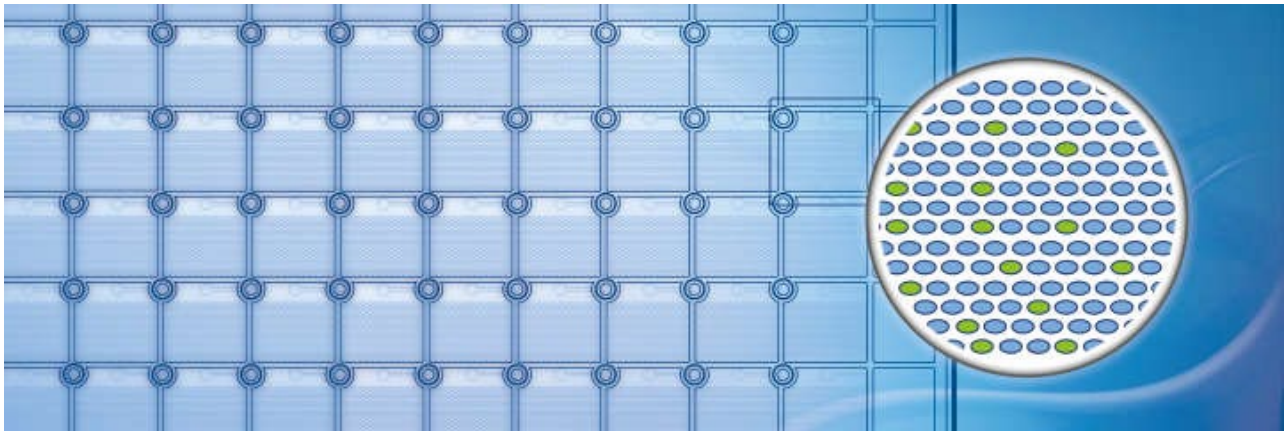
November 2023

QIAcuity® Application Guide

Version 2

QIAcuity User Manual Extension



Sample to Insight

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1 Nanoplate Digital PCR

1.1 Introduction

Digital PCR (dPCR) uses the procedure of end-point PCR but splits the PCR reaction into many single partitions, in which the template is randomly distributed across all available partitions. After PCR, the amplification target is detected by measuring the fluorescence – of either sequence-specific DNA probes or intercalating dyes – in all positive partitions. As the template is distributed randomly, Poisson statistics can be used to calculate the average amount of target DNA per valid, analyzable partition. The total amount of target DNA in all partitions of a well is calculated by multiplying the amount of average target DNA per partition with the number of valid partitions. Calculation of target concentration is determined by referring to the volume in all analyzable partitions, that is, partitions that were filled with reaction mix. The total number of filled partitions is identified by a fluorescent dye present in the reaction mix itself. Absolute quantification by dPCR eliminates the need for standard curves to determine amounts of target DNA in a given sample.

1.2 QIAcuity system and workflow

The QIAcuity is designed as a walk-away instrument that integrates and automates all plate processing steps. Only the plate preparation must be done manually before starting the run. This includes the pipetting of the target, reagents, and master mix in the plate's input wells and the closing of the wells with the Nanoplate Seal. Once the preparation is done and the experiment is set up, the plate is placed in a free plate slot of the instrument tray. By reading the barcode of the plate, the instrument links the plate to the experiment previously defined in the software. After pressing the play button, all further steps are performed fully automated by the instrument.

Figure 1. QIAcuity One, Four, and Eight instruments.

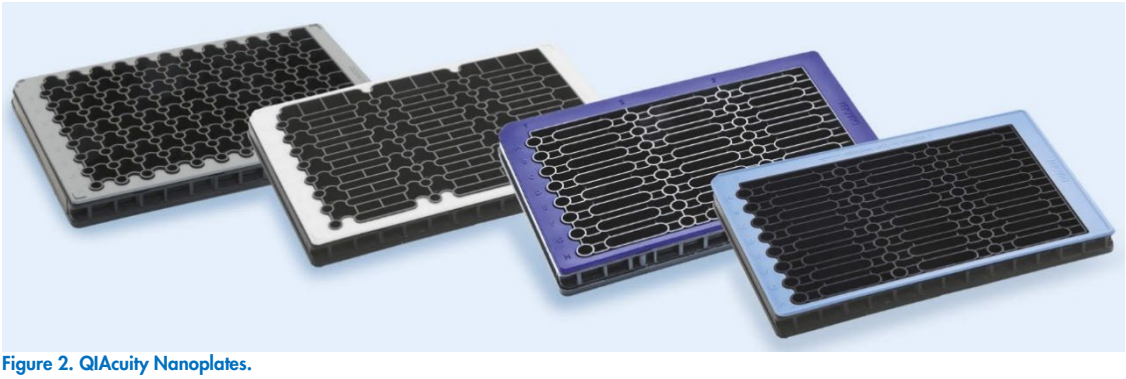


Figure 2. QIAcuity Nanoplates.

1.2.1 Partitioning

In the first step, the plate's microchannels and partitions are filled with the input volume in the wells. This is done by plunging of 8/24/96 pins in the elastic top seal and the input wells. This creates a peristaltic pressure that pumps the input well liquid into the microchannels and partitions. Subsequentially, the connecting channels between the partitions are being closed by a pressure controlled rolling process.

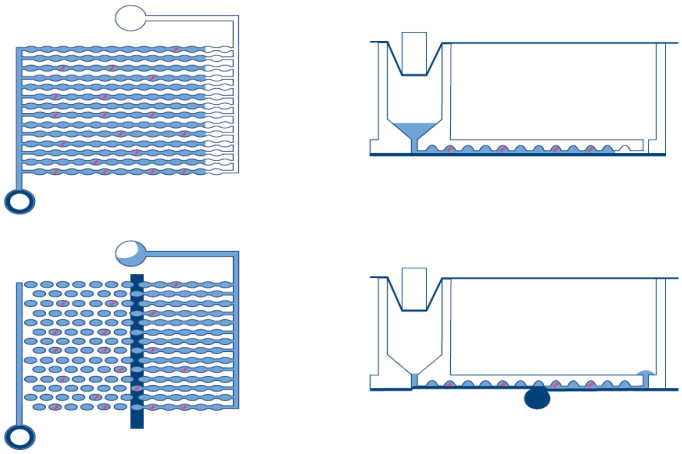


Figure 3. Scheme of filling and partitioning of a well.

1.2.2 Thermocycling

The second step is a high-accuracy plate thermocycler that performs the polymerase chain reaction. The cycling profile can be set in the QIAcuity Software Suite or the instrument software. The thermal cycler of the QIAcuity is a plate thermocycler with high speed and precise temperature control of the various cycling steps. Several Peltier elements are used for temperature generation and control. For optimal thermal contact between plate and thermocycler, the plate is being clamped on the heating surface during cycling. The QIAcuity Eight features two thermocyclers that are operated in parallel.

1.2.3 Imaging

The final step is the image acquisition of all wells. The user can select the detection channels in the experiment setup. The partitions that have a target molecule inside emit fluorescence light and are brighter than those without target (see the image below). For more details and specifications on the imaging system, see the *QIAcuity User Manual* on **www.qiagen.com**.

Table 1. Available channels

Channel	Excitation (nm)	Emission (nm)	Example fluorophores
Green	463–503	518–548	FAM™, EvaGreen®
Yellow	514–535	550–564	HEX, VIC®
Orange	543–565	580–606	TAMRA, ATTO 550
Red	570–596	611–653	ROX, Texas Red
Crimson	590–640	654–692	Cy5, Quasar 670

1.3 Statistics of nanoplate dPCR

1.3.1 Statistics and absolute quantification with copies per microliter and per partition

In dPCR, the PCR reaction is separated into thousands of single partitions, in which the target molecules are randomly distributed across all available partitions. Some partitions will contain no copy of the target molecule, some will contain one copy of the target molecule, and some others will contain more than one copy of the target molecule. As the target molecules are distributed randomly, Poisson distribution can be used to calculate the copies of the target molecule per positive partition.

The Poisson distribution gives probabilities for positive integer random events. The parameter of this distribution, λ, is the expectation value for these events, which means it is the mean probability for a proportion of a counting process or the counting process for the dPCR analysis.

The formula of the Poisson distribution is:

$$P(X = k) = \frac{\lambda^k e^{-\lambda}}{k!}$$

- e: Euler's number (2.718281828…)
- λ: Expectation value
- $k!$: The factorial of k
- k: Copies per partition with k = 0, 1, 2, …

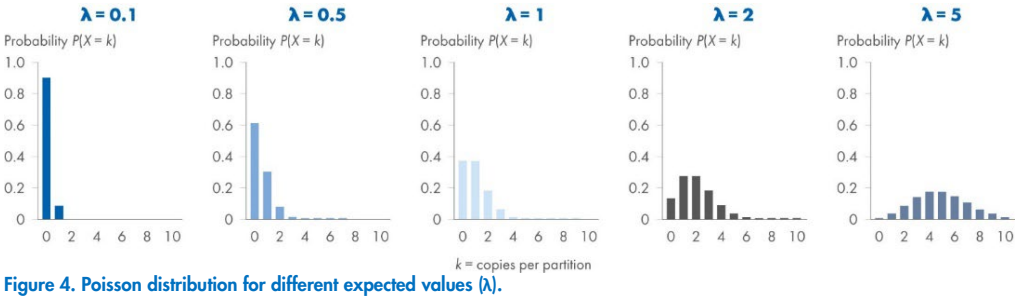


Figure 4. Poisson distribution for different expected values (λ).

The total number of copies of the target molecule in all valid partitions of a well is calculated by multiplying the copies of the target molecule per partition with the number of valid partitions. The calculation of the target concentration is determined by referring to the volume of a valid partition.

Absolute quantification — copies per partition

The total number of copies of the target molecule in all valid partitions of a well is calculated by multiplying the copies of the target molecule per partition with the number of valid partitions. We use an intermediary quantity, λ, interpreted as the average number of target molecules in a single partition.

The estimation of λ is probabilistic in its nature, and the uncertainty of λ is described by the normal distribution centered around the following mean $\hat{\lambda}$.

$$\lambda = -\ln\left(\frac{\text{Number of valid partitions} - \text{number of positive partitions}}{\text{Number of valid partitions}}\right)$$

The 95 % confidence interval of this distribution is a range given by:

$$CI_{low} = \lambda_{low} = -\ln\left(1 - p + 1.96 \cdot \sqrt{\frac{p \cdot (1 - p)}{\text{Number of valid partitions}}}\right)$$

$$CI_{high} = \lambda_{high} = -\ln\left(1 - p - 1.96 \cdot \sqrt{\frac{p \cdot (1 - p)}{\text{Number of valid partitions}}}\right)$$

Where

$$p = \frac{\text{Number of positive partitions}}{\text{Number of valid partitions}}$$

Example:

For simplicity, the assumption is that we have 8500 total partitions of which 8000 are valid and of these, 4000 are positive. The estimated number of copies of the target molecule per positive partition is:

$$\lambda = -\ln\left(\frac{8000 - 4000}{8000}\right) = 0.6931471805599450$$

$\lambda = 0.693$

By using the formula for the Standard Error of $\hat{\lambda}$ the uncertainty can be estimated:

$$CI_{low} = \lambda_{low} = -\ln\left(0.5 + 1.96 \times \cdot \sqrt{\frac{0.5 \times 0.5}{8000}}\right) = 0.67147036342047\ldots$$

$$CI_{high} = \lambda_{high} = -\ln\left(0.5 - 1.96 \times \cdot \sqrt{\frac{0.5 \times 0.5}{8000}}\right) = 0.71530431303236\ldots$$

The CI range is equal to $CI_{high} - CI_{low} = 0.0438\ldots$ This range is used to determine how many digits of the result are significant.

The result is based on detecting the 4000 positive partitions out of 8000 total valid, the λ parameter is distributed with around a value $\lambda = 0.693$ and there is 95% certainty that the true value lays between $\lambda \in 0.671\ldots0.715$. This equals to a CI range of 6.32% compared to mean λ at 0.693…

Estimation of number of copies of the target molecule in the whole well:

$$8500 \times (0.671\ldots0.693\ldots0.715) = (5707\ldots5892\ldots6080)$$

Absolute quantification — copies per microliter

Based on the known number of copies of the target molecule per partition (λ) and the partition volume, the copies per microliter can be calculated using the tripartite calculation.

$$\lambda_{volume} = \frac{\lambda}{V\,[\mu L]}$$

Example:

The number of copies of the target molecule is λ = 0.693.

The estimated partition volume is, for example, V = 0.34 nL.

The copies per microliter is: $\lambda_{volume} = \frac{0.693}{0.34} \times 1000 = 2038$ copies/µL

The 2038 copies/µL is the concentration, which is the standard readout in the dPCR results. To calculate the copies of the target molecule in the reaction volume, you multiply by the input reaction volume. In case the input reaction volume is 12 µL, the copy number in the input reaction is 2038 x 12 = 24,456 copies.

The following example shows how the copies per microliter can be converted to copies per microliter sample or copies per sample. In this example, 5 µL sample (DNA), 3 µL 4x QIAcuity Probe Master Mix, and 4 µL dPCR Primer/Probe Assay sum up to a total dPCR reaction volume of 12 µL. The reported concentration is 2038 copies/µL.

With a reported concentration of 2038 copies/µL and 5 µL sample in 12 µL reaction volume, the 5 µL original sample contains (12/5) x 2038 = 4891 copies/µL. Therefore, the copies in the 5 µL sample are 5 x 4891 = 24,456 copies of target DNA, which is equal to the copies in the total reaction.

Adding the 95% confidence interval in copies per microliter calculation again, we obtain the confidence interval. Again, for this example, λ = 0.693, 2038 copies/µL and a partition volume of 0.34 nL are used.

The confidence bound at a 95% confidence interval can also be shown as confidence values. The confidence values at 95% confidence interval are reported in the QIAcuity Software Suite next to the concentration (copies/µL). The calculation of the confidence values are as follows:

$$CV_{relative} = \frac{\lambda_{high} - \lambda_{low}}{2\lambda} * 100\% = 2.63\%$$

Note: λ value is rounded up to 3 digits for ease of calculations.

1.3.2 Concentration range

Based on the Poisson distribution, the number of copies of the target molecule that a partition contains can be determined. For a low concentration like λ = 0.1, most of the partitions will contain zero copies of the target molecule and nearly all positive partitions will contain only one copy of the target molecule. For medium concentrations like λ = 0.5, some positive partitions will likely contain more than one copy of the target molecule. For higher concentrations like λ = 5, most of the positive partitions will contain more than one copy of the target molecule and nearly no partition will contain zero copies of the target molecule.

Table 2. Percentage of expected target copies per partition for low, medium, and high concentrations

		Likelihood (percentage) of given copies per partition (0–10) at different concentrations (%)										
		0	1	2	3	4	5	6	7	8	9	10
Concentration [cp/partition] (λ)	0.1	90.50	9.00	0.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	0.5	60.70	30.30	7.60	1.30	0.20	0.00	0.00	0.00	0.00	0.00	0.00
	1	36.80	36.80	18.40	6.10	1.50	0.30	0.10	0.00	0.00	0.00	0.00
	1.5	22.30	33.50	25.10	12.60	4.70	1.40	0.40	0.10	0.00	0.00	0.00
	2	13.53	27.07	27.07	18.04	9.02	3.61	1.20	0.34	0.09	0.02	0.00
	5	0.67	3.37	8.42	14.04	17.55	17.55	14.62	10.44	6.53	3.63	1.81

Table 3. Expected number of partitions with different copies per partition count, for 8500 partitions

		Expected number of partitions containing given copies per partition (0–10), at different concentrations										
		0	1	2	3	4	5	6	7	8	9	10
Concentration [cp/partition] (λ)	**0.1**	7691	769	38	1	0	0	0	0	0	0	0
	0.5	5156	2578	644	107	13	1	0	0	0	0	0
	1	3127	3127	1563	521	130	26	4	1	0	0	0
	1.5	1897	2845	2134	1067	400	120	30	6	1	0	0
	2	1150	2301	2301	1534	767	307	102	29	7	2	0
	5	57	286	716	1193	1491	1491	1243	888	555	308	154

Table 4. Expected number of partitions with different copies per partition count, for 26,000 partitions

		Expected number of partitions containing given copies per partition (0–10), at different concentrations										
		0	1	2	3	4	5	6	7	8	9	10
Concentration [cp/partition] (λ)	**0.1**	23,526	2353	118	4	0	0	0	0	0	0	0
	0.5	15,770	7885	1971	329	41	4	0	0	0	0	0
	1	9565	9565	4782	1594	399	80	13	2	0	0	0
	1.5	5801	8702	6527	3263	1224	367	92	20	4	1	0
	2	3519	7037	7037	4692	2346	938	313	89	22	5	1
	5	175	876	2190	3650	4562	4562	3802	2716	1697	943	471

Replicates

Replicates of a sample are identified by having the same: reaction mix, sample name, dilution, and conversion factors (if used). Replicates are detected automatically when the user assigns a sample to a well. If there is another well with the same sample name and reaction mix, this well is identified as a Replicate.

Replicates are analyzed as separate wells, but in addition, the mean concentration value and the CI value of the mean concentration are provided on demand.

On the right side above the table there is a checkbox that allows the user to show mean values for replicates in addition. By default, the button is unchecked and the results are displayed without mean values. When the button is checked, the list still shows independent rows for each selected well but replicates are grouped together. Replicates from the same group are next to each other in the list view. There are two additional columns indicating the mean values:

Mean concentration

Mean concentration for replicates is calculated as a weighted average for concentrations for individual replicates. Weights are the well-specific volume of a single partition within a well, which can be calculated as a volume of the whole well divided by the total number of partitions.

$$\text{Formula for mean concentration (cp per µL)) of 2 replicates} = \frac{(c(1) \times v(1) + c(2) \times v(2))}{(v(1) + v(2))}$$

$$\text{Example for mean concentration (cp per µL) of 2 replicates} = \frac{(6278 \times 2.889 + 6411 \times 2.773)}{(2.889 + 2.773)} = 6343 \text{ cp/µL}$$

CI (95%) — CI of mean concentration assumes that error between individual results has normal distribution. t_{n-1} is defined as critical value of Student's t distribution for 95% and number of degrees of freedom equal number of replicates minus 1.

$$\text{CI for replicates=mean weighted by partition volume } \pm t_{n-1} \times \frac{\text{Standard deviation of the replicate set}}{\sqrt{n}}$$

In this example the critical value for the appropriate t distribution is $t_{n-1} = t_1 = 12.706$. The factor $\frac{t_{n-1}}{\sqrt{n}}$ can be seen as a factor by which the standard error for the replicates is larger than the standard deviation of the concentrations in the observed set of replicates. The factor is fixed for a given number of replicates. In this example, with two replicates, the factor is $\frac{t_{n-1}}{\sqrt{n}} = \frac{12.706}{1.414213} = 8.9845$. We calculate the standard deviation of the replicate set using the formula for sample:

$$\text{Standard deviation of the replicate set} = \sqrt{\frac{\sum (c_i - \bar{c})^2}{n-1}}$$

Standard deviation

$$\text{Example for the standard deviation} = \sqrt{\frac{(6278 - 6343)^2 + (6411 - 6343)^2}{2-1}} = 94.07$$

Coefficient of variation

Coefficient of variance is a ratio of Standard deviation to Mean concentration for replicates, expressed in %:

$$\text{Formula for coefficient of variation of 2 replicates} = \frac{\text{standard deviation}}{\text{mean concentration}} 100\%$$

$$\text{Example for coefficient of variation of 2 replicates} = \frac{94.07}{6343} 100\% = 1.48\%$$

2 Intended Use of the QIAcuity

For detailed information on the intended use, refer to the *QIAcuity User Manual* on **www.qiagen.com**.

3 Requirements for QIAcuity Users

For detailed information on the requirements for QIAcuity users, refer to the *QIAcuity User Manual* on **www.qiagen.com**.

4 Safety Information

For detailed information on the Safety Information, refer to the *QIAcuity User Manual* on **www.qiagen.com**.

5 Experimental Setup and Absolute Quantification

5.1 Assay design guidance

Important factors for success in dPCR include the design of optimal primer pairs and probes, the use of appropriate primer and probe concentrations, and the correct storage of primers and probes.

The guidelines for the optimal design of primers and probes are given below. It is particularly important to minimize nonspecific annealing of primers and probes. This can be achieved through careful assay design.

T_m of primers for TaqMan® assays

- Use specialized design software (e.g., Primer Express® Software) to design primers and probes.
- The T_m of all primers should be 58–62°C and within 2°C of each other.
- The T_m of probes should be 8–10°C higher than the T_m of the primers.
- Avoid a guanidine at the 5′ end of probes, next to the reporter, because this causes quenching.
- Avoid runs of 4 or more of the same nucleotide, especially of guanidine.
- Choose the binding strand so that the probe has more C than G bases.
- All assays should be designed using the same settings to ensure that they will work optimally under the same cycling conditions (60°C annealing/extension).

Primer sequence

- Length: 18–30 nucleotides.
- GC content: 30–70%.
- Always check the specificity of primers by performing a BLAST® search. Ensure that primer sequences are unique for your template sequence.
- Check that primers and probes are not complementary to each other.
- Try to avoid highly repetitive sequences.
- Avoid complementarity of 2 or 3 bases at the 3′ ends of primer pairs to minimize primer–dimer formation.
- Avoid mismatches between the 3′ end of primers and the template sequence.
- Avoid runs of 3 or more Gs and/or Cs at the 3′ end.
- Avoid complementary sequences within a primer sequence and between the primer pair.

Product size

Ensure that the length of PCR products is 60–150 bp, if possible. This is particularly important when multiplexing several assays in dPCR.

5.1.1 Handling and storing of primers and probes

The guidelines for handling and storing primers and probes are given below. For optimal results, we recommend only combining primers of comparable quality.

Storage buffer

Lyophilized primers and probes should be dissolved in a small volume of low-salt buffer to give a concentrated stock solution (e.g., 100 µM). We recommend using Buffer TE (10 mM Tris·Cl, 1 mM EDTA, pH 8.0) for standard primers and probes labeled with most fluorescent dyes.

However, probes labeled with fluorescent dyes such as Cy5 should be stored in Buffer TE, pH 7.0, because they tend to degrade at higher pH.

Storage

Primers should be stored in sterile, nuclease-free Buffer TE in small aliquots at –20°C.

Standard primers are stable under these conditions for at least 1 year. Fluorescently labeled probes are usually stable under these conditions for at least 6–9 months. Repeated freeze–thaw cycles should be avoided because they may lead to degradation. For easy and reproducible handling of primer–probe sets used in multiplex assays, we recommend preparing 20x primer–probe mixes, each containing 2 primers and 1 probe for a particular target at the suggested concentrations (see the QIAcuity Probe and the QIAcuity EG PCR Kits).

Dissolving primers and probes

Before opening a tube containing lyophilized primer or probe, centrifuge the tube briefly to collect all material at the bottom of the tube. To dissolve the primer or the probe, add the required volume of sterile, nuclease-free Buffer TE, mix, and leave for 20 minutes to allow the primer or probe to completely dissolve. Mix again and determine the concentration by spectrophotometry. We do not recommend dissolving primers and probes in water. They are less stable in water than in Buffer TE, and some may not dissolve easily in water.

5.1.2 EvaGreen detection

The fluorescent dye EvaGreen binds all double-stranded DNA molecules, emitting a fluorescent signal of a defined wavelength on binding (Figure 5). The excitation and emission peak of EvaGreen I are at 495 nm and 530 nm, respectively, allowing the detection of the dye in the green channel of the QIAcuity instrument. Signal intensity increases with increasing cycle number due to the accumulation of PCR product. Use of EvaGreen enables analysis of many different targets without having to synthesize target-specific labeled probes. However, nonspecific PCR products and primer–dimers will also contribute to the fluorescent signal and might appear as a separate group of fluorescent signals in dPCR. Therefore, high PCR specificity is required when using EvaGreen.

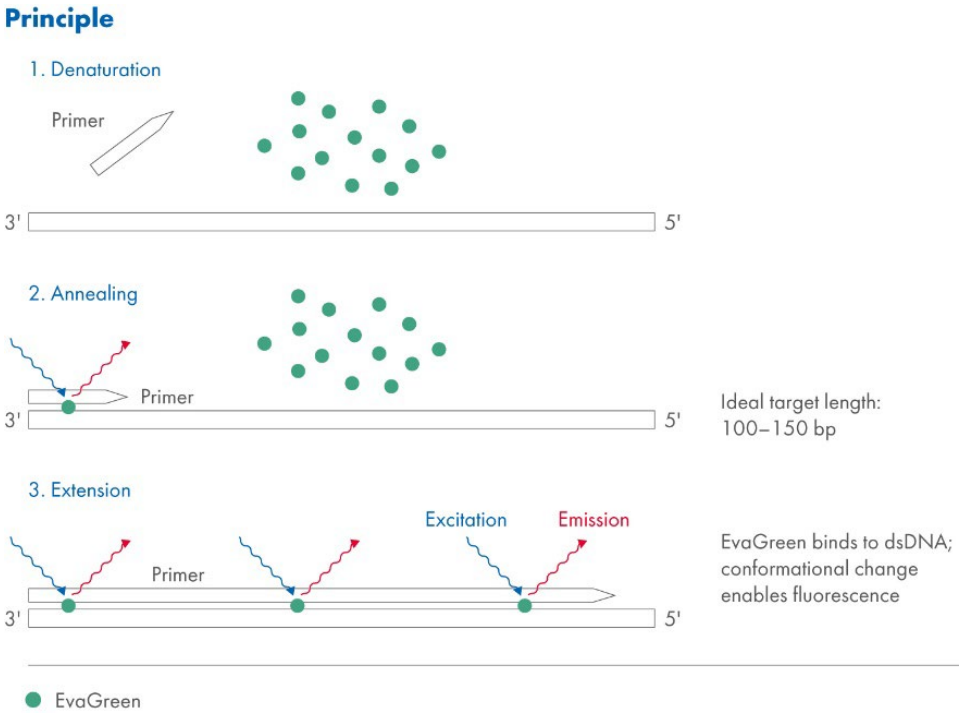


Figure 5. Principle of EvaGreen detection. The PCR primers anneal to the target sequence during the PCR annealing step. During the PCR extension step, Taq DNA polymerase extends the primer and EvaGreen binds to the newly formed double-stranded DNA. Upon DNA binding, a conformation change of the dye is induced and it will start to fluoresce brightly. This signal is proportional to the amount of accumulated PCR product. Please note that, in dPCR on the QIAcuity instrument, the detection of the fluorescence happens after the PCR is completed, that is, post-PCR.

5.1.3 Hydrolysis probe detection

Hydrolysis probes, also known as TaqMan probes, are sequence-specific oligonucleotides with a fluorophore and a quencher moiety attached (Figure 6). The fluorophore is at the 5′ end of the probe, and the quencher moiety is usually located at the 3′ end or internally. During the extension phase of PCR, the probe is cleaved by the 5′→3′ exonuclease activity of Taq DNA polymerase, separating the fluorophore and the quencher moiety. This results in detectable fluorescence that is proportional to the amount of accumulated PCR product.

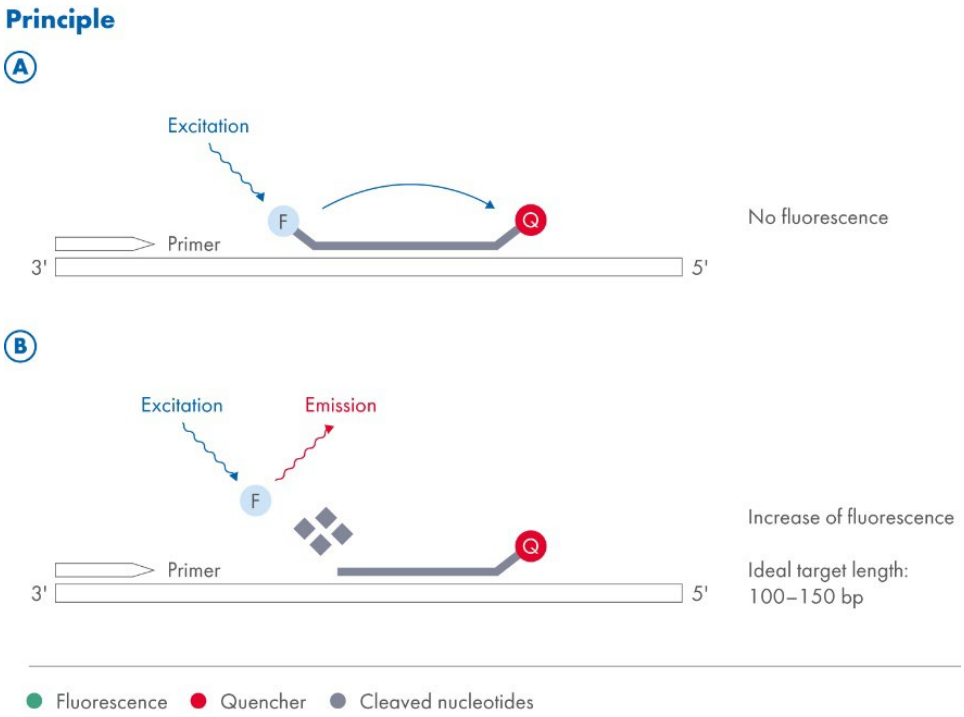


Figure 6. Principle of hydrolysis probe detection. Both the hydrolysis probe and the PCR primers anneal to the target sequence during the combined PCR annealing/extension step. The proximity of the fluorophore with the quencher prevents the fluorophore from fluorescing. During the PCR extension step, Taq DNA polymerase extends the primer. When the enzyme reaches the TaqMan probe, its 5′→3′ exonuclease activity cleaves the fluorophore from the probe. The fluorescent signal from the free fluorophore is measured. This signal is proportional to the amount of accumulated PCR product. Please note, that in dPCR on the QIAcuity instrument, the detection of the fluorescence happens after the PCR is completed, that is, post-PCR.

5.1.4 Transfer of qPCR assays to dPCR

All the rules for proper real-time PCR assay design also apply to dPCR. An assay working well in real-time PCR will most likely do so as well in dPCR. However, care should be taken that the recommended cycling conditions and primer/probe concentrations for dPCR, as outlined in the respective QIAcuity dPCR kits, are selected. For fast optimization of suboptimal performing assays, for example, by running a temperature gradient during the annealing steps, the QIAcuity master mixes can also be run on any real-time PCR instrument.

5.2 Sample preparation

Success in dPCR and RT-PCR depends on the purity and integrity of the template, primers, and probes used. A template can be DNA or RNA, purified from the biological sample being analyzed, or a known amount of DNA or RNA to be used as a standard or positive control. Primers and probes are DNA oligonucleotides which are typically purchased from a commercial supplier.

Because PCR consists of multiple rounds of enzymatic reactions, it is more sensitive to impurities such as proteins, phenol/chloroform, salts, and EDTA than single-step enzyme-catalyzed reactions.

Purity of nucleic acid templates is important for dPCR because contaminants can interfere with fluorescence detection. QIAGEN® offers a complete range of nucleic acid purification systems that provide pure, high-quality templates for PCR and RT-PCR. These include QIAprep® kits for purification of plasmid DNA, QIAamp® and DNeasy® kits for purification of genomic DNA, RNeasy® kits for purification of total RNA, and the PAXgene® Blood RNA System for stabilization and purification of RNA from blood. Phenol and other contaminants can be efficiently removed from crude RNA preps using the RNeasy MinElute® Cleanup Kit to clean up and concentrate RNA for sensitive assays. Details about QIAGEN kits for nucleic acid purification can be found at **www.qiagen.com**.

5.2.1 Sample input amount

Copy numbers, starting from 1 copy up to 217,000 copies per reaction, can be used on the 26K Nanoplate for the QIAcuity instruments, when using probe based detection. Similarly, up to 170,000 copies per reaction can be used on the 8.5K Nanoplates. This is based on the following calculations.

Table 5. Nanoplates and their maximal copy numbers

Plate type	Number of partitions	Upper limit of copies per partition	Analyzed volume (µL)	Total rxn volume (µL)	Max copy number per analyzed volume	Estimated max copy number per reaction
8.5K Nanoplate	Approx. 8500	5 (Probe) 2 (EvaGreen)*	Approx. 2.6	12	42,500 (Probe) 17,000 (EG)	170,000 (Probe) 68,000 (EG)
26K Nanoplate	Approx. 26,000	5 (Probe) 2 (EvaGreen)*	Approx. 22.0	40	130,000 (Probe) 52,000 (EG)	217,000 (Probe) 69,000 (EG)

* The different upper limit for probe and EvaGreen based detection, respectively, is due to that fact that, if human genomic DNA is used as starting template, the dsDNA template itself will cause significant background fluorescence upon intercalation of the EvaGreen dye. The situation will be different if cDNA or gDNA from organisms with smaller genome size is used.

