# Digital LightCycler® System

## Application Guide

Digital LightCycler®

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# 1. The digital PCR principle

Digital PCR (dPCR) is a relatively new PCR-based method for quantification of DNA or RNA targets. It builds on traditional PCR amplification and fluorescent probe-based detection methods to provide precise, absolute quantification of nucleic acids with single molecule resolution.

The sample preparation step is similar to that used for real-time (RT) PCR. The reaction mixture containing target nucleic acid, primers, probe, and other reagents is randomly distributed into thousands of partitions, usually of equal volume, such that each partition contains either zero, one, or a few target molecules. Fluorescent probes are included to detect the amplified target using an endpoint measurement to obtain individual fluorescence brightness values from all partitions **(Figure 1.1)**.

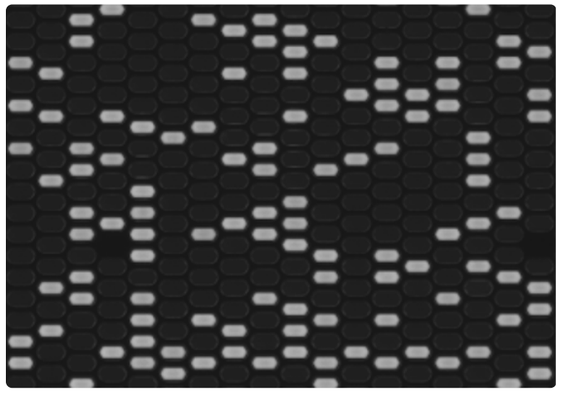


**Figure 1.1:** Image of dPCR nanowell plate after partitioning and amplification. Target-positive partitions are visible as discrete fluorescent wells against a background of non-fluorescent, target-negative wells.

At the endpoint, partitions containing amplified fluorescent target molecules are considered positive, and those with little or no fluorescence, and thus without any amplified target, are considered negative. For each reaction, the proportion of negative partitions provides the basis for quantification of the number of molecules containing the target-amplified sequence using Poisson statistics. This method accounts for the fact that positive partitions may contain more than one target molecule, and is described in further detail in Section 2.

In comparison to quantitative PCR (qPCR), higher precision, sensitivity, and accuracy can be achieved with dPCR[1] since interference by PCR inhibitors and competing background DNA is reduced by partitioning of the reaction mixtures.[2]

The main advantages of dPCR compared with qPCR include:

- **No need for standard curves**
  dPCR quantification is more accurate since it relies on clustering partitions into negative and positive groups, followed by direct counting of negative partitions and calculation of the target concentration without the need for running standard curves. This removes the potential for variation in amplification efficiency to impact target quantification.
- **Increased signal-to-noise ratio**
  Partitioning effectively enriches target concentrations in target-positive partitions relative to those of potential PCR inhibitors and interfering background sequences, allowing for more sensitive detection of rare targets **(Figure 1.2)**.
- **Higher precision**
  Partitioning also allows better precision for target quantification. The more partitions, the greater the precision **(see Section 3, Figure 3.5)**. Precision could be further improved by merging multiple lanes (Section 3.3).

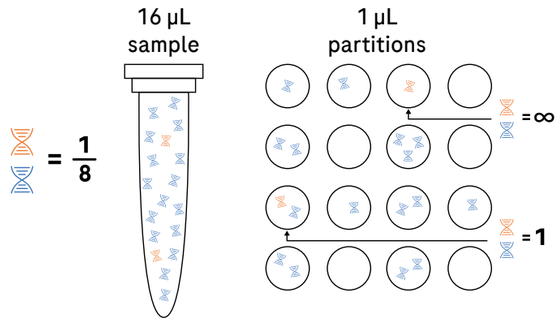



**Figure 1.2:** Illustration of target sequence (orange) enrichment relative to competing background sequences (blue) through partitioning. In the unpartitioned sample (tube on the left), the ratio of target to background is 1:8. In target-positive partitions (circles on the right), the ratio varies from 1:1 to infinite (pure target). Adapted from Basu 2017.[3]

[1]Belmonte FR, et al. Sci Rep 2016;28:25186. doi: 10.1038/srep251 86. [2]Sidstedt M, Rådström P, Hedman J. Anal Bioanal Chem 2020;412:2009–2023. doi: 10.1007/s00216-020-02490-2.
[3]Basu AS. SLAS Technol 2017;22(4):369–386. doi:10.1177/2472630317705680.



# 2. Fundamentals of dPCR statistics

## 2.1 Poisson distribution

The Poisson distribution is the most essential part of dPCR statistics. It is assumed that: (1) target molecules are independently distributed into the partitions with equal probability, and (2) the system employs a large number of partitions, such that the probability of a target molecule entering a particular partition is very small. When these assumptions are true, the number of target molecules in a partition can be approximated by the Poisson distribution (see details in Basu 2017[3]). The number of target molecules in each positive partition can range from one to a few. In contrast, the number of targets in each negative partition is zero with 100% certainty. Therefore, the proportion of negative partitions among all the valid partitions provides a direct measure for the probability that a partition has zero target molecules.

Mathematically, the probability mass function of a Poisson random variable X is:

$$P(X = x) = \frac{\lambda^x e^{-\lambda}}{x!},\ x = 0, 1, 2, \ldots$$

where $\lambda$ is the mean copies per partition, or mean cpp.

The probability that a partition contains zero target molecules, $q$, can be calculated by plugging in $x = 0$ in the previous equation. This simplifies it to $e^{-\lambda}$, in which case:

$$\lambda = -\log(q)$$

The absolute concentration of target molecules can be estimated by replacing $q$ with the proportion of negative partitions in the above equation, divided by the partition volume.

**Figure 2.1** shows the Poisson probability that a partition contains 0, 1, 2,..., 20 target molecules for different values of $\lambda$. When $\lambda$ is very high (e.g. $\lambda = 9$), the probability of observing at least one negative partition is less than 0.012%. The Poisson distribution provides the basis for quantifications and error calculations.

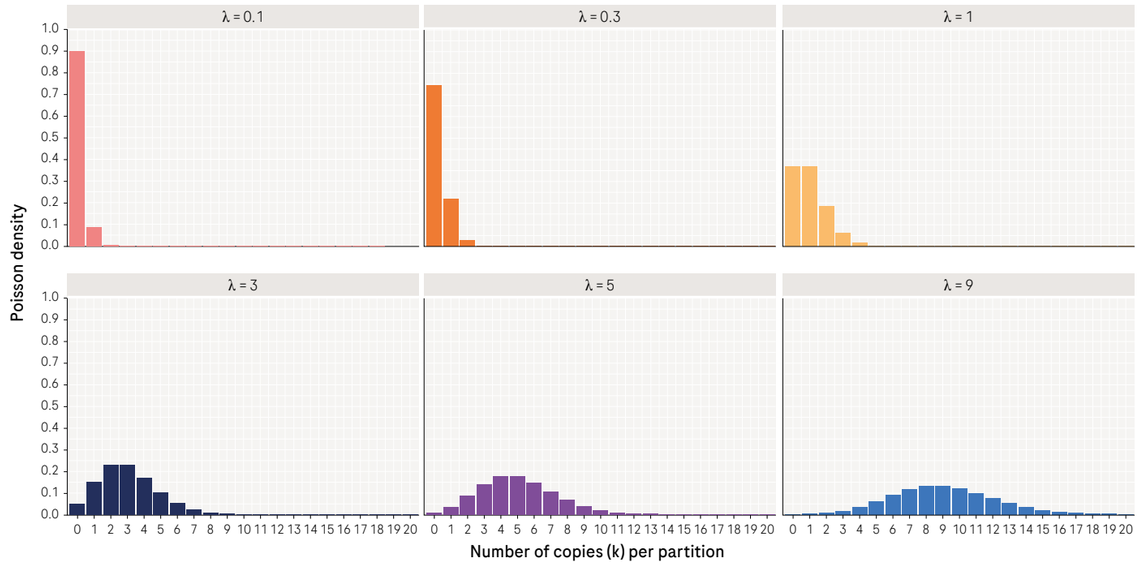



**Figure 2.1:** Poisson probability that a partition contains k target molecules (k = 0, 1, 2,..., 20) for various mean copies per partition.

[3]Basu AS. SLAS Technol 2017;22(4):369–386. doi:10.1177/2472630317705680.

## 2.2 Sources of error

There are several possible sources of inaccurate results from dPCR experiments. These include assay-related features, such as non-specific amplifications, which can lead to false positives, and suboptimal target sequence amplification, which can result in false negatives and errors from processes such as pipetting, etc. For the Poisson statistics to support accurate input target concentration calculations, two other types of errors must also be avoided: subsampling and partitioning errors.

Assuming a perfect dPCR experimental procedure, subsampling and partitioning become the two main sources of error.

### Subsampling error

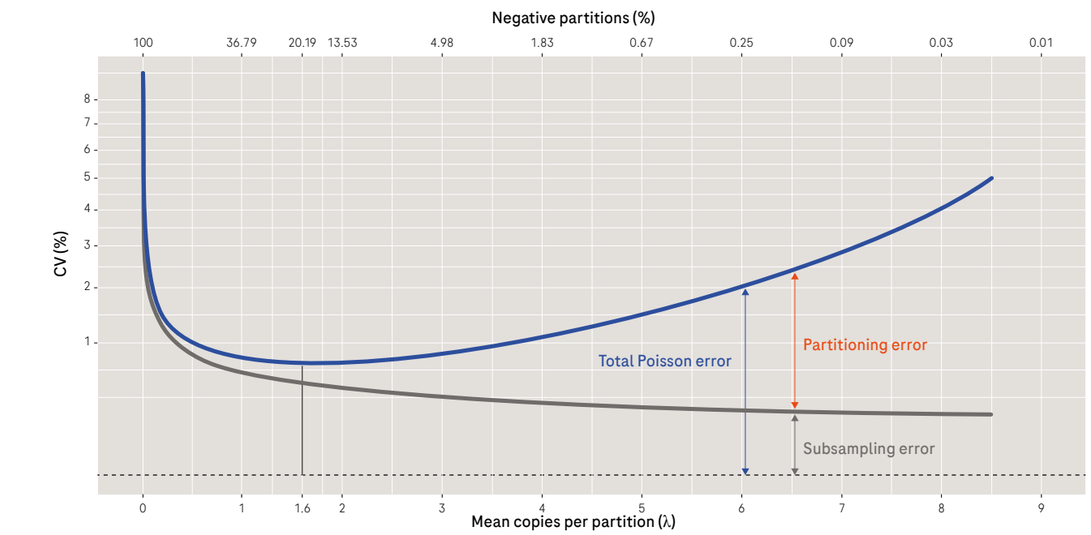
Subsampling error is not specific to dPCR-based assays and can occur in any situation when sampling a fraction of a whole, e.g. a draw of blood from a patient. The total number of target molecules added to the dPCR reaction can be approximated by the Poisson distribution if the target molecules are all independent and have an equal chance of being sampled, and under the condition that the sampling fraction is small (e.g. below 5% when sampling from a total of 100 molecules, see: www.https://tinyurl.com/handbookPMC). One of the most important properties of a Poisson random variable is that its mean and variance are the same. Therefore, the subsampling error, expressed as the % coefficient of variation (CV) of the total number of target molecules added to the dPCR reaction from multiple samplings, is inversely proportional to the square root of the mean total input number of target molecules. The larger the number of target molecules, the lower the %CV, and thus the lower the subsampling error.

The subsampling error is therefore independent of the total number of valid partitions and dominates at lower amounts of input sample nucleic acid. For example, in an adult with 5 L total blood volume containing 10,000 copies of a target molecule, if 5 mL (0.1%) of blood is drawn, the mean number of target molecules is 10, with a %CV of 31.6%. Alternatively, if 100 mL (2%) is drawn, the mean number of target molecules is 200, and the %CV would be 7.1%. **Figure 2.2** shows that the subsampling error decreases with increasing sample input.

### Partitioning error

Partitioning error reflects the statistical uncertainty of the number of negative partitions, and is specific to dPCR. Suppose the same experiment is repeated multiple times, and the total input numbers of target molecules all happen to be identical. If this number is very low, then the probability of multiple occupancy (more than one target molecule in a partition) is low, and there is a high chance that the number of negative partitions will remain constant among repeated experiments, yielding a very small partitioning error. If this number is high, such that there is a significant probability of multiple occupancy, higher variation in the number of negative partitions and a larger partitioning error would be expected. Thus, partitioning error dominates at higher concentrations.

The total Poisson error is the sum of subsampling and partitioning errors, and can be expressed in terms of %CV or % relative confidence **(Figure 2.2)**. The latter is defined as the larger half width of a 95% confidence interval (CI) of concentration, divided by the concentration.[3,4]




CV, coefficient of variation

**Figure 2.2:** Total Poisson, subsampling and partitioning errors, expressed as %CV, for a plate with 28,000 partitions. Partitioning error is total Poisson error minus subsampling error. Total Poisson error achieves its minimum value when the mean copies per partition is ~1.6.

[3]Basu AS. SLAS Technol 2017;22(4):369–386. doi:10.1177/2472630317705680.
[4]Majumdar N, Wessel T, Marks J. PLoS One 2015;10(3):e0118833. doi: 10.1371/journal.pone.0118833.



# 3. dPCR on the Digital LightCycler®

## 3.1 General workflow description

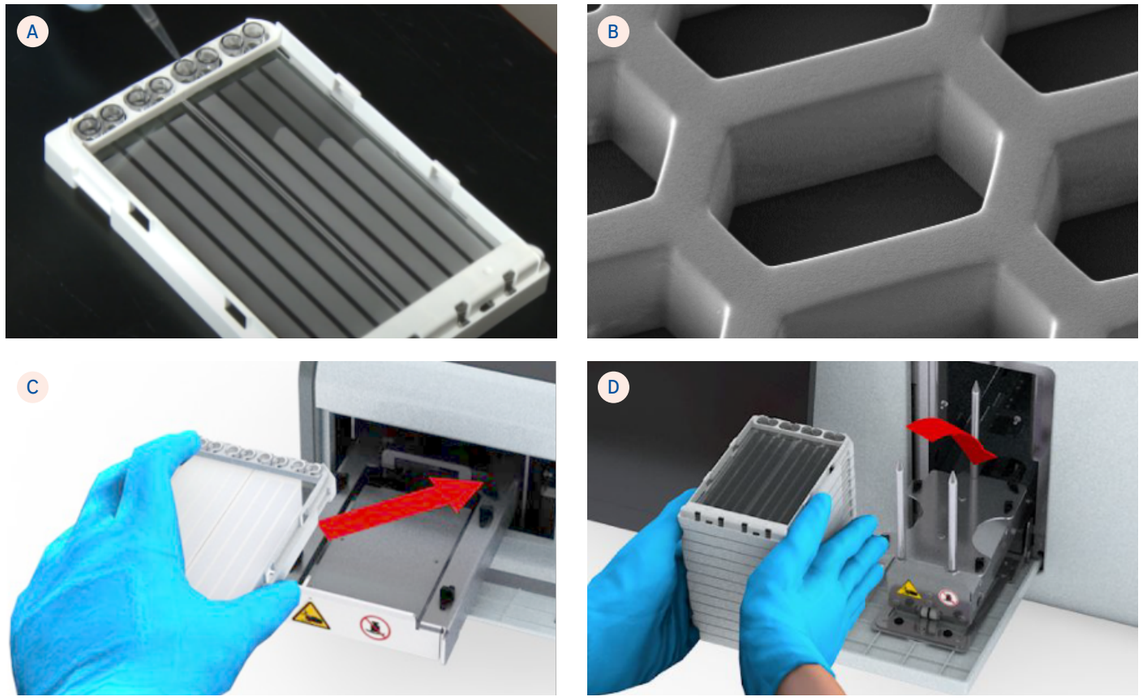
The general, high-level Digital LightCycler® System workflow is described below.

First, dPCR reaction mixtures are prepared by mixing RNA or DNA extracted from a specimen with primers, probes, and one of Roche's exclusive, generic 5× concentrated DNA or RNA master reagents. These are the Digital LightCycler® 5× DNA Master and the Digital LightCycler® 5× RNA Master (Master Material Numbers 09393544001 and 08897115001, respectively). The master reagents provide optimal reaction conditions for amplification and detection reactions and include a control dye that helps validate correct filling of the individual nanowells during partitioning.

The reaction mixtures are then pipetted separately into eight lanes of a nanowell plate and partially enter the lanes via capillary forces **(Figure 3.1A)**.

Three types of nanowell plates are available, featuring ~20,000 (HiSens), ~28,000 (Uni), or ~100,000 (HiRes) nanowells. The nanowells are highly uniform, hexagonal microscopic structures on a chip surface **(Figure 3.1B)**.

Next, the reaction mixtures are loaded into the partitioning engine **(Figure 3.1C)**. After partitioning, these thousands of isolated, individual reaction chambers are ready for simultaneous PCR amplification. Up to 12 plates (96 samples) can be loaded onto the Digital LightCycler® Analyzer **(Figure 3.1D)**.




dPCR, digital PCR

**Figure 3.1:** (A) After preparation, dPCR reaction mixtures are pipetted into an eight-lane nanowell plate, where the samples are partially drawn into the lanes by capillary action. (B) A magnified picture of the nanowells where the individual PCR reactions take place. (C) After pipetting, nanowell plates are inserted into the partitioning engine. (D) Following partitioning, samples undergo PCR amplification in the Digital LightCycler® Analyzer.

During the partitioning process, the reaction mixtures are entirely and equally distributed into the nanowells by the partitioning engine, with virtually no dead volume losses **(Figure 3.2)**. The nanowells are tightly sealed with an oil-based partitioning fluid (yellow in diagram), creating thousands of individual isolated reaction chambers (blue in diagram).

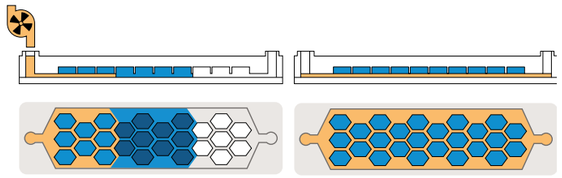


**Figure 3.2:** During partitioning, reaction mixtures are equally distributed between the nanowells and sealed in with a partitioning fluid.

On the analyzer, PCR reactions are carried out according to user-defined thermal cycling protocols on up to six thermal cyclers located within two chambers. These are pressurized to prevent bubble formation in the nanowells. After thermal cycling is finished, the nanowells are imaged by an automated microscopic optic in up to six user-defined fluorescent channels plus one channel for the filling control dye **(Figure 3.3)**.

The microscopic images are then analyzed by an image analysis algorithm. The algorithm performs quality control (QC) and evaluates fluorescent brightness value data for all valid nanowells, which are subsequently stored in result data files. The data are visualized using scatter plots in the Digital LightCycler® Development Software **(Figure 3.4)**. Setting of fluorescence thresholds that define clusters of positive and negative nanowells can be performed manually or by automated, adjustable algorithms.

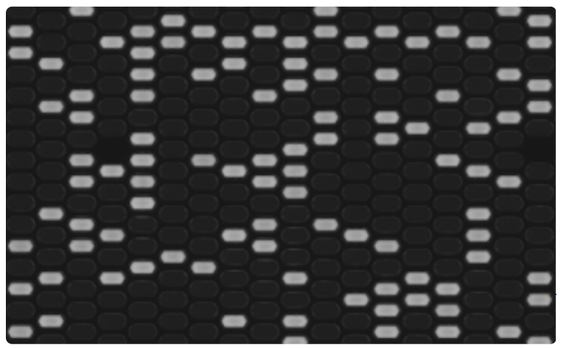


**Figure 3.3:** At the end of the PCR reaction, nanowells containing the amplified target molecules fluoresce, and the resulting image is processed by the Digital LightCycler® analysis algorithm.

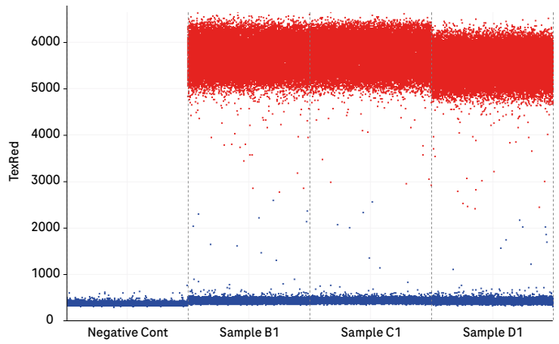



**Figure 3.4:** One-dimensional (1D) scatter plot for one negative control lane (left side) and three positive lanes showing fluorescence intensity in each nanowell. Red dots are defined as positive and blue dots as negative, based on the threshold chosen during the analysis step.

Specific analysis options for absolute quantification ('Abs Quant'), copy number variation ('CNV'), and insertion–deletion ('Indel') assays can be selected for calculating application-specific target values, including target concentrations, genomic copy numbers, or percentages of variant target sequences.

Following optimization and validation of assay-specific parameters, such as run protocols, limits of detection, mandatory controls, and control limits, the development software can be used to create analysis packages. These facilitate the establishment of easy-to-perform diagnostic workflows on the Digital LightCycler® System, using the Web Application. For diagnostic workflows, these analysis packages are assigned to samples either manually or via laboratory information systems (LIS) and provide all required information for the system to perform assay-specific amplification, imaging, and data analysis steps. Final results are reported and can be approved by diagnostic users.

