

Appendix B-1: Infringement Claim Chart for U.S. Patent No. 10,770,170 and QX600

Use of Bio-Rad's QX600 Droplet Digital PCR System ddPCR ("QX600") infringes Claims 1-20 of U.S. Patent No. 10,770,170 ("the '170 Patent").

The accused QX600 includes both the QX600 Droplet Digital PCR System and the QX600 AutoDG Droplet Digital PCR System, including all the various additional devices, software, accessories, and/or components used in connection with the QX600. Reference to QX600 will be understood to refer to both versions of the system.

As non-limiting examples, Plaintiffs identify where and how use of the Accused Product performs each and every step of the below claimed method. Elements from other charts may be used in combination with the below to perform the steps of the claimed method, and Plaintiffs incorporate all other '051 Charts here by reference.

On information and belief, the QX600 is used with other devices, accessories, and components to practice the claimed methods of the '051 Patent. See <https://www.bio-rad.com/en-us/product/qx600-droplet-digital-pcr-system?ID=b07d12ac-0585-fc4c-a586-3ddf20d5c4a0> and <https://www.bio-rad.com/en-us/product/qx600-autodg-droplet-digital-pcr-system?ID=0d9e49a4-cf92-f9a9-5779-ef412180f1ed>. A list of non-limiting examples of such devices, accessories, and/or components include:

- The QX600 Droplet Reader;
- Software, such as the QX Manager Software (Premium and/or Standard Editions);
- A droplet generator, such as the Bio-Rad Automated Droplet Generator and/or QX200 Droplet Generator;
- A thermal cycler, such as the C1000 Touch Thermal Cycler with 96-Deepwell Reaction Module and/or PTC Tempo Deepwell Thermal Cycler;
- A plate sealer, such as the PX1 PCR Plate Sealer;
- ddPCR kits and reagents, such as
 - o PrimePCR ddPCR Assays,
 - o ddPCR Multiplex Supermix,
 - o ddPCR Supermixes for Probes (No dUTP),
 - o ddPCR Supermix for Probes,
 - o One-Step RT-ddPCR Advanced Kit for Probes,
 - o ddPCR Buffer Control for Probes,
 - o Residual DNA Quantification Supermixes,
 - o QX200 ddPCR EvaGreen Supermix,
 - o QX200 Buffer Control for EvaGreen, and

- o ddPCR Library Quantification Kit for Illumina TruSeq;
- Consumables, such as
 - o ddPCR 96-Well Plates,
 - o Droplet Generation Oil for Probes,
 - o QX200 Droplet Generation Oil for EvaGreen,
 - o Droplet Reader Oil,
 - o DG8 Cartridges and DG8 Gaskets,
 - o DG8 Cartridge Holders,
 - o pierceable foil such as the PCR Plate Heat Seal,
 - o DG32 Cartridges for AutoDG,
 - o Pipet Tips for AutoDG,
 - o Pipet Tip Waste Bin for AutoDG;
- PrimePCR Primers, Assays and Arrays;
- Each of the assays identified in these Infringement Contentions; and/or
- Any other processes, procedures, devices, and/or system components used to facilitate the performance of the steps of the Asserted Claims.

See <https://www.bio-rad.com/en-us/product/qx600-droplet-digital-pcr-system?ID=b07d12ac-0585-fc4c-a586-3ddf20d5c4a0>;
<https://www.bio-rad.com/en-us/product/qx600-autodg-droplet-digital-pcr-system?ID=0d9e49a4-cf92-f9a9-5779-ef412180f1ed>.

Bio-Rad directly infringes the Asserted Claims by using the QX600 alone and/or with the above-listed devices, software, accessories, and/or components to perform each and every step of the claim methods below. By way of non-limiting example, Bio-Rad uses the QX600 in connection with (but not exclusively with) the various assays identified in these Infringement Contentions to practice each and every step of the Asserted Claims.

Bio-Rad also indirectly infringes the Asserted Claims by providing the QX600 alone and/or with the accompanying QX Manager Software. Bio-Rad also induces customers and/or end users to use the QX600 and/or QX Manager Software to infringe by inducing them to use these products in a way that performs each step of the Asserted Claims as described below and throughout these Infringement Contentions in violation of 35 U.S.C. § 271(b). By way of non-limiting example, such customers and/or end users use the QX600 in connection with (but not exclusively with) the various assays identified in these Infringement Contentions to practice each and every step of the Asserted Claims.

Bio-Rad also contributes to the infringement of customers and/or end-users by providing the QX600 and/or the QX Manager to the same who then perform each step of the Asserted Claims as described below and throughout these Infringement Contentions in

violation of 35 U.S.C. § 271(c). By way of non-limiting example, such customers and/or end users use the QX600 in connection with (but not exclusively with) the various assays identified in these Infringement Contentions to practice each and every step of the Asserted Claims.

On information and belief, Bio-Rad also supplies or causes to be supplied in or from the United States all or a substantial portion of the QX600 and/or QX Manager Software such that Bio-Rad induces infringe of customers or end users outside the United States in violation of 35 U.S.C. § 271(f)(1). For like reasons as described above, Bio-Rad contributes to the infringement of customers or end users outside the United States in violation of 35 U.S.C. § 271(f)(2).

On information and belief, to the extent Bio-Rad performs the steps of the Asserted Claims outside the United States and subsequently imports, offers to sell, sells, and/or uses the results of the claimed methods within the United States, Bio-Rad infringes in violation of 35 U.S.C. § 271(g).

Plaintiffs allege infringement both literally and under the doctrine of equivalents.

As non-limiting examples, Plaintiffs identify where and how use of the Accused Product performs each and every step of the below claimed method.

Claims	Exemplary Disclosure of Accused Product
[1-PRE] 1. A method of unambiguously detecting any unique combination of presence or absence of at least five polynucleotide analytes in a	To the extent the preamble is found to be limiting, the QX600 Droplet Digital PCR System is used to perform a method of unambiguously detecting any unique combination of presence or absence of at least five polynucleotide analytes in a plurality of droplets the presence or absence of at least seven polynucleotide analytes, in any combination of presence or absence, in a single sample solution volume, both literally and under the doctrine of equivalents. For example:

plurality of droplets, the method comprising:	QX600 Droplet Digital PCR System 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 More
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Overview

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**
 - 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

See <https://www.bio-rad.com/en-us/product/qx600-droplet-digital-pcr-system?ID=b07d12ac-0585-fc4c-a586-3ddf20d5c4a0>.

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

BIO_CC00008190 at BIO_CC00008191

	The ability to quantify 12 targets with 6 colors must include the steps of identifying the presence or absence of at least 7 of those targets. 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. BIO_CC00008190 at BIO_CC00008192 The system allows for clear discrimination of multiple targets and more accurate and reproducible quantification, which allows it to detect the presence or absence of polynucleotide analytes with high accuracy, precision, and reproducibility. 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. BIO_CC00007427 at BIO_CC00007449 The QX600 system provides absolute quantification of nucleic acid target sequences detecting the presence or absence of polynucleotide analytes, such as by counting nucleic acid molecules encapsulated in discrete volumetrically defined water-in-oil droplet partitions. The Automated Droplet Generator is compatible with QX600 Droplet Digital PCR Systems.
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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](#).

BIO_CC00007427 at BIO_CC00007458

The QX600 AutoDG Droplet Digital PCR System (ddPCR) offers workflow automation, delivers advanced six-color multiplexing, and absolute quantification of nucleic acid without sacrificing the best-in-class performance you have come to expect from Bio-Rad's ddPCR systems.

The AutoDG Instrument eliminates user-to-user variability, allowing you to fully exploit the precision of ddPCR technology by providing more consistent droplet counts across users and plates. The AutoDG Instrument also minimizes hands-on time — it takes less than 5 minutes to load the instrument, and no supervision or user activity is required during droplet generation.

Benefits of the QX600 AutoDG Droplet Digital PCR System

The Automated Droplet Generator offers:

- A simplified ddPCR workflow
- Generates droplets for up to 96 ddPCR samples in under 45 minutes
- Compatibility with QX200™ reagents and consumables
- Guided setup and loading with user-friendly touch-screen interface
- Run-to-run flexibility with user-definable plate layout in 8-well increments
- Reduced contamination risk with HEPA-filtered enclosure allowing the system to be placed in any lab environment

See <https://www.bio-rad.com/en-us/product/qx600-autodg-droplet-digital-pcr-system?ID=0d9e49a4-cf92-f9a9-5779-ef412180f1ed>

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

BIO_CC00007427 at BIO_CC00007633

Automated Droplet Generator #1864101



Overview

AutoDG Instrument for automated droplet generation of up to 96 samples, for use with the QX600/QX200 Droplet Digital PCR Systems

List Price: **Inquire**

Your Price: [Log in](#) to see your price

Quantity: [Add to Cart](#) [Add to Quote](#) [Add to Hot List](#)

See <https://www.bio-rad.com/en-us/sku/1864101-automated-droplet-generator?ID=1864101> Such samples may include, but not be limited to, the following applications:

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.

See <https://www.bio-rad.com/en-us/product/qx600-droplet-digital-pcr-system?ID=b07d12ac-0585-fc4c-a586-3ddf20d5c4a0>; see also “Multiplex Assay Design for Epidemiological Wastewater Monitoring with Droplet Digital PCR Technology,” BIO_CC00008198; see also “Reproducible High-Resolution Characterization of AAV Vector Genome Identity Using the QX600,” BIO_CC00008202; see also “Mutation Detection Multiplexing Using the QX600 Droplet Digital PCR System,” BIO_CC00008194.

On information and belief, these applications comprise at least five polynucleotide analytes. For example:

In one example, the QX600 ddPCR System can be used for highly sensitive detection of commonly monitored pathogens in a rapid and accurate manner. The Multiplex Assay Design for Epidemiological Wastewater Monitoring with ddPCR Technology was used to create two 6-plex (e.g., 6 targets) wastewater assays and one single-target control assay for the following pathogens: adenoviruses, noroviruses, enteroviruses, Legionella, influenza A, influenza B, two monkeypox virus (MPXV) variants, and mouse hepatitis virus (MHV) as control.

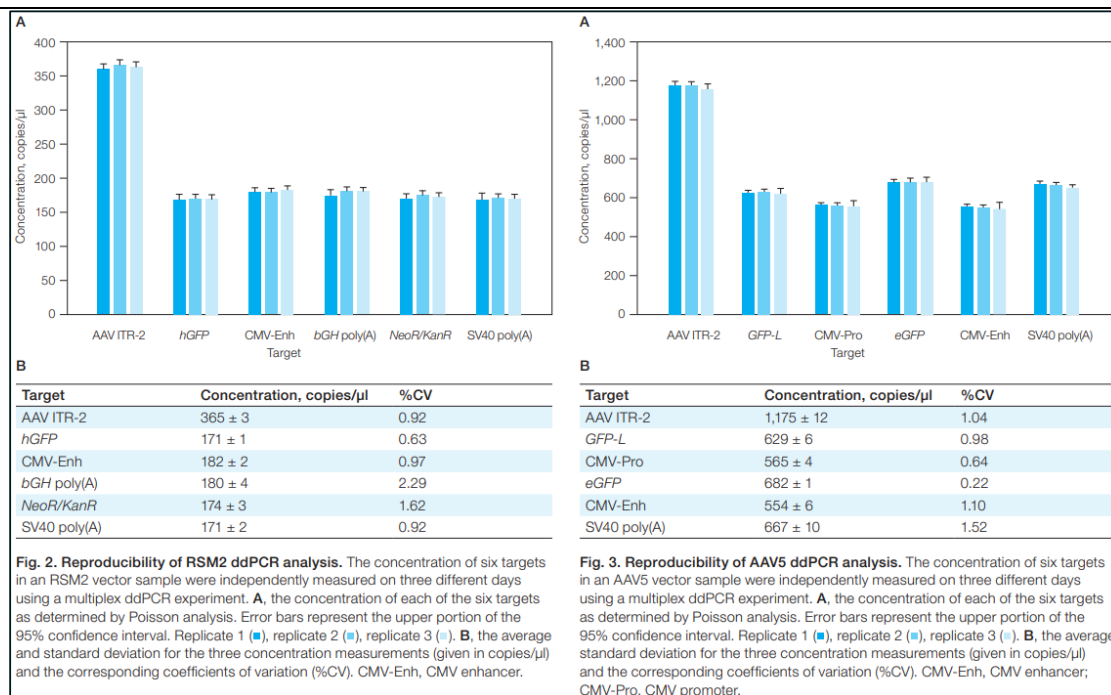
Table 1. Pathogens and assigned fluorophores targeted in assays 1 and 2 and the MHV control assay.							
	FAM	HEX	FAM/HEX	ROX	ATTO 590	Cy5	Cy5.5
Assay 1	Adenovirus	Norovirus	MHV control assay	MPXV (West African Variant 1)	MPXV (West African Variant 2)	Enterovirus	Legionella
Assay 2	Adenovirus	Norovirus	MHV control assay	MPXV (West African Variant 1)	MPXV (West African Variant 2)	Influenza A	Influenza B

MHV, mouse hepatitis virus; MPXV, monkeypox virus.

The Bio-Rad Expert Design service was used to create two 6-plex wastewater assays and one single-target control assay for the following pathogens: adenoviruses, noroviruses, enteroviruses, *Legionella*, influenza A, influenza B, two monkeypox virus (MPXV) variants, and mouse hepatitis virus (MHV) as control (Table 1)

BIO_CC00008198 at 8199

In another example, the QX600 ddPCR System can be used for high-resolution characterization of an AAV vector genome with multiple assays. The high-resolution characterization of AAV vector genome identity assay was used to perform two 6-plex (e.g., 6 targets) assays for the detection of 1. RSM2 ddPCR analysis: AAV ITR-2, hGFP, CMV-Enh, bGH poly(A), NeoR/KanR, SV40 poly(A); and 2. AAV5 ddPCR analysis: AAV ITR-2, GFP-K, CMV-Pro, eGFP, CMV-Enh, SV40 poly(A).



BIO_CC00008202 at 8204

In another example, the QX600 ddPCR System can detect mutations commonly associated with non-small-cell lung cancer in a single-well, 6-plex reaction (e.g., 6 targets). The target mutations detected in this assay includes, at least five polynucleotide analytes: EGFR Exon 19 deletions, EGFR WT, KRAS G12C, EGFR L858r, BRAF V6000E, EGFR T790M.

Abstract

Precise and rapid mutation detection is critical to the advancement of cancer research, as patient outcomes depend on the early and accurate identification of these mutations. Compared to other methods of mutation detection, Droplet Digital™ PCR (ddPCR™) allows researchers to detect rare mutations at very low fractional abundance, with faster turnaround times, and at lower costs. In this study, we demonstrate how the QX600™ ddPCR System can detect mutations commonly associated with non-small-cell lung cancer (NSCLC) in a single-well, 6-plex reaction. While multiplexing cancer mutations is often challenging due to the need to discriminate multiple sets of highly similar sequences in a single well, here we show the capability of the QX600 ddPCR System to detect mutations with simple analysis.

	BIO_CCODE00008194 at 8194 <table border="1"> <caption>Table 1. Mutation detection assays and corresponding detection channels.</caption> <thead> <tr> <th>Assay</th><th>Detection Channel</th></tr> </thead> <tbody> <tr> <td>EGFR Exon 19 Deletions</td><td>FAM</td></tr> <tr> <td>EGFR WT</td><td>HEX</td></tr> <tr> <td>KRAS G12C</td><td>Cy5</td></tr> <tr> <td>EGFR L858R</td><td>Cy5.5</td></tr> <tr> <td>BRAF V600E</td><td>ROX</td></tr> <tr> <td>EGFR T790M</td><td>ATTO 590</td></tr> <tr> <td>WT, wild type.</td><td></td></tr> </tbody> </table> BIO_CCODE00008194 at 8195	Assay	Detection Channel	EGFR Exon 19 Deletions	FAM	EGFR WT	HEX	KRAS G12C	Cy5	EGFR L858R	Cy5.5	BRAF V600E	ROX	EGFR T790M	ATTO 590	WT, wild type.	
Assay	Detection Channel																
EGFR Exon 19 Deletions	FAM																
EGFR WT	HEX																
KRAS G12C	Cy5																
EGFR L858R	Cy5.5																
BRAF V600E	ROX																
EGFR T790M	ATTO 590																
WT, wild type.																	
[1a] (a) providing a sample comprising, or potentially comprising, at least one of said at least five polynucleotide analytes;	See Appendix A-01 at [1a.1] (“providing a sample solution volume comprising or potentially comprising at least one of at least seven polynucleotide analytes”). The QX600 Droplet Digital PCR System is used to perform the step of providing a sample comprising, or potentially comprising, at least one of said at least five polynucleotide analytes, both literally and under the doctrine of equivalents. An exemplary description of the ddPCR Workflow is described in the QX600 Instruction Manual, which also describes a process for sample solution provision:																

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.

BIO_CC00007427 at 7450; *see also* BIO_CC00007643 at 7666.

Samples may be provided by combining DNA or RNA with primers, probes dye, and Bio-Rad ddPCR supermixes. *See id.*

Samples may be further provided using a droplet generator such as the AutoDG or QX200 Droplet Generator. *See, e.g.,* BIO_CC00006235 at -6271 through -6289; BIO_CC00008417-8418; BIO_CC00006175 at -6203 through 7207.

Samples may be further provided by via a thermal cycler. Two exemplary thermal cyclers offered by Bio-Rad include the PTC Tempo Thermal Cycler and the C1000 Touch Thermal Cycler.

Description

The C1000 Touch thermal cycler offers superior performance and a large color touch screen for easy programming. This fully modular platform is able to accommodate different throughput needs with easily interchangeable reaction modules that swap in seconds without tools. Each PCR module has a fully adjustable heated lid that supports a wide range of vessels and sealers, including low-profile and standard-height PCR plates.

Features and Benefits

- Quick and easy protocol programming — 2 programming options, including the **Protocol Autewriter**, which automatically generates customized standard, fast, and ultrafast protocols
- Easy protocol editing — the large color touch screen and redesigned navigation make editing and running protocols easy
- **Flexible platform** — increase throughput capability by linking up to 3 additional S1000 thermal cyclers or adding PC control for up to 32 cyclers
- Convenient file management — keep protocols organized using personalized folders or a USB flash drive
- **Thermal gradient** — quickly and easily identify optimal annealing temperatures by using the programmable temperature gradient

Description

The PTC Tempo Thermal Cycler is Bio-Rad's next-generation conventional PCR instrument. The PTC Tempo Deepwell Thermal Cycler is built with a fixed 96-well reaction block that accommodates reaction volumes of up to 125 µl and offers the same trusted performance as the C1000 Touch Thermal Cycler as well as several new features.

Considering a PTC
Tempo Thermal
Cycler for Your Lab?

[Contact An Expert](#)

Features and Benefits

- Programmable thermal gradient — for optimization of annealing temperatures
- Large touch screen — for streamlined protocol programming and editing
- Heated and automated lid — for use with various vessels, sealer types, and for integration with automation robotics
- Expanded connectivity — WiFi, ethernet, and USB
- Cloud connectivity: Create and send protocols to the PTC Tempo Thermal Cycler from the BR.io cloud platform
- Network storage drive access for excellent data management

<https://www.bio-rad.com/en-us/product/c1000-touch-thermal-cycler?ID=LGTW9415>; <https://www.bio-rad.com/en-us/sku/12015392-ptc-tempo-deepwell-thermal-cycler?ID=12015392>.

During the thermal cycling, a plate sealer such as the PX1 PCR Plate Sealer may optionally be used:

	Description Use the PX1 PCR Plate Sealer for consistent, uniform sealing across the entire plate, minimizing evaporation during cycling. The small-footprint heat sealer is compatible with a wide range of PCR plates and different types of films and foils. Features and Benefits • Ready to seal in less than 3 min • Support block holds both 96- and 384-well plates • Motorized drawer for insertion of plate and seal • Touch-screen interface allowing single button modification of temperature and time • Easy access to previously stored sealing protocols The intuitive touch-screen interface of the PX1 PCR Plate Sealer shows instrument status, platen temperature, protocol name, set temperature, and time. Sealing protocols can be stored and easily edited. The sealer can be programmed to automatically shut down after a user-defined period of idle time. Touching the display after shutdown will reactivate the display and the sealer will begin heating to the last set temperature. The semi-automated PX1 PCR Plate Sealer provides fast, consistent sealing, removing human variability and increasing reproducibility. Uniform sealing over the whole plate is particularly important for low-volume samples where small amounts of evaporation can have significant effect. In qPCR experiments, differential evaporation from wells can lead to large standard deviations in the number of quantification cycles (Cq). Product Components • Heat sealing instrument • Plate support block for both 96- and 384-well plates • Sealing frame Related Products • Optically Clear Heat Seals, for PCR and real-time PCR applications (1814030) • Permanent Clear Heat Seals, nonpeelable, nonpierceable clear seals for PCR, water bath cycling, and sample disposal (1814035) • Pierceable Foil Heat Seals for PCR and QX200™ Droplet Digital™ PCR System applications (1814040) • Peelable Foil Heat Seals laminate/foil peelable seals for PCR and sample storage (1814045) https://www.bio-rad.com/en-us/sku/1814000-px1-pcr-plate-sealer?ID=1814000. Samples may be also provided via a well plate in connection with the QX600 Droplet Reader. See <i>generally</i> BIO_CC00007427, BIO_CC00007643, BIO_CC00008141, BIO_CC00008190, BIO_CC00008194, BIO_CC00008198, and BIO_CC00008202. Exemplary processes for providing samples is described at BIO_CC00007427 at 7477-7493 and BIO_CC00007643 at 7691-7711. The samples provided in connection with the QX600 comprise at least of at least seven polynucleotide analytes (referred to as “targets”). For example:
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	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 BIO_CC00008190 at BIO_CC00008191 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. BIO_CC00007427 at BIO_CC00007449 The QX600 system requires dividing each sample into a sample solution volume (e.g., a water-in-oil droplet) with target and background DNA randomly distributed into the droplets and allowing for quantification of at least seven targets in a single well:
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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 <u>and</u> HEX/VIC and one target in FAM <u>or</u> 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 <u>and</u> Cy5.5, one target in Cy5 <u>or</u> 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.

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 <u>and</u> 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 <u>and</u> 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 <u>and</u> 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 with Channel 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				✓			

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, for 1 to 6 targets	FAM	HEX	Cy5	Cy5.5	ROX	ATTO590
	EvaGreen®	VIC	None	None	None	None
	None	None				
Amplitude multiplex, for 1 to 12 targets	FAM Hi	HEX Hi	Cy5 Hi	Cy5.5 Hi	ROX Hi	ATTO 590 Hi
	FAM Lo	HEX Lo	Cy 5 Lo	Cy 5.5 Lo	ROX Lo	ATTO 590 Lo
	EvaGreen® Hi	VIC Hi	None	None	None	None
	EvaGreen® Lo	VIC Lo				
	None	None				
Probe mix triplex, for 9 targets	FAM	HEX	Cy5	Cy5.5	ROX	ATTO 590
	None	VIC	None	None	None	None
		None				
Advanced classification	FAM	HEX	Cy5	Cy5.5	ROX	ATTO 590
	+1-10	1 to 10	1 to 10	1 to 10	1 to 10	1 to 10
	EvaGreen®	VIC	None	None	None	None
	1 to 10	1 to 10				
	None	None				

BIO_CC00007427 at 7505-7507; *see also* BIO_CC00007643.