Given that the haploid genome size of the organism studied is known, the correlation between mass input of gDNA and the resulting copy number (for a single-copy gene) can be easily calculated, using the following formula:

Size of the genome (bp) x average weight of a single base pair (1.096×10^{-21} g/bp)

For the human genome with a genome size of ca. 3.1×10^{9} bp, the calculation is as follows:

3.1×10^{9} bp x 1.096×10^{-21} g/bp = 3.3×10^{-12} g = 3.4 pg

The genome sizes of many organisms have been published.

A few examples are depicted below.

Table 6. Genomes sizes of selected organisms

	*Homo sapiens*	3.1 x 10E9 bp	3,099,750,718*	**http://www.ensembl.org/Homo_sapiens/Info/Index**
	Rat	2.6 x 10E9 bp	2,647,915,728*	**http://www.ensembl.org/Rattus_norvegicus/Info/Annotation**
	Mouse	2.7 x 10E9 bp	2,728,222,451*	**http://www.ensembl.org/Mus_musculus/Info/Annotation**
	Zebrafish	1.4 x 10E9 bp	1,373,471,384*	**http://www.ensembl.org/Danio_rerio/Info/Annotation**
	*Saccharomyces cerevisiae*	1.2 x 10E7 bp	12,157,105*	**http://www.ensembl.org/Saccharomyces_cerevisiae/Info/Annotation**
	*Escherichia coli*	4.6 x 10E6 bp	4,641,652*	**http://bacteria.ensembl.org/Escherichia_coli_str_k_12_substr_mg1655_gca_000005845/Info/Annotation/#assembly**
	Standard plasmid DNA	3.5 x 10E3 bp	3500*	

* Number as of November 2023. The numbers might change frequently upon updates of the respective databases.

When looking for a single-copy gene (i.e., 1 copy/haploid genome), 10 ng of gDNA as template correspond to:

- Human: approximately 3000 copies
- Zebrafish: approximately 5400 copies
- Yeast: approximately 760,500 copies
- *E. coli*: approximately 2,000,000 copies
- Plasmid DNA (3.5 kb): 2,600,000,000 copies

Not to forget:

- In dPCR, the average number of copies per partition must not exceed 5, ideally in the range of 0.5–3.
- This means ideally:
    - 8.5K Nanoplate: approximately 17,000–100,000 copies per 12 µL reaction, 2.6 µL analyzed (approx. 56–330 ng of human gDNA for single-copy gene)
    - 26K Nanoplate: approximately 22,000–130,000 copies per 40 µL reaction, 22 µL analyzed (approx. 73–430 ng of human gDNA for single-copy gene)

If the copy number input cannot be determined before starting the experiment, it is recommended to perform an initial titration experiment, using the unknown template in 2–4 tenfold dilutions to determine to optimal range for subsequent analyses.

5.2.2 DNA sample digestion

Random template partitioning is essential for accurate quantification in dPCR systems. For the vast majority of QIAcuity dPCR applications, template DNA is uniformly distributed throughout the QIAcuity Nanoplate reaction chambers. In QIAcuity reactions using PCR products, formalin-fixed, paraffin-embedded (FFPE) DNA, circulating cell-free DNA (cfDNA), or complementary DNA (cDNA) as template, a uniform distribution of PCR signal is observed. However, DNA molecules >30 kb are unevenly partitioned, which leads to overquantification of template concentration.

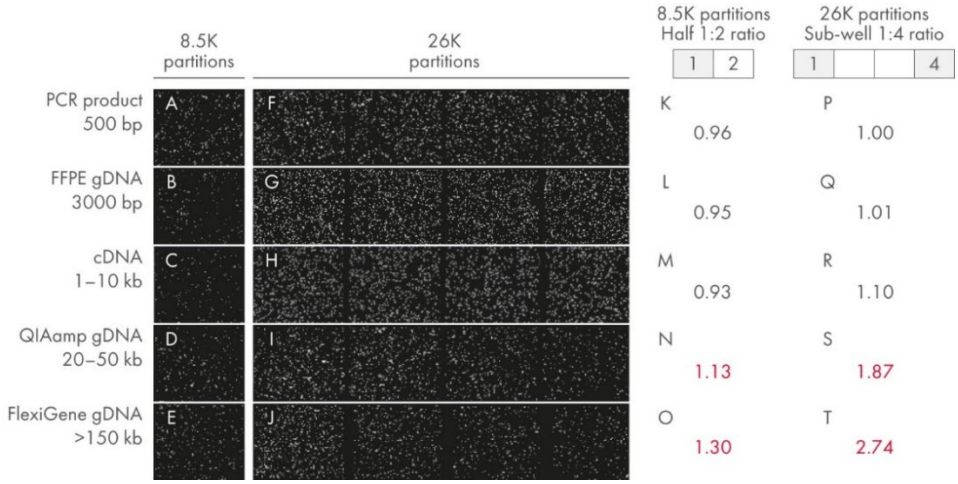


Figure 7. Uniformity of DNA distribution in QIAcuity Nanoplates is size dependent. Typical QIAcuity samples ranging from 500 to >150 kb in size served as template in 8.5K and 26K QIAcuity dPCR reactions. Images of PCR signal from 8.5K **(A–C)** and 26K **(F–H)** Nanoplates indicate that smaller DNA templates are evenly distributed, while larger templates like intact human gDNA accumulate across a left-to-right gradient **(D, E, I, J)**. For smaller DNA templates, the ratios of positive partitions between the first and second half (half 1:half 2 ratio) of 8.5K Nanoplate wells **(K–M)** and the first and fourth subwell (subwell 1:4 ratio) of 26K Nanoplate wells **(P–R)** are close to 1, indicating even template partitioning. For larger DNAs, half 1: half 2 ratios and subwell 1:4 ratios exceed 1 and reveal that distribution bias is more severe for DNA >150 kb **(O, T)** than for DNA 20–50 kb in size **(N, S)**.

By adding restriction enzymes directly to the QIAcuity reaction mixes, large templates can be fragmented to smaller sizes, which results in even template distribution and accurate quantification. When adding restriction enzymes to reaction mixes, users must be sure that the enzymes do not cut within the amplicon sequence.

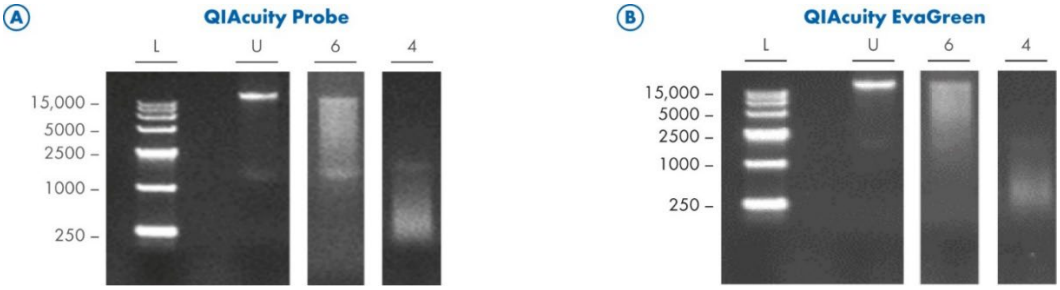


Figure 8. Restriction enzymes fragment DNA directly in QIAcuity Probe and QIAcuity EvaGreen mixes. The activity of two restriction enzymes was assayed in 12 µL QIAcuity Probe **(A)** or QIAcuity EvaGreen **(B)** reaction mixes containing 210 ng of FlexiGene® gDNA. Untreated DNA (U) appears as two high molecular weight bands on a 0.8% agarose gel. Addition of EcoRI-HF, a 6-cutter (6) restriction enzyme, or AluI, a 4-cutter (4) restriction enzyme, fragmented the FlexiGene DNA to average sizes of approximately 4096 and approximately 256 bp, respectively. Reactions were carried out at room temperature (15–25°C) for 10 minutes. EcoRI-HF and AluI were added at concentrations of 0.25 U/µL and 0.025 U/µL, respectively. GelPilot High Range Ladder (100) was used as size ladder (L).

5.3 Absolute quantification and analysis

5.3.1 Data analysis overview

The QIAcuity Software Suite enables you to analyze plates that have been processed by the instrument. The following analysis options are available in the software:

- Absolute quantification
- Mutation detection
- Genome editing
- Copy number variation
- Gene expression

For detailed information on the different analysis options, see the following chapters in this document or refer to the *QIAcuity User Manual* on **www.qiagen.com**.

5.3.2 Accessing the analysis environment

Please refer to the *QIAcuity User Manual* for more information on the analysis environment.

5.3.3 Images

Please refer to the *QIAcuity User Manual* more information about single and multi-imaging steps.

5.3.4 Thresholding in QIAcuity Software Suite

Please refer to the *QIAcuity User Manual* for more information on the thresholding in QIAcuity Software Suite.

5.3.5 Hyperwell function of the QIAcuity Software

The QIAcuity software allows the combination of several wells to one analytic unit, provided that the selected wells contain only replicates, that is, the same assay and template, and have therefore the same sample name assigned to them. Any number of wells can be combined; however, the natural maximum are the 96 wells on a 8.5K Nanoplate. This function is mainly used for ultra-rare mutation detection, where the same genomic DNA containing a low number of mutation positive DNA fragments is tested across several wells. In the example in Figure 9, row A contains replicates of the same assay and template. The selected samples can then be grouped as a hyperwell (Figure 9, left panel). The grouped wells then appear with a red background (Figure 9, right panel). In the analysis view, the 1D plots still display the individual wells; however in the list view, only one well appears. All the analytic parameters (partitions, CI, etc.) are then calculated as if there was only one well on the plate. In the example here, all wells had been combined to a hyperwell with 97,000 single partitions, of which 28,000 had been positive. Of course, the hyperwelling can be undone and each well analyzed individually.

Figure 9. Example of a 96 well 8.5K plate with the first row in hyperwell view.

5.3.6 Multiplexing using the QIAcuity

In PCR, the term multiplexing describes the simultaneous run of multiple PCR reactions in the same reaction chamber. Each PCR reaction can have its own pair of primers and detection method. In qPCR, multiplexing is commonly performed using TaqMan probe chemistry, where each assay has its own probe labelled with a different fluorophore. Therefore, each assay emits light in a different wavelength and can therefore be differentiated from the other assays. However, all assays share the same pool of reagents (dNTPS, polymerase, etc.) and templates, competing and hindering each other to reach optimum performance. This is different to dPCR, where on the QIAcuity each reaction is split into small partitions, ideally with only one template target in each partition. Because the QIAcuity has 5 optical channels, 5 assays can be run at the same time. Each assay is detected by a different fluorophore and therefore is only visible in the respective channel.

When planning a multiplex reaction, certain considerations should be made beforehand:

- The annealing temperature of each assay should be similar.
- The amplicon length should be in the same range.
- Each assay should not interact with the other assays.

When starting to setup a multiplex reaction, it is advised to start with a duplex reaction and increase the number of assays. After each step check the performance of each assay by checking:

- NTC or Lower limit of Blank
- Clearly separated signals
- Correct quantification with each assay

6 CNV Analysis

6.1 Introduction

CNVs (copy number variations, or copy number alterations, CNAs) are structural changes in genome (such as deletions, insertions, duplications, translocations, and inversions) that lead to gain or loss of copy numbers of a region, ranging from few hundred base pairs up to whole chromosomes. CNVs are either inherited or the results of de novo somatic mutations. Responsible for up to 10–20% variation in the genome, CNVs are a source of natural genetic diversity as well as biological dysfunction in humans. CNVs often result in disruption of gene function, dosage imbalances, and positional effects, which are associated with complex diseases and traits such as cancer, obesity, and neurodegenerative and autoimmune diseases. The quantitative analyses of CNVs at disease-associated loci, therefore, provide insights into molecular mechanisms of diseases and offer potential for the discovery of novel biomarkers.

QIAGEN's dPCR Copy Number Assays provide highly sensitive and accurate detection of copy number changes for individual genes or regions of interest. QIAcuity CNV dPCR assays can discriminate as low as 10% variation, owing to exceptional precision and resolution of copy number detection by QIAcuity dPCR technology. Other standard methods for CNV detection, such as microarrays, next-generation sequencing, and RT-PCR, lack the accuracy of absolute quantification and sensitivity of copy number detection achievable by dPCR. These approaches fail to detect small changes in CNVs reliably, particularly at very low and very high DNA template concentrations.

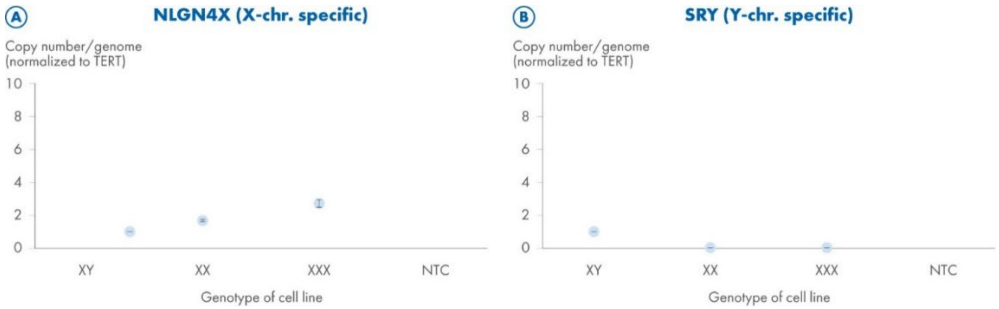


Figure 10. dPCR Copy Number Assays accurately identify aneuploidy. dPCR Copy Number Assays designed to target NLGN4X and SRY genes, which are on X and Y chromosomes, were tested using 3 Coriell templates with XY, XX, and XXX genotypes. TERT assay was used as reference assay, with stable copy numbers across all three XY, XX, and XXX genotypes. dPCR Copy Number Assays accurately measured single-copy changes and detected 1 copy, 2 copies, and 3 copies of NLGN4X gene in XY, XX, and XXX templates, respectively. Single copy of SRY gene was only detected in XY template but not in others, as expected.

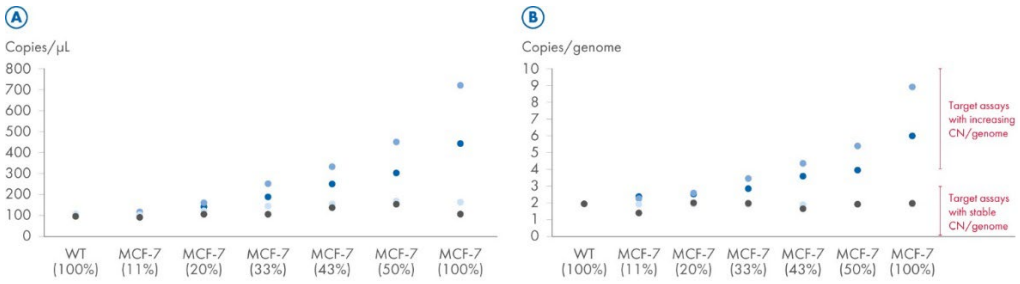
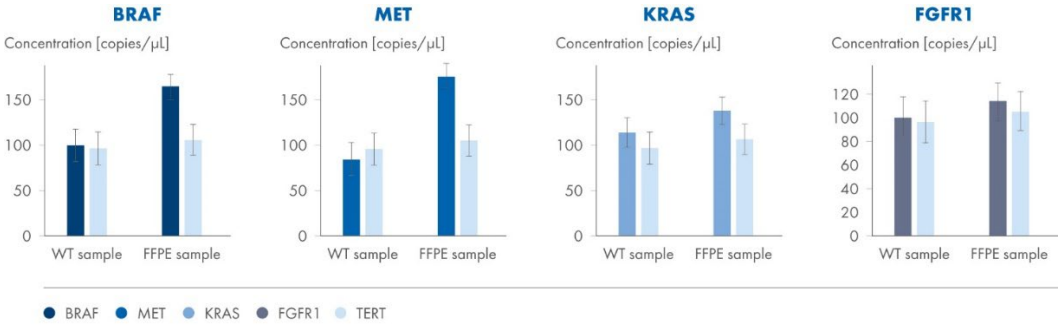


Figure 11. dPCR Copy Number Assays accurately detect small changes in copy number. dPCR Copy Number Assays targeting MYC and NRAS were tested using heterogeneous samples. Samples were prepared by mixing MCF-7 cell line genomic DNA (100% MCF7) and WT genomic DNA from healthy donor (100% WT) in different proportions: 10%, 20%, 33%, 43%, and 50%. dPCR Copy Number Assays accurately measured the increase in CN of MYC and NRAS genes in MCF-7 cell line as reported previously. The small ~10% incremental changes in copy numbers/microliter in samples as well as the changes in copy number/genome were captured. TERT and RPP30 assays were used as reference assays and showed stable copy numbers across all heterogenous mixtures as expected.

The outstanding performance of the QIAcuity dPCR Copy Number Assays allows accurate and reproducible detection of CNVs in very low quantity and quality of sample material. DNA samples extracted from FFPEs, ctDNAs, liquid biopsies, and various other biosamples in limited amounts can be used to assess CNVs present in these samples.



	CN/genome			
Sample	BRAF	MET	KRAS	FGFR1
FFPE	2.99	3.82	2.22	2.06
WT	2	2	2	2

Figure 12. dPCR Copy Number Assays can be used to detect CNVs in low quantities of compromised material. Formalin-damaged and fragmented reference standard template from Horizon™ was tested for CNVs using dPCR Copy Number Assays. Assays targeting BRAF, MET, KRAS, and FGFR1 genes were tested as targets, and TERT assay (pink columns) was used as reference assay for normalization. Wild-type genomic DNA, 3 ng, from healthy donors and 3 ng of genomic DNA from FFPE samples were tested per reaction. dPCR Copy Number Assays detected copy number changes for BRAF and MET genes but not for KRAS and FGFR1 genes in these templates.

6.2 CNV calculation and normalization

CNV analysis involves the assessment of copy numbers of a gene of interest (GOI) or target of interest (TOI) in a given sample. This requires (1) absolute quantification of copy amount of GOI (or TOI) present in the sample and (2) calculation of copy numbers of GOI (or TOI) per genome in relation to either reference sample(s) or reference assay(s).

Reference target assay(s) with known copy numbers/genome can be used for normalization of copy number of GOI (or TOI). For CNV analysis, the concentration of GOI (or TOI) (copies/µL) will be compared to the concentration of reference assay (copies/µL) obtained for the same target sample and the ratio will represent the ratio of copy number of GOI to copy number of reference assay within the genome. This approach is particularly useful for CNV analysis of novel target assays as it requires no prior knowledge of the copy number of GOI per genome under normal conditions.

Copy number of GOI: ($Conc^{TARGET}$/$Conc^{REF.ASSAY}$) * Copy no. of Ref. Assay

$Conc^{TARGET}$: concentration of GOI (or TOI) (copies/µL)
$Conc^{REF.ASSAY}$: concentration of Ref. Assay (copies/µL)

Reference sample(s) with a known copy number for GOI or a known CNV can also be used to determine the copy numbers present in the target samples. First, concentrations of reference and target assays are determined in the reference sample. Their ratio establishes the relation between the copy number of GOI and reference assay per genome in the reference sample. This ratio can then be used to calculate the copy number of GOI in target samples based on the assumption that the copy number of reference assay per genome remains identical among all target and reference samples. These calculations also calibrate for the

concentration differences in the starting material of target and reference samples, making this approach particularly useful for CNV analysis of target samples with varying DNA concentrations or quality.

Copy number of $GOI^{TARGET\ SAMPLE}$:Conc $Ratio^{TARGET\ SAMPLE}$/Conc $Ratio^{REF.\ SAMPLE}$ * Copy number of $GOI^{REF.\ SAMPLE}$

Copy number of $GOI^{REF.\ SAMPLE}$: already known

QIAcuity Software Suite 2.2 allows normalization using reference sample(s) or reference targets(s).

The CNV analysis in QIAcuity Software Suite consists of (1) absolute quantification of copies/microliter of each target present in samples and (2) quantification of changes in copy number/genome of each target assay in relation to a defined reference sample and/or reference assays(s). CNV analysis integrated into the QIAcuity Software Suite workflow allows for determination of CNVs (copy number [CN]/genome) in target samples in simple, quick, and intuitive steps.

Apart from the selection of ideal reference assays and samples for normalization, heterogeneity of biological samples has an impact on the accuracy of CNV determination. In typical cases, the biological samples are obtained from uniform cell populations such as clonal cell lines derived from patients and exhibit a single type of CNV. The challenges in CNV calculations arise when biological samples are comprised of heterogenic populations of cells and tissues, such as mixed cell cultures, collection of samples from blood, bone marrow, and most commonly tumor biopsies from cancer patients. Observed CNVs in such samples can originate from a single subpopulation of cells exhibiting drastic changes in copy numbers. Given the limited amount of biopsy samples, these cells can be present in very low quantities in the samples.

Conversely, CNVs can originate from different subpopulations exhibiting various degrees and directionality of changes, such as some cells harboring deletions and copy number loss, whereas others harboring duplications and copy number gains for a region of interest (Figure 13). In both cases, it is not possible to differentiate between different CNVs and CNV analysis is often biased toward the copy number of the abundant cell type population present in the biopsy material. For accurate analysis of CNVs, the complexity of sample heterogeneity and the degree of fluctuations in copy numbers must be taken into consideration. Multiple reference samples and assays that help determine the relative amount of each cell type and thus copy number variant can be included in the experimental designs. If the percentage of cells with CNV can be estimated, a GOI copy can also be determined (Table 7).

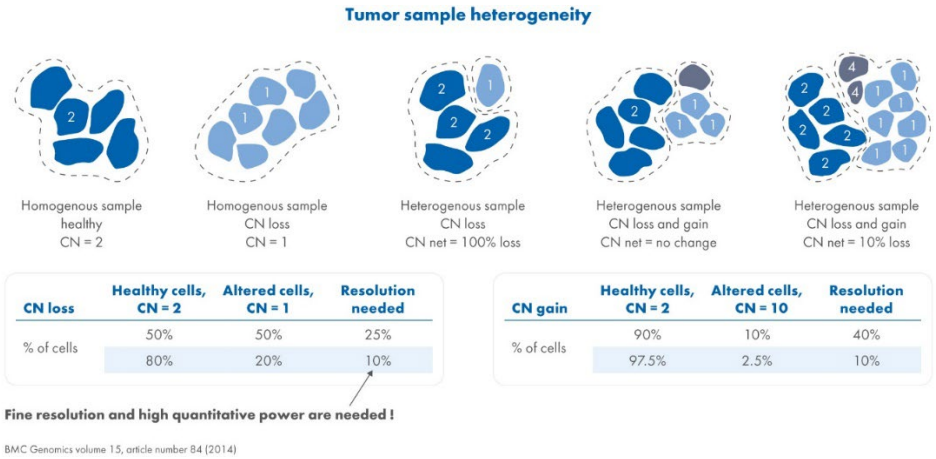


Figure 13. Sample heterogeneity affects copy number analysis.

Table 7. Copy number analysis in heterogeneous samples

	Expected CNV of total sample				
	% of "CNV-harboring" cells				
CN of CNV-harboring cells	87.5	75	50	25	12.5
0	0.25	0.5	1	1.5	1.75
1	1.1	1.3	1.5	1.8	1.9
3	2.9	2.8	2.5	2.3	2.1
4	3.8	3.5	3.0	2.5	2.3
8	7.3	6.5	5.0	3.5	2.8
16	14.3	12.5	9.0	5.5	3.8
32	28.3	24.5	17.0	9.5	5.8

Table 7 shows copy numbers calculated for a mixed population of CNV-harboring tumor and nontumor cells with the normal diploid genome. Copy number calculations depend on the target gene's copy number in the tumor cells and its percentage in the heterogeneous sample.

6.2.1 Copy number reference assays selection

Reference assays capture the relative differences in input DNA concentration as well as copy numbers in target samples. The selection of appropriate reference assays is important for accurate analysis of CNVs in samples.

Good reference assays should give the same copy number across different experiments and samples in a reproducible manner, given that the reaction parameters and setup stay the same. Ideal reference targets should have no known pseudogenes, duplications, or deletions, showing low inter-replicate variability as well as stable copy numbers across different tissues, cell types, etc.

Most widely used reference targets are present in 2 copies per genome; however, reference targets present in multiple copies must be taken into consideration when targets are expected in high copy numbers (Table 8). Reference assays that amplify invariant or stable genome sequences are less prone to polymorphisms or structural changes and are often present in multiple copies. Reference targets that are unrelated to the biological phenotype in question, thus most likely to stay unaltered in target samples, can also be used as alternative references.

Table 8. Use of a multicopy reference assay increases the accuracy of CNV estimation

Reference assay		Target assay		CNV analysis
Expected CN/genome	Measured CN/genome	True CN/genome	Measured CN/genome	
2	2	30	30	Correct
2	3	30	20	Incorrect (–30%)
2	1	30	60	Incorrect (+100%)
10	10	30	30	Correct
10	12	30	25	Incorrect (–16%)
10	8	30	37.5	Incorrect (+25%)

Table 6 shows how unexpected loss or gain of copies of the reference target influence CN calculations and the final CNV analysis outcome.

It is recommended to use multiple reference assays (preferably 2) in each CNV experiment. When selecting multiple reference assays, low variability among reference assay performances is desired. Good reference assays will provide CNV estimations that are in agreement with each other. This will help the user find out if any reference assays show unexpected copy number changes in target samples, which will alter copy number calculations. When multiple reference assays are used, the most "stable" reference assay can be selected for downstream CNV analysis. Multiple reference assays can be used as both calibrators and positive or negative controls for CNV among the target samples. Experimental results from different runs, plates, and even laboratories can be compared more easily as the number of reference targets increases. The use of reference assays with different copy numbers will provide higher sensitivity to CNV discrimination. Thus, using multiple reference assays will result in higher accuracy and reproducibility of CNV determination in target samples.

For an optimal CNV analysis, consideration of multiple reference assays and reference samples in experimental design is highly recommended. A reference sample with a normal copy number and another one with CNV (such as duplication, deletion, or high copy numbers) similar to the expected outcome in target samples (when prior knowledge available) are often reference samples of choice (Figure 14).

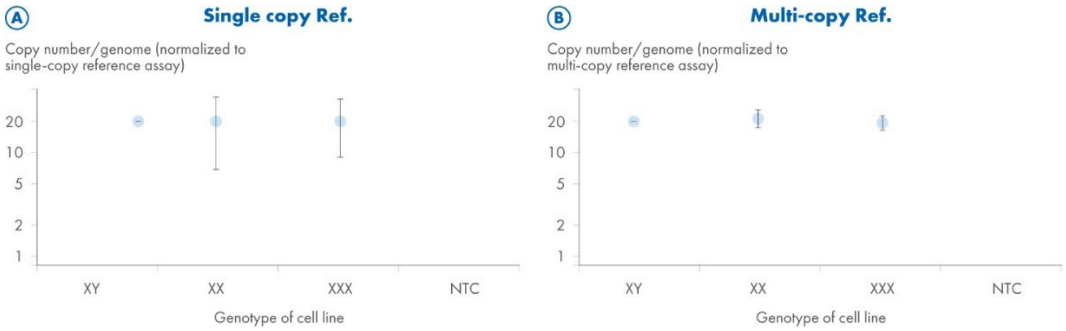


Figure 14. When target assays are expected to have high copy number/genome, normalization using multicopy reference assays provide more accurate results. dPCR Copy Number Assay targeting ACTB gene, which has 20 copies in genome, was tested using a single copy reference assay (left) and multicopy reference assay (right) under exact same experimental conditions.

When reference samples are used for normalization of copy numbers, it is recommended that, if possible, the reference samples should come from the same type of tissue as the target samples. Thus, the DNA quality of both the reference and target samples matches. For fresh or frozen tissue samples, the DNA from fresh or frozen tissues should be used as reference samples. When using target samples originated from FFPE tissue material, calibrator DNA from FFPE material should be used as a reference sample. Problems might arise when genomic DNA purified from fresh or frozen tissue samples are used as reference samples in the latter case. Degraded, crosslinked, or fragmented samples might have a disadvantage at the level of PCR amplification. Upon fragmentation, amplicons might be lost, resulting in underestimating total copies of any target or reference assay in the fragmented samples. Therefore, the final CNV call is mostly influenced by the differences in the fragmentation level between the reference and target samples. When uncertain of sample quality, it is recommended to check DNA quality first.

6.3 Experiment planning

The QIAcuity Digital PCR platform provides easy-to-interpret, reproducible, and accurate CNV analysis results for individual GOIs or TOIs using QIAGEN's portfolio of dPCR Copy Number (EvaGreen or Probe based) assays and the QIAcuity chemistries (QIAcuity EG and Probe PCR Kit). Simplicity of the workflow and large number of assays available in dPCR Copy Number Assays portfolio allow fast and efficient routine CNV profiling in any research laboratory (see the workflow in the next page).

The QIAcuity CNV dPCR procedure starts with genomic DNA purification from starting material (or already purified DNA sample). After quality control, each purified DNA sample is mixed with ready-to-use QIAcuity (EG or Probe) PCR master mix and dPCR Copy Number Assays according to the experimental design. The reaction mixtures are then aliquoted into each well of QIAcuity Nanoplates. After the dPCR run (see section "QIAcuity system and workflow", page 7), CNVs in target samples are calculated based on absolute copy numbers detected in samples using QIAcuity Software Suite.

QIAcuity CNV dPCR workflow overview

Sample preparation
DNA purification (QIAamp DNA Mini Kit, DNAeasy Blood & Tissue Kit, and QIAamp DNA FFPE Tissue Kit)
DNA concentration and purity check (UV spectrophotometry, or using DNA QC standards, such as QIAxpert®)

↓

Reaction setup using dPCR Copy Number Assays from QIAGEN's portfolio
Considerations for template preparation: restriction enzyme selection
Considerations for plate selection: number of total reactions per plate, number of partitions required for desired sensitivity, and accuracy of detection

↓

Plate setup and run in QIAcuity Digital PCR instrument

↓

CNV data analysis using QIAcuity Software Suite

6.3.1 Considerations

We recommend the following precautions:

- Use sterile pipet tips with filters.
- Decontaminate workspace and labware to avoid any foreign DNA contamination.
- Store DNA-containing material separately from all other reagents.
- Prepare DNA-containing reaction mixes in spatially separated rooms or facilities.
- Separate dPCR sample/plate setup and dPCR analysis.
- Thaw all components before preparing reaction mixes. When thawed, mix components thoroughly for obtaining homogenous solutions and centrifuge briefly to avoid spill overs.
- Pipetting accuracy and precision affect the consistency of results. Be sure that all pipettes and instruments have been checked and calibrated according to the manufacturer's recommendations.
- Make sure no bubbles are introduced into the wells of the dPCR nanoplate during sample setup.

6.3.2 Sample isolation and preparation

Sample DNA quantity and purity can affect the performance of QIAcuity dPCR Assays for CNV analysis. It is highly recommended to check the DNA quantity and quality prior to setting up dPCR reactions.

DNA purification

When starting with purification of genomic DNA, an appropriate purification method should be used depending on the starting material and desired amount of purified genomic DNA. The kits listed below are recommended for genomic DNA purification from various types of starting material for use with QIAGEN's dPCR Copy Number Assays (Table 9). DNA purification can be carried out according to the kit instructions.

Table 9. Recommended DNA purification kits for use with QIAcuity CNV dPCR Assays

Starting material	Purification method	Cat. no.
Fresh or frozen tissues, cultured cells	QIAamp DNA Mini Kits	51304 and 51306
	DNeasy Blood & Tissue Kits	69506, 69504, 69582, and 69581
	EZ1 DNA Investigator Kit	952034
	EZ1 DNA Tissue Kit	953034
	Blood & Cell Culture DNA Mini, Midi, or Maxi Kit	13323, 13343, or 13362, respectively
	Flexigene DNA Kit	51206
	PAXgene Tissue DNA Kit	767134
FFPE tissues	QIAamp DNA FFPE Tissue Kit	56404
	GeneRead DNA FFPE Kit	180134
cfDNA from human plasma or serum	QIAamp DNA Blood Mini or Maxi Kits	51106, or 51194 and 51192, respectively
	PAXgene Blood DNA Kit	761133
	QIAamp ccfDNA/RNA Kit	55184
	EZ1 ccfDNA Mini or Midi Kit	954134 or 954154, respectively
	QIAamp MinElute ccfDNA Midi and Mini Kits	55284 and 55204, respectively
Other biological samples	Contact QIAGEN Technical Service	

Important: Do not use diethyl pyrocarbonate (DEPC)–treated water.
Important: Presence of RNA in the sample preparations will result in inaccurate DNA concentration measurements. Removing RNA contamination with an RNase A digestion is highly recommended.
Important: Including no template control samples (NTCs) in the experimental design will provide information about presence of any DNA contaminants.