Users should confirm the stability of their prepared reaction mixtures, prepared plates, and partitioned plates after determining the inherent variability of their assay. Clinical Laboratory and Standards Institute (CLSI) EP25-A guidelines[5] may serve as a guide. Ideally, the stability of prepared reaction mixtures, prepared plates, and partitioned plates for specimens should be compared to a control processed directly through the complete Digital LightCycler® workflow.

## 3.2 Nanowell plates

The Digital LightCycler® System features three different plate types, which differ in reaction mixture input volumes and nanowell (partition) numbers **(Table 3.1)**.

Plate-specific performance considerations are described below to assist with plate selection.

### Higher input volume for higher sensitivity

Higher input dPCR reaction volumes allow for higher amounts of target nucleic acid and increased sensitivity for detection of variants present at low abundance. Thus, for detection of rare targets requiring maximal sensitivity, the use of Digital LightCycler® HiSens Plates is recommended.

Sensitivity can also be increased by distributing an even larger volume of reaction mixture across several lanes of a nanowell plate and using the software to merge the lanes. For samples distributed over multiple lanes that are later merged, the software calculates only one result based on the total target copies detected in all merged lanes.

### Higher nanowell numbers for increased precision

Poisson statistics are fundamental to target concentration determination by dPCR and for its precision. Using a higher number of partitions (nanowells) results in improved precision. For applications requiring high precision, the use of Digital LightCycler® HiRes Plates is recommended.

| Plate type | Reaction volume (µL) | Maximum sample input volume (µL) | Approximate number of nanowells | Product number |
|---|---|---|---|---|
| Digital LC **HiSens** Plate | 45 | 31.5 | 20,000 | 09033718001 |
| Digital LC **Uni** Plate | 30 | 21.0 | 28,000 | 09033696001 |
| Digital LC **HiRes** Plate | 15 | 10.5 | 100,000 | 09033700001 |

LC, LightCycler.

**Table 3.1:** Digital LightCycler® plate types.

Precision also depends on absolute target concentrations. In dPCR, concentrations are expressed as $\lambda$ (mean copies per partition [cpp])). **Figure 3.5** shows the relative confidence for all three plate types in relation to $\lambda$.

Optimal precision is generally achieved at $\lambda$~1.6, which corresponds to ~20% negative partitions. Precision decreases at lower values of $\lambda$ due to increasing subsampling error. At higher $\lambda$ values, partitioning error increases because of uncertainty in the number of negative partitions, which also decreases precision. For applications utilizing specimens with relatively high target nucleic acid concentrations that do not require extreme sensitivity, and do not require high resolution to discriminate small differences, the use of Digital LightCycler® Uni Plates is recommended.

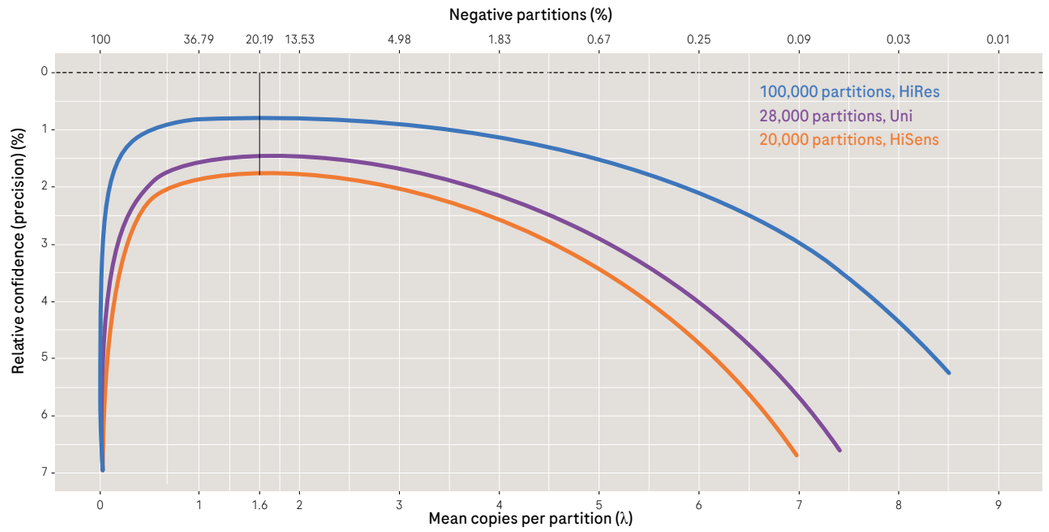



**Figure 3.5:** Precision, represented by percentage relative confidence calculated from the 95% confidence interval (CI), for the three plate types. For all three plate types, precision is highest at $\lambda$~1.6. Relative confidence is defined as the larger half width of a 95% CI of concentration, divided by the concentration.

[5]Clinical and Laboratory Standards Institute (CLSI). Evaluation of Stability of in vitro Diagnostic Reagents; Approved Guideline. 1st edition. CLSI document EP25-A (ISBN 1-56238-706-5 [Electronic]); Wayne, PA 19087, USA: Clinical and Laboratory Standards Institute; 2009.

## 3.3 General assay optimization considerations

### Assay design tools

For primer and probe design, as well as associated assay parameters, Primer 3 Plus[6] (https://www.primer3plus.com), OligoArchitect™ (Sigma-Aldrich®; http://www.oligoarchitect.com/LoginServlet), or other assay design tools can be used. For the Digital LightCycler® 5× DNA Master, concentrations of monovalent ions, divalent ions, and deoxynucleotide triphosphates (dNTPs) are 100 mM, 4.5 mM, and 1.2 mM, respectively. For the Digital LightCycler® 5× RNA Master, concentrations of monovalent ions, divalent ions, and dNTPs are 100 mM, 3.2 mM, and 1.5 mM, respectively.

### Primer design

*General recommendations for primers*

Primer sequences should meet the following general characteristics:

- Optimal amplicon length: less than 120 and no longer than 200 base pairs. For cell-free DNA (cfDNA) and formalin-fixed paraffin embedded tissue (FFPET) specimens, shorter amplicons will generally improve sensitivity.
- Primer length: 18–25 nucleotides with no or very low internal secondary structure.
  - Avoid mismatches close to the 3' end of the primer, especially for reverse transcription primers. Base pair mismatches can reduce the efficiency of complementary DNA (cDNA) synthesis in RT-PCR assays. It is critical to avoid mismatches at the three 3' bases.
  - The type of primer mismatch correlates with the reverse transcription efficiency. G:T mismatches are better tolerated than other mismatches.
  - Mismatch tolerance is higher for non-reverse transcription primers. The closer a mismatch is to the 3' end, the larger is its effect.
- Check for possible non-specific binding and extension reactions through *in silico* sequence analysis.

If possible, design the primers according to the following rules:

- Primers *should* have:
  - A or C at the 3' end.
  - 35–65% GC content.
  - A melting temperature ($T_m$) of 60–70°C that is at least 3–5°C higher than the annealing temperature. The annealing step then occurs at a temperature of 55–65°C.
- Primers should *avoid*:
  - Sequences of identical nucleotides (e.g. four or more consecutive residues, such as CCCC or GGGG).
  - Being complementary to each other or themselves at their 3' ends.

### Probe design

*General recommendations for probes*

- Probe length: 15–30 nucleotides; can reduce to 12 nucleotides if $T_m$ enhancers are used.
- 45–65% GC content.
- Avoid formation of internal secondary structures, including G-quadruplexes.
- Avoid primer/probe 3' dimer formation (primer–probe non-overlap or maximum two base overlap).
- Avoid runs of identical nucleotides (e.g. four or more consecutive residues, such as CCCC or GGGG).
- Avoid having a G at the 5' end for the fluorescein amidite (FAM)/hexachloro-fluorescein (HEX) label since it has a quenching effect if the fluorescent dye is located at the 5' end.
- The $T_m$ of the probe must be at least 5°C higher than the $T_m$ of the primer. If the probe does not bind before the annealing of primers, the DNA polymerase extends the primers before the probe anneals. The consequence is inefficient or negligible probe cleavage.
- Dark quencher dyes (e.g. black hole quencher [BHQ] and iowa black [IB]) result in better fluorescent signals.
- Hydrolysis probes with an internal rather than a 3' terminal quencher show lower background and higher relative fluorescence intensity (RFI) values. Probes labeled with an internal quencher have advantages for assay design and production, but have low tolerance for mismatched targets.
- To increase mismatch tolerance for probes with internally placed quenchers, use wider spacing (>12 nucleotides) between the quencher and reporter.
- To reduce potential interference with reverse transcription efficiency in RT-PCR-based assays, avoid probes that can bind to the target RNA, i.e. are anti-sense.
- Mismatches between reporter and quencher can lead to hybridization with 5' flap formation and non-productive cleavage of the probe.
- For probes with an internal quencher, use the BHQ2 dye as an insertion and not as a substitution for a base.
- 3' ends of probes should be blocked to prevent extension, e.g. with a 3' spacer or 3' phosphate.
- Commonly used modifications, such as minor groove binder (MGB), locked nucleic acid (LNA), etc. are compatible with the Digital LightCycler® 5× DNA and 5× RNA Masters.
- Consider the most appropriate reporter dye and quencher dye **(see Table 3.2)**.

[6]Untergasser A, et al. Nucleic Acids Res 2007;35:W71–4. doi: 10.1093/nar/gkm306.



### Supported dyes

**Table 3.2** lists the dyes that are compatible with the Digital LightCycler® System for multiplexing applications due to the fixed, built-in automated color-correction algorithm.

| Channel | Reporter dye(s) | Quencher dye |
|---|---|---|
| 1 | Coumarin/Cyan500/Atto425 | BHQ |
| 2 | FAM | BHQ/IB |
| 3 | HEX/VIC | BHQ/IB |
| 4 | LC610/TexRed/CFR610 | BHQ/IB |
| 5 | CY5/JA270/LC640 | BHQ/IB |
| 6 | CY5.5 | BHQ/IB |

BHQ, black hole quencher; FAM, fluorescein amidite; HEX, hexachloro-fluorescein; IB, Iowa black; VIC, Victoria.

**Table 3.2:** Digital LightCycler®-compatible dyes.

### Assay design

*General considerations*

- Target sequence: 35–65% GC content.
- Recommended oligo concentration: 0.2–1.6 µM (0.9 µM starting point) for primers; 0.125–0.5 µM (0.25 µM starting point) for probes.
- Specificity (inclusivity and exclusivity).

*Specimen types and nucleic acid extraction*

The Digital LightCycler® System is compatible with commonly used specimen types and extraction methods that deliver high-quality nucleic acids. Tested specimen types include cell culture, plasmids, cfDNA, FFPET, whole blood, nasopharyngeal swabs, and stool. Stool specimens are likely to require pre-treatment (e.g. as described for MagNA pure extraction kits); total sample volumes should be kept below 50% of the total reaction mixture volumes.

Other recommended pre-treatments include:

- Restriction enzyme digestion for large/bulky genomic DNA (gDNA) to reduce sample viscosity.
- A denaturation step (e.g. 80°C for 15 minutes) for RNA samples with a protein coating (e.g. armored RNA).
- Additional carriers (e.g. polyriboadenylic acid [poly-rA]) in sample dilution buffer when handling and storing low-concentration samples.
- For stool samples, unformed stool is the only acceptable specimen type.
- See Appendix for more details.

### The Digital LightCycler® 5× DNA and 5× RNA Masters

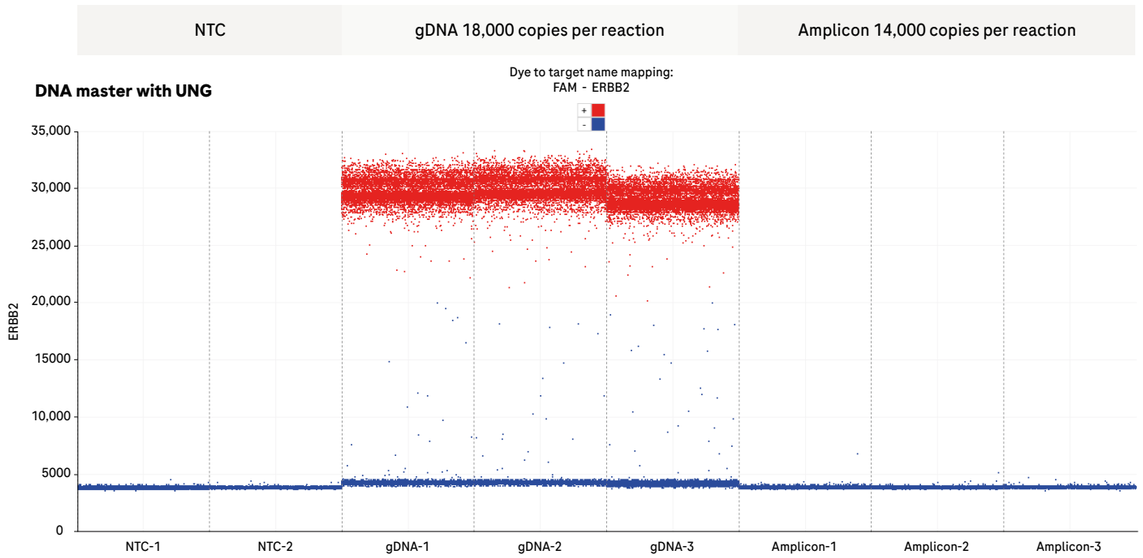
The Digital LightCycler® 5× DNA Master contains:

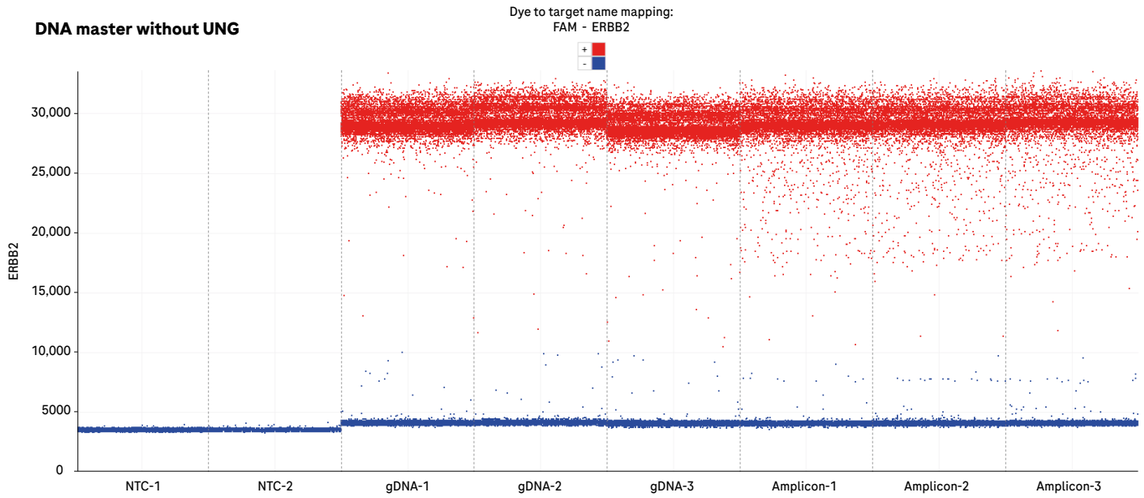
- dPCR Reaction Buffer
- Taq Polymerase
- AmpErase enzyme
- deoxyadenosine triphosphate (dATP)
- deoxycytidine triphosphate (dCTP)
- deoxyguanosine triphosphate (dGTP)
- deoxyuridine triphosphate (dUTP)
- Sodium azide
- Proprietary additives

The Digital LightCycler® 5× RNA Master includes:

- dPCR Reaction Buffer
- A blend of thermostable DNA polymerase enzymes
- AmpErase enzyme
- dATP, dCTP, dGTP, dTTP, dUTP
- Sodium azide
- Proprietary additives

The use of dUTP and AmpErase enzyme allows for the utilization of a decontamination step in the PCR thermal profile **(Figure 3.6 overleaf)**.




FAM, fluorescein amidite; gDNA, genomic DNA; NTC, no template control; UNG, uracil N-glycosylase.

**Figure 3.6:** Amplicons were spiked into the DNA template at ~14,000 copies/reaction. The UNG in the Digital LightCycler® 5× DNA Master was able to decontaminate 100% of the amplicons without affecting the amplification of a gDNA target at 18,000 copies/reaction.

### Optimal input target dilutions

For general absolute quantification purposes, the optimal input concentration for all three plate types is ~1.6 mean cpp since, at this concentration, dPCR has the highest precision. There is a maximum input limit that still allows DNA to be quantified with precision of 2.5% CV, and the limits are different on the three plate types. Refer to **Table 3.3** to estimate the optimal and maximum sample input when planning an experiment.

#### *Application-specific considerations*

For applications such as CNV or multiplex gene expression analysis, the optimal and maximum input DNA concentration needs specific consideration since the targets may be present in different concentrations. In this case, for optimal sample input the concentration of all targets should be considered. See Section 3.5 for more information.

Mutation detection assays may not tolerate a high concentration sample input as this can interfere with clear threshold and cluster definition, which is important for distinction of related variants in the same amplified sequence. See Section 3.6 for detailed recommendations.

### Merged lanes

The Digital LightCycler® Development Software offers the option to define merged lanes. If two lanes are merged, the software treats them as one reaction mixture (sample). This feature can be applied where a very high sample input volume is required due to low target concentrations or very high precision requirements (e.g. determination of the limit of blank for rare template detection).

### Controls

#### *No template controls (NTC)*

NTCs are negative controls that are used to monitor potential contamination in the system, workflow, and reagents. In NTCs the sample material is typically replaced by water or sample dilution buffer.

#### *Positive controls*

Positive controls should be introduced to check for potential inhibitory components and as a guide for the typical appearance of positive nanowell clusters during thresholding. Plasmids, synthetic DNA (e.g. gblocks, integrated DNA technologies [IDT], ultramers), synthetic transcripts, or any positive sample reference material can be used. The use of multiple types of positive control (e.g. synthetic DNA at known copy number in buffer and spiked into the specimen matrix of matching unknown samples) can be helpful for troubleshooting when unexpected results are obtained with one or more controls.

| | | High sensitivity (HiSens plate) | Universal (Uni Plate) | High resolution (HiRes Plate) |
|---|---|---|---|---|
| Number of partitions per reaction | | 20,000 | 28,000 | 100,000 |
| Optimal sample input | CPP | 1.6 | 1.6 | 1.6 |
| | Copies per reaction | 32,000 | 44,800 | 160,000 |
| | Human gDNA (ng/reaction) | 106 | 148 | 528 |
| Maximum sample input with Poisson precision of 2.5% CV | CPP | 6.15 | 6.64 | 8.38 |
| | Copies per reaction | 123,000 | 185,920 | 838,000 |
| | Human gDNA (ng/reaction) | 406 | 614 | 2765 |

CPP, copies per partition; CV, coefficient of variation; gDNA, genomic deoxyribonucleic acid.

**Table 3.3:** Optimal maximal target inputs with <2.5% Poisson precision for the three plate types. Calculation of gDNA amount assumes two copies per genome. See Section 3.5 for details regarding more than two copy targets.

*Application-specific controls*

- **Wild-type control:** For the detection of rare variants, controls based on background or wild-type only samples ('wild-type control') are useful, especially for the purpose of optimizing thresholds. These controls provide a realistic picture of the background signal to be expected from negative nanowells.
- **Copy number control:** For CNV applications, 'normal' copy number samples can be used.
- **Mutation variant control:** For mutation-specific assays, potential cross-reactive species (e.g. variants that are intentionally excluded by design) can be used to verify assay specificity.
- **Variant control:** For the detection of genetic variants (Indel assay), controls containing a known percentage of variant sequences (variant controls) can be introduced.

### Run protocols

In general, the default, generic run profiles included in the Digital LightCycler® Analyzer Software provide a good starting point for optimization **(Table 3.4)**.

Run profile-related optimization considerations:

- The default annealing/extension hold time is 20 seconds. An increase to 30 or 40 seconds may improve long amplicon and highly multiplexed assays.
- Three-step amplification cycles may improve performance for long amplicon assays.
- The default denaturation temperature is 95°C. An increase by 2–3°C may significantly improve GC-rich and/or secondary structure-heavy assays.