The QX600 system requires the introduction of a sample to the system in order to perform an analysis, including preparing the samples for PCR by combining DNA or RNA with primers, probes dye, and Bio-Rad ddPCR supermix. The QX600 system requires dividing each sample into a sample (e.g., a water-in-oil droplet) with target and background DNA randomly distributed into the droplets and allowing for quantification of 12 targets in a single well. Thus, each sample can comprise at least one of at least five polynucleotide analytes (e.g., quantification of up to 12 targets).

Such samples may include, but not be limited to, the following applications:

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.

See <https://www.bio-rad.com/en-us/product/qx600-droplet-digital-pcr-system?ID=b07d12ac-0585-fc4c-a586-3ddf20d5c4a0>; see also “Multiplex Assay Design for Epidemiological Wastewater Monitoring with Droplet Digital PCR Technology,” BIO_CC00008198; see also “Reproducible High-Resolution Characterization of AAV Vector Genome Identity Using the QX600,” BIO_CC00008202; see also “Mutation Detection Multiplexing Using the QX600 Droplet Digital PCR System,” BIO_CC00008194.

On information and belief, these applications comprise at least five polynucleotide analytes. For example:

In one example, the QX600 ddPCR System can be used for highly sensitive detection of commonly monitored pathogens in a rapid and accurate manner. The Multiplex Assay Design for Epidemiological Wastewater Monitoring with ddPCR Technology was used to create two 6-plex (e.g., 6 targets) wastewater assays and one single-target control assay for the following pathogens: adenoviruses, noroviruses, enteroviruses, Legionella, influenza A, influenza B, two monkeypox virus (MPXV) variants, and mouse hepatitis virus (MHV) as control.

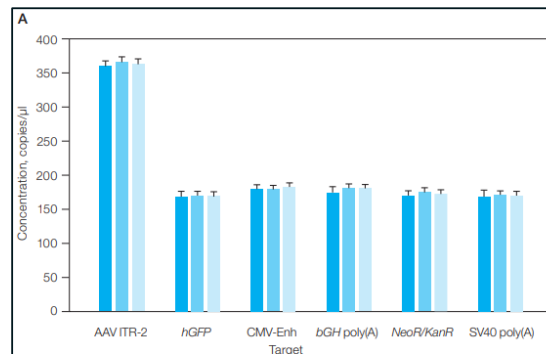
Table 1. Pathogens and assigned fluorophores targeted in assays 1 and 2 and the MHV control assay.							
	FAM	HEX	FAM/HEX	ROX	ATTO 590	Cy5	Cy5.5
Assay 1	Adenovirus	Norovirus	MHV control assay	MPXV (West African Variant 1)	MPXV (West African Variant 2)	Enterovirus	Legionella
Assay 2	Adenovirus	Norovirus	MHV control assay	MPXV (West African Variant 1)	MPXV (West African Variant 2)	Influenza A	Influenza B

MHV, mouse hepatitis virus; MPXV, monkeypox virus.

The Bio-Rad Expert Design service was used to create two 6-plex wastewater assays and one single-target control assay for the following pathogens: adenoviruses, noroviruses, enteroviruses, *Legionella*, influenza A, influenza B, two monkeypox virus (MPXV) variants, and mouse hepatitis virus (MHV) as control (Table 1)

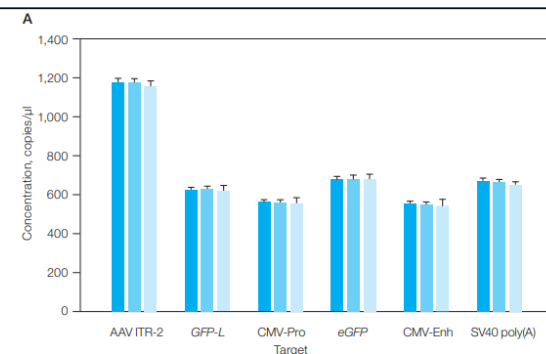
BIO_CC00008198 at 8199

In another example, the QX600 ddPCR System can be used for high-resolution characterization of an AAV vector genome with multiple assays. The high-resolution characterization of AAV vector genome identity assay was used to perform two 6-plex (e.g., 6 targets) assays for the detection of 1. RSM2 ddPCR analysis: AAV ITR-2, hGFP, CMV-Enh, bGH poly(A), NeoR/KanR, SV40 poly(A); and 2. AAV5 ddPCR analysis: AAV ITR-2, GFP-K, CMV-Pro, eGFP, CMV-Enh, SV40 poly(A).



Target	Concentration, copies/μl	%CV
AAV ITR-2	365 ± 3	0.92
hGFP	171 ± 1	0.63
CMV-Enh	182 ± 2	0.97
bGH poly(A)	180 ± 4	2.29
NeoR/KanR	174 ± 3	1.62
SV40 poly(A)	171 ± 2	0.92

Fig. 2. Reproducibility of RSM2 ddPCR analysis. The concentration of six targets in an RSM2 vector sample were independently measured on three different days using a multiplex ddPCR experiment. **A**, the concentration of each of the six targets as determined by Poisson analysis. Error bars represent the upper portion of the 95% confidence interval. Replicate 1 (■), replicate 2 (■), replicate 3 (■). **B**, the average and standard deviation for the three concentration measurements (given in copies/μl) and the corresponding coefficients of variation (%CV). CMV-Enh, CMV enhancer.



Target	Concentration, copies/μl	%CV
AAV ITR-2	1,175 ± 12	1.04
GFP-L	629 ± 6	0.98
CMV-Pro	565 ± 4	0.64
eGFP	682 ± 1	0.22
CMV-Enh	554 ± 6	1.10
SV40 poly(A)	667 ± 10	1.52

Fig. 3. Reproducibility of AAV5 ddPCR analysis. The concentration of six targets in an AAV5 vector sample were independently measured on three different days using a multiplex ddPCR experiment. **A**, the concentration of each of the six targets as determined by Poisson analysis. Error bars represent the upper portion of the 95% confidence interval. Replicate 1 (■), replicate 2 (■), replicate 3 (■). **B**, the average standard deviation for the three concentration measurements (given in copies/μl) and the corresponding coefficients of variation (%CV). CMV-Enh, CMV enhancer; CMV-Pro, CMV promoter.

BIO_CCODE00008202 at 8204.

In another example, the QX600 ddPCR System can detect mutations commonly associated with non-small-cell lung cancer in a single-well, 6-plex reaction (e.g., 6 targets). The target mutations detected in this assay includes, at least five polynucleotide analytes: EGFR Exon 19 deletions, EGFR WT, KRAS G12C, EGFR L858r, BRAF V6000E, EGFR T790M.

	Abstract Precise and rapid mutation detection is critical to the advancement of cancer research, as patient outcomes depend on the early and accurate identification of these mutations. Compared to other methods of mutation detection, Droplet Digital™ PCR (ddPCR™) allows researchers to detect rare mutations at very low fractional abundance, with faster turnaround times, and at lower costs. In this study, we demonstrate how the QX600™ ddPCR System can detect mutations commonly associated with non–small-cell lung cancer (NSCLC) in a single-well, 6-plex reaction. While multiplexing cancer mutations is often challenging due to the need to discriminate multiple sets of highly similar sequences in a single well, here we show the capability of the QX600 ddPCR System to detect mutations with simple analysis. BIO_CC00008194 at 8194. Table 1. Mutation detection assays and corresponding detection channels. <table> <tr> <th>Assay</th><th>Detection Channel</th></tr> <tr> <td>EGFR Exon 19 Deletions</td><td>FAM</td></tr> <tr> <td>EGFR WT</td><td>HEX</td></tr> <tr> <td>KRAS G12C</td><td>Cy5</td></tr> <tr> <td>EGFR L858R</td><td>Cy5.5</td></tr> <tr> <td>BRAF V600E</td><td>ROX</td></tr> <tr> <td>EGFR T790M</td><td>ATTO 590</td></tr> </table> WT, wild type. BIO_CC00008194 at 8195.	Assay	Detection Channel	EGFR Exon 19 Deletions	FAM	EGFR WT	HEX	KRAS G12C	Cy5	EGFR L858R	Cy5.5	BRAF V600E	ROX	EGFR T790M	ATTO 590
Assay	Detection Channel														
EGFR Exon 19 Deletions	FAM														
EGFR WT	HEX														
KRAS G12C	Cy5														
EGFR L858R	Cy5.5														
BRAF V600E	ROX														
EGFR T790M	ATTO 590														
[1b.1] (b) forming a mixture of said sample and at least five hybridization probes,	When the QX600 is used to perform ddPCR analysis, the step of forming a mixture of said sample and at least five hybridization probes is performed. <i>See</i> step 1a above, which describes how a mixture is formed. <i>See also</i> Appendix A-01 at Step 1a.1, 1a.4, and 1a.5 which describe the step of forming the mixture using the hybridization probes. Plaintiffs incorporate those steps here by reference. As described in those steps, the QX600 is able to be used with over seven hybridization probes, meaning it can also be used with “at least five hybridization probes.” The QX600 Droplet Digital PCR System is used to perform the step of forming a mixture of said sample and at least five hybridization probes, both literally and under the doctrine of equivalents.														

For example:

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

BIO_CCODE00008190 at BIO_CCODE00008191

Chapter 1 Introduction to Droplet Digital PCR

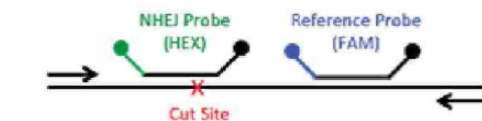
Droplet Digital™ PCR (ddPCR™) is a digital polymerase chain reaction method based on water-oil emulsion droplet technology. ddPCR uses a combination of microfluidics and proprietary surfactant chemistries to divide each sample into water-in-oil droplets. The technology uses reagents and workflows similar 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.

BIO_CCODE00007427 at BIO_CCODE00007449

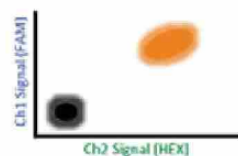
The QX600 Droplet Digital PCR System uses kits that use hybridization probes for the detection of targets, including existing ddPCR Assays for hydrolysis probe chemistry such as TaqMan probe-based assays.

Drop-Off Probe in HEX

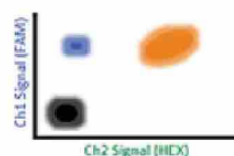
NHEJ Probe = HEX and Reference Probe = FAM



Wild Type Sample

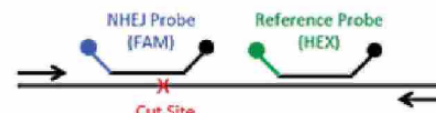


NHEJ Sample

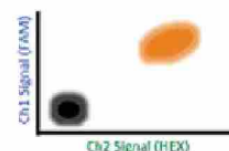


Drop-Off Probe in FAM

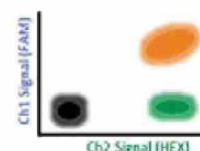
NHEJ Probe = FAM and Reference Probe = HEX



Wild Type Sample



NHEJ Sample



BIO_CCODE00007427 at BIO_CCODE00007618

As shown in the example figure above, each probe corresponds to one of the polynucleotide analytes: the NHEJ probe corresponds to NHEJ, and the reference probe corresponds to the reference. Each of the probes also corresponds to a specific fluorophore or a combination of fluorophores (e.g. NHEJ Probe = HEX and Reference Probe = FAM, or NHEJ Probe = FAM and Reference Probe = HEX), with each corresponding to a color indicated in the figure.

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.

BIO_CC00007427 at BIO_CC00007505

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
Positive Cy5 and Cy5.5	=	Light Blue
Positive ROX	=	Maroon
Positive ATTO 590	=	Gold
Positive ROX and ATTO 590	=	Aqua
Double negative	=	Gray

	BIO_CC00007427 at BIO_CC00007561 The QX600 system can be used to perform a probe mix triplex assay that has 9 hybridization probes (e.g., probes that correspond to FAM, HEX, FAM/HEX, Cy5, Cy5.5, Cy5/Cy5.5, ROX, ATTO590, and ROX/ATTO590), with each corresponding to one polynucleotide analyte targets for a total of 9 targets (e.g., three groups of three targets each). 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. BIO_CC00007427 at BIO_CC00007450 The samples are mixed together with hybridization probes, primers, probe dyes, and Bio-Rad ddPCR supermix for the ddPCR reaction. As explained above, there are at least five hybridization probes (e.g., 9). Thus, the QX600 system is used to form a sample mixture and perform an assay that has 9 polynucleotide analytes which each have a unique probe assigned to each analyte, demonstrating that 9 unique probes are used in the methodology.
[1b.2] wherein each of said at least five hybridization probes further comprises at least one fluorophore and at most four fluorophores;	When the QX600 is used to perform ddPCR analysis, the step of 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) is performed. <i>See</i> step 1a above, which describes how a mixture is formed. <i>See also</i> Appendix A-01 at Step 1a.3, which describes the process by which each hybridization probe comprises at least one fluorophore selected from four fluorophore. That step is materially the same as this step, and Plaintiffs incorporate all references steps here by reference. As described in those steps, the QX600 is able to be used with over seven hybridization probes as well as four fluorophores comprising the list, meaning it can also be used with “at least five hybridization probes” and with at least one fluorophore and at most four fluorophores. Additionally, on information and belief the QX ONE is able to filter certain fluorophores. Further:

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](#).

BIO_CCODE00007427 at 7451; *see also* BIO_CCODE00007643.

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-glycosylase (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.

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 <u>and</u> HEX/VIC and one target in FAM <u>or</u> 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 <u>and</u> Cy5.5, one target in Cy5 <u>or</u> 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.

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 with Channel 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				✓			

QX Manager Software automatically populates the fluorophore in each channel for each target but you can change the default selections.

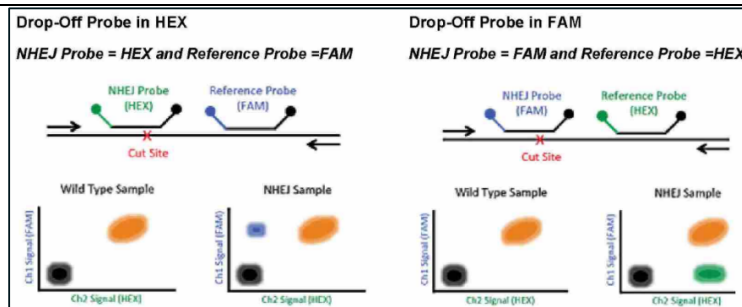
Use Table 21 to select from the fluorophore options for each assay method listed.

Table 21. Fluorophore options

Assay method	Signal Ch1	Signal Ch2	Signal Ch3	Signal Ch4	Signal Ch5	Signal Ch6
Single target per channel, for 1 to 6 targets	FAM	HEX	Cy5	Cy5.5	ROX	ATTO590
	EvaGreen®	VIC	None	None	None	None
	None	None				
Amplitude multiplex, for 1 to 12 targets	FAM Hi	HEX Hi	Cy5 Hi	Cy5.5 Hi	ROX Hi	ATTO 590 Hi
	FAM Lo	HEX Lo	Cy 5 Lo	Cy 5.5 Lo	ROX Lo	ATTO 590 Lo
	EvaGreen® Hi	VIC Hi	None	None	None	None
	EvaGreen® Lo	VIC Lo				
	None	None				
Probe mix triplex, for 9 targets	FAM	HEX	Cy5	Cy5.5	ROX	ATTO 590
	None	VIC	None	None	None	None
		None				
Advanced classification	FAM	HEX	Cy5	Cy5.5	ROX	ATTO 590
	+1-10	1 to 10	1 to 10	1 to 10	1 to 10	1 to 10
	EvaGreen®	VIC	None	None	None	None
	1 to 10	1 to 10				
	None	None				

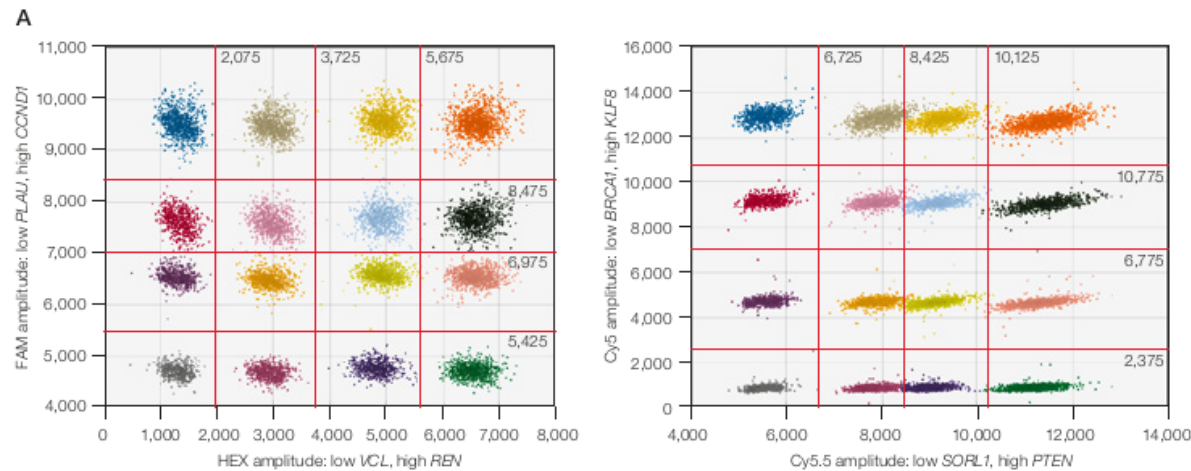
BIO_CC00007427 at 7504-7507; *see also* BIO_CC00007643.

The QX600 Droplet Digital PCR System uses assays and kits that use hybridization probes for the detection of targets. The following provides a non-limiting example disclosure for how the hybridization probes correspond to at least one fluorophore from the available fluorophore:



BIO_CCODE00007427 at 7618; *see also* BIO_CCODE00007643; *see* additional examples in Appendix A of the user guides. As shown in the example figure above, each probe corresponds to a specific fluorophore or a combination of fluorophores.

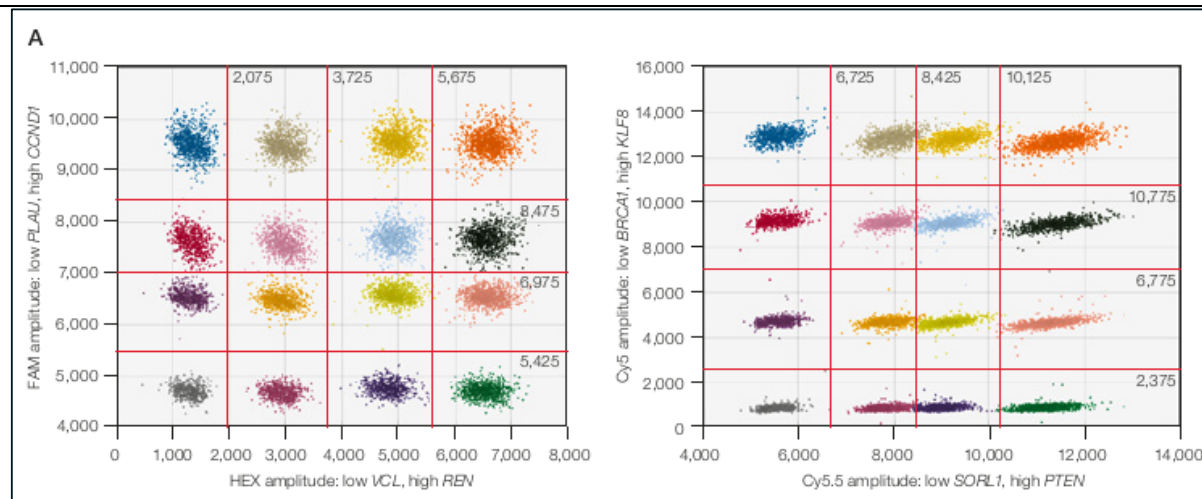
The following provides an additional example of each probe comprising at least one fluorophore selected from a group of four fluorophores:



BIO_CCODE00008190 at 8192.

	Default color assignments for 2D amplitude plots are:<table><tr><td>Positive FAM</td><td>=</td><td>Blue</td></tr><tr><td>Positive HEX/VIC</td><td>=</td><td>Green</td></tr><tr><td>Positive FAM and HEX/VIC</td><td>=</td><td>Orange (may appear brown on some monitors)</td></tr><tr><td>Positive Cy5</td><td>=</td><td>Red</td></tr><tr><td>Positive Cy5.5</td><td>=</td><td>Violet</td></tr><tr><td>Positive Cy5 and Cy5.5</td><td>=</td><td>Light Blue</td></tr><tr><td>Positive ROX</td><td>=</td><td>Maroon</td></tr><tr><td>Positive ATTO 590</td><td>=</td><td>Gold</td></tr><tr><td>Positive ROX and ATTO 590</td><td>=</td><td>Aqua</td></tr><tr><td>Double negative</td><td>=</td><td>Gray</td></tr></table>	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	Positive Cy5 and Cy5.5	=	Light Blue	Positive ROX	=	Maroon	Positive ATTO 590	=	Gold	Positive ROX and ATTO 590	=	Aqua	Double negative	=	Gray
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																													
Positive Cy5 and Cy5.5	=	Light Blue																													
Positive ROX	=	Maroon																													
Positive ATTO 590	=	Gold																													
Positive ROX and ATTO 590	=	Aqua																													
Double negative	=	Gray																													
	BIO_CC00007427 at 7561.																														
[1c] (c) partitioning said mixture into said plurality of droplets;	The QX600 Droplet Digital PCR System is used to perform the step of partitioning said mixture into said plurality of droplets. For example: 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. BIO_CC00007427 at 7449. As discussed above in the preamble and Step 1a, a sample is prepared by separating the mixture into a plurality of droplets within a well. This can occur using a droplet generator such as the QX200 droplet generator or the autodroplet generator. The QX600 Droplet Digital PCR System is used to perform digital PCR, which requires that samples be partition into droplets for analysis (e.g., divide each sample into water-in-oil droplets). The QX600 system requires and enables the partitioning of the sample into droplets prior to analysis.																														

[1d] (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;	Plaintiffs incorporate by reference its analysis from Appendix A-01 at [1a.4], [1a.5], and [1b]. In those elements, Plaintiffs explained how the QX600 is used to excite at least one fluorophore to generate at least one signals when the target analytes is present in the sample. Also as explained there, the signal is created by the excited fluorophore when the hybridization probe contacts the analyte. In this chart, in Step [1c] is explained how that sample is split into the droplets. Those elements taken together show how this step is performed. Additionally: 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. BIO_CC00007427 at 7451 The QX600 system determines which droplets contain molecules by determining the positive reading (contains target molecules) or negative reading (does not contain target molecules) in a six-color detector. The QX600 system detects multiple targets in at least one fluorophore (e.g., six fluorophores) using interrogation points across defined spectrums, and is accomplished by means of light emitting diodes on the QX600.
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BIO_CC00008190 at 8192.

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

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

BIO_CC00008190 at 8191

The QX600 system is used to perform the method of generating the signals of up to 12 targets in a single well. The QX600 system collects binary data (e.g., positive reading for droplets containing target molecules, and negative reading for droplets that do not contain target molecules) for analysis as it singulates the 20,000 nanoliter-sized droplets in each sample.