DNA quality

All DNA samples used in reaction mixes should show similar quality and quantity, which can easily be assessed using ultraviolet (UV) spectrophotometry.

Upon UV spectrophotometry measurement DNA samples should meet the following criteria:

- Concentration determined by A_{260} should be >10 µg/mL.
- A_{260}:A_{230} ratio should be greater than 1.7.
- A_{260}:A_{280} ratio should be greater than 1.8.

Given that the samples are diluted, and their absorbance is measured in 10mM Tris Cl, pH 8.0, an absorbance reading of 1.0 at 260 nm in a 1 cm detection path corresponds to a DNA concentration of 50 ug/µL.

Template digestion

For optimal performance, high purity gDNA digested with appropriate restriction enzymes should be used as templates in dPCR reaction mixes whenever possible (see section "DNA sample digestion"). Restriction digestion of genomic DNA larger than 20 kb in size produces DNA fragments that segregate and distribute in the partitions evenly. Furthermore, if intact gDNA is used for CNV analysis, the copy number of GOI or TOI that harbor tandem repeats or repetitive elements may be underestimated, as the amplicons may be physically linked and migrate into the same partition of the nanoplate well. Restriction digestion of genomic DNA separates tandem repeats or repetitive elements present in region of interests, ensuring their random distribution within partitions and making them accessible for PCR amplification. Therefore, fragmentation of gDNA results in higher accuracy of copy number estimations and CNV calculations.

Important: Up to 200 ng (EvaGreen-based detection) and up to 450 ng (Probe-based detection) of human genomic DNA should be used per dPCR reaction when detecting CNV assays present in 2 copies/diploid genome. In EvaGreen based detection, the double-stranded non-amplified template DNA itself will result in an increased background signal in the negative partitions; therefore, the maximal template is reduced compared to probe-based detection. Loading amounts should be adjusted according to the expected copy number of targets to avoid saturation of partitions. DNA samples should be digested to ensure uniform distribution of templates within partitions, according to recommendations in the assay product sheet.

Copies/haploid genome	Copies in 10 ng gDNA
1	2,777
5	13,885
10	27,770
20	55,540
50	138,850

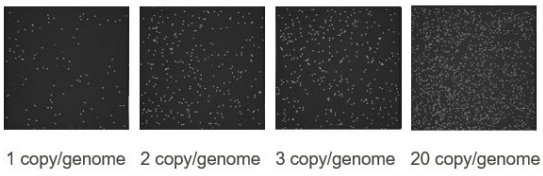
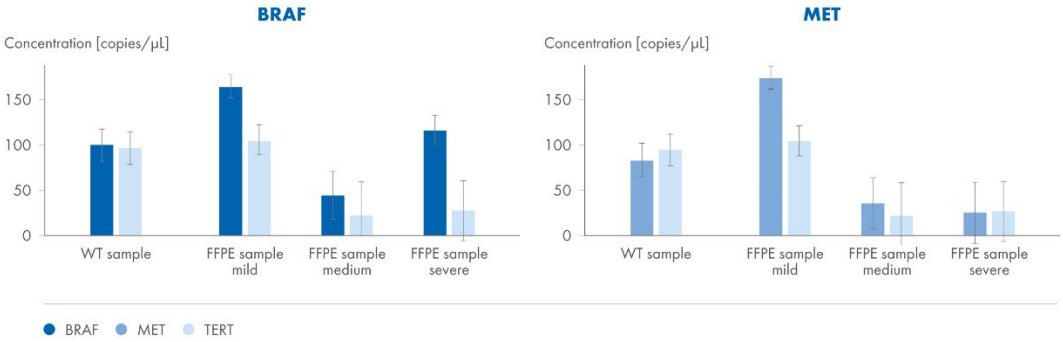


Figure 15. Increasing copy number of target assays in the genome can result in increasing no of positive partitions in dPCR at equal loading amounts.

Quality-compromised input material, such as DNA samples extracted from FFPE samples with varying degrees of crosslinking and fragmentation, do not require additional restriction digestion step. However, these samples may result in suboptimal CNV detection due to possible fragmentation of the genomic region that harbors the GOI or TOI. Choosing reference and target assays with similar designs (amplicon length, GC content, and primer annealing temperatures) will help minimize the extent to which the residual crosslinking and DNA fragmentation in the assayed FFPE samples biases amplification efficiency. This will help to ensure that reference and target amplicons are evenly quantified.



	CN/genome	
Sample	**BRAF**	**MET**
WT	2	2
FFPE mild	2.99	3.82
FFPE medium	3.79	3.69
FFPE severe	8.24	2.07

Figure 16. Use of DNA templates with compromised quality (here, fragmented FFPE material) might result in inaccurate CNV detection. Examples given here are target assays BRAF and MET, which were tested using genomic DNA extracted from FFPE tissue material with varying levels of fragmentation and formalin damage (mild, medium, and severe). With increasing levels of fixation and fragmentation, CN/genome of both BRAF and MET target assays changed drastically. These results indicate that copy number determination is inaccurate and inconsistent in "severely" fragmented and formalin-compromised FFPE material, when compared to FFPE material with "mild" and "medium" levels of fragmentation and fixation.

Important considerations for restriction digestion of template DNA:

- Restriction digestion can be carried out directly in the nanoplates according to the Quick-Start Protocol for dPCR Copy Number Assays. For more details, see section "DNA sample digestion".
- Incubate the nanoplate 10 minutes at room temperature (15–25°C).
- DNA purification is not necessary after the restriction digestion.
- Heat inactivation of the restriction enzyme will be performed during the dPCR run.
- DNA digested prior to the experiment can be stored at –20°C.

Table 10. List of recommended enzymes

6-Cutter restriction enzymes		4-Cutter restriction enzymes	
*Eco*RI	0.25 U/µL EcoRI-HF®, NEB® 0.025 U/µL Anza™ 11 EcoRI, TFS	*Alu*I	0.025 U/µL AluI, NEB 0.025 U/µL Anza 44 AluI, TFS
*Pvu*II	0.025 U/µL PvuII, NEB 0.025 U/µL Anza 52 PvuII, TFS	*Cvi*QI	0.025 U/ µL CviQI, NEB 0.025 U/µL Csp6I (CviQI), TFS
*Xba*I	0.025 U/µL Anza 12 XbaI, TFS	*Hae*III	0.025 U/µL BsuRI (HaeIII), TFS

NEB, New England Biolabs; TFS, Thermo Fisher Scientific. Please note that restriction enzymes from other suppliers might perform differently.

6.3.3 Reference assays selection

Digital PCR allows accurate estimation of copy number changes for target genes based on the ratio of absolute concentrations of target and reference genes in samples. Including reference assays in dPCR tests serves other purposes as well, such as normalization of input templates due to (1) variation in the quality and quantity of DNA from sample to sample, especially when the origin of samples is unknown, and (2) pipetting errors. Multiple reference assays can be quantitated simultaneously and independently, eliminating any sample variation or negative impact from potential pipetting errors. The dPCR Copy Number Assay portfolio offers multiple reference assays to choose from. Commonly used reference targets RPP30, TERT, APB3B, and AGO1 are present in 2 copies per genome. However, reference assays that are present in multiple copies per genome or that are located in centromeric regions can also be used. Reference assays should be selected according to expected CNV in target samples and desired resolution of CNV detection (if prior knowledge available). Use of a combination of reference assays with different copy numbers per genome is highly recommended (Table 11).

Table 11. List of recommended reference assays

Assay name	Copy number (diploid genome)	GeneGlobe® cat. no.	Detection Method	Assay portfolio
RPP30	2	DCH110-0463302A	EvaGreen-based detection	dPCR Copy Number Assay
TERT	2	DCH105-0006472A		
AP3B1	2	DCH105-0386794A		
R6	12	DCH101-0003124A		
R10	20	DCH101-0003135A		
RPP30	2	DCR0000181	Probe-based detection	dPCR CNV Probe Assay
TERT	2	DCR0000186		
AP3B1	2	DCR0000238		
AGO1	2	DCR0000536		
Centromeric reference assays	2	Various		

6.3.4 Sample loading amount (DNA loading)

For an optimal CNV analysis, expected copy numbers for targets and references should be taken into consideration when setting up reactions (when prior information available). When loading samples into the wells, total expected copies must lie within upper and lower limits of dynamic detection range of the QIAcuity Digital PCR instrument (see section "Sample input amount", page 20). Although as low as a single copy of the target can be reliably detected, it is recommended to set up reactions with template input amounts that contain 0.5–2 DNA copies/partition. In addition, template amounts should range between 30 ng/µL and 100 ng/µL and should not exceed 200 ng total DNA (EvaGreen detection) or 450ng (Probe based detection) for optimal loading of a single-copy target in a single dPCR reaction. Table 12 summarizes relative copies present in an 8.5K 96-well Nanoplate depending on the copy number of targets in relation to a standard reference assay with 2 copies in genome.

Table 12. Copy number effect on loading amounts of samples in nanoplates

Assay Type	Copy number/diploid genome	Loading amount (0.005 copies/partitions)	Loading amount (3 copies/partitions)
Reference	2	0.3 copies/µL	180 copies/µL
Target 1	10	1.5 copies/µL	900 copies/µL
Target 2	20	3 copies/µL	1800 copies/µL
Target 3	50	15 copies/µL	9000 copies/µL

6.3.5 Using dPCR Copy Number Assays for copy number analysis

Over 100,000 assays targeting each gene covering whole human genome are available in the GeneGlobe dPCR Copy Number Assay portfolio. Each assay is designed strategically to optimize coverage of CNVs within the target amplicon. Use of assays targeting neighboring amplicon regions allows detection of CNVs with high spatial resolution.

All QIAcuity CNV dPCR Assays are in silico validated and ready for use in QIAcuity Digital PCR. More than 200 assays targeting key CNVs are validated experimentally on the QIAcuity Digital PCR instrument. These targets are carefully selected from peer-reviewed publications based on their function and their association with a cancer-related gene, pathway, or genetic disease phenotype.

6.3.6 Using QIAcuity EG PCR Kit and dPCR Copy Number Assays for copy number analysis on QIAcuity Digital PCR platform

For optimal performance, QIAcuity EG PCR Kit is highly recommended for setting up reactions using dPCR Copy Number Assays. The QIAcuity EG PCR Kit contains hot-start QuantiNova® DNA polymerase, which is kept in an inactive state by the QuantiNova antibody and is active only after heat treatment. This allows reaction setup at room temperature (15–25°C) without the risk of primer–dimer formation. The stringency of hot-start, along with other proprietary chemical components in the QIAcuity EG PCR Kit, is essential for delivering highest performance in copy number determination. The QIAcuity EG PCR Kit and dPCR Copy Number Assays are compatible for use in all dPCR nanoplates and can be ordered through **geneglobe.qiagen.com/**

Important: The QIAcuity EG PCR Kit was developed for optimum performance of dPCR Copy Number Assays on the QIAcuity Digital PCR platform. Do not add any additional reagents in QIAcuity EG PCR master mix.

6.3.7 Using dPCR CNV Probe assays for copy number analysis

Over 200 dPCR CNV Probe assays are validated for use in QIAcuity dPCR and are available in the GeneGlobe dPCR CNV Probe Assay portfolio. These targets are carefully selected from peer-reviewed publications based on their function and their association with a cancer-related gene, pathway, or genetic disease phenotype. In addition to target assays, the dPCR CNV Probe Assay portfolio includes assays for reference genes as well as centromeric reference assays within the centromeric region for optimal normalization.

dPCR CNV Probe assays are LNA-enhanced probes and primers for providing the highest specificity of detection and are available in different fluorescence labels. This allows flexibility when designing a multiplexing setup, by using target and reference assays in different color combinations. Up to 5-channels can be utilized for full exploitation of multiplexing capacity using dPCR CNV Probe assays. Figure 17 shows flexibility of 2- to-5-plex reaction setups using same dPCR CNV Probe assays.

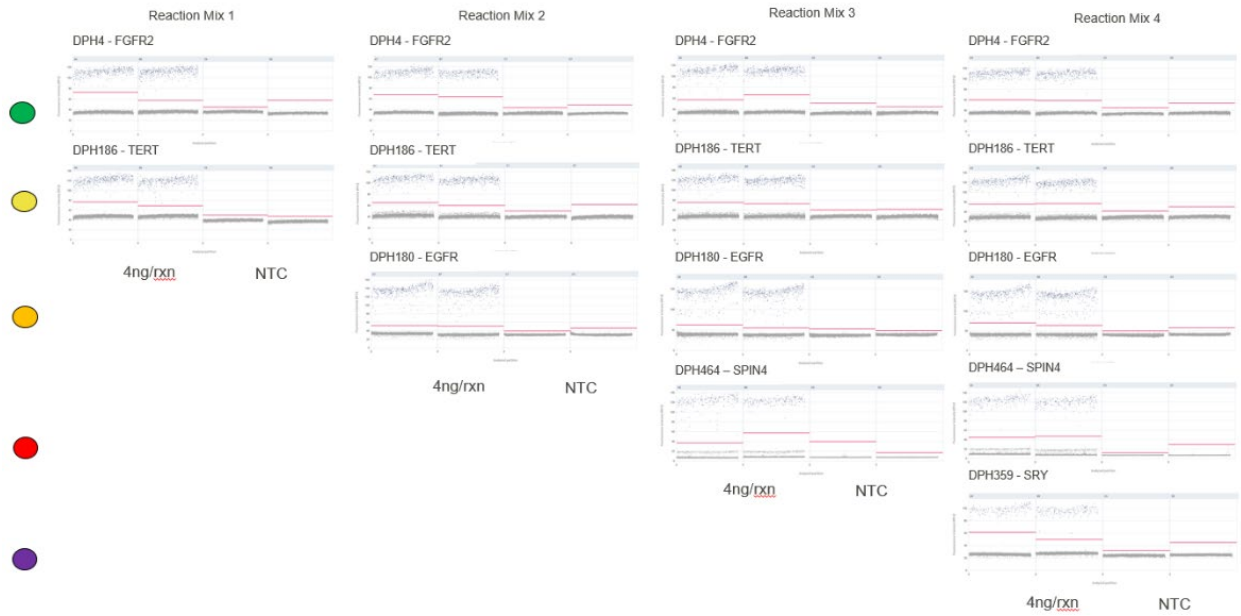


Figure 17. Multiplex CNV detection. dPCR CNV Probe Assays allow for simultaneous detection of up to 5 target and reference genes within a single reaction.

6.3.8 Using QIAcuity Probe PCR Kit and dPCR CNV Probe Assays for Copy Number Analysis on QIAcuity dPCR platform

For optimal performance, QIAcuity Probe PCR Kit is highly recommended for setting up reactions using dPCR CNV Probe Assays. QIAcuity Probe PCR Kit contains hot-start QuantiNova® DNA polymerase, which is kept in an inactive state by the QuantiNova antibody and is active only after heat treatment. This allows reaction setup at room temperature (15–25°C) without the risk of primer–dimer formation. The stringency of hot-start, along with other proprietary chemical components in QIAcuity Probe PCR Kit, is essential for delivering highest performance in copy number determination. The QIAcuity Probe PCR Kit and dPCR CNV Probe Assays are compatible for use in all dPCR nanoplates and can be ordered through **geneglobe.qiagen.com/**

Important: QIAcuity Probe PCR Kit is developed for optimum performance of dPCR CNV Probe Assays in QIAcuity dPCR platform. Do not add any additional reagents in QIAcuity Probe PCR Master Mix.

6.3.9 Recommendation of a QIAcuity Nanoplate

In addition to the appropriate selection of dPCR Assays and QIAcuity chemistry, selection of appropriate QIAcuity Nanoplates is crucial for reaching desired sensitivity and range of copy number detection. The QIAcuity Nanoplates come in three different sizes, 8-well, 24-well and 96-well plates, with 26K and 8.5K partitions, respectively. Use of 8.5K 96-well plates is recommended for high-throughput routine CNV screening where large number of samples can be tested using low-input volumes. However, 26K Nanoplates can also be used according to experimental needs where high reaction volumes are ideal for sensitive detection of rare CNV events in heterogenous samples. Ideal reaction volumes and partition sizes must be selected according to the experimental design, such as total number of samples, replicates, and template concentrations.

For detailed information, see **www.qiagen.com/qiacuity-nanoplates-and-accessories-productdetails**

6.3.10 CNV analysis in QIAcuity Software Suite

The QIAcuity Software Suite analyzes the CNV in target samples. The analysis consists of absolute quantification of copies of each target present in samples, followed by quantification of changes in copy number/genome of each target assay in relation to a defined reference sample or target (second-level analysis).

Setting up CNV analysis

The first step is absolute quantification of samples to determine absolute copies per microliter of GOI. Results can be analyzed using QIAcuity Software Suite by selecting Analyse option from left section bars. A new window will open. In the top left, Absolute Quantification and, in top right, Copy Number Variation plugins can be found (Figure 18).

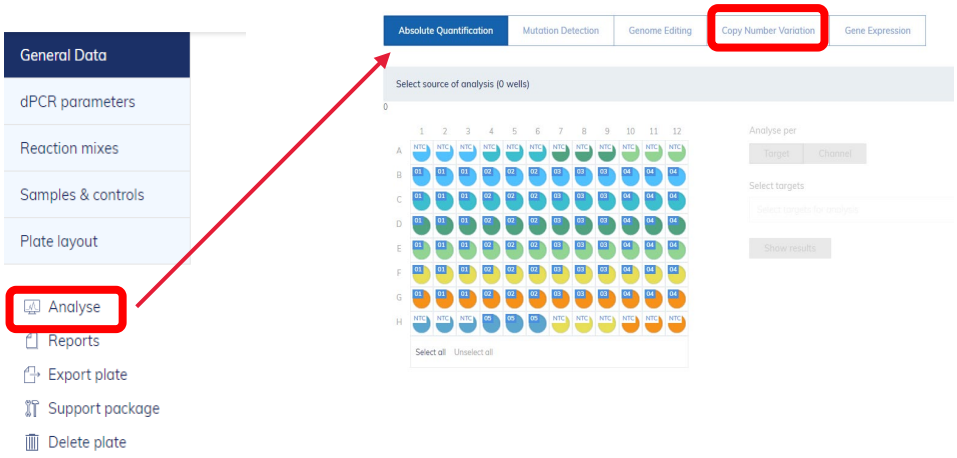


Figure 18. QIAcuity Software Suite has integrated workflow for absolute quantification as well as CNV analysis.

Select wells and TOIs. Select **Show results**.

The analysis Absolute Quantification and corresponding analysis views and options are described in *QIAcuity User Manual*, section "Absolute quantification and analysis".

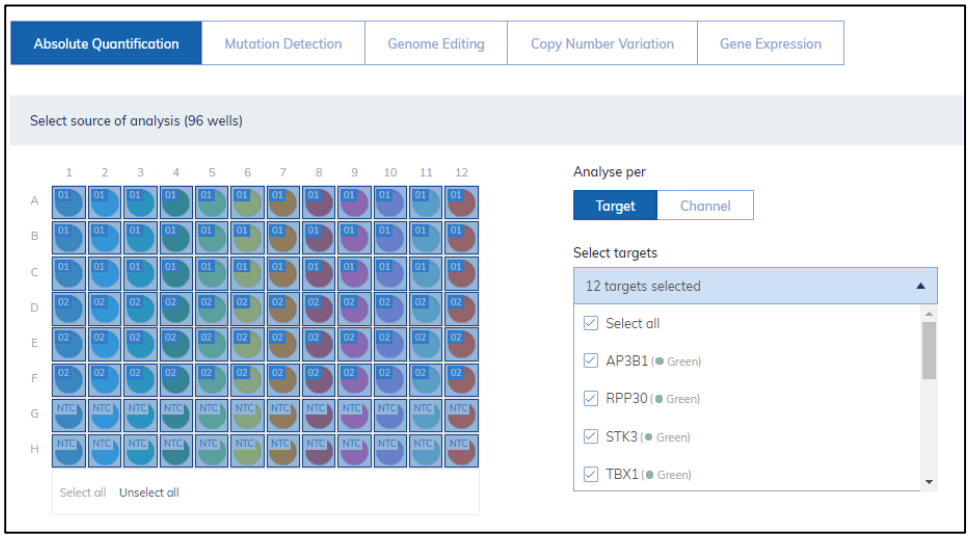
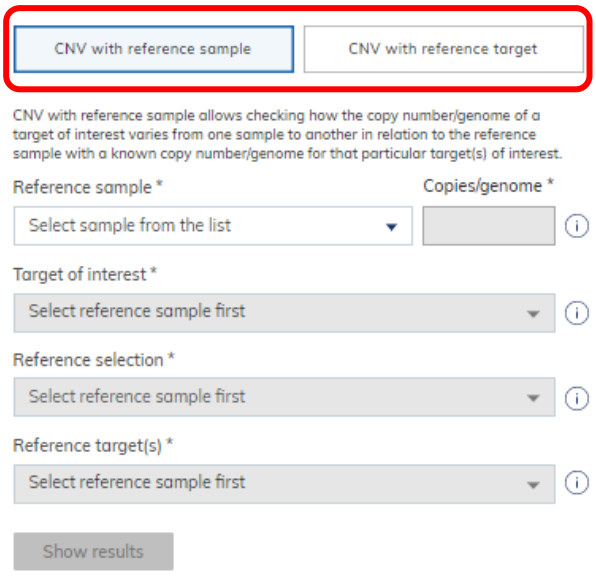


Figure 19. Absolute quantification of multiple targets can be done by selecting multiple wells and relevant TOIs.

For CNV analysis, switch to Copy Number Variation tab (top right). To view the contents of the tab, click the tab title. For more information, see *QIAcuity User Manual*, section "Copy number variation".

There are two ways of CNV Analysis:

- CNV with reference sample allows checking how the copy number/genome of a target of interest varies from one sample to another in relation to the reference sample with a known copy number/genome for that particular target(s) of interest.
- CNV with reference target allows checking how the copy number/genome of a particular target of interest varies from one sample to another in relation to the reference target present in the selected samples. The copy number per genome of the reference target is considered to remain constant among all samples to be analyzed and has to be known for proper analysis.



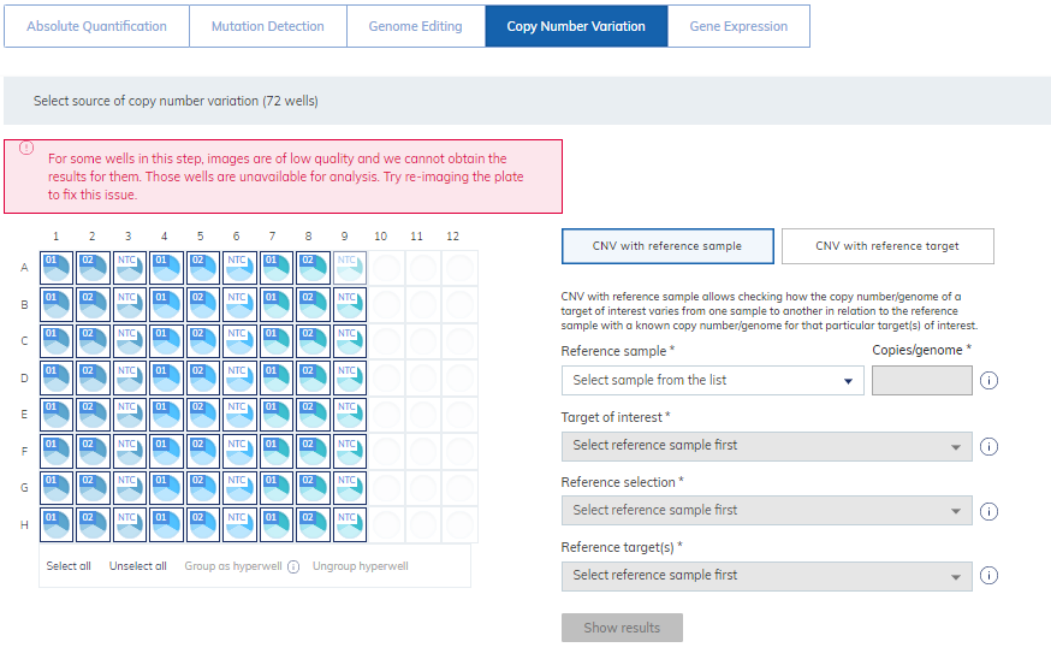
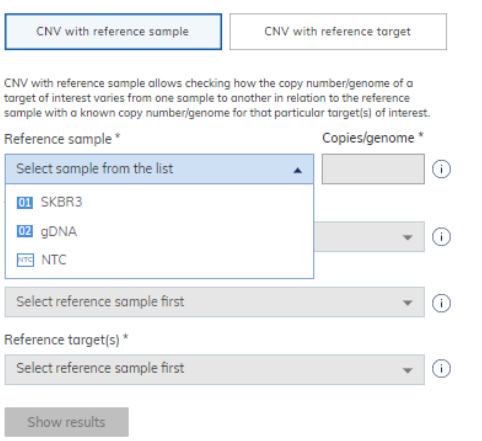


Figure 20. Second-level analysis referencing. CNV with reference sample **(left, A)**. CNV with reference target **(right, B)**.

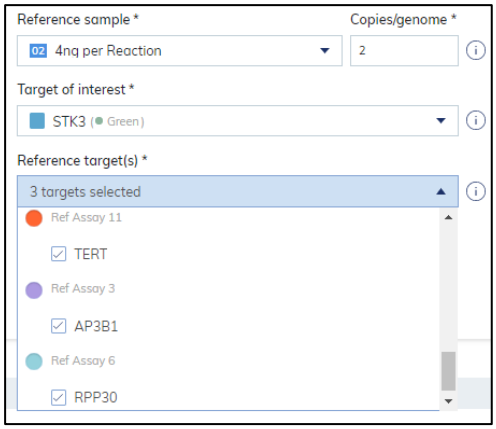
Despite the referencing method, all wells to be analyzed need to be selected.

CNV analysis with reference sample

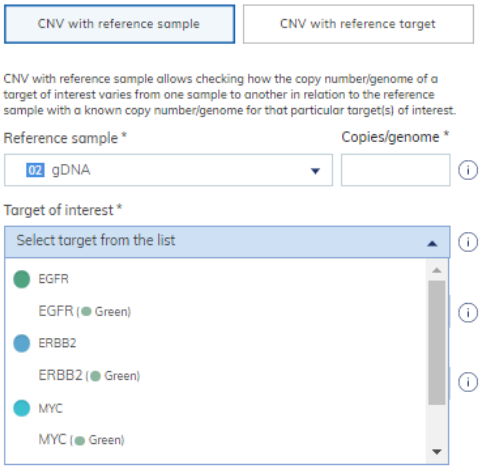
1. Select reference sample.



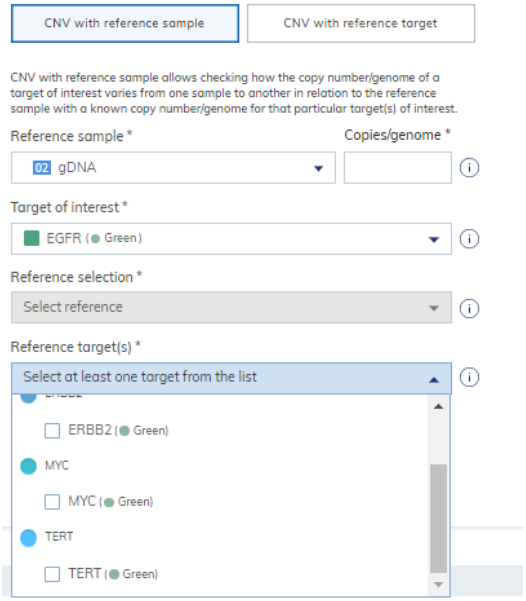
2. Enter the appropriate copies/genome for selected reference sample.



3. Select the desired target in the Target of interest field.



4. Select a reference target in the Reference target(s) field. You can select more than one reference target.



5. To view the results of the analysis, click **Show results**.

   The results are divided into several tabs: List view, Heatmap, Point diagram, and Concentration diagram. To view the contents of the tab, click the tab title.

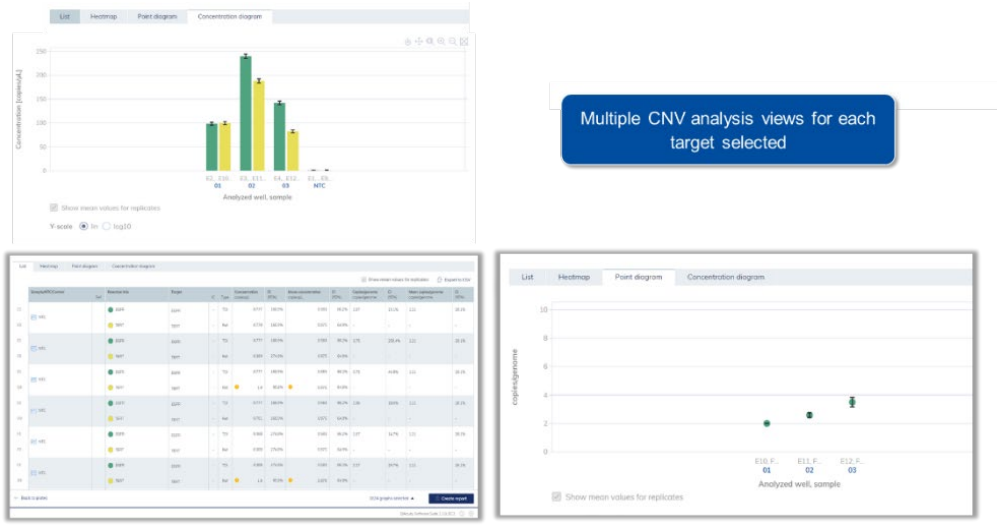


Figure 21. Multiple CNV views for each target selected.

List

The List tab contains a table with an overview of the analyzed wells. These columns are available in the table:

- **Sample/NTC/Control Name** – This column shows the sample, NTC, or control name with its corresponding icon that identifies the sample or indicates whether the entry is an NTC or control.
- **Sample/NTC/Control Reference** – This column shows your reference sample.
- **Reaction mix** – This column contains the icon and the name of the well.
- **Target name** – This column shows the target name.
- **Target type** – This column shows the target type: reference target(s) or TOI.
- **Copy number of target** – This column shows the copy number assigned to each target.
- **CI (95%)** – This column shows the value of the confidence interval for the copy numbers/genome.

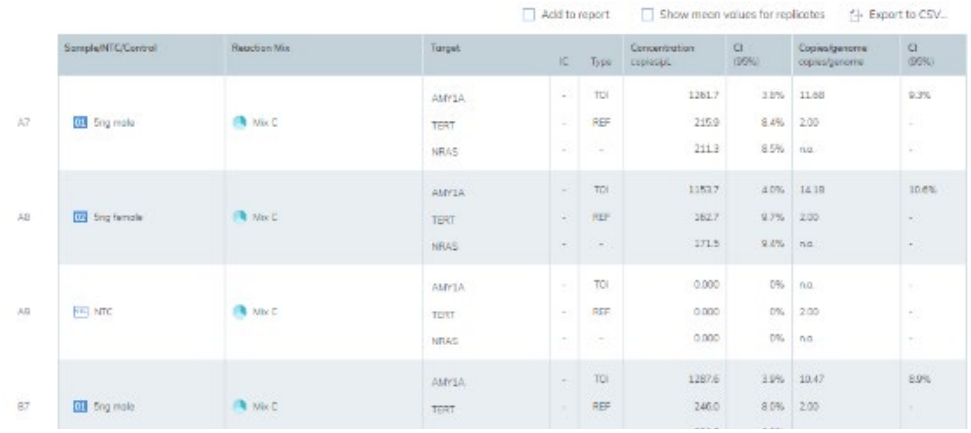


Figure 22. List tab for copy number analysis.

For more information, see *QIAcuity User Manual*, section "List tab for copy number variation".

Heatmap

The Heatmap tab contains a heatmap that shows the number of copies per genome in each of the wells.