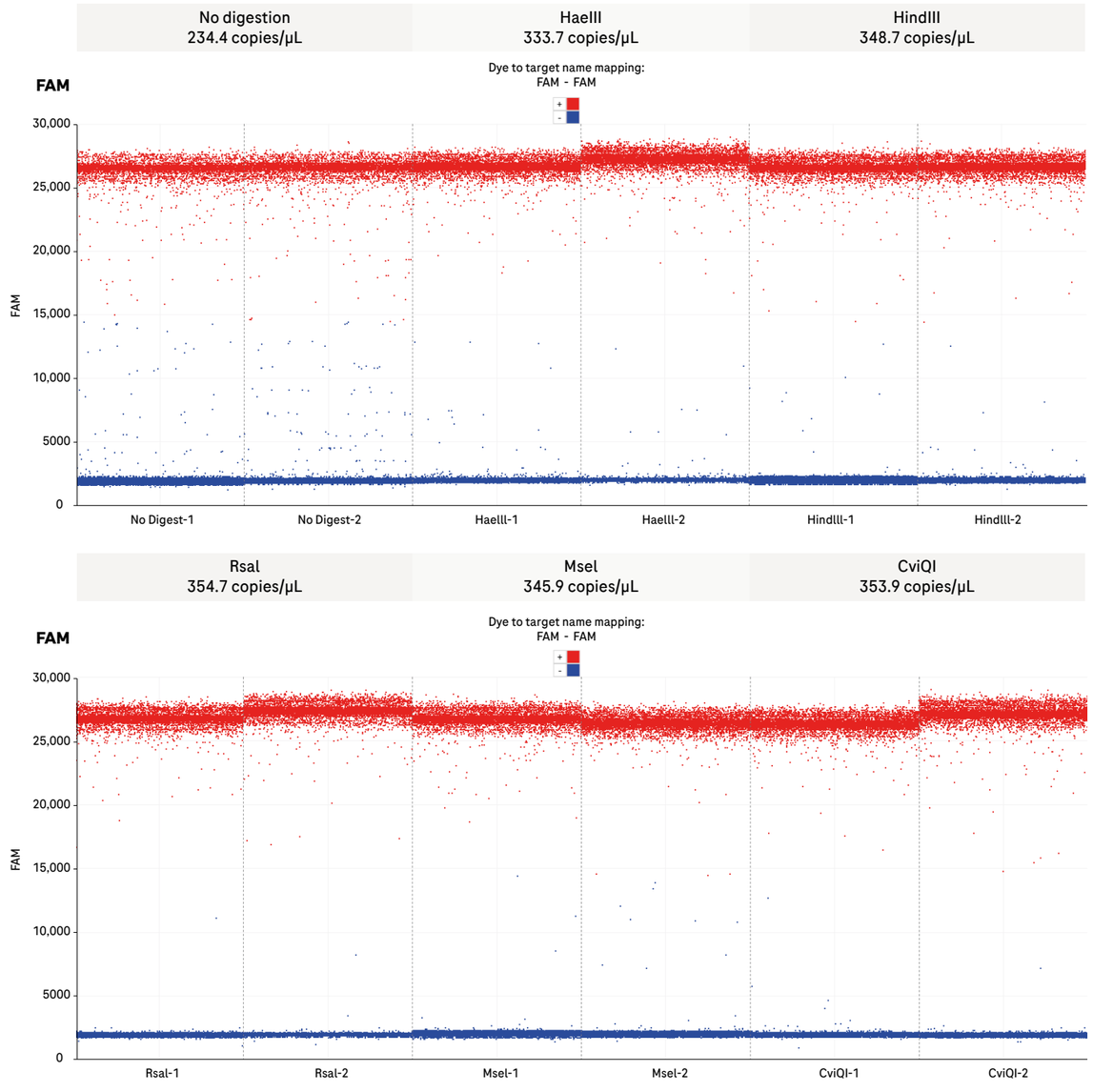
| Program name | Cycles | Target (°C) | Hold (s) |
|---|---|---|---|
| Default DNA run protocol | | | |
| UNG | 1 | 50 | 120 |
| Denaturation | 1 | 95 | 120 |
| Amplification | 40 | 95 | 10 |
| | | 55–65 | 20 |
| Cooling | | 40 | 30 |
| Default RNA run protocol | | | |
| UNG | 1 | 50 | 120 |
| Denaturation | 1 | 95 | 20 |
| RT step 1 | 1 | 55 | 180 |
| RT step 2 | 1 | 65 | 720 |
| RT-inactivation | 1 | 95 | 600 |
| Amplification | 40 | 95 | 15 |
| | | 56–65 | 30 |
| Cooling | 1 | 40 | 30 |

RT, reverse transcription; UNG, uracil N-glycosylase.

**Table 3.4:** The default generic Digital LightCycler® DNA and RNA run profiles.

### Restriction digestion

When using gDNA templates, the use of restriction enzymes to improve assay performance and accurate quantitation is recommended. gDNA extracted from cell lines is bulky and viscous, and thus may not partition homogeneously across the nanowell plate lanes. In addition, some extraction methods yield intact and structured gDNA, and target regions may be difficult for primers and probes to access during the initial PCR cycles, resulting in reduced assay performance. Restriction enzymes should be chosen carefully, avoiding those that can digest the target sequences. In the example in **Figure 3.7**, a DNA assay was tested on the same gDNA sample with different restriction enzymes added during setup. The reactions without added enzymes showed more nanowells with intermediate fluorescence ('rain') and significantly lower concentration calls (copies/µL) than any of the digested reactions.




FAM, fluorescein amidite.

**Figure 3.7:** Five commonly used restriction enzymes that recognize four- or six-base sequences (no cutting into amplicon sequences) were spiked into the reaction mixtures at 5 U/reaction with no additional incubation step and compared with reactions without added restriction enzymes.

For CNV applications, the target of interest can be present in tandem repeats. If not separated, the target concentration and its copy number will be underestimated, as shown in **Figure 3.8**.

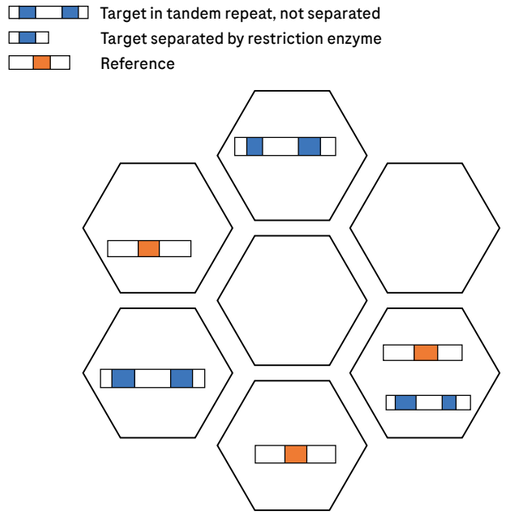
*Pre-digestion of gDNA for dPCR*
Considerations for choosing assay-compatible restriction enzymes:

- The enzymes should not cut within the amplicon sequences.
- Enzymes that recognize four-base sequences are preferred over those that recognize six-base sequences because they generate smaller fragments.
- One enzyme is enough for general applications.
- More than one enzyme can be used in CNV applications for optimal outcomes.

Recommendations for the restriction enzyme digestion process:

- Pre-digestion in the restriction enzyme buffer in a separate reaction is recommended for complete separation of tandem repeats or for linearization of circular plasmids.
- Heat inactivation is recommended if the digested DNA will be stored long term.
- Please refer to the restriction enzyme vendor's instructions for use for optimization.
- Generally, restriction digestion product volumes should not exceed 20% of the volume of the final dPCR reaction to avoid interference by the digestion buffer components.
- Additional optimization experiments may be needed to identify the optimal input range of undiluted restriction digestion products.
- No inhibition of target DNA quantitation was observed when as much as 33% of the dPCR reaction volume was the rCutSmart™ Buffer from New England Biolabs (www.neb.com/products/b6004-rcutsmart-buffer).

For general applications without any need to separate tandem repeat sequences and linearize circular sequences, restriction enzymes can be added directly to the dPCR reactions as part of the workflow. No additional incubation is needed, as long as the enzyme retains at least partial activity under these conditions. Between 0.1 and 0.25 enzyme units per µL in the reaction mixture works well in general. Optimization may be needed for some clinical samples showing sensitivity to restriction digestion.




CPP, copies per partition.

**Figure 3.8:** Targets in tandem repeat need to be separated for accurate quantification.

## 3.4 Absolute quantification assays

### Assay principle

Absolute quantification represents the basis for all dPCR applications. In contrast to qPCR, target concentrations are directly determined in copies/µL without the need to generate standard curves.

### Specific considerations for absolute quantification setups

*Linearization of circular DNA template (plasmid)*
Plasmid samples should be pre-linearized using a restriction digestion reaction prior to any PCR amplification. Circular and supercoiled structures will result in heavy 'rain' in one-dimensional (1D) scatter plots, difficulty in clustering, and incorrect quantification results.

*Protected nucleic acids*
If DNA and/or RNA is in a protected format (for example, armored RNA, nucleic acid in liposomes, MS2-coated RNA, etc.), pre-denaturation or additional purification/extraction steps should be considered to ensure accessibility of the template and reproducibility of quantification.

*Handling samples with low concentrations*
cfDNA and cfRNA should be stored at -80°C in low-binding tubes and frequent freeze–thaw cycles should be avoided. The addition of carriers (e.g. poly-rA) to the dilution buffer is recommended.

*Potential inhibition from samples and preparation methods*
Clinical samples may contain PCR inhibitors originating from the specimen matrix and/or from carry-over of the extraction reagents. The use of a 'clean sample' comprising material with known copy number lacking any trace of specimen matrix from the extraction reagents and a control containing the same material spiked into the specimen matrix can be considered to ensure that assay performance is not affected. In some cases, higher sample dilution may be required to lower the impact of PCR inhibitors.

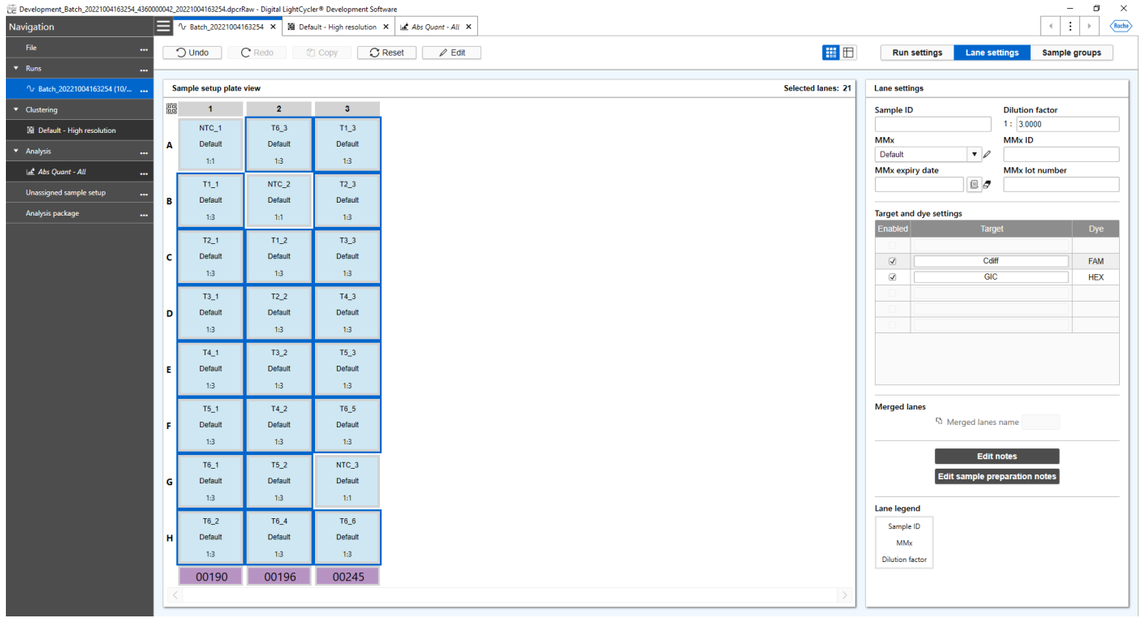
*Sample input and plate selection*
When planning an absolute quantification experiment, it is important to determine target concentrations in advance to ensure the target input is within the dynamic measurement range. As discussed previously (Section 3.2), an average of ~1.6 cpp will result in the highest precision of the concentration measurement. Due to the different measurement principles, commonly used nucleic acid quantification methods, such as spectrophotometry (e.g. NanoDrop) or fluorometry (e.g. Bioanalyzer or Qubit), could be inaccurate with respect to actual copy number. Therefore, especially for highly concentrated samples, consider establishing the DNA or RNA target concentration range using a dilution series on the Digital LightCycler®.

**Application example: Absolute quantification of *Clostridioides difficile (C. difficile)* samples for validation of measurement range and linearity**

*Setup*

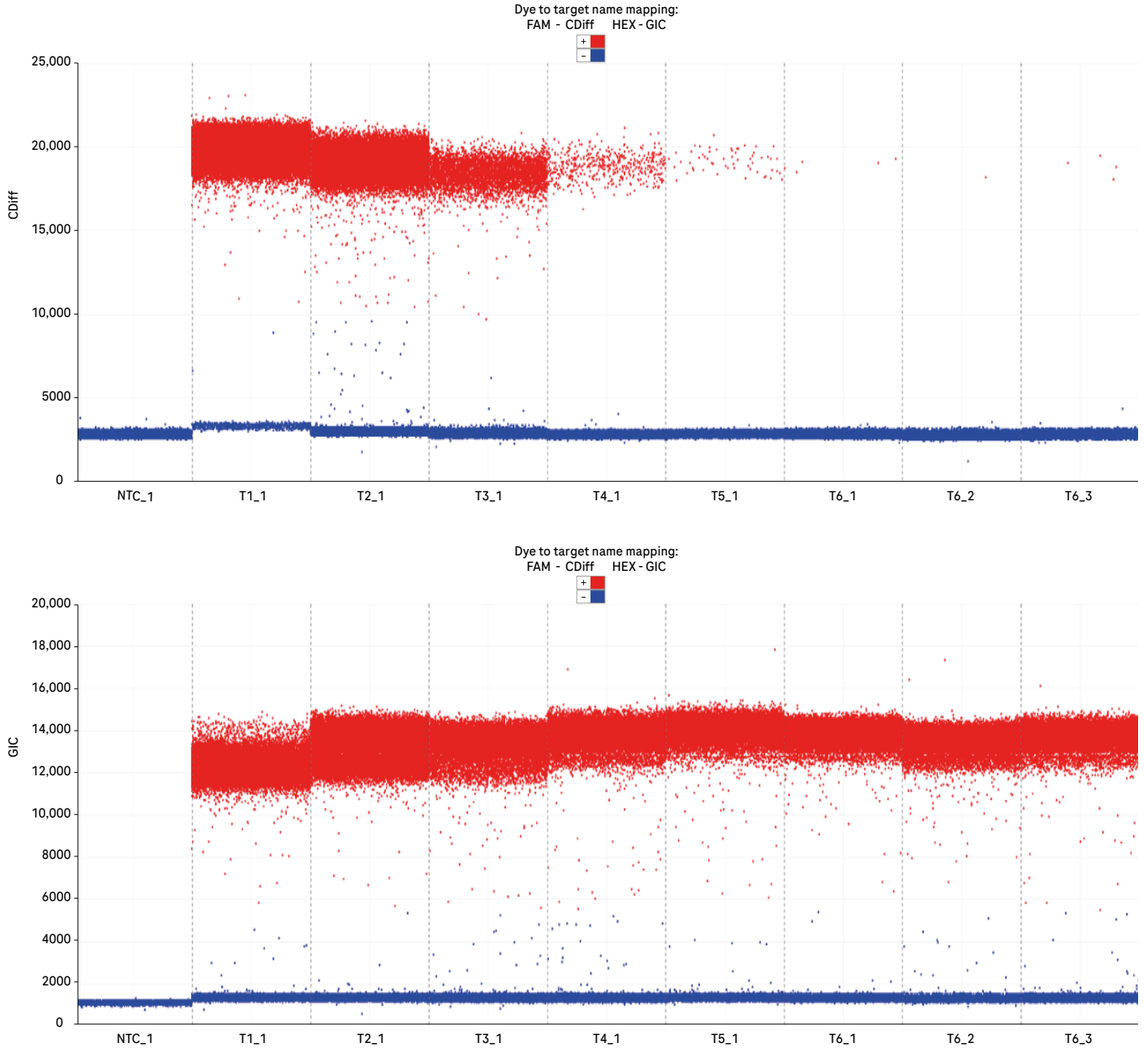
To validate the measuring range and linearity of a *C. difficile* assay, a set of serially diluted samples containing approximately 150,000, 15,000, 1500, 150, 15, and 1.5 copies/µL (samples labeled as T1, T2, T3, T4, T5, and T6) of synthetic gblock *C. difficile* template in a constant background of a generic internal control (GIC) plasmid template was generated. Samples were tested at 5 µL per 15 µL input (dilution factor 1:3) with a duplex assay – *C. difficile* (FAM)/ GIC (HEX) – on HiRes plates. Three replicates were used for the first five concentration levels and the NTC condition, while for the lowest level (1.5 copy/µL), six replicates were used to increase precision. **Figure 3.9** shows the settings applied for each reaction in the Digital LightCycler® Development Software.

For clustering, the 'automatic-custom' default settings were applied. The resulting duplex 1D scatter plots are shown in **Figure 3.10.**




CDiff, *Clostridioides difficile*; cps, copies; FAM, fluorescein amidite; GIC, generic internal control; HEX, hexachloro-fluorescein; MMx, master mix; NTC, no template control.

**Figure 3.9:** Development software lane settings for application example. Each cell in the table on the left corresponds to a dPCR reaction and corresponding lane on the plate.




1-D, one-dimensional; CDiff, *Clostridioides difficile*; cps, copies; dPCR, digital PCR; FAM, fluorescein amidite; GIC, generic internal control; HEX, hexachloro-fluorescein; NTC, no template control.

**Figure 3.10:** Development software 1D scatter plots for the *C. difficile* (top) and GIC (bottom) targets after clustering using the 'automatic-custom' default settings.

Results

< Custom view > Searching...

Filter by

Configure columns Delete Save Apply

| Sample ID | Target | MMx | Lane | Dilution factor | Concentration [copies/µL] | Conc. 95% CI min. | Conc. 95% CI max. | Total copies | Sample type | Final result | Flags |
|---|---|---|---|---|---|---|---|---|---|---|---|
| NTC_1 | Cdiff | Default | A1 | 1 | 0.0000 | N/A | N/A | 0 | | Valid | |
| T1_1 | Cdiff | Default | B1 | 3 | 143593.7891 | 141685.5156 | 145527.7638 | 717968.9453 | | Positive | |
| T2_1 | Cdiff | Default | C1 | 3 | 14286.8505 | 14149.9914 | 14425.0334 | 71434.2525 | | Positive | |
| T3_1 | Cdiff | Default | D1 | 3 | 1425.3535 | 1386.9446 | 1464.8261 | 7126.7676 | | Positive | |
| T4_1 | Cdiff | Default | E1 | 3 | 139.7241 | 128.1737 | 152.3152 | 698.6203 | | Positive | |
| T5_1 | Cdiff | Default | F1 | 3 | 15.8560 | 12.2851 | 20.4650 | 79.2801 | | Positive | |
| T6_1 | Cdiff | Default | G1 | 3 | 1.3437 | 0.5593 | 3.2282 | 6.7183 | | Positive | |
| T6_2 | Cdiff | Default | H1 | 3 | 0.2704 | N/A | N/A | 1.352 | | Positive | |
| T6_3 | Cdiff | Default | A2 | 3 | 1.0851 | N/A | N/A | 5.4254 | | Positive | |
| NTC_2 | Cdiff | Default | B2 | 1 | 0.0000 | N/A | N/A | 0 | | Valid | |
| T1_2 | Cdiff | Default | C2 | 3 | 144187.4249 | 142261.3564 | 146139.5704 | 720937.1246 | | Positive | |
| T2_2 | Cdiff | Default | D2 | 3 | 14356.0396 | 14218.4808 | 14494.9291 | 71780.1978 | | Positive | |
| T3_2 | Cdiff | Default | E2 | 3 | 1423.8311 | 1385.4706 | 1463.2538 | 7119.1557 | | Positive | |
| T4_2 | Cdiff | Default | F2 | 3 | 157.6136 | 145.3453 | 170.9175 | 788.0681 | | Positive | |
| T5_2 | Cdiff | Default | G2 | 3 | 12.9107 | 9.7295 | 17.1321 | 64.5537 | | Positive | |
| T6_4 | Cdiff | Default | H2 | 3 | 0.5410 | N/A | N/A | 2.705 | | Positive | |
| T1_3 | Cdiff | Default | A3 | 3 | 141634.2666 | 139781.3645 | 143511.7303 | 708171.333 | | Positive | |
| T2_3 | Cdiff | Default | B3 | 3 | 14357.7455 | 14220.4738 | 14496.3423 | 71788.7273 | | Positive | |
| T3_3 | Cdiff | Default | C3 | 3 | 1456.8782 | 1418.1031 | 1496.7134 | 7284.3908 | | Positive | |
| T4_3 | Cdiff | Default | D3 | 3 | 146.4837 | 134.6460 | 159.3623 | 732.4187 | | Positive | |
| T5_3 | Cdiff | Default | E3 | 3 | 13.2301 | 9.9991 | 17.5050 | 66.1504 | | Positive | |
| T6_5 | Cdiff | Default | F3 | 3 | 1.0754 | N/A | N/A | 5.3771 | | Positive | |
| NTC_3 | Cdiff | Default | G3 | 1 | 0.0000 | N/A | N/A | 0 | | Valid | |
| T6_6 | Cdiff | Default | H3 | 3 | 1.0805 | N/A | N/A | 5.4024 | | Positive | |
| NTC_1 | GIC | Default | A1 | 1 | 0.0000 | N/A | N/A | 0 | | Valid | |
| T1_1 | GIC | Default | B1 | 3 | 13788.8659 | 13654.9314 | 13924.1141 | 68944.3295 | | Positive | |

CDiff, *Clostridioides difficile*; CI, confidence interval; cps, copies; Conc, concentration; GIC, generic internal control; MMx, master mix; NTC, no template control.

**Figure 3.11:** Development software result table for *C. difficile* application example. Note that for the lowest concentration sample (1.5 copy/µL, T6), the 95% CI cannot be calculated because of the extremely low concentrations (<4 positive nanowells per reaction).

### *Data analysis*

In the Analysis settings, NTC reactions were set as negative control samples with an upper limit of 0.2 copies/µL for both channels (no more than three positives per reaction). **Figure 3.11** shows the development software results table after calculation based on these settings. The results were exported for further analysis in a linearity plot of expected vs measured sample concentrations **(Figure 3.12)** based on CLSI EP06-ED2[7] guidelines.

