experiment involving six targets. Each plot shows individual cluster representations for first and second target positive droplets, droplets containing both targets, and droplets containing neither target.

The following graphic shows six colors in standard-size droplets.



When you select the 2D Amplitude tab, the toolbar options allow you to change thresholds, manually cluster wells, and show or hide the heat map above the plots.



For detailed information on each toolbar option, see Amplitude Plot Analysis Options on page 122.

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Default color assignments for 2D amplitude plots are:

Positive FAM	=	Blue
Positive HEX/VIC	=	Green
Positive FAM and HEX/VIC	=	Orange (may appear brown on some monitors)
Positive Cy5	=	Red
Positive Cy5.5	=	Violet
Postive Cy5 and Cy5.5	=	Light Blue
Positive ROX	=	Maroon
Positive ATTO 590	=	Gold
Positive ROX and ATTO 590	=	Aqua
Double negative	=	Gray

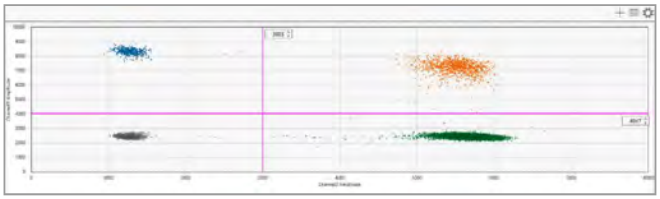
Note: Well data must meet certain quality metrics (minimum of 10,000 droplets) before QX Manager Software will automatically calculate a threshold above which droplets are considered positive. You can use the manual thresholding tools to ensure correct quantification of positive and negative droplets.

To use the 2D amplitude tools

1. Open your data file in the Analysis module and select the 2D Amplitude tab.
2. Choose a threshold line or cluster option.

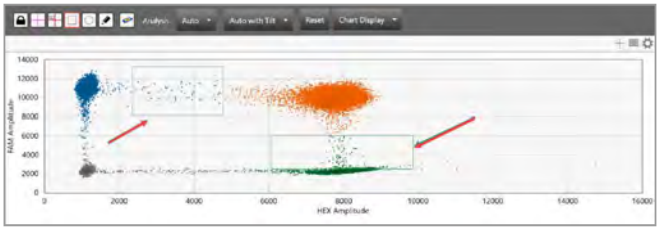
   Note: Use the cluster option when the data is not orthogonal and the threshold lines do not adequately divide the populations of droplets.

   - To adjust threshold lines, select a threshold line type () and then click in the plot to apply crosshair lines. Use any of the following methods:
     - Drag the lines horizontally or vertically in the plot area.
     - Enter a new threshold value in the boxes at the end of each threshold line.
     - Tap the up and down arrows to incrementally adjust the numbers.



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- To adjust thresholds using the cluster option, select a cluster type ( ) and draw the chosen shape around the cluster of droplets to classify.



3. (Optional) To reset to the automatic threshold, click Auto.
4. (Optional) To adjust the automatic threshold to consider tilt correction, click Auto with Tilt.
5. (Optional) To reset everything to the original display,click Reset.

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Data Visualization Chart Analysis Options

QX Manager Software also provides chart displays where you can view the data from the original calculations, or choose different options to recalculate the results.

In charts reflecting Data Visualization distributions, you can

- Show individual or merged well data
- Change the error model and confidence level

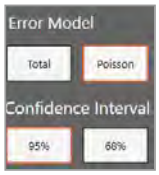
Note: For event counts, you can also alter the view mode to see positive, negative, or all droplet counts.

Table 35 explains the available options.

Table 35. Chart tool options for Data Visualization distribution charts

Icon	Mode	Description
	Show both	Shows the calculated total or percentage as a merged calculation for all selected wells and as an individual calculation for each selected well
	Show merged	Shows the calculated total or percentage as a combined number for all selected wells with the same parameters **Note:** Well setup must match for each well before the software can merge them.
	Show individual	Shows the calculated total or percentage as an individual calculation for each selected well
	X-axis well	Shows the well number on the x axis

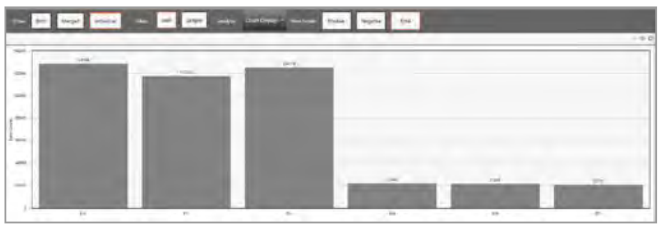
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Icon	Mode	Description
	X-axis sample	Shows a partial sample name on the x axis **Note:** You can select Well and Sample simultaneously.
	Error model and confidence interval	Select an error model and confidence level **Note:** The default choices are Poisson and 95%, to yield the highest confidence in the results.  **Important:** The software uses the following multipliers in its calculations: ■ For 95% confidence interval, the sigma multiplier is 1.96 ■ For 68% confidence interval, the sigma multiplier is 1.0

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Table 36 identifies the additional options available in the Event Count window. These options are available for event counts only.

Table 36. Additional options for event counts

Icon	Mode	Description
Positive	View mode positive	Displays in a bar chart the number of positive droplets for each target in each well 
Negative	View mode negative	Displays in a bar chart the number of negative droplets for each target in each well 
Total	View mode total	Displays in a bar chart the number of total droplets in each well 

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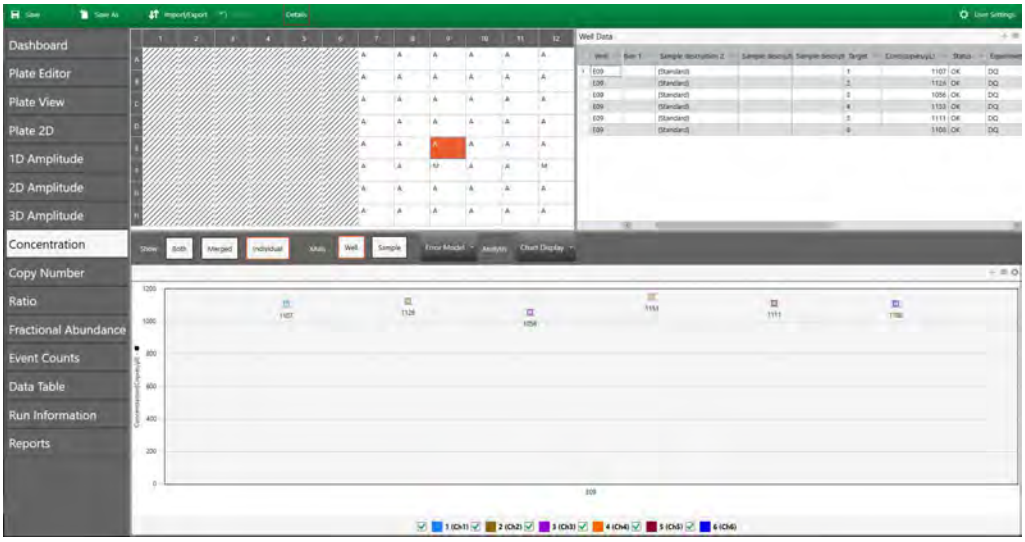
Concentration

Concentration measures the number of copies of the target molecules in each µl of sample based on the default settings. Use the toolbar to merge or separate the calculated results for each well, and to recalculate your results based on a different error model and confidence interval.

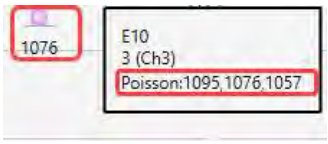
For information on toolbar options, see Data Visualization Chart Analysis Options on page 135.



The following graphic shows the concentration for six colors in standard-size droplets.



QX Manager Software plots the averaged concentration for each target as a data point. Pause on a data point to see the high, median, and low numbers.



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Concentration Calculation

The software uses the formula and variables shown below.

$$c = -\ln\left(\frac{N_{neg}}{N}\right) / V_{droplet}$$

Where:

-ln	Negative natural logarithm
$\mathbf{V_{droplet}}$	Volume of droplet
$\mathbf{N_{neg}}$	Number of negative droplets
N	Total number of droplets

Confidence Interval Calculation

Note: If you select a 68% confidence interval, the multiplier is changed from 1.96 to 1.00.

$$p = \frac{N_{neg}}{N}$$

$$\partial_{N_{neg}} = \sqrt{Np(1-p)}$$

$$N_{neg,\min} = N_{neg} - 1.96 * \partial_{N_{neg}}$$

$$N_{neg,\max} = N_{neg} + 1.96 * \partial_{N_{neg}}$$

Where:

$\mathbf{V_{droplet}}$	Volume of droplet
$\mathbf{N_{neg}}$	Number of negative droplets
N	Total number of droplets
p	p-value (probability of finding the target in the sample)
∂	Delta

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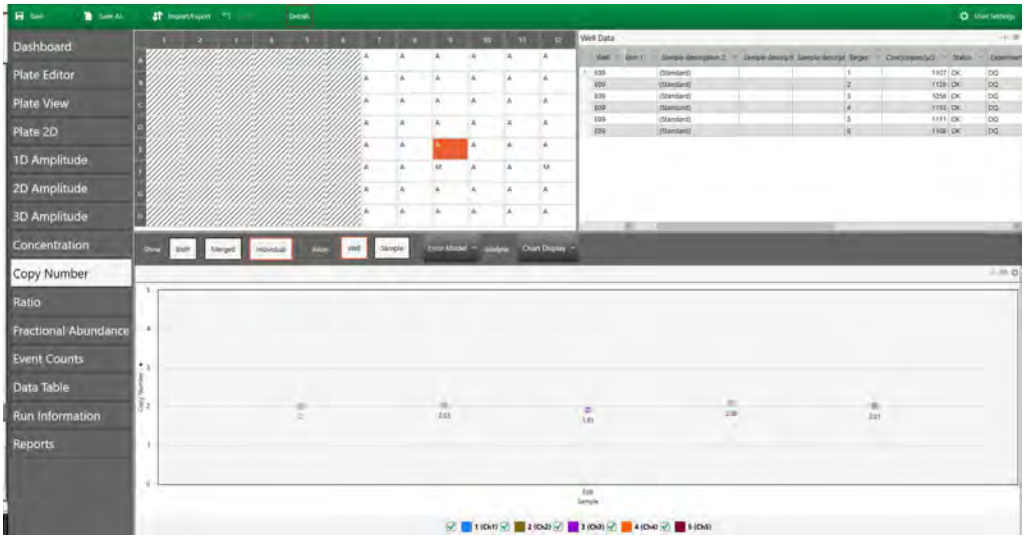
Copy Number

Important: To see copy number variation data, you must run a copy number variation (CNV) ddPCR experiment.

Copy number measures the count of the target gene, relative to the reference gene, to calculate the number gene targets per genome. Use the toolbar to merge or separate the calculated results for each well, and to recalculate your results based on a different error model and confidence interval. For information on toolbar options, see Data Visualization Chart Analysis Options on page 135.



The following graphic shows copy number data for six colors in standard-size droplets.



QX Manager Software plots each copy number variation as a data point. Pause on a data point to see the high, median, and low numbers.

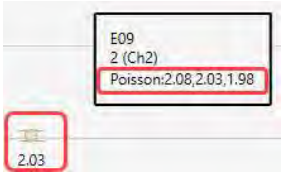


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Copy Number Calculation

The software uses the formula and variables shown below.

$$CNV = \frac{A}{B} N_B$$

Where:

A	Target concentration
B	Reference concentration
N_B	Number of reference copies in genome (usually 2)

Confidence Interval Calculations

Note: If you specify the lesser confidence interval when you define your error model, the software uses 68% in its calculations instead of 95%.

$$CI_{CNV} = N_B \cdot \frac{A}{B} \sqrt{\frac{CI_A^2}{A^2} + \frac{CI_B^2}{B^2}}$$

.

$$(CNV_{low\_95\%}, CNV_{hi\_95\%}) = (CNV - (1/2)CI_{CNV}, CNV + (1/2)CI_{CNV})$$

Where:

A	Target concentration
B	Reference concentration
N_B	Number of reference copies in genome (usually 2)
CI_A	Poisson confidence interval of target (95% max - 95% min)
CI_B	Poisson confidence interval of reference (95% max - 95% min)

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Notes:

Copy Number analysis involves determining the number of copies of a given target DNA sequence as compared to an invariant reference DNA sequence. Use the CNV analysis to determine structural variations, such as ploidy changes (addition or deletion of chromosomes), deletions (missing partial chromosomes) or duplications (repeated target sequences) in the genome.

The ability to discriminate between consecutive CN states has been a technical challenge in copy number assessment. Fundamentally, as CN state increases, the percentage difference in target genomic material between states decreases. For example, for a given target locus, a CN of 3 is 50% more abundant in concentration per genome than a CN of 2, while a CN of 5 is only 25% more abundant per genome than a CN of 4, and so on.

Due to the massive partitioning of the reaction across up to 20,000 droplets per well, and the absolute nature and precision of the ddPCR concentration measurement, ddPCR CNV analysis enables the quantitative discrimination required to resolve consecutive copy number states beyond CN 3. For example, a 20% difference in target concentration between a CN 5 versus CN 6 sample can be reproducibly resolved using ddPCR. This discrimination is both a function of concentration differences between consecutive CN states, as well as the amount of DNA assayed per well.

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Ratio

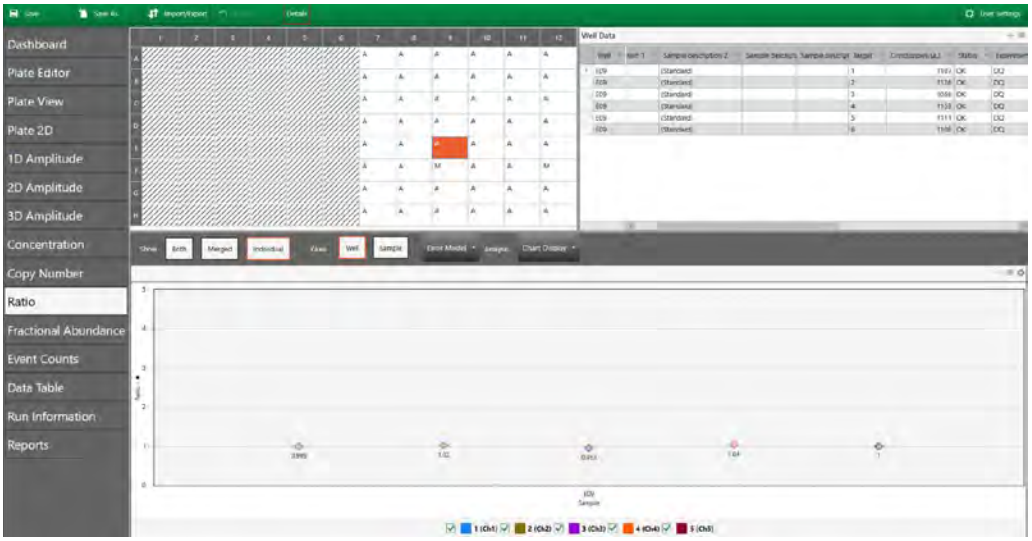
Ratio determines the quotient of two target molecules in each µl of sample based on the default settings.

T1:T2

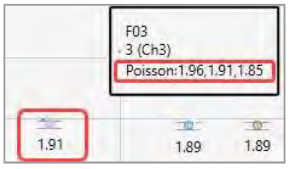
Use the toolbar to merge or separate the calculated results for each well, and to recalculate your results based on a different error model and confidence interval. For information on toolbar options, see Data Visualization Chart Analysis Options on page 135.



The following graphic shows the ratio for standard-size droplets.



QX Manager Software plots each ratio as a data point. To see the confidence level numbers, pause on the data point.



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Ratio Calculation

The software uses the formula and variables shown below.

$$r = \frac{A}{B}$$

Where:

A	Target concentration
B	Reference concentration

Note: If no reference is selected, the target with the highest dye concentration in the highest channel will automatically be considered as the reference. If multiple references are selected, the geometric mean is used.