- Select a well for the analysis, and the value will be displayed on the heatmap.
- To view an additional toolbar that enables actions related to the diagram, such as downloading the plot, hold the pointer over the diagram. For more details about the toolbar, refer to Diagram options.
- To view detailed information about a particular well, point the cursor over the well. A tooltip with detailed information opens.
- To view the mean values for replicates in multiplex tests, click **Show mean values for replicates**.

For more information, see *QIAcuity User Manual*, section "Heatmap tab for copy number variation".

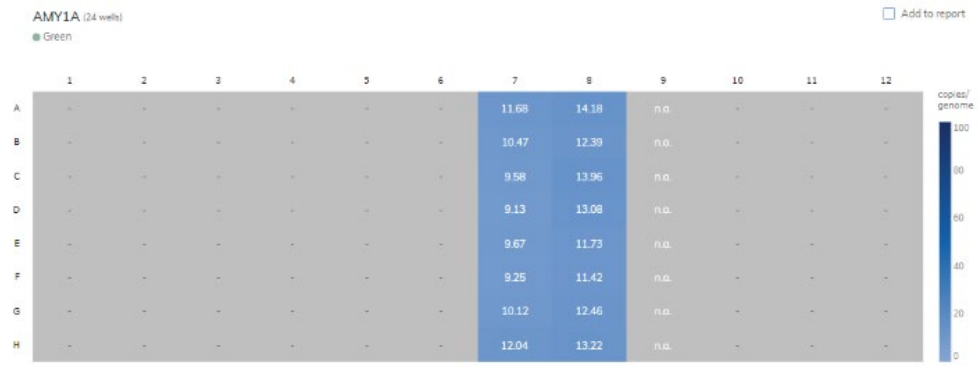


Figure 23. Heatmap tab for copy number analysis.

To add any of the heatmaps to the report, click **Add to report** next to the relevant diagram. For more information on reports, refer to the *QIAcuity User Manual*, section "Reports".

Point diagram

The Point diagram tab shows a diagram that displays number of copies/genome and its confidence interval related to the samples analyzed. A point diagram has two axes: *x*-axis shows the analyzed wells and samples, whereas the *y*-axis represents the number of copies in each genome. A confidence interval displayed as an error bar is shown for each of the points on the diagram.

For more information, see *QIAcuity User Manual*, section "Point diagram tab for copy number variation".

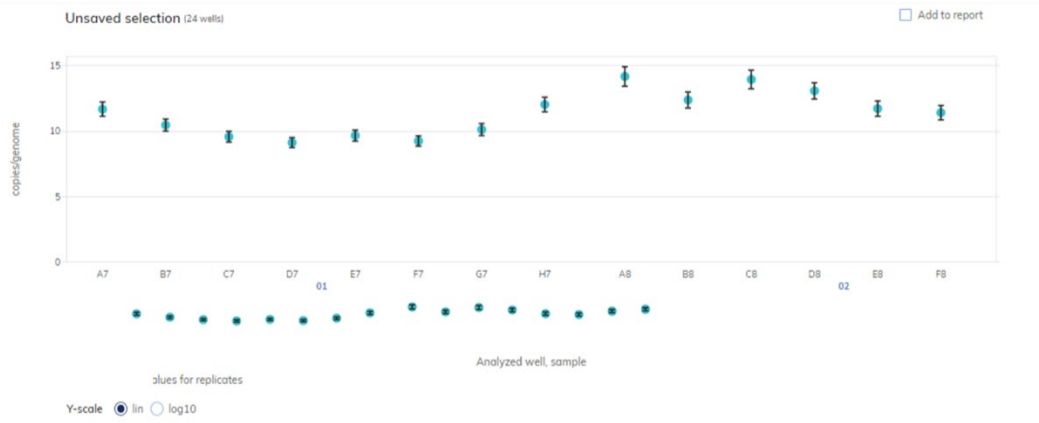


Figure 24. Point diagram tab for copy number analysis.

To add any of the point diagrams to the report, click **Add to report** next to the relevant diagram. For more information on reports, refer to the *QIAcuity User Manual*, section "Reports".

Concentration diagram tab for copy number variation

The Concentration diagram tab shows the diagram that displays the concentration values of the configured copy number variation test and the confidence intervals related to every value. A concentration diagram has two axes: *x*-axis represents samples, whereas *y*-axis represents the concentration values and their confidence intervals for targets or channels from selected wells, which are displayed as column bars and error bars, respectively.

For more information, see the *QIAcuity User Manual*, section "Concentration diagram tab for Copy number variation".

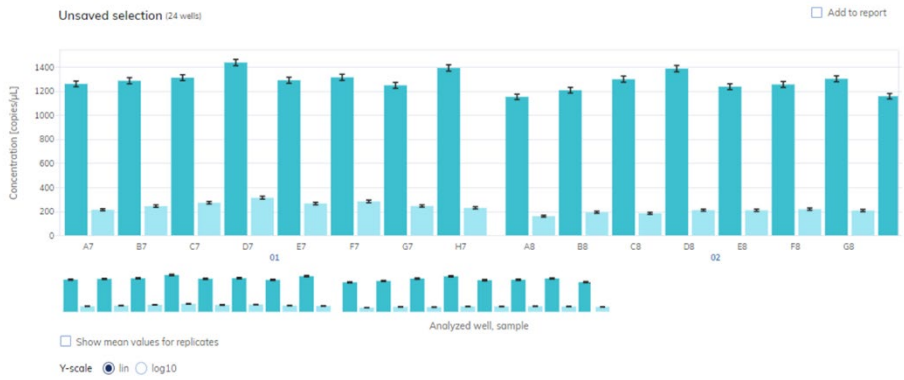


Figure 25. Concentration diagram tab for copy number analysis.

To add the Concentration diagram to the report, click **Add to report**. For more information on reports, refer to the *QIAcuity User Manual*, section "Reports".

7 Mutation Detection

7.1 Introduction

A main area of interest in many biological and pharmacological studies is the detection of mutations for the diagnosis of genetic disorders and diseases, such as cancer. The majority of genomic alterations leading to cancer or other diseases are not inherited via the germline. Rather, they are acquired in somatic cells. This results in cells or tissues harboring unique sets of mutations in an otherwise normal surrounding context. By capturing and characterizing these somatic mutations, the molecular mechanisms that underlie disease may be further elucidated, and in turn help to identify suitable drug targets.

Reliable and efficient detection of mutations present at low quantities in samples is a major challenge for researchers. Patient samples, patient-derived cell lines, and tumor biopsies are often heterogenous mixtures of cells. Therefore, the number of cells carrying a mutation of interest can be very small, which limits the abundance of mutant DNA in the samples. Thus, it is essential that the tools researchers choose for their investigations can accurately and sensitively detect mutations even at very low frequencies.

The dPCR LNA Mutation Assays combined with the QIAcuity Digital PCR platform enable very sensitive detection of rare mutations, even within a strong wildtype (WT) background. The random partitioning of template molecules in a dPCR reaction separates mutant DNA from its WT counterpart. As a result, both WT and mutant targets are efficiently amplified and accurately quantified. This is in striking contrast to traditional PCR reactions, where a single reaction mix contains highly abundant WT and rare mutant sequences, which can suppress the amplification efficiency of mutant template species, thereby leading to inaccurate quantification.

In dPCR, the frequency of a rare mutation is calculated based on the absolute number of mutant copies detected in relation to the total number of copies in a given reaction. No additional references or endogenous controls are needed for calculating mutation frequency. The very simple calculations are illustrated below.

$Conc^{MUTANT}$: concentration of mutant sequence (copies/µL)
$Conc^{WILD\ TYPE}$: concentration of WT sequence (copies/µL)

$Conc^{TOTAL}$: $Conc^{MUTANT\ ASSAY}$+ $Conc^{WILD\ TYPE.\ ASSAY}$

$Frequency^{MUTATION}$: $Conc^{MUTANT}/Conc^{TOTAL}$

7.2 Experiment planning

The convenient workflow and large number of assays available in the dPCR LNA Mutation Assays portfolio allow for fast, accurate, and highly sensitive detection of target mutation frequencies in a wide range of samples. The QIAcuity mutation detection dPCR workflow starts with purifying genomic DNA from the sample (e.g., tissue biopsy). After quality control, each purified DNA sample is mixed with the ready-to-use QIAcuity Probe PCR master mix and appropriate dPCR LNA Mutation Assays. The reaction mixtures are then aliquoted into the wells of QIAcuity Nanoplates. After the dPCR run (see section "QIAcuity system and workflow", on page 7), mutation frequencies in each sample are calculated in the QIAcuity Software Suite based on the relation of absolute copy numbers of WT and mutant molecules detected.

7.3 Considerations

We recommend the following precautions:

- Always wear a suitable lab coat, disposable gloves, and protective goggles. It is crucial to avoid contamination of DNA samples with foreign DNA, especially from previous test material.
- Use sterile pipet tips with filters.
- Decontaminate workspace and labware to avoid any foreign DNA contamination.
- Store DNA-containing material separately from all other reagents.
- Prepare DNA-containing reaction mixes in spatially separated rooms or facilities.
- Separate dPCR sample/plate setup and dPCR analysis.
- Thaw all components before preparing reaction mixes. When thawed, mix components thoroughly for obtaining homogenous solutions and centrifuge briefly to avoid spill overs.
- Pipetting accuracy and precision affect the consistency of results. Be sure that all the pipets and instruments have been checked and calibrated according to the manufacturer's recommendations.
- Use of 8-channel pipette is highly recommended for dispensing the reaction mixes into reaction wells.
- Make sure no bubbles are introduced into the wells of dPCR nanoplate during sample setup.

7.4 Sample isolation and preparation

Sample DNA quantity and purity can affect the performance of dPCR LNA Mutation Assays. It is highly recommended to check the DNA quantity and quality prior to setting up dPCR reactions.

7.4.1 DNA purification

The kits listed below are recommended for genomic DNA purification from various types of starting material for use with QIAcuity Mutation Detection dPCR Assays (Table 13). DNA purification can be carried out according to the kit instructions.

Table 13. Recommended DNA purification kits for use with QIAcuity LNA Mutation Assays

Starting material	Purification method	Cat. no.
Fresh or frozen tissues, cultured cells	QIAamp DNA Mini Kits	51304 and 51306
	DNeasy Blood & Tissue Kits	69506, 69504, 69582, and 69581
	EZ1 DNA Investigator Kit	952034
	EZ1 DNA Tissue Kit	953034
	Blood & Cell Culture DNA Mini, Midi, or Maxi Kit	13323, 13343, or 13362, respectively
	Flexigene DNA Kit	51206
	PAXgene Tissue DNA Kit	767134
FFPE tissues	QIAamp DNA FFPE Tissue Kit	56404
	GeneRead DNA FFPE Kit	180134
cfDNA from human plasma or serum	QIAamp DNA Blood Mini or Maxi Kits	51106, or 51194 and 51192, respectively
	PAXgene Blood DNA Kit	761133
	QIAamp ccfDNA/RNA Kit	55184
	EZ1 ccfDNA Mini or Midi Kit	954134 or 954154, respectively
	QIAamp MinElute ccfDNA Midi and Mini Kits	55284 and 55204, respectively
Other biological samples	Contact QIAGEN Technical Service	

Important: Do not use DEPC-treated water.
Important: Presence of RNA in the sample preparations will result in inaccurate DNA concentration measurements. Removing of RNA contamination using RNase A digest is highly recommended.
Important: Including NTCs in the experimental design will provide information about presence of any DNA contaminants.

7.5 LoD and amount of starting material

dPCR LNA Mutation Assays can be used to identify rare mutations in a wide variety of samples, including fresh frozen cell lines and tissues, FFPE samples, cfDNA samples, and tissue biopsies. Sample quality is often compromised from crosslinking treatments as well as from DNA fragmentation. Some samples contain only very low amounts of genomic DNA. Although restriction digestion of templates is not necessary for detection with dPCR, we recommend checking the quality and quantity of DNA samples using UV spectrophotometry before setting up the reactions (for details, see section “Sample preparation”, page 20).

For obtaining accurate results using QIAcuity Mutation Detection dPCR Assays, see section “Sample input amount” on page 20 for more information.

7.6 Using QIAGEN's dPCR LNA Mutation Assays for rare mutation detection

The dPCR LNA Mutation Assays portfolio offers sensitive and accurate detection of a wide range of mutations arising from base-pair exchanges, insertions, or deletions. Each assay is designed strategically to optimize discrimination of mutated sequences from their nonmutated counterparts or from similar sequences. Locked nucleic acid (LNA) bases are placed within PCR amplification primers and target-specific probes, ideally positioned at or adjacent to the mutation sites. Incorporation of LNAs provides multiple advantages compared to standard nucleic acids, such as (1) specific detection of shorter DNA targets, (2) higher affinity of binding to complementary DNA sequence, (3) superior single nucleotide discrimination, and (4) increased target specificity and discrimination.

dPCR LNA Mutation Assays are available in an easy-to-handle single tube format. Each assay tube comes with primers to amplify the genomic region bearing the mutation and two probes for detecting mutant and WT sequences in the same reaction. Users have two choices for detection dye combinations: FAM + HEX or ATTO550 + ROX. Mutant sequences are detected with FAM or ATTO550 labeled probes, while WT sequences are detected with HEX or ROX labeled probes. For detection of two unique mutations simultaneously, users can multiplex two assays with compatible dyes in one reaction (e.g., FAM + HEX dyes for one assay, and ATTO550 + ROX dyes for the second assay.).

All these features make QIAGEN's LNA-enhanced mutation assays optimum tools for detection of low abundance rare mutation events with high levels of discrimination.

7.7 dPCR LNA Mutation Assays and dPCR Probe Kit on the QIAcuity

The QIAcuity Digital PCR platform allows for highly sensitive and accurate detection of low frequency mutations. The QIAcuity instrument has been tested with a wide range of mutation detection assays from different manufacturers. For best usability and performance, we recommend using the QIAcuity Probe PCR Kit and QIAcuity dPCR LNA Mutation Assays on the QIAcuity Digital PCR Platform. The QIAcuity Probe PCR Kit is developed for optimal performance of the QIAcuity dPCR LNA Mutation Assays on the QIAcuity Digital PCR platform. The hot-start QuantiNova DNA Polymerase enzyme, along with other proprietary chemical components in QIAcuity Probe PCR Kit, is essential for delivering the highest performance in mutation detection.

Important: The QIAcuity Probe PCR Kit is developed for optimum performance of dPCR LNA Mutation Assays in the QIAcuity Digital PCR platform. Do not add any additional reagents in the QIAcuity Probe PCR master mix.

The dPCR LNA Mutation Assays can be ordered through **geneglobe.qiagen.com**. All assays are in-silico validated for use in dPCR. A subset of key assays targeting highly studied mutation sites has also been validated experimentally on the QIAcuity Digital PCR instrument. These targets are carefully selected from peer-reviewed publications based on their function and based on their association with a cancer-related gene, pathway, or genetic disease phenotypes.

The QIAcuity Probe PCR Kit is developed for optimal performance of the QIAcuity dPCR LNA Mutation Assays in QIAcuity Digital PCR platform. The hot-start QuantiNova DNA Polymerase enzyme, along with other proprietary chemical components in QIAcuity Probe PCR Kit, is essential for delivering highest performance in mutation detection.

7.8 Recommendation of a QIAcuity Nanoplate

For highly sensitive and accurate detection of mutations using the QIAcuity Digital PCR platform, we recommend using 26K Nanoplates. In this nanoplate format, the reaction mix and template are evenly distributed across approximately 26,000 partitions. The large number of partitions in each well increases the accuracy of copy number determination. However, 8.5K Nanoplates can also be used according to experimental needs. These may include high throughput analyses where large number of samples must be tested using low input volumes. For detailed information, see **www.qiagen.com/qiacuity-nanoplates-and-accessories-productdetails**

7.9 Mutation analysis in QIAcuity Software Suite

The QIAcuity Software Suite allows users to easily determine the mutation frequencies in their target samples. The analysis begins with absolute quantification of WT and mutant target present in a reaction, followed by calculating the frequency of mutation in a reaction.. For additional information regarding setting up the plates and samples, see *QIAcuity User Manual*, section "Setting up an experiment".

7.9.1 Setting up a mutation detection analysis

1. Setup plate configuration: general data, dPCR parameters, reaction mixes, sample, and plate layout. Analyze the results after completion of dPCR run.
2. Carry out absolute quantification of selected or all wells.
3. Select wells and TOIs in the Select wells panel.
4. Select the relevant WT and mutant target present in the wells chosen for analysis.
5. If you want to save the analysis, enter a name in the Test name field. Click **Save** as test to save the analysis.
6. To view the results of the analysis, click **Show results**.

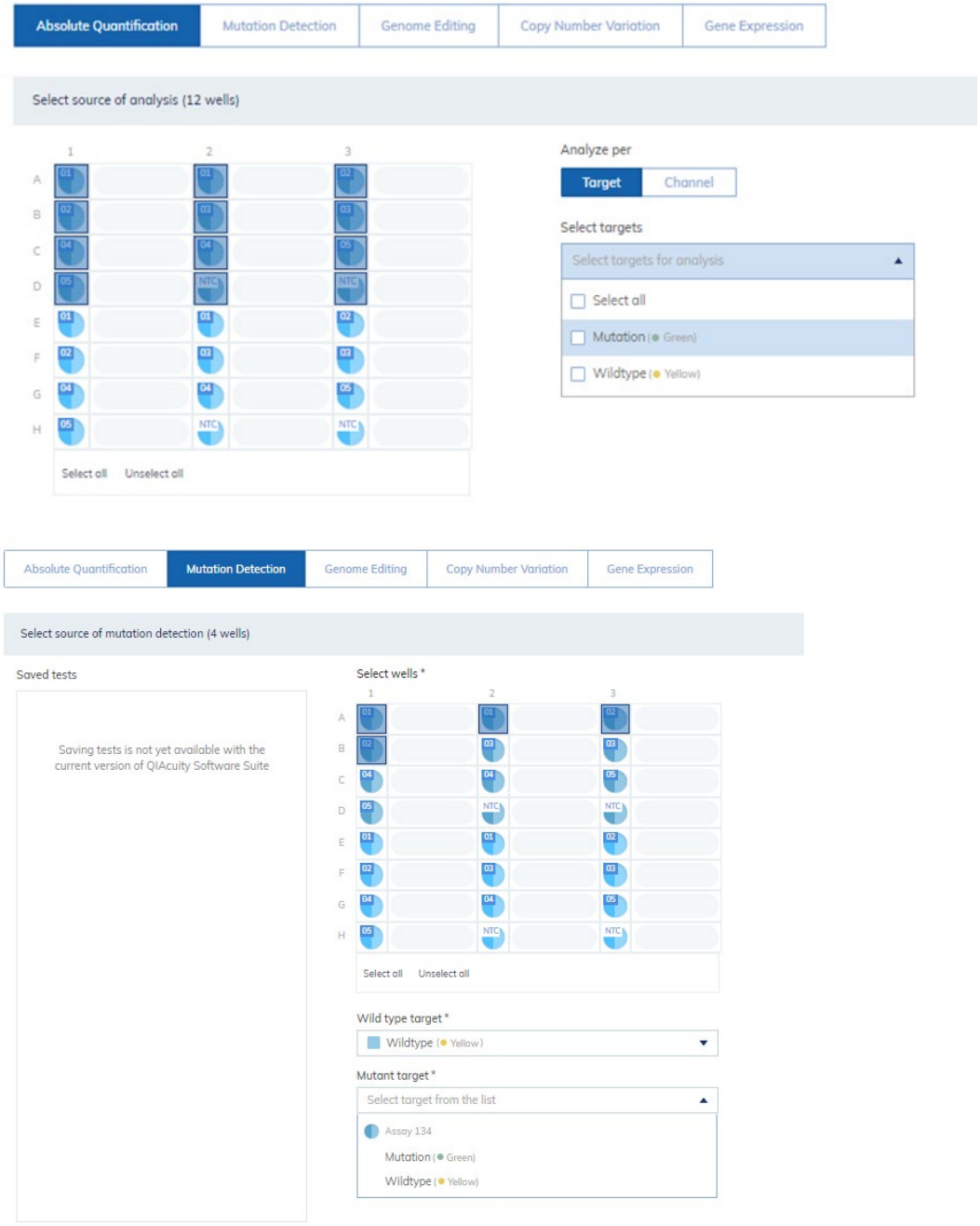


Figure 26. Second level mutation detection analysis.

The results are divided into several tabs: List view, Signalmap, Heatmap, Histogram, 1D and 2D Scatterplots, as well as Concentration diagram. To view the contents of each tab, click the tab title. For additional information regarding absolute quantification analysis, see *QIAcuity User Manual*, section "Analysis".

An important consideration for accurate mutation analysis is the correct threshold setting in 1D and 2D Scatterplots. Auto thresholding or manual thresholding can be performed to separate positive and negative partitions correctly. Pressing the **Auto-threshold** button will set the threshold automatically. Individual thresholds can be adjusted manually by clicking into the individual wells and moving the red threshold line vertically. Once the threshold is changed, analysis can be updated by pressing **Recalculate**.

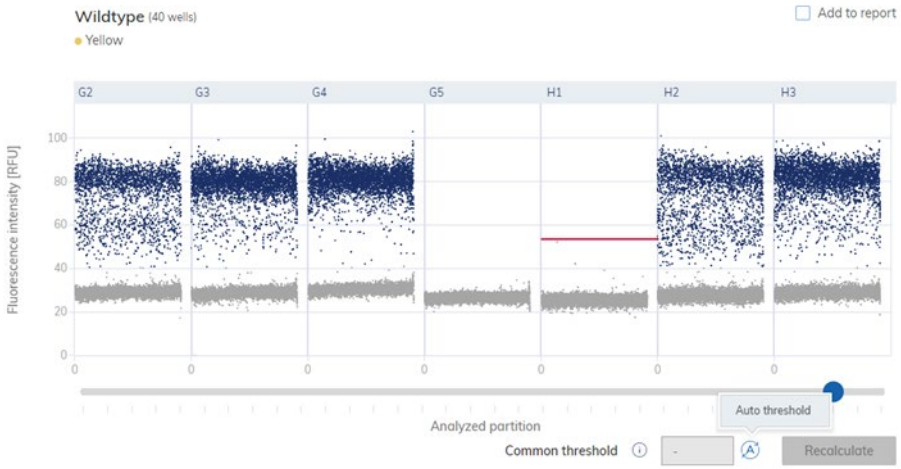
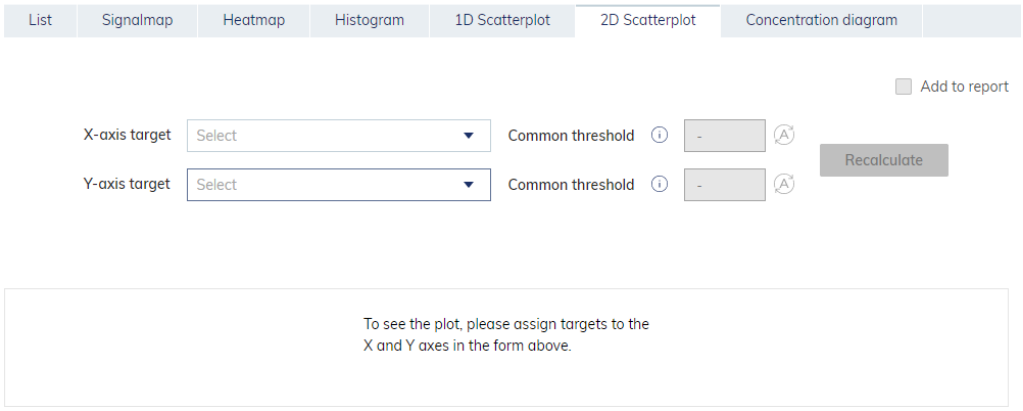
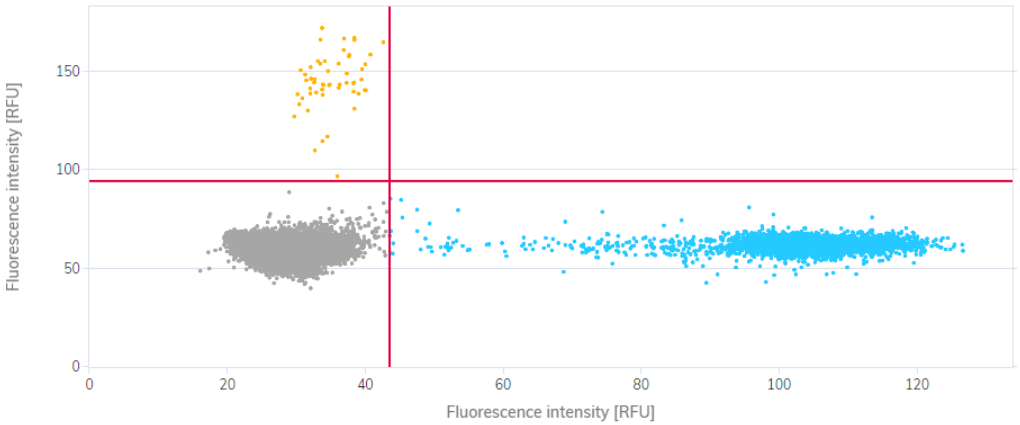


Figure 27. Exemplary 1D scatterplot for WT probe and yellow channel.

Two dimensional scatterplots can be created by selecting the *x*- and *y*-axis targets from drop-down menu. Pressing the **Auto-threshold** button will set the threshold manually. The threshold can also be changed manually by inserting axis values. Analysis can be updated with respect to a new threshold by pressing **Recalculate**.

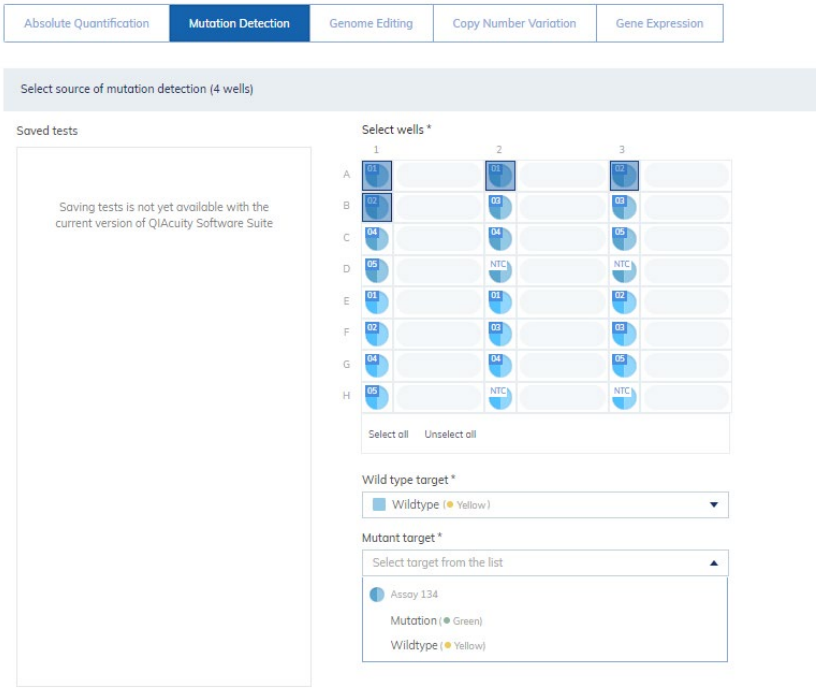




Upon assigning the MUT and WT targets to *x*- and *y*-axes, different clusters of positive and negative partitions can be visualized. Two dimensional scatterplots provide a visual readout for analysis: each colored dot represents readout from WT and MUT probes in each single partition. Navy, orange, blue, and gray colors represent WT+/MUT+, WT+/MUT–, MUT+/WT–, and MUT–/WT– populations, respectively. For free hand selection, a lasso tool can be used to assign partitions to individual populations.

For mutation analysis, switch to Mutation Detection tab. To view the contents of the tab, click the tab title.

1. Select wells. Select relevant WT and mutant target.



2. Click **Show Results**. The results are divided into several tabs: List view, Heatmap, Point diagram, and Concentration diagram. To view the contents of each tab, click the tab title.
3. Add desired results views and graphs by clicking **Add to report**.
4. Create report.

7.9.2 List

The List tab contains a table with an overview of the analyzed wells. These columns are available in the table:

- **Sample/NTC/Control** – This column shows the sample, NTC, or control name with its corresponding icon that identifies the sample or indicates whether the entry is an NTC or control.
- **Reaction mix** – This column contains the icon and the name of the reaction mix.
- **Target type** – This column shows the target type: MUT or WT.
- **Concentration (copies/µL)** – This column shows the concentration assigned to each MUT and WT target.
- **CI (95%)** – This column shows the value of the confidence interval.
- **Mutant fraction** – This column shows the percentage of mutant copies per sample.
- **CI (95%)** – This column shows the value of the confidence interval for the copies per genome.

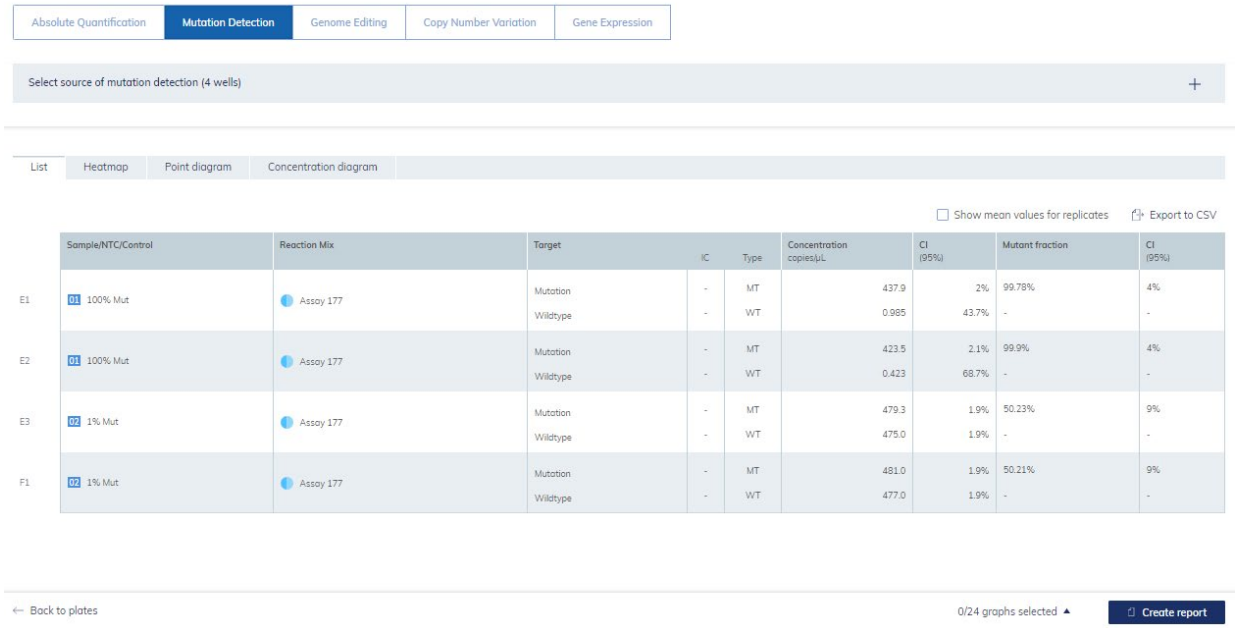


Figure 28. List tab for Mutation Detection.

7.9.3 Heatmap

The Heatmap tab contains heatmaps that show the fraction of mutation for each sample. One heatmap is created for each test that was done.

If a well is not selected as a source for the analysis, the value is not displayed on the heatmap and its background color is gray.

For more details about the toolbar, refer to the *QIAcuity User Manual*, section "Heatmap tab for mutation detection".

With the mouse, hover over a well to see a more detailed information.

To view the mean values for replicates, click **Show mean values for replicates**.