### *Merging replicate lanes*

When technical replicates are given the same sample ID during sample setup, the development software will virtually merge these lanes for quantification and handle the lanes as one sample. For low concentration samples, this can improve the quantification. For example, if the six technical replicates of the lowest concentration samples for this application example are merged for analysis, a total of 27 copies per merged reaction are calculated. This higher number now allows for the calculation of the 95% CI **(Figure 3.13)**.

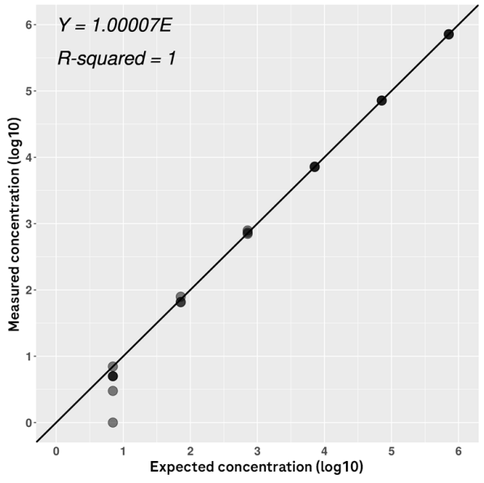



**Figure 3.12:** Linearity plot of expected vs measured sample concentrations for the *C. difficile* application example.

[7]Clinical and Laboratory Standards Institute (CLSI). Evaluation of Linearity of Quantitative Measurement Procedures. 2nd edition. CLSI document EP06 (ISBN 978-1-68440-096-6 [Print]; ISBN 978-1-68440-097-3 [Electronic]). Wayne, PA 19087, USA: Clinical and Laboratory Standards Institute; 2020.



| Sample ID | Target | MMx | Lane | Dilution factor | Concentration [copies/µL] | Conc. 95% CI min. | Conc. 95% CI max. | Total copies | Sample type | Final result | Flags |
|---|---|---|---|---|---|---|---|---|---|---|---|
| NTC | Cdiff | Default | A1 | 1 | 0.0000 | N/A | N/A | 0 | | Valid | |
| T1 | Cdiff | Default | B1 | 3 | 143142.3524 | 142044.8373 | 144248.3474 | 2147135.2853 | | Positive | |
| T2 | Cdiff | Default | C1 | 3 | 14333.5161 | 14254.1256 | 14413.3487 | 215002.7413 | | Positive | |
| T3 | Cdiff | Default | D1 | 3 | 1435.3758 | 1413.0110 | 1458.0946 | 21530.6373 | | Positive | |
| T4 | Cdiff | Default | E1 | 3 | 147.9576 | 140.9714 | 155.2899 | 2219.3636 | | Positive | |
| T5 | Cdiff | Default | F1 | 3 | 14.0005 | 11.9672 | 16.3792 | 210.0072 | | Positive | |
| T6 | Cdiff | Default | G1 | 3 | 0.8999 | 0.5806 | 1.3949 | 26.9977 | | Positive | |

CDiff, *Clostridioides difficile*; CI, confidence interval; cps, copies; Conc, concentration; MMx, master mix; NTC, no template control.

**Figure 3.13:** After merging the replicate lanes, the 95% CI minimum and maximum concentrations can be calculated because the total copies per 90 µL (total of six reactions) has increased from four to 27.

## 3.5 Copy number variation assays

CNV is a major cause of structural variation in the genome, including amplification or deletion of a genomic sequence in comparison to a reference genome. Digital PCR is particularly suitable for CNV applications due to its high accuracy and precision.

### Assay principle

Typically, CNVs are detected using a non-competing duplex assay, with one target and one reference assay in two optical channels **(Figure 3.14)**. The two assays need to have similar amplification efficiency and compatible optimal reaction conditions. These are represented by an orthogonal cluster distribution on a two-dimensional (2D) scatter plot and result in comparable quantification between the singleplex and duplex reactions. In some cases, using multiple reference assays can improve accuracy and precision. The copy number of a target gene is calculated using the following formula, where concentrations are copies/µL:

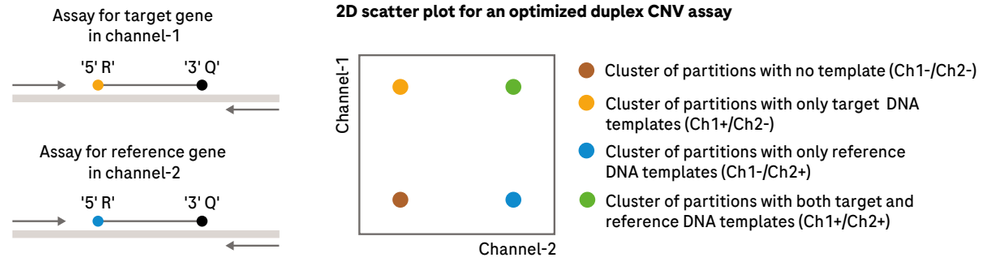
$$\text{copy number (target)} = \frac{\text{concentration of target}}{\text{concentration of reference}} \times \text{number of reference copies per genome}$$

For human gDNA, the typical reference target is present in two copies per genome.

### Specific considerations for CNV setups

#### *Pre-treatment*

Restriction digestion of sample DNA is recommended before CNV experiments because any target present in multiple copies on the same DNA strand will lead to under-quantification in dPCR if not separated before partitioning (see Section 3.3, **Figure 3.7**). For complete restriction digestion, consider a separate reaction with an optimal buffer using vendor-recommended incubation conditions, rather than adding the enzyme to the dPCR reaction. For details about restriction digestion, please refer to Section 3.3. If the DNA is already fragmented (e.g. cfDNA, FFPET eluates), there is no need to further fragment the DNA by restriction digestion.




2-D, two-dimensional; Ch, channel; CNV, copy number variation; Q, quencher; R, reporter.

**Figure 3.14:** Schematic representation of CNV assay setup (left) and a 2D scatter plot for an optimized CNV assay (right).

### *Reference targets*

Choose one or more reference assays when multiplexing target assays. In general, stable regions in the genome should be used for reference assays. Commonly used reference assays are ribonuclease P protein subunit 30 (RPP30), eukaryotic translation initiation factor 2C (EIF2C), human beta-globin, etc. For oncology applications, assessment of the copy number of candidate reference genes in clinical samples is recommended, to inform the selection of the most stable one(s) in the context of the specific disease background. The target and reference assays should be designed with the same oligo $T_m$ and similar amplicon length so that they can be duplexed in one reaction with the same optimized thermal cycling profile.

### *Optimal input copy number for CNV*

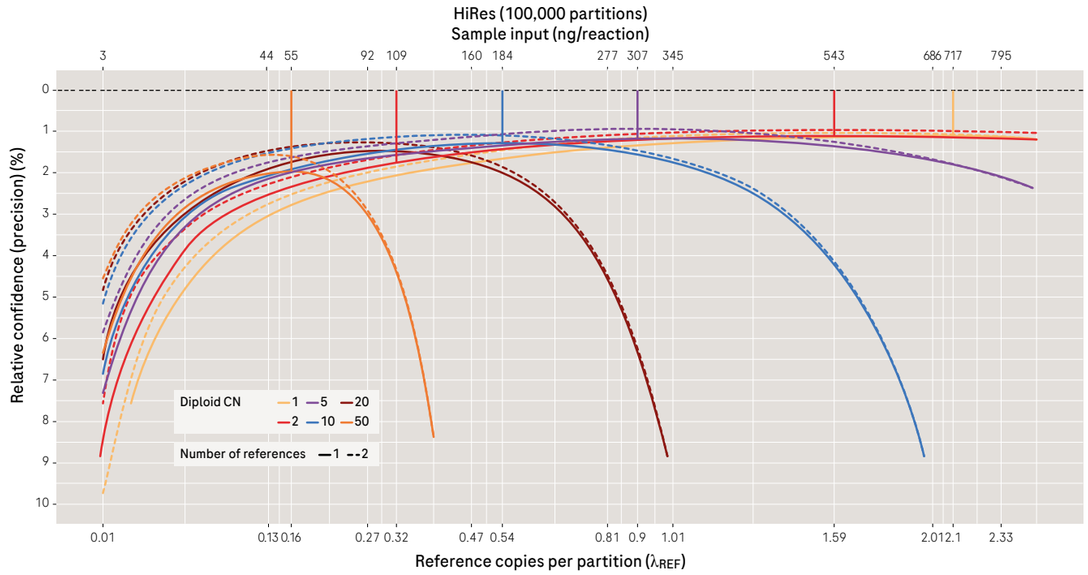
As discussed previously, the optimal input for absolute quantification by dPCR with the highest precision is ~1.6 cpp because the total Poisson error is minimized at this level, independent of the number of partitions. For CNV applications, due to the potential differences in copy numbers between the target and reference, it may be challenging to optimize the amount of input DNA and achieve maximal precision for both. For example, the optimal input for a reference gene with a copy number of 2 (1.6 cpp) would contain 8 cpp of a target present in 10 copies per genome, and thus the calculated CNV would suffer from suboptimal precision.

The optimal sample input level (in reference cpp or ng/µL of amplifiable gDNA) for targets with copy number of 1, 2, 5, 10, 20, or 50 when using a HiRes plate (100,000 partitions per reaction) is depicted in **Figure 3.15**. When the estimated target copy number is low (five or less), there is a relatively wide range of sample input that still allows for good precision. However, when the target copy number increases (10 and above), the sample input range for optimal precision is significantly narrower.

The recommended amount of sample input for precise CNV calling is summarized in **Table 3.5**. For example, if the target is expected to be present in about 10 copies per genome, the optimal amount of amplifiable human gDNA to add to the reaction is approximately 49 ng if using a Uni plate, 38 ng on a HiSens plate, and 184 ng on a HiRes plate.

### *Non-integer copy number readout*

When testing control samples (e.g. plasmid, gDNA extracted from stable cell lines), CNV calls are expected to be an integer value. However, CNV in real clinical samples may not always result in integer copy numbers.




CN, copy number.

**Figure 3.15:** Precision curves at various input DNA amounts (3–853 ng/reaction) with HiRes plates for targets with different copy numbers per genome. When using only one reference gene, the sample inputs (ng/reaction) with the greatest precision are 717, 543, 307, 184, 109, and 55 (vertical solid lines) for copy numbers 1, 2, 5, 10, 20, and 50, respectively.

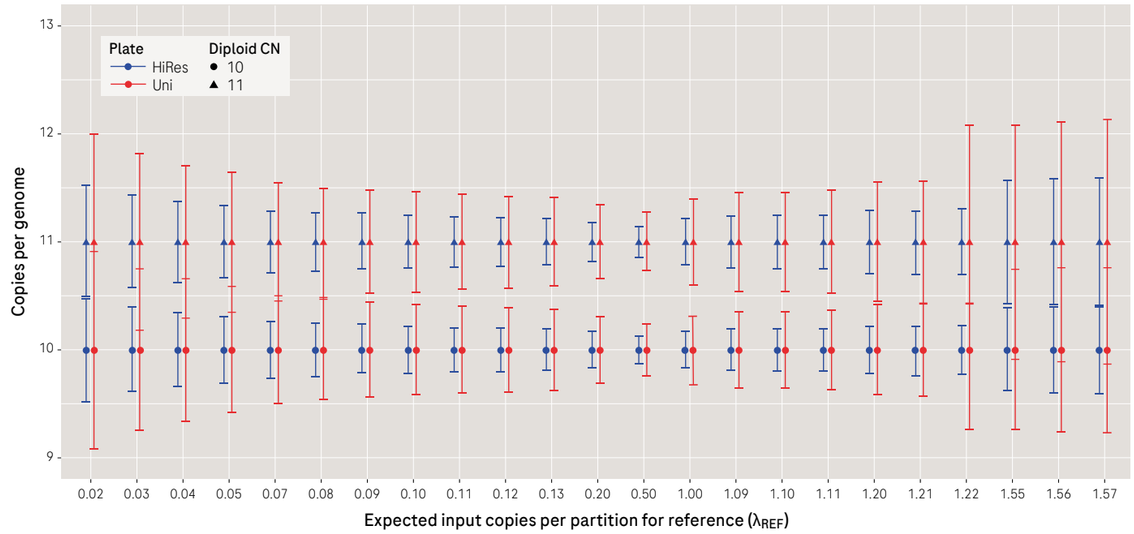
*Possible factors that contribute to non-integer copy number include:*

- Fragmentation and damage to the sample DNA, which is frequently seen with FFPET samples
- Mixed populations of cells, for example normal tissue mixed with tumor tissue
- Non-optimal DNA input leading to decreased precision

| Expected target copy number in the genome | Optimal sample input for CNV calling* | | | | |
|---|---|---|---|---|---|
| | Reference cpp | Target cpp | Amplifiable human gDNA (ng/reaction) | | |
| | | | Uni plate | HiSens plate | HiRes plate |
| 1 | 2.10 | 1.05 | 192 | 149 | 717 |
| 2 | 1.59 | 1.59 | 145 | 113 | 543 |
| 5 | 0.90 | 2.25 | 82 | 64 | 307 |
| 10 | 0.54 | 2.70 | 49 | 38 | 184 |
| 20 | 0.32 | 3.20 | 29 | 23 | 109 |
| 50 | 0.16 | 4.00 | 15 | 11 | 55 |

CNV, copy number variation; cpp, copies per partition; gDNA, genomic DNA. *Calculation of gDNA amount assumes use of a single reference present at two copies per genome and genome mass of 3.3 pg.

**Table 3.5:** Optimal sample input of gDNA (ng/reaction) for different target copy numbers for the three different plate types.




CI, confidence interval; CN, copy number.

**Figure 3.16:** Precision (95% CI) for copy number measurement in cell lines with 10 or 11 copies per genome of a target, at different amounts of input DNA, in two different types of plate.

### Resolving small differences in CNV

For resolving small differences in CNV between two samples, the HiRes plate with ~100,000 partitions is the best plate type to use since it enables clear discrimination over a wide range of DNA sample input. **Figure 3.16** shows simulated predictions of copy number values with 95% CI for diploid cell lines with 10 or 11 copies of a target per genome over a range of DNA input corresponding to 0.01–1.6 cpp of a reference with two copies per genome. The optimal sample input for best precision and discrimination between cell lines is around 46 ng/reaction (reference 0.5 cpp) for Uni plates (28,000 nanowells) and 171 ng/reaction for HiRes plates (100,000 nanowells). Non-overlapping error bars represent successful discrimination between the two cell lines.

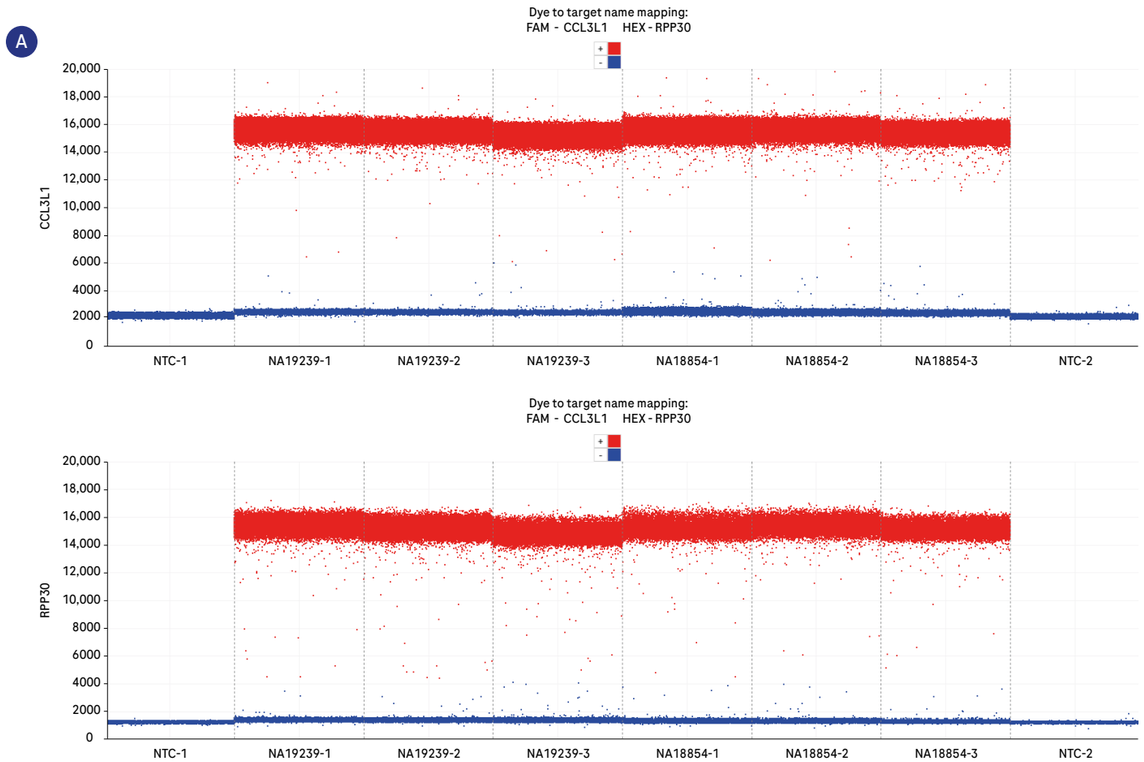
HiRes plates support the ability to discriminate over a wider range of DNA input concentrations (approximately 7–529 ng/reaction) compared to Uni plates (7–110 ng/reaction).

**Application example: Discriminating CNV 10 from 11**

In a wet-lab experiment, a duplex CNV assay using chemokine ligand 3-like 1 (CCL3L1) (FAM labeled) as the target, RPP30 (HEX labeled) as the reference, and two cell lines with estimated target copy number of 10 and 11 (Coriell, Camden, New Jersey) was used to demonstrate resolving a 10% copy number difference on HiRes plates. The cell line gDNA was pre-digested using RsaI and MseI, and DNA input was around 175 ng per reaction. Each sample was run in triplicate reactions, and two NTCs were used to ensure a contamination-free workflow and reagents.

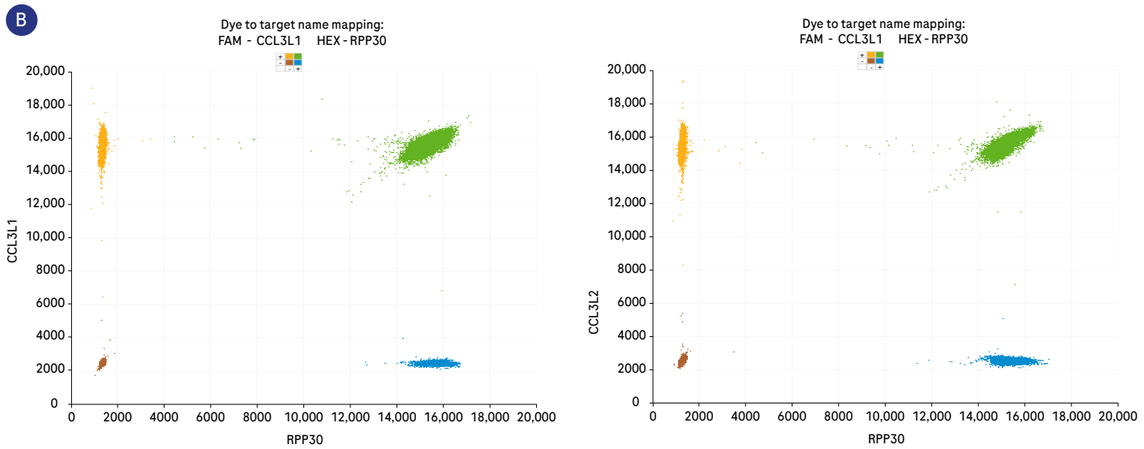
*Data analysis*

1D scatter plots from FAM and HEX channels **(Figure 3.17A)** showed clear separation of positive and negative clusters using automatic clustering with standard settings. 2D scatter plots (for one replicate per cell line; **Figure 3.17B)** showed an orthogonal cluster pattern, indicating the assays were compatible with each other without competition or interactions.



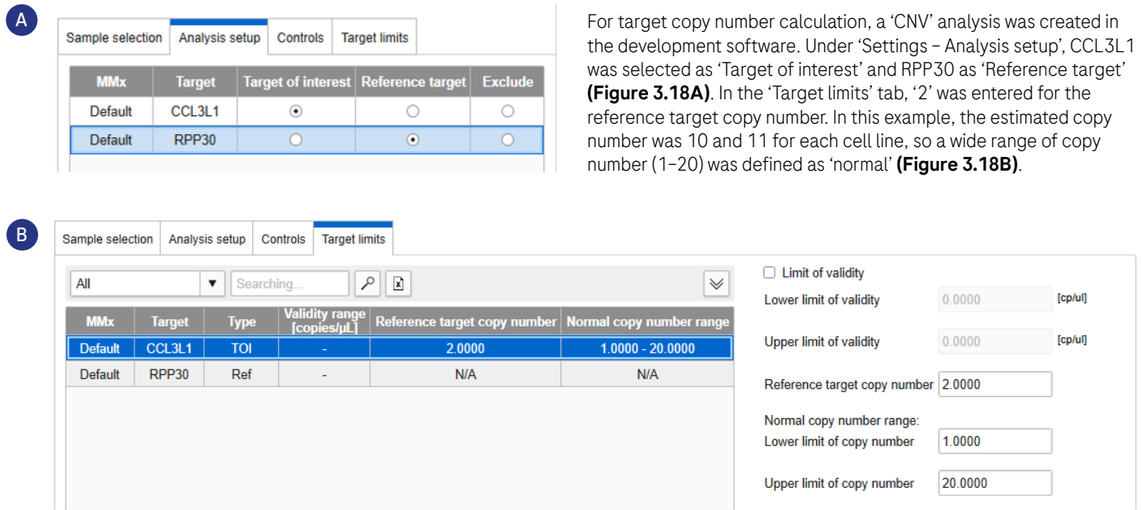

CCL3L1, chemokine ligand 3-like 1; FAM, fluorescein amidite; HEX, hexachloro-fluorescein; NTC, no template control; RPP30, ribonuclease P protein subunit 30.