Confidence Interval Calculations

Note: f you specify the lesser confidence interval when you define your error model, the software uses 68% in its calculations instead of 95%.

$$CI_r = \frac{A}{B}\sqrt{\frac{CI_A^2}{A^2} + \frac{CI_B^2}{B^2}}$$

$$(r_{low\_95\%}, r_{hi\_95\%}) = (r - (1/2)CI_r, r + (1/2)CI_r)$$

Where:

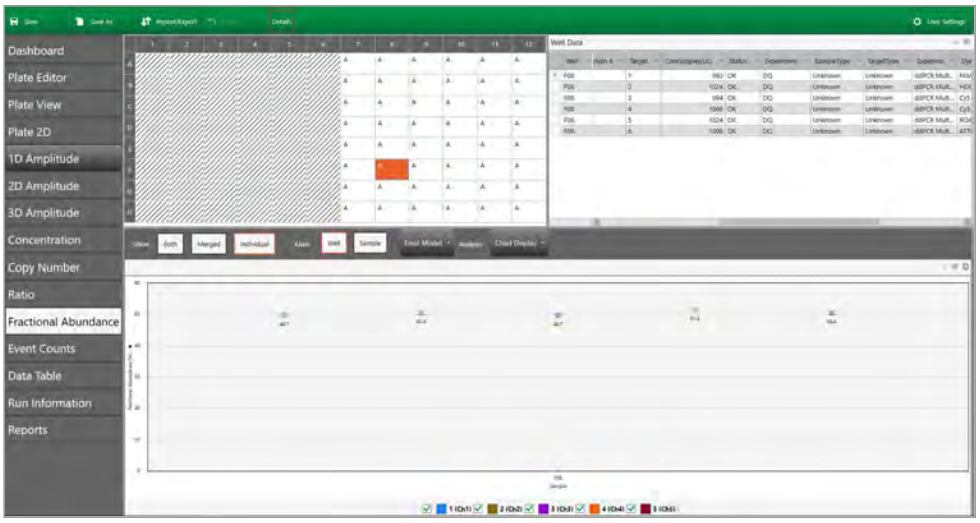
A	Channel 1 target concentration
B	Channel 2 target concentration
CI_A	Confidence interval of Channel 1 target (95% max - 95% min)
CI_B	Confidence interval of Channel 2 target (95% max - 95% min)

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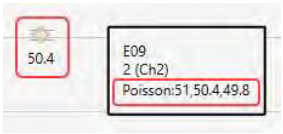
Fractional Abundance

Fractional Abundance determines the relative abundance of your targets in each µl of sample based on the default settings. Use the toolbar to merge or separate the calculated results for each well, and to recalculate your results based on a different error model and confidence interval. For information on toolbar options, see Data Visualization Chart Analysis Options on page 135.





QX Manager Software plots the fractional abundance as a data point. To see the high and low confidence interval numbers, pause on the data point.



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Fractional Abundance Calculation

The software uses the formula and variables shown below.

$$f = \frac{A}{A+B}$$

Where:

A	Target concentration
B	Reference concentration

Note: If no reference is selected, the target with the highest dye concentration in the highest channel will automatically be considered as the reference. If multiple references are selected, the geometric mean is used.

Confidence Interval Calculations

Note: If you specify the lesser confidence interval when you define your error model, the software uses 68% in its calculations instead of 95%.

$$CI_f = \frac{1}{(A+B)^2}\sqrt{B^2CI_A^2 + A^2CI_B^2}$$

$$(f_{low\_95\%}, f_{hi\_95\%}) = (f - (1/2)CI_f, f + (1/2)CI_f)$$

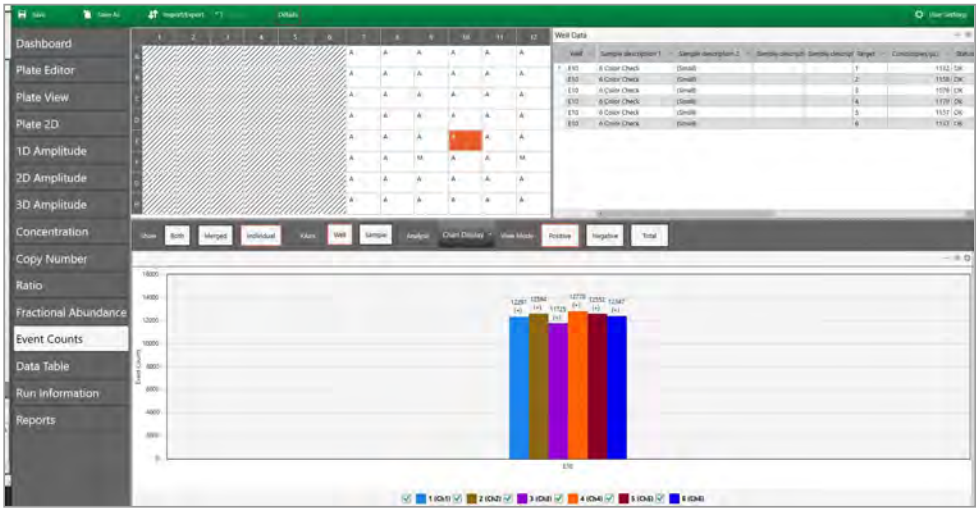
Where:

A	Channel 1 target concentration
B	Channel 2 target concentration
CI_A	Confidence interval of Channel 1 target (95% max - 95% min)
CI_B	Confidence interval of Channel 2 target (95% max - 95% min)

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Event Counts

The Event Counts tab shows a bar chart view of the number of positive and negative droplets for each of the targets in each of the selected wells.



Use the toolbar to change the display by combining or separating data for each well. You can also show data by well name or by sample name, and you can show any combination of positive, negative, or combined droplet counts.



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Linkage Analysis

In ddPCR, physical linkage of two targets can occur in one of the following patterns:

- Tandem repeats if the same target sequence
- Proximity of two target sequences physically linked on the same piece of DNA

QX Manager Software uses the formulas and variables shown below.

- The following formula presents the linkage in copies per µL:

$$linkage = \ln(N) - \ln\left(n_{00} + n_{01} + n_{10} + \frac{n_{01}n_{10}}{n_{00}}\right)\left(\frac{1000}{droplet\ volume}\right)$$

- The following formula presents the linkage in copies per droplet.

$$linkage = \ln(N) - \ln\left(n_{00} + n_{01} + n_{10} + \frac{n_{01}n_{10}}{n_{00}}\right)$$

Where:

N	Total number of droplets
$\mathbf{n_{00}}$	Number of negative droplets for both channels
$\mathbf{n_{01}}$	Number of negative droplets in channel 1 and positive droplets in channel 2
$\mathbf{n_{10}}$	Number of positive droplets in channel 1 and negative droplets in channel 2
p	p-value (calculated probability)
∂	Delta

Where:

Confidence Interval Calculation

$$CI_{linkage} = (linkage - (1.96)\sigma_{linkage}, linkage + (1.96)\sigma_{linkage})$$

Where:

σ	Standard deviation

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Chapter 9 Analysis Module Reporting

Use the Reporting function to create a variety of reports in the QX Manager Software Analysis module.

You can create reports on the fly from any analysis file or, if you need to generate specific reports on a regular basis, you can set up report templates for reuse.

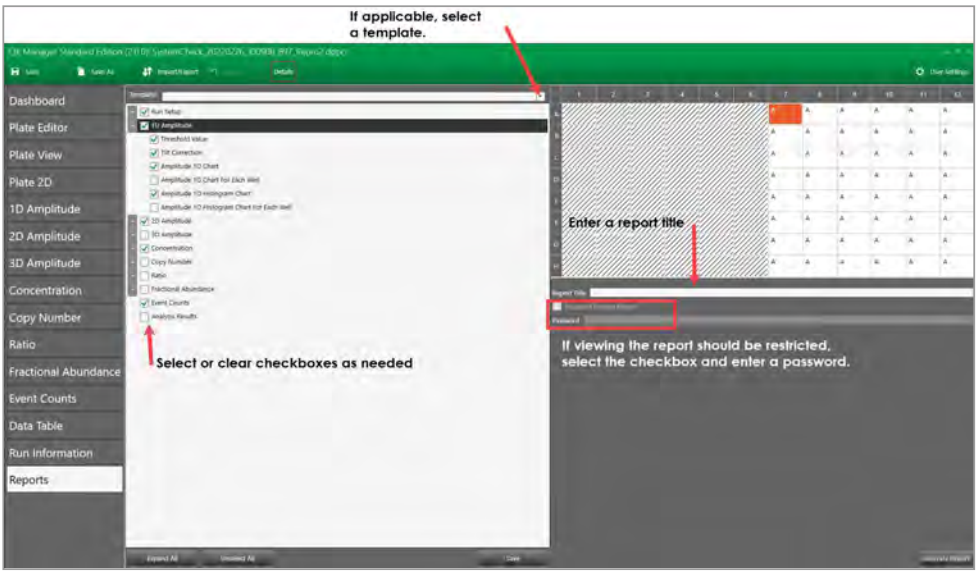
This section contains information on how to create analysis reports.

Creating Analysis Reports

You can create reports with or without a template. For information on creating templates for reports you generate often, see Creating or Editing Report Templates on page 80.

To create a report

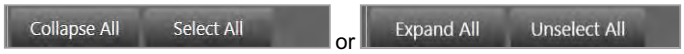
1. Select the Reports tab to display the Reports window.



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2. Do one of the following:
   - To create a report from a template, click the Template dropdown arrow and select a template from the list
   - To create a custom report, click the arrows to expand reporting categories, and then select the checkbox for each report element to include, and clear checkboxes for elements to eliminate.

     Note: Use the buttons at the bottom of the screen to expand or collapse all categories at once, or to select or clear all checkboxes at once. You can also drag and drop the section to rearrange the order of appearance in the report, and drag and drop items in the section to reorder them.

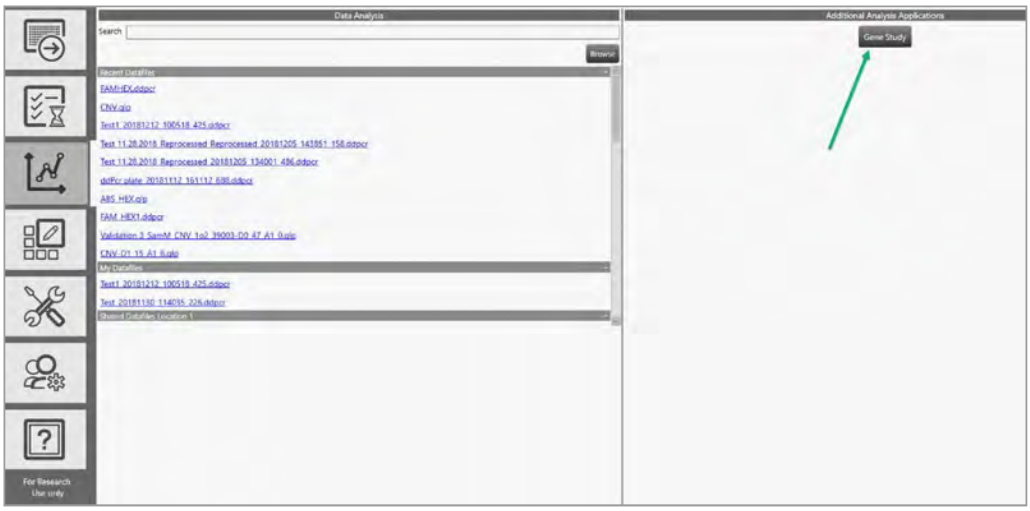
3. Enter a report title.
4. (Optional) To restrict viewing, select the Password Encrypt Report checkbox and then enter a password.
5. Click Generate Report to generate a PDF report.
6. To save the report, click Save.

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Chapter 10 Gene Study Module

You access the Gene Study module from the Data Analysis window.

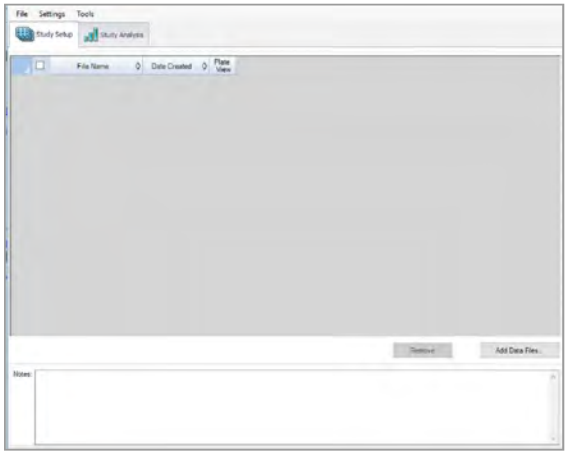


To create a gene study, you add one or more data files and then compare gene expression data from one or more ddPCR experiments.

Note: The maximum number of samples you can analyze in a gene study is limited by the size of the computer's RAM and virtual memory.

To open the Gene Study module

▶ Click Gene Study.



The Gene Study dialog box includes two tabs:

- **Study Setup** — manages the run files in the gene study

  Important: Adding or removing data files in a gene study does not change the data in the original file.

- **Study Analysis** — displays the gene expression data for the combined runs.

From these tabs you can add files to set up a gene study, analyze your data in charts and plots, and run reports on the gene study data.

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Gene Study Options

The Gene Study module provides the menus and corresponding options described below.

- **File** — Open, save, and close the gene study, and add files
- **Settings** — Restore the default layout
- **Tools** — Create a report

To open a gene study file

1. Click File → Open.
2. In the Open dialog box, navigate to and select the data file, and then click Open.

To save a gene study file

1. Click File and select Save or Save As.
2. If prompted, enter a file name and click Save.
3. If prompted to overwrite an existing file, click Yes or No.

To close a gene study file

▶ Click File → Close.

Note: If you have not saved the file, an advisory prompt appears.

To add data files

1. Click File → Add Data Files.
2. In the Open dialog box, navigate to the data files to be added to the study.
3. Select one or more files and then click Open to add the files to the Gene Study window.

To restore default settings

▶ Click Settings → Restore default settings.

Note: At least one modified data file must be open.

To generate a report

▶ See Gene Study Reports on page 161.

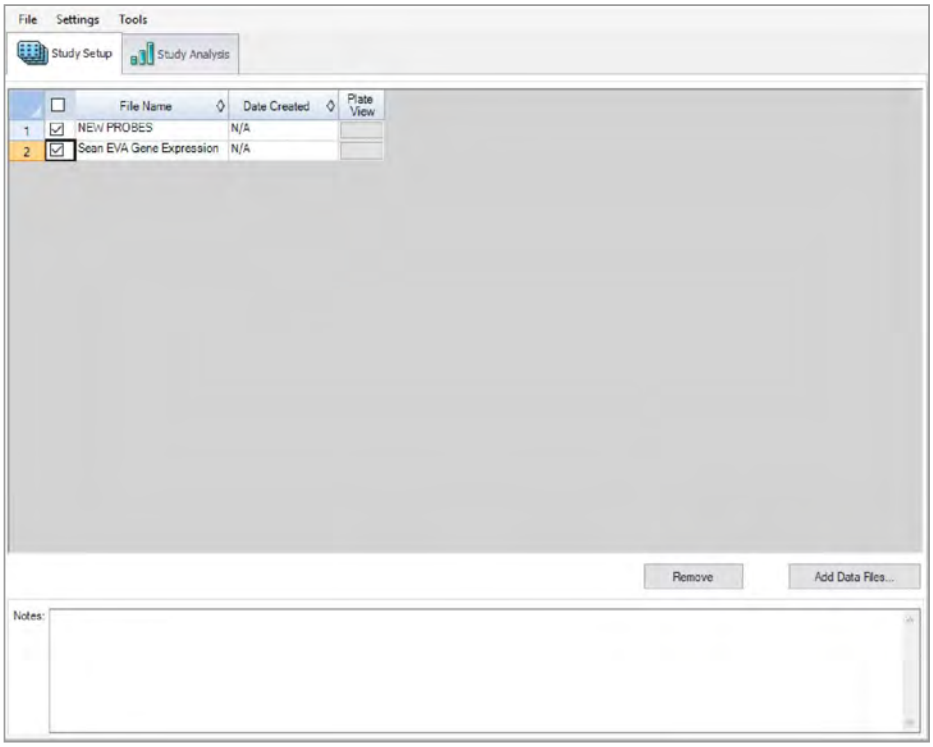
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Setting up the Gene Study

Use the Study Setup window to

- Add data files to include in the study
- Remove data files from the study
- Select data files for viewing and analysis
- Show the plate view for a data file



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Table 37 defines each column in Study Setup.

Table 37. Gene Study Setup Fields

Column Title	Description
File Name	Name of the data file
Date Created	Date the file was imported into the Gene Study module
Plate View	Opens a map of the plate with the data for each dye in each well included in the Gene Study 

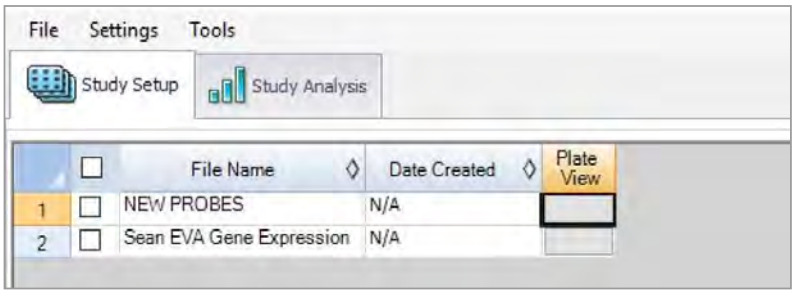
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Adding Data Files

To add data files

1. On the Study Setup tab, click Add Data Files.
2. In the Open dialog box, navigate to the data files to be added to the study.
3. Select one or more files and then click Open.

   The files are added to the Study Setup window.



Removing Data Files

To remove data files

▶ Select the checkbox next to the file and then click Remove.

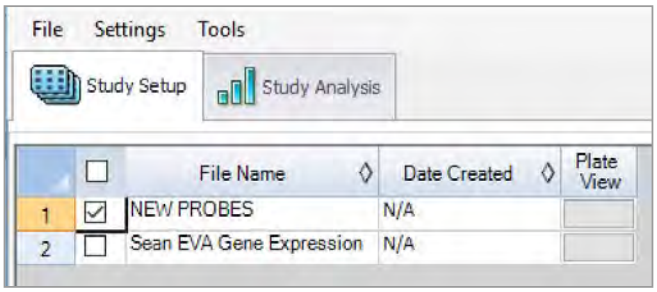
The file is removed from the Study Setup window.