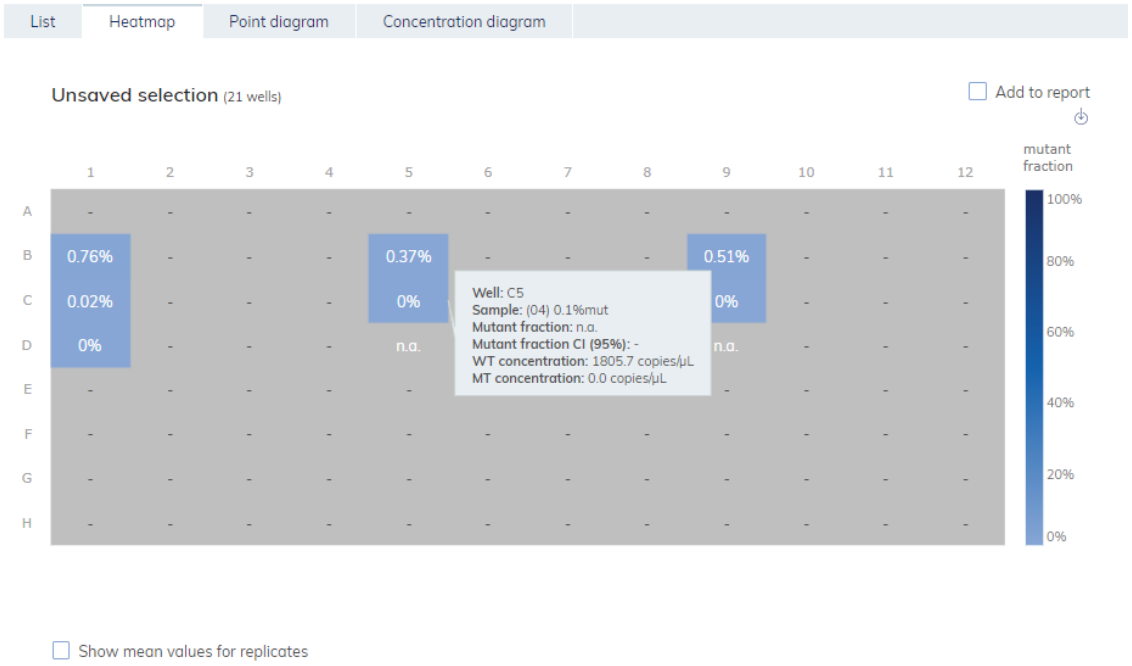


Figure 29. The Heatmap tab.

To add any of the heatmaps to the report, click **Add to report** next to the relevant diagram. For more information on reports, refer to the *QIAcuity User Manual*, section "Reports".

7.9.4 Point diagram

The Point diagram tab shows the diagrams that display mutant fraction per sample in each analyzed well and the confidence intervals related to every value. One diagram is created for each test that was done. A point diagram has two axes. The *x*-axis shows the analyzed wells and samples, and the *y*-axis represents the edited fraction, shown as a percentage. A confidence interval displayed as an error bar is shown for each of the points on the diagram.

With the mouse, hover over the diagram to view an additional toolbar that enables you to download the plot. For more details about the toolbar, refer to the *QIAcuity User Manual*, section "Point diagram tab for mutation detection".

With the mouse, hover over a well to see a more detailed information about the concentration and fraction of each MUT and WT target per sample.

To view the mean values for replicates, click **Show mean values for replicates**.

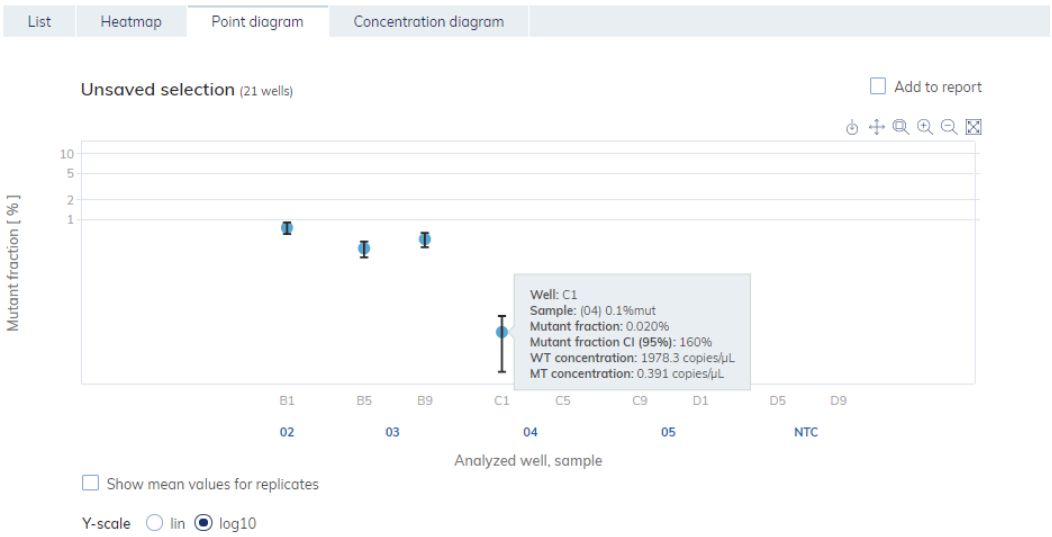


Figure 30. The Point diagram tab.

To add any of the point diagrams to the report, click **Add to report** next to the relevant diagram. For more information on reports, refer to the *QIAcuity User Manual*, section "Reports".

7.9.5 Concentration diagram

The Concentration diagram tab shows the diagrams that display the distribution of concentration values in the wells and confidence intervals. One diagram is created for each test that was run. A concentration diagram has two axes. The *x*-axis shows the analyzed wells and samples, and the *y*-axis represents the concentration values. The values on the *y*-axis have two available scales – linear and logarithmic.

With the mouse, hover over the diagram to view the additional options. An additional toolbar and the threshold value are shown. For more information about the toolbar, refer to the *QIAcuity User Manual*, section "Concentration diagram tab for mutation detection".

The *y*-axis scale can be modified using the radio buttons located below each graph. To view the values on a linear scale, click **lin**. To view the values on a logarithmic scale, click **log10**.

Each diagram presents two values for each well – the concentration value, displayed as a bar, and the confidence interval, displayed as an error bar. To view the exact values, hover over to one of the bars with the mouse. This will also reveal more detailed information about concentration and fraction of each MUT and WT target per sample.

To view the mean values for replicates on the diagram, click **Show mean values for replicates**. To add any of the concentration diagrams to the report, click **Add to report** next to the relevant diagram.

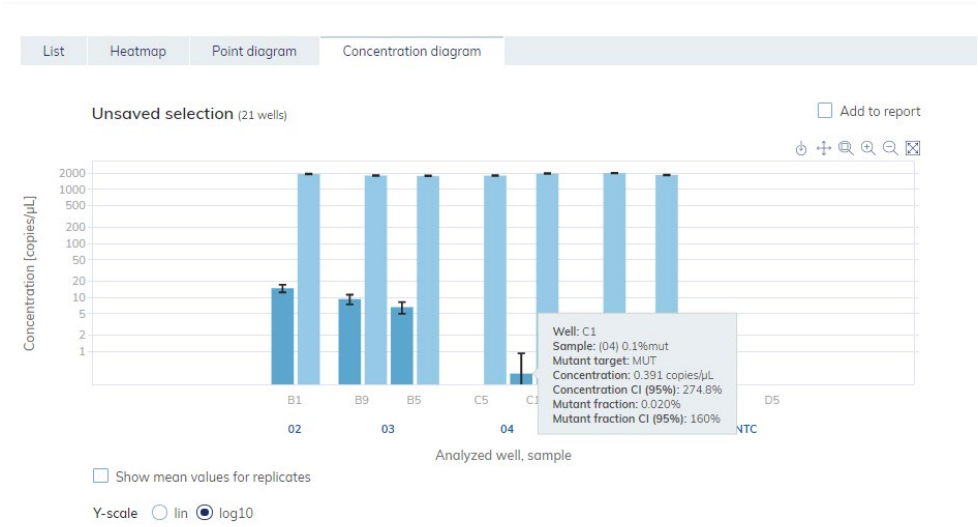


Figure 31. The Concentration diagram tab.

8 Gene Expression Analysis Using the QIAcuity

8.1 Introduction

Gene expression analysis is based on the quantification of mRNA. The general assumption is that higher quantification reflects a higher level of gene expression. Unlike with DNA, RNA requires a pre-processing step to be PCR amplified, commonly referred to as cDNA synthesis. In this step, the RNA is used as a template and a complementary strand of DNA is being synthesized.

Today there are two common ways to facilitate cDNA synthesis – one step and two step RT-PCR.

In a two-step protocol, cDNA synthesis and PCR amplification are separate reactions. First, RNA is transcribed into cDNA usually using a universal oligo(dT) primer, random hexamers, or a mixture of both as starting points. The cDNA reaction is then used as a template in further PCR reactions. This approach offers the advantage of generating a separate template that can be used repeatedly across multiple experiments. This lets users determine optimal PCR conditions and different detection chemistries (EvaGreen or Probe based).

In a one-step protocol, cDNA synthesis and PCR are combined in a single reaction containing RNA template. Importantly, the cDNA synthesis is performed using the same primers as those that are later used in the PCR step. This ensures only the relevant mRNA fragment is being transcribed into cDNA and subsequently amplified.

A hybrid approach can be used for highly damaged RNA extracted from FFPE samples. The basis for this approach is the two-step protocol. However, unlike the classic two-step method, gene-specific primers are used for the reverse transcription. Up to 20 different primers can be used to generate target specific cDNA fragments. Following reverse transcription, standard PCR is performed with one of the gene specific primer pairs.

Perhaps the most common PCR method for studying gene expression with two-step or one-step approaches is quantitative PCR (RT-qPCR). RT-qPCR has a broad linear range and is capable of quantifying low to high abundant transcripts. However, one disadvantage of RT-qPCR is that it only provides relative expression levels that are normalized to a reference target (e.g., housekeeping gene). Additionally, small changes in expression, in the range of 10–20%, cannot be reliably detected with RT-qPCR.

These limitations can be overcome by using two-step or one-step RT-dPCR. When using the correct dilution factor (see section "General considerations for performing gene expression analysis on the QIAcuity Digital PCR instrument", page 63), each transcript of interest will lead to a positive partition in the QIAcuity Nanoplate. As random distribution can lead to more than one target molecule in a single partition, the absolute number of target molecules is extrapolated from the number of positive partitions using Poisson statistics (see section "Statistics of nanoplate dPCR", page 9) without the need for using a reference target. It is nevertheless necessary to use reference targets in case the concentration of the input template differs between samples.

8.2 QIAcuity solutions for gene expression analysis

8.2.1 Two-step RT-dPCR with the QIAcuity EG PCR Kit and the QIAcuity Probe PCR Kit

The QIAcuity EG PCR Kit and the QIAcuity Probe PCR Kit were developed as universal QIAcuity dPCR kits capable of delivering the highest performance in gene expression analysis.

High specificity and sensitivity in dPCR are achieved by a hot-start procedure. This allows for room-temperature setup of the PCR reaction setup without the risk of primer–dimer formation. The hot start is achieved using QuantiNova DNA Polymerase, which is a novel hot-start enzyme, and the additive QuantiNova Guard. These unique components further improve the stringency of the antibody-mediated hot start.

8.2.2 One-step RT-dPCR with the QIAcuity OneStep Advanced EG and QIAcuity OneStep Advanced Probe Kits

The QIAcuity OneStep Advanced EG Kit and QIAcuity OneStep Advanced Probe Kit were developed to provide users with the convenience of one-step RT-dPCR while ensuring accurate, sensitive, and specific amplification. In addition to a hot-start DNA polymerase, both kits contain a novel hot-start reverse transcriptase for heat-mediated activation of the reverse-transcription step. The hot-start reverse transcriptase comes loaded with an RT-blocker, rendering the enzyme almost inactive at ambient temperatures. Upon starting the RT-dPCR protocol with the RT step at 50°C, the inhibitor is released from the reverse transcriptase and cDNA synthesis is initiated. This allows for room-temperature RT-PCR reaction setup. It also lets users take full advantage of the QIAcuity Four and QIAcuity Eight instruments. Users can run up to four and eight plates in parallel on the QIAcuity Four and QIAcuity Eight instruments, respectively, and obtain the same accurate, sensitive, and specific quantification across all plates.

8.2.3 Using QuantiNova LNA PCR Assays with EvaGreen (EG) QIAcuity chemistries

The QIAcuity EG PCR Kit and QIAcuity OneStep Advanced EG Kit have been tested with a wide range of gene expression assays from different manufacturers. For best performance, we recommend using the QuantiNova LNA PCR Assays.

The LNA-enhanced QuantiNova LNA PCR Assays and Panels deliver highly sensitive and accurate quantification of mRNA and lncRNA targets in an easy-to-handle format. They are designed to work in two-step or one-step workflows that use either qPCR or dPCR. Thus, the QuantiNova LNA PCR Assays provide a versatile and flexible tool for gene expression analysis across multiple platforms.

The QuantiNova LNA PCR Assays can be ordered through **geneglobe.qiagen.com**. All assays are in silico validated for use in dPCR. A subset of key assays has also been validated experimentally on the QIAcuity instrument.

8.2.4 Using hydrolysis probe assays on the QIAcuity

The QIAcuity Probe PCR Kit and QIAcuity OneStep Advanced Probe Kit have been tested with probe-based gene expression assays from different manufacturers. Across the board, third party assays showed compatibility with both the QIAcuity Probe PCR Kit and QIAcuity OneStep Advanced Probe Kit.

For custom assays, we recommend following the same rules used to design probe-based gene expression assays for qPCR. Therefore, the vast majority of existing qPCR assays can be directly used in dPCR on the QIAcuity instrument with little to no adjustment required (see section "Assay design guidance", page 16).

8.3 RNA isolation

The most important prerequisite for any gene expression analysis experiment is consistent, high quality RNA for every experimental sample. Residual traces of proteins, salts, or other contaminants may degrade the RNA or decrease the efficiency of enzyme activities necessary for optimal RT and PCR performance.

8.3.1 Recommended RNA preparation methods

High-quality total RNA for your digital RT-PCR experiment should be prepared using one of the methods described below, depending on the biological sample. For optimal results, RNA samples should be eluted in RNase-free water.

Important: Do not use DEPC-treated water.

Table 14. Recommended RNA preparation methods

Sample type	Recommendation
Cultured cells	Use the QIAwave RNA Mini Kit (cat. no. 74536) or the RNeasy Plus Mini Kit (cat. no. 74134)
Tissue samples	Use the QIAwave RNA Mini Kit or the RNeasy Plus Mini Kit for RNA purification. **Note**: Some tissues, such as spleen and thymus, contain very high amounts of DNA, which will overload the gDNA Eliminator spin column. For these tissues, we recommend using the RNeasy Mini Kit (cat. no. 74104) in combination with the RNase-Free DNase Set (cat. no. 79254).
FFPE samples	RNeasy FFPE Kit (cat. no. 73504)
Small samples yielding <100 ng total RNA	RNeasy Plus Micro Kit (cat no. 74034)
Whole blood samples	PAXgene Blood RNA Kit (cat. no. 762164) or the QIAamp RNA Blood Mini Kit (cat. no. 52304)
Total RNA isolated using a phenol-based method (e.g., QIAzol® Lysis Reagent, TRIzol Reagent, RNAzol® Reagent)	Purify further using the RNeasy Mini Kit. **Important**: Perform the on-column DNase digestion step described in the *RNeasy Mini Handbook*, **www.qiagen.com/HB-0435**.
Other biological samples	Contact QIAGEN Technical Service

8.3.2 Storage of RNA

Purified RNA may be stored at –30°C to –15°C or –90°C to –65°C in RNase-free Water. Under these conditions, no degradation of RNA is detectable after 1 year.

8.3.3 Quantification of RNA

For best results from the QuantiNova PCR Assays and Panels, all RNA samples should also demonstrate consistent quality according to the following criteria for concentration and purity, as determined by UV spectrophotometry and gel electrophoresis.

The concentration of RNA should be determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer (see "Spectrophotometric quantification of RNA", below). For small amounts of RNA, however, it may be difficult to determine amounts photometrically. Small amounts of RNA can be accurately quantified using QIAxcel®, Agilent® 2100 Bioanalyzer, or fluorometric quantification.

8.3.4 Spectrophotometric quantification of RNA

To ensure significance, A_{260} readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 44 µg of RNA per mL (A_{260} =1 → 44 µg/mL). This relation is valid only for measurements at a neutral pH. Therefore, if it is necessary to dilute the RNA sample, this should be done in a buffer with neutral pH. The ratio between the absorbance values at 260 nm and 280 nm gives an estimate of RNA purity. As discussed below (see "Purity of RNA"), the ratio between absorbance values at 260 nm and 280 nm gives an estimate of RNA purity.

When measuring RNA samples, be certain that cuvettes are RNase free, especially if the RNA is to be recovered after spectrophotometry. This can be accomplished by washing cuvettes with 0.1 M NaOH, 1 mm EDTA, followed by washing with RNase-Free Water. Use the buffer in which the RNA is diluted to zero the spectrophotometer. An example of the calculation involved in RNA quantification is shown below:

Volume of RNA sample = 100 µL
Dilution = 10 µL of RNA sample + 490 µL of 10 mm Tris·Cl, pH 7.0 (1/50 dilution)

Measure absorbance of diluted sample in a 1 mL cuvette (RNase free).

A_{260} = 0.2
Concentration of RNA sample = 44 µg/mL x A_{260} x dilution factor
= 44 µg/mL x 0.2 x 50
= 440 µg/mL
Total amount = concentration x volume in milliliters
= 440 µg/mL x 0.1 mL
= 44 µg of RNA

8.3.5 Purity of RNA

The ratio of the readings at 260 and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV spectrum, such as protein. However, the A_{260}/A_{280} ratio is influenced considerably by pH. Because water is not buffered, the pH and the resulting A_{260}/A_{280} ratio can vary greatly. Lower pH results in a lower A_{260}/A_{280} ratio and reduced sensitivity to protein contamination (1). For accurate values, we recommend measuring absorbance in 10 mm Tris·Cl, pH 7.5. Pure RNA has an A_{260}/A_{280} ratio of 1.9–2.1* in 10 mm Tris·Cl, pH 7.5. Always be sure to calibrate the spectrophotometer with the same solution used for dilution.

For determination of RNA concentration, however, we recommend dilution of the sample in a buffer with neutral pH since the relationship between absorbance and concentration (A_{260} reading of 1 = 44 µg/mL RNA) is based on an extinction coefficient calculated for RNA at neutral pH (see "Spectrophotometric quantification of RNA").

8.3.6 Integrity of RNA

Run an aliquot of each RNA sample on a QIAxcel Connect instrument (cat. no. 9003110) with the QIAxcel RNA QC Kit v2.0 (cat. no. 929104), on an Agilent Bioanalyzer using an RNA 6000 Nano LabChip®, or on a denaturing agarose gel (Figure 32). For RNA derived from biological samples (e.g., human cells), verify that there are sharp bands/peaks present for the appropriate ribosomal RNAs (e.g., human 18S and 28S). For synthetic RNA samples (e.g., in vitro transcripts), verify that there are sharp peaks at the size expected for that RNA molecule. Any smearing of the RNA bands or shoulders on the RNA peaks indicate that degradation has occurred in the RNA sample.

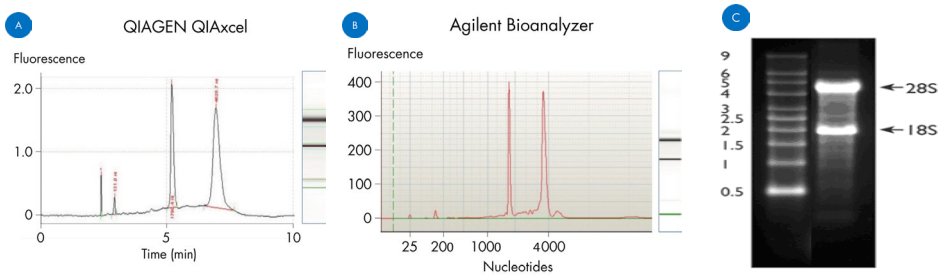


Figure 32. Ribosomal RNA integrity. (A) QIAxcel and **(B)** Agilent Bioanalyzer electropherogram of high-quality total RNA from human cells showing sharp peaks for the 18S (left) and 28S (right) ribosomal RNA. Due to the high quality of the RNA, peaks do not have shoulders (especially to the left of each peak). **(C)** Agarose gel electrophoresis shows sharp bands (especially at the bottom of each band) for 28S and 18S ribosomal RNA.

* Values up to 2.3 are routinely obtained for pure RNA (in 10 mM Tris·Cl, pH 7.5) with some spectrophotometers.

8.3.7 Genomic DNA contamination

Unwanted signals generated by contaminating genomic DNA in a sample is limited by the design of the QuantiNova LNA PCR Assays. If the gene structure allows, QuantiNova LNA PCR Assays make use of primers that target different exons while spanning a large intron in the mRNA (intron-spanning assay). GeneGlobe indicates if intron-spanning assay design is not possible by the warning “Important: this assay may detect gDNA”.

To remove genomic DNA contamination from your RNA samples, we strongly recommend RNA purification using the RNeasy/RNeasy Plus products, including the optional on-column DNase digestion step.

8.3.8 cDNA synthesis for two-step RT-dPCR

Use of the QuantiTect Reverse Transcription Kit for cDNA synthesis is critical for obtaining optimal results with QuantiNova LNA PCR Assays or Panels. Please follow the instructions provided in the *QuantiTect Reverse Transcription Kit Handbook*.

8.4 General considerations for performing gene expression analysis on the QIAcuity Digital PCR instrument

8.4.1 Amount of sample input and dilutions

The number of transcripts is calculated using Poisson statistics based upon the total number of positive partitions. In extreme cases, that is:

- Samples with low number of transcripts: expression values might vary from the true expression as a rare transcript could be lost in the dead volume or as the rare transcript might not be correctly represented in the fraction of the original cDNA that was used for quantification.
- Samples with high number of transcripts: with increasing number of transcripts being randomly distributed over the partitions, a partition could contain multiple transcripts. In extreme cases, when all partitions are positive, it will no longer be possible to conduct any quantification.

Table 15 provides guidance on the dynamic range of the different QIAcuity Nanoplates for gene expression analyses.

Table 15. Calculated dynamic range of template input amounts for fold change spectra in gene expression assays with the QIAcuity Nanoplates

Nanoplate	Reaction volume/ well	Fold change spectrum	Lower limit	Sweet spot (copies/partition)					
			1 Positive partition	0.001	0.01	0.1	0.5	2	
8.5K	12 µL		4.1	35.7	351.7	3517	17,586	70,344	Number of input template molecules per reaction well
		5 fold					X	X	
		20 fold				X	X	X	
		200 fold			X	X	X	X	
		2000 fold		X	X	X	X	X	
		17,000 fold	X	X	X	X	X	X	
26K	40 µL		1.7	43.33	433.3	4333	21,667	86,667	Number of input template molecules per reaction well
		5 fold					X	X	
		20 fold				X	X	X	
		200 fold			X	X	X	X	
		2000 fold		X	X	X	X	X	
		50,980 fold	X	X	X	X	X	X	

Calculations are based on 8500 partitions with 0.34 nL per partition for the 8.5K Nanoplate and 26,000 partitions with 0.91 nL per partition for 26K Nanoplates. Depending on the nanoplate used, 8.5K or 26K, fold change spectra cover different amounts of input template molecules per well. With a maximum of 2 copies per partition the upper limits for input amounts per well are 70,344 template molecules for the 8.5K Nanoplate and 86,666 template molecules for the 26K Nanoplate. The maximum lower limit of a single positive partition translates into 4.1 and 1.7 template molecules per well and a coverage of fold changes of 17,000 and 50,980 for the 8.5K and 26K Nanoplates, respectively.

cDNA input amount for two-step RT-dPCR

In case the expression level of your gene of interest is unknown, we recommend diluting the cDNA 1:10 and 1:100 after using the QuantiTect Reverse Transcription Kit for two-step RT-dPCR.

RNA input amount for one-step RT-dPCR

When evaluating gene expression in biological systems like cultured cells or animal models, the abundance of transcript from a particular gene of interest is apt to be unknown. Therefore, using between 0.5 and 5 ng total RNA per reaction is recommended as a starting point.

In particular scenarios, low abundant transcripts may require large amounts of input RNA in order to be detected. When using the QIAcuity OneStep Advanced EG Kit, users are advised to add no more than 200 ng of total RNA per 8.5K Nanoplate reaction (Figure 33) or 500 ng of total RNA per 26K Nanoplate reaction (Figure 34). Adding more than the recommended amount of RNA to a reaction will generate background fluorescence that makes thresholding between positive and negative partitions difficult or impossible.

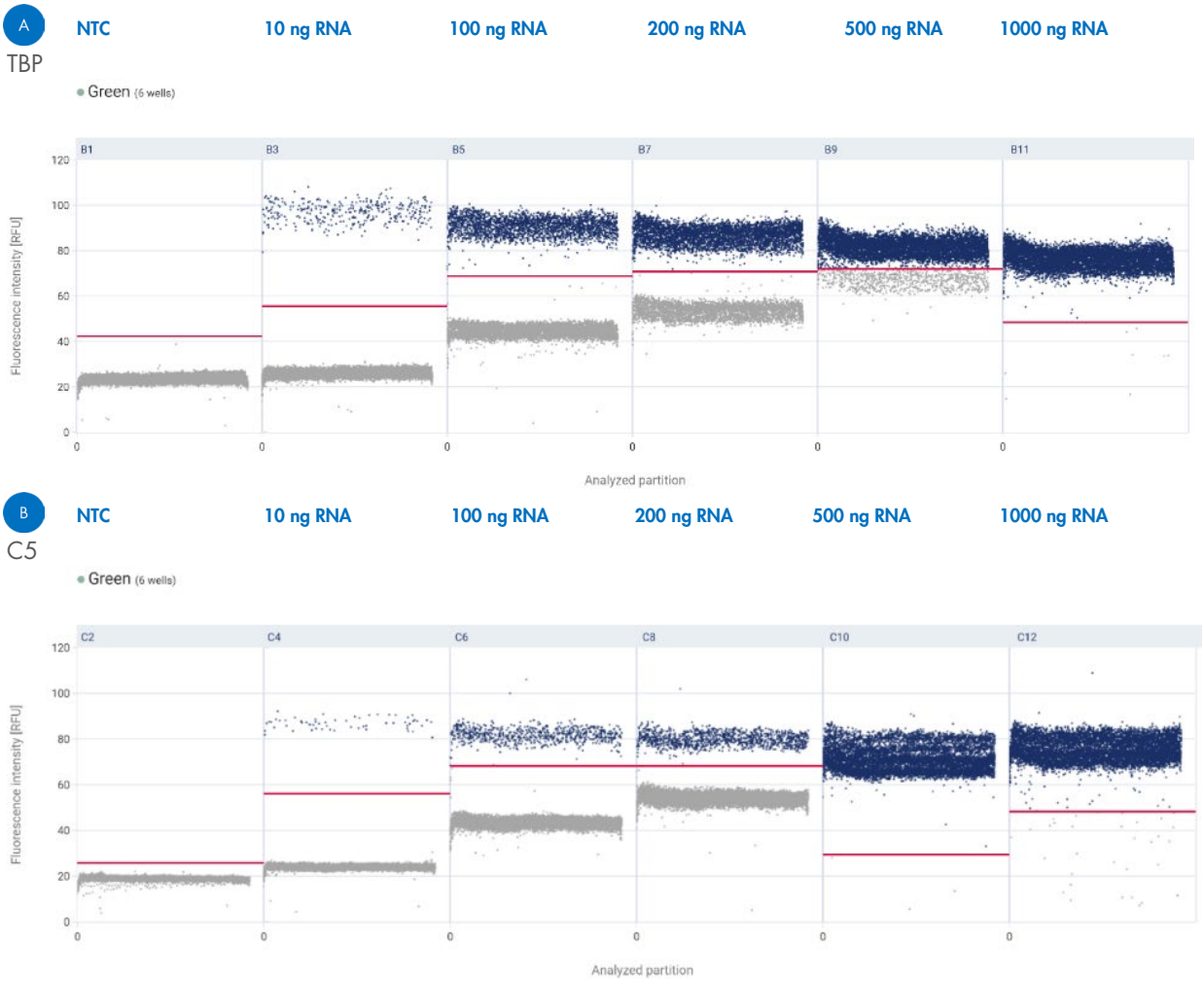


Figure 33.Total RNA amounts should not exceed 200 ng per reaction when using 8.5K Nanoplates. QuantiNova LNA Assays targeting TBP (Figure 30A) and C5 (Figure 30B) mRNA were used in QIAcuity OneStep Advanced EvaGreen reactions in 8.5K Nanoplates following recommended cycling protocols. Between 10 and 1000 ng of total RNA isolated from cultured human leukocytes were used as template per reaction. In reactions containing more than 200 ng total RNA, the separation between negative partitions (gray) and positive partitions (dark blue) decreased such that thresholding between positive and negative partitions became **(A)** difficult or **(B)** impossible.

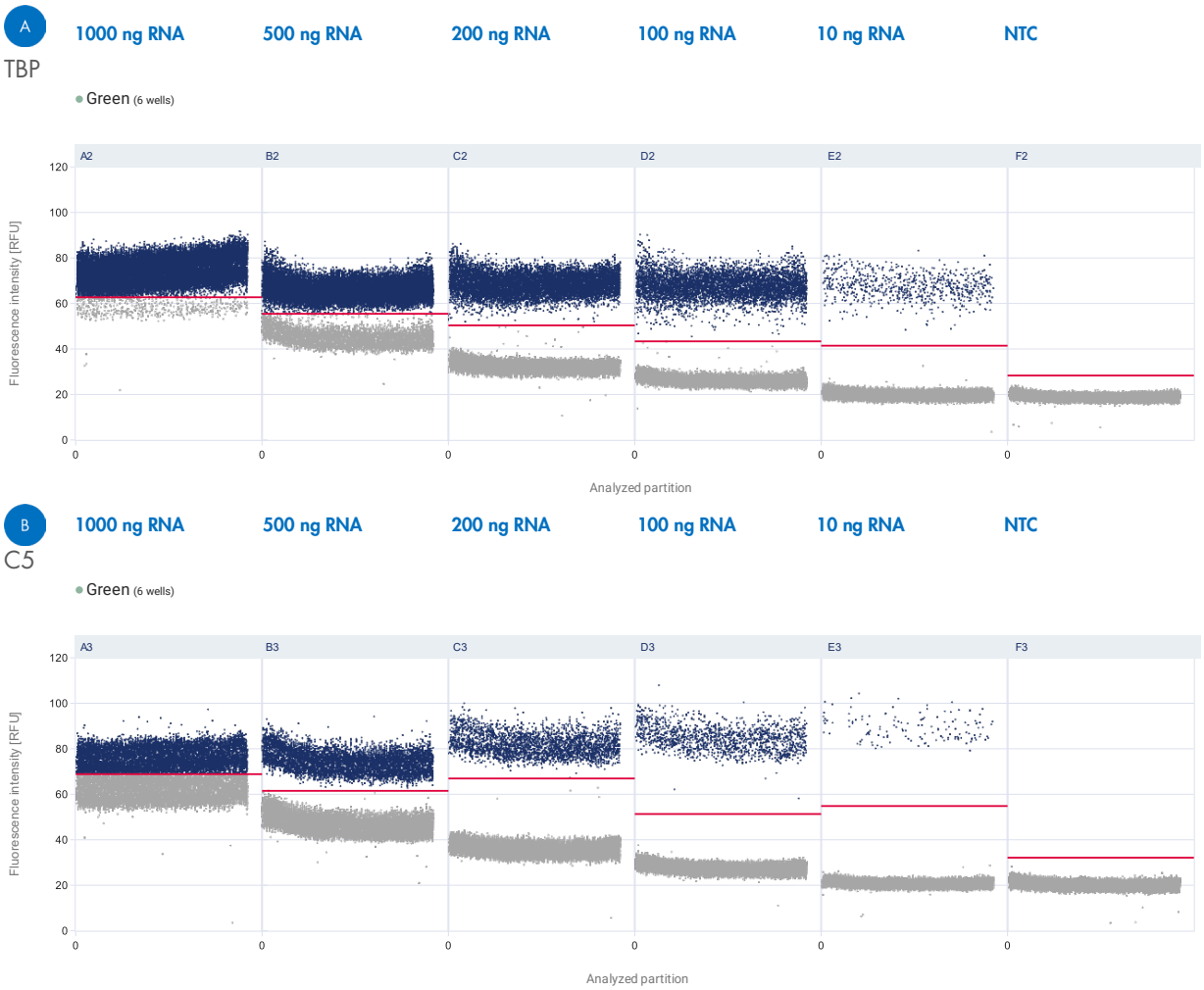


Figure 34.Total RNA amounts should not exceed 500 ng per reaction when using 26K Nanoplates. QuantiNova LNA Assays targeting TBP (Figure 31A) and C5 (Figure 31B) mRNA were used in QIAcuity OneStep Advanced EvaGreen reactions in 26K Nanoplates following recommended cycling protocols. Between 10 and 1000 ng of total RNA isolated from cultured human leukocytes were used as template per reaction. In reactions containing more than 500 ng total RNA, the separation between negative partitions (gray) and positive partitions (dark blue) decreased such that thresholding between positive and negative partitions became **(A)** difficult or **(B)** impossible.