The QX600 system is used to perform common hydrolysis probe chemistry where signal from the hydrolysis probes is generated if they become attached to the analytes targets and is generated by means of an extension reaction where one or more polynucleotide analytes is present in said plurality of droplets. The QX600 system performs the step to generate one or more signals if one or more of said at least give polynucleotide analytes is present in said plurality of droplets, for example, the 2D fluorescence multiplex amplitude plots shows at least five polynucleotide analytes (e.g., 8 targets: PLAUI, CCND1, VCL, REN, BRCA1, KLF8, SORL1, and PTEN) present in plurality of droplets to generate one or more signals as seen by the fluorescence signals in the 2D fluorescence multiplex amplitude plots. Such samples may include, but not be limited to, the following applications:

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.

See <https://www.bio-rad.com/en-us/product/qx600-droplet-digital-pcr-system?ID=b07d12ac-0585-fc4c-a586-3ddf20d5c4a0>; see also “Multiplex Assay Design for Epidemiological Wastewater Monitoring with Droplet Digital PCR Technology,” BIO_CC00008198; see also “Reproducible High-Resolution Characterization of AAV Vector Genome Identity Using the QX600,” BIO_CC00008202; see also “Mutation Detection Multiplexing Using the QX600 Droplet Digital PCR System,” BIO_CC00008194.

On information and belief, these applications comprise at least five polynucleotide analytes. For example:

In one example, the QX600 ddPCR System can be used for highly sensitive detection of commonly monitored pathogens in a rapid and accurate manner. The Multiplex Assay Design for Epidemiological Wastewater Monitoring with ddPCR Technology was used to create two 6-plex (e.g., 6 targets) wastewater assays and one single-target control assay for the following pathogens: adenoviruses, noroviruses, enteroviruses, Legionella, influenza A, influenza B, two monkeypox virus (MPXV) variants, and mouse hepatitis virus (MHV) as control.

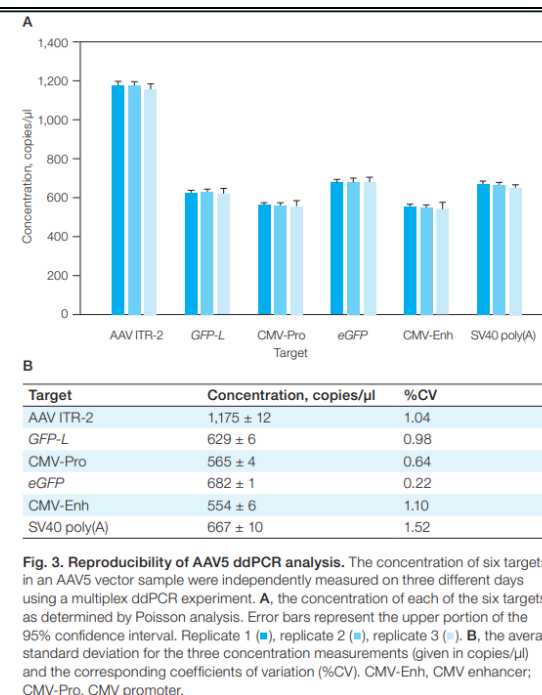
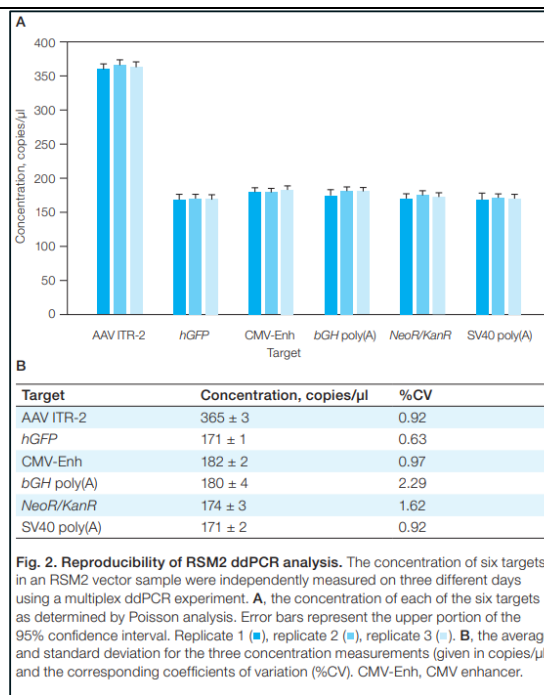
Table 1. Pathogens and assigned fluorophores targeted in assays 1 and 2 and the MHV control assay.							
	FAM	HEX	FAM/HEX	ROX	ATTO 590	Cy5	Cy5.5
Assay 1	Adenovirus	Norovirus	MHV control assay	MPXV (West African Variant 1)	MPXV (West African Variant 2)	Enterovirus	Legionella
Assay 2	Adenovirus	Norovirus	MHV control assay	MPXV (West African Variant 1)	MPXV (West African Variant 2)	Influenza A	Influenza B

MHV, mouse hepatitis virus; MPXV, monkeypox virus.

The Bio-Rad Expert Design service was used to create two 6-plex wastewater assays and one single-target control assay for the following pathogens: adenoviruses, noroviruses, enteroviruses, Legionella, influenza A, influenza B, two monkeypox virus (MPXV) variants, and mouse hepatitis virus (MHV) as control (Table 1)

BIO_CC00008198 at 8199.

In another example, the QX600 ddPCR System can be used for high-resolution characterization of an AAV vector genome with multiple assays. The high-resolution characterization of AAV vector genome identity assay was used to perform two 6-plex (e.g., 6 targets) assays for the detection of 1. RSM2 ddPCR analysis: AAV ITR-2, hGFP, CMV-Enh, bGH poly(A), NeoR/KanR, SV40 poly(A); and 2. AAV5 ddPCR analysis: AAV ITR-2, GFP-K, CMV-Pro, eGFP, CMV-Enh, SV40 poly(A).



BIO_CC00008202 at 8204.

In another example, the QX600 ddPCR System can detect mutations commonly associated with non-small-cell lung cancer in a single-well, 6-plex reaction (e.g., 6 targets). The target mutations detected in this assay includes, at least five polynucleotide analytes: EGFR Exon 19 deletions, EGFR WT, KRAS G12C, EGFR L858r, BRAF V6000E, EGFR T790M.

	Abstract Precise and rapid mutation detection is critical to the advancement of cancer research, as patient outcomes depend on the early and accurate identification of these mutations. Compared to other methods of mutation detection, Droplet Digital™ PCR (ddPCR™) allows researchers to detect rare mutations at very low fractional abundance, with faster turnaround times, and at lower costs. In this study, we demonstrate how the QX600™ ddPCR System can detect mutations commonly associated with non-small-cell lung cancer (NSCLC) in a single-well, 6-plex reaction. While multiplexing cancer mutations is often challenging due to the need to discriminate multiple sets of highly similar sequences in a single well, here we show the capability of the QX600 ddPCR System to detect mutations with simple analysis. BIO_CC00008194 at 8194 Table 1. Mutation detection assays and corresponding detection channels. <table> <tr> <th>Assay</th><th>Detection Channel</th></tr> <tr> <td>EGFR Exon 19 Deletions</td><td>FAM</td></tr> <tr> <td>EGFR WT</td><td>HEX</td></tr> <tr> <td>KRAS G12C</td><td>Cy5</td></tr> <tr> <td>EGFR L858R</td><td>Cy5.5</td></tr> <tr> <td>BRAF V600E</td><td>ROX</td></tr> <tr> <td>EGFR T790M</td><td>ATTO 590</td></tr> </table> WT, wild type. BIO_CC00008194 at 8195 The QX600 Droplet Digital PCR System is used to perform the step wherein said one or more signals comprise at least one signal generated by excitement of said at least one fluorophore, both literally and under the doctrine of equivalents.	Assay	Detection Channel	EGFR Exon 19 Deletions	FAM	EGFR WT	HEX	KRAS G12C	Cy5	EGFR L858R	Cy5.5	BRAF V600E	ROX	EGFR T790M	ATTO 590
Assay	Detection Channel														
EGFR Exon 19 Deletions	FAM														
EGFR WT	HEX														
KRAS G12C	Cy5														
EGFR L858R	Cy5.5														
BRAF V600E	ROX														
EGFR T790M	ATTO 590														
[1e] (e) measuring said one or more signals to	Plaintiffs incorporate by reference its analysis from Appendix A-01 at [1c.1] and [1c.2]. In those elements, Plaintiff explained how the QX600 is used to measure the 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. For the same reasons, that explanation also shows how the system may indicate the presence or absence of at least														

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	five polynucleotide analytes. Likewise, the signal that is generated is a unique combination of the presence or absence of at least five polynucleotide analytes. Additionally: 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 $\pm 10\%$ 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. See BIO_CC00007427 at 7449-7450; see also BIO_CC00007643.
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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](#).

See BIO_CCODE00007427 at 7451; see also BIO_CCODE00007643.

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.

See BIO_CCODE00007427 at 7492; see also BIO_CCODE00007643.

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-glycosylase (UNG) decontamination protocols to prevent the reamplification of carryover PCR products between experiments.

BIO_CC00007427 at 7504; *see also* BIO_CC00007643.

The QX600 system provides ways of measuring the cumulative intensity signals, for example, signal from the hydrolysis probes is generated, for example, when they become attached to the analytes targets and is generated by means of an extension reaction by common hydrolysis probe chemistry in using hydrolysis probe-based assays.

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.

BIO_CC00007427 at 7501; *see also* BIO_CC00007643.

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 <u>and</u> HEX/VIC and one target in FAM <u>or</u> 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 <u>and</u> Cy5.5, one target in Cy5 <u>or</u> 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.

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 <u>and</u> 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 <u>and</u> 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 <u>and</u> 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 with Channel 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				✓			

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, for 1 to 6 targets	FAM	HEX	Cy5	Cy5.5	ROX	ATTO590
	EvaGreen®	VIC	None	None	None	None
	None	None				
Amplitude multiplex, for 1 to 12 targets	FAM Hi	HEX Hi	Cy5 Hi	Cy5.5 Hi	ROX Hi	ATTO 590 Hi
	FAM Lo	HEX Lo	Cy 5 Lo	Cy 5.5 Lo	ROX Lo	ATTO 590 Lo
	EvaGreen® Hi	VIC Hi	None	None	None	None
	EvaGreen® Lo	VIC Lo				
	None	None				
Probe mix triplex, for 9 targets	FAM	HEX	Cy5	Cy5.5	ROX	ATTO 590
	None	VIC	None	None	None	None
		None				
Advanced classification	FAM	HEX	Cy5	Cy5.5	ROX	ATTO 590
	+1-10	1 to 10	1 to 10	1 to 10	1 to 10	1 to 10
	EvaGreen®	VIC	None	None	None	None
	1 to 10	1 to 10				
	None	None				

BIO_CCODE00007427 at 7505-7507; *see also* BIO_CCODE00007643.

Also:

To set experiment parameters

- Tip:** For automatic thresholding of positive control wells, you must select Pos Ctrl.

- 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.

- 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

9. Click Apply.

The QX600 System 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. Thus, on information and belief, each target is encoded with a specific fluorophore corresponding to a cumulative signal measurement to non-degenerately indicate the presence or absence of each target.

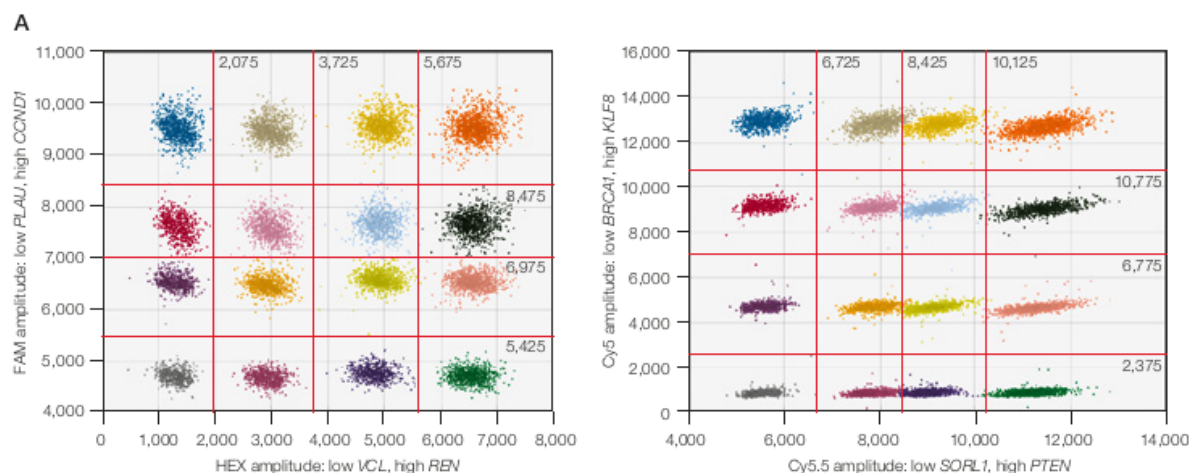
QX Manager Software automatically populates the fluorophore in each channel for each target but you can change the default selections.

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, for 1 to 6 targets	FAM	HEX	Cy5	Cy5.5	ROX	ATTO590
	EvaGreen®	VIC	None	None	None	None
	None	None				
Amplitude multiplex, for 1 to 12 targets	FAM Hi	HEX Hi	Cy5 Hi	Cy5.5 Hi	ROX Hi	ATTO 590 Hi
	FAM Lo	HEX Lo	Cy 5 Lo	Cy 5.5 Lo	ROX Lo	ATTO 590 Lo
	EvaGreen® Hi	VIC Hi	None	None	None	None
	EvaGreen® Lo	VIC Lo				
	None	None				
Probe mix triplex, for 9 targets	FAM	HEX	Cy5	Cy5.5	ROX	ATTO 590
	None	VIC	None	None	None	None
		None				
Advanced classification	FAM	HEX	Cy5	Cy5.5	ROX	ATTO 590
	+1-10	1 to 10	1 to 10	1 to 10	1 to 10	1 to 10
	EvaGreen®	VIC	None	None	None	None
	1 to 10	1 to 10				
	None	None				

BIO CCODE00007427 at 7507; *see also* BIO_CCODE00007643.



BIO_CC00008190 at 8192.

As seen in the exemplary plots above, when the QX600 is used, each cumulative signal measurement gives a unique pattern that, on information and belief, non-degenerately indicates the presence or absence of the at least seven polynucleotide analytes (e.g., eight targets), in any combination of presence or absence. On information and belief, this may be based on the specific encoding scheme chosen.

Also, on information and belief, the specific exemplary plots above were selected to non-degenerately indicate the presence or absence of eight targets in any combination presence or absence. The x and y axes of the two 2D amplitude plots indicate the targets and their corresponding fluorophore components (e.g., “HEX amplitude: low VCL, high REN” indicates VCL target corresponds to low HEX signal, REN target corresponds to high HEX signal). Low is represented by 1 (e.g., low HEX for VCL indicated as 1), and high is represented by 2 (e.g., high HEX for REN indicated as 2). The assay as reproduced above shows the non-degenerate identification of 8 analytes across four channels. Each graph represents 2^4 possibilities for each of the 4 analytes, thus giving 16 possibilities. Each will then also have a value in the paired graph for the second set of fluorophores. This gives a cumulative signal measurement non-degenerately indicates the presence or absence of the at least seven polynucleotide analytes (e.g., eight targets), in any combination of presence or absence.

Analyte	FAM	HEX	Cy5	Cy5.5
PLAU	1	0	0	0
CCND1	2	0	0	0
VCL	0	1	0	0
REN	0	2	0	0
BRCA1	0	0	1	0
KLF8	0	0	2	0
SORL1	0	0	0	1
PTEN	0	0	0	2

The QX600 system is used to perform different experiment and assay types to measure the cumulative intensity signals, such as amplitude multiplex and probe mix triplex experiments and assays.

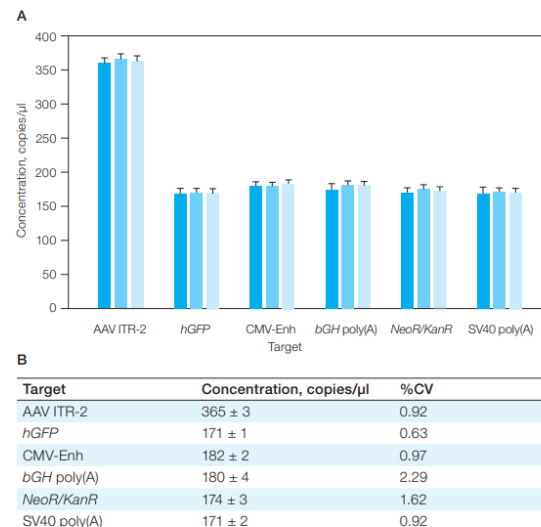


Fig. 2. Reproducibility of RSM2 ddPCR analysis. The concentration of six targets in an RSM2 vector sample were independently measured on three different days using a multiplex ddPCR experiment. **A**, the concentration of each of the six targets as determined by Poisson analysis. Error bars represent the upper portion of the 95% confidence interval. Replicate 1 (■), replicate 2 (■), replicate 3 (■). **B**, the average and standard deviation for the three concentration measurements (given in copies/μl) and the corresponding coefficients of variation (%CV). CMV-Enh, CMV enhancer.

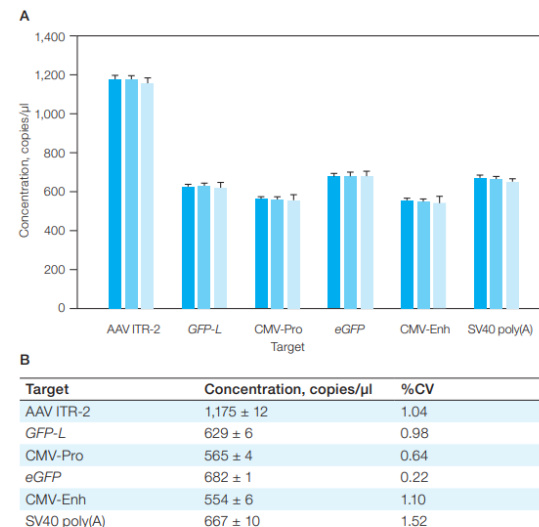


Fig. 3. Reproducibility of AAV5 ddPCR analysis. The concentration of six targets in an AAV5 vector sample were independently measured on three different days using a multiplex ddPCR experiment. **A**, the concentration of each of the six targets as determined by Poisson analysis. Error bars represent the upper portion of the 95% confidence interval. Replicate 1 (■), replicate 2 (■), replicate 3 (■). **B**, the average standard deviation for the three concentration measurements (given in copies/μl) and the corresponding coefficients of variation (%CV). CMV-Enh, CMV enhancer; CMV-Pro, CMV promoter.

BIO_CC00008202 at BIO_CC00008204

In another example, the QX600 ddPCR System can detect mutations commonly associated with non-small-cell lung cancer in a single-well, 6-plex reaction (e.g., 6 targets). The target mutations detected in this assay includes, at least five polynucleotide analytes: EGFR Exon 19 deletions, EGFR WT, KRAS G12C, EGFR L858r, BRAF V600E, EGFR T790M.

Abstract

Precise and rapid mutation detection is critical to the advancement of cancer research, as patient outcomes depend on the early and accurate identification of these mutations. Compared to other methods of mutation detection, Droplet Digital™ PCR (ddPCR™) allows researchers to detect rare mutations at very low fractional abundance, with faster turnaround times, and at lower costs. In this study, we demonstrate how the QX600™ ddPCR System can detect mutations commonly associated with non-small-cell lung cancer (NSCLC) in a single-well, 6-plex reaction. While multiplexing cancer mutations is often challenging due to the need to discriminate multiple sets of highly similar sequences in a single well, here we show the capability of the QX600 ddPCR System to detect mutations with simple analysis.

BIO_CC00008194 at BIO_CC00008194

Table 1. Mutation detection assays and corresponding detection channels.

Assay	Detection Channel
EGFR Exon 19 Deletions	FAM
EGFR WT	HEX
KRAS G12C	Cy5
EGFR L858R	Cy5.5
BRAF V600E	ROX
EGFR T790M	ATTO 590

WT, wild type.

BIO_CC00008194 at BIO_CC00008195

[1f]

(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,

The QX600 Droplet Digital PCR System is used to perform the step 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, both literally and under the doctrine of equivalents.

For example:

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.

	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 $\pm 10\%$ 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. BIO CCODE00007427 at 7449-7450; <i>see also</i> BIO_CCODE00007643; <i>see also id.</i> at 7451.
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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 <table><tr><th>Type</th><th>Description</th></tr><tr><td>Direct quantification (DQ)</td><td>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.</td></tr><tr><td>Copy number variation (CNV)</td><td>CNV determines concentration and calculates the copy number of an unknown (CNV) target relative to a known reference or references within the same well.</td></tr><tr><td>Mutation detection (MUT)</td><td>MUT determines concentration and calculates the fractional abundance of an unknown mutant present at a low frequency in a wild-type background.</td></tr><tr><td>Rare event detection (RED)</td><td>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.</td></tr><tr><td>Drop-off (DOF)</td><td>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.</td></tr><tr><td>Gene expression (GEX)</td><td>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.</td></tr></table>	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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	<i>Id. at 7501-7502, 7504.</i>														

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.

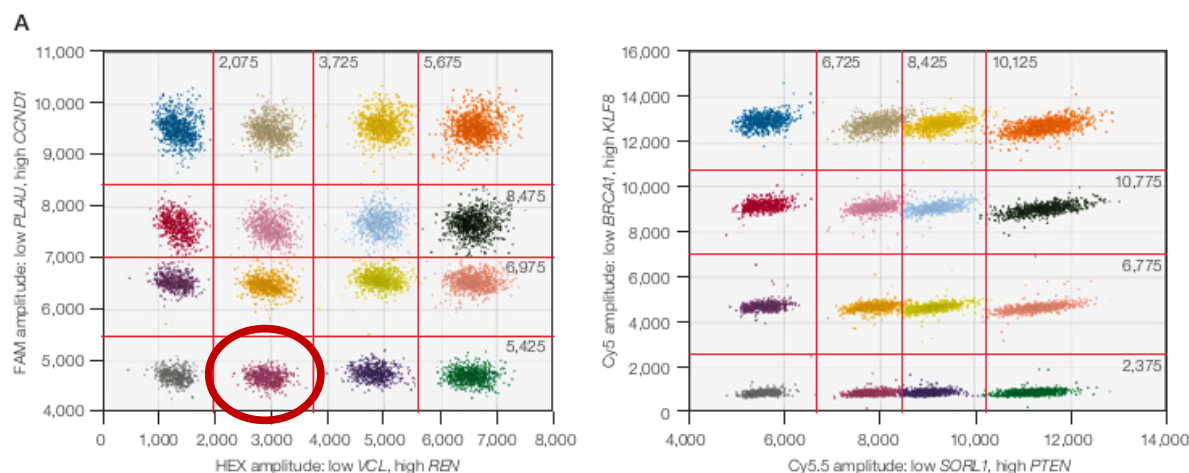
8. (Optional) Change the high and low target identifiers.

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.

Id. at 7612-7614, 7621. Quantification kits discussed at *id.* 7639.