Note: The Remove button is disabled until you select a file.

Selecting Data Files for Analysis

To select data files for analysis

1. Select the checkboxes next to the files to be included in the analysis.



2. Select the Study Analysis tab to see the analysis results. For information, see Using the Study Analysis Features on page 159.

Showing the Plate View

To show the plate view

1. In the Plate View column, click the button for the plate view to be displayed.



The Plate view appears.



2. Click Close to close the view.

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Using the Study Analysis Features

The Study Analysis tab displays the data from all runs in the gene study in the views described in Table 38.

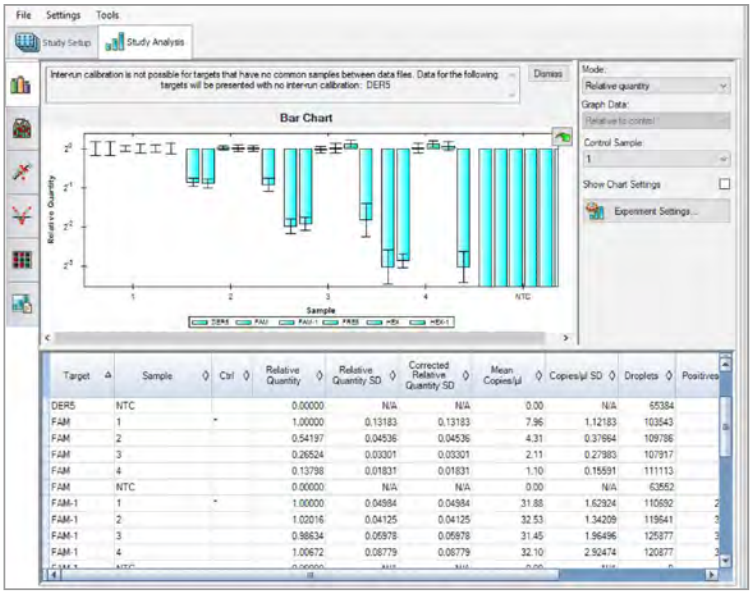


Table 38. Analysis Toolbar

Button	Description
Bar Chart 	Size of bars correspond to normalized expression or relative quantity
Clustergram 	Data is arranged in a hierarchy based on the degree of similarity of expression for different targets and samples
Scatter Plot 	Dot plot of normalized expression of targets for a control versus an experimental sample

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Table 38. Analysis Toolbar, continued

Button	Description
Volcano Plot 	Dot plot of change in expression (regulation) of a target for an experimental sample compared to a control
Heat Map 	Plate layout grid showing target regulation for an experimental compared to a control sample based on relative normalized expression **Note:** If the same targets are in the same location on multiple plates but with different samples, use the dropdown menu to select a particular plate for analysis.
Results Table 	Provides results in a tabular format

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Gene Study Reports

Use the Gene Study Report dialog box to arrange the gene study data into a report. Table 34 lists all the options available for a gene study report.

Table 39. Gene Study Report Categories

Category	Option	Description
Header		
	Report Information	Date, user name, data file name, data file path, and the selected well group
	Gene Study File List	List of all the data files in the gene study
	Notes	Notes about the data report
Study Analysis Bar Chart		
	Analysis Settings	List of the selected parameters
	Chart	Gene expression bar chart showing the data
	Target Names	List of targets in the gene study
	Sample Names	List of samples in the gene study
	Data	Spreadsheet that shows the data
	Target Stability	Target stability data
	Inter-run Calibration	Inter-run calibration data
Study Analysis Clustergram, Scatter Plot, Volcano Plot, and Heat Map		
	Analysis Settings	Settings for each chart type
	Chart	Gene expression chart showing the data
	Data	Spreadsheet listing the data in each target

Creating a Gene Study Report

Before creating a report, make any needed adjustments to the gene study data and charts.

To create a gene study report

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1. Select Tools → Reports in the Gene Study menu to open the Report dialog box.
2. Select the options you want to include in the report.

   The report opens with default options selected. Select or clear the checkboxes to change whole categories or individual options within a category.

   For information on the available options to display, see Gene Study Reports on page 161.
3. Change the order of categories and items in a report.

   Drag the options to the required position. Note that Items can be reordered only within the categories to which they belong.
4. Click Update Report to update the Report Preview window with any changes.
5. Print or save the report:
   a. Click the Print Report button on the toolbar to print the current report.
   b. Select File → Save to save the report in PDF, MHT or MHTML file format and select a location to save the file.

      Note: MHT and MHTML are Microsoft formats.
   c. Select File → Save As to save the report with a new name or in a new location.
6. (Optional) To save the current report settings as a template, select Template → Save or Save As.

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Chapter 11 System Utilities

From the System tab, you can access the following tabs for managing tasks and data related to the instrument, software, data, and storage:

- **Datafile and Template Storage**
  - **System Settings** — View shared template and data file locations
  - **Event Log** — View logged data about all software activities
  - **Maintenance Log** — Record maintenance activities
  - **Maintenance Reports** — PDF maintenance records from Bio-Rad maintenance
  - **System Data** — archive data to clear up space in primary storage areas, access troubleshooting data, and use the data recovery function
  - **Reprocess Data** — Reprocess a .data file using new calibration values
  - **Tools** — Flush or prime the instrument
- **Instrument**
  - Change the instrument

Note: The Instrument Calibration tab is enabled only for the Bio-Rad service engineer user account.

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System Settings Tab

When you select the System tab, the System Settings window opens by default.



The window contains the following data file and template storage options set by your software administrator:

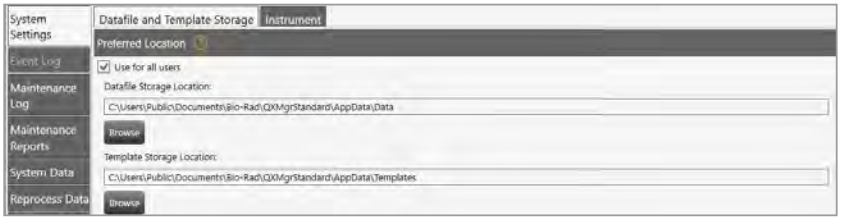
- **Preferred Locations** — if the checkbox selected, storage for all users is limited to a single folder for data files and a single folder for templates.
- **Shared Settings** — if the Preferred Locations checkbox remains cleared, then shared locations can be defined, but remain optional. Users can choose to save their files to their personal folders specified in User Preferences or to the shared location.

  Note: The software administrator can also define a backup location for data file storage if the priority folder is full.

Preferred Location

If the administrator sets Preferred Location file paths, then data files and templates for all users are automatically saved to the specified folders. Preferred Locations override selections in individual user preferences.

Important: Only users assigned the System Settings user privilege can set preferred locations.



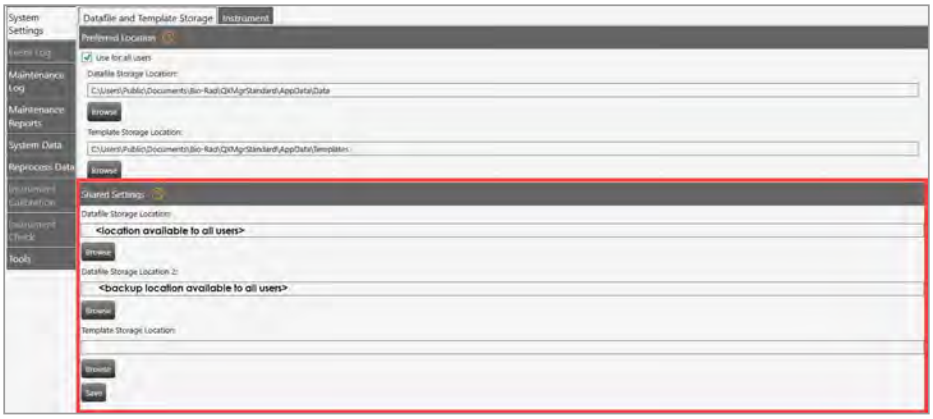
To specify preferred locations

1. Select the Use for all users checkbox.
2. (Optional) Keep the default paths or modify them to alternative secure storage locations.
3. Click Save.
4. When the confirmation message appears, click OK.

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Shared Settings

Under Shared Settings, you can choose to save your files to locations that are available to all users.



The software allows the software administrator to set a primary storage folder and a backup storage folder. Bio-Rad recommends routinely checking available storage space and if necessary, using the Archive function.

Important: Only users assigned the System Settings user privilege (typically your software administrator) can change storage locations in the System Settings window.

To specify shared folder locations

1. In each of the storage path fields, enter a file path or click Browse to search for the folder.
2. Click Save.
3. When the confirmation message appears, click OK.

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Changing Instruments

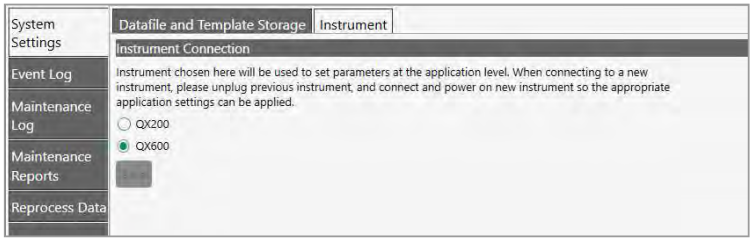
When you open QX Manager Software for the first time after installation, the Instrument connection window appears and you must select the instrument you will be using. If both the QX200 and QX600 droplet readers are used in your laboratory, you can select the alternate instrument on the Instrument tab in System Settings.

To change the instrument in the software

1. Select the System Settings tab.



2. In the System Settings window (default), select the Instrument tab.



3. Select the instrument and then click Save.

   Features associated with the selected instrument become available when the instrument is connected.

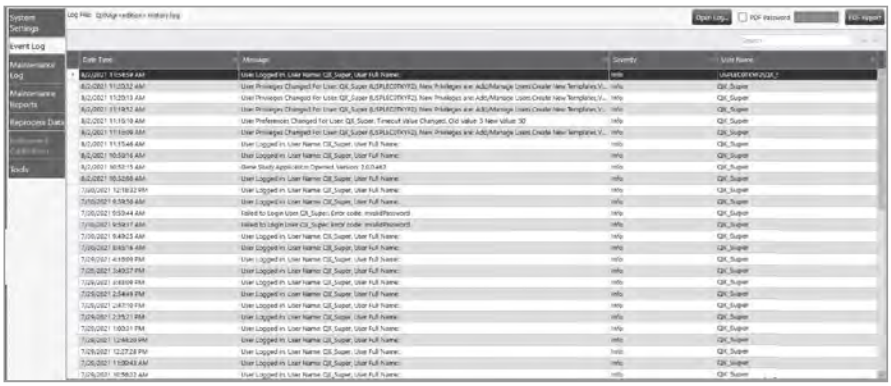
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System Log Files

From the System tab, you can access log files for system and software events (event log), and maintenance activities (maintenance log).

Event Log

Users who are assigned the Maintenance user privilege can view the system event log, which contains timestamped information on all software activities.



To view the Event Log

1. Select the System Utilities tab.



2. Select Event Log.
3. Click Open Log to choose either the debug log or the history log.
4. Review the log entries.
5. (Optional) To automatically generate and display a PDF document, click PDF Report.

   Tip: To restrict access to the file, you can assign a password. Select the PDF Password check box and then enter a password in the field. The file is saved in the Log Reports folder and can be opened only by entering the password.

Maintenance Log

All users can view the maintenance log, which contains a list of maintenance records. Users who are assigned the Maintenance user privilege can set up maintenance records, specify associated activities, and generate PDF reports. This guide also contains a form on which you can document completion of maintenance activities. For information, see Instrument Maintenance Log on page 201.



To add or view an activity

1. Select the System Utilities tab.



2. Select Maintenance Log. Note that activities that are already scheduled appear in the grid, sorted by newest activity first.
3. Do one of the following:
   - Scroll to view a particular activity.

     Important: You can view or add static activities; you cannot modify them.
   - Add an activity; click the + icon next to New Activity.

   A line item appears in the bottom section, with all fields except Activity completed automatically.
4. In the Activity field in the bottom section, type the activity into the Activity field, and click Save.
5. Repeat to add another activity.
6. (Optional) Click PDF Report to create a report of the added activities.

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Maintenance Reports

Reports generated from the Bio-Rad service engineer when performing maintenance on your instrument are stored on the Maintenance Reports tab.

▶ To open a report, click the list item.



Following is an example of a Color Calibration report:

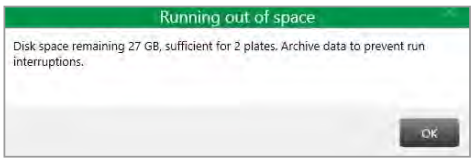


System Data

Select the System Data tab to archive raw data and access troubleshooting data.

Archiving Raw Data

When the storage space on the computer connected to the QX600 Droplet Reader approaches the limit displayed under Disk Space, you can archive raw data to an archive storage folder in a different location . The software also displays an advisory message, as shown in the following graphic:



Important: You must be assigned the System Data user privilege to archive data.

To archive files

1. Select the System Utilities tab.



2. Select System Data.

   The window opens to the Archive Raw Data screen by default.



3. In the Archive Progress section, click the calendar icons and select a From date and a To date.
4. Click Query to search within the defined date span.
5. Click Browse to locate the archive location on your network, and then click Archive.

Troubleshooting Data

If issues occur with the instrument or software, you can access data to assist in troubleshooting the problem.

Important: You must be assigned the System Data user privilege to access troubleshooting data.

To collect data for troubleshooting

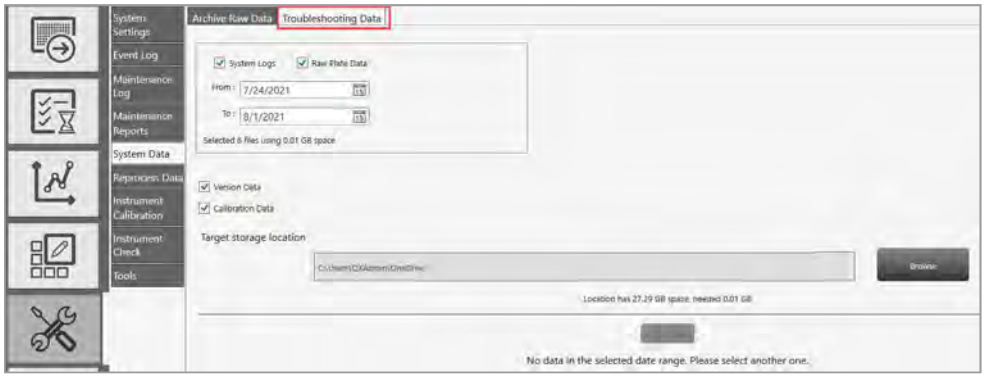
1. Select the System Settings tab.



2. Select System Data.

   The window opens to the Archive Raw Data screen by default.

3. Select the Troubleshooting Tab.



4. Click the From and To calendar icons to select a date range.
5. Select any of the following checkboxes:
   - System Logs
   - Raw Plate Data
   - Version Data
   - Calibration Data

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6. Click Browse to navigate to a folder where the data will be stored.

   QX Manager Software advises how much space you need and how much is available, and also advises if there is no data from your specified date range.

7. Click Collect Data.

   A progress bar appears.



   QX Manager Software creates a subfolder containing the data you specified and places it in the target location.

Reprocessing Data

After your instrument has been recalibrated, you can reprocess a data file from a run using different color calibration values.

Note: The instrument must be in an idle state before you can reprocess the data inQX Manager Software If the file was created in an older software version, the software cannot reprocess the file and displays an advisory message.

To reprocess a data file

1. Select the System Utilities tab.

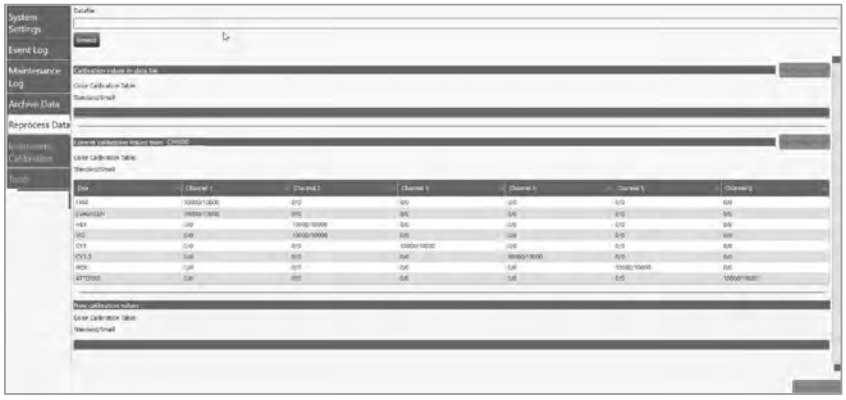


2. Click Reprocess Data.
3. Under Datafile, click Browse and navigate to the analysis data file.

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4. In the Select Datafile dialog box, select the file and click Open.

   The calibration values appear in the first Color Calibration table.



5. In the table, enternew values for the fluorophores.
6. Click Reprocess Data.

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System Calibration and Tools

You cannot execute runs on the QX600 Droplet Reader unless the instrument is properly calibrated.

Only a Bio-Rad service engineer can calibrate your instrument and use the Instrument Calibration options in QX Manager Software.