8.4.2 Recommendations for usage of QIAcuity Nanoplates in gene expression analyses

The different QIAcuity Nanoplates offer a wide range of applications, from 24-well plates with high volumes for high sensitivity and broad dynamic range to 96-well plates with low volumes for high throughput.

For selecting the ideal QIAcuity Nanoplate for your application, please refer to Table 15.

8.4.3 Normalization and recommended controls

The efficiency of RNA extraction, cDNA synthesis, or reverse transcription can vary from sample to sample and from experiment to experiment. It is therefore crucial to normalize expression values obtained for a sample against a reference gene.

Note: It is assumed that reference genes do not change from sample to sample. It is nevertheless recommended to confirm that the chosen reference gene that you are using does not change between the different conditions being analyzed.

The reference gene for normalization should exhibit a similar expression level as the gene being analyzed. In this way, users can assess the expression values of both gene of interest and reference gene using the same sample dilution. This minimizes concentration differences caused by pipetting errors or the variability conferred by the pipette that you are using.

Table 16 provides a list of recommended reference genes for different expression ranges.

Table 16. List of reference genes recommended for normalization

Assay Name	Species			Expression level
ACTB	Hs	Mm	Rn	High
B2M	Hs	Mm	Rn	High
GAPDH	Hs	Mm		High
GUSB	Hs	Mm	Rn	Medium high
HPRT	Hs	Mm	Rn	Medium high
HSP90AB1	Hs	Mm	Rn	Medium
LDHA	Hs	Mm		Medium
LOC108351137	Hs		Rn	Medium
MALAT1				Medium
MTOR		Mm	Rn	Medium
NONO				Medium
PGK1		Mm		High
PPIA			Rn	High
PPIH		Mm		Low
PTEN		Mm		Medium
RN7SK				Low-high
RPLP0		Mm	Rn	High
RPLP1		Mm	Rn	Medium
SNORA73A				Low
TBP			Rn	Low

Hs: *H. sapiens* (human); Mm: *Mus musculus* (house mouse); Rn: *Rattus norvegicus* (rat).

8.5 Conducting a gene expression analysis in the QIAcuity Software Suite

The Gene Expression analysis feature of the QIAcuity Software Suite can be accessed by selecting the Analysis option. A new window will open. In the top right you will find the button for the Gene Expression Analysis Plugin (Figure 35).

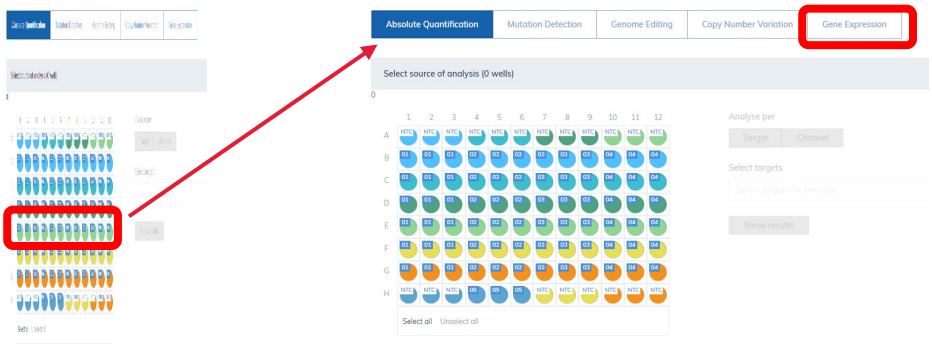


Figure 35. Navigation to second-level analysis for gene expression in the QIAcuity Software Suite.

First select the wells that you would like to analyze. Wells can be selected or unselected by a mouse click or you can also select multiple wells by moving over the wells while clicking the left mouse button (Figure 36).

Next, specify the reference sample in the first pull-down menu. All other gene expression data generated with the remaining samples will be compared against this sample. Fold changes/gene regulation will be reported in the final output.

After that, a target of interest (TOI) needs to be selected. TOIs are all specific assays that are not used as a reference gene. Once a TOI has been selected, users must select one or more “Reference targets”.

If the selected “Reference sample” is comprised of wells and hyperwells with the same sample ID, the “Reference selection” drop-down menu will become active. Users will then need to choose between the hyperwell or “Non-hyperwell, all replicates” for their reference sample.

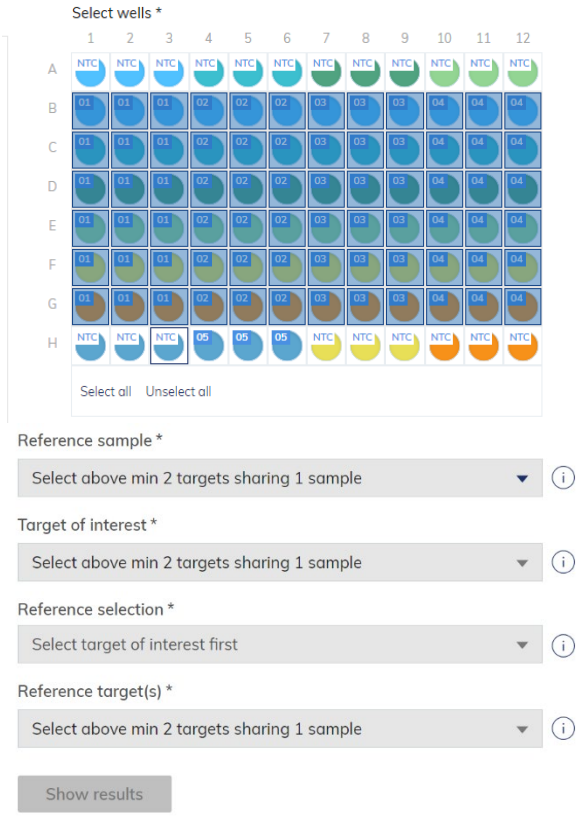


Figure 36. Gene expression can be quantified by selecting multiple wells, TOIs, and reference targets.

Once you click **Show results**, four different analysis results will be provided:

- **List**: A list showing the difference in gene expression of the Target of interest for all sample combinations (fold change and fold regulation). This list can be exported as a CSV file.
- **Concentration diagram**: A bar chart showing the expression level for the Target of interest and Reference target in all samples.
- **Point diagram**: A diagram showing the fold change of the Target of interest in all samples.
- **Heatmap**: A heatmap showing the differences in gene expression in each well of the nanoplate.

For detailed information on the different analysis options and applied calculations, refer to the *QIAcuity User Manual* on **www.qiagen.com**

9 Further Applications of Nanoplate dPCR

The use of dPCR spans many DNA, RNA, and epigenetic applications. The partitioning of the sample into thousands of individual reactions is the key element of the dPCR workflow. This allows sensitive and specific detection of single molecules and the most precise quantification. The partitioning step results in a dilution effect that a rare target molecule in a high background of competing molecules can be detected and quantified with the highest sensitivity. Beyond the described applications that are part of the analysis toolbox of the QIAcuity Software Suite, there are a further number of applications that benefit from advantages of dPCR.

10 miRNA Expression Analysis

10.1 Introduction

microRNAs (miRNAs) are short non-coding RNAs shown to post-transcriptionally regulate gene expression via either translational repression or mRNA degradation.

Because miRNAs are present in tissues as well as body fluids, miRNAs show high potential as non-invasive diagnostic and prognostic biomarkers. Deregulated miRNA expression has been identified to significantly contribute to pathogenesis, progression, and prognosis of serious human diseases such as various types of cancers. miRNA expression in malignant cells is often attributed to alterations in genomic miRNA copy numbers and gene locations. In the past decades, research into miRNA involvement in cancer has made tangible progress. However, robust miRNA quantification is not always easy, especially when analyzing samples with a high inhibitory burden or low amount of nucleic acid.

Digital PCR using the QIAcuity Digital PCR System allows you to overcome these hurdles and to detect and quantify miRNAs with high sensitivity and precision without the need for pre-amplification. Using dedicated miRCURY® LNA® miRNA PCR Assays that contain LNA modifications increases specificity allowing for a highly accurate miRNA quantification.

The fusion of digital PCR with the miRCURY LNA miRNA Assays enables reliable miRNA detection from a wide range of sample types. Outstanding sensitivity allows for quantification of low-abundance miRNAs without pre-amplification even from low-miRNA containing samples such as urine and cerebrospinal fluid (CSF) samples.

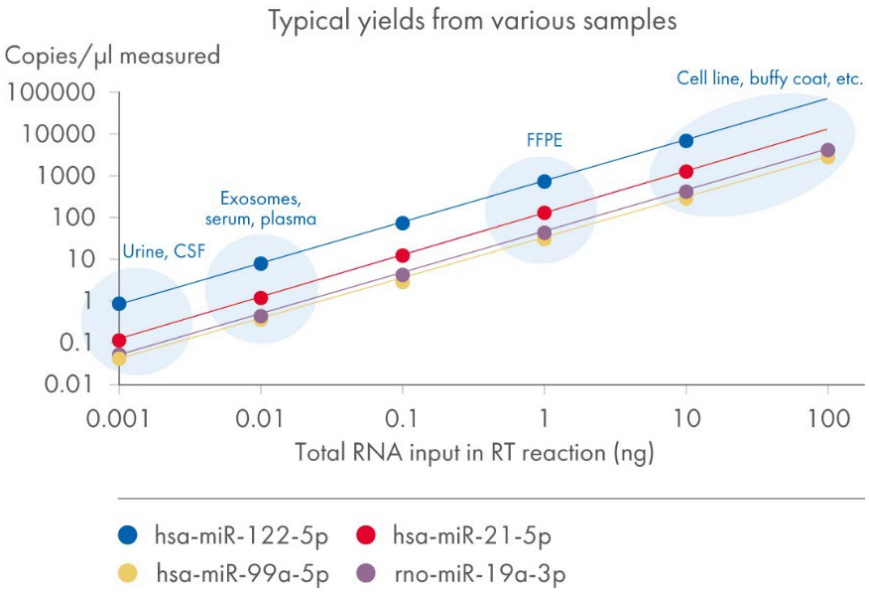


Figure 37. Excellent results with a broad range of total RNA input into the RT reaction. 1 pg – 100 ng of total RNA was used as input into the RT reaction. Data from serial dilutions of AM6000 total RNA are shown. All miRNA assays exhibited linear readout with R2 > 0.99.

10.2 General consideration for performing miRNA analyses on the QIAcuity dPCR instrument

The number of miRNA copies present in a sample is calculated in dPCR using Poisson statistics based on the number of valid, positive, and negative partitions. When dealing with samples with very low number of miRNAs to be detected, the number of copies might vary from the true expression since not the whole reaction volume is analyzed. Additional information can be found in section "Experimental Setup and Absolute Quantification".

Samples with highly abundant miRNAs of interest might lead in cases of reaction overload to partitions containing multiple copies of the miRNA of interest and eventually to loss of negative partitions. In case that all valid partitions are positive, it will no longer be possible to conduct absolute quantification following Poisson statistics.

Detailed information on how to setup a miRNA dPCR and how to perform proper input dilution can be found in **www.qiagen.com/HB-2947**

10.3 Recommendation for usage of QIAcuity Nanoplates in miRNA analyses

The different QIAcuity Nanoplates offer a wide range of applications, from 24-well plates with high volumes for high sensitivity and broad dynamic range to 96-well plates with low volumes for high throughput. The broad dynamic range allows quantification of low- and high-expressed miRNAs. The QIAcuity Digital PCR System enables miRNA quantification of up to 200,000 copies per reaction with high accuracy and precision. Precise thresholding is important for absolute quantification of miRNAs (see Section 5.2.1, Sample input amount).

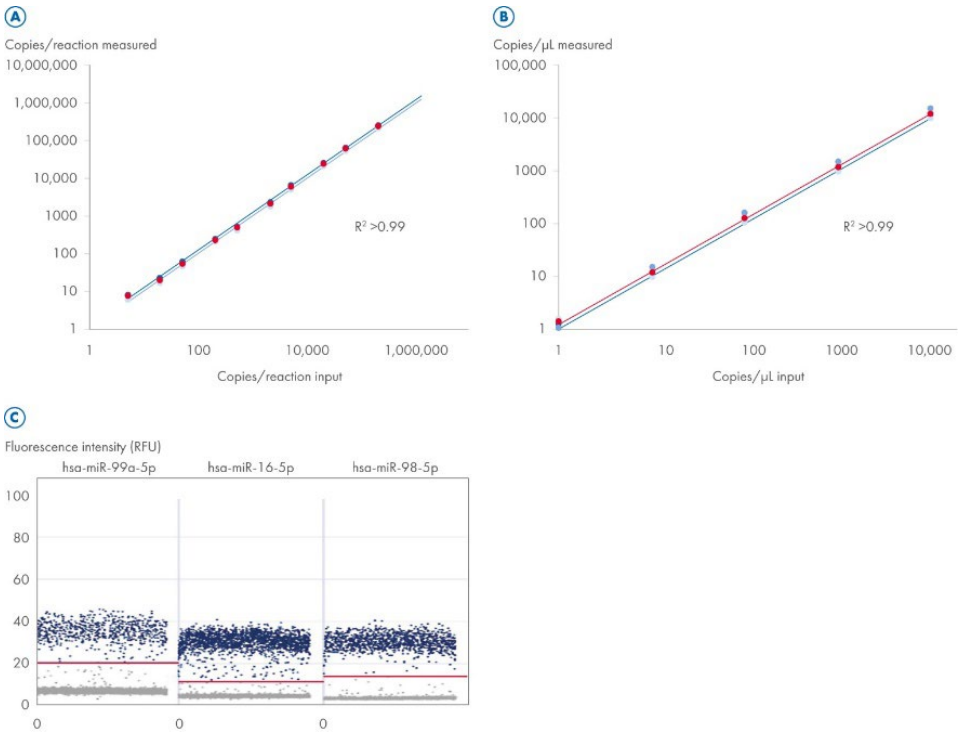


Figure 38. QIAcuity dPCR dynamic range on 26K and 8.5K Nanoplates. (A) Serial dilutions of synthetic cDNAs were performed from 5 copies/reaction to 200,000 copies/reaction on 26K Nanoplates and **(B)** from 12 copies/reaction up to 120,000 copies/reaction on 8.5K Nanoplates. **(C)** 1D scatterplot of 3 human miRNAs with a high signal-to-noise ratio. miRCURY LNA miRNA PCR Assays together with QIAcuity EG MM were used for miRNA quantification.

10.4 Experimental planning and considerations

The most important prerequisite for any miRNA analysis experiment is consistent, high quality RNA from every experimental sample. Residual traces of proteins, salts, or other contaminants may degrade the RNA or decrease the efficiency of enzyme activities necessary for optimal RT and PCR performance. Next, the generation of cDNA from RNA with high efficiency and accuracy is crucial and well-chosen controls are essential for reliable data analysis and interpretation.

10.5 Recommended miRNA preparation methods

RNA is not protected after harvesting until the sample is treated with RNAprotect® Cell Reagent (cultured cells only) or RNAprotect Tissue Reagent (animal tissues only), flash-frozen or disrupted and homogenized in the presence of RNase-inhibiting or denaturing reagents. Otherwise, unwanted changes in the gene expression profile will occur. It is therefore important that samples are immediately frozen in liquid nitrogen and stored at –90°C to –65°C (animal tissues only), processed as soon as harvested, or immediately immersed in RNAprotect Cell Reagent or RNAprotect Tissue Reagent. Animal cells can be pelleted and then stored at –90°C to –65°C until required for RNA purification. An alternative to RNAprotect Tissue Reagent is Allprotect® Tissue Reagent, which provides immediate stabilization of DNA, RNA and protein in tissues samples at room temperature (15–25°C).

The procedures for harvesting and RNA protection should be carried out as quickly as possible. Frozen samples should not be allowed to thaw during handling or weighing. After disruption and homogenization in QIAzol Lysis Reagent, samples can be stored at –90°C to –65°C for months.

It is not necessary to enrich for small RNAs. QIAGEN provides a range of solutions for purification of total RNA containing miRNA. For more information on miRNA purification, visit **www.qiagen.com/miRNA**

Recommended kits are listed in Table 17.

Table 17. Recommended kits

Product	Contents	Cat. no.
miRNeasy Mini Kit (50)	50 RNeasy Mini Spin Columns, collection tubes (1.5 mL and 2 mL), QIAzol Lysis Reagent, RNase-free reagents and buffers.	217004
miRNeasy Serum/Plasma Advanced Kit (50)	50 RNeasy UCP MinElute Spin Columns, collection tubes (1.5 mL and 2 mL), QIAzol Lysis Reagent, RNase-free reagents and buffers.	217204
miRNeasy Micro Kit (50)	50 RNeasy UCP MinElute spin columns, collection tubes (1.5 mL and 2 mL), QIAzol Lysis Reagent, RNase-free reagents and buffers.	217084
miRNeasy FFPE Kit	50 RNeasy MinElute Spin Columns, collection tubes, proteinase K, RNase-free DNase I, DNase Booster Buffer, RNase-free buffers, RNase-free water.	217504
PAXgene® Tissue RNA/miRNA Kit (50)	PAXgene RNA MinElute Spin Columns, PAXgene Shredder Spin Columns, processing tubes, microcentrifuge tubes, carrier RNA, RNase-free DNase and RNase-free buffers, to be used with PAXgene Tissue Containers.	766134
PAXgene Tissue Container (10)	For collection, fixation and stabilization of 10 samples: 10 prefilled reagent containers containing PAXgene Tissue Fix and PAXgene Tissue Fix and PAXgene Tissue Stabilizer.	765112

10.6 Storage of miRNA

Purified RNA may be stored at temperatures between –70°C and –15°C in RNase-free water. Under these conditions, no degradation of RNA is detectable after 1 year.

10.7 RNA input amount

The miRCURY LNA miRNA PCR protocol is optimized for use of 10 ng total RNA per 10 µL cDNA synthesis reaction. The exact amount of total RNA needed may vary depending on the miRNA expression levels in the cells or tissue to be analyzed. For highly expressed miRNAs, it is possible to use down to 10 pg total RNA. For weakly expressed miRNAs, up to 200 ng total RNA may be used provided that the dilution of the cDNA is kept as described in the corresponding handbook. However, in samples with high amounts of PCR inhibitors such as FFPE tissue samples, this may not be feasible. Furthermore, inhibitors may be present in the RNA preparations from certain samples such as serum and plasma. Prior to conducting a larger miRNA profiling study, it is recommended to optimize the amount of input RNA to the RT reaction, to avoid conducting a larger study in which inhibition occurs sporadically throughout the dataset.

10.8 cDNA synthesis

Mature miRNAs are naturally occurring, 22-nucleotide, noncoding RNAs that mediate post-transcriptional gene regulation. Unlike mRNAs, miRNAs are not polyadenylated in nature. Mature miRNAs can be polyadenylated by poly(A) polymerase and reverse transcribed into cDNA using oligo-dT primers. It is highly recommended to use the miRCURY LNA RT Kit (cat. no. 339340) for cDNA synthesis.

Polyadenylation and reverse transcription are performed in parallel in the same tube. The reaction takes place at 42°C for 60 minutes and is then inactivated at 95°C. Only cDNA generated using the miRCURY LNA RT Lit can be analyzed using the miRCURY LNA miRNA PCR assays and panels.

Detailed information on how to setup a cDNA reaction can be found in **www.qiagen.com/HB-2947**

10.9 Reference assays and reference candidates

Reference assays detect small noncoding RNAs – small nuclear RNA, small nucleolar RNA, or miRNA – which are frequently found to be stably expressed across different cells or tissues. Reference assays may therefore be candidate assays for normalization in a profiling study with several samples. Although this is a good and recommended approach, great caution should be taken in the selection of reference genes. The danger of using endogenous reference genes lies in the assumption that a specific gene is expressed at the exact same level in all sample types. This is rarely true. The selection of reference genes should therefore be made with care and should be specific to the sample set of interest. The actual selection of reference genes to be used for normalization should always be based on a determination of the most stably expressed gene(s). Whenever applicable, it is recommended to use miRNA rather than small nuclear or nucleolar RNA for normalization. Firstly, those RNAs are longer than miRNA and may purify differently from miRNA. Moreover, small nuclear and nucleolar RNA have entirely different functions and subcellular locations. Finally, certain samples, such as blood plasma, do not contain small nuclear and nucleolar RNAs.

10.10 RNA spike-ins (synthetic control templates)

RNA spike-ins and the matching primer pairs enable to control quality of the RNA isolation, the cDNA synthesis reaction, and the PCR amplification. RNA isolations may vary in yield, purity, and integrity. Some sample types may contain compounds that inhibit the cDNA synthesis or the PCR amplification, even if the RNA was purified using the best standard procedures. This might result in different efficiencies of the reverse transcription or PCR between compared samples. One way to control for differences in efficiencies at each experimental level (isolation, cDNA synthesis, and PCR) is by adding known RNA spike-ins to the sample prior to isolation and cDNA synthesis. Use of the RNA spike-ins may also reveal if nucleases are present. After conducting the PCR, but before initiating the data analysis, wells detecting RNA spike-ins are compared and outlier samples identified and considered for exclusion from further data analysis. For this purpose a collection of RNA spike-ins exist. The UniSp6 RNA spike-in is one of those. The spike.in template is provided with the miRCURY LNA RT Kit. The cel-miR-39-3p RNA template is provided in a separate vial in the RNA Spike-in Kit; it can be mixed with the UniSp6 template from the miRCURY LNA RT Kit to obtain two different template concentrations. This combination can be added during the cDNA synthesis.

10.11 Using the QIAcuity EG PCR Kit for miRNA analysis on the QIAcuity

The QIAcuity EG PCR Kit was developed as universal QIAcuity dPCR Kit capable of delivering highest performance in various applications such as gene expression or CNV analysis. High specificity and sensitivity in dPCR are achieved by a hot-start procedure. This allows room-temperature setup of the PCR reaction without the risk of primer–dimer formation. The hot-start is achieved using QuantiNova DNA Polymerase, which is a novel hot-start enzyme, and the additive QuantiNova Guard. These unique components further improve the stringency of the antibody-mediated hot-start. The combination of the QIAcuity EG PCR chemistry with the miRCURY LNA miRNA PCR assays and panels enable miRNA quantification with unmatched specificity and sensitivity.

10.12 Using the miRCURY LNA miRNA PCR assays and panels on the QIAcuity

The miRCURY LNA miRNA PCR Assays provide highly sensitive and accurate LNA-enhanced dPCR quantification assays for miRNA targets in an easy-to-handle format. They are designed for use with universal RT, followed by PCR amplification. To obtain optimal results in dPCR, the use of miRCURY LNA miRNA PCR Assay products in combination with the miRCURY LNA RT Kit and the QIAcuity EG PCR Kit is recommended. The miRCURY LNA miRNA PCR Assays and the QIAcuity instrument form a unique system for miRNA profiling that offers the best combination of performance and ease-of-use tools on the miRNA dPCR market.

- cDNA synthesis using a universal RT: One first-strand cDNA synthesis reaction provides the template for all miRNA PCR assays. This saves precious sample, reduces technical variation, consumes less reagents, and saves time in the laboratory. The same cDNA synthesis can be used across all assay formats.
- LNA-enhanced dPCR amplification: Both PCR amplification primers (forward and reverse) are miRNA specific with the LNAs placed intelligently in the primers to fully optimize the primer performance. The result is exceptional sensitivity and specificity with extremely low background, enabling reliable quantification of very low levels of miRNA. The highly specific assays allow discrimination between closely related miRNA sequences. The miRCURY LNA miRNA PCR Assays and the outstanding performance of the QIAcuity EG PCR Kit offer solutions for both high-throughput miRNA expression profiling and for quantification of individual miRNAs.

10.13 miRNA analysis in the QIAcuity Software Suite

Please follow step-by-step instructions provided for gene expression analyses in Section 8, Gene Expression Analysis Using the , on page 58.

10.14 Challenges in miRNA detection and quantification

The small sizes and widely varying GC content (5–95%) of miRNAs make them challenging to analyze using traditional methods. DNA- or RNA-based methods for miRNA analysis can introduce high uncertainty and low robustness because the Tm of the oligonucleotide/miRNA duplex will vary greatly depending on the GC content of the sequences. This is especially problematic in applications such as microarray profiling and high-throughput experiments in which many miRNA targets are analyzed under the same experimental conditions.

Use of LNA-enhanced oligonucleotides overcomes these challenges. By simply varying the LNA content, oligonucleotides with specific duplex melting temperatures can be designed, regardless of the GC content of the miRNA. T_m-normalized primers perform well under the same experimental conditions.

Another challenge of studying miRNAs is the high degree of similarity between the sequences. Some miRNA family members vary by only a single nucleotide. LNA can be used to enhance the discriminatory power of primers to allow excellent discrimination of closely related miRNA sequences. LNA offers significant improvement in sensitivity and specificity and ensures optimal performance for all miRNA targets.

An LNA oligonucleotide offers substantially increased affinity for its complementary strand, compared to traditional DNA or RNA oligonucleotides. This results in unprecedented sensitivity and specificity and makes LNA oligonucleotides ideal for the detection of small or highly similar DNA or RNA target.

11 Cell and Gene Therapy Applications

11.1 Introduction AAV detection

Cell and gene therapies seek to target previously untreatable diseases at their source using individualized treatments. However, developing safe and effective cell and gene therapies requires strict monitoring at all stages of the development process. Adeno-associated virus (AAV) has turned into a primary modality for efficient gene therapy applications. The process of generation and purification of the viral vectors require precise quality control to enable safe and reliable dosing during clinical studies or patient care. The ability to accurately and reproducibly quantify vector titers is essential for safe and effective AAV-based gene therapies.

qPCR is a widely used method for AAV quantitation due to its sensitivity and ease of use. Nevertheless, well-characterized DNA standards and assays are needed for accurate quantification. The ability to accurately quantify vector titers as well as to determine contaminations is key for safe and effective AAV-based gene therapies. The QIAcuity Digital PCR System, with its dedicated cell and gene therapy (CGT) dPCR assays and its standardized viral vector lysis kits, enables vector genome titration with outstanding accuracy, reproducibility and speed with an easy workflow comparable to qPCR.

The AAV genome is a key component for vector quantification. HEK293 cells are typical producers of AAVs of various serotypes. After production of AAVs, the particles must be extracted and processed. Upstream and downstream processes are strictly monitored. Vector genome titration is performed at various steps throughout the process. Digital PCR enables robust titer determination of AAV samples of different purities and concentrations.

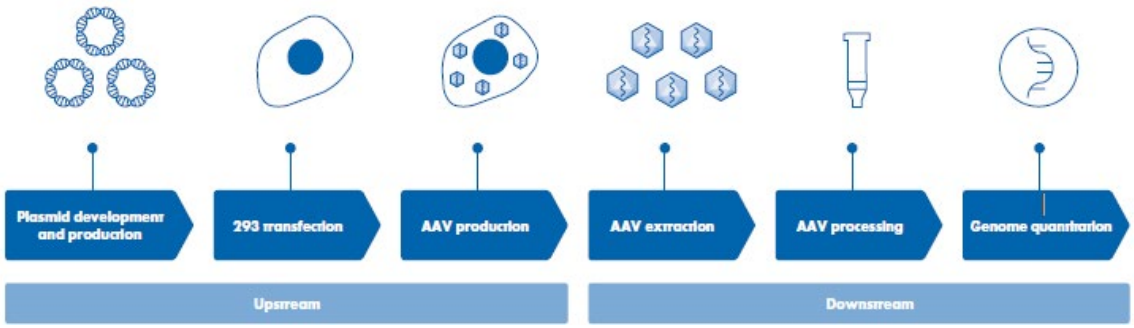


Figure 39. Schematic illustration of AAV production, processing, and quantification.

11.2 Using the CGT dPCR assays on the QIAcuity

Viral vector titer determination and monitoring are important tools for, for example, Biopharma in CGT for process quality control and safe and effective therapeutic performance. The viral vector titer, also referred to as physical titer, is directly related to dosage and thus of critical importance. The physical titer is typically calculated by PCR-based methods, one of which is digital PCR. Several decades of research has shown that AAVs appear to be the safest and most effective vectors for gene therapeutic applications. The encapsulated AAV vector genome is a key mediator and indicator of therapeutic effect. For reliable titration the gene of interest or other generic regulatory elements encoded on the viral genome such as promoter or poly-A can be used. Primers and probes targeting the ITR region can be used as well.

Titers of viral vectors such as AAVs can be accurately and precisely determined using digital PCR and QIAGEN's dedicated CGT dPCR assays without the need for references or standard curves.

The CGT dPCR assays in combination with the QIAcuity dPCR system provide a robust quantification with high repeatability between operators.

CGT dPCR assays show accurate quantification independent of fluorophores and operators. Therefore, higher multiplexing is possible without affecting titration. Titration with more than one assay gives a complete picture on the 'real' vector titer.

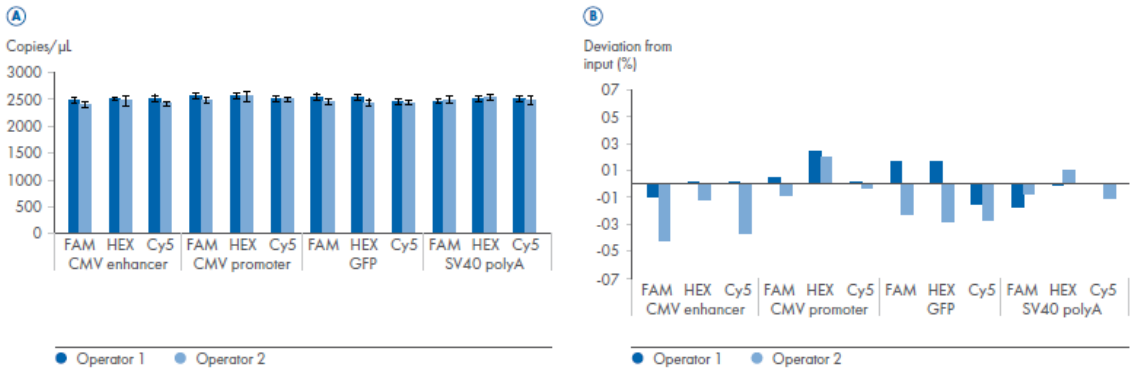


Figure 40. Highly accurate dPCR. (A) The CGT dPCR assays were run with AAV DNA. A total of 2500 copies/µL was used as input on an 8.5K Nanoplate. Mean number of copies of at least 13 replicates per operator are shown. Mean CV among all assays and operators ~2%. **(B)** Mean values of deviation from expected copies of at least 13 replicates per operator are shown.

11.3 Challenges in viral vector processing and titer determination

Accurate and precise titer determination and characterization are key to develop safe and effective AAV-based gene therapies. Quality control assays to measure the concentration and functional activity of AAV vectors are required to define investigational product dosing and assure consistent lot-to-lot functional activity. Assays to quantify vector genomes must include a nuclease step performed prior to denaturation of the vector so that only AAV packaged DNA is measured. Non-packaged vector DNA can result in an overestimation of vector genomes. Due to the lack of tools available to help increase the number of viral particles containing full-length genomes, the final viral vector product represents a mixture of capsids with different degrees of fragmentation. Targeting multiple regions of the viral vector genome (e.g., in a multiplex dPCR reaction) helps to provide an

additional layer of information regarding genome integrity. Because only intact genome contribute to, for example, the efficacy of a gene-therapy product, it is important to assess vector genome integrity.

Besides vector genome integrity, the choice of the target to be quantified is crucial as well. An AAV viral genome typically includes two ITR sequences with a characteristic T-shaped hairpin structure forming energetically stable double-stranded regions with sequence repeats and a high GC content. Those structures may limit accessibility of targets during PCR. Through enzymatic digest within the ITR regions, the secondary structures can be released improving accessibility for target amplification. It has been shown that the secondary structures within the ITR regions impact ITR quantifications as well as the quantification of target regions in close vicinity. Amplifying distal region to the ITR could increase VG titers. Nevertheless, vector genome titers determined from ITR or ITR proximal amplification could be overestimated due to the amplification of truncated vector DNA containing terminal genome fragments that are usually found in vector preparations containing both full and empty particles.