**Figure 3.17A:** Scatterplots for the target CCL3L1 (FAM) and reference RPP30 (HEX) in cell lines with 10 or 11 copies of the CCL3L1 target. 1D scatter plot of samples for FAM (top) and HEX (bottom).




CCL3L1, chemokine ligand 3-like 1; FAM, fluorescein amidite; HEX, hexachloro-fluorescein; NTC, no template control; RPP30, ribonuclease P protein subunit 30.

**Figure 3.17B:** Scatterplots for the target CCL3L1 (FAM) and reference RPP30 (HEX) in cell lines with 10 or 11 copies of the CCL3L1 target. 2D scatter plot for FAM and HEX with one replicate of each cell line.




For target copy number calculation, a 'CNV' analysis was created in the development software. Under 'Settings – Analysis setup', CCL3L1 was selected as 'Target of interest' and RPP30 as 'Reference target' **(Figure 3.18A)**. In the 'Target limits' tab, '2' was entered for the reference target copy number. In this example, the estimated copy number was 10 and 11 for each cell line, so a wide range of copy number (1–20) was defined as 'normal' **(Figure 3.18B)**.

CCL3L1, chemokine ligand 3-like 1; MMx, master mix; RPP30, ribonuclease P protein subunit 30; TOI, target of interest.

**Figure 3.18:** A) Definition of target of interest and reference target under 'Settings – Analysis setup'. (B) Definition of 'reference target copy number' and 'normal copy number range' in the 'Target limits' tab.

After calculation, the 'Results' tab displayed the measured copy number and 95% Poisson CI **(Figure 3.19)**. All three replicates showed measured copy number values close to the expected values of 10 and 11 with non-overlapping CIs between the two cell lines.

## 3.6 Rare mutation assays

### Assay principle

Rare mutation detection is one of the most important dPCR applications in oncology. There are two main considerations when designing a rare mutation detection assay:

- The mutant target sequence may differ by as little as a single base from the wild-type sequence. Thus, assay design needs to provide high specificity in order to discriminate a single nucleotide difference.
- The target sequence is often at a much lower concentration than the background (wild-type) sequence in a sample. Thus, assay design needs to provide high sensitivity to detect low-abundance mutant targets.

Unlike allele-specific priming in qPCR, the more commonly used design strategy in dPCR relies on the TaqMan hydrolysis probe to confer specificity **(Figure 3.20)**.

To achieve single nucleotide discrimination, probe lengths need to be relatively short. In this case, single base pair mismatches result in considerably higher destabilization of the probe–template duplex during the annealing step, and thus result in much reduced or no probe hydrolysis.

Two commonly used probe design strategies are:

- Standard (non-modified) short probes: Need to have a relatively low annealing temperature for optimal cleaving efficiency. Sometimes it can be difficult to design short probes for AT-rich sequences. Moreover, the low annealing temperature may not be optimal for PCR amplification.
- Modified, $T_m$-enhanced short probes: Various $T_m$ enhancers can be used to increase the probe $T_m$ while keeping it short. However, the positioning of modified bases can be challenging and may not always give robust assay performance.

Results | Result overview | Statistics

**Results**

< Custom view > | Searching...

| Sample ID | TOI | MMx (TOI) | Lane | Copy number | Final result | CN 95% CI min. | CN 95% CI max. | Flags | Sample type |
|---|---|---|---|---|---|---|---|---|---|
| NA19239-1 | CCL3L1 | Default | B1 | 10.1713 | Normal | 10.0447 | 10.2980 | | |
| NA19239-2 | CCL3L1 | Default | C1 | 10.0575 | Normal | 9.9323 | 10.1827 | | |
| NA19239-3 | CCL3L1 | Default | D1 | 10.0842 | Normal | 9.9583 | 10.2102 | | |
| NA18854-1 | CCL3L1 | Default | E1 | 10.8349 | Normal | 10.6784 | 10.9915 | | |
| NA18854-2 | CCL3L1 | Default | F1 | 10.9512 | Normal | 10.7938 | 11.1086 | | |
| NA18854-3 | CCL3L1 | Default | G1 | 10.9024 | Normal | 10.7452 | 11.0596 | | |

CCL3L1, chemokine ligand 3-like 1; CI, confidence interval; CN, copy number; MMx, master mix; RPP30, ribonuclease P protein subunit 30; TOI, target of interest.

**Figure 3.19:** The development software Results tab displays the calculated copy numbers and corresponding Poisson CI (copy number 95% CI minimum and copy number 95% CI maximum).

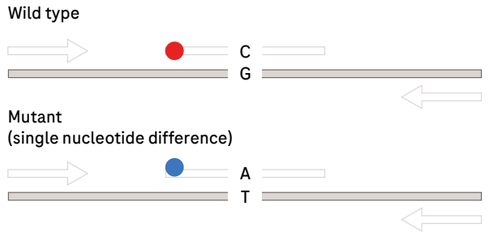
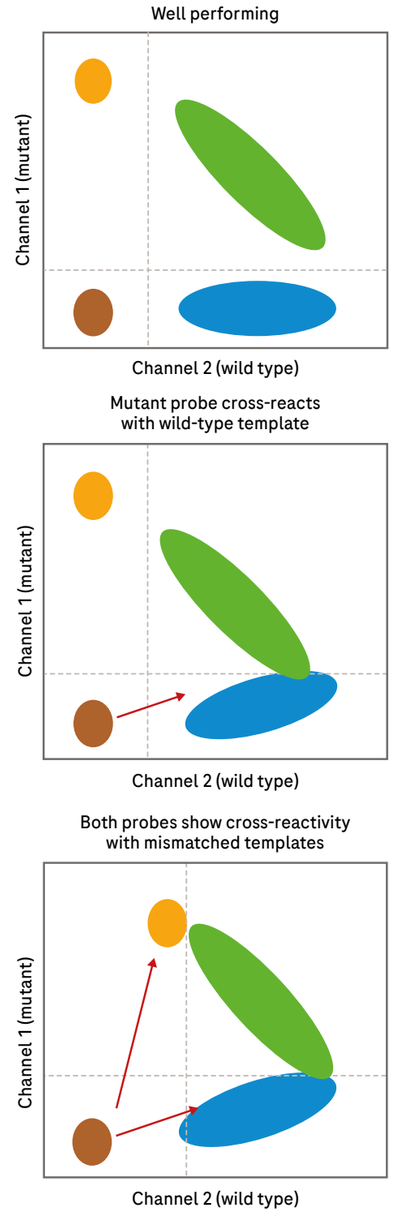



**Figure 3.20:** Schematic representation of a rare mutation assay design. The red and blue dots represent two different fluorophores.

To evaluate the performance of a mutation detection assay, 2D scatter plots are used to visualize cluster distribution. Compared with a non-competing duplex assay, a mutation detection assay has a very distinct cluster pattern in a 2D scatter plot, where the double-positive (mutant) partitions are clustered as an arc, with a lower endpoint fluorescence than the single-positive partitions **(Figures 3.21 and 3.22)**. This pattern is due to the competition for the shared amplification primers, which limits the total number of double-stranded amplicons that can be generated.

In mutation detection assays, a clear separation between the double-positive and single-positive wild-type clusters indicates a successful assay design. Cross-reactivity of the probes may occur if the probe design or amount of input DNA is not optimal. For example, if the mutant probe binds more tightly than the wild-type probe, it will bind to wild-type amplicons and cause increased mutant channel fluorescence in the wild-type-only cluster. On a 2D scatter plot, this is reflected by the non-orthogonal position of the single-positive wild-type cluster resulting in insufficient separation and inability to determine an appropriate threshold. In general, the mutant and wild-type probes should be matched in their footprint, have a similar $T_m$, and have similarly modified bases.




2-D, two-dimensional.

**Figure 3.21:** Schematic representation of 2D scatter plots with the expected cluster positions for a rare mutation assay with different levels of probe cross-reactivity.

The arc-shaped double-positive cluster can change its location depending on the amount of input DNA and the abundance of the mutant target. **Figure 3.22** shows a double-positive cluster (green) moving closer to the single-positive wild-type cluster (blue) as sample input increases from 46 to 457 ng amplifiable gDNA per reaction (0.5 to 5 cpp on Uni plate, fixed mutation allele frequency at 1%). This is due to the increased wild-type-to-mutant ratio in co-occupied partitions suppressing the mutant probe signal. Some non-optimized assays may even lose separation between the two clusters at high amounts of input DNA. Therefore, evaluation of a rare mutation assay at all clinically relevant sample concentrations is recommended during the assay validation experiments.

### Specific considerations for rare mutation setups

#### *False-positive rate*

One important aspect of rare mutation detection is the false-positive rate, which can limit assay sensitivity. False-positive results can be detected using wild-type-only control samples in the clinically relevant specimen matrix at the relevant concentrations.

The sources of false positives are:

- Polymerases, which can make infrequent errors and misincorporate bases during amplification, potentially converting a wild-type to a mutant sequence. If this happens in early cycles, the nanowell will be double positive after amplification and will increase the readout in mutant concentration. Some mutations may have a higher polymerase error rate than others (e.g. C to T, G to A).
- Old or damaged samples. For example, FFPET samples that frequently contain deaminated bases can result in a C to U base change, which can lead to false-positive results in wild-type templates. Since the false-positive rate is closely linked to the total template input (in a non-linear way), it should be evaluated using the most relevant template concentrations and sample matrix.
- Contamination from other samples or controls.

Commonly used controls in the rare mutation detection setup include:

- NTCs, which are used to monitor for contaminants or artifacts related to the workflow, reagents, and the system.
- Samples with wild-type-only templates at the relevant concentration and in the relevant clinical sample matrix (negative control for mutant detection).
- Contrived positive control samples with both mutant and wild-type templates at a fractional abundance that allows representation of all four clusters to guide the selection of thresholds.

Considerations when choosing plates for mutation detection applications:

- If the sample contains moderate-to-high concentrations of DNA (e.g. tissue samples, whole-blood eluates, etc.), the Uni plate is generally recommended.
- If the sample contains relatively low concentrations of DNA (e.g. cfDNA samples) and mutation allele frequency is very low, the HiSens plate is recommended to accommodate high sample volume to capture rare species.
- If the sample is pre-amplified to a very high DNA concentration, but contains very low mutant fractional abundance, the HiRes plate can be considered.

### Application example: A *BRAF*-V600E mutation detection assay

#### *Setup*

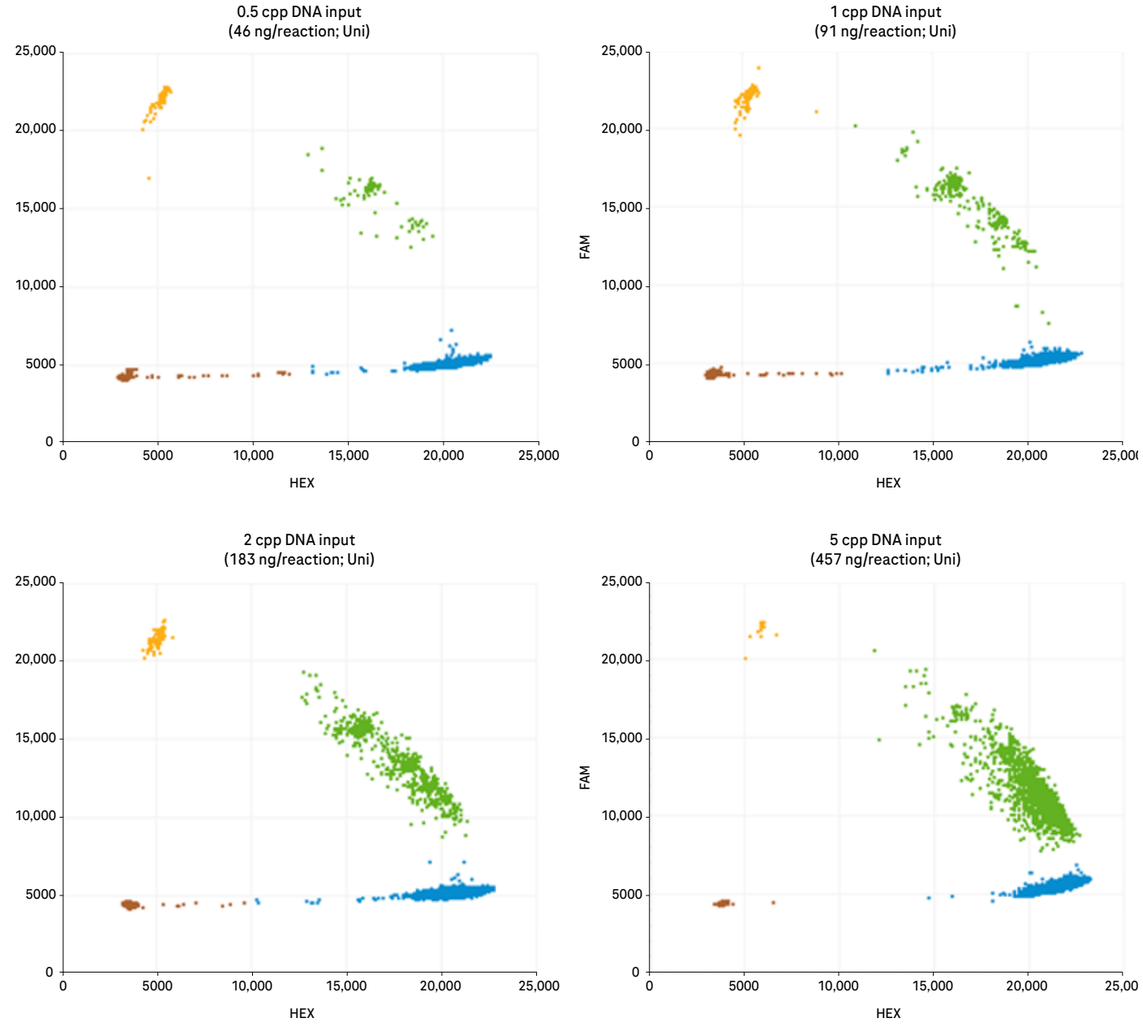
The following samples were run on a Uni plate on the Digital LightCycler® Analyzer using a matching run profile. The mutant probe was FAM labeled and the wild-type probe was HEX labeled. The sample setup for the plate is described below:

**Lanes A, B:**
Positive control (Custom Singleplex gDNA Reference Standard, 1% B-Raf V600E, Catalog ID HD-C2309, Batch ID 50104, 50 ng/µL; Horizon Discovery, Boulder, Colorado)

**Lanes C, D:**
A sample with unknown mutant concentration

**Lanes E, F:**
Negative control (Singleplex gDNA Reference Standard, 100% B-Raf wild type, Catalog ID HD249, Batch ID 42820, 50 ng/µL; Horizon Discovery, Boulder, Colorado)

**Lanes G, H:**
NTC




cpp, copies per partition; FAM, fluorescein amidite; HEX, hexachloro-fluorescein.

**Figure 3.22:** The double-positive cluster (green) position shifts towards the single-positive wild-type cluster (blue) with increasing amounts of DNA input.

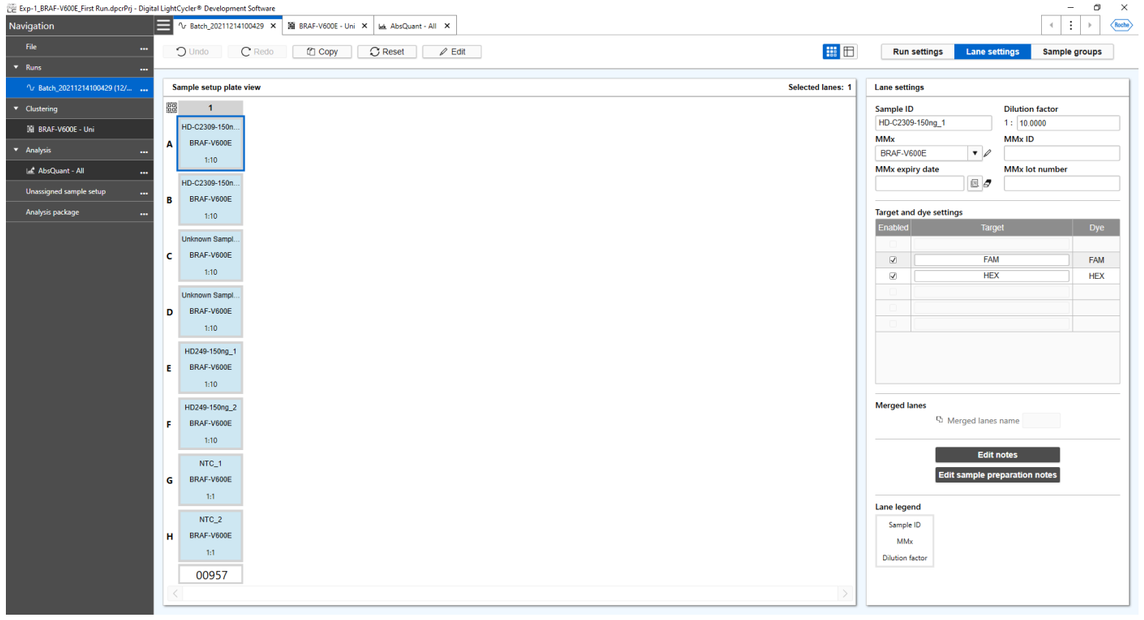
After opening the raw data (.dpcrRaw) in the development software, each reaction was annotated with master mix (MMx) name, sample ID, and dilution factor information **(Figure 3.23)**. Technical replicates were given different sample IDs so that they were analyzed as individual reactions. If the replicates are given the same sample ID, they will be merged and analyzed as one reaction.

### *Data analysis*

Next, 'Automatic – Rare Event Detection' was selected in the clustering settings and cluster classification was examined using 2D scatter plots **(Figure 3.24)**.

After validating correct clustering for all samples, an Abs Quant analysis was created and the control limits were defined in the analysis settings **(Figure 3.25)**:

- **Wild-type-only negative control:** The upper limit was set to 1 copy/µL in the mutant (FAM) channel, to account for the 10-fold dilution factor of 3 µL specimen into a 30 µL total reaction volume, which would correspond to a maximum of three positive nanowells per lane.
- **NTC:** This accounts for any positive nanowells from the entire 30 µL reaction (i.e. specimen and reagents), so there is no 10-fold dilution factor and would therefore still correspond to a maximum of three positive nanowells per lane.
- **Positive control:** The lower and upper limits for the FAM (mutant) channel were set to 75 and 225 mutant copies/µL and to 12,000–18,000 copies/µL for the HEX (wild-type) channel.
- **Optional:** If previously established, users can choose to input the lower limit of detection (LLoD) in the 'Target limits' field, to help define a positive vs negative result (user defined: for example, 0.5 copies/µL).




FAM, fluorescein amidite; HEX, hexachloro-fluorescein; MMx, master mix; NTC, no template control.