If your instrument requires service, contact Bio-Rad Technical Support.

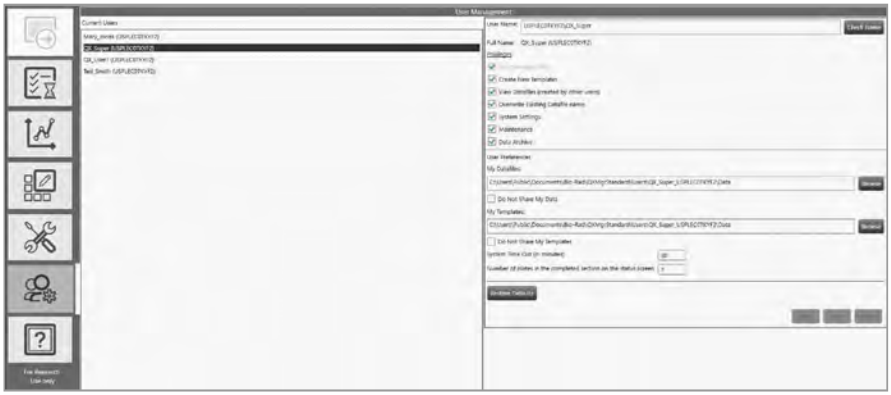
The Tools functions are currently applicable only to instrument maintenance. For information, see Appendix B, Instrument Maintenance.

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Chapter 12 Managing Users

Important: This chapter applies only if you enabled the User Management module during the installation of QX Manager Software Standard Edition. If you did not enable the module, QX Manager Software creates a generic user with the ability to set up, run, and analyze experiments.

Use the User Management window to add, change, or remove QX Manager Software users, and set or change user privileges and preferences.



An administrative user account, with all privileges, should be set up first. You will use this user account to create additional users and assign user privileges. If you need assistance, contact Bio-Rad Technical Support. Users who are not assigned any of the specified privileges can still execute runs and use the Analysis module.

You can set up local or domain accounts. If the computer connected to the QX600 Droplet Reader

- Cannot access your company's network, your IT department or system adminisrator can set up user names as local Windows accounts.
- Can access your company's network, QX Manager Software can recognize the Active Directory user names.

You can create user accounts with any user name convention and password character structure, as long as it complies with your organizational requirements. The software is designed to validate each user against the name it finds in either the Active Directory or local domain, so users must log in with the proper domain and user name.

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Adding Users

This section applies only if the User Management module was enabled during the installation of QX Manager Software Standard Edition.

Before you can add users to QX Manager Software, you must be logged in with the initial superuser account, or another account that is assigned the Add/Manage Users privilege.

Important: Before you begin, ensure that all users either have local Windows user accounts on the local computer or user accounts in Active Directory (network domain).

To add a user

1. Open QX Manager Software and sign into the application.
2. Click the User Management tab on the left



3. In the User Name field on the right, enter the user name as follows:
   - If the computer is connected to a network and you are using Active Directory accounts, enter <domain name>/<user name>. For example, **global/johnsmith**.

     Important: Active Directory users must log in every time with the domain name preceding the user name.
   - If the computer is not connected to a network, enter <local computer name>/<user name>. For example, **analysiscomputer/johnsmith**.

     Important: To locate the computer name, right-click on the yellow folder in the lower menu bar and select File Explorer. When the dialog box appears, the computer name is located at the top of the left panel. Local users are not required to reenter the computer name after the initial login.

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4. Click Check Name.

   One of the following occurs:

   - If the software recognizes the network domain or computer name and the user name, the software displays a validation message.
   - If the user is not recognized, an error message appears. Do one of the following:
     - For network users, ensure there is a working connection to the network and then verify your entry is accurate.
     - For local users, verify the entry is accurate. If the user is not set up locally or on the network, contact your system administrator for assistance.

5. When the This user is valid message appears, click OK, and then click Add at the bottom of the pane.

   An Are you sure you want to add this user? message appears.

6. Click Yes.

   A confirmation message appears.

7. Click OK.
8. Continue until all users are added.

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Adding or Removing User Privileges

This section applies only if the User Management module was enabled during the installation of QX Manager Software Standard Edition.

Only a user assigned the Add/Manage users privilege can add or remove user privileges. By default, individual users without privileges can set up and execute a run, open files, view and analyze results.

For descriptions of all user privileges, see Viewing User Privileges on page 41.



Table 40 contains a sample set of privileges assigned by user roles common to a laboratory. User roles can be defined and used in any scenario, but might be required in regulatory environments.

Table 40. Example User Roles

Privilege	Superuser	Lab Manager	Group Lead	Technician/ Student Intern
Add/manage users	✓	✓		
Create new templates	✓	✓	✓	
View data files created by other users	✓	✓	✓	✓
Override existing ddPCR file name	✓	✓	✓	
System settings	✓			
Maintenance	✓			

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Table 40. Example User Roles, continued

Privilege	Superuser	Lab Manager	Group Lead	Technician/ Student Intern
Archive data files	✓	✓	✓	✓
Access the module recovery tool	✓	✓	✓	✓
Manage Plates	✓	✓	✓	

You can assign the Create new templates privilege to non-administrative roles if anyone can create and save a template. If template formats are restricted, then you should assign this permission to administrative or supervisory roles only.

Note: Bio-Rad recommends defining at least one additional superuser as a backup, as well as other administrative and standard roles based on your needs. Guest users can open shared templates, execute runs, and perform analysis but are not typically assigned other privileges.

To add or modify privileges

1. Click the Add/Manage Users tab to open the User Management window.
2. Select a user from the Current Users list. The name appears in the User Name field. A network or computer name and backslash might appear before the user name.
3. Select or clear the checkbox for each privilege in accordance with the user's role in using the software. You can assign user privileges in any combination.
4. When the confirmation message appears, click Yes to save the changes, and then click OK to close the pop-up.

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Modifying User Preferences

This section applies only if the User Management module was enabled during the installation of QX Manager Software Standard Edition. If User Management was not enabled, only the options for system timeout duration and the number of completed plates displayed in the Runs window are available. For information, see Partial Preferences on page 44.

Users assigned the Add/Manage Users privilege can change the preferences for other users. Individual users can also change their own preferences.

To modify a user's preferences

1. Click the Add/Manage Users tab, and then enter the user name.
2. Change any of the following preferences for the user:
   - Enter a different file path for user data files and templates, and choose to keep them private.

     Important: You can also specify Preferred Locations in System Settings, to override all file paths specified in individual preferences. For information, see System Settings Tab on page 164.
   - Enter a different default system timeout period.
   - Enter a different number of completed plates to show in the Status section, up to a maximum of 100.
3. Click Save. QX Manager Software displays a confirmation message.
4. Click Yes to save the changes, and then click OK to close the dialog box.

Removing Users

This section applies only if the User Management module was enabled during the installation of QX Manager Software Standard Edition.

Only a user assigned the Add/Manage Users privilege can remove users. Removing a user from the software does not remove the user from other systems, such as Windows, or databases

1. Click the Add/Manage Users tab to open the User Management window.
2. In the Current Users pane, select the user and click Remove. QX Manager Software displays a confirmation message.
3. Click Yes to remove the user, and then click OK to close the dialog box.

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Appendix A Experiment Examples

This section contains setup and analysis information for the following sample experiments:

- Direct Quantification, using the Amplitude Multiplex assay type
- Copy Number Variation, using the Probe Mix Triplex assay type
- Drop-Off, using the Basic Drop-off Assay type for genome edit detection
- Direct Quantification, using the Advanced Classification Method assay type

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Direct Quantification Multiplexed

You can increase the number of targets to be evaluated by using the Amplitude Multiplex option. Optionally, to expedite plate setup you can copy and paste data from one well to another, and you can click Undo to undo an applied well setting. You cannot change the supermix that was used during data collection.

Setting Up Experiment Parameters

To set experiment parameters

1. Open the analysis data file and select the Plate Editor tab.
2. Select one or more wells to be assigned well analysis attributes.
3. Change the experiment type to Direct Quantification (DQ), and then click Apply.
4. (Optional) Change the sample description identifiers.
5. (Optional) Change the sample type. The default type is Unknown.

   Tip: For automatic thresholding of positive control wells, you must select Pos Ctrl.
6. Under Assay Type, select Amplitude Multiplex and click Apply. Twelve target rows appear.
7. Change the target name and type (unknown or reference) for each target in the well.

   The software defaults to FAM Lo, FAM Hi, HEX Lo, HEX Hi, Cy5 Lo, Cy5 Hi, Cy5.5 Lo, Cy5.5 Hi, ROX Lo, ROX Hi, ATTO 590 Lo, and ATTO 590 Hi, respectively, for each target.
8. (Optional) Change the high and low target identifiers.

   Amplitude Multiplex allows for up to two assays in each channel, indicated by Lo and Hi labels. In this multiplex mode, each target may have a signal for only one channel. You can

   - Select a different dye, if the channel supports two dyes (for example change HEX to VIC or VIC to HEX)
   - Select a different channel for the target (for example, change FAM to HEX)
   - Change Hi to Lo or Lo to Hi
9. Click Apply.

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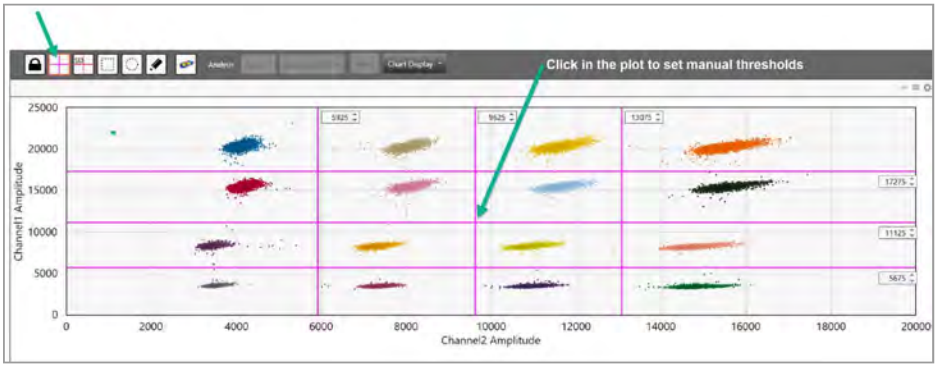
Viewing and Adjusting Results

Automatic thresholding is not available for Amplitude Multiplex experiments.

For clusters that are easily identifiable across the chart, use Threshold Lines mode to set manual thresholds. If the data is not orthogonal and the threshold lines do not adequately divide the populations of droplets, use Threshold Clusters mode to set manual thresholds.

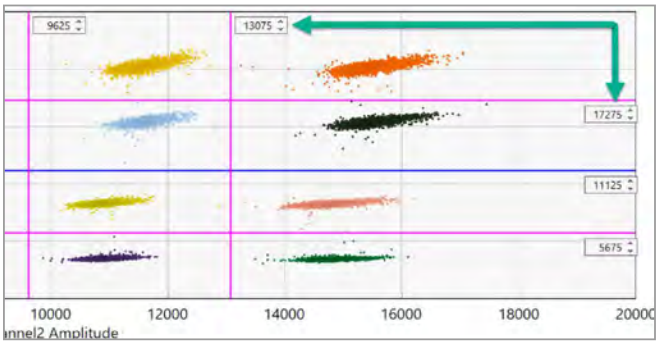
To set thresholds in the 2D Amplitude window

1. Select the 2D Amplitude tab.



2. On the toolbar, click the Threshold Line Mode button.
3. Click anywhere on the plot to apply “best fit” threshold lines to the data.
4. To adjust the thresholds, drag the lines horizontally or vertically in the plot area.

   Tip: You can also enter a specific threshold value in the boxes at the end of each threshold line.



To apply and adjust manual clusters

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1. Click any of the three cluster mode buttons (square, circle, or freehand).
2. Press the left mouse button and draw the chosen shape around the cluster of droplets to be classified first.

   Tip: When you release the mouse button, a pop-up tool appears to help you select the correct target combination. Target names are shown along the two axis based on the signal values assigned during plate setup.



3. To classify droplets as positive or negative for each given target, select the colored box that best represents the location of the cluster of droplets.

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Copy Number Variation Triplexed

A CNV experiment with probe mix triplexing uses two targets and a reference in three groups of three to produce analysis data. Optionally, to expedite plate setup you can copy and paste data from one well to another, and you can click Undo to undo an applied well setting.

Setting Up Experiment Parameters

To set experiment parameters

1. Open the analysis data file and select the Plate Editor tab.
2. Select one or more wells to be assigned well analysis attributes.
3. Set the experiment type to Copy Number Variation (CNV), and click Apply.
4. (Optional) Change the sample description identifiers.
5. (Optional) Change the sample type. The default type is Unknown.

   Tip: For automatic thresholding of positive control wells, you must select Pos Ctrl.

   Note: You cannot change the supermix that was used during data collection.
6. Click Apply.
7. From the Assay Type dropdown list, select Probe Mix Triplex and click Apply.

   QX Manager Software displays three targets.

   Important: You cannot add or delete triplex targets.
8. Enter target names and select the target type (unknown or reference) for each target in the well.

   This creates three groups of three targets each.

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9. Assign the targets as follows:

Group 1

Target	Target Type	Signal Ch1	Signal Ch2	Ref Copies	Plot?
Target 1	Unknown	FAM	None	N/A	N/A
Target 2	Unknown	None	HEX/VIC	N/A	N/A
Target 3	Reference	FAM	HEX/VIC	2	Yes

Group 2

Target	Target Type	Signal Ch3	Signal Ch4	Ref Copies	Plot?
Target 4	Unknown	Cy5	None	N/A	N/A
Target 5	Unknown	None	Cy5.5	N/A	N/A
Target 6	Reference	Cy5	Cy5.5	2	Yes

Group 3

Target	Target Type	Signal Ch3	Signal Ch4	Ref Copies	Plot?
Target 7	Unknown	ROX	None	N/A	N/A
Target 8	Unknown	None	ATTO 590	N/A	N/A
Target 9	Reference	ROX	ATTO 590	2	Yes

10. Click Apply.

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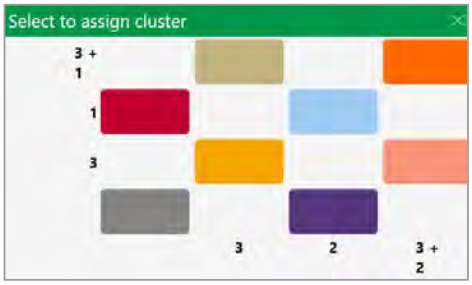
Viewing and Adjusting Results

Note: Linear thresholding is not available for Probe Mix Triplex experiments.

To see the results

1. After all wells are configured, select the 2D Amplitude tab to evaluate the data and manually set clusters.
2. Click any of the three cluster mode buttons (square, circle, or freehand).
3. Press the left mouse button and draw the chosen shape around the cluster of droplets to be classified first.

   Tip: When you release the mouse button, a pop-up tool appears to help you select the correct target combination. Target names are shown along the two axis based on the signal values assigned during plate setup.



4. To classify droplets as positive or negative for each given target, select the colored box that best represents the location of the cluster of droplets.

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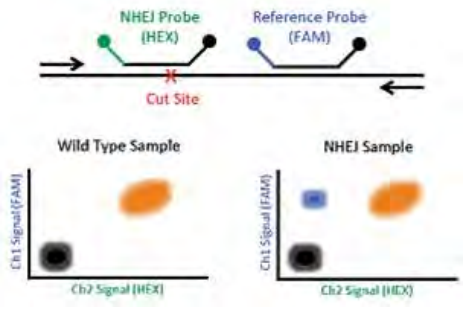
Drop-Off Assays for Genome Edit Detection

Optionally, to expedite plate setup you can copy and paste data from one well to another, and you can click Undo to undo an applied well setting.

Two configurations of drop-off assays are possible and are shown below. Assign a signal (expected cluster position) to wild-type and NHEJ indel alleles. The signal produced by a wild-type allele will always be positive for both FAM and HEX, while the signal produced by an NHEJ indel allele depends on the assay configuration used (as shown below left and right).

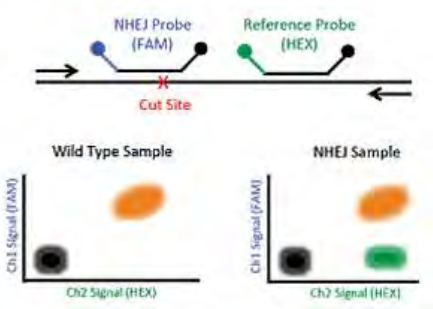
Drop-Off Probe in HEX

NHEJ Probe = HEX and Reference Probe =FAM



Drop-Off Probe in FAM

NHEJ Probe = FAM and Reference Probe =HEX



This type allows three groups of two targets per well; Group 1, shown by default, with 1 target in FAM and HEX (reference), and 1 target in FAM or HEX (unknown). You can add a second group with 1 target in Cy5 and Cy5.5 (reference) and 1 target in Cy5 or Cy5.5 (unknown) and a third group with 1 target in ROX and ATTO 590 (reference) and 1 target in ROX and ATTO 590 (unknown).

Note: The channels within each group are not interchangeable therefore FAM is always paired with HEX/VIC. Cy5 with Cy5.5. and ROX with ATTO 590.