BIO_CCODE00008190 at BIO_CCODE00008192

As seen in the 2D fluorescence amplitude plots, each cumulative intensity measurement corresponds to the presence of a unique combination of presence or absence of the at least five polynucleotide analytes (*e.g.*, eight targets) in said sample. For example, the bottom left magenta cluster in the left graph (circled in magenta) corresponds to the VCL target probe excited and contacted with its corresponding VCL target to generate a cumulative intensity measurement comprising low HEX fluorophore shown as a magenta cluster on the 2D amplitude plot.

From the above, it appears the following coding scheme was selected to correspond to the presence of a unique combination of presence or absence of eight targets. The x and y axes of the two 2D amplitude plots indicate the targets and their corresponding fluorophore components (*e.g.*, “HEX amplitude: low VCL, high REN” indicates VCL target corresponds to low HEX signal, REN target corresponds to high HEX signal). Low is represented by 1 (*e.g.*, low HEX for VCL indicated as 1), and high is represented by 2 (*e.g.*, high HEX for REN indicated as 2). The assay as reproduced above shows the non-degenerate identification of 8 analytes across four channels. Each graph represents 2^4 possibilities for each of the 4 analytes, thus giving 16 possible locations. Each will then also have a value in the paired graph for the second set of fluorophores. This gives a cumulative intensity measurement that corresponds to the presence of a unique

	combination of presence or absence of the at least five polynucleotide analytes (e.g., eight targets) in the sample. By accounting for every unique combination of presence or absence of the at least five polynucleotide analytes, the QX600 system can be used to determine whether each of the at least five polynucleotide analytes (e.g., PLAU or CCND1 or PLAU/CCND1) is present in any unique combination of presence or absence, based on the cumulative intensity measurement indicated by the 2D fluorescence amplitude plots and the coding scheme. <table><tr><td>Analyte</td><td>FAM</td><td>HEX</td><td>Cy5</td><td>Cy5.5</td></tr><tr><td>PLAU</td><td>1</td><td>0</td><td>0</td><td>0</td></tr><tr><td>CCND1</td><td>2</td><td>0</td><td>0</td><td>0</td></tr><tr><td>VCL</td><td>0</td><td>1</td><td>0</td><td>0</td></tr><tr><td>REN</td><td>0</td><td>2</td><td>0</td><td>0</td></tr><tr><td>BRCA1</td><td>0</td><td>0</td><td>1</td><td>0</td></tr><tr><td>KLF8</td><td>0</td><td>0</td><td>2</td><td>0</td></tr><tr><td>SORL1</td><td>0</td><td>0</td><td>0</td><td>1</td></tr><tr><td>PTEN</td><td>0</td><td>0</td><td>0</td><td>2</td></tr></table>	Analyte	FAM	HEX	Cy5	Cy5.5	PLAU	1	0	0	0	CCND1	2	0	0	0	VCL	0	1	0	0	REN	0	2	0	0	BRCA1	0	0	1	0	KLF8	0	0	2	0	SORL1	0	0	0	1	PTEN	0	0	0	2
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PTEN	0	0	0	2																																										
[1g] wherein the method does not require any step of	See Appendix A-01 at [1d.2].																																													

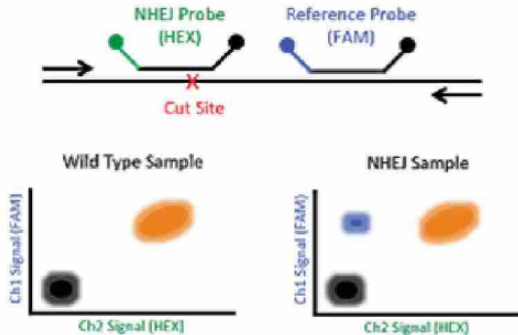
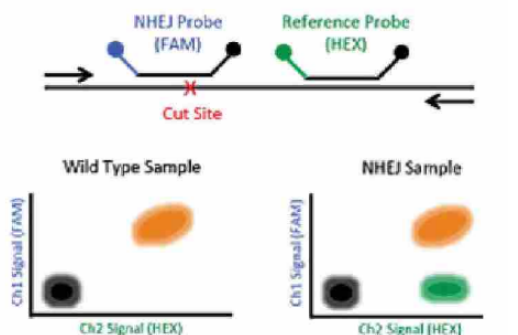
immobilization of said at least five polynucleotide analytes or mass spectrometry.	
2. The method of claim 1, wherein each of said at least five hybridization probes is complementary to one of said at least five polynucleotide analytes.	The QX600 Droplet Digital PCR System is used to perform the step wherein each of said at least five hybridization probes is complementary to one of said at least five polynucleotide analytes, both literally and under the doctrine of equivalents. For example: Drop-Off Probe in HEX <i>NHEJ Probe = HEX and Reference Probe =FAM</i>  Drop-Off Probe in FAM <i>NHEJ Probe = FAM and Reference Probe =HEX</i>  BIO_CC00007427 at BIO_CC00007618 As shown in the example figure above, each probe corresponds to one of the polynucleotide analytes: the NHEJ probe corresponds to NHEJ, and the reference probe corresponds to the reference. Each of the probes also corresponds to a specific fluorophore or a combination of fluorophores (e.g. NHEJ Probe = HEX and Reference Probe =FAM, or NHEJ Probe = FAM and Reference Probe = HEX), with each corresponding to a color indicated in the figure.

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.

BIO_CCODE00007427 at BIO_CCODE00007505

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
Positive Cy5 and Cy5.5	=	Light Blue
Positive ROX	=	Maroon
Positive ATTO 590	=	Gold
Positive ROX and ATTO 590	=	Aqua
Double negative	=	Gray

	BIO_CC00007427 at BIO_CC00007561 The QX600 system can be used to perform a probe mix triplex assay that has 9 hybridization probes (e.g., probes that correspond to FAM, HEX, FAM/HEX, Cy5, Cy5.5, Cy5/Cy5.5, ROX, ATTO590, and ROX/ATTO590), with each corresponding to one polynucleotide analyte targets for a total of 9 targets (e.g., three groups of three targets each). Thus, each probe of said at least five hybridization probes (e.g., 9 probes) is complementary to one of said at least five polynucleotide analytes (e.g., 9 analytes). Such samples may include, but not be limited to, the following applications:
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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.

See <https://www.bio-rad.com/en-us/product/qx600-droplet-digital-pcr-system?ID=b07d12ac-0585-fc4c-a586-3ddf20d5c4a0>; see also “Multiplex Assay Design for Epidemiological Wastewater Monitoring with Droplet Digital PCR Technology,” BIO_CC00008198; see also “Reproducible High-Resolution Characterization of AAV Vector Genome Identity Using the QX600,” BIO_CC00008202; see also “Mutation Detection Multiplexing Using the QX600 Droplet Digital PCR System,” BIO_CC00008194.

On information and belief, these applications comprise at least five polynucleotide analytes. For example:

In one example, the QX600 ddPCR System can be used for highly sensitive detection of commonly monitored pathogens in a rapid and accurate manner. The Multiplex Assay Design for Epidemiological Wastewater Monitoring with ddPCR Technology was used to create two 6-plex (e.g., 6 targets) wastewater assays and one single-target control assay for the following pathogens: adenoviruses, noroviruses, enteroviruses, *Legionella*, influenza A, influenza B, two monkeypox virus (MPXV) variants, and mouse hepatitis virus (MHV) as control.

Table 1. Pathogens and assigned fluorophores targeted in assays 1 and 2 and the MHV control assay.

	FAM	HEX	FAM/HEX	ROX	ATTO 590	Cy5	Cy5.5
Assay 1	Adenovirus	Norovirus	MHV control assay	MPXV (West African Variant 1)	MPXV (West African Variant 2)	Enterovirus	<i>Legionella</i>
Assay 2	Adenovirus	Norovirus	MHV control assay	MPXV (West African Variant 1)	MPXV (West African Variant 2)	Influenza A	Influenza B

MHV, mouse hepatitis virus; MPXV, monkeypox virus.

The Bio-Rad Expert Design service was used to create two 6-plex wastewater assays and one single-target control assay for the following pathogens: adenoviruses, noroviruses, enteroviruses, *Legionella*, influenza A, influenza B, two monkeypox virus (MPXV) variants, and mouse hepatitis virus (MHV) as control (Table 1)

BIO_CC00008198 at BIO_CC00008199

In another example, the QX600 ddPCR System can be used for high-resolution characterization of an AAV vector genome with multiple assays. The high-resolution characterization of AAV vector genome identity assay was used to perform two 6-plex (e.g., 6 targets) assays for the detection of 1. RSM2 ddPCR analysis: AAV ITR-2, hGFP, CMV-Enh, bGH poly(A), NeoR/KanR, SV40 poly(A); and 2. AAV5 ddPCR analysis: AAV ITR-2, GFP-K, CMV-Pro, eGFP, CMV-Enh, SV40 poly(A).

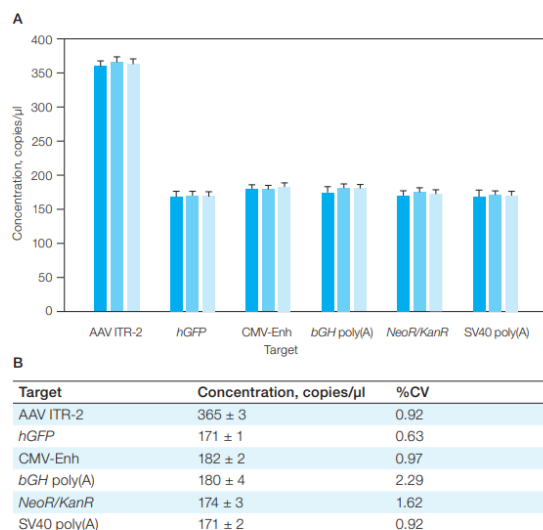


Fig. 2. Reproducibility of RSM2 ddPCR analysis. The concentration of six targets in an RSM2 vector sample were independently measured on three different days using a multiplex ddPCR experiment. **A**, the concentration of each of the six targets as determined by Poisson analysis. Error bars represent the upper portion of the 95% confidence interval. Replicate 1 (■), replicate 2 (■), replicate 3 (■). **B**, the average and standard deviation for the three concentration measurements (given in copies/μl) and the corresponding coefficients of variation (%CV). CMV-Enh, CMV enhancer.

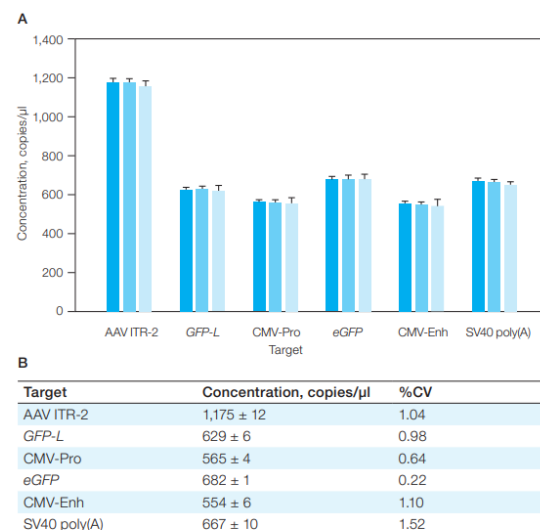


Fig. 3. Reproducibility of AAV5 ddPCR analysis. The concentration of six targets in an AAV5 vector sample were independently measured on three different days using a multiplex ddPCR experiment. **A**, the concentration of each of the six targets as determined by Poisson analysis. Error bars represent the upper portion of the 95% confidence interval. Replicate 1 (■), replicate 2 (■), replicate 3 (■). **B**, the average standard deviation for the three concentration measurements (given in copies/μl) and the corresponding coefficients of variation (%CV). CMV-Enh, CMV enhancer; CMV-Pro, CMV promoter.

BIO_CC00008202 at BIO_CC00008204

In another example, the QX600 ddPCR System can detect mutations commonly associated with non-small-cell lung cancer in a single-well, 6-plex reaction (e.g., 6 targets). The target mutations detected in this assay includes, at least five polynucleotide analytes: EGFR Exon 19 deletions, EGFR WT, KRAS G12C, EGFR L858r, BRAF V6000E, EGFR T790M.

Abstract

Precise and rapid mutation detection is critical to the advancement of cancer research, as patient outcomes depend on the early and accurate identification of these mutations. Compared to other methods of mutation detection, Droplet Digital™ PCR (ddPCR™) allows researchers to detect rare mutations at very low fractional abundance, with faster turnaround times, and at lower costs. In this study, we demonstrate how the QX600™ ddPCR System can detect mutations commonly associated with non-small-cell lung cancer (NSCLC) in a single-well, 6-plex reaction. While multiplexing cancer mutations is often challenging due to the need to discriminate multiple sets of highly similar sequences in a single well, here we show the capability of the QX600 ddPCR System to detect mutations with simple analysis.

BIO_CC CODE00008194 at BIO_CC CODE00008194

Table 1. Mutation detection assays and corresponding detection channels.

Assay	Detection Channel
EGFR Exon 19 Deletions	FAM
EGFR WT	HEX
KRAS G12C	Cy5
EGFR L858R	Cy5.5
BRAF V600E	ROX
EGFR T790M	ATTO 590
WT, wild type.	

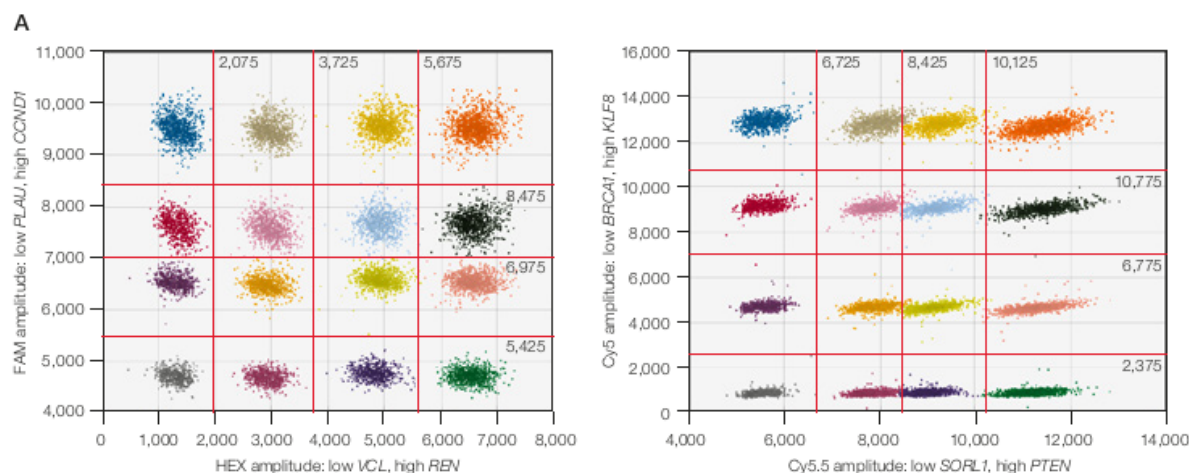
BIO_CC CODE00008194 at BIO_CC CODE00008195

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

The QX600 Droplet Digital PCR System is used to perform the step wherein said at least five hybridization probes comprise one or more fluorophores selected from one, two, three, or four fluorophores, both literally and under the doctrine of equivalents.

For example:

two, three, or four fluorophores.	Default color assignments for 2D amplitude plots are: <table><tr><td>Positive FAM</td><td>=</td><td>Blue</td></tr><tr><td>Positive HEX/VIC</td><td>=</td><td>Green</td></tr><tr><td>Positive FAM and HEX/VIC</td><td>=</td><td>Orange (may appear brown on some monitors)</td></tr><tr><td>Positive Cy5</td><td>=</td><td>Red</td></tr><tr><td>Positive Cy5.5</td><td>=</td><td>Violet</td></tr><tr><td>Positive Cy5 and Cy5.5</td><td>=</td><td>Light Blue</td></tr><tr><td>Positive ROX</td><td>=</td><td>Maroon</td></tr><tr><td>Positive ATTO 590</td><td>=</td><td>Gold</td></tr><tr><td>Positive ROX and ATTO 590</td><td>=</td><td>Aqua</td></tr><tr><td>Double negative</td><td>=</td><td>Gray</td></tr></table> BIO_CCODE00007427 at BIO_CCODE00007561 The QX600 system can be used to perform a probe mix triplex assay that has 9 hybridization probes (e.g., probes that correspond to FAM, HEX, FAM/HEX, Cy5, Cy5.5, Cy5/Cy5.5, ROX, ATTO590, and ROX/ATTO590), with each corresponding to one polynucleotide analyte targets for a total of 9 targets (e.g., three groups of three targets each). Thus, each probe of said at least five hybridization probe (e.g., 9 probes) is complementary to one of said at least five polynucleotide analytes (e.g., 9 analytes).	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	Positive Cy5 and Cy5.5	=	Light Blue	Positive ROX	=	Maroon	Positive ATTO 590	=	Gold	Positive ROX and ATTO 590	=	Aqua	Double negative	=	Gray
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Double negative	=	Gray																													



BIO_CCODE00008190 at BIO_CCODE00008192

From the above, it appears the following coding scheme was selected to correspond to the presence of a unique combination of presence or absence of eight targets. The x and y axes of the two 2D amplitude plots indicate the targets and their corresponding fluorophore components (e.g., “HEX amplitude: low *VCL*, high *REN*” indicates *VCL* target corresponds to low HEX signal, *REN* target corresponds to high HEX signal). Low is represented by 1 (e.g., low HEX for *VCL* indicated as 1), and high is represented by 2 (e.g., high HEX for *REN* indicated as 2).

Analyte	FAM	HEX	Cy5	Cy5.5
<i>PLAU</i>	1	0	0	0
<i>CCND1</i>	2	0	0	0
<i>VCL</i>	0	1	0	0
<i>REN</i>	0	2	0	0
<i>BRCA1</i>	0	0	1	0
<i>KLF8</i>	0	0	2	0
<i>SORL1</i>	0	0	0	1

	<table><tr><td>PTEN</td><td>0</td><td>0</td><td>0</td><td>2</td></tr></table> From the 2D fluorescence amplitude plots and the coding table indicates each hybridization probe, which corresponds to an analyte as explained, comprise one or more fluorophores selected from one, two, three, or four fluorophores (e.g., FAM, HEX, Cy5, Cy5.5). The assay described uses four different fluorophores combined with 8 different probes. Therefore, each of the probes comprises one fluorophore selected from the list of 4 fluorophores: FAM, HEX, Cy5, Cy5.5.	PTEN	0	0	0	2
PTEN	0	0	0	2		
4. The method of claim 1, wherein, in (b), a droplet of said plurality of droplets comprises said at least five hybridization probes.	The QX600 Droplet Digital PCR System is used to perform the step wherein, in (b), a droplet of said plurality of droplets comprises said at least five hybridization probes, both literally and under the doctrine of equivalents. For example: 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. BIO_CC00007427 AT BIO_CC00007449 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 BIO_CC00008190 at BIO_CC00008191 The QX600 system is used to perform and designed to be used in a droplet digital system, thus it is inherently designed and requires to function that the sample is separated into a plurality of droplets as					

	described above, and the probes being contained within at least one droplet to enable detection of the targets. Since the QX600 system can detect up to 12 targets in a single well (e.g., a droplet), and as explained above, each target contains a hybridization probe, the QX600 system is used to perform a droplet of said plurality of droplets comprises said at least five hybridization probes (e.g., 12 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.	The QX600 Droplet Digital PCR System is used to perform the step wherein, in (b), a droplet of said plurality of droplets comprises one or more of said at least five polynucleotide analytes, both literally and under the doctrine of equivalents. For example: 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. BIO_CCODE00007427 AT BIO_CCODE00007449 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 BIO_CCODE00008190 at BIO_CCODE00008191 The QX600 is used to perform a method where each sample is divided into droplets as described. The QX600 is used to perform a droplet (e.g., a sample) comprise one or more of said at least five

polynucleotide analytes (e.g., 12 targets). Such samples may include, but not be limited to, the following applications:

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.

See <https://www.bio-rad.com/en-us/product/qx600-droplet-digital-pcr-system?ID=b07d12ac-0585-fc4c-a586-3ddf20d5c4a0>; see also “Multiplex Assay Design for Epidemiological Wastewater Monitoring with Droplet Digital PCR Technology,” BIO_CC00008198; see also “Reproducible High-Resolution Characterization of AAV Vector Genome Identity Using the QX600,” BIO_CC00008202; see also

“Mutation Detection Multiplexing Using the QX600 Droplet Digital PCR System,”
BIO_CCODE00008194.

On information and belief, these applications comprise at least five polynucleotide analytes. For example:

In one example, the QX600 ddPCR System can be used for highly sensitive detection of commonly monitored pathogens in a rapid and accurate manner. The Multiplex Assay Design for Epidemiological Wastewater Monitoring with ddPCR Technology was used to create two 6-plex (e.g., 6 targets) wastewater assays and one single-target control assay for the following pathogens: adenoviruses, noroviruses, enteroviruses, *Legionella*, influenza A, influenza B, two monkeypox virus (MPXV) variants, and mouse hepatitis virus (MHV) as control.

Table 1. Pathogens and assigned fluorophores targeted in assays 1 and 2 and the MHV control assay.

	FAM	HEX	FAM/HEX	ROX	ATTO 590	Cy5	Cy5.5
Assay 1	Adenovirus	Norovirus	MHV control assay	MPXV (West African Variant 1)	MPXV (West African Variant 2)	Enterovirus	<i>Legionella</i>
Assay 2	Adenovirus	Norovirus	MHV control assay	MPXV (West African Variant 1)	MPXV (West African Variant 2)	Influenza A	Influenza B

MHV, mouse hepatitis virus; MPXV, monkeypox virus.

The Bio-Rad Expert Design service was used to create two 6-plex wastewater assays and one single-target control assay for the following pathogens: adenoviruses, noroviruses, enteroviruses, *Legionella*, influenza A, influenza B, two monkeypox virus (MPXV) variants, and mouse hepatitis virus (MHV) as control (Table 1)

BIO_CCODE00008198 at BIO_CCODE00008199

In another example, the QX600 ddPCR System can be used for high-resolution characterization of an AAV vector genome with multiple assays. The high-resolution characterization of AAV vector genome identity assay was used to perform two 6-plex (e.g., 6 targets) assays for the detection of 1. RSM2 ddPCR analysis: AAV ITR-2, hGFP, CMV-Enh, bGH poly(A), NeoR/KanR, SV40 poly(A); and 2. AAV5 ddPCR analysis: AAV ITR-2, GFP-K, CMV-Pro, eGFP, CMV-Enh, SV40 poly(A).

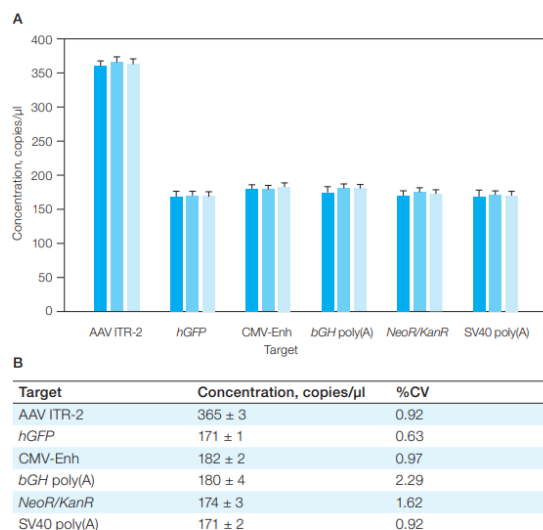


Fig. 2. Reproducibility of RSM2 ddPCR analysis. The concentration of six targets in an RSM2 vector sample were independently measured on three different days using a multiplex ddPCR experiment. **A**, the concentration of each of the six targets as determined by Poisson analysis. Error bars represent the upper portion of the 95% confidence interval. Replicate 1 (■), replicate 2 (■), replicate 3 (■). **B**, the average and standard deviation for the three concentration measurements (given in copies/μl) and the corresponding coefficients of variation (%CV). CMV-Enh, CMV enhancer.