**Figure 3.23:** Annotating the lane settings in the development software.

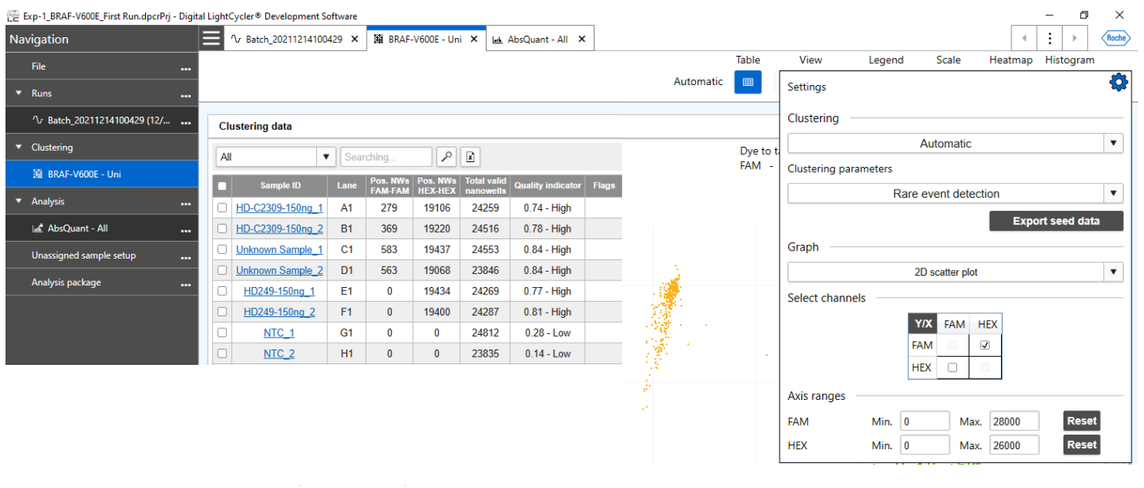
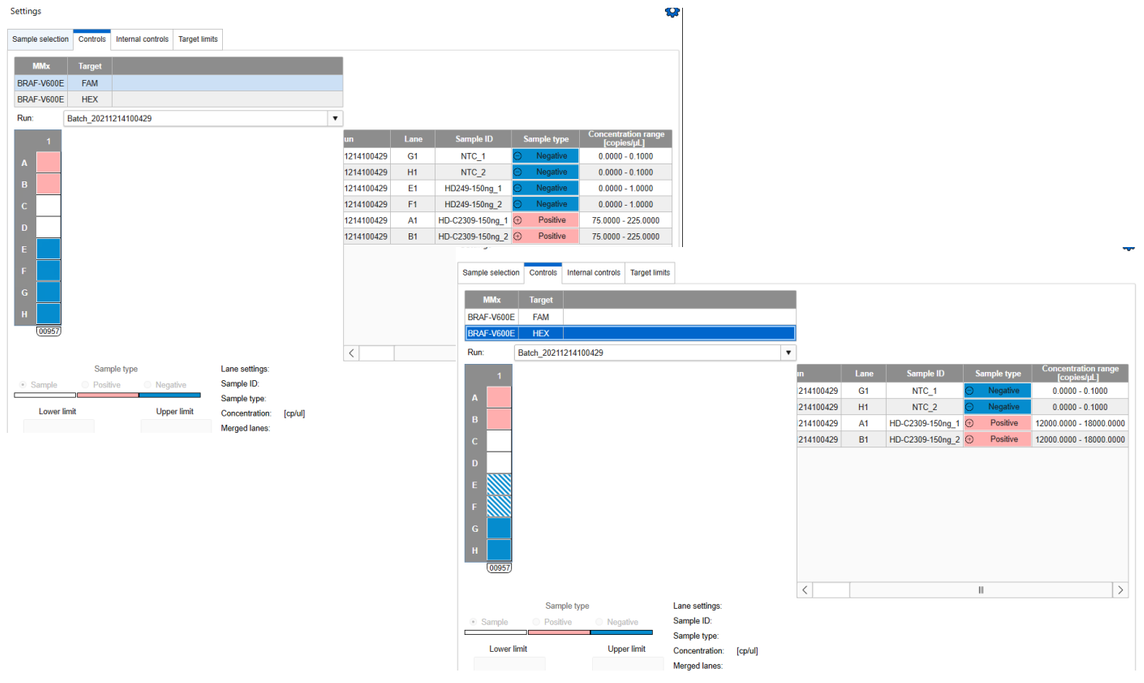



**Figure 3.24:** Selecting the clustering settings for rare event detection in the development software.




FAM, fluorescein amidite; HEX, hexachloro-fluorescein; MMx, master mix; NTC, no template control.

**Figure 3.25:** Definition of the control limits for the mutant (FAM) and wild-type (HEX) channels in the development software 'Analysis settings' panel.

| Sample ID | Target | MMx | Lane | Dilution factor | Concentration [copies/µL] | Final result | Conc. 95% CI min. | Conc. 95% CI max. | Flags | Sample type | Total copies | Copies per nanowell |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| HD-C2309-150ng_1 | FAM | BRAF-V600E | A1 | 10 | 106.9088 | Valid | 95.0721 | 120.2192 | | ⊕ | 320.7264 | 0.0116 |
| HD-C2309-150ng_2 | FAM | BRAF-V600E | B1 | 10 | 140.1647 | Valid | 126.5687 | 155.2211 | | ⊕ | 420.494 | 0.0152 |
| Unknown Sample_1 | FAM | BRAF-V600E | C1 | 10 | 222.0979 | Positive | 204.7814 | 240.8788 | | | 666.2938 | 0.0240 |
| Unknown Sample_2 | FAM | BRAF-V600E | D1 | 10 | 220.8226 | Positive | 203.3147 | 239.8382 | | | 662.4679 | 0.0239 |
| HD249-150ng_1 | FAM | BRAF-V600E | E1 | 10 | 0.0000 | Valid | N/A | N/A | | ⊖ | 0 | 0.0000 |
| HD249-150ng_2 | FAM | BRAF-V600E | F1 | 10 | 0.0000 | Valid | N/A | N/A | | ⊖ | 0 | 0.0000 |
| NTC_1 | FAM | BRAF-V600E | G1 | 1 | 0.0000 | Valid | N/A | N/A | | ⊖ | 0 | 0.0000 |
| NTC_2 | FAM | BRAF-V600E | H1 | 1 | 0.0000 | Valid | N/A | N/A | | ⊖ | 0 | 0.0000 |
| HD-C2309-150ng_1 | HEX | BRAF-V600E | A1 | 10 | 14318.0094 | Valid | 14095.8077 | 14543.7137 | | ⊕ | 42954.0281 | 1.5492 |
| HD-C2309-150ng_2 | HEX | BRAF-V600E | B1 | 10 | 14162.4228 | Valid | 13943.7353 | 14384.5401 | | ⊕ | 42487.2684 | 1.5324 |
| Unknown Sample_1 | HEX | BRAF-V600E | C1 | 10 | 14495.9442 | Positive | 14272.3570 | 14723.0340 | | | 43487.8326 | 1.5685 |
| Unknown Sample_2 | HEX | BRAF-V600E | D1 | 10 | 14857.6196 | Positive | 14625.1200 | 15093.8153 | | | 44572.8588 | 1.6076 |
| HD249-150ng_1 | HEX | BRAF-V600E | E1 | 10 | 14910.5239 | Positive | 14679.2178 | 15145.4748 | | | 44731.5717 | 1.6133 |
| HD249-150ng_2 | HEX | BRAF-V600E | F1 | 10 | 14818.5084 | Positive | 14588.7220 | 15051.9142 | | | 44455.5252 | 1.6034 |
| NTC_1 | HEX | BRAF-V600E | G1 | 1 | 0.0000 | Valid | N/A | N/A | | ⊖ | 0 | 0.0000 |
| NTC_2 | HEX | BRAF-V600E | H1 | 1 | 0.0000 | Valid | N/A | N/A | | ⊖ | 0 | 0.0000 |

CI, confidence interval; conc, concentration; FAM, fluorescein amidite; HEX, hexachloro-fluorescein; NTC, no template control; MMx, master mix.

**Figure 3.26:** Results tab displaying mutant and wild-type concentrations with 95% CI.

In the results tab, mutant and wild-type concentrations with 95% CI limits are reported for each reaction **(Figure 3.26)**. Users can customize the report with additional information in 'Configure columns' (e.g. total copies, copies per nanowell) and export results in csv.

The mutant fractional abundance for each sample was calculated manually with the reported concentrations according to the following formula:

$$\textit{mutant fractional abundance (\%)} = 100 \times \frac{\textit{mutant concentration}}{\textit{mutant concentration + wild-type concentration}}$$

### Example of limit of detection (LoD) determination for the *BRAF*-V600E assay on Uni plates according to CLSI EP17-A2 guidelines[8]

*Experimental setup*

- 60 negative control replicates (wild-type gDNA, ~183 ng per reaction).
- Contrived sample panel, each panel member tested in 24 replicates **(Table 3.6)**.

| Sample | Expected mutant copies per reaction | Expected wild-type copies per reaction | Expected mutant fractional abundance (%) |
|---|---|---|---|
| 1 | 25 | 55,450 | 0.045 |
| 2 | 20 | 55,450 | 0.036 |
| 3 | 15 | 55,450 | 0.027 |
| 4 | 10 | 55,450 | 0.018 |
| 5 | 5 | 55,450 | 0.009 |

**Table 3.6:** Contrived sample panel.

*Data analysis*

**Cut-off setting:** Based on the measured copy numbers for the 60 negative controls in the mutant (FAM) channel, the cut-off value was set to eight copies per lane using the Poisson distribution described in Section 2 and based on the worst-case replicate (3.38 copies) and a target specificity of 95%.

**Probit analysis:** The measured copy number per reaction for each replicate of the contrived samples was compared to the cut-off value and determined as 'positive' if the measured value was higher than the cut-off.

The hit rate for each sample **(Table 3.7)** was calculated according to the following formula:

$$\textit{\% Hit rate} = 100 \times \frac{\textit{number of positive replicates}}{\textit{total number of replicates}}$$

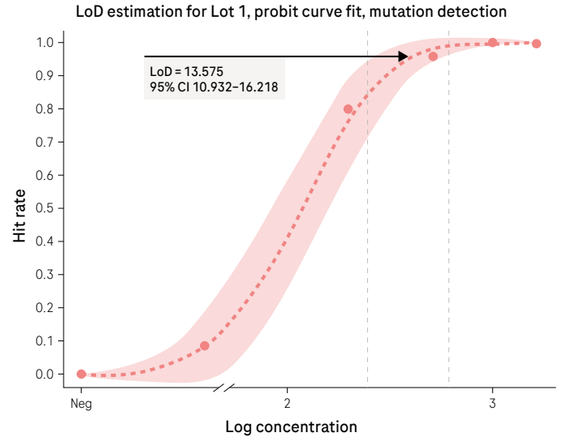
The resulting probit curve fit **(Figure 3.27)** predicted a LoD of 13.6 copies per reaction (95% CI 10.9–16.2), without any evidence for a lack of fit. The observed average wild-type concentration was 51,463 copies per reaction.

| Concentration (number of mutant copies per reaction) | Number of positive replicates | Total number of replicates | Hit rate (%) |
|---|---|---|---|
| 25 | 24 | 24 | 100.00 |
| 20 | 24 | 24 | 100.00 |
| 15 | 23 | 24 | 95.83 |
| 10 | 19 | 24 | 79.17 |
| 5 | 2 | 24 | 8.33 |
| 0 | 0 | 60 | 0.00 |

**Table 3.7:** Calculated hit rates for the six samples.

[8]Clinical and Laboratory Standards Institute (CLSI). Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures. 2nd edition. CLSI document EP17-A2 (ISBN 1-56238-795-2 [Print]; ISBN 1-56238-796-0 [Electronic]). Wayne, PA 19087, USA: Clinical and Laboratory Standards Institute; 2012.






CI, confidence interval; LoD, limit of detection.

**Figure 3.27:** Probit curve fit for the determination of LoD.

The LoD for **mutant fractional abundance** was calculated as:

$$\textit{LoD mutant fractional abundance} = 100 \times \frac{\textit{LoD (copies per reaction)}}{\textit{average wild type (copies per reaction)}}$$

$$= 100 \times \frac{13.6}{51{,}463} = 0.026\%$$

## 3.7 Insertion–deletion (Indel) assays

### Assay principle

To detect small insertions or deletions, two design strategies are commonly used:

1. If the variant sequence is known, a strategy equivalent to the rare mutation detection scheme described in Section 3.6 can be used. A pair of primers amplifies the sequence region of interest, and a pair of differently labeled probes is used to discriminate between wild-type and variant targets.

2. If there are multiple variants in the same region that need to be detected without the need to distinguish between them, a variant probe and a reference probe are used in combination to detect the presence of the variant sequences.[9] The reference probe is designed to match a conserved sequence within the amplicon and will detect all amplicons. The variant probe, however, is designed to only match the wild-type sequence at the positions where deletions or insertions may occur. Thus, the loss of variant probe signal indicates the presence of variant(s) **(Figure 3.28)**.

[9]Yung TKF, et al. Clin Cancer Res 2009;15:2076–2084. doi: 10.1158/1078-0432.CCR-08-2622.

The assay is analyzed using a 2D scatter plot, and is expected to show three clusters for a variant positive sample **(Figure 3.28)**:

- A double-negative cluster: Nanowells without either template (brown cluster);
- A single-positive cluster (reference probe fluorescence only): Nanowells containing only the variant (blue cluster);
- A double-positive cluster: Nanowells containing wild-type only and both wild type and variant (green cluster).

The calculation of variant copy number is based on the variant-only nanowells (single-positive cluster) without specifically counting the wild type and variant co-occupied nanowells, and a statistical correction for variant target quantification. The Digital LightCycler® Development Software Indel Analysis Module performs this calculation automatically after the clustering is defined. The variant concentration (copies/µL), wild-type concentration (copies/µL), and variant allele frequency (%) are reported.

### Specific considerations for Indel setups

*DNA input considerations*

Similar to most mutation detection assays, Indel assays often target variants that are present at very low frequencies.

While increasing the template concentration usually improves the sensitivity for detection of rare variants, this does not apply to Indel assays that rely on variant-only, single-positive partitions for quantification. Increased template concentrations lead to higher wild-type backgrounds, an increased fraction of co-occupied partitions, and a reduced variant-only fraction.

The relationship between the number of variant-only partitions

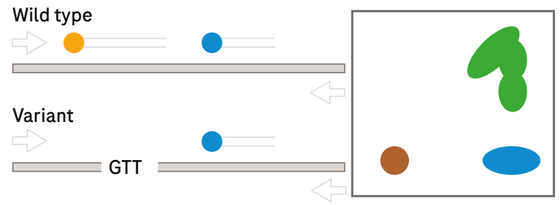



**Figure 3.28:** Insertion–deletion (Indel) assay design principle (left) and cluster pattern expected in 2D scatter plot (right). Blue and orange dots represent different fluorophores.

and the wild-type DNA concentration is shown in **Figure 3.29** overleaf. (see page 29). The maximum number of variant-only partitions is observed with wild-type DNA concentrations around 1 cpp (vertical green dashed line), which corresponds to 92 ng, 66 ng, and 330 ng of amplifiable human gDNA per reactions on Uni, HiSens, and HiRes plates, respectively.

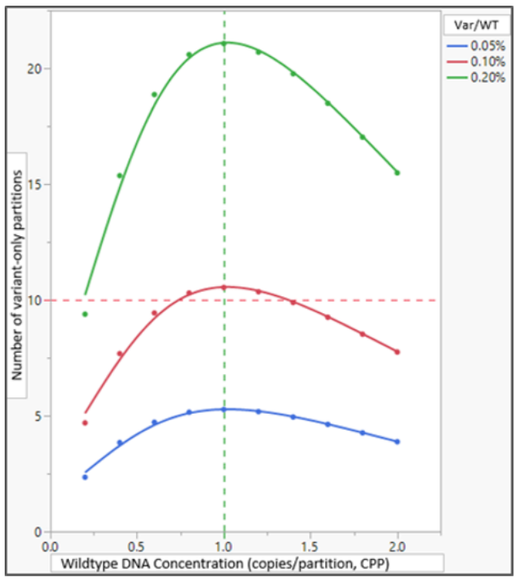
Using an example of 10 variant-only partitions as a realistic cut-off for definition of a positive result, there is only a very narrow range of wild-type DNA input (0.8–1.2 cpp) that allows reliable detection of variant positive samples at the 0.1% variant allele frequency level (red curve). It is therefore recommended to keep the wild-type DNA concentrations close to amounts that correspond to 1 cpp for all variant allele frequencies to optimize the number of variant-only partitions for sensitive and precise quantification.

### *Unexpected single-positive partitions*

In some cases, nanowells that are singly positive for the variant probe may appear in the 2D scatter plots. Theoretically this is not expected because the reference probe should always bind and generate fluorescence. However, this can occur in the following scenarios:

- The presence of polymorphic sequence variation in the reference probe binding site. In this case, alternative reference probe designs, or the usage of multiple reference probes, should be considered to ensure inclusivity.

- Clinical samples may have fragmented or damaged DNA sequences. For example, old FFPET eluates may show a few single-positive nanowells in both channels even for wild-type-only samples. In this case, the false positives in the variant probe channel can be ignored since they do not affect the quantification. The false positives in the reference probe channel should be evaluated with respect to the thresholds used to define positive and negative nanowells, to ensure accuracy of true positive calls.




CPP, copies per partition; Var, variant; WT, wild type.

**Figure 3.29:** Dependence of the number of variant-only partitions on the input DNA target concentrations for different levels of variant allele frequencies.

## Analyzing Indel assays

### *Controls*

- **NTCs:** Generally used to monitor for any contamination in the consumables, reagent, instrument, or workflows.
- **Wild-type-only negative control:** Wild-type-only samples at the relevant concentration and ideally in the relevant clinical specimen matrix.
- **Positive controls:** Contrived (spiked) samples containing wild-type and variant templates at defined fractional abundances that represent all three expected clusters in order to guide threshold setting, at the relevant concentrations and in the relevant clinical specimen matrix.

### *Clustering*

Indel assay clustering should be performed on 2D scatter plots in the development software. ‘Manual’ clustering using the ‘Lasso’ option is recommended for optimal results **(Figure 3.30)**. Depending on the assay, manual clustering using the ‘Threshold’ or ‘Spider’ options may also work well.

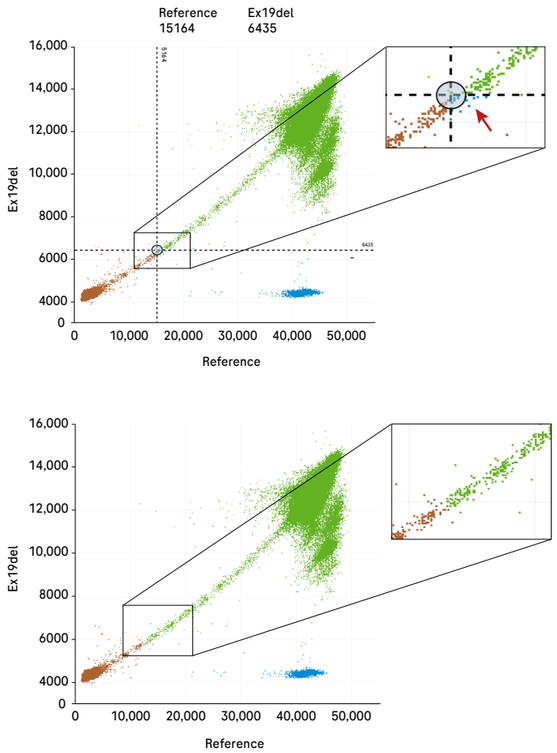
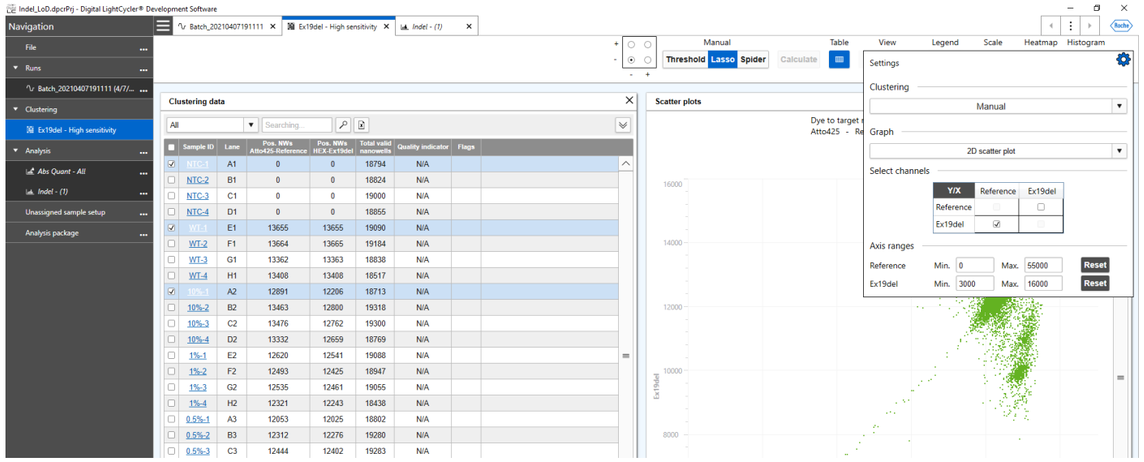



**Figure 3.31:** Classification of nanowells on the diagonal axis between the negative and double-positive clusters as belonging to the variant-only, single-positive cluster (in this example, the blue color in the lower panel) should be avoided or minimized.

In this context, it is important to ensure that nanowells on the diagonal axis between the negative and double-positive clusters are classified as double-negative or double-positive **(Figure 3.31)**. Misclassification of these nanowells as part of the variant-only, single-positive cluster affects the accurate quantification of the variant-only fraction.

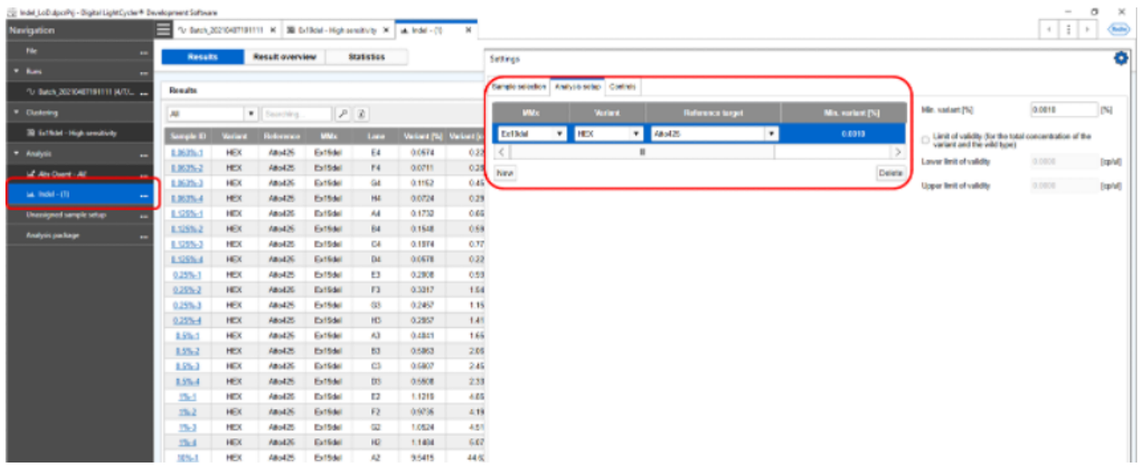
## Analysis settings

After creation of an ‘Indel’ analysis, under ‘Analysis settings’ in the ‘Analysis setup’ tab, assign the ‘variant’ to the variant probe channel and the ‘reference target’ to the reference probe channel. Set the minimum variant (%) value to ‘0’ or any predetermined LoD value from previous assay validation studies **(Figure 3.32)**.