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Setting Up Experiment Parameters

To set experiment parameters

1. Open the analysis data file and select the Plate Editor tab.
2. Select one or more wells to be assigned well analysis attributes.
3. Set the experiment type to Drop-Off (DOF) and click Apply.



4. (Optional) Change the sample description identifiers.
5. (Optional) Change the sample type. The default type is Unknown.

   Tip: For automatic thresholding of positive control wells, you must select Pos Ctrl.

   Note: You cannot change the supermix that was used during data collection.
6. Click Apply.

   QX Manager Software automatically assigns the assay method as Basic Drop-Off.
7. Enter the target names, and then designate one target as Reference and the other as Unknown.

   The Reference target enables ratio calculation of results.
8. Click Apply.

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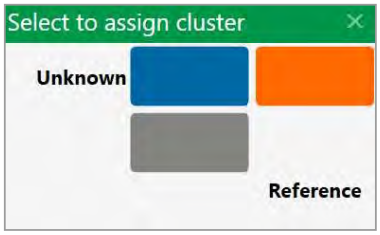
Viewing and Adjusting Results

Thresholding is not available for Drop Off experiments.

To apply and adjust clusters

1. After all wells are configured, select the 2D Amplitude tab to evaluate the data and manually set clusters.
2. Click any of the three cluster mode buttons (square, circle, or freehand).
3. Press the left mouse button and draw the chosen shape around the cluster of droplets to be classified first.

   Tip: When you release the mouse button, a pop-up tool appears to help you select the available cluster assignments. Target names are shown along the two axis based on the signal values assigned during plate setup.



4. To classify droplets as positive or negative for each given target, select the colored box that best represents the location of the cluster of droplets.

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Direct Quantification with the Advanced Classification Method

The Advanced Classification Method analysis mode allows the user complete control of droplet identification, and does not force or preassign any clusters. This allows a higher degree of multiplexing through radial dye mixing. Optionally, to expedite plate setup you can copy and paste data from one well to another, and you can click Undo to undo an applied well setting.

Important: This mode is intended for experienced users of Droplet Digital PCR.

Setting Up Experiment Parameters

To use the advanced classification method

1. Open the analysis file and select the Plate Editor tab.
2. Select one or more wells to be assigned well analysis attributes.
3. Set the experiment type to Direct Quantification (DQ) or RED and click Apply.
4. (Optional) Change the sample description identifiers.
5. (Optional) Change the sample type. The default type is Unknown.

   Tip: For automatic thresholding of positive control wells, you must select Pos Ctrl.

   Note: You cannot change the supermix that was used during data collection.
6. Click Apply.
7. Under Assay Type, select Advanced Classification Method and click Apply.
8. Use the + icon to add up to ten targets.
9. Enter the target name and type (unknown or reference) for each target in the well.
10. (Optional) If you select Reference, you can change the number of reference copies. The default is 2.

    - If no reference is selected, the target with the highest dye concentration in the highest channel is used as the reference. For example, if there are targets in all six channels, the target in Channel 6 is used; however, if there are targets only in the first five channels, then Channel 5 is the reference target.
    - If more than one reference is selected in the well, checkboxes appear to the right for using the reference in the plot.

    Note: Each Reference target enables ratio calculations in the results.
11. Assign the first target an expected signal in a channel.

12. Assign your last target a signal in a channel that was not selected for your first target.

    Each target in between will be assigned a signal depending on mixture ratio of the target.

13. Click Apply to confirm all well settings.

Viewing and Adjusting Results

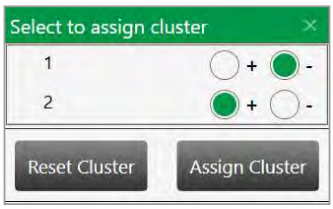
To view and adjust results

1. Click the 2D Amplitude tab.

   All droplets are unclassified by default and are colored red. Only the Threshold Cluster mode is available for setting thresholds in Advanced Classification experiments in the 2D plot.

2. Use one of the Threshold Cluster mode tools (box, circle or freehand) to assign droplet clusters to targets.

   When you select a droplet cluster, a cluster assignment box appears.



3. Select a radio button to identify the composition of the cluster.

   For example, if the cluster is composed of positive droplets for Target-2, select the positive radio button for Target-2 and the negative radio buttons for the remainder.

   Important: For calculations to occur, you must have at least one positive cluster for each target, as well as the assigned negative cluster.

   Note: The software sets aside unassigned for calculation purposes. If you are unsure of droplet/cluster composition, leave it unassigned (red).

   You can view the concentration results in the data table and in the Concentration tab.

   If one of your samples was identified as a Reference under AssayType, you can view a ratio of concentration for unknown versus reference in the Ratio tab.

Appendix B Instrument Maintenance

Bio-Rad recommends regular maintenance of your equipment, which includes

- Keeping the surface areas clean, post-run or as necessary

  Note: Wipe down external panels and the plate holder with 70% ethanol. See Cleaning or Decontaminating the Instrument on page 200 for information.

- Regularly checking the fluid levels
- Inspecting the equipment for damaged parts or wiring

Bio-Rad also recommends that a Bio-Rad service engineer perform annual preventive maintenance on your instrument. Contact Bio-Rad if your instrument requires service or recalibration.

Fluid Maintenance and Waste Management

You must ensure there is enough oil for at least one full run, and you must empty the waste container when it approaches a full state.

You can verify your fluid levels by checking the following:

- Instrument status bar
- Instrument indicator lights
- The oil and waste bottles

Table 41 explains fluid level maintenance for your instrument.

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Table 41. Fluid Levels

Fluid Level	Status
Oil greater than 40% or waste less than 70%	Bottle levels OK. You can start your run.
Oil less than 40% or waste greater than 70%	You may be able to start a run with fewer than 96 wells. If there is not enough oil in the dispensers to perform a run on the number of wells you set up in the Plate Editor, the software prevents the run until you fill the oil dispenser and empty the waste container. **Note:** Pay attention to the fluid level by checking instrument status bar in the QX Manager Software or by checking the oil bottles.
Oil less than 10% or waste greater than 90%	The software prevents the run until you fill the oil dispenser and empty the waste container.

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Changing the Droplet Reader Oil Bottle

To replace the oil bottle

1. Slide the container carriage out using the handle on the side of the instrument.
2. Remove the oil bottle and set it aside, to use when changing the waste bottle.
3. Insert a new bottle of oil and tighten the cap into place.
4. Prime and flush the instrument at least once after the new oil bottle is installed. See Priming and Flushing the Instrument on page 199 for information.

Emptying the Waste Container

Important: Use each empty oil bottle as a new waste bottle once and then discard it. Before discarding, add 50 mL of 10% bleach solution to the bottle to prevent microbial growth, and then label the bottle as waste.

To replace the waste bottle

1. Slide the container carriage out using the handle on the side of the instrument and remove the waste bottle.
2. Place the new Waste label over the Oil label on the oil bottle.
3. Insert the last empty oil bottle into the waste container slot.
4. Flush the instrument after the new waste bottle is installed. See Priming and Flushing the Instrument on page 199 for information.

Managing Waste

You must apply standard Material Safety Data Sheet (MSDS) and OSHA practices when handling and disposing of generated waste. A typical waste profile should contain the following:

Table 42. Managing Waste

Chemical name	Weight %
Fluorinated fluids	>50%
Water	<50%
Bleach	<10%
Other materials (proteins, nucleic acids, fluorescent dye)	<10%

Note: The chemical identities and exact percentages in the above table are withheld as trade secrets.

Bio-Rad droplet generation and reader fluids are based on fluorinated hydrocarbon chemistry, and should be disposed of in accordance with institutional, state and local regulations. Avoid release to the environment and prevent entry into sewer systems or bodies of water. As a disposal alternative, incinerate in a permitted high-temperature waste incineration facility.

These nonflammable fluids are inert and have low environmental impact and low toxicity.

Toxicological Information

Chemical name: (mixture)
Oral LD50: >5000 mg/kg (Rat)
Dermal LC50: >5000 mg/kg (Rabbit)
Inhalation LD50: >8838 ppm (Rat/8-hr)

Ecological Information

Acute aquatic hazard: LC50 > 500 mg/l (96-hr./static; Pimephales promelas/Fathead minnow)
Chronic aquatic hazard: No information available (GHS Chronic 4)

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Priming and Flushing the Instrument

You should prime the instrument after you replace the oil bottle and you should prime and flush the instrument when you change the waste bottle.

Important: Before you initiate a prime or flush, *the lid must be closed*. Do NOT press the button to open the lid while a prime or flush is in progress.

To prime the instrument

1. Select the System Utilities tab.
2. Tap Prime.

   A message appears, asking if you want to prime the instrument.
3. Tap Yes to begin.
4. A “Performing Prime” message appears.

   When the priming process is finished, an operation complete message appears.
5. Tap OK.

To flush the instrument

1. Select the System Utilities tab.
2. Tap Flush.

   A message appears, asking if you want to flush the instrument.
3. Tap Yes to begin.
4. A “Performing Flush” message appears.

   When the flushing process is finished, an operation complete message appears.
5. Tap OK.

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Recovery From Idle Mode

Complete the steps below if the instrument has been idle:

1. Check the expiration date on the oil bottle. If expired, replace the oil bottle.
2. Ensure the waste bottle is not full.
3. If needed, add 50 mL of 10% bleach to the waste bottle.
4. If you have changed the bottles, or if you have not used the system for a week or more, perform the prime and flush processes as specified in Priming and Flushing the Instrument on page 199.

Cleaning or Decontaminating the Instrument

Use the protocols specified below for the following:

- Regular post-run instrument cleanup
- Instrument decontamination, as needed

If you have questions regarding the use of cleaning agents other than those specified, contact Bio-Rad Technical Support.

To clean the instrument

1. Remove the plate after a run is complete and ensure it is properly discarded.
2. Using 70% ethanol, wipe down the external panels, as well as the plate holder and its compartment.

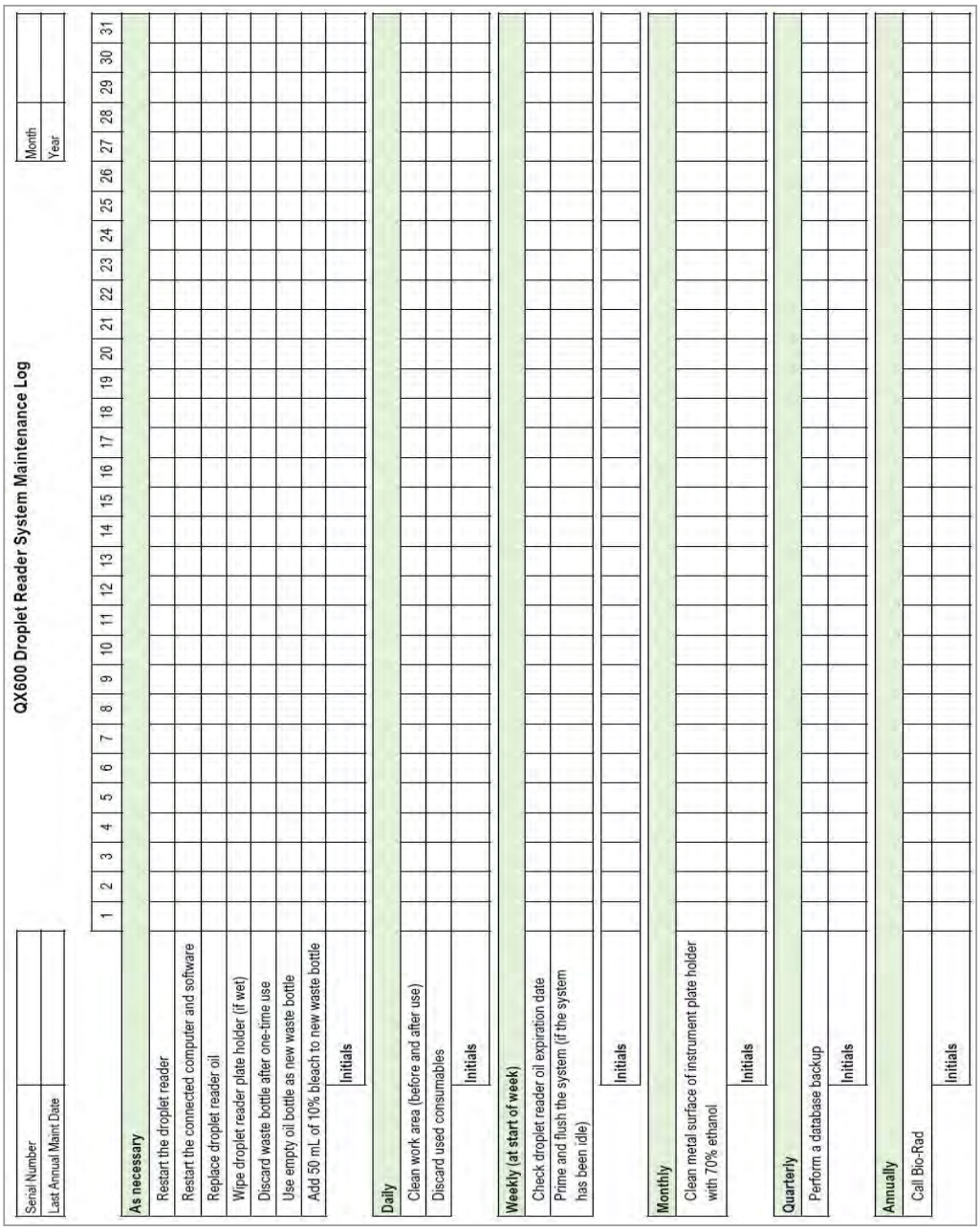
To decontaminate the instrument

1. Remove the plate after a run is complete and ensure it is properly discarded.
2. Wipe down the plate holder and its compartment with 10% bleach.

   Important: Do not use bleach inside the system.
3. Wipe down all external surfaces with 10% bleach.
4. Identify the equipment as *Decontaminated*.

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Instrument Maintenance Log



QX600 Droplet Reader System Maintenance Log

Serial Number
Last Annual Maint Date

Month
Year

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
As necessary																															
Restart the droplet reader																															
Restart the connected computer and software																															
Replace droplet reader oil																															
Wipe droplet reader plate holder (if wet)																															
Discard waste bottle after one-time use																															
Use empty oil bottle as new waste bottle																															
Add 50 mL of 10% bleach to new waste bottle																															
Initials																															
Daily																															
Clean work area (before and after use)																															
Discard used consumables																															
Initials																															
Weekly (at start of week)																															
Check droplet reader oil expiration date																															
Prime and flush the system (if the system has been idle)																															
Initials																															
Monthly																															
Clean metal surface of instrument plate holder with 70% ethanol																															
Initials																															
Quarterly																															
Perform a database backup																															
Initials																															
Annually																															
Call Bio-Rad																															
Initials																															

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Appendix C Troubleshooting

This section contains information on troubleshooting and correcting minor issues you may experience with your instrument or software.

Event Log

The Event Log contains records for all system and user activities, including system errors. If you do not understand, or cannot find an immediate remedy to, the error event, contact Bio-Rad Technical Support.

Instrument Errors

If an error occurs during a run, the software cancels the run and displays an X in the Status column, along with a Run Aborted by System message as the Run End status. Table 43 contains error grouped by type.

1. Tap the X to display the corresponding error code and description.
2. Perform the steps in the solution column before starting a new run.
3. If the suggested solution does not resolve the problem, contact Bio-Rad Technical Support immediately.

   Note: The error code and description are also displayed in the Run Information window .

Communication errors can be caused by instrument, connectivity, or power failures.

Table 43. Communication errors

Error Code	Description	Solution
102, 103, 104*, 110*, 111	Communication loss between the instrument and software, detector board error, loss during a run, or unable to reestablish a connection **Note:** For codes marked with an asterisk (*), some data may be usable.	Disconnect and reconnect the USB cable, and then power cycle the instrument.
302, 308	Communication error involving pump **Note:** Some data might be usable.	Power cycle the instrument.

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Table 43. Communication errors, continued

Error Code	Description	Solution
502	Invalid checksum value	Retry.
900	Invalid command	

An electrical error is typically triggered by an invalid setting.

Table 44. Electrical error codes

Error Code	Description	Solution
406	Electrical Error; invalid power value setting. **Note:** Some data might be usable.	Power cycle the instrument.

Mechanical errors can be caused by fluid issues (low oil, high waste), instrument operation errors, or binary data collection errors.

Table 45. Mechanical error codes

Error Code	Description	Solution
105, 106	Fluidics or motion response was invalid. **Note:** Some data may be usable.	Power cycle the instrument.
201	Motors disabled **Note:** Some data may be usable.	1. Remove and reinsert the plate and plate lid. 2. Check for incorrect or multiple heat seal foil layers. 3. Check for strain on all motor axes. 4. Power cycle the instrument.
205, 207, 210, 211, 212, 218	X, Y, or Z-axis, unable to reach target location or home in allotted time. **Note:** Some data might be usable.	Check for motor obstructions, and then power cycle the instrument.
301, 303, 304, 305, 307, 312, 316, 317	Pump errors **Note:** Some data might be usable.	Power cycle the instrument.