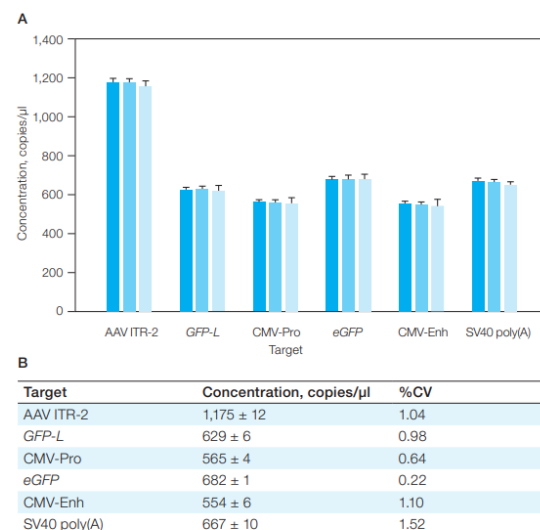


Fig. 3. Reproducibility of AAV5 ddPCR analysis. The concentration of six targets in an AAV5 vector sample were independently measured on three different days using a multiplex ddPCR experiment. **A**, the concentration of each of the six targets as determined by Poisson analysis. Error bars represent the upper portion of the 95% confidence interval. Replicate 1 (■), replicate 2 (■), replicate 3 (■). **B**, the average standard deviation for the three concentration measurements (given in copies/μl) and the corresponding coefficients of variation (%CV). CMV-Enh, CMV enhancer; CMV-Pro, CMV promoter.

BIO_CC00008202 at BIO_CC00008204

In another example, the QX600 ddPCR System can detect mutations commonly associated with non-small-cell lung cancer in a single-well, 6-plex reaction (e.g., 6 targets). The target mutations detected in this assay includes, at least five polynucleotide analytes: EGFR Exon 19 deletions, EGFR WT, KRAS G12C, EGFR L858r, BRAF V6000E, EGFR T790M.

Abstract

Precise and rapid mutation detection is critical to the advancement of cancer research, as patient outcomes depend on the early and accurate identification of these mutations. Compared to other methods of mutation detection, Droplet Digital™ PCR (ddPCR™) allows researchers to detect rare mutations at very low fractional abundance, with faster turnaround times, and at lower costs. In this study, we demonstrate how the QX600™ ddPCR System can detect mutations commonly associated with non-small-cell lung cancer (NSCLC) in a single-well, 6-plex reaction. While multiplexing cancer mutations is often challenging due to the need to discriminate multiple sets of highly similar sequences in a single well, here we show the capability of the QX600 ddPCR System to detect mutations with simple analysis.

BIO_CC00008194 at BIO_CC00008194

Table 1. Mutation detection assays and corresponding detection channels.

Assay	Detection Channel
EGFR Exon 19 Deletions	FAM
EGFR WT	HEX
KRAS G12C	Cy5
EGFR L858R	Cy5.5
BRAF V600E	ROX
EGFR T790M	ATTO 590

WT, wild type.

BIO_CC00008194 at BIO_CC00008195

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

The QX600 Droplet Digital PCR System is used to perform the step wherein, in (b), a droplet of said plurality of droplets comprises at most one of said at least five polynucleotide analytes, both literally and under the doctrine of equivalents.

For example:

polynucleotide analytes.	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. BIO_CCODE00007427 AT BIO_CCODE00007449 The QX600 system is used to divide each sample (<i>e.g.</i>, one of said at least five polynucleotide analytes) into droplets, thus a droplet (<i>e.g.</i>, a water-in-oil droplet) can comprise at most one of the at least five polynucleotide analytes (<i>e.g.</i>, divide each sample into water-in-oil droplets). Such samples may include, but not be limited to, the following applications:
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Applications of the QX600 Droplet Digital PCR System

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 - 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.
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- **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.
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See <https://www.bio-rad.com/en-us/product/qx600-droplet-digital-pcr-system?ID=b07d12ac-0585-fc4c-a586-3ddf20d5c4a0>; see also “Multiplex Assay Design for Epidemiological Wastewater Monitoring with Droplet Digital PCR Technology,” BIO_CCODE00008198; see also “Reproducible High-Resolution Characterization of AAV Vector Genome Identity Using the QX600,” BIO_CCODE00008202; see also “Mutation Detection Multiplexing Using the QX600 Droplet Digital PCR System,” BIO_CCODE00008194.

On information and belief, these applications comprise at least five polynucleotide analytes. For example:

In one example, the QX600 ddPCR System can be used for highly sensitive detection of commonly monitored pathogens in a rapid and accurate manner. The Multiplex Assay Design for Epidemiological Wastewater Monitoring with ddPCR Technology was used to create two 6-plex (e.g., 6 targets) wastewater assays and one single-target control assay for the following pathogens: adenoviruses, noroviruses, enteroviruses, *Legionella*, influenza A, influenza B, two monkeypox virus (MPXV) variants, and mouse hepatitis virus (MHV) as control.

Table 1. Pathogens and assigned fluorophores targeted in assays 1 and 2 and the MHV control assay.

	FAM	HEX	FAM/HEX	ROX	ATTO 590	Cy5	Cy5.5
Assay 1	Adenovirus	Norovirus	MHV control assay	MPXV (West African Variant 1)	MPXV (West African Variant 2)	Enterovirus	<i>Legionella</i>
Assay 2	Adenovirus	Norovirus	MHV control assay	MPXV (West African Variant 1)	MPXV (West African Variant 2)	Influenza A	Influenza B

MHV, mouse hepatitis virus; MPXV, monkeypox virus.

The Bio-Rad Expert Design service was used to create two 6-plex wastewater assays and one single-target control assay for the following pathogens: adenoviruses, noroviruses, enteroviruses, *Legionella*, influenza A, influenza B, two monkeypox virus (MPXV) variants, and mouse hepatitis virus (MHV) as control (Table 1)

BIO_CC00008198 at BIO_CC00008199

In another example, the QX600 ddPCR System can be used for high-resolution characterization of an AAV vector genome with multiple assays. The high-resolution characterization of AAV vector genome identity assay was used to perform two 6-plex (e.g., 6 targets) assays for the detection of 1. RSM2 ddPCR analysis: AAV ITR-2, hGFP, CMV-Enh, bGH poly(A), NeoR/KanR, SV40 poly(A); and 2. AAV5 ddPCR analysis: AAV ITR-2, GFP-K, CMV-Pro, eGFP, CMV-Enh, SV40 poly(A).

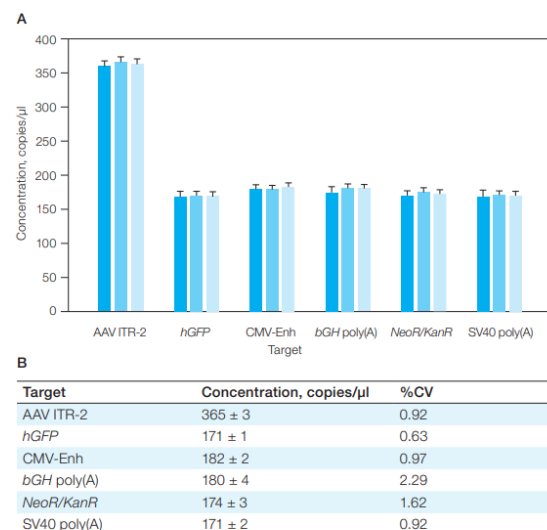


Fig. 2. Reproducibility of RSM2 ddPCR analysis. The concentration of six targets in an RSM2 vector sample were independently measured on three different days using a multiplex ddPCR experiment. **A**, the concentration of each of the six targets as determined by Poisson analysis. Error bars represent the upper portion of the 95% confidence interval. Replicate 1 (■), replicate 2 (■), replicate 3 (■). **B**, the average and standard deviation for the three concentration measurements (given in copies/μl) and the corresponding coefficients of variation (%CV). CMV-Enh, CMV enhancer.

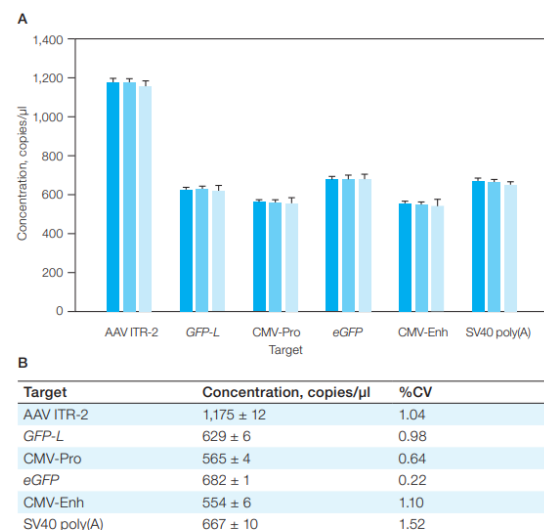


Fig. 3. Reproducibility of AAV5 ddPCR analysis. The concentration of six targets in an AAV5 vector sample were independently measured on three different days using a multiplex ddPCR experiment. **A**, the concentration of each of the six targets as determined by Poisson analysis. Error bars represent the upper portion of the 95% confidence interval. Replicate 1 (■), replicate 2 (■), replicate 3 (■). **B**, the average standard deviation for the three concentration measurements (given in copies/μl) and the corresponding coefficients of variation (%CV). CMV-Enh, CMV enhancer; CMV-Pro, CMV promoter.

BIO_CC00008202 at BIO_CC00008204

In another example, the QX600 ddPCR System can detect mutations commonly associated with non-small-cell lung cancer in a single-well, 6-plex reaction (e.g., 6 targets). The target mutations detected in this assay includes, at least five polynucleotide analytes: EGFR Exon 19 deletions, EGFR WT, KRAS G12C, EGFR L858r, BRAF V6000E, EGFR T790M.

Abstract

Precise and rapid mutation detection is critical to the advancement of cancer research, as patient outcomes depend on the early and accurate identification of these mutations. Compared to other methods of mutation detection, Droplet Digital™ PCR (ddPCR™) allows researchers to detect rare mutations at very low fractional abundance, with faster turnaround times, and at lower costs. In this study, we demonstrate how the QX600™ ddPCR System can detect mutations commonly associated with non–small-cell lung cancer (NSCLC) in a single-well, 6-plex reaction. While multiplexing cancer mutations is often challenging due to the need to discriminate multiple sets of highly similar sequences in a single well, here we show the capability of the QX600 ddPCR System to detect mutations with simple analysis.

BIO_CC00008194 at BIO_CC00008194

Table 1. Mutation detection assays and corresponding detection channels.

Assay	Detection Channel
EGFR Exon 19 Deletions	FAM
EGFR WT	HEX
KRAS G12C	Cy5
EGFR L858R	Cy5.5
BRAF V600E	ROX
EGFR T790M	ATTO 590

WT, wild type.

BIO_CC00008194 at BIO_CC00008195

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

See Appendix A-01 at Claim 4.

8. The method of claim 1, wherein said at least five polynucleotide analytes are at least seven polynucleotide analytes.	See Appendix A-01 at Claim 1, which describes the method with respect to at least seven polynucleotide analytes. As described there, the QX600 is able to quantify as many as 12 targets.
9. The method of claim 1, wherein said at least five hybridization probes are at least seven hybridization probes.	See Appendix A-01 at Claim 1 (in particular 1a.2), which describes the method with respect to at least seven hybridization probes. As described there, the QX600 is able to use at least seven 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.	The QX600 Droplet Digital PCR System is used to perform the step 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, both literally and under the doctrine of equivalents. For example:

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.

BIO_CCODE00007427 AT BIO_CCODE00007449

	 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. BIO_CC00008190 at BIO_CC00008193. On information and belief, the analytes will also include cell-free nucleic acid, fetal nucleic acid, viral nucleic acid, and/or some combination of those with the above reference to tumor nucleic acid. The QX600 system is used to perform detections of genomic alterations, detections of rare sequences, such as nucleic acid of tumor cells. It can also be used to perform mutation detection with circulating tumor nucleic acid (<i>e.g.</i>, DNA) analysis.
11. The method of claim 1, wherein said sample is a biological sample derived from a subject.	The QX600 Droplet Digital PCR System is used to perform the step wherein said sample is a biological sample derived from a subject, both literally and under the doctrine of equivalents. For example:

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.
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- **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.
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- **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.

BIO_CCODE00007427 AT BIO_CCODE00007449

	Abstract Precise and rapid mutation detection is critical to the advancement of cancer research, as patient outcomes depend on the early and accurate identification of these mutations. Compared to other methods of mutation detection, Droplet Digital™ PCR (ddPCR™) allows researchers to detect rare mutations at very low fractional abundance, with faster turnaround times, and at lower costs. In this study, we demonstrate how the QX600™ ddPCR System can detect mutations commonly associated with non–small-cell lung cancer (NSCLC) in a single-well, 6-plex reaction. While multiplexing cancer mutations is often challenging due to the need to discriminate multiple sets of highly similar sequences in a single well, here we show the capability of the QX600 ddPCR System to detect mutations with simple analysis. BIO_CC00008190 at BIO_CC00008194 The QX600 system is used to perform detection of genomic alterations, detection of rare diseases, next-generation sequencing, which comprise biological sample derived from a subject. The system is used to detect mutations commonly associated with non-small-cell lung cancer, which tests patient samples for improved patient outcomes.
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.	The QX600 Droplet Digital PCR System is used to perform the step 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, both literally and under the doctrine of equivalents. For example:

ddPCR is highly effective in the following areas:

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- **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.

BIO_CCODE00007427 AT BIO_CCODE00007449

	 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. BIO_CC00008190 at BIO_CC00008193 Abstract Precise and rapid mutation detection is critical to the advancement of cancer research, as patient outcomes depend on the early and accurate identification of these mutations. Compared to other methods of mutation detection, Droplet Digital™ PCR (ddPCR™) allows researchers to detect rare mutations at very low fractional abundance, with faster turnaround times, and at lower costs. In this study, we demonstrate how the QX600™ ddPCR System can detect mutations commonly associated with non–small-cell lung cancer (NSCLC) in a single-well, 6-plex reaction. While multiplexing cancer mutations is often challenging due to the need to discriminate multiple sets of highly similar sequences in a single well, here we show the capability of the QX600 ddPCR System to detect mutations with simple analysis. BIO_CC00008190 at BIO_CC00008194 The QX600 system is used to perform detections of genomic alterations, detections of rare sequences, such as nucleic acid of tumor cells. It can also be used to perform mutation detection with circulating tumor nucleic acid (<i>e.g.</i>, DNA) analysis. Detection of tumor targets indicates the presence of cancer or an infection with a pathogen in said subject. The system is used to detect the presence of mutations commonly associated with non-small-cell lung cancer.
13. The method of claim 1, further comprising	See Appendix A-01 at Claim 5.

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.	See Appendix A-01 at Claim 6.
15. The method of claim 14, wherein said clinical	The QX600 Droplet Digital PCR System is used to perform the step wherein said clinical decision is a treatment decision, both literally and under the doctrine of equivalents.

decision is a treatment decision.	For example: Precise and reproducible characterization of adeno-associated virus (AAV) vectors is critical for both preclinical and clinical applications of AAV-based gene therapy. An AAV vector genome may contain the inverted terminal repeat (ITR), an upstream control element, a downstream regulatory element, and a transgene. Because there are several potential target sequences within the AAV vector genome, multiple Droplet Digital PCR (ddPCR™) assays distributed throughout the genome can provide a more confident estimate of the encapsidated AAV vector genome concentration and genome identity. In this article, we demonstrate that high-resolution characterization of an AAV vector genome with multiple assays can be easily and reproducibly accomplished with the QX600 ddPCR System. BIO_CC00008202 Abstract Precise and rapid mutation detection is critical to the advancement of cancer research, as patient outcomes depend on the early and accurate identification of these mutations. Compared to other methods of mutation detection, Droplet Digital™ PCR (ddPCR™) allows researchers to detect rare mutations at very low fractional abundance, with faster turnaround times, and at lower costs. In this study, we demonstrate how the QX600™ ddPCR System can detect mutations commonly associated with non-small-cell lung cancer (NSCLC) in a single-well, 6-plex reaction. While multiplexing cancer mutations is often challenging due to the need to discriminate multiple sets of highly similar sequences in a single well, here we show the capability of the QX600 ddPCR System to detect mutations with simple analysis. BIO_CC00008190 at BIO_CC00008194 The QX600 system is used in to perform characterization of AAV vectors for preclinical and clinical applications of AAV-based gene therapy from where treatment decisions can be made. The QX600 system is used to detect mutations commonly associated with non-small-cell lung cancer, and such information is be used to improve patient outcomes where a health care professional makes a treatment decision based on accurate identification of these mutations.
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16. The method of claim 15, wherein said treatment decision comprises treatment with an antibiotic or an anticancer therapeutic.	The QX600 Droplet Digital PCR System is used to perform the step wherein said treatment decision comprises treatment with an antibiotic or an anticancer therapeutic, both literally and under the doctrine of equivalents. For example: 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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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.

BIO_CC00008190 at BIO_CC00008193

Abstract

Precise and rapid mutation detection is critical to the advancement of cancer research, as patient outcomes depend on the early and accurate identification of these mutations. Compared to other methods of mutation detection, Droplet Digital™ PCR (ddPCR™) allows researchers to detect rare mutations at very low fractional abundance, with faster turnaround times, and at lower costs. In this study, we demonstrate how the QX600™ ddPCR System can detect mutations commonly associated with non–small-cell lung cancer (NSCLC) in a single-well, 6-plex reaction. While multiplexing cancer mutations is often challenging due to the need to discriminate multiple sets of highly similar sequences in a single well, here we show the capability of the QX600 ddPCR System to detect mutations with simple analysis.

Systems over NGS include faster turnaround time, less hands-on-time, and lower cost. These combined benefits allow ddPCR technology to advance discoveries and improve outcomes across the patient journey. It is currently utilized in targeted mutational screening and monitoring, therapy response tracking, and molecular relapse monitoring, and appears in over 2,000 peer-reviewed publications related to mutation detection and liquid biopsy (bio-rad.com/ddPCR/publications).

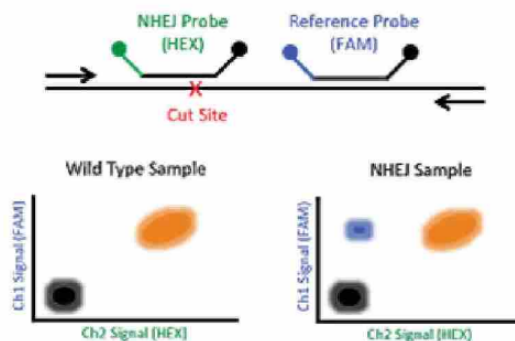
	BIO_CC00008190 at BIO_CC00008194 The QX600 system is used to perform detections of genomic alterations, detections of rare sequences, such as nucleic acid of tumor cells. It can also be used to perform mutation detection with circulating tumor nucleic acid (<i>e.g.</i>, DNA) analysis. Detection of tumor targets is used in treatment decisions comprising treatment with an antibiotic or an anticancer therapeutic. The QX600 system is used to detect mutations commonly associated with non-small-cell lung cancer, and such information is be used to improve patient outcomes including providing anticancer therapeutic and therapy response tracking.
17. The method of claim 1, wherein at least part of said method is performed using a computer.	See Appendix A-01 at Claim 7.
18. The method of claim 17, wherein said computer is located on a remote server.	See Appendix A-01 at Claim 8.
19. The method of claim 17, wherein said computer is located on a droplet reader.	See Appendix A-01 at Claim 7. As discussed there, this claim is satisfied when the QX600 is the computer. Alternatively, the claim may be satisfied by the computer running the QX Manager software which is operating as a part of the droplet reader.
20. The method of claim 1, wherein each of said at least five hybridization	The QX600 Droplet Digital PCR System is used to perform the step 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, both literally and under the doctrine of equivalents.

probes are provided at a given concentration such that said cumulative intensity measurement corresponds to said presence of said unique combination.

For example:

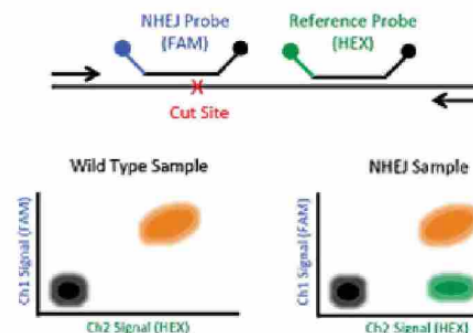
Drop-Off Probe in HEX

NHEJ Probe = HEX and Reference Probe = FAM



Drop-Off Probe in FAM

NHEJ Probe = FAM and Reference Probe = HEX



BIO_CCODE00007427 at BIO_CCODE00007618

As shown in the example figure above, each probe corresponds to one of the polynucleotide analytes: the NHEJ probe corresponds to NHEJ, and the reference probe corresponds to the reference. Each of the probes also corresponds to a specific fluorophore or a combination of fluorophores (e.g. NHEJ Probe = HEX and Reference Probe = FAM, or NHEJ Probe = FAM and Reference Probe = HEX), with each corresponding to a color indicated in the figure.

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.

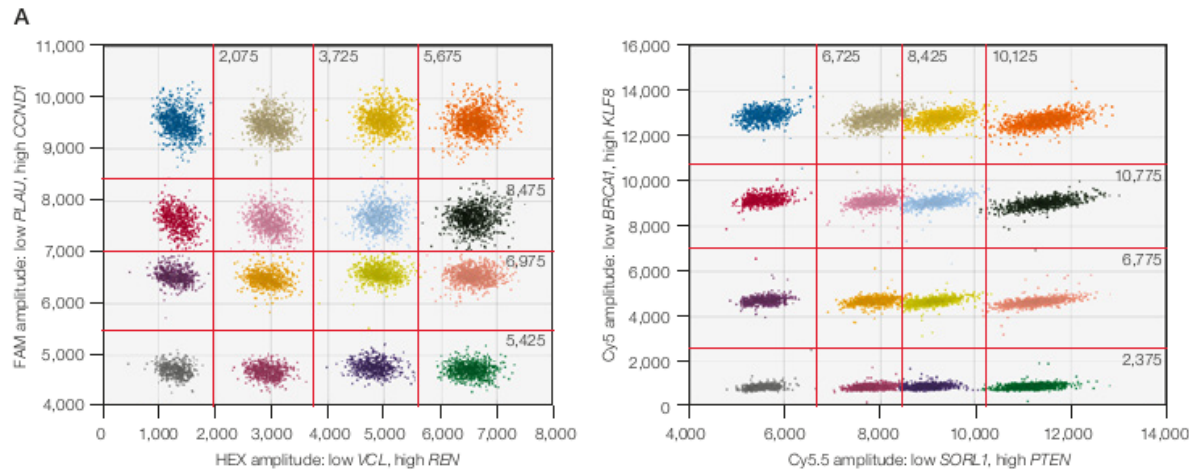
BIO_CCODE00007427 at BIO_CCODE00007505

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
Positive Cy5 and Cy5.5	=	Light Blue
Positive ROX	=	Maroon
Positive ATTO 590	=	Gold
Positive ROX and ATTO 590	=	Aqua
Double negative	=	Gray

BIO_CCODE00007427 at BIO_CCODE00007561

The QX600 system can be used to perform a probe mix triplex assay that has 9 hybridization probes (e.g., probes that correspond to FAM, HEX, FAM/HEX, Cy5, Cy5.5, Cy5/Cy5.5, ROX, ATTO590, and ROX/ATTO590), with each corresponding to one polynucleotide analyte targets for a total of 9 targets (e.g., three groups of three targets each).