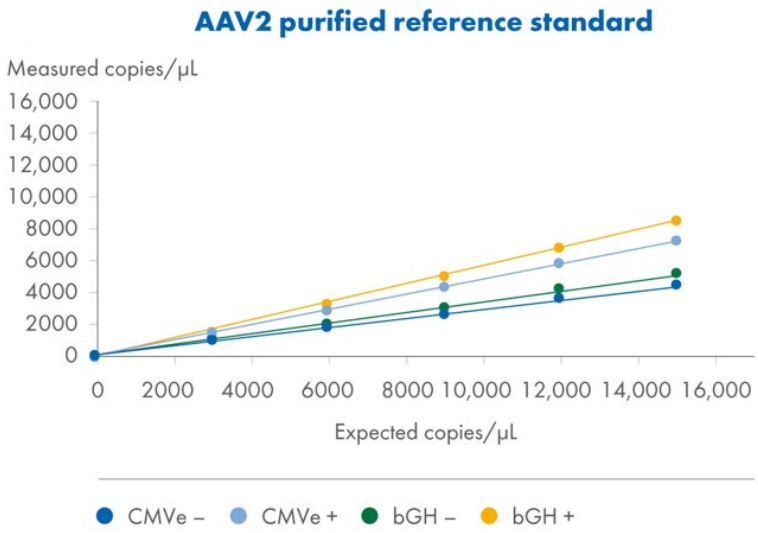


Figure 41. ITR digestion improves the quantification of ITR and non-ITR targets. AAV2 samples were processed using the CGT Viral Vector Lysis Kit and quantified using the QIAcuity Digital PCR instrument with 8.5K Nanoplates and the CGT dPCR Assays. The CGT dPCR Assays were run in triplex reactions in the FAM,HEX and Cy5 channels. The samples were serially diluted in 6 steps from 15,000 copies/µL down to 2.5 copies/µL with an R^2 = 1.0 on 8.5K Nanoplates. Each dilution was measured in technical triplicates. Quantifications were performed with (+) and without (–) restriction digest of the ITR regions. For the titration of a purified AAV2 reference standard sample, the CGT dPCR assays targeting the CMV enhancer bGH polyA regions were used. The expected copies are based on an ITR estimate determined by qPCR measurements from the reference standard supplier and not directly comparable to dPCR measurements.

For viral vector titer determination of, for example, AAV samples, the genomic DNA must be extracted from the capsids to be able to efficiently amplify the region of interest in a PCR reaction. The thermal stability of the viral capsids is known to be serotype dependent. For some serotypes it is sufficient to break open the viral capsids and release the target DNA within the thermal cycling of the PCR, whereas for other serotypes an additional enzymatic digest might be required. It is difficult to find a standardized AAV processing protocol applicable to multiple serotypes. It is also important to consider potential PCR inhibitors introduced into the processing workflow. When lysing AAV capsids using Proteinase K, it is important to make sure that the enzyme is inactivated prior to PCR to protect the polymerase from an enzymatic digestion. When using detergents, it is essential to dilute the sample sufficiently to prevent PCR inhibition and guarantee optimal PCR efficiency. The CGT Viral Vector Lysis Kit (**www.qiagen.com/CGT-Viral-Vector-Lysis-Kit**) provides an efficient standardized workflow for capsid lysis independent of its capsid nature and the associated melting temperature, purity and vector concentration.

11.4 Experimental planning and considerations

A robust sample preparation method and standardized quantification procedure is key to robust and highly repeatable titer determination with high accuracy and precision. A standardized procedure also helps with reproducibility of the quantification with different lots, between multiple operators and laboratories.

Additionally, the sample processing and quantification methods should be robust towards potential inhibitors since the viral vector titer needs to be determined at different stages along the purification workflow.

11.4.1 Input amount of viral particles

The CGT Viral Vector Lysis Kits are optimized for processing of AAV particles. The amount of starting material can vary greatly depending on upstream processes (production system, purification and enrichment of particles). AAV samples can be stored in various storage buffers. The processing and quantification workflow is very robust towards various buffer components (detergents, high salt, carrier) as well as against potential inhibitors such as coating agents, secreted intracellular and extracellular material carried over after particle extraction from producer cell lines such as HEK 293 cells.

A wide range of particle titers can be processed using the CGT Viral Vector Lysis Kits. In cases where a high viral titer is expected, samples can be diluted before transfer into the DNase I digest and/or throughout the whole processing workflow.

11.4.2 Processing of in-process samples for titer determination

Since a plethora of viral vector formulations exist, many of which interfere with analytical techniques, a reliable sample processing and quantification method is required.

The CGT Viral Vector Lysis Kit (**www.qiagen.com/CGT-Viral-Vector-Lysis-Kit**) is compatible with AAV samples of different purities and allows reliable AAV titer determination on a QIAcuity dPCR System. For example, clarified cell lysates from Sf9 (insect cells), Tnms42 (insect cells), HEK293 and BHK-21 do not significantly affect sample processing and the downstream viral vector titer quantification. The largely standardized workflow enables reliable titration with high inter- and intra-assay precision (less than +/–10%) independent of assays, operators and laboratories.

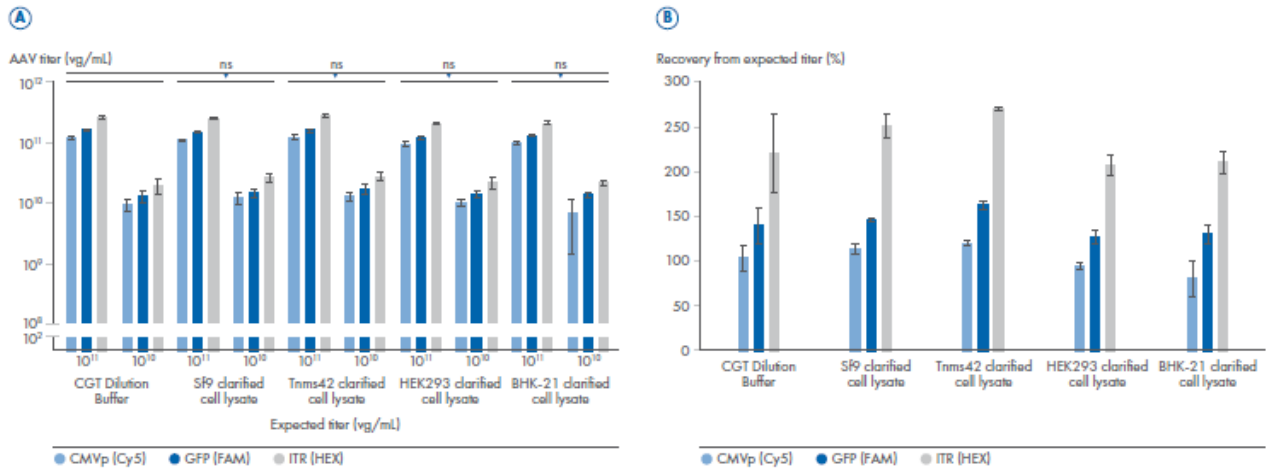
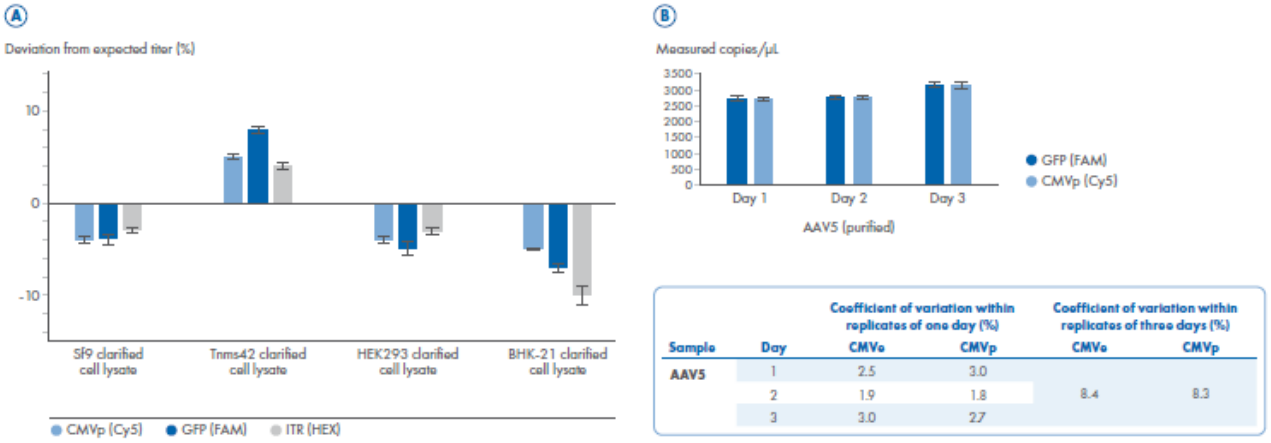


Figure 42. Robust AAV titer determination. (A) A purified AAV2 reference standard (10^{11} vg/mL and 10^{10} vg/mL; Vigene) was spiked into CGT Dilution Buffer as well as clarified cell lysates derived from Sf9 (insect cells), Tnms42 (insect cells), HEK293, and BHK-21. The particles were processed using the CGT Viral Vector Lysis Kit. After capsid lysis, the samples were serially diluted (10x, 5x, 4x, 4x). The last three dilution steps were used for titer determination. Quantification was performed in technical duplicates using 8.5K Nanoplates and the CGT dPCR CMV promoter, GFP, and ITR assays in the FAM, HEX, and Cy5 channels of the QIAcuity dPCR System. The expected AAV titer is based on titer information provided by the supplier. A paired student's t-test was performed to determine significance (ns not significant *, $P < 0.05$ **, $P < 0.01$***, $P < 0.001$). **(B)** The percentage of measured titer from the expected titer was calculated using all data points from 10^{11} vg/mL and 10^{10} vg/mL AAV spike-in samples.



Sample	Day	Coefficient of variation within replicates of one day (%) CMVo	CMVp	Coefficient of variation within replicates of three days (%) CMVo	CMVp
AAV5	1	2.5	3.0		
	2	1.9	1.8	8.4	8.3
	3	3.0	2.7		

Figure 43. Reproducible AAV titer determination independent of background matrices and operator. (A) An AAV5 reference standard (Vector Biolabs) was processed using the CGT Viral Vector Lysis Kit and quantified using the CGT dPCR assays targeting the CMV promoter, GFP and ITR regions on a QIAcuity dPCR System. The determined vector genome titers for every target were set as expected titer. AAV5 particles were spiked into clarified cell lysates derived from Sf9 (insect cells), Tnms42 (insect cells), HEK293 and BHK-21. The particles were processed using the CGT Viral Vector Lysis Kit. After capsid lysis, the samples were serially diluted (10x, 5x, 4x, 4x). The last three dilution steps were used for titer determination. Quantification was performed in technical duplicates using 8.5K Nanoplates and the CGT dPCR CMV promoter, GFP and ITR assays in the FAM, HEX and Cy5 channels of the QIAcuity dPCR System. The deviation from the measured titers to the expected titers was calculated for each target separately. **(B)** An AAV5 reference standard was processed over 3 days by one operator. Mean quantification of 15 replicates and standard deviation is shown. Coefficient of variations (CV) within the replicates of one day as well as between the replicates of 3 or 4 days are shown.

11.5 General considerations for viral vector titer determination on the QIAcuity dPCR instrument

The number of viral vector genome copies present in a sample is calculated in dPCR using Poisson statistics based on the number of valid, positive and negative partitions.

Highly concentrated viral vector samples might lead in cases of reaction overload to partitions containing multiple copies of the genome target of interest and eventually to loss of negative partitions. In case that all valid partitions are positive, it will no longer be possible to conduct absolute quantification following Poisson statistics.

When dealing with in-process samples in matrices that might contain PCR inhibitors, additional dilution steps will be required. In order to check for linear readout, a dilution series can be loaded on the nanoplates. The coefficient of determination is a useful measure and indicator of the strength of the linear relationship between two different variables (e.g., expected quantification vs measured values).

Detailed information on how to setup a viral vector titration and how to perform proper input dilution can be found in **www.qiagen.com/HB-3362**

11.5.1 Recommendation for usage of QIAcuity Nanoplates in viral vector titration

The broad dynamic range of the QIAcuity Nanoplates allow titration of samples with high and low titers. This is particularly important for the analysis of in-process samples that did not undergo the process of advanced purification and enrichment.

High linearity from 2.5 copies/µL to 15,000 copies/µL on 8.5K Nanoplates enables titer determination of AAV samples originating from different in-process steps. Samples from early in the purification process are expected to have lower titers, whereas close to the release, product titers are expected to be highest.

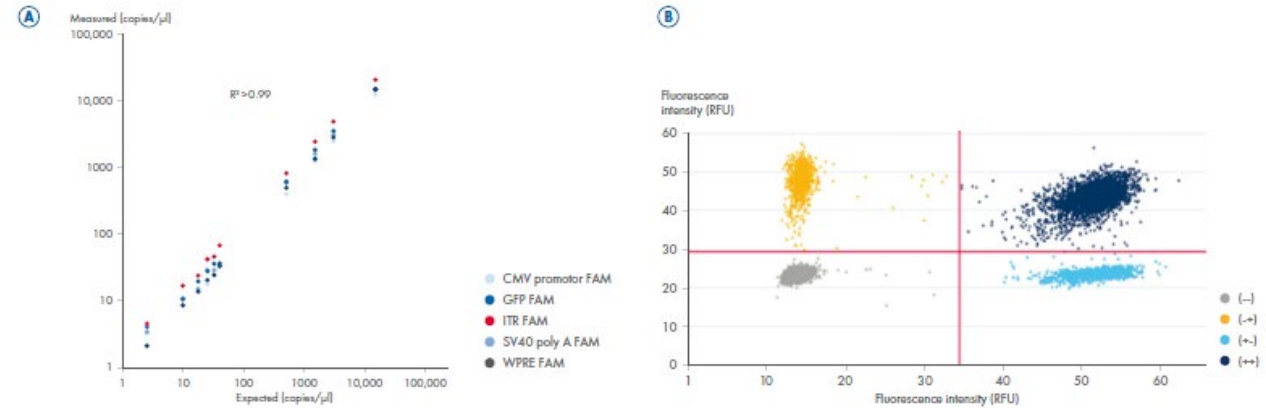


Figure 44. Highly accurate dPCR. (A) The CGT dPCR FAM assays were run using a serial dilution of DNA extracted from AAV2 samples on a 8.5K Nanoplate. The DNA input ranged from 2.5 to 15,000 copies/µL. The coefficients of determination were >0.99. **(B)** The CGT dPCR ITR FAM assay was run in a multiplex reaction with the CGT dPCR CMV promoter HEX assay on AAV DNA on a 8.5K Nanoplate. The fluorescence intensity (RFU) of the ITR FAM assay (x-axis) was plotted against the fluorescence intensity of the CMV promoter HEX assay (y-axis) as shown in the 2D scatter plot.

26K Nanoplates can also be used for titration of viral vectors with only minimal deviation between the two nanoplate types. When quantifying just a few copies of target of interest, the 26K Nanoplate is preferred over the 8.5K Nanoplate. Detailed information on the nanoplates and their dynamic ranges can be found in Section 1, Table 3 and Table 4.

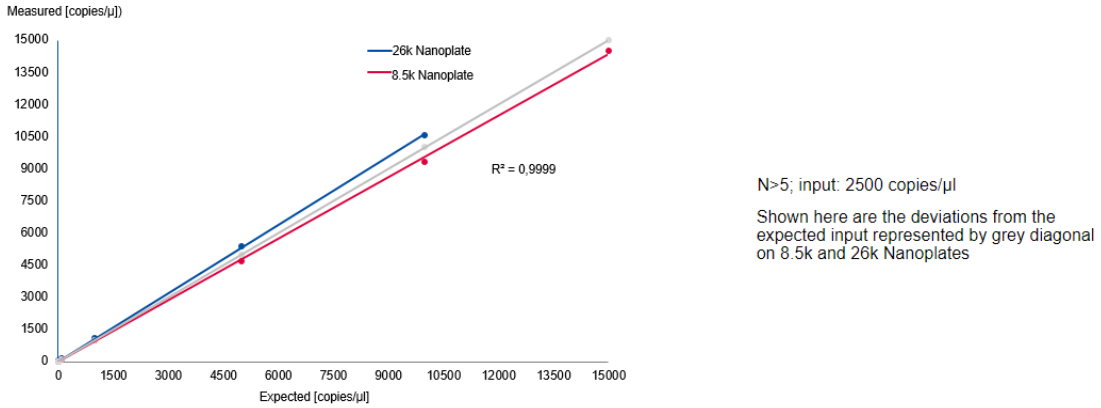


Figure 45. Accurate quantification on 8.5K and 26K Nanoplates. A serial dilution of AAV DNA ranging from 2.5 up to 15,00 copies/µL was loaded on 8.5K and 26K Nanoplates. Gray diagonal represents expected quantification. At least 5 technical replicates were run on both plate types.

11.5.2 Viral vector titer analysis in the QIAcuity Software Suite

Please refer to section "Absolute quantification and analysis" for step-by-step instructions on how to analyze plates and interpret quantification results.

The readout of dPCR is target copies per microliter without using a standard curve or reference standard. Dilution factors that were applied during sample processing must be factored in separately as well as the conversion from copies/µL to vector genomes (vg)/mL.

12 Wastewater-based Epidemiology Using QIAcuity dPCR Workflows

12.1 Introduction

12.1.1 Why use wastewater in epidemiology?

Following the global SARS-CoV-2 pandemic, wastewater-based epidemiology has been established as a powerful tool for tracking the spread of pathogens within large populations. However, sensitively detecting trace amounts of viruses and bacteria in wastewater samples comes with significant challenges. The physical and chemical properties of wastewater make nucleic acid extraction difficult, while PCR inhibitors inherent to environmental samples can interfere with PCR detection. Therefore, a carefully considered workflow is crucial to maximizing the sensitivity and accuracy of viral detection in wastewater with PCR.

12.1.2 QIAGEN workflow for wastewater-based epidemiology

QIAGEN extraction technologies dedicated for environmental samples paired with QIAcuity dPCR are the ideal combination for detecting pathogens in wastewater (

Figure 46).

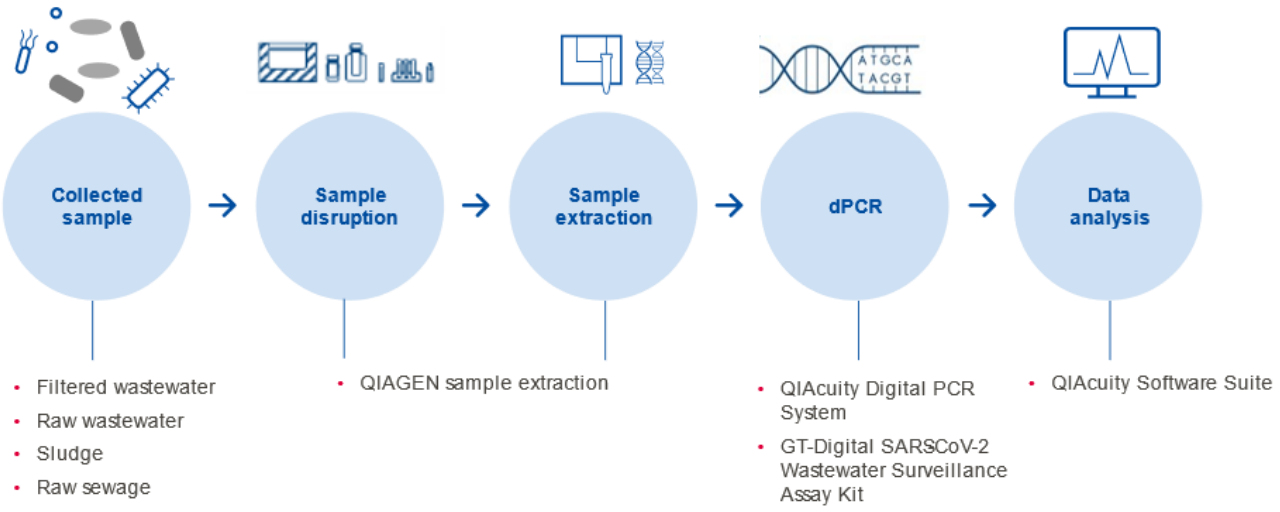


Figure 46. QIAGEN workflow for wastewater-based epidemiology.

12.1.3 QIAGEN provides sample extraction kits tailored for wastewater samples

Viruses like SARS-CoV-2 are enriched in wastewater solids compared to the aqueous phase. However, nucleic acids extracted from wastewater using standard methods strongly inhibit PCR and dramatically reduce workflow sensitivity. In contrast, QIAGEN sample extraction technologies containing proprietary Inhibitor Removal Technology® (IRT) increase workflow sensitivity by letting researchers confidently interrogate wastewater solids without the concern of PCR inhibition.

12.1.4 Advantages of QIAcuity dPCR for wastewater-based epidemiology

Compared with qPCR, QIAcuity dPCR does not require a prequalified standard curve for quantification and is less sensitive to PCR inhibitors from environmental samples. QIAcuity dPCR chemistries tailored to accommodate environmental samples, such as the QIAcuity OneStep Advanced Probe Kit, can bolster PCR sensitivity even further.

12.1.5 A workflow beyond SARS-CoV-2

While developed to address the SARS-CoV-2 pandemic, the QIAcuity workflow described here can also be extended to further targets, such as influenza, norovirus, monkeypox and polio, among others, providing a powerful tool for understanding the prevalence and spread of these pathogens in the environment.

12.2 Proof of concept

Sample extraction methods with IRT are key to workflow sensitivity.

Publications have shown that enveloped viruses such as SARS-CoV-2 are enriched in the solid fraction of wastewater. However, the solid fractions of wastewater harbor significant levels of RT-dPCR inhibitors. Without the use of IRT in sample extraction (e.g., QIAGEN QIAamp Viral RNA Mini Kit, Zymo Quick-RNA Viral Kit), eluates from wastewater solids are severely inhibitory to RT-dPCR when compared to samples extracted with kits that use IRT – such as the QIAGEN AllPrep PowerViral DNA/RNA Kit (Figure 47). A list of QIAGEN extraction kits that are suited for wastewater or similar environmental probes can be found in Table 18.

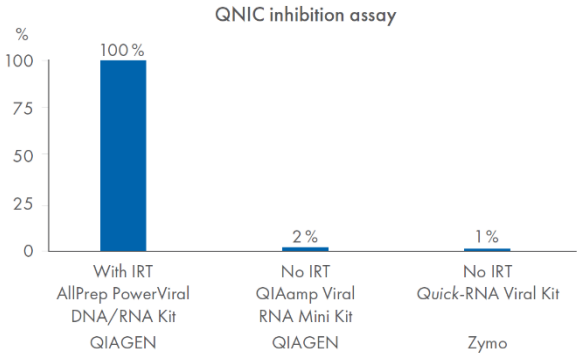


Figure 47. Relative quantification of a spike-in RNA target (QNIC) added to QIAcuity RT-dPCR reactions shows severe inhibition in wastewater samples extracted without IRT.

Table 18. QIAGEN sample extraction solution for wastewater samples

Wastewater products	Starting material
AllPrep PowerFecal Pro DNA/RNA Kit AllPrep PowerViral DNA/RNA Kit	Wastewater, stool, biosolids, and gut material
RNeasy PowerFecal Pro RNeasy PowerMicrobiome Kit	Stool, sludge, raw wastewater, and sediments
RNeasy PowerWater Kit	Filtered ocean water, fresh water, brackish water, ground water, wastewater bacteria (gram +/–)
RNeasy PowerSoil Total RNA Kit	Soil samples, including compost, sediment, and manure

12.2.1 SARS-CoV-2 detection using QIAGEN dPCR workflow for wastewater

The QIAGEN dPCR workflow for pathogen detection in wastewater was used to quantify SARS-CoV-2 in real wastewater. For comparison, the ddPCR wastewater workflow from Bio-Rad was run in parallel. To account for the heterogenous nature of wastewater, samples were obtained from two separate treatment facilities (H and M). For each nucleic acid extraction, 40 mL of wastewater were used as input.

The QIAGEN workflow used the RNeasy PowerFecal Pro Kit to extract RNA from pelleted wastewater solids. To demonstrate the benefit of dPCR chemistries specifically tailored for wastewater applications, the current QIAcuity OneStep Advanced Probe chemistry, which was specifically developed for wastewater applications, was run in parallel against the legacy QIAcuity OneStep Viral PCR Kit. Assays from GT Molecular were used to detect SARS-CoV-2 in 26K Nanoplate reactions using 9 µL of eluate per reaction.

In the Bio-Rad workflow, RNA was extracted from clarified wastewater supernatant with the Zymo Quick-RNA Viral Kit, which is the sample extraction kit recommended by Bio-Rad for wastewater samples. The PREvalence SARS-CoV-2 Wastewater Kit was used to detect SARS-CoV-2 with 9 µL of eluate in 20 µl ddPCR reactions.

Compared to the Bio-Rad workflow, higher levels of SARS-CoV-2 were detected with the QIAGEN workflow when either the legacy QIAcuity OneStep Viral PCR chemistry or the current QIAcuity OneStep Advanced Probe chemistry were used (Figure 48). These results highlight the significant benefits that QIAGEN sample extraction kits with IRT bring to wastewater workflows. Furthermore, the higher quantification of SARS-CoV-2 with the QIAcuity OneStep Advanced Probe chemistry underscores the important role that reaction chemistry plays in maximizing workflow sensitivity.

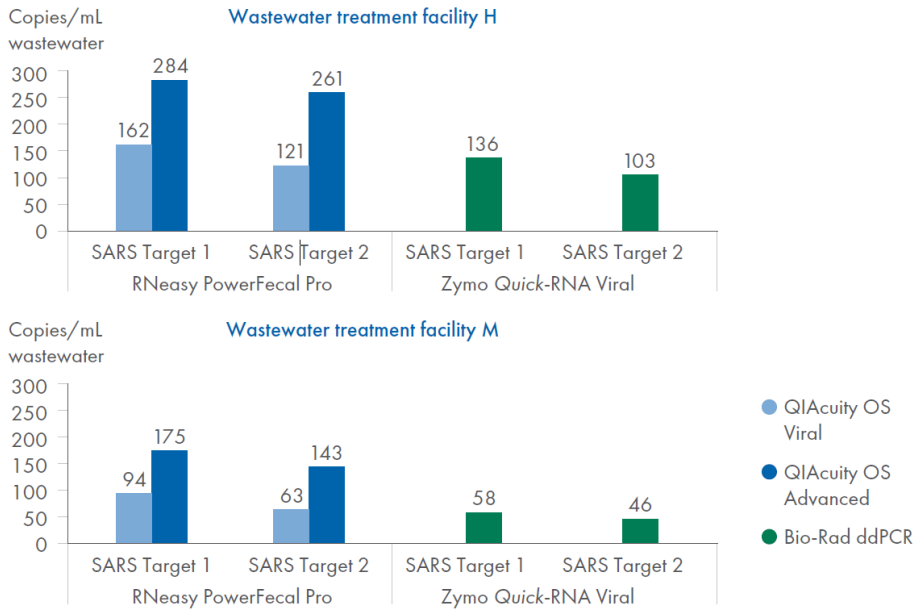


Figure 48. QIAGEN dPCR workflow for wastewater-based epidemiology outperforms the ddPCR wastewater workflow from Bio-Rad. GT Molecular SARS Assays used with QIAGEN RT-dPCR chemsitry: SARS Target 1 (N2:FAM); SARS Target 2 (N1:ROX). PREvalence SARS-CoV-2 Wastewater Kit Assays used with Bio-Rad RT-ddPCR chemistry: SARS Target 1 (N2: FAM); SARS Target 2 (E:HEX).

12.3 Summary

QIAGEN offers an end-to-end, Sample to Insight® workflow for wastewater-based epidemiology (WWBE) that addresses two critical aspects for success: Sample extraction and PCR chemistry. Wastewater solids are enriched for viruses of interest, but they contain high levels of RT-dPCR inhibitors. Extraction methods with IRT like those from QIAGEN are key to unlocking the potential of wastewater solids in WWBE. Similarly, the choice of RT-dPCR chemistry is key to maximizing sensitivity. Compared with the QIAcuity OneStep Viral RT-PCR Kit, the QIAcuity OneStep Advanced Probe Kit is designed to tolerate inhibitors found in wastewater. When combined, sample extraction and RT-dPCR chemistries from QIAGEN provide a WWBE workflow with superior sensitivity compared to other commercially available solutions.

12.3.1 Why combine QIAcuity Digital PCR and wastewater for use in epidemiology?

Cost

- Worldwide wastewater monitoring could save up to 1 billion USD for national monitoring programs depending on frequency of sampling and population.

Sensitivity

- One infected individual among 10,000 persons could potentially be detected.

Coverage

- Everyone "opts in" for testing.
- 2.1 billion people could be monitored globally in 105,600 sewage treatment plans.

Precise and absolute quantification

- QIAcuity Digital PCR allows for end-point detection as in conventional PCR.
- A standard curve is not required – no need to rely on the correctness of the concentration of the standard.
- Higher precision than qPCR made possible by the thousands of partitions interrogated in a single reaction.

Robust

- Increased reproducibility and reduced variability leads to be better inter-laboratory comparability.

Resistant to inhibitors

- Higher robustness for viral detection from complex samples – wastewater samples are diverse and contain high levels of PCR inhibitors.

13 Design and Optimization of dPCR Assays

The most common nucleic acid detection and quantification approach is real-time quantitative PCR (qPCR). However, dPCR, in its various formats (chips, droplets, etc.), is a rapidly evolving technology in this area. The two techniques share similarities and differences, and it is due to those differences, several applications benefit from dPCR over qPCR. This includes partitioning the samples into thousands of individual reactions, absolute quantification without standard curves and the reduced susceptibility to PCR inhibitors. Digital PCR follows end-point amplification, and each partition can be positive or negative, depending on the presence or absence of a target sequence counted by a fluorescence measurement, resulting in a binary readout. Poisson statistics is then applied to determine the absolute quantity of target DNA in a sample. Moreover, the end-point measurement enables quantification independent of the amplification efficiency, thereby allowing dPCR to be used for low-abundance target quantification even in complex samples or highly precise quantification of very low DNA copy numbers.

Quantitative PCR is a well-established technique with optimized protocols in most molecular biology laboratories, so when establishing dPCR for the first time or migrating existing assays from qPCR to dPCR, different aspects need to be considered. These include, but are not limited to, primer and probe concentrations and cycling conditions (e.g., time, temperature, and cycle number). However, note that the fundamental detection chemistries and assay formats of dPCR (e.g., LNA probes, hydrolysis probes and DNA-binding dyes, primer design, one- step or two-step reaction, etc.) will seem highly familiar to an existing qPCR user (Figure 49 and Figure 50). Here, we demonstrate how to optimize your assays on a microfluidic nanoplate-based dPCR system, the QIAcuity, and provide recommendations for a seamless transfer. Moreover, the QIAcuity dPCR workflow is very similar to qPCR.

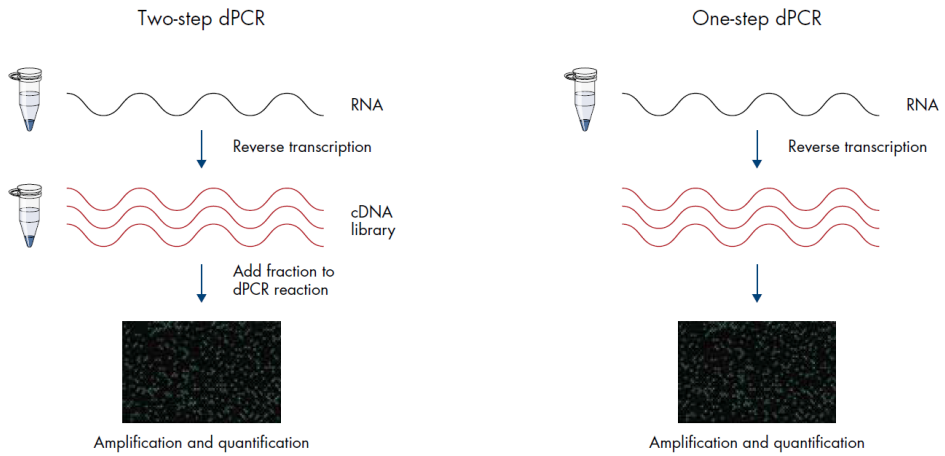


Figure 49. Familiar assay formats for dPCR: one-step reaction (left) and two-step reaction (right). cDNA synthesis from RNA input and PCR amplification of cDNA is performed in the same reaction well in one-step dPCR. cDNA is synthesized from RNA in bulk, and PCR amplification is done on a sub-sample of the cDNA in two-step dPCR.