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Appendix D Ordering Information

This appendix contains descriptions and catalog numbers for new or replacement instruments, accessories, and consumables for Bio-Rad ddPCR products, including your QX600 Droplet Reader.

ddPCR System and Instrument Packages

Table 46. ddPCR system and instrument packages

Product	Description	Catalog Number
QX200 Droplet Digital PCR System	Includes the QX200 Droplet Generator, QX200 Droplet Reader, computer, software, and consumables	1864001
QX600 Droplet Digital PCR System	Includes the QX200 Droplet Generator, QX600 Droplet Reader, computer, software, and consumables	17007769
QX200 AutoDG™ Droplet Digital PCR System	Includes the AutoDG, QX200 Droplet Reader, computer, and QX Manager Software Standard Edition	1864100
QX600 AutoDG Droplet Digital PCR System	Includes the AutoDG, QX600 Droplet Reader, computer, QX Manager Software Standard Edition, and consumables	17008371
Automated Droplet Generator	Includes the AutoDG (1), cooling block accessory (1), oil purge reservoir (1), and power cord (1)	1864101
QX200 Droplet Generator	Includes the droplet generator (1), cartridges and gaskets (24 each), cartridge holders (2), and power cord (1)	1864002
QX200 Droplet Reader	Includes the droplet reader, two plate holders, USB cable, and power cord	1864003
QX600 Droplet Reader	Includes the droplet reader, two plate holders, USB cable, and power cord	12013328

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Thermal Cyclers and Plate Sealer

Table 47. Thermal cycler and plate sealer

Product	Description	Catalog Number
C1000™ Touch Thermal Cycler with 96–Deepwell Reaction Module	Includes the C1000 Touch Thermal Cycler chassis, 96–deepwell reaction module, USB flash drive	1851197
PTC Tempo Deepwell Thermal Cycler	Includes the thermal cycler, power cord, USB cable, and Ethernet cable	12015392
PX1 PCR Plate Sealer	PCR plate sealer, includes heat sealing instrument, plate support block that holds 96-well and 384-well plates, sealing frame, power cord	1814000
Piercable Foil Heat Seal	Heat seal for the PX1 PCR Plate Sealer	1814040

AutoDG Accessories

Table 48. Automated Droplet Generator accessories

Product	Description	Catalog Number
Cooling block accessory	Prevents evaporation during droplet generation	12002819
Oil purge reservoir	Collects oil waste from priming and flushing **Note:** Contact Bio-Rad Technical Support for replacement information.	N/A
Power cord	Connects the AutoDG to the power source **Note:** Contact Bio-Rad Technical Support for replacement information.	N/A

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QX200 Droplet Generator Accessories

Product	Description	Catalog Number
DG8 droplet generator cartridges and gaskets	Microfluidic cartridges used to mix sample and oil for droplet generation, and gaskets to seal the cartridges **Note:** You must seal the cartridge to prevent evaporation and to apply required pressure for droplet generation.	1864007
Droplet generator cartridge holder	Positions and holds the droplet generator cartridge in the instrument compartment	1863051
Power cord	Connects the QX200 Droplet Generator to the power source **Note:** Contact Bio-Rad Technical Support for replacement information.	N/A

QX600 Droplet Reader Accessories

Table 49. QX600 Droplet Reader accessories

Product	Description	Catalog Number
Computer	Computer that connects to the QX600 Droplet Reader for data collection and analysis	12017458
USB cable and power cord	Cable connecting the computer to the instrument and power cord connecting the instrument to the power source **Note:** Contact Bio-Rad Technical Support for replacement information.	Included
Droplet reader plate holders (2)	Used to position the 96-well plate in the droplet reader plate compartment	12006834

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QX200 Droplet Reader Accessories

Table 50. QX200 Droplet Reader accessories

Product	Description	Catalog Number
Computer	Computer that connects to the QX200 Droplet Reader for data collection and analysis	12017458
USB cable and power cord	Cable connecting the computer to the instrument and power cord connecting the instrument to the power source **Note:** Contact Bio-Rad Technical Support for replacement information.	Included
Droplet reader plate holders (2)	Used to position the 96-well plate in the droplet reader plate compartment	12006834

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ddPCR Consumables and Other Materials

Table 51. Consumables and other materials

Product	Description	Catalog Number
ddPCR 96-Well Plates	Cear well/clear shell semi-skirted plates	12001925 (pkg of 25)
DG32 Droplet Generation Cartridges	Microfluidic cartridge with gasket for the AutoDG	1864108 (pkg of 30) 1864109 (pkg of 60)
Pipet Tips	Pipet tips for the AutoDG	1864120 (pkg of 20) 1864121 (pkg of 40)
Pipet Tip Bins	Pipet tip waste bins for the AutoDG	1864125
Automated Droplet Generation Oils	Automated droplet generation oils for the AutoDG: ■ Automated Droplet Generation Oil for Probes ■ Automated Droplet Generation Oil for EvaGreen®	 1864110 1864112
Droplet Generation Oils	Droplet generation oil for the QX200 Droplet Generator: ■ Droplet Generation Oil for Probes, 10 x 7 ml ■ Droplet Generation Oil for EvaGreen®, 2 x 7 ml ■ Droplet Generation Oil for EvaGreen®, 10 x 7 ml	 1863005 1864005 1864006
Rainin Pipets	■ 20 µl for sample loading ■ 50 µl for droplet transfer ■ 8-channel, 200 µl for oil	L-20, L8-20 L-50, L8-50 L8-200
Rainin Pipet Tips	Filtered	GP-L10F GP-L200F
Foil Plate Seals for PX1	Pierceable foil plate seals for PX1 Plate Sealer	1814040
ddPCR Droplet Reader Oil	Droplet reader oil for the QX600 Droplet Reader and QX200 Droplet Reader	1863004
Droplet reader waste bottle	You can use an empty droplet reader oil bottle to collect the waste from droplet reading.	N/A

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ddPCR Supermixes

Table 52. ddPCR supermixes

Supermix	Description	Catalog number	
ddPCR EvaGreen® Supermix	2x supermix, for use in nucleic acid sample preparation with the QX600 and QX200 ddPCR systems	1864033 1864034 1864035 1864036	2ml (2 x 1ml) 5ml (5 x 1ml) 25ml (25 x 1ml) 50ml (50 x 1ml)
ddPCR Supermix for Probes (no dUTP)	2x supermix, for use in nucleic acid sample preparation with the QX600 and QX200 ddPCR systems	1863023 1863024 1863025	2ml (2 x 1ml) 5ml (5 x 1ml) 25ml (25 x 1)
ddPCR Supermix for Probes	2x supermix, for use in sample preparation with the QX600 and QX200 ddPCR systems	1863026 1863010 1863027 1863028	2ml (2 x 1ml) 5ml (5 x 1ml) 25ml (25 x 1ml) 50ml (50 x 1ml)
ddPCR Multiplex Supermix	4x supermix especially suited for probe-based detection of multiple targets in DNA samples using the QX600 and QX200 ddPCR systems	12005909 12005910 12005911	1.2ml (2 x 0.6ml) 3 ml (5 x 0.6 ml) 12.5 ml (5 x 2.5 ml),
ddPCR Supermix for Residual DNA Quantification	2x supermix, for use in residual DNA detection with the QX600 and QX200 ddPCR systems	1864037 1864038 1864039 1864040	2ml (2 x 1ml) 5ml (5 x 1ml) 25ml (25 x 1ml) 50ml (50 x 1ml)
One-step RT-ddPCR Advanced Kit for Probes	200 or 500x 20 µl reaction kit, for absolute quantification of target RNA in a one-step format with the QX600 and QX200 ddPCR systems	1864021 1864022	2ml (2 x 1ml) 5ml (5 x 1ml)

Buffer Controls

Table 53. Buffer controls

Buffer	Catalog Number
ddPCR Buffer Control Kit for Probes	1863052
QX200 Buffer Control Kit for EvaGreen®	1864052

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ddPCR Kits

Table 54. ddPCR Kits

Product	Description	Catalog number
ddPCR Mutation Screening Kits		
ddPCR KRAS G12/G13 Screening Kit		1863506
ddPCR KRAS Q61 Screening Kit		12001626
ddPCR BRAF V600 Screening Kit		12001037
ddPCR NRAS Q61 Screening Kit		12001006
ddPCR NRAS G12 Screening Kit		12001094
ddPCR NRAS G12/G13 Screening Kit		12001627
ddPCR EGFR Exon 19 Deletions Screening Kit		12002392
All kits include 20x multiplex assay and 2x ddPCR supermix for probes (no dUTP).		
ddPCR Residual DNA Quantification Kits		
ddPCR CHO Residual DNA Quantification Kit		17000031
ddPCR E.coli Residual DNA Quantification Kit		17000032
200x 20 µl reactions, includes 20x CHO or E.coli RDQ assay and 2x ddPCR Supermix for Residual DNA Quantification.		
ddPCR Copy Number Determination Kits		
ddPCR SMN1 Copy Number Determination Kit		1863500
ddPCR SMN2 Copy Number Determination Kit		1863503
200x 20 µl reactions, includes assay at 20x concentration, 2x ddPCR Supermix for Probes (no dUTP) and positive controls		
ddPCR Library Quantification Kit		
ddPCR Library Quantification Kit for Illumina TruSeq		1863040
200x 20 µl reactions, includes 1 vial of primers and probes at 20x concentration, 2x ddPCR Supermix for Probes (no dUTP) and positive controls		

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ddPCR Assays

Table 55. ddPCR Assays

Product	Catalog number	
ddPCR HDR Gene Edit Assay	12002312	100 rxns
ddPCR HDR Gene Edit Assay	12002313	500 rxns
ddPCR HDR Gene Edit Package	12003796	1,000 rxns
ddPCR HDR Ref Assay, Predesigned	12003805	100 rxns
ddPCR HDR Ref Assay, Predesigned	12003806	500 rxns
ddPCR HDR Ref Package, Predesigned	12003793	1,000 rxns
ddPCR NHEJ Gene Edit Assay	12002314	100 rxns
ddPCR NHEJ Gene Edit Assay	12002315	500 rxns
ddPCR NHEJ Gene Edit Package	12003794	1,000 rxns

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BIO-RAD Bio-Rad Laboratories, Inc.

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Website bio-rad.com USA 1 800 424 6723 Australia 61 2 9914 2800 Austria 00 800 00 24 67 23 Belgium 00 800 00 24 67 23 Brazil 4003 0399
Canada 1 905 364 3435 China 86 21 6169 8500 Czech Republic 00 800 00 24 67 23 Denmark 00 800 00 24 67 23 Finland 00 800 00 24 67 23
France 00 800 00 24 67 23 Germany 00 800 00 24 67 23 Hong Kong 852 2789 3300 Hungary 00 800 00 24 67 23 India 91 124 4029300 Israel 0 3 9636050
Italy 00 800 00 24 67 23 Japan 81 3 6361 7000 Korea 82 080 007 7373 Luxembourg 00 800 00 24 67 23 Mexico 52 555 488 7670
The Netherlands 00 800 00 24 67 23 New Zealand 64 9 415 2280 Norway 00 800 00 24 67 23 Poland 00 800 00 24 67 23 Portugal 00 800 00 24 67 23
Russian Federation 00 800 00 24 67 23 Singapore 65 6415 3188 South Africa 00 800 00 24 67 23 Spain 00 800 00 24 67 23 Sweden 00 800 00 24 67 23
Switzerland 00 800 00 24 67 23 Taiwan 886 2 2578 7189 Thailand 66 2 651 8311 United Arab Emirates 36 1 459 6150 United Kingdom 00 800 00 24 67 23

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Droplet Digital PCR

QX600™ Droplet Digital™ PCR System



Visit bio-rad.com/QX600 for more information.

BIO-RAD

MULTIPLEXING SIMPLIFIED — GET MORE DATA FROM YOUR PRECIOUS SAMPLES

The QX600 Droplet Digital PCR (ddPCR™) System provides advanced, six-color multiplexing to maximize the number of biomarkers interrogated per well, allowing the preservation of precious samples and reducing laboratory burden. For maximum flexibility, the QX200™ Droplet Digital PCR System assays, consumables, and reagents can be used on the QX600 ddPCR System.



FEATURES AND BENEFITS

SENSITIVE MULTIPLEXING

- 6-color detection capability (FAM/EvaGreen®, HEX/VIC, Cy5, Cy5.5, ROX, and ATTO 590)
- Quantification of 12 targets in a single well
- Absolute quantification with 0.1% or better sensitivity

ACCURATE AND REPRODUCIBLE RESULTS

- Confidence in your results — the system has been qualified by traceable material from the National Measurement Institute of Australia (NMIA)
- Reproducible results — market-leading ddPCR technology has been cited in reproducibility studies

PORTFOLIO COMPATIBILITY

- Same assays, reagents, and consumables as QX200 Droplet Digital PCR System
- Extensive menu of existing ddPCR Assays for EvaGreen® and hydrolysis probe chemistry

POWERFUL SOFTWARE

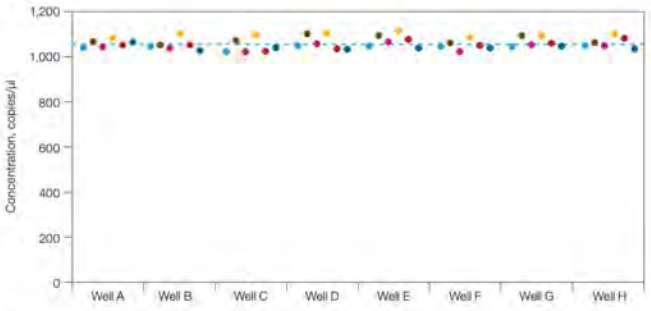
- Results that are easy to analyze
- Premium edition available featuring audit trails and tools for U.S. FDA 21 CFR Part 11 compliance
- Premium edition supports assay protocol files for automated data analysis and reporting

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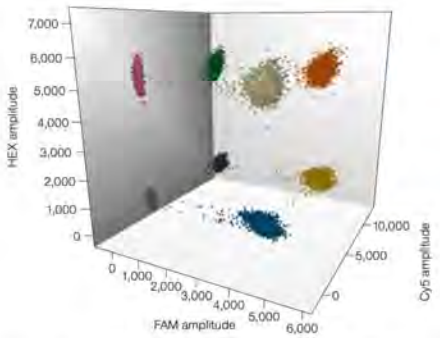
SIX-COLOR DETECTION CAPABILITY WITH HIGH ACCURACY, PRECISION, AND REPRODUCIBILITY

The QX600 ddPCR System offers six-color multiplexing for the clear discrimination of multiple targets and more accurate and reproducible quantification with six-color detection capability (FAM/EvaGreen®, HEX/VIC, Cy5, Cy5.5, ROX, and ATTO 590). It has been qualified by traceable material from the National Measurement Institute of Australia.

A



B



C

Metric	QX600 ddPCR System, %*	Other Digital PCR Platform, %
Accuracy, difference from NMIA target concentration	0.7	3.2**
Precision, % CV, mean concentration across a plate	1.7	3.5–3.8***

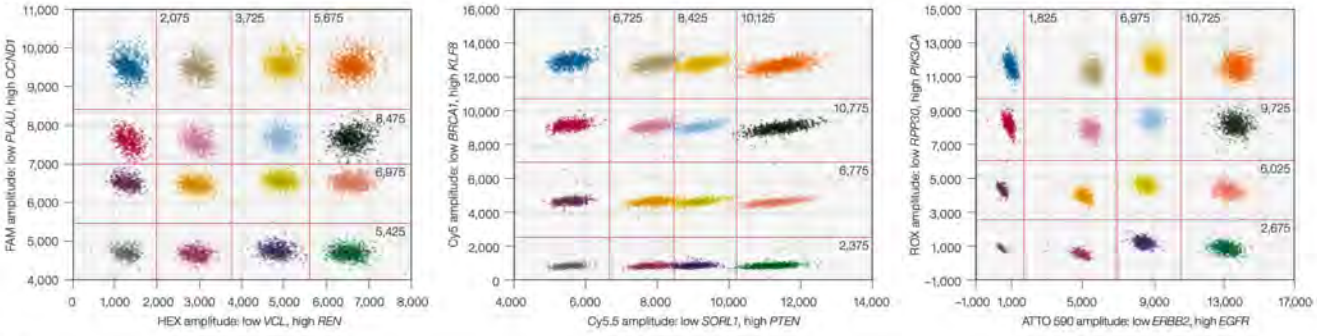
* NMIA standard, data shown, 2023.
** NIST standard, as published by competitor, 2023.
*** *ERBB2* assay, as published by competitor, 2022.

Accurate, precise, and reproducible data of a known metrology standard for six targets. A, concentration plot shows accurate, precise, and reproducible values across eight wells of the 6-plex assay. NMIA standard target concentration is 1,060 copies/µl (---). FAM (■), HEX (■), Cy5 (■), Cy5.5 (■), ROX (■), ATTO 590 (■). **B**, 3-D fluorescence amplitude plots of a 6-plex NMIA standard. **C**, the concentration calls for the NMIA standard were accurate and precise on the QX600 Droplet Reader, with no greater than 1% difference compared to the NMIA standard concentration and with CV <2.0%. Data were generated on a QX600 Droplet Reader using ddPCR Multiplex Supermix and a Bio-Rad performance qualification test. CV, coefficient of variation; NMIA, National Measurement Institute of Australia.