BIO_CCODE00008190 at BIO_CCODE00008192

As seen in the 2D fluorescence amplitude plots, each cumulative intensity measurement corresponds to the presence of a unique combination of presence or absence of the at least five polynucleotide analytes (e.g., eight targets) in said sample.

From the above, it appears the following coding scheme was selected to correspond to the presence of a unique combination of presence or absence of eight targets. The x and y axes of the two 2D amplitude plots indicate the targets and their corresponding fluorophore components (e.g., “HEX amplitude: low VCL, high REN” indicates VCL target corresponds to low HEX signal, REN target corresponds to high HEX signal). Low is represented by 1 (e.g., low HEX for VCL indicated as 1), and high is represented by 2 (e.g., high HEX for REN indicated as 2).

Analyte	FAM	HEX	Cy5	Cy5.5
PLAU	1	0	0	0
CCND1	2	0	0	0
VCL	0	1	0	0
REN	0	2	0	0
BRCA1	0	0	1	0
KLF8	0	0	2	0
SORL1	0	0	0	1
PTEN	0	0	0	2

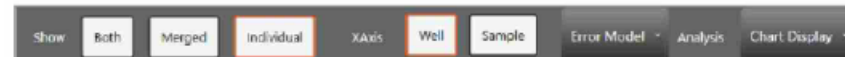
The QX600 system is used to perform ddPCR reactions such that the cumulative intensity in each channel of the system corresponds to the unique combination of analytes in that channel. Because the intensities are either “1” as low or “2” as high, 4 unique cumulative positions are possible in each channel. By combining the results of each of the 4 channels, each of the 256 different possible combinations of the 8 analytes possible in any droplet are uniquely represented:

$$(4 \times 4 \times 4 \times 4) = (2^8)$$

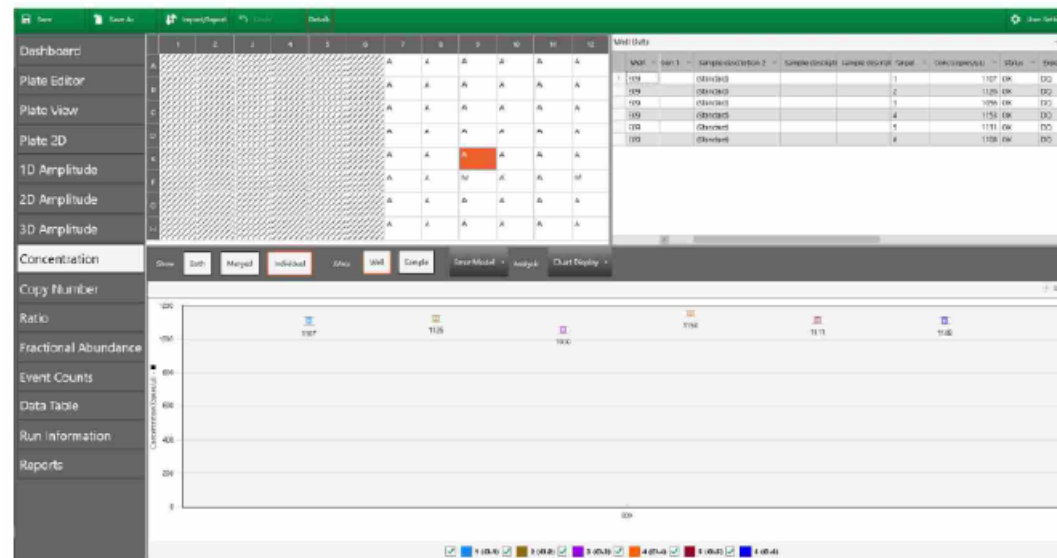
Concentration

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.

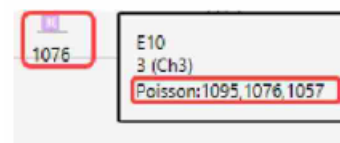
For information on toolbar options, see [Data Visualization Chart Analysis Options on page 135](#).



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.



BIO_CC00007427 at BIO_CC00007566

Thus, combining the disclosures stated above, the QX600 system can be used to perform wherein each of said at least five hybridization probes (e.g., 9 probes) are provided at a given concentration (e.g., a concentration indicating the number of copies of the target molecules in each ul of sample) such that said cumulative intensity measurement corresponds to said presence of said unique combination (e.g., each of the 256 different possible combinations of the 8 analytes possible in any droplet are uniquely represented).

See also Claim Element 1f describing the various disclosures for quantifying the presence of analytes.

Appendix B-2: Infringement Claim Chart for U.S. Patent No. 10,770,170 and QX ONE

Use of Bio-Rad's QX ONE Droplet Digital PCR (ddPCR) System ("QX ONE") (includes both standard and premium editions) infringes Claims 1-20 of U.S. Patent No. 10,770,170 ("the '170 Patent"). As non-limiting examples, Plaintiffs identify where and how use of the Accused Product performs each and every step of the below claimed method. Elements from other charts may be used in combination with the below to perform the steps of the claimed method, and Plaintiffs incorporate all other '170 Charts here by reference.

As non-limiting examples, Plaintiffs identify where and how use of the Accused Product performs each and every step of the below claimed method. Elements from other charts may be used in combination with the below to perform the steps of the claimed method, and Plaintiffs incorporate all other '170 Charts here by reference.

On information and belief, the QX600 is used with devices, accessories, and components to practice the methods of the '170 Patent. See <https://www.bio-rad.com/en-us/life-science/digital-pcr/qx-one-droplet-digital-pcr-ddpcr-system>. A list of non-limiting examples of such devices, accessories, and/or components include:

- The QX ONE system;
- Software, such as the QX ONE Software (Regulatory and Standard editions);
- Consumables such as
 - o GCR96 Cartridges,
 - o QX ONE Droplet Generation Oil for Probes,
 - o QX ONE Droplet Reader Oil,
 - o GCR96 Foil Heat Seal,
 - o QX ONE ddPCR System Waste Bottle;
 - o the ddPCR 96-Well Plates,
 - o Droplet Generation Oil for Probes,
 - o QX200 Droplet Generation Oil for EvaGreen,
 - o Droplet Reader Oil,
 - o DG8 Cartridges and DG8 Gaskets,
 - o DG8 Cartridge Holders,
 - o pierceable foil such as the PCR Plate Heat Seal,
 - o DG32 Cartridges for AutoDG,
 - o Pipet Tips for AutoDG, and/or
 - o Pipet Tip Waste Bin for AutoDG;
- A plate sealer, such as the PX1 PCR Plate Sealer;

- ddPCR kits and reagents, such as
 - o PrimePCR ddPCR Assays,
 - o ddPCR Multiplex Supermix,
 - o ddPCR Supermixes for Probes (No dUTP),
 - o ddPCR Supermix for Probes,
 - o One-Step RT-ddPCR Advanced Kit for Probes,
 - o ddPCR Buffer Control for Probes,
 - o Residual DNA Quantification Supermixes,
 - o QX200 ddPCR EvaGreen Supermix,
 - o QX200 Buffer Control for EvaGreen, and
 - o ddPCR Library Quantification Kit for Illumina TruSeq;
- PrimePCR Primers, Assays and Arrays;
- Each of the assays identified in these Infringement Contentions; to include:
 - o dHsaEXD41810842
 - o RCAAV Kit
 - o HEK293 Kit, and/or
- Any other processes, procedures, devices, and/or system components used to facilitate the performance of the steps of the Asserted Claims.

Bio-Rad directly infringes the Asserted Claims by using the QX One alone and/or with the above-listed devices, software, accessories, and/or components to perform each and every step of the claim methods below. By way of non-limiting example, Bio-Rad uses the QX One in connection with (but not exclusively with) the various assays identified in these Infringement Contentions to practice each and every step of the Asserted Claims.

Bio-Rad also indirectly infringes the Asserted Claims by providing the QX One alone and/or with the accompanying QX One Software. Bio-Rad also induces customers and/or end users to use the QX One and/or QX One Software to infringe by inducing them to use these products in a way that performs each step of the Asserted Claims as described below and throughout these Infringement Contentions in violation of 35 U.S.C. § 271(b). By way of non-limiting example, such customers and/or end users use the QX One in connection with (but not exclusively with) the various assays identified in these Infringement Contentions to practice each and every step of the Asserted Claims.

Bio-Rad also contributes to the infringement of customers and/or end-users by providing the QX One and/or the QX One Software to the same who then perform each step of the Asserted Claims as described below and throughout these Infringement Contentions in violation of

35 U.S.C. § 271(c). By way of non-limiting example, such customers and/or end users use the QX One in connection with (but not exclusively with) the various assays identified in these Infringement Contentions to practice each and every step of the Asserted Claims.

On information and belief, Bio-Rad also supplies or causes to be supplied in or from the United States all or a substantial portion of the QX One and/or QX One Software such that Bio-Rad induces infringe of customers or end users outside the United States in violation of 35 U.S.C. § 271(f)(1). For like reasons as described above, Bio-Rad contributes to the infringement of customers or end users outside the United States in violation of 35 U.S.C. § 271(f)(2).

On information and belief, to the extent Bio-Rad performs the steps of the Asserted Claims outside the United States and subsequently imports, offers to sell, sells, and/or uses the results of the claimed methods within the United States, Bio-Rad infringes in violation of 35 U.S.C. § 271(g).

Plaintiffs allege infringement both literally and under the doctrine of equivalents.

Claims	Exemplary Disclosure of Accused Product
[1-pre] 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:	The QX One System is used to perform 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. To the extent the preamble is limiting, the QX ONE is used to detect the unique combination of presence or absence of up to eight polynucleotide analytes (called “targets”) as described below. Performs this detection based on droplets that are prepared from a sample volume using the QX ONE’s droplet generator. The system allows for clear discrimination of multiple targets and more accurate and reproducible quantification, which allows it to detect the presence or absence of polynucleotide analytes with high accuracy, precision, and reproducibility. The QX ONE System comprises multiple components and kits.

QX ONE Droplet Digital PCR System Components

The QX ONE Droplet Digital PCR System ships with the following items:

- The instrument, which performs the sequential ddPCR processes
- Attached touch screen, which is a computer that displays the software interface and connects to the instrument
- QX ONE Software, which is installed on the instrument touch screen computer, and provides a user interface to
 - Control the instrument
 - Set up assays
 - Collect analysis data
 - Recover plates from run failures
 - Store analysis data
 - Display analysis data
- QX ONE Droplet Digital PCR System Accessory Kit, which includes the following:
 - PX1 GCR Sealer Support Block
 - QX ONE Droplet Digital PCR System Waste Bottles
 - Keyboard
 - Power cord (compatible within North America)
 - Warranty card

For information on additional ddPCR components and supplies, see [Appendix B, Ordering Information](#).

QX ONE Droplet Digital PCR System and QX ONE Software Instrument Guide Version 1.4,
BIO_CC00006554 at 6577.

Experiment Types

QX ONE Software offers the experiment types described in Table 9, each enabling different assay options and analysis tools. You can assign multiple experiment types across the plate, but you can assign only one experiment type to each well.

Table 9. Experiment types

Type	Description
Direct quantification (DQ)	DQ uses absolute quantification to determine the concentration (copies/ μ l) 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 (as with DQ) 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 (as with DQ) 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.
Residual DNA quantification (RDQ)	RDQ provides a precise method for residual host cell DNA quantification. Note: Users can enter a conversion factor to calculate the mass for a well, up to three decimals. The software displays the result in the Molecular Weight column of the Data Table.

BIO_CC00006618.

The QX One System can detect up to eight targets using four fluorophores.

Assay Types and Fluorophores

QX ONE Software offers the assay methods explained in [Table 10](#).

Table 10. Assay types

Assay type	Description
Single target per channel	Method assuming up to four probe colors (FAM, HEX/VIC, Cy5 and Cy5.5) and up to four targets per well, with a single target detected per channel. This is the default assay type, with the maximum four rows displayed. You can delete up to three rows.
Amplitude multiplex	Method to increase multiplexing up to eight 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 two 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. You can delete a group, but not the individual targets within the group. Note: The channels within a 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 a group are not interchangeable, therefore FAM is always paired with HEX/VIC and so forth.

Table 10. Assay types, continued

Assay type	Description
Double drop-off	This assay type is available only for the DOF experiment type, and allows two groups of three targets each. The first group appears by default, and looks for one target in FAM and in HEXV/C (target type reference) one target in FAM (target type unknown), and one target in HEXV/C (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). 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 HEXV/C, Channel 3 Cy5 is always paired with Channel 4 Cy5.5.)

The experiment type you select determines the available assay methods. [Table 11](#) displays the sample type associated with each assay method.

Table 11. Assay types by experiment type

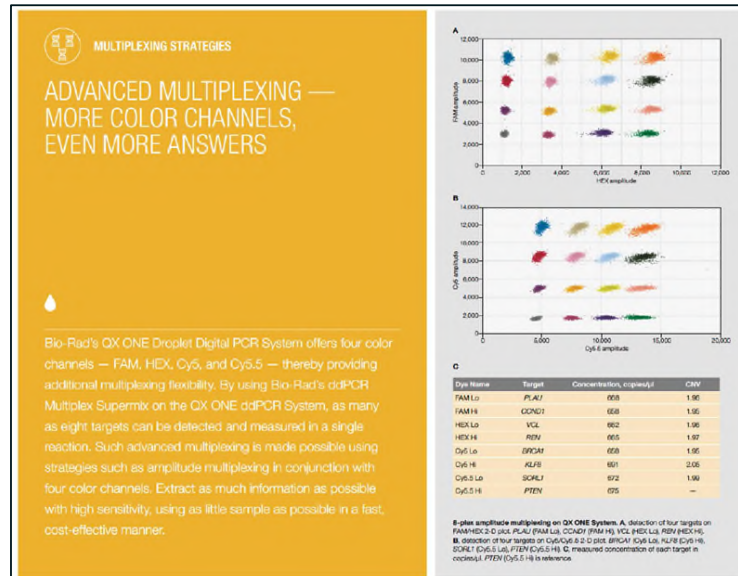
Assay type	Experiment type						
	DQ	CNV	MUT	DOF	GEX	RDQ	RED
Single target per channel	✓	✓	✓		✓	✓	✓
Amplitude multiplex	✓	✓	✓		✓	✓	✓
Probe mix triplex	✓	✓	✓		✓	✓	✓
Advanced classification	✓						✓
Basic drop-off				✓			
Double drop-off				✓			

QX ONE Software automatically populates the fluorophore in each channel for each target but you can change the default selections.

Table 12. Fluorophore options

Assay method	Signal Ch1	Signal Ch2	Signal Ch3	Signal Ch4
Single target per channel, for 1 to 4 targets	FAM	HEX	Cy5	Cy5.5
	None	VIC	None	None
Amplitude multiplex, for 1 to 8 targets	FAM Hi	HEX Hi	Cy5 Hi	Cy5.5 Hi
	FAM Lo	HEX Lo	Cy5 Lo	Cy5.5 Lo
	None	VIC Hi	None	None
		VIC Lo		
Probe mix triplex, for 6 targets	FAM	HEX	Cy5	Cy5.5
	None	VIC	None	None
Advanced classification	FAM	HEX	Cy5	Cy5.5
	+1-10	1 to 10	1 to 10	1 to 10
	None	VIC	None	None
		1 to 10		

BIO_CC00006622-6624



BIO_CC00008407

The QX One System analyzes multiple targets (up to 8 targets) using amplitude multiplexing and probe mixing strategies.

	Data Analysis Module The Data Analysis module functionality allows you to ■ View your droplet data in 1D, 2D, and 3D amplitude □ View thresholds automatically assigned by the software based on the distance of the threshold from the means and standard deviations of the positive and negative clusters □ Manually adjust thresholds or classify droplets using cluster modes □ View data in amplitude plots, histograms, and heat maps □ Apply tilt correction to automatic analysis ■ View charts reflecting calculated data visualizations for concentration, copy number, ratio, and fractional abundance ■ 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 BIO_CC00006651
[1a] (a) providing a sample comprising, or potentially comprising, at least one of said at least five polynucleotide analytes;	See Appendix A-02 at [1a.1] ("providing a sample solution volume comprising or potentially comprising at least one of at least seven polynucleotide analytes"). The QX One System is used to perform providing a sample comprising, or potentially comprising, at least one of said at least five polynucleotide analytes. The QX One System requires the samples to contain the targeted DNA or RNA.

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.

BIO_CCODE00006573

The QX One System can detect up to eight targets using four fluorophores.

Assay Types and Fluorophores

QX ONE Software offers the assay methods explained in [Table 10](#).

Table 10. Assay types

Assay type	Description
Single target per channel	Method assuming up to four probe colors (FAM, HEX/VIC, Cy5 and Cy5.5) and up to four targets per well, with a single target detected per channel. This is the default assay type, with the maximum four rows displayed. You can delete up to three rows.
Amplitude multiplex	Method to increase multiplexing up to eight 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 two 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. You can delete a group, but not the individual targets within the group. Note: The channels within a 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 <u>and</u> HEX/VIC and one target in FAM <u>or</u> 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 <u>and</u> Cy5.5, one target in Cy5 <u>or</u> Cy5.5. You can delete a group, but not the individual targets in the group. Note: The channels within a group are not interchangeable; therefore FAM is always paired with HEX/VIC and so forth.

Table 10. Assay types, continued

Assay type	Description
Double drop-off	This assay type is available only for the DOF experiment type, and allows two 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). 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..

The experiment type you select determines the available assay methods. [Table 11](#) displays the sample type associated with each assay method.

Table 11. 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				✓			

QX ONE Software automatically populates the fluorophore in each channel for each target but you can change the default selections.

Table 12. Fluorophore options

Assay method	Signal Ch1	Signal Ch2	Signal Ch3	Signal Ch4
Single target per channel, for 1 to 4 targets	FAM	HEX	Cy5	Cy5.5
	None	VIC	None	None
		None		
Amplitude multiplex, for 1 to 8 targets	FAM Hi	HEX Hi	Cy5 Hi	Cy5.5 Hi
	FAM Lo	HEX Lo	Cy 5 Lo	Cy 5.5 Lo
	None	VIC Hi	None	None
		VIC Lo		
Probe mix triplex, for 6 targets	FAM	HEX	Cy5	Cy5.5
	None	VIC	None	None
		None		
Advanced classification	FAM	HEX	Cy5	Cy5.5
	+1-10	1 to 10	1 to 10	1 to 10
	None	VIC	None	None
		1 to 10		
		None		

BIO_CC00006622- BIO_CC00006624

[1b.1]

(b) forming a mixture of said sample and at least five hybridization probes,

See Appendix A-02 at [1a.1], [1a.4], and [1a.5], which describe the step of forming the mixture using the hybridization probes. Plaintiffs incorporate those steps here by reference. As described in those steps, the QX ONE is able to be used with over seven hybridization probes, meaning it can also be used with “at least five hybridization probes.”

Additionally:

The QX One System is used to perform a mixture of said sample and at least five hybridization probes either literally or under the doctrine of equivalents.

The QX One System offers different types of supermixes based on different experiments.

	Supermixes QX ONE 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. <table border="1"> <thead> <tr> <th>ddPCR Supermix</th><th>Description</th></tr> </thead> <tbody> <tr> <td>Multiplex Supermix</td><td>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.</td></tr> <tr> <td>Supermix for Probes (No dUTP)</td><td>For use in nucleic acid sample preparations, with sensitivity for the amplification and detection of DNA targets using hydrolysis probe-based assays.</td></tr> <tr> <td>Supermix for Probes</td><td>For use in sample preparation with uracil N-glycosylase (UNG) decontamination protocols to prevent the reamplification of carryover PCR products between experiments.</td></tr> <tr> <td>Supermix for Residual DNA Quantification</td><td>For use in residual DNA detection.</td></tr> <tr> <td>One-Step RT Advanced Kit for Probes</td><td>For use in absolute quantification of target RNA molecules. Important: Load only one (1) one-step RT plate, using the first slot in the QX ONE Droplet Digital PCR System plate Inbox. For more information, see Using the One Step RT ddPCR Advanced Kit for Probes.</td></tr> <tr> <td>EvaGreen® Supermix</td><td>For use in gene expression.</td></tr> </tbody> </table> BIO_CC00006620	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-glycosylase (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 Probes	For use in absolute quantification of target RNA molecules. Important: Load only one (1) one-step RT plate, using the first slot in the QX ONE Droplet Digital PCR System plate Inbox. For more information, see Using the One Step RT ddPCR Advanced Kit for Probes .	EvaGreen® Supermix	For use in gene expression.
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-glycosylase (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 Probes	For use in absolute quantification of target RNA molecules. Important: Load only one (1) one-step RT plate, using the first slot in the QX ONE Droplet Digital PCR System plate Inbox. For more information, see Using the One Step RT ddPCR Advanced Kit for Probes .														
EvaGreen® Supermix	For use in gene expression.														
[1b.2] wherein each of said at least five hybridization probes further comprises at least one	See step 1a above, which describes how a mixture is formed. See also Appendix A-02 at Step [1a.3] (“wherein each hybridization probe comprises at least one fluorophore selected from four fluorophores”), which describes the process by which each hybridization probe comprises at least one fluorophore selected from four fluorophore. That step is materially the same as this step, and Plaintiffs incorporate all references steps here by reference. As described in those steps, the QX600 is able to be used with over seven hybridization probes as well as four fluorophores comprising the list, meaning it can also be														

fluorophore and at most four fluorophores;

used with “at least five hybridization probes” and with at least one fluorophore and at most four fluorophores.

Additionally, on information and belief the QX ONE is able to filter certain fluorophores. Further:

The QX One System is used to perform the step of wherein each of said at least five hybridization probes further comprises at least one fluorophore.

The QX One System has four channels, each channel contains at least one fluorophore (from FAM, HEX/VIC, Cy5 and Cy5.5).

FEATURES AND BENEFITS	
 WALK-AWAY OPERATION	▪ The QX ONE System automates a fully integrated ddPCR workflow, allowing for walk-away operation ▪ A 5-plate capacity allows 480 samples to be analyzed in a single day
 EASY TO USE	▪ Simple, intuitive run setup using a large onboard touch screen ▪ Optimized plate setup means fewer handling steps, fewer operator errors, and faster onboarding
 ADVANCED MULTIPLEXING	▪ Four color channels combined with amplitude multiplexing and/or radial multiplexing allow for multiple unique targets to be detected in a single reaction ▪ Specially formulated ddPCR Multiplex Supermix is optimized to empower advanced multiplexing in probe-based assays
 REGULATORY COMPLIANT	▪ QX ONE Software, Regulatory Edition is a robust package that enables U.S. FDA 21 CFR Part 11 compliance and offers audit trails with tracked protocol changes for regulatory requirements ▪ RFID barcoded consumables allow lot management and traceability

BIO_CC00008490

Assay Types and Fluorophores

QX ONE Software offers the assay methods explained in Table 10.