Indel, Insertion–deletion; NTC, no template control; NW, nanowell; pos, positive; WT, wild type.

**Figure 3.30:** Using ‘Manual’ 2D clustering with the ‘Lasso’ thresholding option for optimal Indel results.




Indel, Insertion–deletion; NTC, no template control; WT, wild type.

**Figure 3.32:** Assigning the channels for the variant and the reference targets in the Indel analysis settings of the development software.

**Application example: LoD determination for an *epidermal growth factor receptor (EGFR)* exon19 deletion assay on HiSens Plates according to CLSI EP17-A2 guidelines[8]**

*Setup*

- 60 negative control replicates (wild-type gDNA, ~64 ng per reaction)
- Contrived sample panel, each panel member tested in 20 replicates

*Data analysis*

**Cut-off setting:** Based on the measured copy numbers for the 60 negative controls, the cut-off value was set to 16 copies per lane using the Poisson distribution described in Section 2 and based on the worst-case replicate (6.4 copies) and a target specificity of 95%.

**Probit analysis:** The measured copy number per reaction for each replicate of the contrived samples **(Table 3.8)** was compared to the cut-off value and determined as 'positive' if the measured value was higher than the cut-off **(Table 3.9)**. The hit rate for each sample was calculated according to the following formula:

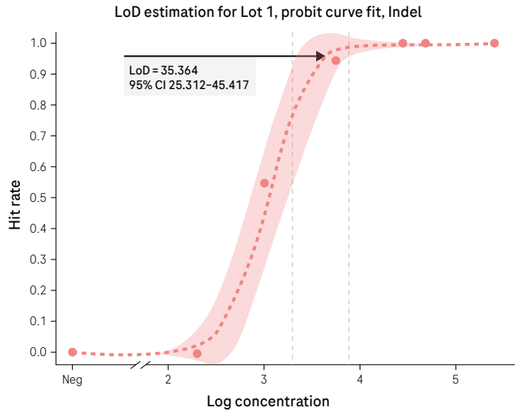
$$\%\ Hit\ rate = 100 \times \frac{number\ of\ positive\ replicates}{total\ number\ of\ replicates}$$

The resulting probit curve fit **(Figure 3.33)** predicted an LoD of 35.4 copies per reaction (95% CI 25.3–45.4), without any evidence for a lack of fit. The observed average wild-type concentration was 21,087 copies per reaction.

The LoD for **mutant fractional abundance** was calculated as:

$$LoD\ mutant\ fractional\ abundance = 100 \times \frac{LoD\ (copies\ per\ reaction)}{average\ wild\ type\ (copies\ per\ reaction)}$$

$$= 100 \times \frac{35.4}{21{,}087} = 0.168\%$$




CI, confidence interval; LoD, limit of detection.

**Figure 3.33:** Probit curve fit for the LoD setup.

| Sample | Expected variant copies per reaction | Expected wild-type copies per reaction | Expected variant fractional abundance (%) |
|---|---|---|---|
| 1 | 195 | 19,497 | 0.99 |
| 2 | 97.5 | 19,497 | 0.50 |
| 3 | 78 | 19,497 | 0.40 |
| 4 | 39 | 19,497 | 0.20 |
| 5 | 19.5 | 19,497 | 0.10 |
| 6 | 9.7 | 19,497 | 0.05 |

**Table 3.8:** Contrived sample panel.

| Concentration | Number of positive replicates | Total number of replicates | Hit rate (%) |
|---|---|---|---|
| 195.00 | 20 | 20 | 100.00 |
| 97.50 | 20 | 20 | 100.00 |
| 78.00 | 20 | 20 | 100.00 |
| 39.00 | 19 | 20 | 95.00 |
| 19.50 | 11 | 20 | 55.00 |
| 9.70 | 0 | 20 | 0.00 |
| 0.00 | 0 | 60 | 0.00 |

**Table 3.9:** Calculated hit rates for the six samples and the negative control.

## 3.8 Gene expression and RNA assays

### Assay principle

For gene expression analysis, RT-PCR assays have proven invaluable. RNA is first converted to cDNA during the reverse transcription step. The cDNA is then used as the template during the PCR step. During the PCR step, the assay-specific primers and probe amplifies the target of interest for easy detection.

The Digital LightCycler® 5× RNA Master allows rapid, easy, and sensitive one-step RT-PCR detection of RNA without the need for a buffer change or addition of any other reagents. It features a hot-start and uses a blend of thermostable enzymes for both the reverse transcription and PCR steps. The use of a thermostable/thermoactive DNA polymerase for reverse transcription allows for increased specificity of primer binding and alleviates potential interference by secondary structures in the RNA template, thus decreasing the chance of premature termination by the reverse transcriptase. AmpErase enzyme and deoxyuridine triphosphate (dUTP) are included in the Digital LightCycler® 5× RNA Master to prevent PCR carryover contamination. The Digital LightCycler® 5× RNA Master was developed and optimized for multiplex performance for up to six assays at once.

Verifying accuracy of results using dPCR for RNA expression is challenging. Reverse transcription efficiencies are highly dependent on the reverse transcription enzyme used, the specific buffer conditions, and the secondary structures formed by a given RNA template, all of which can vary across dPCR platforms. In addition, the lack of any commercially available dPCR RNA standards makes comparisons between platforms challenging. It is therefore recommended to use relative quantification through normalization by using reference(s) (e.g. housekeeping genes) for RNA applications.

*RNA diluent*

For diluting RNA, an RNase/DNase-free buffer, such as TE (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA), is recommended.

*Carrier RNA*

For samples with low concentrations or short fragments, use of carrier nucleic acid (e.g. poly-rA) in the sample buffer is recommended to reduce nucleic acid loss due to binding to the sample tubes. If the sample buffer does not contain a carrier nucleic acid, it may be added to prevent loss of RNA during preparation and storage.

### Assay design

Assay designs should follow guidelines outlined in Section 3.3, in addition to the following:

- If possible, primers should span intron/exon boundaries to amplify exclusively from cDNA transcribed from RNA and not from any contaminating gDNA.
- Oligo(dT)N, anchored oligo(dT)N, and random hexamers cannot be used directly with the Digital LightCycler® 5× RNA Master because they do not hybridize well at the elevated temperatures required by the thermostable DNA polymerase used for the reverse transcription step. If these oligo types are required for the application, a two-step RT-PCR design must be used. A cDNA synthesis kit will be required to generate the cDNA, and the Digital LightCycler® DNA Master is used for the dPCR step.

*Considerations specific to RNA setups*

- When extracting RNA using commercial kits, DNAse treatment during the purification is essential to ensure any positive results are from RNA only.
- The inclusion of carrier RNA, such as poly(A), helps prevent binding to tubes, especially at low concentrations of RNA.

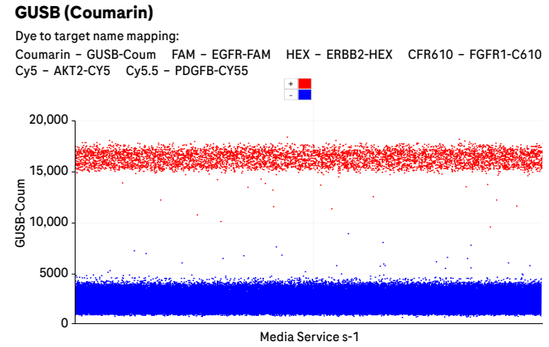
### Analyzing RNA setups

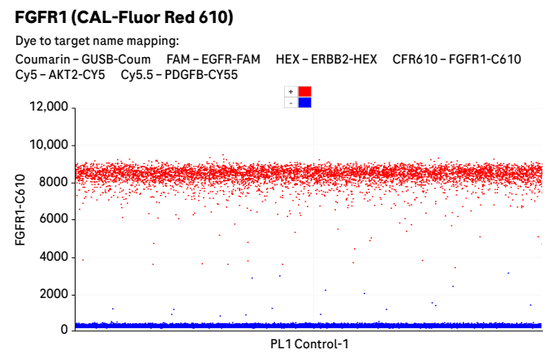
There are no specific suggestions or requirements with respect to analyzing RNA data in the development software. RNA applications usually require absolute quantification analysis; refer to Section 3.4 for details.

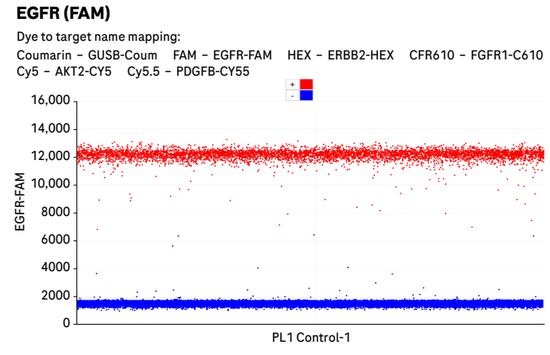
The development software for CNV analysis can also be used for general relative quantification applications.

*Example data*

An example of results from an RNA six-channel multiplex assay performed on the Digital LightCycler® System is shown in **Figure 3.34A ,** and **Figure 3.34B** overleaf.



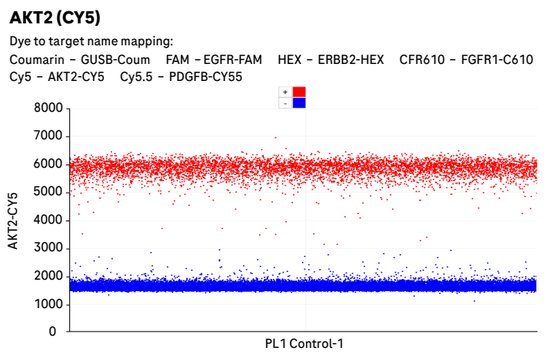




AKT2, serine/threonine kinase 2; FAM, fluorescein amidite; EGFR, epidermal growth factor receptor; ERBB2, Erb-B2 receptor tyrosine kinase 2; FGFR1, fibroblast growth factor receptor 1; GUSB, β-glucuronidase; HEX, hexachloro-fluorescein; PDGFB, platelet-derived growth factor subunit B.

**Figure 3.34A:** 1D scatter plots of the six RNA assays in each channel on the Digital LightCycler® System.

[8]Clinical and Laboratory Standards Institute (CLSI). Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures. 2nd edition. CLSI document EP17-A2 (ISBN 1-56238-795-2 [Print]; ISBN 1-56238-796-0 [Electronic]). Wayne, PA 19087, USA: Clinical and Laboratory Standards Institute; 2012.




AKT2, serine/threonine kinase 2; FAM, fluorescein amidite; EGFR, epidermal growth factor receptor; ERBB2, Erb-B2 receptor tyrosine kinase 2; FGFR1, fibroblast growth factor receptor 1; GUSB, β-glucuronidase; HEX, hexachloro-fluorescein; PDGFB, platelet-derived growth factor subunit B.

**Figure 3.34B:** 1D scatter plots of the six RNA assays in each channel on the Digital LightCycler® System.

## 3.9 Multiplexing

### Assay principle

The majority of dPCR users are still using platforms with two optical channels and rely on complicated methods (e.g. titrating and mixing differently labeled probes) for multiplexing. These methods require significant assay design expertise, time-consuming optimization processes, manual clustering by experienced users, and sometimes generate results that are difficult to interpret. Multichannel systems greatly simplify the way multiplex assays are performed, by assigning each target to its own optical channel(s).

The benefits of multiplexing include:

- **Testing for multiple targets of interest in one reaction.** For example, a hotspot panel with *EGFR* point mutations, deletions, and insertions; multiple break/fusion points in breakpoint cluster region-tyrosine-protein kinase ABL1 (BCR-ABL); multiple informative single nucleotide polymorphisms (SNPs) in transplant rejection monitoring.
- **Optional use of additional control(s) or references.** For example, multiple housekeeping assays in expression applications; multiple reference assays in CNV.
- **Reduced sample volume requirements.** For example, for low concentration samples with limited specimen volume that otherwise would have to be split into multiple reactions, i.e. cfDNA samples for rare mutation detection.
- No pipetting variations between the otherwise required multiple reaction mixtures.
- Shortened turnaround times.
- Lower costs.

In principle, there are two different multiplex assay formats:

1. **Non-discriminating multiplex:** Detects several targets without discriminating one from another. This type of multiplex design is commonly used for biomarkers with the same clinical implications and/or if the biomarkers are mutually exclusive. This multiplex can be further specified based on the probe design:

   a) Multiple targets detected using one probe: e.g. the *EGFR* exon 19 deletion assay can use one variant-specific probe for detection of multiple deletions located in the same region.

   b) Targets are detected by individual probes, but all probes are labeled with the same reporter fluorophore: e.g. Kirsten rat sarcoma virus (KRAS) G12X assay, where multiple probes specific for individual loci are labeled with the same dye.

2. **Discriminating multiplex:** Detects and discriminates between multiple targets. Depending on the targets and applications, a discriminating multiplex assay can be assembled using the same type of designs and using similar clustering methods for data analysis (e.g. gene expression and CNV), but it can also incorporate different types of designs and requires separate clustering thresholds for data analysis (e.g. detection of single-nucleotide mutations and deletions in the same reaction).

### Specific considerations for multiplexing assays

#### *Supported dyes*

The Digital LightCycler® System supports discriminative multiplexing for up to six targets/channels. Each target is assigned to an optical channel. See **Table 3.2** (on page 11) for a list of supported dyes for each channel. Multiplexing is limited to these dyes because the implemented, automated color crosstalk correction algorithm is specifically designed to work for these dyes only.

Currently, the Digital LightCycler® software does not support data analysis for radius multiplexing (probe mixing) or amplitude multiplexing (probe titration).

#### *Compatibility of the assays*

a) **Minimize potential oligo interactions:** Oligo-related interactions that may lead to non-specific amplification, primer dimer formation, and probe signal suppression should be examined *in silico* before multiplexing.

b) **Assay efficiency:** Non-matching $T_m$(s) and bigger differencesin amplicon lengths can result in suppression of weaker assays. Therefore, assays should be designed for identical or similar efficiency-related parameters.

#### *Accurate quantification*

If assays in a multiplex do not interact with each other, quantification results should be similar when comparing single plex vs multiplex setups.

#### *Template input*

In multiplex CNV and gene expression multiplex applications, some variants may be present at an amplified copy number or expressed at a much higher level than other targets or the reference targets. In this case, preliminary experiments with representative samples may be required to guide optimal input DNA or RNA concentrations and keep measured copy numbers within the dynamic range of the system.

#### *False positives in non-discriminating mutation detection*

For non-discriminating mutation multiplexing using several probes labeled with the same dye, the false-positive rate is usually higher than for an individual 'singleplex' and may approach that of the sum of all singleplexes. In this case, the analytical sensitivity of the multiplex assay can be different or much worse than for the corresponding singleplex assays.

#### *Increased baseline and low fluorescence in non-discriminating mutation detection*

For non-discriminating mutation multiplex assays using several probes labeled with the same dye, the baseline fluorescence of nanowells in the negative partition is usually higher and may even be the sum of the baseline intensities of the individual mutation assays. However, the fluorescence signal of nanowells in the positive partitions remains similar to that of nanowells in the individual mutation assays due to the 'mutual exclusivity' of the mutations. In these cases, additional optimization of probe concentrations may be required.

### Multiplex example data 1: Six-plex copy number variation assay

**Figure 3.35** (overleaf) shows an example of a well-optimized discriminating multiplex for CNV (please see also **Table 3.10**). It is a six-plex assay with two references and four gene targets that are frequently amplified in cancer, each in a distinct optical channel. Individual assay primer and probes were designed using similar parameters and showed no interactions from each other, as demonstrated in 1D, 2D and three-dimensional (3D) fluorescence plots.

### Multiplex example data 2: *EGFR* multiplex combining mutation and Indel detection

**Figure 3.36** (on page 42) shows another example of a well-optimized discriminating multiplex, this time for multiplexed *EGFR* targets (please see also **Table 3.11**). In this six-plex assay, individual assay primer and probes showed no interactions with each other, as demonstrated in 1D and 2D fluorescence plots.

| Optical channel | Dye | Gene of interest | Alteration in cancer |
|---|---|---|---|
| 1 | Atto425 | *HOGA1* | Reference |
| 2 | FAM | *MET* | Amplification |
| 3 | HEX | *B2M* | Reference |
| 4 | Texas Red | *FGFR1* | Amplification |
| 5 | Cy5 | *FGFR2* | Amplification |
| 6 | Cy5.5 | *EGFR* | Amplification |

B2M, β2-microglobulin; EGFR, epidermal growth factor receptor; FAM, fluorescein amidite; FGFR1/2, fibroblast growth factor receptor 1/2; HEX, hexachloro-fluorescein; HOGA1, 4-hydroxy-2-oxoglutarate aldolase 1; MET, mesenchymal-epithelial transition.

**Table 3.10:** Channel assignment for targets of interest and reference targets of the example six-plex assay.

| Optical channel | Dye | Exon | Mutation/indel |
|---|---|---|---|
| 1 | Atto425 | Exon18 | G719S; G719A; G719C* |
| 2 | FAM | Exon20 | C797S (G>C; T>A) |
| 3 | HEX | Exon19 | Exon19 deletion (27**) |
| 4 | Texas Red | Exon20 | T790M |
| 5 | Cy5 | Exon21 | L858R (T>G; TG>GT) |
| 6 | Cy5.5 | Exon19 | Exon19 reference |

FAM and HEX definitions: FAM, fluorescein amidite; HEX, hexachloro-fluorescein.
* Three G719 mutations are detected without separate identification from one another.
** A total of 27 Exon19 deletions (deletion from position 2233 to 2253) are covered by this assay design, without separate identification from one another.

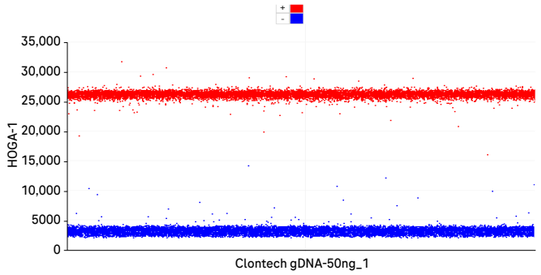
**Table 3.11:** Channel assignment for mutation and indel targets of interest and reference of the example EGFR multiplex.