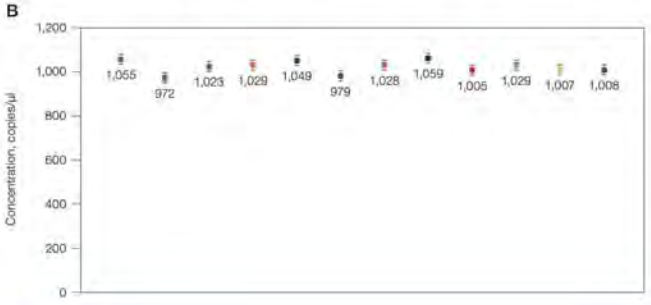
QUANTIFICATION OF 12 TARGETS IN A SINGLE WELL

The QX600 ddPCR System provides unprecedented multiplexing capability for 12 copy number targets in a single well using amplitude multiplexing.

A



B



Detection of 12 copy number targets in a single well. A, 2-D fluorescence amplitude plots show a single well with 12 targets across six fluorescence channels using amplitude multiplexing; **B**, concentration plot shows measured concentration of 12 genes (*PLAU, CCND1, VCL, REN, BRCA1, KLF8, SORL1, PTEN, RPP30, PIK3CA, ERBB2,* and *EGFR*) for which the expected concentration was 1,000 copies/µl. Data were generated on a QX600 Droplet Reader using ddPCR Multiplex Supermix and ddPCR Copy Number Assays (available at bio-rad.com/digital-assays).

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WHY BIO-RAD?

Bio-Rad has more than 70 years' experience supporting life science research and biopharma. We are a leader in digital PCR and other technologies. Our innovative products are available as stand-alone solutions or combined into workflows that provide the right answer the first time, every time with minimized downtime.

KEY APPLICATIONS

The QX600 ddPCR System's six-color detection capabilities set a new standard in user friendliness when developing highly multiplexed assays in the following key applications.

Mutation Detection
The QX600 ddPCR System enables six-color mutation detection with ultrasensitive, cost-effective circulating tumor DNA analysis and a rapid turnaround time — unlike next-generation sequencing and other digital PCR platforms.

Viral Titer and Vector Identity
The QX600 ddPCR System enables rapid viral vector titer characterization of multiple critical targets like inverted terminal repeat, target gene of interest, control elements, and terminator, all in the same well, saving time and cost.

Wastewater Surveillance
The QX600 ddPCR System can quantify multiple pathogens in wastewater with high precision and sensitivity.

DIGITAL ASSAYS

Bio-Rad has a large menu of off-the-shelf assays, kits, and design services. The digital assays website allows ddPCR users to find and buy assays across a variety of applications. You can search curated lists of predesigned and validated assays, or design and customize your own assay by using one of the assay design engines. Built by ddPCR experts, the design engines customize a ready-to-use assay optimized for Droplet Digital PCR.

Go to **bio-rad.com/digital-assays** to find and buy ddPCR Assays.

SPECIFICATIONS

Parameter	QX600 Droplet Reader	Automated Droplet Generator	QX200 Droplet Generator
Dimensions (W x D x H)	66 x 52 x 29 cm (26 x 20 x 11 in.)	66 x 56 x 66 cm (26 x 22 x 26 in.)	28 x 36 x 13 cm (11 x 14 x 5 in.)
Weight	26 kg (56.6 lb)	45.5 kg (100 lb)	4.5 kg (10 lb)
Capacity	1–96 samples/run	1–96 samples/run	1–8 samples/run
Droplets per 20 µl sample	20,000	20,000	20,000
Detection channels	FAM/EvaGreen®, HEX/VIC, Cy5, Cy5.5, ROX, and ATTO 590	—	—
Sample Illumination	Light-emitting diodes	—	—
Sample detection	Avalanche photodiodes	—	—

ORDERING INFORMATION

Catalog #	Description
QX600 Droplet Digital PCR Systems	
17007769	**QX600 Droplet Digital PCR System**
17008371	**QX600 AutoDG™ Droplet Digital PCR System**
12013328	**QX600 Droplet Reader**
1864002	**QX200 Droplet Generator**
1864101	**Automated Droplet Generator**

Visit **bio-rad.com/QX600** for more information.

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Purchase of Digital PCR and/or Single-Cell NGS Sample Preparation products (the "Products") from Bio-Rad Laboratories is subject to Bio-Rad Laboratories, Inc. Standard Terms and Conditions of Sale, which can be accessed at https://www.bio-rad.com/en-us/terms-conditions. Unless we expressly state otherwise in additional Terms and Conditions, no rights are granted for you to distribute or resell the Products. Unless we expressly state otherwise in additional Terms and Conditions, no rights are granted for the development or commercialization of diagnostic assays for use with the Products without a license from Bio-Rad. It is the user's obligation to obtain a commercial license from Bio-Rad for (i) all commercial uses (not just diagnostic uses) and (ii) sale of assays for use on Bio-Rad's dPCR and ddSEQ instruments. The Products and/or their use are covered by U.S. and foreign patents and/or pending patent applications owned by or under license to Bio-Rad Laboratories, Inc. See https://www.bio-rad.com/en-us/trademarks.

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France 00 800 00 24 67 23 **Germany** 00 800 00 24 67 23 **Hong Kong** 852 2789 3300 **Hungary** 00 800 00 24 67 23 **India** 91 124 4029300 **Israel** 0 3 9636050
Italy 00 800 00 24 67 23 **Japan** 81 3 6361 7000 **Korea** 82 080 007 7373 **Luxembourg** 00 800 00 24 67 23 **Mexico** 52 555 488 7670
The Netherlands 00 800 00 24 67 23 **New Zealand** 64 9 415 2280 **Norway** 00 800 00 24 67 23 **Poland** 00 800 00 24 67 23 **Portugal** 00 800 00 24 67 23
Russian Federation 00 800 00 24 67 23 **Singapore** 65 6415 3188 **South Africa** 00 800 00 24 67 23 **Spain** 00 800 00 24 67 23 **Sweden** 00 800 00 24 67 23
Switzerland 00 800 00 24 67 23 **Taiwan** 886 2 2578 7189 **Thailand** 66 2 651 8311 **United Arab Emirates** 36 1 459 6150 **United Kingdom** 00 800 00 24 67 23

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Assays for Droplet Digital PCR

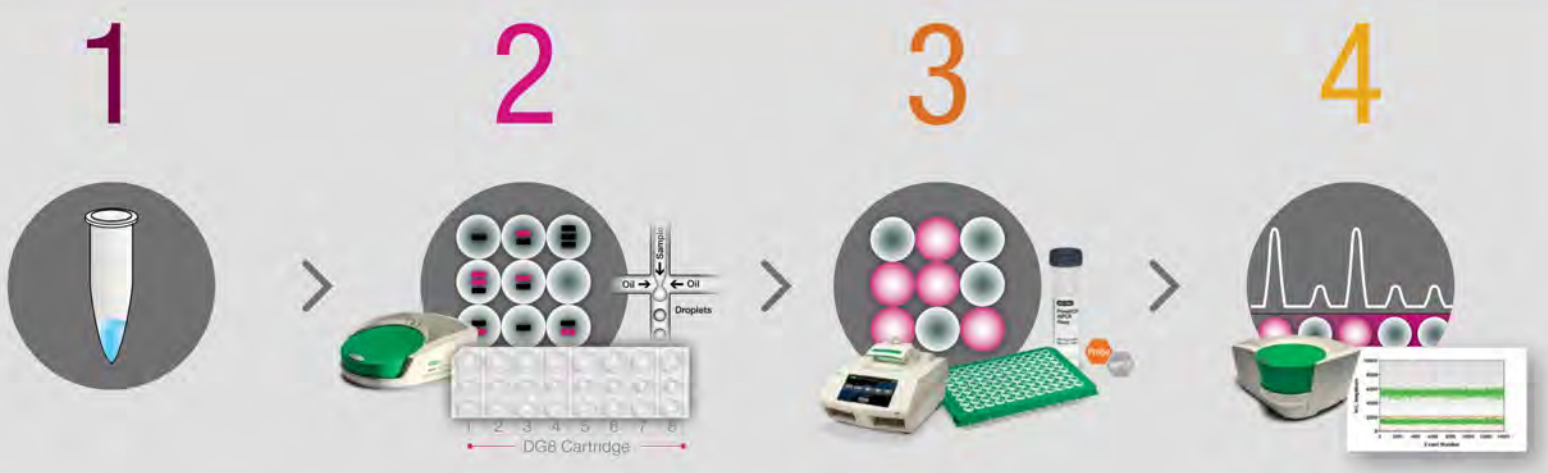
BIO-RAD

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WORKFLOW

A VERSATILE AND SCALABLE WORKFLOW

Bio-Rad's Droplet Digital PCR (ddPCR) technology is based on water-oil emulsion droplet technology to partition the sample. The ddPCR technology is composed of a droplet generator, a droplet reader, and associated consumables. The droplet generator partitions each sample into 20,000 uniform nanoliter-sized droplets containing target and background DNA in random distribution. Each droplet contains a separate reaction. Droplets are transferred to a 96-well PCR plate and PCR is performed to end point in a thermal cycler. The droplets are singulated and pass by an optical detection system. Up to 96 samples can be processed per run.



Prepare ddPCR reaction mix

- Combine DNA/RNA sample, primers, and/or probes with one of Bio-Rad's ddPCR supermixes
- Fully validated ddPCR assays can be used

Generate droplets

- Load the ddPCR reaction mix into the wells of a droplet generator cartridge
- 8 x 20,000 droplets are generated from each run in the QX200 Droplet Generator
- Target DNA (▬) and background DNA (▬) are randomly distributed in droplets

Perform PCR

- Transfer the droplets to a 96-well PCR plate and seal the plate
- Run the PCR protocol

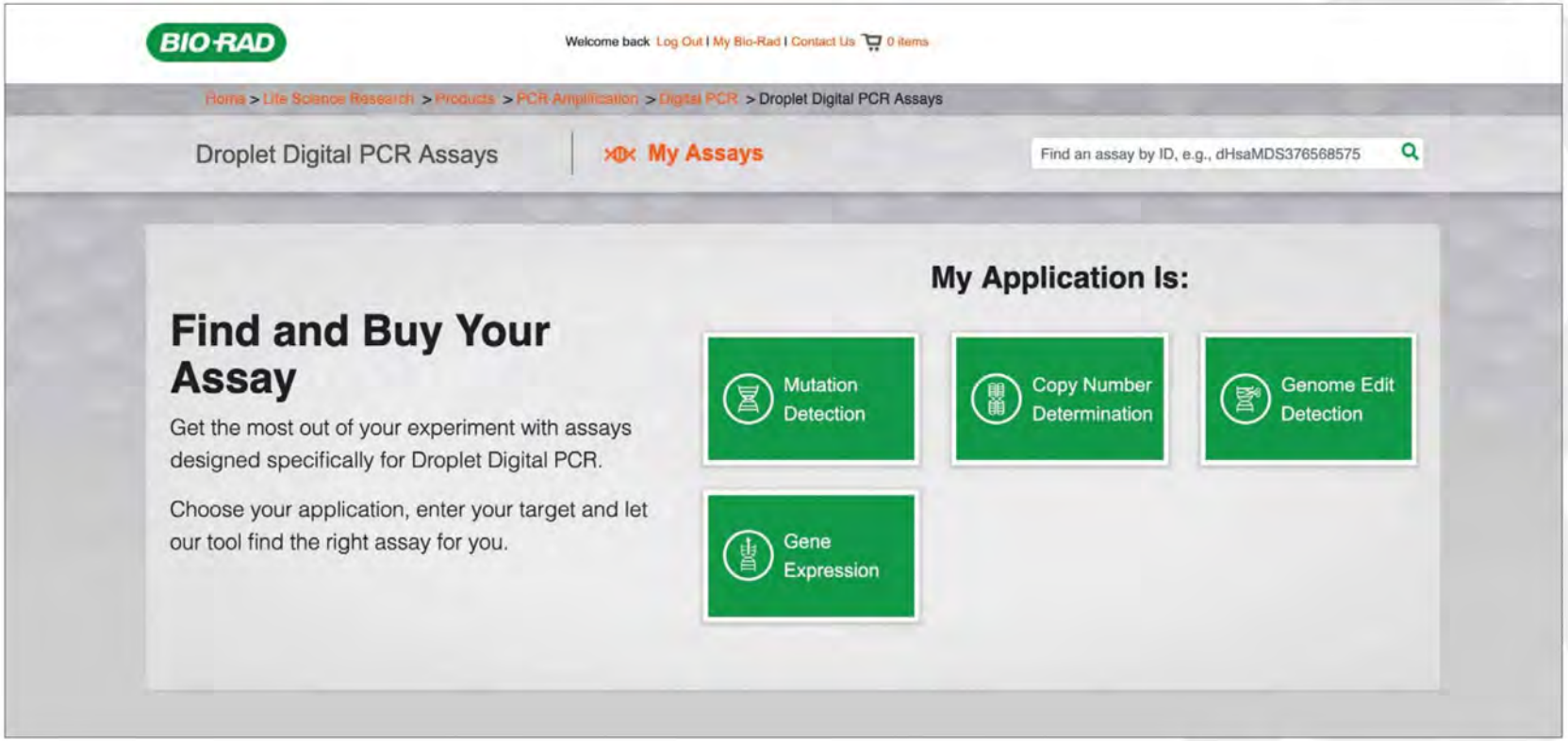
Read and analyze results

- After PCR, load the 96-well PCR plate into the QX200 Droplet Reader
- Positive and negative droplets in each sample are read
- Analyze concentrations

ASSAYS FOR DROPLET DIGITAL PCR

EXPERTLY DESIGNED ASSAYS ARE A CLICK AWAY THROUGH OUR ASSAY DESIGN ENGINE

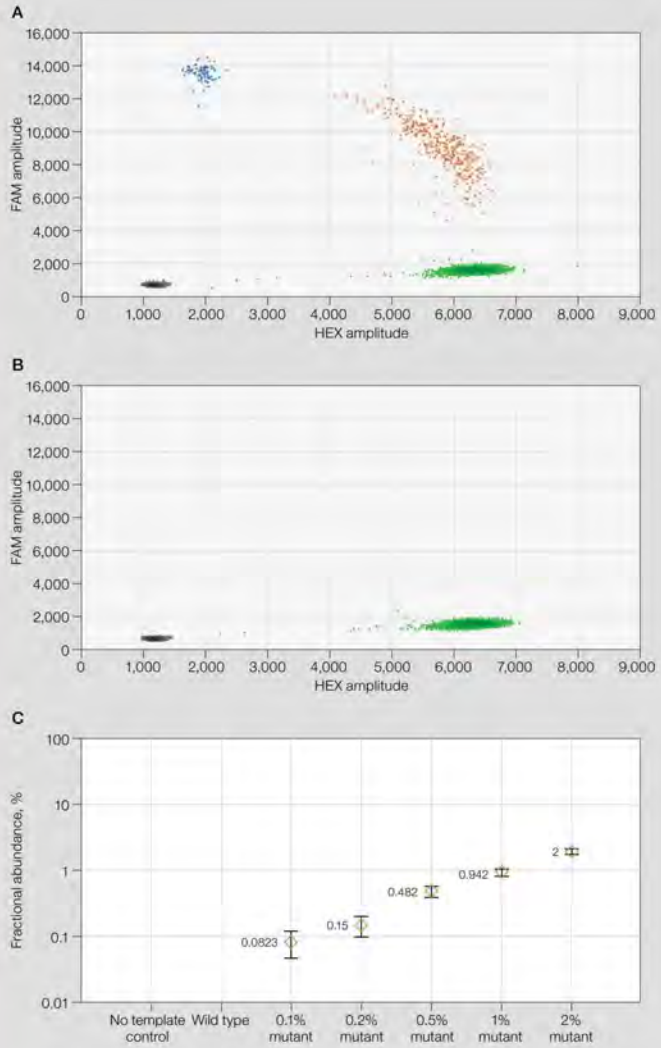
Go to **bio-rad.com/Digital-Assays** for more information.



MUTATION DETECTION ASSAYS

THE NEW GOLD STANDARD IN QUANTIFYING RARE CANCER MUTATIONS

One application that harnesses the power of ddPCR is mutation detection, where a biomarker exists within a background of a highly abundant counterpart that can differ by only a single nucleotide. Many methods for mutation analysis have poor selectivity and fail to detect mutant sequences with abundances of less than one in 100 wild-type sequences. Mutation detection assays on the ddPCR platform enable detection of minute mutant fractions without pre-amp because partitioning increases sensitivity by isolating the target signal from competing background.



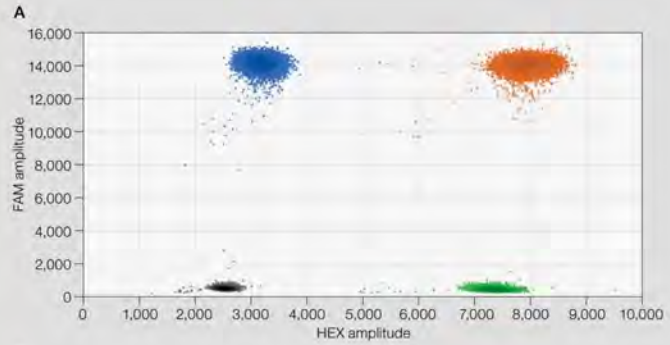
High sensitivity of ddPCR mutation detection assays provides precise quantification of *JAK2 V617F* mutation in the presence of wild-type DNA. A, 2-D fluorescence amplitude plot shows duplicate wells of a mixed mutant: wild-type sample across multiple percentage mutant blends. The black cluster on the plot represents the negative droplets, the green cluster represents the droplets that are positive for wild-type DNA only, the blue cluster represents the droplets that are positive for mutant DNA only, and the orange cluster represents the droplets that are positive for both targets. **B**, 2-D fluorescence amplitude plot shows duplicate wells of a wild-type–only sample. **C**, fractional abundance plot shows the percentage frequency (orange markers) of the mutant DNA in a wild-type DNA background. All error bars represent the 95% confidence interval.