Table 10. Assay types

Assay type	Description
Single target per channel	Method assuming up to four probe colors (FAM, HEX/VIC, Cy5 and Cy5.5) and up to four targets per well, with a single target detected per channel. This is the default assay type, with the maximum four rows displayed. You can delete up to three rows.
Amplitude multiplex	Method to increase multiplexing up to eight 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.

BIO_CC00006622

Table 12. Fluorophore options

Assay method	Signal Ch1	Signal Ch2	Signal Ch3	Signal Ch4
Single target per channel, for 1 to 4 targets	FAM	HEX	Cy5	Cy5.5
	None	VIC None	None	None
Amplitude multiplex, for 1 to 8 targets	FAM Hi	HEX Hi	Cy5 Hi	Cy5.5 Hi
	FAM Lo	HEX Lo	Cy 5 Lo	Cy 5.5 Lo
	None	VIC Hi	None	None
		VIC Lo None		
Probe mix triplex, for 6 targets	FAM	HEX	Cy5	Cy5.5
	None	VIC None	None	None
Advanced classification	FAM	HEX	Cy5	Cy5.5
	+1-10	1 to 10	1 to 10	1 to 10
	None	VIC 1 to 10 None	None	None

BIO_CC00006624

The QX One System is used to perform the step of wherein each of said at least five hybridization probes further comprises at most four fluorophores either literally or under the doctrine of equivalents.

The QX One System has four channels, which correspond to at most four fluorophores (FAM, HEX/VIC, Cy5 and Cy5.5).

FEATURES AND BENEFITS		
	WALK-AWAY OPERATION	- The QX ONE System automates a fully integrated ddPCR workflow, allowing for walk-away operation - A 5-plate capacity allows 480 samples to be analyzed in a single day
	EASY TO USE	- Simple, intuitive run setup using a large onboard touch screen - Optimized plate setup means fewer handling steps, fewer operator errors, and faster onboarding
	ADVANCED MULTIPLEXING	- Four color channels combined with amplitude multiplexing and/or radial multiplexing allow for multiple unique targets to be detected in a single reaction - Specially formulated ddPCR Multiplex Supermix is optimized to empower advanced multiplexing in probe-based assays
	REGULATORY COMPLIANT	- QX ONE Software, Regulatory Edition is a robust package that enables U.S. FDA 21 CFR Part 11 compliance and offers audit trails with tracked protocol changes for regulatory requirements - RFID barcoded consumables allow lot management and traceability

BIO_CC00008490

Assay Types and Fluorophores

QX ONE Software offers the assay methods explained in [Table 10](#).

Table 10. Assay types

Assay type	Description
Single target per channel	Method assuming up to four probe colors (FAM, HEX/VIC, Cy5 and Cy5.5) and up to four targets per well, with a single target detected per channel. This is the default assay type, with the maximum four rows displayed. You can delete up to three rows.
Amplitude multiplex	Method to increase multiplexing up to eight 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.

BIO_CC00006622

	Table 12. Fluorophore options <table><tr><th>Assay method</th><th>Signal Ch1</th><th>Signal Ch2</th><th>Signal Ch3</th><th>Signal Ch4</th></tr><tr><td rowspan="3">Single target per channel, for 1 to 4 targets</td><td>FAM</td><td>HEX</td><td>Cy5</td><td>Cy5.5</td></tr><tr><td>None</td><td>VIC</td><td>None</td><td>None</td></tr><tr><td></td><td>None</td><td></td><td></td></tr><tr><td rowspan="5">Amplitude multiplex, for 1 to 8 targets</td><td>FAM Hi</td><td>HEX Hi</td><td>Cy5 Hi</td><td>Cy5.5 Hi</td></tr><tr><td>FAM Lo</td><td>HEX Lo</td><td>Cy 5 Lo</td><td>Cy 5.5 Lo</td></tr><tr><td>None</td><td>VIC Hi</td><td>None</td><td>None</td></tr><tr><td></td><td>VIC Lo</td><td></td><td></td></tr><tr><td></td><td>None</td><td></td><td></td></tr><tr><td rowspan="3">Probe mix triplex, for 6 targets</td><td>FAM</td><td>HEX</td><td>Cy5</td><td>Cy5.5</td></tr><tr><td>None</td><td>VIC</td><td>None</td><td>None</td></tr><tr><td></td><td>None</td><td></td><td></td></tr><tr><td rowspan="5">Advanced classification</td><td>FAM</td><td>HEX</td><td>Cy5</td><td>Cy5.5</td></tr><tr><td>+1-10</td><td>1 to 10</td><td>1 to 10</td><td>1 to 10</td></tr><tr><td>None</td><td>VIC</td><td>None</td><td>None</td></tr><tr><td></td><td>1 to 10</td><td></td><td></td></tr><tr><td></td><td>None</td><td></td><td></td></tr></table> BIO_CC00006624	Assay method	Signal Ch1	Signal Ch2	Signal Ch3	Signal Ch4	Single target per channel, for 1 to 4 targets	FAM	HEX	Cy5	Cy5.5	None	VIC	None	None		None			Amplitude multiplex, for 1 to 8 targets	FAM Hi	HEX Hi	Cy5 Hi	Cy5.5 Hi	FAM Lo	HEX Lo	Cy 5 Lo	Cy 5.5 Lo	None	VIC Hi	None	None		VIC Lo				None			Probe mix triplex, for 6 targets	FAM	HEX	Cy5	Cy5.5	None	VIC	None	None		None			Advanced classification	FAM	HEX	Cy5	Cy5.5	+1-10	1 to 10	1 to 10	1 to 10	None	VIC	None	None		1 to 10				None		
Assay method	Signal Ch1	Signal Ch2	Signal Ch3	Signal Ch4																																																																						
Single target per channel, for 1 to 4 targets	FAM	HEX	Cy5	Cy5.5																																																																						
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Amplitude multiplex, for 1 to 8 targets	FAM Hi	HEX Hi	Cy5 Hi	Cy5.5 Hi																																																																						
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Probe mix triplex, for 6 targets	FAM	HEX	Cy5	Cy5.5																																																																						
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	None	VIC	None	None																																																																						
		1 to 10																																																																								
		None																																																																								
[1c] (c) partitioning said mixture into said plurality of droplets;	The QX One System is used to perform the step of partitioning said mixture into said plurality of droplets. The QX One System follows the ddPCR Workflow which fractionates a sample into 20,000 nanoliter-sized droplets with target and background DNA distributed randomly into droplets during the partitioning process.																																																																									

	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. BIO_CC00006573. As describe above, the QX ONE has an integrate droplet generator which is used to perform this function.
[1d] (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;	Plaintiffs incorporate by reference its analysis from Appendix A-02 at [1a.4], [1a.5], and [1b]. In those elements, Plaintiffs explained how the QX ONE is used to excite at least one fluorophore to generate at least one signals when the target analytes is present in the sample. Also as explained there, the signal is created by the excited fluorophore when the hybridization probe contacts the analyte. In this chart, in Step [1c] is explained how that sample is split into the droplets. Those elements taken together show how this step is performed. Additionally: The QX One System is used to perform the step of exciting said at least one fluorophore. The Droplet Reader exhibit droplets with increased fluorescence compared to negative droplets.

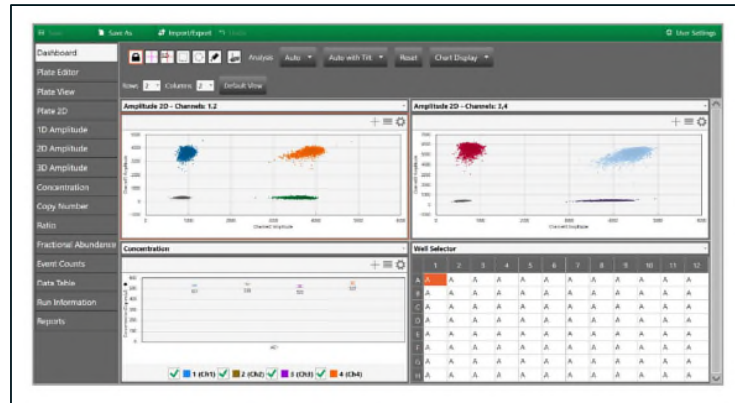
Droplet Reader

Following PCR amplification of the nucleic acid target in the droplets, the plate moves to the Droplet Reader, which analyzes each droplet individually using a four-color detection system, depending on the number of channels specified in the experiment.

The autosampler in the Droplet Reader picks up the droplets from each well in the plate. The droplets are spaced out individually for fluorescence reading and fluorescence is then measured in each droplet. Positive droplets, which contain at least one copy of the target DNA molecule, exhibit increased fluorescence compared to negative droplets and are presented in color in the analysis displays. Negative droplets are presented in grayscale.

BIO_CC00006574

The detection of positive targets corresponds to different color on the 2D Amplitude chart:



BIO_CC00006652

In Amplitude Multiplex method, the four channels can detect up to eight targets, which will be shown in different color on the 2D Amplitude Graph:

Table 12. Fluorophore options

Assay method	Signal Ch1	Signal Ch2	Signal Ch3	Signal Ch4
Single target per channel, for 1 to 4 targets	FAM	HEX	Cy5	Cy5.5
	None	VIC	None	None
Amplitude multiplex, for 1 to 8 targets	FAM Hi	HEX Hi	Cy5 Hi	Cy5.5 Hi
	FAM Lo	HEX Lo	Cy5 Lo	Cy5.5 Lo
	None	VIC Hi	None	None
		VIC Lo		
Probe mix triplex, for 6 targets	FAM	HEX	Cy5	Cy5.5
	None	VIC	None	None
Advanced classification	FAM	HEX	Cy5	Cy5.5
	+1-10	1 to 10	1 to 10	1 to 10
	None	VIC	None	None
		1 to 10		

BIO_CC00006622-6624

The QX One System is used to perform the step of generating one or more signals if one or more of said at least five polynucleotide analytes is present in said plurality of droplets.

The signals are generated based on the presence (or absence) of the analytes in the droplets.

Assay Types and Fluorophores

QX ONE Software offers the assay methods explained in [Table 10](#).

Table 10. Assay types

Assay type	Description
Single target per channel	Method assuming up to four probe colors (FAM, HEX/VIC, Cy5 and Cy5.5) and up to four targets per well, with a single target detected per channel. This is the default assay type, with the maximum four rows displayed. You can delete up to three rows.
Amplitude multiplex	Method to increase multiplexing up to eight 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 two 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. You can delete a group, but not the individual targets within the group. Note: The channels within a 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 <u>and</u> HEX/VIC and one target in FAM <u>or</u> 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 <u>and</u> Cy5.5, one target in Cy5 <u>or</u> Cy5.5. You can delete a group, but not the individual targets in the group. Note: The channels within a group are not interchangeable; therefore FAM is always paired with HEX/VIC and so forth.

Table 10. Assay types, continued

Assay type	Description
Double drop-off	This assay type is available only for the DOF experiment type, and allows two 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). 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..

The experiment type you select determines the available assay methods. [Table 11](#) displays the sample type associated with each assay method.

Table 11. 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				✓			

QX ONE Software automatically populates the fluorophore in each channel for each target but you can change the default selections.

Use [Table 12](#) to select from the fluorophore options for each assay method listed.

Table 12. Fluorophore options

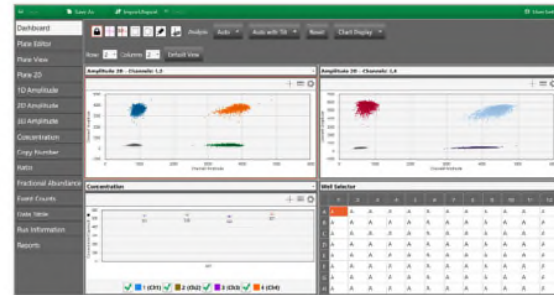
Assay method	Signal Ch1	Signal Ch2	Signal Ch3	Signal Ch4
Single target per channel, for 1 to 4 targets	FAM	HEX	Cy5	Cy5.5
	None	VIC	None	None
Amplitude multiplex, for 1 to 8 targets	FAM Hi	HEX Hi	Cy5 Hi	Cy5.5 Hi
	FAM Lo	HEX Lo	Cy5 Lo	Cy5.5 Lo
	None	VIC Hi	None	None
		VIC Lo		
		None		
Probe mix triplex, for 6 targets	FAM	HEX	Cy5	Cy5.5
	None	VIC	None	None
		None		
Advanced classification	FAM	HEX	Cy5	Cy5.5
	+1-10	1 to 10	1 to 10	1 to 10
	None	VIC	None	None
		1 to 10		
		None		

BIO_CCODE00006622-BIO_CCODE00006624

All determinations are shown as a unique color pattern on the analysis dashboard.

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. For information on the available options in the Dashboard view, see [Dashboard Options on page 84](#). For more information on the Analysis Dashboard, refer to the QX ONE Software User Guide.



Note: If you open a .qlo 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.

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.

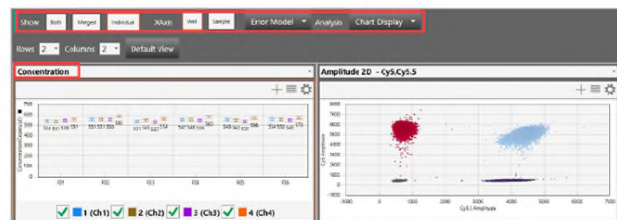


	Table 15 describes the option sets identified in the following graphic.  BIO_CC00006652- BIO_CC00006654.
[1e] (e) measuring said one or more signals to generate a cumulative intensity measurement, wherein said cumulative intensity measurement corresponds to the presence of a unique combination of presence or absence of said at least five polynucleotide analytes in said sample; and	Plaintiffs incorporate by reference its analysis from Appendix A-02 at [1c.1] and [1c.2]. In those elements, Plaintiffs explained how the QX ONE is used to measure the 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. For the same reasons, that explanation also shows how the system may indicate the presence or absence of at least five polynucleotide analytes. Likewise, the signal that is generated is a unique combination of the presence or absence of at least five polynucleotide analytes. Additionally: The QX One System is used to perform the step of measuring said one or more signals to generate a cumulative intensity measurement. The QX One System can support different experiment types.

Experiment Types

QX ONE Software offers the experiment types described in Table 9, each enabling different assay options and analysis tools. You can assign multiple experiment types across the plate, but you can assign only one experiment type to each well.

Table 9. Experiment types

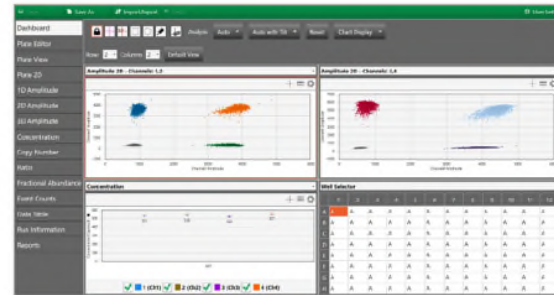
Type	Description
Direct quantification (DQ)	DQ uses absolute quantification to determine the concentration (copies/μl) of target DNA copies in a sample. DQ measures the number of positive and negative droplets for each target in a well based on their fluorescence amplitude, and uses a Poisson algorithm 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 (as with DQ) 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 (as with DQ) 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.
Residual DNA quantification (RDQ)	RDQ provides a precise method for residual host cell DNA quantification. Note: Users can enter a conversion factor to calculate the mass for a well, up to three decimals. The software displays the result in the Molecular Weight column of the Data Table.

BIO_CC00006618

The Analysis Dashboard shows the result of measuring intensity.

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. For information on the available options in the Dashboard view, see [Dashboard Options on page 84](#). For more information on the Analysis Dashboard, refer to the QX ONE Software User Guide.



Note: If you open a .qlo 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.

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.

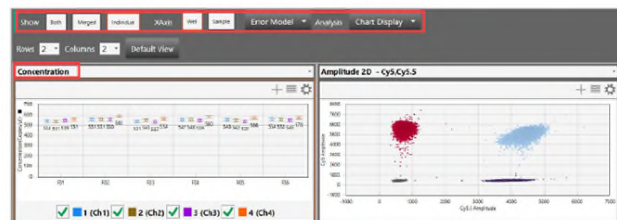
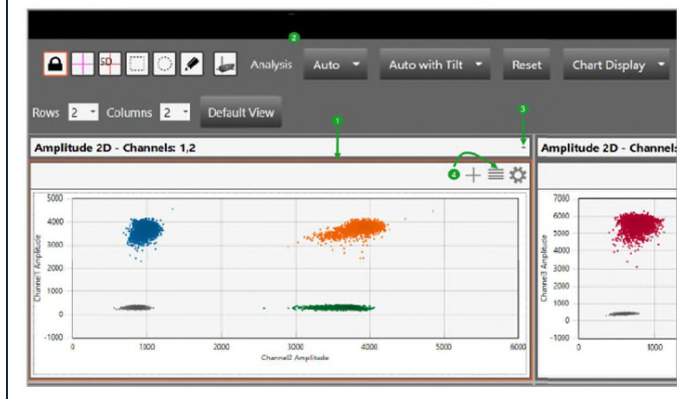


Table 15 describes the option sets identified in the following graphic.



BIO_CCODE00006652- BIO_CCODE00006654

The QX One System is also used to perform the step of 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. The cumulative intensity measurement indicates the presence or absence of the targeted analytes, based on the experiment types.

“

Assay Types and Fluorophores

QX ONE Software offers the assay methods explained in [Table 10](#).

Table 10. Assay types

Assay type	Description
Single target per channel	Method assuming up to four probe colors (FAM, HEX/VIC, Cy5 and Cy5.5) and up to four targets per well, with a single target detected per channel. This is the default assay type, with the maximum four rows displayed. You can delete up to three rows.
Amplitude multiplex	Method to increase multiplexing up to eight 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 two 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. You can delete a group, but not the individual targets within the group. Note: The channels within a 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 <u>and</u> HEX/VIC and one target in FAM <u>or</u> 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 <u>and</u> Cy5.5, one target in Cy5 <u>or</u> Cy5.5. You can delete a group, but not the individual targets in the group. Note: The channels within a group are not interchangeable; therefore FAM is always paired with HEX/VIC and so forth.

Table 10. Assay types, continued

Assay type	Description
Double drop-off	This assay type is available only for the DOF experiment type, and allows two 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). 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..

The experiment type you select determines the available assay methods. [Table 11](#) displays the sample type associated with each assay method.

Table 11. 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				✓			

QX ONE Software automatically populates the fluorophore in each channel for each target but you can change the default selections.

Use [Table 12](#) to select from the fluorophore options for each assay method listed.

	Table 12. Fluorophore options <table><tr><th>Assay method</th><th>Signal Ch1</th><th>Signal Ch2</th><th>Signal Ch3</th><th>Signal Ch4</th></tr><tr><td rowspan="2">Single target per channel, for 1 to 4 targets</td><td>FAM</td><td>HEX</td><td>Cy5</td><td>Cy5.5</td></tr><tr><td>None</td><td>VIC None</td><td>None</td><td>None</td></tr><tr><td rowspan="5">Amplitude multiplex, for 1 to 8 targets</td><td>FAM Hi</td><td>HEX Hi</td><td>Cy5 Hi</td><td>Cy5.5 Hi</td></tr><tr><td>FAM Lo</td><td>HEX Lo</td><td>Cy 5 Lo</td><td>Cy 5.5 Lo</td></tr><tr><td>None</td><td>VIC Hi</td><td>None</td><td>None</td></tr><tr><td></td><td>VIC Lo</td><td></td><td></td></tr><tr><td></td><td>None</td><td></td><td></td></tr><tr><td rowspan="3">Probe mix triplex, for 6 targets</td><td>FAM</td><td>HEX</td><td>Cy5</td><td>Cy5.5</td></tr><tr><td>None</td><td>VIC</td><td>None</td><td>None</td></tr><tr><td></td><td>None</td><td></td><td></td></tr><tr><td rowspan="5">Advanced classification</td><td>FAM</td><td>HEX</td><td>Cy5</td><td>Cy5.5</td></tr><tr><td>+1-10</td><td>1 to 10</td><td>1 to 10</td><td>1 to 10</td></tr><tr><td>None</td><td>VIC</td><td>None</td><td>None</td></tr><tr><td></td><td>1 to 10</td><td></td><td></td></tr><tr><td></td><td>None</td><td></td><td></td></tr></table> BIO_CC00006622-BIO_CC00006624	Assay method	Signal Ch1	Signal Ch2	Signal Ch3	Signal Ch4	Single target per channel, for 1 to 4 targets	FAM	HEX	Cy5	Cy5.5	None	VIC None	None	None	Amplitude multiplex, for 1 to 8 targets	FAM Hi	HEX Hi	Cy5 Hi	Cy5.5 Hi	FAM Lo	HEX Lo	Cy 5 Lo	Cy 5.5 Lo	None	VIC Hi	None	None		VIC Lo				None			Probe mix triplex, for 6 targets	FAM	HEX	Cy5	Cy5.5	None	VIC	None	None		None			Advanced classification	FAM	HEX	Cy5	Cy5.5	+1-10	1 to 10	1 to 10	1 to 10	None	VIC	None	None		1 to 10				None		
Assay method	Signal Ch1	Signal Ch2	Signal Ch3	Signal Ch4																																																																		
Single target per channel, for 1 to 4 targets	FAM	HEX	Cy5	Cy5.5																																																																		
	None	VIC None	None	None																																																																		
Amplitude multiplex, for 1 to 8 targets	FAM Hi	HEX Hi	Cy5 Hi	Cy5.5 Hi																																																																		
	FAM Lo	HEX Lo	Cy 5 Lo	Cy 5.5 Lo																																																																		
	None	VIC Hi	None	None																																																																		
		VIC Lo																																																																				
		None																																																																				
Probe mix triplex, for 6 targets	FAM	HEX	Cy5	Cy5.5																																																																		
	None	VIC	None	None																																																																		
		None																																																																				
Advanced classification	FAM	HEX	Cy5	Cy5.5																																																																		
	+1-10	1 to 10	1 to 10	1 to 10																																																																		
	None	VIC	None	None																																																																		
		1 to 10																																																																				
		None																																																																				
[1f] (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,	The QX One System is used to perform the step of 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, For example:																																																																					

Assay Types and Fluorophores

QX ONE Software offers the assay methods explained in [Table 10](#).

Table 10. Assay types

Assay type	Description
Single target per channel	Method assuming up to four probe colors (FAM, HEX/VIC, Cy5 and Cy5.5) and up to four targets per well, with a single target detected per channel. This is the default assay type, with the maximum four rows displayed. You can delete up to three rows.
Amplitude multiplex	Method to increase multiplexing up to eight 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 two 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. You can delete a group, but not the individual targets within the group. Note: The channels within a 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 a group are not interchangeable; therefore FAM is always paired with HEX/VIC and so forth.

Table 10. Assay types, continued

Assay type	Description
Double drop-off	This assay type is available only for the DOF experiment type, and allows two groups of three targets each. The first group appears by default, and looks for one target in FAM <u>and</u> 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 <u>and</u> 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). 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..