A

**Atto425 - HOGA1**

Dye to target name mapping:

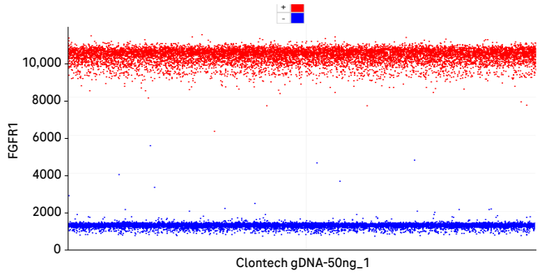
Atto425 - HOGA-1 FAM - MET HEX - B2M TexRed - FGFR1 Cy5 - FGFR2 Cy5.5 - EGFR




**Texas Red - FGFR1**

Dye to target name mapping:

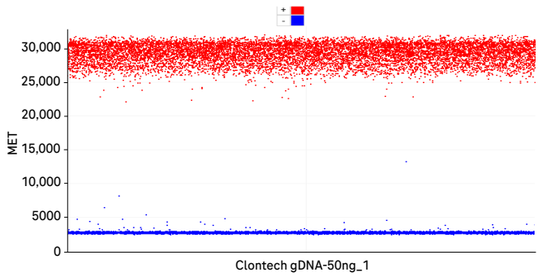
Atto425 - HOGA-1 FAM - MET HEX - B2M TexRed - FGFR1 Cy5 - FGFR2 Cy5.5 - EGFR




**FAM - MET**

Dye to target name mapping:

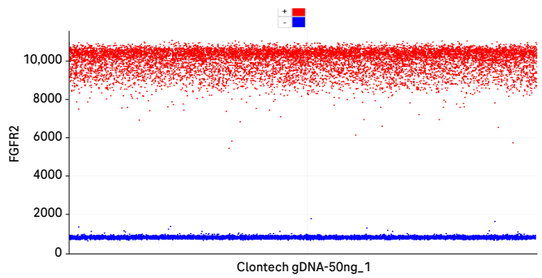
Atto425 - HOGA-1 FAM - MET HEX - B2M TexRed - FGFR1 Cy5 - FGFR2 Cy5.5 - EGFR




**Cy5 - FGFR2**

Dye to target name mapping:

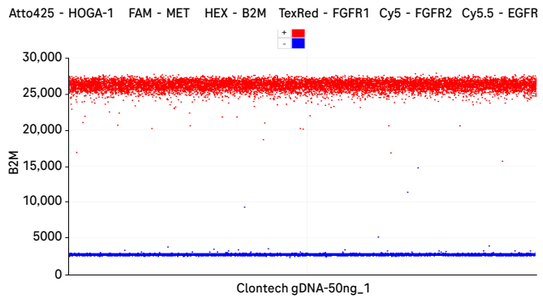
Atto425 - HOGA-1 FAM - MET HEX - B2M TexRed - FGFR1 Cy5 - FGFR2 Cy5.5 - EGFR




**HEX - B2M**

Dye to target name mapping:

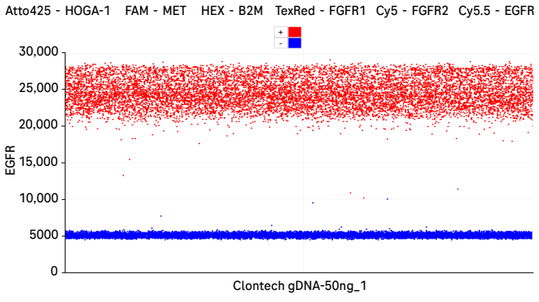
Atto425 - HOGA-1 FAM - MET HEX - B2M TexRed - FGFR1 Cy5 - FGFR2 Cy5.5 - EGFR



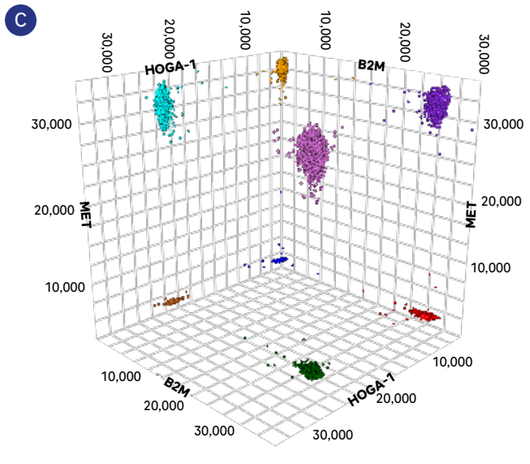

**Cy5.5 - EGFR**

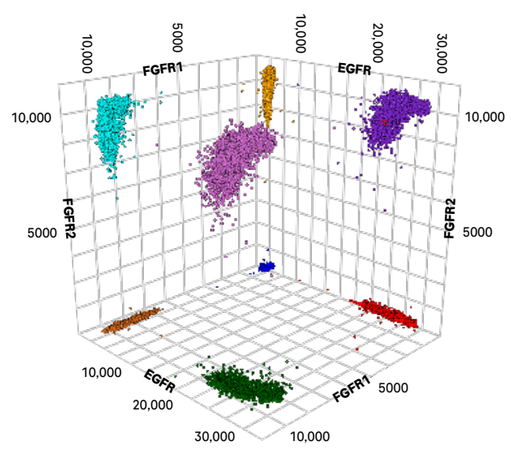
Dye to target name mapping:

Atto425 - HOGA-1 FAM - MET HEX - B2M TexRed - FGFR1 Cy5 - FGFR2 Cy5.5 - EGFR




C

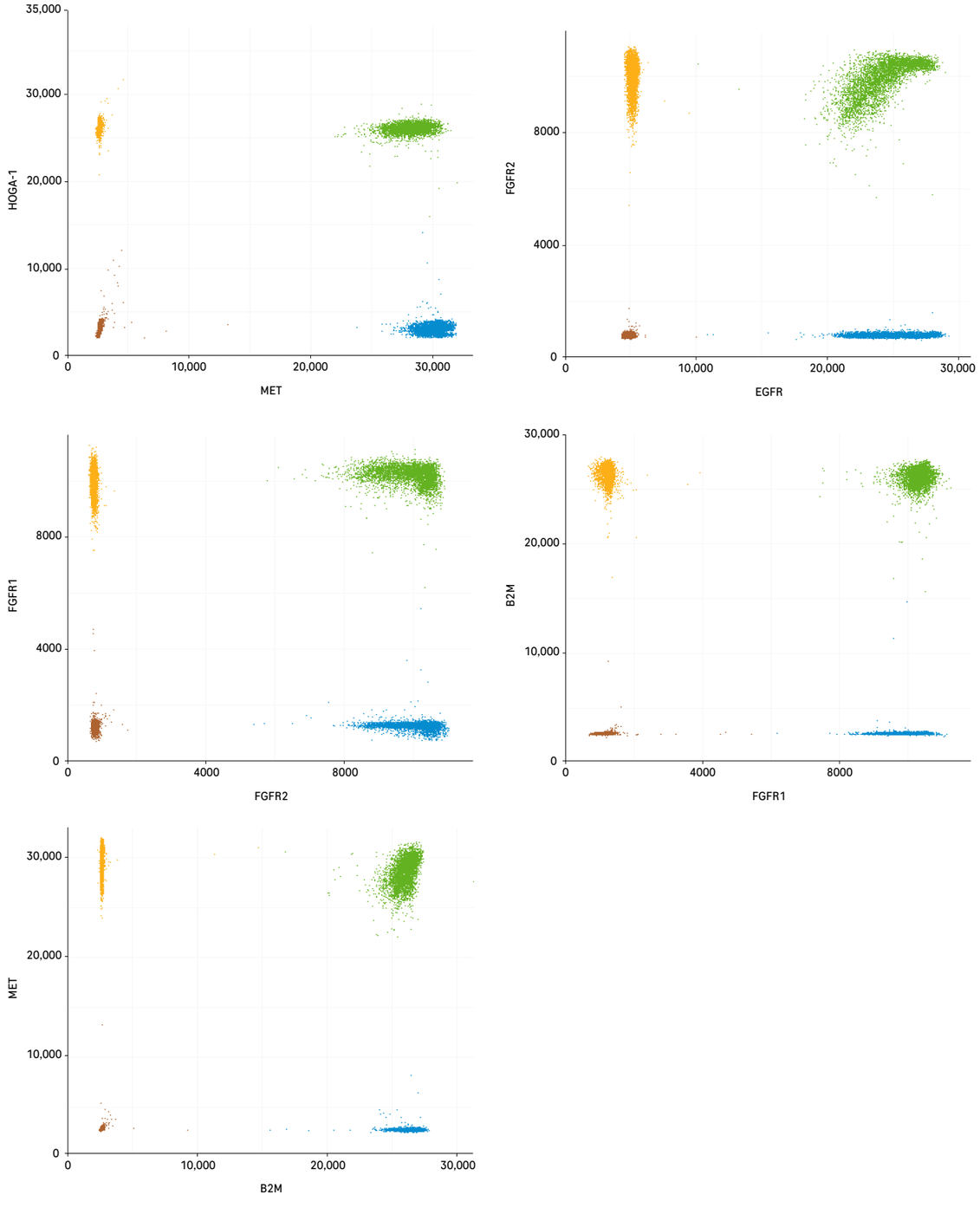



B

Dye to target name mapping:

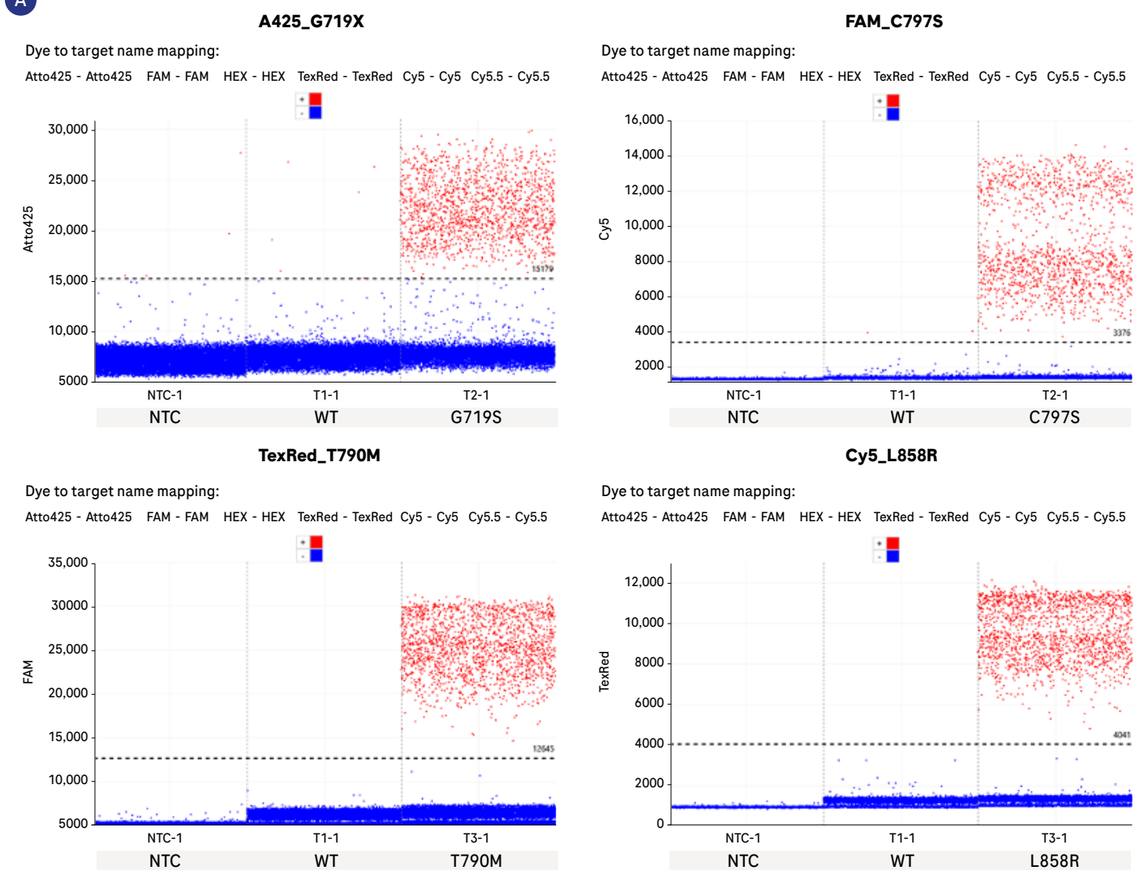
Atto425 - HOGA-1 FAM - MET HEX - B2M TexRed - FGFR1 Cy5 - FGFR2 Cy5.5 - EGFR



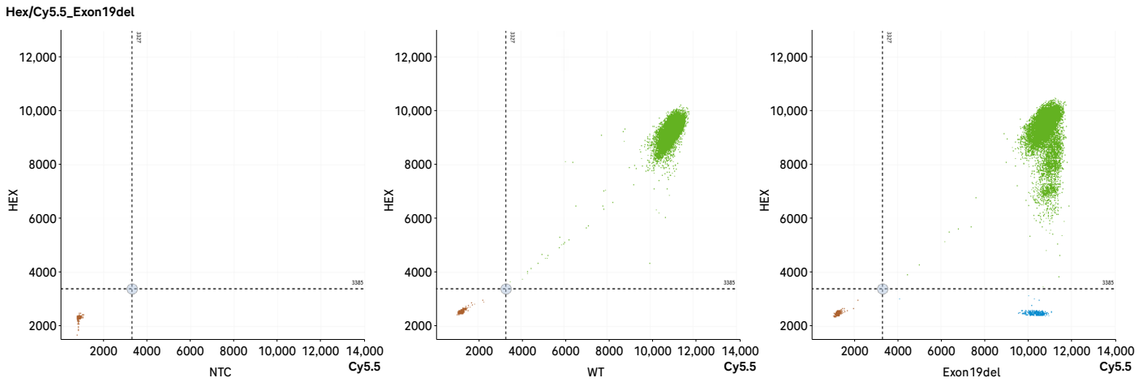

B2M, β2-microglobulin; EGFR, epidermal growth factor receptor; FAM, fluorescein amidite; FGFR1/2, fibroblast growth factor receptor 1/2; HEX, hexachloro-fluorescein; HOGA1, 4-hydroxy-2-oxoglutarate aldolase 1; MET, mesenchymal-epithelial transition.

**Figure 3.35:** 1D (A), 2D (B), and 3D (C) scatter plots demonstrating robust performance and no interference between target amplifications of the example six-plex assay.

A




B




FAM, fluorescein amidite; HEX, hexachloro-fluorescein; NTC, no template control; WT, wild type.

**Figure 3.36:** 1D (A) and 2D (B) scatter plots demonstrating robust performance and no interference between the multiplexed *EGFR* targets.



# 4.0 Troubleshooting

| Category | Description of phenotype | Possible causes | Recommended solution |
|---|---|---|---|
| Partition fluorescence | 'Rain': Partitions with intermediate endpoint fluorescence and it is difficult to determine whether they are positive or negative. | Assay design: Mismatches between template sequence and primer/probe sequences can cause inhibited amplification and thus lower endpoint fluorescence. | Check possible SNP variations; shift primer/probe sequences to avoid mismatches if possible; use multiple primer/probes to include variant species. |
| | | Assay design: Two amplifications are close in position and generate long amplicons that result in lower efficiency with low fluorescence. | For DNA assays, restriction enzymes may be able to cut the template into two pieces and thus no bridging product forms from the distal primers. If possible, redesign the assays so that two amplification locations are not too close. |
| | | Assay designs: Use of modified oligos may result in poor performance. | Schoenbrunner NJ, et al. Biol Methods Protoc 2017;2:bpx011. doi: 10.1093/biomethods/bpx011. |
| | | Non-optimal thermal profile: Denature or annealing temperatures are non-optimal; anneal/extension hold time is non-optimal. | For GC-rich amplification, increasing the denature temperature may reduce rain. For annealing step, test ± 2°C of the targeted temperature. For long-amplicon assays and highly multiplexed assays, consider extending the annealing/extension hold time, or a three-step PCR profile. |
| | | Fragmented or damaged samples. | Shorter amplicon assay design may help with fragmented samples. |
| | | Complicated secondary structures or tandem repeat targets. | Perform restriction digestions: Use multiple enzymes and execute as independent reactions. |
| | | Sample matrix: PCR inhibitors were carried over during nucleic acid extraction. | Dilute the eluate until positive clusters are clearly separated from negative. |
| Cluster position | Negative and/or positive clusters showed variations in endpoint fluorescence, compared with a positive control sample or a previously established result. | Slight differences might be seen due to variations in consumables, instrument, and reagents (lane-to-lane, plate-to-plate, run-to-run, and lot-to-lot variations). | Perform studies to set reasonable ranges of variation. |
| | | Changes to assay: Changes in number or concentration of the probes used in that channel; addition of other probes that may cause oligo interactions. | Use a control with the same primer/probe configuration. Check oligo interactions. |
| | | In multiplex assays (more than two amplifications in one tube): Increasing the template concentration may result in a higher fraction of partitions with multi-occupancy, which can lower the endpoint fluorescence in the channel where assay is relatively less efficient. | Identify the least efficient assay and improve assay performance by increasing the primer/probe concentration; optimize the annealing temperature for the least efficient assay. |
| | | In competing duplex assays (genotyping, mutation assays, etc.): Co-occupancy partitions (double-positive) are expected to have a lower fluorescence than the single-occupancy partition (single-positive). | It is normal to see multiple populations on 1D scatter plot: Users are expected to cluster with the assistance of a 2D scatter plot. As long as the positive populations are separated clearly from negative populations, results should still be considered valid. |

| Category | Description of phenotype | Possible causes | Recommended solution |
|---|---|---|---|
| Cluster position | Changes in the relative cluster position on a 1D or 2D scatter plot. | In competing duplex assays (genotyping, mutation assays, etc.): Some probes may exhibit cross-reactivity and show an additional negative cluster with low fluorescence. | It is normal to see multiple populations on 1D scatter plot: Users are expected to cluster with the assistance of a 2D scatter plot. As long as the positive populations are separated clearly from negative populations, results should still be considered valid. |
| | | Sample matrix: PCR inhibitors were carried over during nucleic acid extraction. | Dilute the eluate until positive clusters are clearly separated from negative. |
| Cluster density | Duplex: No double-positive. | Assay interference: Amplification of one target suppresses the other. | Confirm by comparing singleplex vs duplex concentrations, then check oligo interactions. |
| | Duplex: Double-positive population is higher than expected. | Two closely located targets are in *cis* (linked). | It is normal to see a significantly reduced or even absent single-positive cluster, while having a denser double-positive cluster. Check the linkage tab in the development software. |
| Quantification | No or low counts. | Check clustering. | Always check in 1D or 2D to make sure automatic clustering did not cause the issue. |
| | | RNAse and/or DNAse contamination. | Use a positive control assay to identify nuclease contamination. |
| | | Sample matrix: PCR inhibitors were carried over during nucleic acid extraction. | Dilute the eluate until positive clusters are clearly separated from negative. |
| | | Template quality and accessibility. | Fully denature the protein coat if using synthetic template, such as armored RNA. |
| | | Lack of carrier in low concentration samples. | Use a carrier (Poly-rA) in the buffer and low-bind tubes during handling and storage for low-concentration samples. |
| | False positives in NTC. | Contaminations. | Check possible contaminations in consumables, reagents, and workflow. |
| | False positives in negative control. | Contaminations in negative-control samples; assay-related false positives. | Check negative-control samples with an appropriate reference method. It is normal to see false positives with mutation detection assay (refer to mutation detection assay chapter). |
| | Significant variation in absolute quantification result from expectation. | Variations in quantification methods; differences in amplicon length when measuring short-fragment samples. | It is normal to observe dPCR concentrations off by up to 60% compared with results generated by spectrophotometers (e.g. NanoDrop). If comparing with another dPCR assay, amplicon length may cause discrepancies in concentrations, especially when samples are fragmented (FFPET, cfDNA). In these cases, shorter amplicon assays report higher concentrations. |
| | Poor linearity. | Errors in serial-dilution protocol. | Good laboratory practices. |
| | | Lack of carrier in low-concentration samples. | Use a carrier (Poly-rA) in the buffer and use low-bind tubes during handling and storage for low-concentration samples. |



# 5.0 Appendices

### Appendix A: Tested sample materials and extraction methods

Below is a list of sample materials and extraction methods that were successfully tested on the system. For extraction protocol details, please consult the respective instructions for use for the commercial extraction methods listed below.

| Sample type | Nucleic acid | Tested extraction methods |
|---|---|---|
| Cell culture | DNA | Commercial extraction kit (e.g. Qiagen, Zymo) |
| Plasmid | DNA | Commercial extraction kit (e.g. Qiagen, Zymo) |
| cfDNA | DNA | cobas cfDNA Sample Preparation Kit; MagNA Pure 24/96 |
| FFPET | DNA, RNA | cobas cfDNA Sample Preparation Kit; MagNA Pure 24/96 |
| Whole blood | DNA, RNA | MagNA Pure 24/96 |
| Nasopharyngeal swab | DNA | MagNA Pure 24 |
| Stool | DNA, RNA | MagNA Pure 24 with Total Nucleic Acid Kit |

### Appendix B: Acronyms and abbreviations

| | | | |
|---|---|---|---|
| **1D** | One-dimensional | **FGFR1** | Fibroblast growth factor receptor 1 |
| **2D** | Two-dimensional | **gDNA** | Genomic DNA |
| **3D** | Three-dimensional | **GIC** | Generic internal control |
| **AKT2** | Serine/threonine kinase 2 | **GUSB** | β-glucuronidase |
| **BCR-ABL** | Breakpoint cluster region-tyrosine-protein kinase ABL1 | **HEX** | Hexachloro-fluorescein |
| **BHQ** | Black hole quencher | **IB** | Iowa Black |
| **CCL3L1** | Chemokine ligand 3-like 1 | **IDT** | Integrated DNA technologies |
| **cDNA** | Complementary DNA | **Indel** | Insertion–deletion |
| **cfDNA** | Cell-free DNA | **KRAS** | Kirsten rat sarcoma virus |
| **cfRNA** | Cell-free RNA | **LIS** | Laboratory information systems |
| **Ch** | Channel | **LLoD** | Lower limit of detection |
| **CI** | Confidence interval | **LoD** | Limit of detection |
| **CLSI** | Clinical and Laboratory Standards Institute | **LNA** | Locked nucleic acid |
| **CNV** | Copy number variation | **MGB** | Minor groove binder |
| **Conc** | Concentration | **MMx** | Master mix |
| **cpp** | Copies per partition | **NTC** | No template control |
| **cps** | Copies | **Oligo(dT)N** | Oligo deoxythymidine primer |
| **CV** | Coefficient of variation | **PCR** | Polymerase chain reaction |
| **dATP** | Deoxyadenosine triphosphate | **Poly-rA** | Polyriboadenylic acid |
| **dCTP** | Deoxycytidine triphosphate | **Pos** | Positive |
| **dGTP** | Deoxyguanosine triphosphate | **QC** | Quality control |
| **DNA** | Deoxyribonucleic acid | **qPCR** | Quantitative PCR |
| **dPCR** | Digital PCR | **RFI** | Relative fluorescence intensity |
| **dTTP** | Deoxythymidine triphosphate | **RNA** | Ribonucleic acid |
| **dUTP** | Deoxyuridine triphosphate | **RPP30** | Ribonuclease P protein subunit 30 |
| **EDTA** | Ethylenediaminetetraacetic acid | **RT** | Real time |
| **EIF2C** | Eukaryotic translation initiation factor 2C | **Tm** | Melting temperature |
| **EGFR** | Epidermal growth factor receptor | **Tris** | Trisaminomethane |
| **ERBB2** | Erb-B2 receptor tyrosine kinase 2 | **SNP** | Single nucleotide polymorphism |
| **FAM** | Fluorescein amidite | **UNG** | Uracil N-glycosylase |
| **FFPET** | Formalin-fixed, paraffin-embedded tissue | **VIC** | Victoria |

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