Exhibit G

COPY NUMBER ASSAYS

AT LAST — IDENTIFYING COPY NUMBER VARIATIONS WITH CONFIDENCE

The major technical challenge in copy number assessment is the ability to discriminate, with statistical confidence, between consecutive copy number states. Current methods to analyze copy number variation (CNV) include array comparative genomic hybridization (aCGH), quantitative PCR (qPCR), and sequencing. These methods lack the sensitivity and resolution needed for this fine degree of quantitative discrimination in CNV analysis. The massive partitioning of a ddPCR reaction into 20,000 droplets enables the discrimination required to resolve small fold changes.



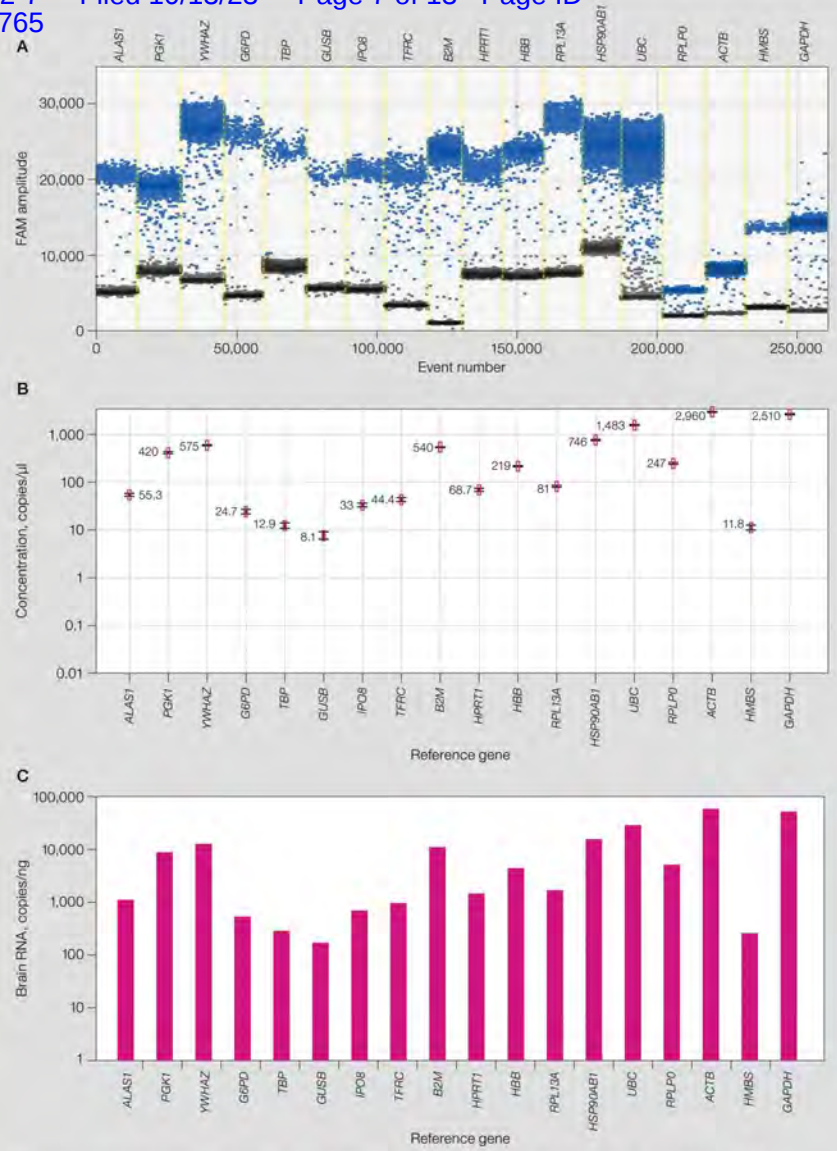
Droplet Digital PCR copy number assays provide superior resolution and precision. **A**, 2-D fluorescence amplitude plot shows four replicate wells of a copy number sample duplexed with *ERBB2* and *RPP30* assays. The black cluster on the plot represents the negative droplets, the green cluster represents the droplets that are positive for *RPP30* reference only, the blue cluster represents the droplets that are positive for *ERBB2* only, and the orange cluster represents the droplets that are positive for both *ERBB2* and *RPP30* targets. **B**, copy number plot shows multiple wells at copy number 2 with precise replicate and merged well values. **C**, copy number plot shows multiple oncogene copy number determinations for various genes with the same NCI-H358 lung cancer sample. All error bars represent the 95% confidence interval.

GENE EXPRESSION ASSAYS

BETTER GENE EXPRESSION DATA FOR SIMILAR COST

With its high precision, ddPCR can be used to detect small fold changes in expression of a target gene between samples. Choose from over 40,000 existing human and mouse gene expression assays, which can be used with QX200 ddPCR EvaGreen® Supermix and modified thermal cycling conditions.

- Contrary to expectations, multiplexed ddPCR gene expression studies have similar costs to singleplex SYBR® qPCR studies
- Singlicate multiplexed ddPCR studies make much better use of rare samples than SYBR® qPCR
- Droplet Digital PCR allows advanced new strategies such as SELFIE Digital PCR



Robust gene expression assays used in ddPCR with EvaGreen® provide precise quantification of 18 reference genes. A, 1-D fluorescence amplitude plot shows results of a two-step reverse transcription ddPCR reference assay panel run on human brain RNA. The black clusters on the plot represent the negative droplets and the blue clusters represent the droplets that are positive for the respective reference assay. **B**, concentration plot shows various gene concentrations of cDNA calculated as copies/µl on a log10 scale. **C**, calculated RNA concentrations in the original human brain RNA sample for each reference gene. All error bars represent the 95% confidence interval.

Exhibit G

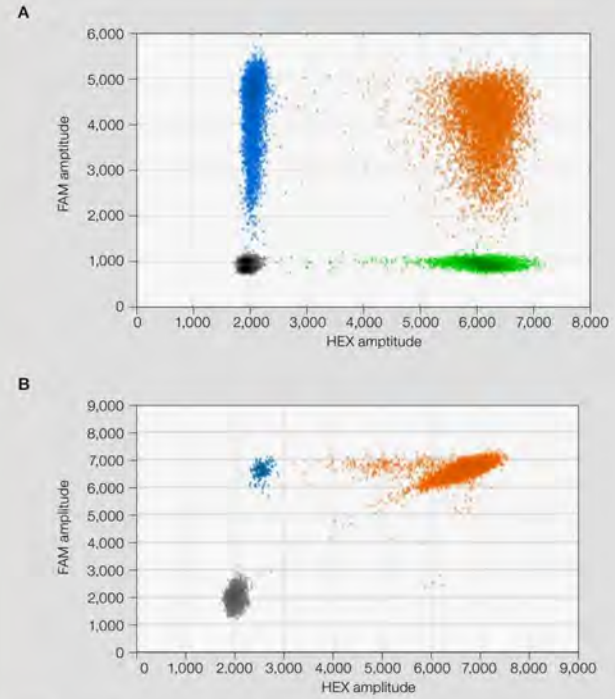
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GENOME EDIT DETECTION ASSAYS

FAST. SIMPLE. PRECISE.

Droplet Digital PCR enables a rapid, cost-effective, and ultrasensitive method for detecting genome editing events created using nucleases that cause double-stranded breaks in DNA, such as CRISPR-Cas9 or other gene editing tools.

- Detect HDR (homology directed repair) and NHEJ (non-homologous end joining) events present at frequencies of ≤0.5%
- Provide absolute quantification of genome editing from as little as 5 ng of total gDNA
- Design and order ddPCR Assays to detect HDR and NHEJ edit events for any target on Bio-Rad's Digital Assay Site



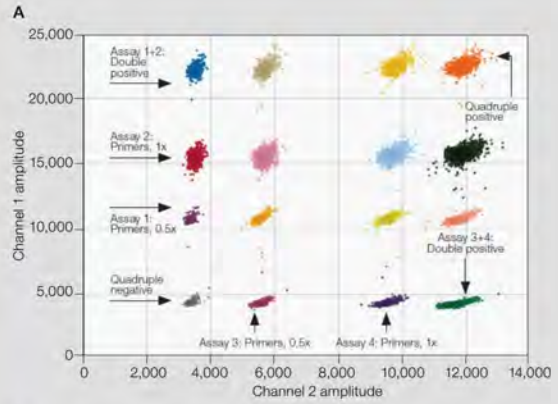
HDR and NHEJ assay readouts. A, HDR mutation-positive control (WT + 20% gblocks gene fragment containing a single base pair substitution) with HDR GED + HDR REF Assay. The HDR edits are FAM+HEX–, while the HEX+ and FAM+HEX+ positive clusters represent the total number of copies, including WT, NHEJ, and HDR edits. **B**, NHEJ mutation-positive control (WT + 1% gblocks gene fragment containing a one base pair deletion at the predicted cut site). The WT cluster is positive for both FAM and HEX, while the NHEJ mutant single-positive droplets are positive for FAM only.

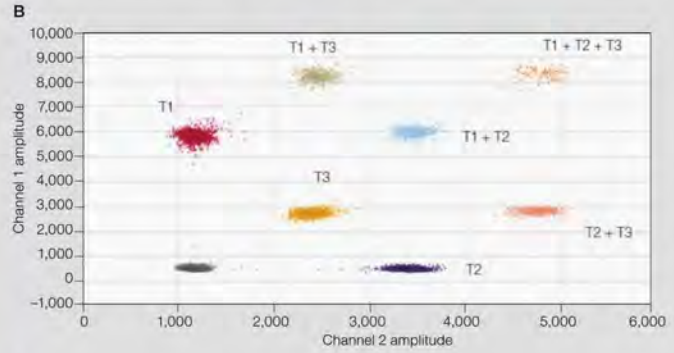
MULTIPLEXING STRATEGIES

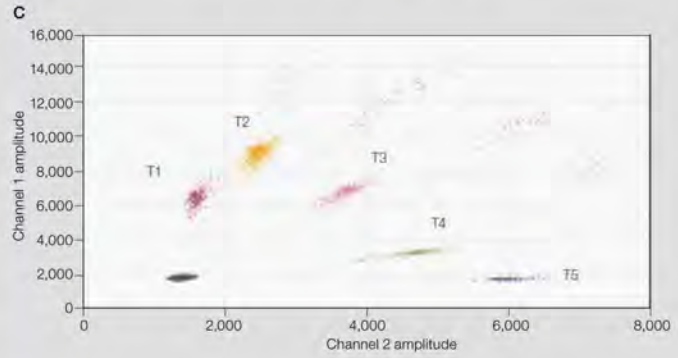
HIGHER-ORDER MULTIPLEXING — SAME COLOR CHANNELS, MORE ANSWERS

The number of targets measured in an assay need not be limited to the number of color channels available on Bio-Rad's Droplet Digital PCR Systems. Multiple targets can be measured in a single reaction, enabling higher-order multiplexing. Various strategies can be employed to maximize data generated from each channel:

- **Amplitude-Based Multiplexing** — targets are detected via probes conjugated with a single dye at different final concentrations in an endpoint reaction
- **Radial/Ratio-Based Multiplexing** — targets are detected using probes mixed at different probe ratios to create unique endpoint fluorescence.
- **Nondiscriminating Multiplexing** — targets are detected but not uniquely identified







Multiplexing with two color channels: A, detection of four targets by amplitude multiplexing. **B**, detection of three targets by radial/ratio-based multiplexing. Assay used the following ratios of FAM to HEX: T1, 1:0; T2, 0:1; T3, 1:1. **C**, detection of five targets by radial/ratio-based multiplexing. Assay used the following ratios of FAM to HEX: T1, 1:0; T2, 3:1; T3, 1:1; T4, 1:3; T5, 0:1.

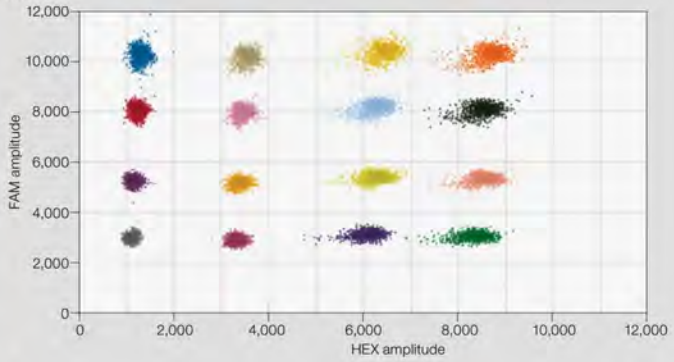
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MULTIPLEXING STRATEGIES

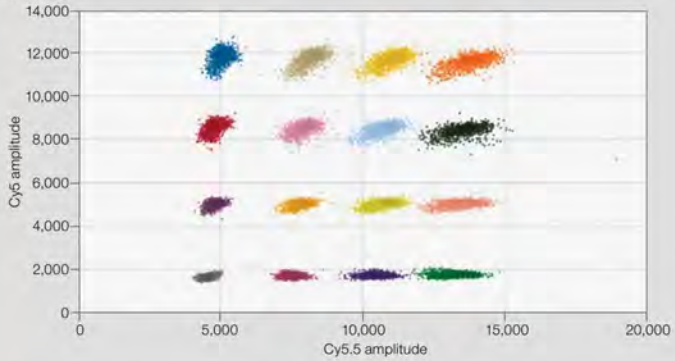
ADVANCED MULTIPLEXING — MORE COLOR CHANNELS, EVEN MORE ANSWERS

Bio-Rad's QX ONE Droplet Digital PCR System offers four color channels — FAM, HEX, Cy5, and Cy5.5 — thereby providing additional multiplexing flexibility. By using Bio-Rad's ddPCR Multiplex Supermix on the QX ONE ddPCR System, as many as eight targets can be detected and measured in a single reaction. Such advanced multiplexing is made possible using strategies such as amplitude multiplexing in conjunction with four color channels. Extract as much information as possible with high sensitivity, using as little sample as possible in a fast, cost-effective manner.

A



B



C

Dye Name	Target	Concentration, copies/µl	CNV
FAM Lo	*PLAU*	668	1.96
FAM Hi	*CCND1*	658	1.95
HEX Lo	*VCL*	662	1.96
HEX Hi	*REN*	665	1.97
Cy5 Lo	*BRCA1*	658	1.95
Cy5 Hi	*KLF8*	691	2.05
Cy5.5 Lo	*SORL1*	672	1.99
Cy5.5 Hi	*PTEN*	675	–

8-plex amplitude multiplexing on QX ONE System. A, detection of four targets on FAM/HEX 2-D plot. *PLAU* (FAM Lo), *CCND1* (FAM Hi), *VCL* (HEX Lo), *REN* (HEX Hi). **B**, detection of four targets on Cy5/Cy5.5 2-D plot. *BRCA1* (Cy5 Lo), *KLF8* (Cy5 Hi), *SORL1* (Cy5.5 Lo), *PTEN* (Cy5.5 Hi). **C**, measured concentration of each target in copies/µl. *PTEN* (Cy5.5 Hi) is reference.

Exhibit G
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COMPREHENSIVE ASSAY PORTFOLIO

SOLUTIONS OFFERED FOR NUMEROUS APPLICATIONS

Droplet Digital PCR Assays and Kits are made for numerous applications, including mutation detection, copy number determination, genome edit detection, gene expression, residual DNA quantification, and library quantification.

Multiplex Mutation Screening

For rapid screening of several key cancer mutations in a single reaction.

- High sensitivity (limit of detection ≤ 0.5%)
- Work with low amounts of input DNA without pre-amplification
- Assays available for *BRAF V600*, *KRAS G12/G13*, *KRAS Q61*, *NRAS G12*, *NRAS G12/G13*, *RNAS Q61*, *EGFT* exon 19 deletions

Copy Number

For the detection of *SMN1* and *SMN2* gene copy number.

- Achieve superior performance with accurate and reproducible copy number calls for the gene of interest
- Perform high-throughput screening by processing several hundred samples in a single day

Residual DNA Quantification

For detection of CHO and *E. coli* host cell DNA.

- Highly precise, femtogram-level quantification of residual CHO or *E. coli* DNA
- Direct quantification without DNA purification step

Library Quantification

For the quantification of NGS sequencing libraries.

- Provide information about library quality, such as adapter dimers, and indicate library insert size
- Provide more efficient and consistent loading of libraries for sequencing runs
- Enable balancing of pooled library samples

AN ASSAY PRODUCT LINE FOR EVERY NEED

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Assay Design Service

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Expert Design

See a list of our most sought after specialty assays, such as the Microsatellite Instability Assay for detection of MSI markers (*BAT25/BAT26*, *NR21/NR24*, and *Mono27*).

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