The experiment type you select determines the available assay methods. [Table 11](#) displays the sample type associated with each assay method.

Table 11. 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				✓			

QX ONE Software automatically populates the fluorophore in each channel for each target but you can change the default selections.

Use [Table 12](#) to select from the fluorophore options for each assay method listed.

Table 12. Fluorophore options

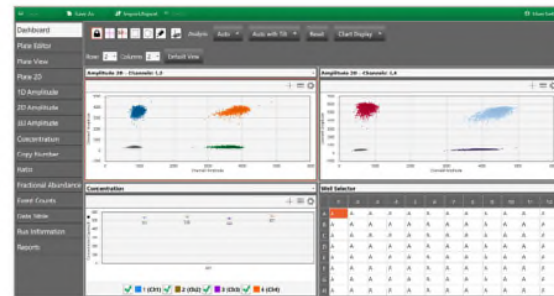
Assay method	Signal Ch1	Signal Ch2	Signal Ch3	Signal Ch4
Single target per channel, for 1 to 4 targets	FAM	HEX	Cy5	Cy5.5
	None	VIC	None	None
		None		
Amplitude multiplex, for 1 to 8 targets	FAM Hi	HEX Hi	Cy5 Hi	Cy5.5 Hi
	FAM Lo	HEX Lo	Cy5 Lo	Cy5.5 Lo
	None	VIC Hi	None	None
		VIC Lo		
		None		
Probe mix triplex, for 6 targets	FAM	HEX	Cy5	Cy5.5
	None	VIC	None	None
		None		
Advanced classification	FAM	HEX	Cy5	Cy5.5
	+1-10	1 to 10	1 to 10	1 to 10
	None	VIC	None	None
		1 to 10		
		None		

BIO_CCODE00006622-BIO_CCODE00006624

All determinations are shown as a unique color pattern on the analysis dashboard.

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. For information on the available options in the Dashboard view, see [Dashboard Options on page 84](#). For more information on the Analysis Dashboard, refer to the QX ONE Software User Guide.



Note: If you open a .qlo 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.

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.

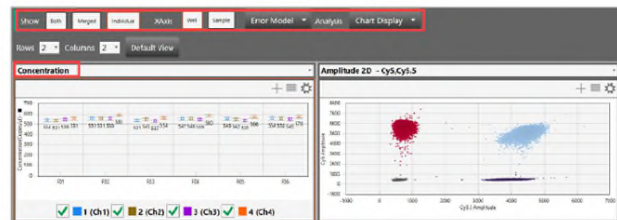
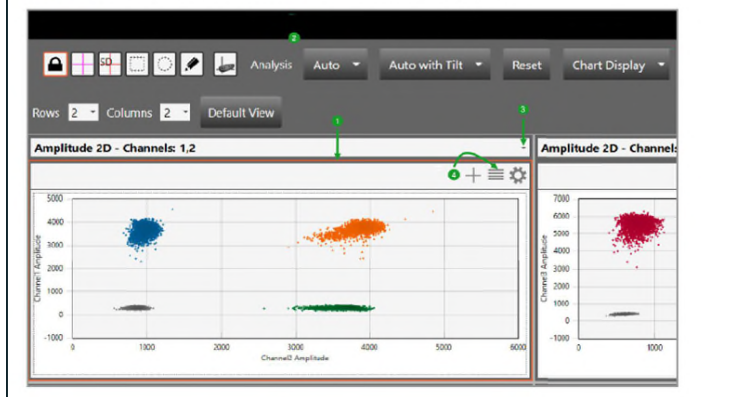


Table 15 describes the option sets identified in the following graphic.



BIO_CCODE00006652- BIO_CCODE00006654.

The cumulative intensity measurement may also be used to quantify the polynucleotide analytes, which will also determine their presence or absence:

Chapter 1 Introduction to Droplet Digital PCR

Droplet Digital™ PCR is a digital polymerase chain reaction method based on water-oil emulsion droplet technology. Droplet Digital PCR™ (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.

BIO_CC00006554 at 6572-6574.

Chapter 2 QX ONE Droplet Digital PCR System

The QX ONE™ Droplet Digital PCR System combines droplet generation, thermal cycling, and droplet reading technologies into a single instrument, as described below:

- The droplet generator partitions samples into approximately 20,000 uniform droplets.
- The internal thermal cycler performs repeated heating and cooling processes to amplify one or more copies of a particular DNA segment in each droplet.
- The droplet reader streams the droplets in single file, and then counts the fluorescent positive and negative droplets to calculate and quantify target DNA concentration.

The QX ONE Droplet Digital PCR System also features a touch screen computer, which is integrated onto the instrument and has QX ONE Software already installed.



When you open QX ONE Software from the touch screen, the software automatically recognizes the QX ONE Droplet Digital PCR System.

Note: You cannot directly connect standalone computers to the instrument. However, from a standalone computer you can access files stored on the touch screen computer's internal drive, or from another shared location, as long as all units are connected through the same company network. You can also access the data with a USB drive connected to the touch screen.

Id. at 6576.

	Experiment Types QX ONE Software offers the experiment types described in Table 9, each enabling different assay options and analysis tools. You can assign multiple experiment types across the plate, but you can assign only one experiment type to each well. Table 9. Experiment types <table> <tr> <th>Type</th><th>Description</th></tr> <tr> <td>Direct quantification (DQ)</td><td>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.</td></tr> <tr> <td>Copy number variation (CNV)</td><td>CNV determines concentration (as with DQ) and calculates the copy number of an unknown (CNV) target relative to a known reference or references within the same well.</td></tr> <tr> <td>Mutation detection (MUT)</td><td>MUT determines concentration (as with DQ) and calculates the fractional abundance of an unknown mutant present at a low frequency in a wild-type background.</td></tr> <tr> <td>Rare event detection (RED)</td><td>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.</td></tr> <tr> <td>Drop-off (DOF)</td><td>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.</td></tr> <tr> <td>Gene expression (GEX)</td><td>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.</td></tr> <tr> <td>Residual DNA quantification (RDQ)</td><td>RDQ provides a precise method for residual host cell DNA quantification. Note: Users can enter a conversion factor to calculate the mass for a well, up to three decimals. The software displays the result in the Molecular Weight column of the Data Table.</td></tr> </table> <i>Id.</i> at 6618-6620.	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 (as with DQ) 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 (as with DQ) 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.	Residual DNA quantification (RDQ)	RDQ provides a precise method for residual host cell DNA quantification. Note: Users can enter a conversion factor to calculate the mass for a well, up to three decimals. The software displays the result in the Molecular Weight column of the Data Table.
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 (as with DQ) 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 (as with DQ) 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.																
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[1g] wherein the method does not require any step of immobilization of said at least	See Appendix A-02 at [1d.2].																

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.	The QX One System is used to perform the step of wherein each of said at least five hybridization probes is complementary to one of said at least five polynucleotide analytes. The probes are complementary to analytes and emits different intensity and/or wavelengths to detect analytes. Assay Types and Fluorophores QX ONE Software offers the assay methods explained in Table 10. Table 10. Assay types <table> <tr> <th>Assay type</th><th>Description</th></tr> <tr> <td>Single target per channel</td><td>Method assuming up to four probe colors (FAM, HEX/VIC, Cy5 and Cy5.5) and up to four targets per well, with a single target detected per channel. This is the default assay type, with the maximum four rows displayed. You can delete up to three rows.</td></tr> <tr> <td>Amplitude multiplex</td><td>Method to increase multiplexing up to eight 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.</td></tr> <tr> <td>Probe mix triplex</td><td>Triplex mode that allows two 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. You can delete a group, but not the individual targets within the group. Note: The channels within a group are not interchangeable; therefore FAM is always paired with HEX/VIC and so forth.</td></tr> <tr> <td>Advanced classification</td><td>Method to increase multiplexing rate in any combination of targets detected per channel. This method requires manual classification of droplets.</td></tr> <tr> <td>Basic drop-off</td><td>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 a group are not interchangeable; therefore FAM is always paired with HEX/VIC and so forth.</td></tr> </table>	Assay type	Description	Single target per channel	Method assuming up to four probe colors (FAM, HEX/VIC, Cy5 and Cy5.5) and up to four targets per well, with a single target detected per channel. This is the default assay type, with the maximum four rows displayed. You can delete up to three rows.	Amplitude multiplex	Method to increase multiplexing up to eight 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 two 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. You can delete a group, but not the individual targets within the group. Note: The channels within a 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 a group are not interchangeable; therefore FAM is always paired with HEX/VIC and so forth.
Assay type	Description												
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Table 10. Assay types, continued

Assay type	Description
Double drop-off	This assay type is available only for the DOF experiment type, and allows two groups of three targets each. The first group appears by default, and looks for one target in FAM <u>and</u> 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 <u>and</u> 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). 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..

The experiment type you select determines the available assay methods. [Table 11](#) displays the sample type associated with each assay method.

Table 11. 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				✓			

QX ONE Software automatically populates the fluorophore in each channel for each target but you can change the default selections.

Use [Table 12](#) to select from the fluorophore options for each assay method listed.

	Table 12. Fluorophore options <table><tr><th>Assay method</th><th>Signal Ch1</th><th>Signal Ch2</th><th>Signal Ch3</th><th>Signal Ch4</th></tr><tr><td rowspan="3">Single target per channel, for 1 to 4 targets</td><td>FAM</td><td>HEX</td><td>Cy5</td><td>Cy5.5</td></tr><tr><td>None</td><td>VIC</td><td>None</td><td>None</td></tr><tr><td></td><td>None</td><td></td><td></td></tr><tr><td rowspan="5">Amplitude multiplex, for 1 to 8 targets</td><td>FAM Hi</td><td>HEX Hi</td><td>Cy5 Hi</td><td>Cy5.5 Hi</td></tr><tr><td>FAM Lo</td><td>HEX Lo</td><td>Cy 5 Lo</td><td>Cy 5.5 Lo</td></tr><tr><td>None</td><td>VIC Hi</td><td>None</td><td>None</td></tr><tr><td></td><td>VIC Lo</td><td></td><td></td></tr><tr><td></td><td>None</td><td></td><td></td></tr><tr><td rowspan="3">Probe mix triplex, for 6 targets</td><td>FAM</td><td>HEX</td><td>Cy5</td><td>Cy5.5</td></tr><tr><td>None</td><td>VIC</td><td>None</td><td>None</td></tr><tr><td></td><td>None</td><td></td><td></td></tr><tr><td rowspan="4">Advanced classification</td><td>FAM</td><td>HEX</td><td>Cy5</td><td>Cy5.5</td></tr><tr><td>+1-10</td><td>1 to 10</td><td>1 to 10</td><td>1 to 10</td></tr><tr><td>None</td><td>VIC</td><td>None</td><td>None</td></tr><tr><td></td><td>1 to 10</td><td></td><td></td></tr><tr><td></td><td></td><td>None</td><td></td><td></td></tr></table> BIO_CC00006622-BIO_CC00006624	Assay method	Signal Ch1	Signal Ch2	Signal Ch3	Signal Ch4	Single target per channel, for 1 to 4 targets	FAM	HEX	Cy5	Cy5.5	None	VIC	None	None		None			Amplitude multiplex, for 1 to 8 targets	FAM Hi	HEX Hi	Cy5 Hi	Cy5.5 Hi	FAM Lo	HEX Lo	Cy 5 Lo	Cy 5.5 Lo	None	VIC Hi	None	None		VIC Lo				None			Probe mix triplex, for 6 targets	FAM	HEX	Cy5	Cy5.5	None	VIC	None	None		None			Advanced classification	FAM	HEX	Cy5	Cy5.5	+1-10	1 to 10	1 to 10	1 to 10	None	VIC	None	None		1 to 10					None		
Assay method	Signal Ch1	Signal Ch2	Signal Ch3	Signal Ch4																																																																							
Single target per channel, for 1 to 4 targets	FAM	HEX	Cy5	Cy5.5																																																																							
	None	VIC	None	None																																																																							
		None																																																																									
Amplitude multiplex, for 1 to 8 targets	FAM Hi	HEX Hi	Cy5 Hi	Cy5.5 Hi																																																																							
	FAM Lo	HEX Lo	Cy 5 Lo	Cy 5.5 Lo																																																																							
	None	VIC Hi	None	None																																																																							
		VIC Lo																																																																									
		None																																																																									
Probe mix triplex, for 6 targets	FAM	HEX	Cy5	Cy5.5																																																																							
	None	VIC	None	None																																																																							
		None																																																																									
Advanced classification	FAM	HEX	Cy5	Cy5.5																																																																							
	+1-10	1 to 10	1 to 10	1 to 10																																																																							
	None	VIC	None	None																																																																							
		1 to 10																																																																									
		None																																																																									
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.	The QX One System is used to perform the step of wherein at least five hybridization probes comprise one or more fluorophores selected from one, two, three, or four fluorophores. The hybridization probes comprise fluorophores from FAM, HEX, Cy5 and Cy5.5.																																																																										

Assay Types and Fluorophores

QX ONE Software offers the assay methods explained in [Table 10](#).

Table 10. Assay types

Assay type	Description
Single target per channel	Method assuming up to four probe colors (FAM, HEX/VIC, Cy5 and Cy5.5) and up to four targets per well, with a single target detected per channel. This is the default assay type, with the maximum four rows displayed. You can delete up to three rows.
Amplitude multiplex	Method to increase multiplexing up to eight 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 two 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. You can delete a group, but not the individual targets within the group. Note: The channels within a 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 a group are not interchangeable, therefore FAM is always paired with HEX/VIC and so forth.

Table 10. Assay types, continued

Assay type	Description
Double drop-off	This assay type is available only for the DOF experiment type, and allows two groups of three targets each. The first group appears by default, and looks for one target in FAM and in HEXV/C (target type reference) one target in FAM (target type unknown), and one target in HEXV/C (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). 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 HEXV/C, Channel 3 Cy5 is always paired with Channel 4 Cy5.5.)

The experiment type you select determines the available assay methods. [Table 11](#) displays the sample type associated with each assay method.

Table 11. 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				✓			

QX ONE Software automatically populates the fluorophore in each channel for each target but you can change the default selections.

Table 12. Fluorophore options

Assay method	Signal Ch1	Signal Ch2	Signal Ch3	Signal Ch4
Single target per channel, for 1 to 4 targets	FAM	HEX	Cy5	Cy5.5
	None	VIC	None	None
Amplitude multiplex, for 1 to 8 targets	FAM Hi	HEX Hi	Cy5 Hi	Cy5.5 Hi
	FAM Lo	HEX Lo	Cy5 Lo	Cy5.5 Lo
	None	VIC Hi	None	None
		VIC Lo		
Probe mix triplex, for 6 targets	FAM	HEX	Cy5	Cy5.5
	None	VIC	None	None
Advanced classification	FAM	HEX	Cy5	Cy5.5
	+1-10	1 to 10	1 to 10	1 to 10
	None	VIC	None	None
		1 to 10		

BIO_CC00006622- BIO_CC00006624

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

The QX One System is used to perform the step of a droplet of said plurality of droplets comprises said at least five hybridization probes.

See Element 1(b).

The QX One System follows the ddPCR Workflow which fractionates a sample into 20,000 nanoliter-sized droplets with target and background DNA distributed randomly into droplets during the partitioning process.

	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. BIO_CC00006573 Droplet Generation Before droplet generation, ddPCR reactions are prepared in a similar manner as real-time PCR reactions that use TaqMan hydrolysis probes labeled with reporter fluorophores or an intercalating dye. ddPCR is performed with Bio-Rad's proprietary reagents that were developed specifically for droplet generation. The QX ONE™ Droplet Digital PCR System runs the plates containing your DNA samples through the Droplet Generator as the first component in the three-part process. The Droplet Generator uses specially developed reagents and microfluidics to partition each sample into approximately 20,000 nanoliter-sized droplets. Target and background DNA are distributed randomly into the droplets during the partitioning process. Droplet generation produces uniform droplets for the sample, enabling precise target quantification. BIO_CC00006574
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.	The QX One System is used to perform the step that wherein, in (b), a droplet of said plurality of droplets comprises one or more of said at least five polynucleotide analytes. See Element [1b]. The QX One System follows the ddPCR Workflow which fractionates a sample into 20,000 nanoliter-sized droplets with target and background DNA distributed randomly into droplets during the partitioning process.

	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. BIO_CC00006573 Droplet Generation Before droplet generation, ddPCR reactions are prepared in a similar manner as real-time PCR reactions that use TaqMan hydrolysis probes labeled with reporter fluorophores or an intercalating dye. ddPCR is performed with Bio-Rad's proprietary reagents that were developed specifically for droplet generation. The QX ONE™ Droplet Digital PCR System runs the plates containing your DNA samples through the Droplet Generator as the first component in the three-part process. The Droplet Generator uses specially developed reagents and microfluidics to partition each sample into approximately 20,000 nanoliter-sized droplets. Target and background DNA are distributed randomly into the droplets during the partitioning process. Droplet generation produces uniform droplets for the sample, enabling precise target quantification. BIO_CC00006574
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.	The QX One System is used to perform the step that wherein, in (b), wherein, in (b), a droplet of said plurality of droplets comprises at most one of said at least five polynucleotide analytes. See Element [1b]. The QX One System follows the ddPCR Workflow which fractionates a sample into 20,000 nanoliter-sized droplets with target and background DNA distributed randomly into droplets during the partitioning process.

	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. BIO_CC00006573 Droplet Generation Before droplet generation, ddPCR reactions are prepared in a similar manner as real-time PCR reactions that use TaqMan hydrolysis probes labeled with reporter fluorophores or an intercalating dye. ddPCR is performed with Bio-Rad's proprietary reagents that were developed specifically for droplet generation. The QX ONE™ Droplet Digital PCR System runs the plates containing your DNA samples through the Droplet Generator as the first component in the three-part process. The Droplet Generator uses specially developed reagents and microfluidics to partition each sample into approximately 20,000 nanoliter-sized droplets. Target and background DNA are distributed randomly into the droplets during the partitioning process. Droplet generation produces uniform droplets for the sample, enabling precise target quantification. BIO_CC00006574
7. The method of claim 1, wherein said one or more signals is generated during a polymerase chain reaction.	See Appendix A-02 at Claim 4.
8. The method of claim 1, wherein said at least five polynucleotide analytes are at	See Appendix A-02 at Claim 1, which describes the method with respect to at least seven polynucleotide analytes. As described there, the QX ONE is able to quantify at least seven targets.

least seven polynucleotide analytes.	
9. The method of claim 1, wherein said at least five hybridization probes are at least seven hybridization probes.	See Appendix A-02 at Claim 1 (in particular 1a.2), which describes the method with respect to at least seven hybridization probes. As described there, the QX ONE is able to use 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.	The QX One System is used to perform the method 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. The QX ONE can detect viruses, tumors, microbes, wild-type backgrounds, and mutations. See BIO_CCODE00006554 at 6572; also see BIO_CCODE00006617.
11. The method of claim 1, wherein said sample is a biological sample derived from a subject.	The QX One System is used to perform the step that wherein said sample is a biological sample derived from a subject. The QX One System can be used to detect viruses, tumors, microbes, wild-type backgrounds, mutations, HSV and B2M from biological samples. See BIO_CCODE00006554 at 6572; also see BIO_CCODE00006617.
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.	The QX One System is used to perform the step that 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. The QX ONE can detect viruses, tumors, microbes, wild-type backgrounds, and mutations. See BIO_CCODE00006554 at 6572; also see BIO_CCODE00006617. Such detection can detect 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.	See Appendix A-02 at Claim 5.
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.	See Appendix A-02 at Claim 6.
15. The method of claim 14, wherein said clinical decision is a treatment decision.	See Appendix A-02 at Claim 6. On information and belief, when the QX ONE system is used in a method, that method may include a clinical decision on treatment.
16. The method of claim 15, wherein said treatment decision comprises treatment with an antibiotic or an anticancer therapeutic.	See Appendix A-02 at Claim 6. On information and belief, when the QX ONE system is used in a method, that method may include a clinical decision on treatment. The QX ONE can detect viruses, tumors, microbes, wild-type backgrounds, and mutations. See BIO_CCODE00006554 at 6572; also see BIO_CCODE00006617. Such detection can detect the presence of cancer or an infection with a pathogen in said subject. As such, that treatment decision may include treatment of an antibiotic to combat the detected microbe or virus or an anticancer therapeutic to combat the detected tumor.

17. The method of claim 1, wherein at least part of said method is performed using a computer.	See Appendix A-02 at Claim 7.
18. The method of claim 17, wherein said computer is located on a remote server.	See Appendix A-02 at Claim 8.
19. The method of claim 17, wherein said computer is located on a droplet reader.	See Appendix A-02 at Claim 7. As discussed in Appendix A-02 and above, the QX ONE includes a droplet reader and is also a computer. As such, this claim is satisfied when the QX ONE is used.
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.	The QX One System is used to perform the step that 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. The probes are provided at a given concentration, such as FAM Hi and FAM Lo.

Assay Types and Fluorophores

QX ONE Software offers the assay methods explained in [Table 10](#).

Table 10. Assay types

Assay type	Description
Single target per channel	Method assuming up to four probe colors (FAM, HEX/VIC, Cy5 and Cy5.5) and up to four targets per well, with a single target detected per channel. This is the default assay type, with the maximum four rows displayed. You can delete up to three rows.
Amplitude multiplex	Method to increase multiplexing up to eight 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 two 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. You can delete a group, but not the individual targets within the group. Note: The channels within a 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 a group are not interchangeable, therefore FAM is always paired with HEX/VIC and so forth.

Table 10. Assay types, continued

Assay type	Description
Double drop-off	This assay type is available only for the DOF experiment type, and allows two groups of three targets each. The first group appears by default, and looks for one target in FAM and in HEXV/C (target type reference) one target in FAM (target type unknown), and one target in HEXV/C (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). 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 HEXV/C, Channel 3 Cy5 is always paired with Channel 4 Cy5.5.)

The experiment type you select determines the available assay methods. [Table 11](#) displays the sample type associated with each assay method.

Table 11. 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				✓			

QX ONE Software automatically populates the fluorophore in each channel for each target but you can change the default selections.

Table 12. Fluorophore options

Assay method	Signal Ch1	Signal Ch2	Signal Ch3	Signal Ch4
Single target per channel, for 1 to 4 targets	FAM	HEX	Cy5	Cy5.5
	None	VIC None	None	None
Amplitude multiplex, for 1 to 8 targets	FAM Hi	HEX Hi	Cy5 Hi	Cy5.5 Hi
	FAM Lo	HEX Lo	Cy5 Lo	Cy5.5 Lo
	None	VIC Hi	None	None
		VIC Lo None		
Probe mix triplex, for 6 targets	FAM	HEX	Cy5	Cy5.5
	None	VIC None	None	None
Advanced classification	FAM	HEX	Cy5	Cy5.5
	+1-10	1 to 10	1 to 10	1 to 10
	None	VIC 1 to 10 None	None	None

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Table 9. Experiment types

Type	Description
Direct quantification (DQ)	DQ uses absolute quantification to determine the concentration (copies/ μ l) 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 (as with DQ) 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 (as with DQ) 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.

BIO_CCODE00006554 at BIO_CCODE00006618

Thus, combining the disclosures stated above, wherein the probes are provided at a different concentration (e.g., a concentration indicating target DNA copies in a sample) such that the cumulative intensity measurement that results corresponds to the presence of the unique combination of targets tested.