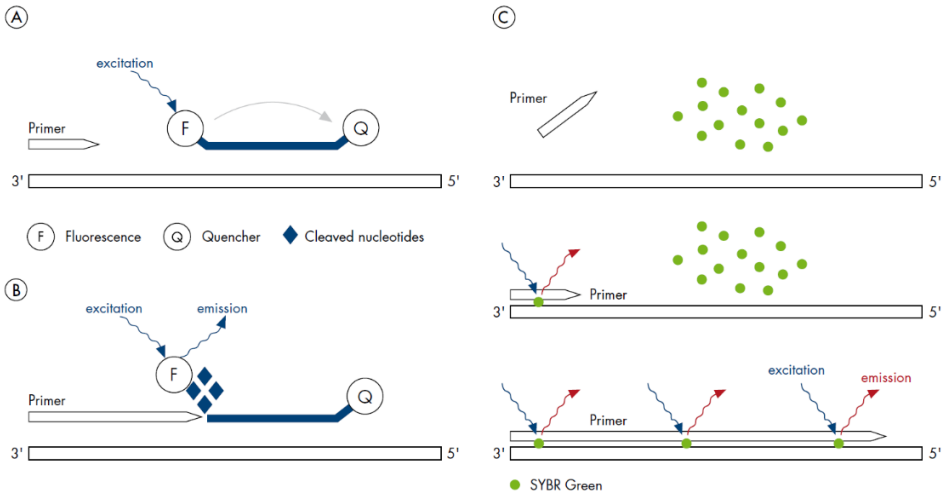


Figure 50. Familiar assay and detection chemistry options: working principle of hydrolysis probes (left) and DNA-binding dyes (right). Three oligos (two PCR primers and one detection probe with fluorophore and quencher) are shown on the left. Primers and probe anneal to target sequence during thermal cycling. When intact, the probe will have minimal fluorescence. The polymerase extends from primer, encounters bound probe and hydrolyzes. Cleavage of the fluorophore from the distal quencher activates the fluorescent signal. This signal is proportional to the amount of accumulated PCR product. On the right, two oligos (forward and reverse PCR primers) and a sequence-agnostic dsDNA binding dye (e.g., EvaGreen) are shown. The PCR primers anneal to the target sequence during the PCR annealing step. Taq DNA polymerase extends the primer during the PCR extension step, and EvaGreen binds to the newly formed double-stranded DNA. Upon DNA binding, a conformational change of the dye is induced, and it starts to fluoresce brightly. This signal is proportional to the amount of accumulated PCR product.

Standardization in qPCR came at a high cost of irreproducible data. In 2009, a group of qPCR experts published the MIQE guidelines for reproducible experiments (1). Those guidelines shaped present-day qPCR and RT-qPCR, which is considered a gold standard technique in gene expression (2). Fast forward, dPCR also took advantage of the publication of the dMIQE guidelines (3) to ensure global standardization.

The best practices of assay design and optimization provided here also ensure adhering to the latest dMIQE guidelines (4) for standardizing experimental protocols, maximizing efficient utilization of resources and further enhancing the impact of this powerful technology.

When no assay is available in hand but there is a need for high resolution target quantification, our general recommendation is as follows:

- Obtain pre-designed and conditionally validated assays from commercial sources – these are usually dMIQE-compliant and tailored to unique master mixes and kinetics of the thermocyclers specific to a system
- Published, peer-reviewed designs – review literature for assays and assess dMIQE compliance. While this practice is common in the research community, we advise checking for the specificity of the published primers.
- Design in-house assays following the recommended conditions mentioned below for the QIAcuity.

Regardless of the starting point – no prior assay, established qPCR assay, or established dPCR assay on another platform, ensure meeting the following primer and probe design criteria when using the QIAcuity.

13.1 Primers

- Use specialized design software (e.g., Primer3Plus or Primer Express)
- Amplicon ideally ≤150 bp
- 18–30 nucleotides in length with 30–70% GC
- Tm of the primers should be 58–62°C and within 2°C of each other
- Avoid
    - Highly repetitive sequences
    - 3'-end cross-complementarity
    - Within or across primer complementarity
    - 3' template mismatch
    - ≥3 Gs or Cs at 3' end
    - Regions with secondary structure specifically at the binding sites of the primers
- Ensure that primer sequences are unique for your template sequence with bioinformatics (e.g., BLAST search)

13.2 Probes

- Use specialized design software (e.g., Primer3Plus or Primer Express)
- The T_m of probes should be 8–10°C higher than the T_m of the primers
- Avoid a G at the 5'-end of probes and runs of ≥4 G nucleotides
- Choose the binding strand so that the probe has more C than G bases
- Ensure that primers and probes are not complementary to each other
- Design using the same settings to ensure that they will work optimally under the same cycling conditions (60°C annealing/extension)

It is important that the fluorescent label attached to the probe is compatible with one of the detection channels of the QIAcuity instrument (Table 19).

Table 19. Fluorophore compatibility

Channel	Excitation (nm)	Emission (nm)	Example fluorophores
Green	463–503	518–548	FAM
Yellow	514–535	550–564	HEX, VIC
Orange	543–565	580–606	TAMRA, ATTO 550
Red	570–596	611–653	ROX, Texas Red
Crimson	590–640	654–692	Cy5, Quasar 670

It is also worth considering the following reaction (primer-probe concentrations) and thermal cycling conditions for the respective QIAcuity PCR Mix for best results (Table 20 and Table 21).

Table 20. Recommended reaction conditions

Component	Volume/reaction		
	Nanoplate 8.5K (24-well, 96-well)	Nanoplate 26K (24-well)	Final concentration
4x Probe PCR Master Mix	3 µL	10 µL	1x
10x primer–probe mix 1	1.2 µL*	4 µL*	0.8 µM forward primer 0.8 µM reverse primer 0.4 µM probe
10x primer–probe mix 2, 3, 4, 5 (for multiplex)	1.2 µL*	µL*	0.8 µM forward primer 0.8 µM reverse primer 0.4 µM probe
Restriction Enzyme (optional)	Up to 1.2 µL	Up to 1.2 µL	0.025–0.25 U/µL
RNase-free water	Variable	Variable	
Template DNA or cDNA (added at step 4)	Variable†	Variable†	
Total reaction volume	12 µL	40 µL	

* Volume might vary, depending on concentration of the primer/probe mix used.
† Appropriate template amount depends on various parameters.

Table 21. Recommended thermal cycling conditions

Step	Time	Temperature (°C)
PCR initial heat activation	2 min	95
2-step cycling (40 cycles)		
Denaturation	15 s	95
Combined annealing/extension	30 s	60*

* Temperature during annealing/extension and number of cycles might vary depending on assay type.

Whether following the recommended assay conditions for the QIAcuity or those used on your current platform during migration, it is advisable to perform pilot runs to determine further optimization.

Pilot runs should be performed by:

- Including a well-characterized positive control, negative control and a no template control (NTC)
- Modifying thermal cycling parameters outside of annealing/extension step, or the hot start if required
- Creating template dilutions to ensure staying within the dynamic range
- Running a dPCR reaction on the QIAcuity using the recommended and your currently used concentration of assay components
- Assessing their initial performance (Figure 51 A–B)

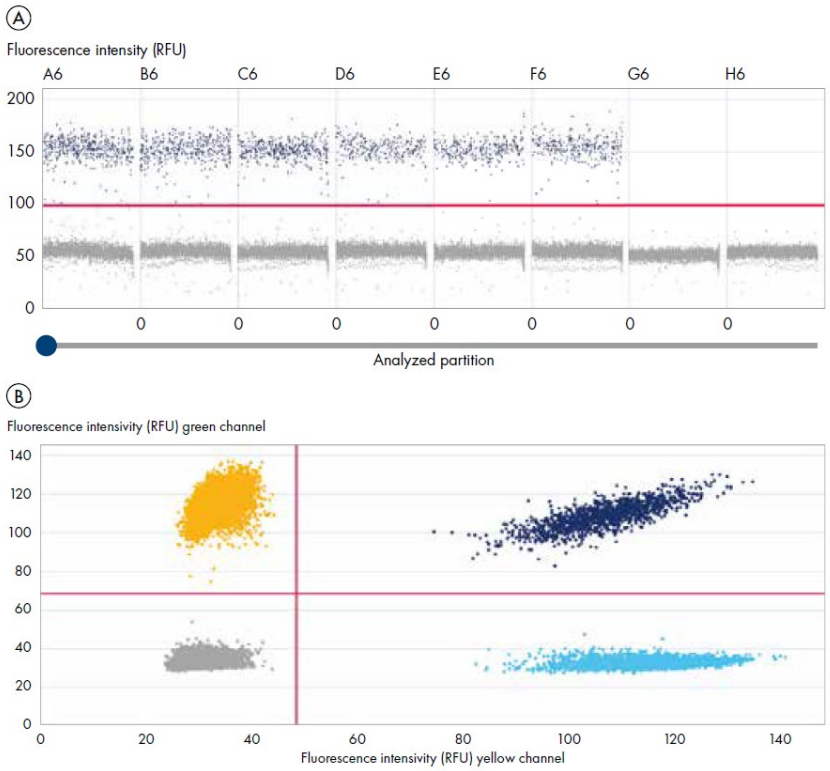


Figure 51. Representative (A)1D and (B) 2D data evaluating the initial performance of a dPCR assay. Criteria for 1D data include the presence of two populations (positive and negative), sufficiently large signal:noise ratio, meaning high RFU of positive population, low RFU of negative population, and the ability to identify the threshold between two populations, and clean NTCs. Criteria for 2D data obtained from a dPCR LNA mutation detection/BRAF V600E assay include the presence of four partition populations (single positives, dual positives and dual negatives), sufficient signal:noise ratio, meaning high RFU of positive populations, low RFU of negative populations and the ability to identify the threshold between four populations, and clean NTCs.

Should the performance be suboptimal, optimizing the following parameters can help separate the positive and negative partition signals better, improve PCR efficiency, increase probe specificity and selectivity, reduce assay artifacts, and resolve intermediate partition signal.

1. Modifying annealing temperature, depending on the use of dye or probe (Figure 52 A–B).

   Even well-working temperature conditions established for other qPCR and dPCR reagents might be suboptimal because primer and probe annealing properties strongly depend on the composition of the master mixes.

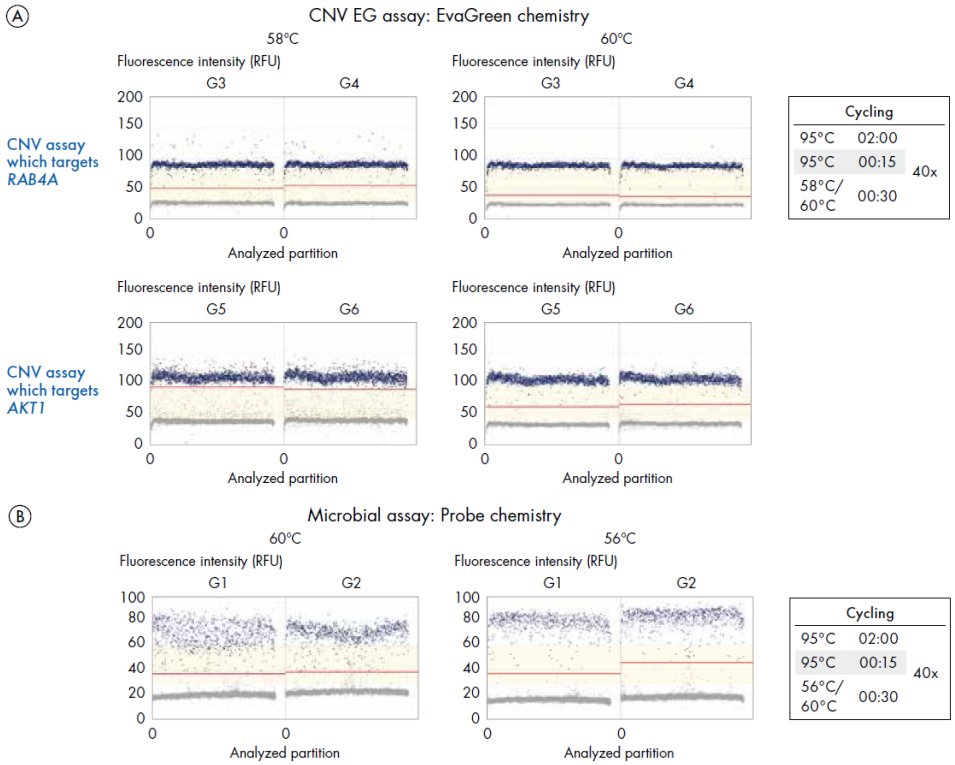


Figure 52. Annealing temperature change resolves intermediate partitions. (A) dPCR Copy Number Assays targeting RAB4A and AKT1 were run using WT gDNA samples as the template and QIAcuity EG PCR master mix. 4 ng of the sample was loaded per reaction, per condition in QIAcuity Nanoplate 8.5K, 96-well. Standard QIAcuity instrument and imaging setup were used. The adaptation in annealing temperature resulted in a reduction of rain and an increase in the signal-to-noise ratio. **(B)** A single-plex Microbial DNA qPCR Assay targeting Lactobacillus plantarum was run using Microbial DNA Positive Control V2 (cat. no. 338135) as the template and QIAcuity Probe PCR master mix. Samples were run in duplicates per condition in QIAcuity Nanoplate 8.5K, 96-well. Standard QIAcuity instrument and imaging setup were used. The adaptation in annealing temperature resulted in a reduction of rain, due to increased primer and probe annealing efficiency.

2. Modifying annealing/extension time (Figure 53).

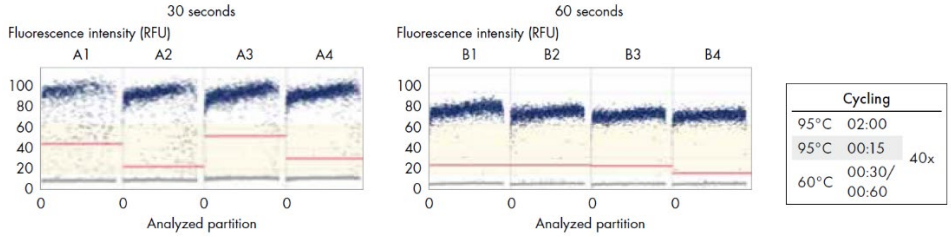


Figure 53. Annealing/extension time change resolves intermediate partitions. dPCR reactions were performed on the QIAcuity system in QIAcuity Nanoplate 8.5K, 96-well using a TaqMan PCR (hydrolysis probe PCR) assay targeting the single-copy human ERBB2 gene from genomic DNA. The cycling parameters used are as indicated in the table. Standard QIAcuity instrument and imaging setup were used. The increase in annealing/extension time resulted in a reduction of rain and an increase in the signal-to-noise ratio.

3. Performing a temperature gradient to determine the optimal annealing temperature.

   Optimal annealing temperatures can be easily determined by a quick temperature gradient experiment on any real-time cycler because all QIAcuity kits are compatible with any real-time cycler. Adhere to the recommended cycling conditions outlined in the respective manuals. From software version 2.5 on, temperature gradients can be run directly on the QIAcuity instrument.

4. Performing restriction enzyme digestion of dsDNA template to improve template accessibility.

5. Adapting the primer-probe concentration (Figure 54).

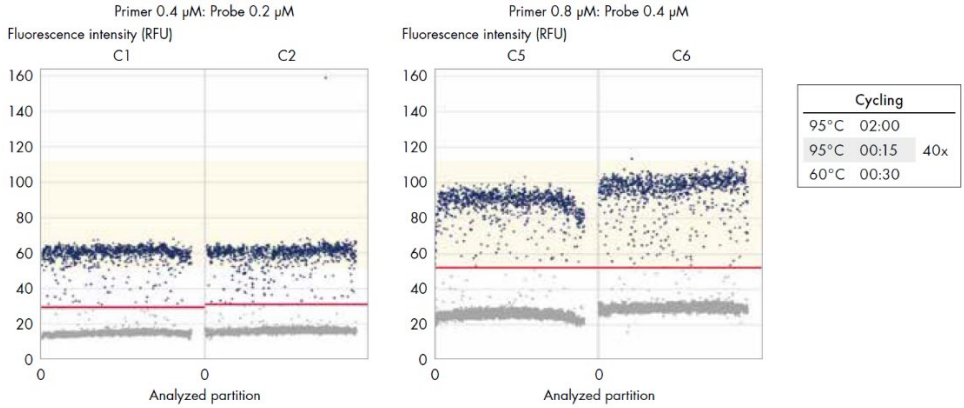
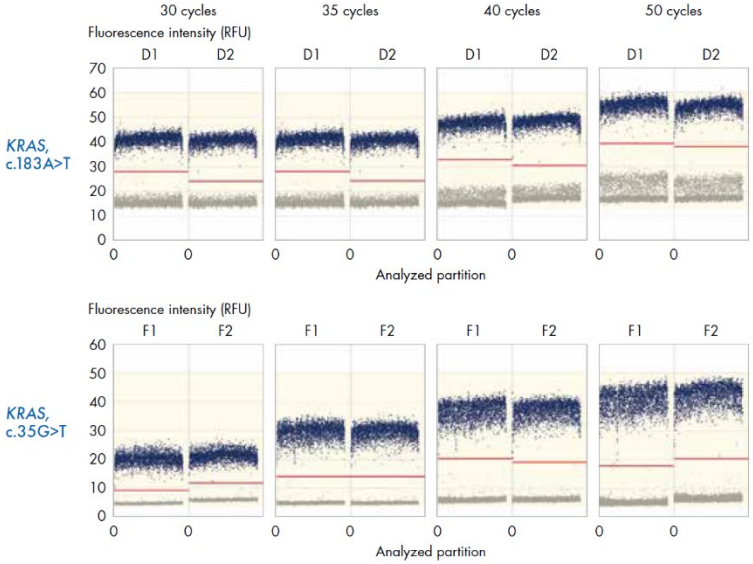


Figure 54. Primer–probe optimization increases positive partition signal. A single-plex Microbial DNA qPCR Assay targeting *Faecalibacterium prausnitzii* was run using Microbial DNA Positive Control V2 (cat. no. 338135) as the template and QIAcuity Probe PCR master mix. Samples were run in duplicates per condition in QIAcuity Nanoplate 8.5K, 96-well. Standard QIAcuity instrument and imaging setup were used. The adaptation resulted in better separation of positive and negative partitions, increasing positive partition signal.

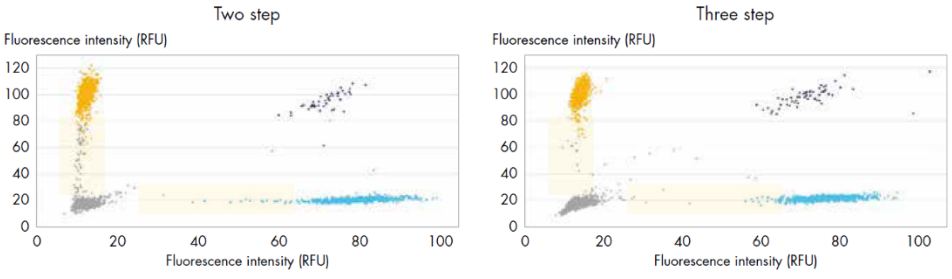
6. Modifying cycle numbers, increasing from 40 to 50 or even 60 (Figure 55).



Cycling		
95°C	02:00	1x
95°C	00:15	30x, 35x, 40x, 50x
60°C	00:30	

Figure 55. Cycle number optimization can increase separation. Digital PCR reactions were performed on the QIAcuity system in QIAcuity Nanoplate 26K, 24-well using LNA mutation assays specific for KRAS mutations. KRAS-specific duplex mutation assay. WT gDNA at 1000 copies/µL was used as the sample in duplicates. Standard cycling and imaging conditions were used. The positive populations detected by the HEX-labeled WT probe in the Yellow channel are shown here. The adaptation increased positive signal, thus demonstrating better separation of positive and negative partitions.

7. Converting 2-step cycling into 3-step cycling with separate steps for annealing and extension at optimal temperatures (Figure 56).

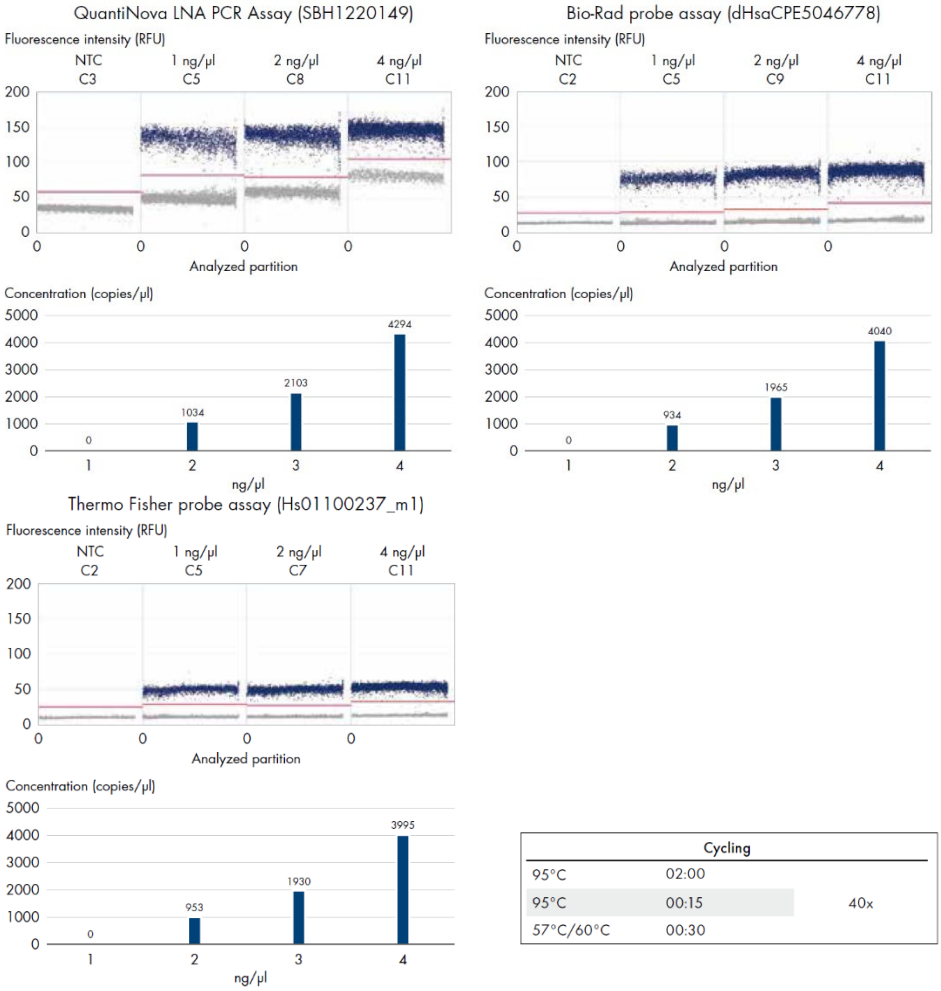


Cycling		
95°C	02:00	
95°C	00:15	40x
60°C	00:30	

Cycling		
95°C	02:00	
95°C	00:15	40x
60°C	00:15	
72°C	00:15	

Figure 56. Transition from two- to three-step thermal cycling can improve separation. 100 copies/µL wild-type gDNA samples were tested with CDH1 (FAM)/TERT (HEX) in a duplex reaction. QIAcuity Probe PCR master mix was used. The cycling parameters are as indicated in the table, and the standard QIAcuity imaging setup was used. This adaptation improves the cluster separation.

Commercial assays may perform well with minor modifications (Figure 57). Commercial, premixed real-time PCR assays often come with final concentrations of primer and probes, deviating from the recommendations for the QIAcuity mixes. Nonetheless, without further adaptation, they might work with the concentration recommended by the assay supplier. If results using the 1x concentration are not satisfactory, adapt the concentration of the commercial assay to the recommended primer-probe concentration for the respective QIAcuity mix. This is easily achievable for probe-containing assays with a primer:probe ratio of 2:1. If other primer-probe ratios are used for the assay, adapt the concentration of the assay so that it will have the primer concentration recommended for the respective QIAcuity mix. For more details, please refer to Section 14.



Cycling		
95°C	02:00	
95°C	00:15	40x
57°C/60°C	00:30	

Figure 57. Commercial assays may perform well with minor modifications. This example assay compared the Eva-Green based QuantiNova LNA PCR Assay for KAT8 with probe-based assays for the same target gene from Bio-Rad and Thermo Fisher, revealing very similar quantification results. Three individual gene expression assays run in QIAcuity Nanoplate 8.5K, 96-well on a QIAcuity dPCR instrument. cDNA template was prepared from UHR (universal human reference RNA from Thermo Fisher Scientific) using the QuantiTect RT Kit (cat. no. 205313). QuantiNova LNA PCR Assay used the QIAcuity EG PCR Kit. The Bio-Rad and Thermo Fisher probe assays used the QIAcuity Probe PCR Kit (cat. no. 250102). 1D Scatterplot depicts one representative of triplicates for each concentration. The numbers above bars show mean concentrations of 3 replicates. Each well was loaded with a 12 µL sample containing the template concentration. Standard QIAcuity instrument and imaging setup were used. The experiment demonstrates the easy adaptation of commercial assays to QIAcuity Nanoplate dPCR.

13.3 Conclusion

A combination of experimental factors and processes should be considered when migrating home-brew or commercial qPCR and dPCR assays from other suppliers into dPCR assays on the QIAcuity, making that transition highly robust. A summary of the recommendations is provided here.

- Ensure assay design meets criteria described in this chapter
- Follow recommended conditions for respective QIAcuity PCR and OneStep Advanced Mixes.
- Check the compatibility of fluorophores.
- Perform pilot tests and assess their initial performance prior to further optimization.

Ultimately, a successful transition to digital PCR can overcome interlaboratory variations in precision and accuracy and lead to global standardization.

14 Transfer of Commercial qPCR Assays onto QIAcuity

Even though dPCR offers a number of advantages over quantitative PCR in various applications, a well-established commercially available qPCR assay is still the first choice for many researchers. The transfer of these assays to dPCR is often thought to be difficult and resource intensive. Here, we demonstrate how predesigned assays for GEX, CNV, and SNP analyses can be easily transferred from a qPCR platform to the QIAcuity dPCR System.

14.1 Gene expression qPCR assays

We selected 20 target genes and ordered an assay for each target from 3 different suppliers (Applied biosystems, Bio-Rad, and IDT). All assays were run in qPCR with either QIAcuity Probe or suppliers' recommended master mixes on thermocyclers as recommended by the assay supplier (ThermoFisher, IDT: QuantStudio 5, Bio-Rad: CFX96). For dPCR all assays were run on the QIAcuity either with concentration as recommended by supplier (i.e. ,1x in final reaction) or adapted to QIAcuity requirements.

Outcome

Out of 60 assays, 59 worked comparably in qPCR with the recommended master mix and the QIAcuity Probe PCR mix (Cq value, efficiency). One assay failed even when using the recommended supplier's master mix. All remaining 59 assays worked well in dPCR with the suppliers' recommended concentration and can be used without any restrictions. Figure 58 shows exemplary a qPCR TaqMan assay (YWHAZ) run with the recommended mastermix (A), the QIAcuity probe master mix (B) and the QIAcuity master mix on the QIAcuity system (C) with two different amounts of starting material.

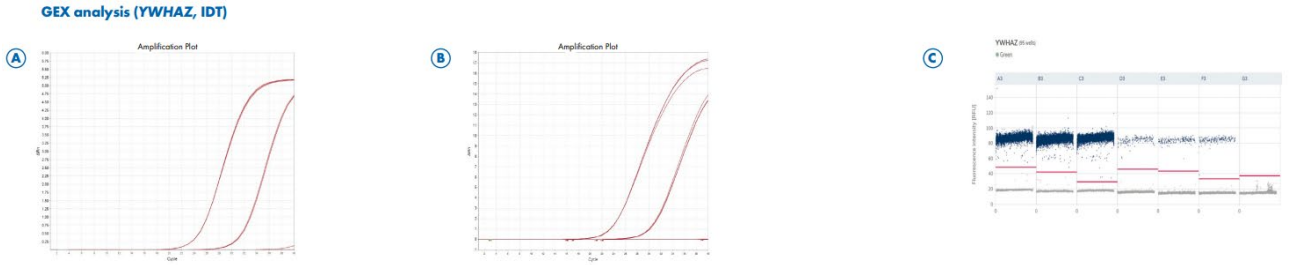


Figure 58. qPCR assays shown were tested (A) using qPCR and assay supplier's master mix, (B) using qPCR and QIAcuity Probe PCR master mix, and (C) using dPCR on the QIAcuity Four System with QIAcuity Probe PCR master mix.

To further show the high accuracy of the QIAcuity system we obtained cDNA from the Universal human reference RNA and spiked the RNA with increasing number of an artificial template. Here we see, that the accuracy in a low template environment is similar between qPCR and dPCR. When the template amounts increase, the QIAcuity remains close to the expected values, whereas qPCR overestimates the spiked in copies (Table 22).

Table 22. Comparison of qPCR assays for gene expression analysis in qPCR and dPCR

Upregulation expected: *UBE2D2*	QuantStudio	QIAcuity
1.2	1.1	1.2
1.4	1.3	1.3
1.6	1.7	1.6
1.8	1.9	1.8
2.0	2.2	2.0
5.0	6.0	5.1
7.0	7.9	6.8
10.0	12.7	10.1

Table 1 shows the expected ratio of the UBE2D2 spiked in target of interest in a constant background of reference cDNA quantified by the reference gene YWHAZ in both qPCR (analyzed with the QuantStudio software) and dPCR (analyzed with QIAcuity software). With increasing template numbers, qPCR becomes inaccurate whereas dPCR results stay close to the calculated gene expression level.

14.2 CNV qPCR assays

Here, 20 TaqMan qPCR Assays were obtained from Thermo Fisher. The assays were first tested using the recommended master mix as well as the QIAcuity dPCR probe master mix on qPCR and then subsequently on dPCR. All 20 assays were transferred successfully to the QIAcuity. We observed some crosstalk into FAM channel, derived from the VIC-labelled reference assay. The observed crosstalk had no effect on the quantification and could be removed completely by decreasing the reference probe assay concentration to 0.25x in the final master mix with no negative impact on the quantification. To demonstrate the accuracy of the QIAcuity system, we spiked in up to 9 genome equivalents of a synthetic template encoding for the MET gene into a background of genomic DNA (quantified by a 2 copy per genome gene). The CNV assays used were of the 20 TaqMAN CNV assays obtained from Thermo Fisher (target of interest MET, FAM labelled, 1x) and RNase P (reference target, VIC labelled and reduced to 0.25x for dPCR).

Table 23. Comparison of qPCR assays for CNV analysis in qPCR and dPCR

Extended copy number	QIAcuity	QuantStudio 5
2	2	2
3	2.8	3.6
4	4.1	4.4
5	4.9	6.1
6	6.7	6.8
7	7.0	7.8
8	7.5	8.6
9	9.0	11.1

Table 23 shows the expected copy numbers in the left column as well as qPCR and dPCR numbers in the second and third column. The additional gene copies were spiked in using an artificial template in increasing numbers. The numbers represent the calculated gene copy numbers based on a 2 copy reference gene (RNAse P). Results obtained on the qPCR platform (rightmost column) deviate from the expected values especially at higher copy numbers (cn > 7) whereas dPCR (middle column) accurately quantifies the expected copy number.

14.3 SNP qPCR assays

To demonstrate the easy transferability of established qPCR assays to the QIAcuity platform, we ordered 20 commercially available SNP qPCR assays (Thermo Fisher). All assays were then tested on qPCR using either QIAcuity Probe or Thermo's recommended master mix on QuantStudio5 qPCR system. All assays were then transferred onto the QIAcuity system using with concentrations as recommended by supplier (i.e., 1x in final reaction) or adapted to QIAcuity requirements. Taken together, all tested assay were successfully transferred to the QIAcuity dPCR platform after a minor modification of the cycling protocol. In order to reduce crosstalk of the VIC signal into the FAM detection channel and cross-hybridization, cycle number were reduced from 40 to 30. All other parameters were kept the same. Figure 59 shows an example of 2 assays for the same SNP with different fluorescence labels.

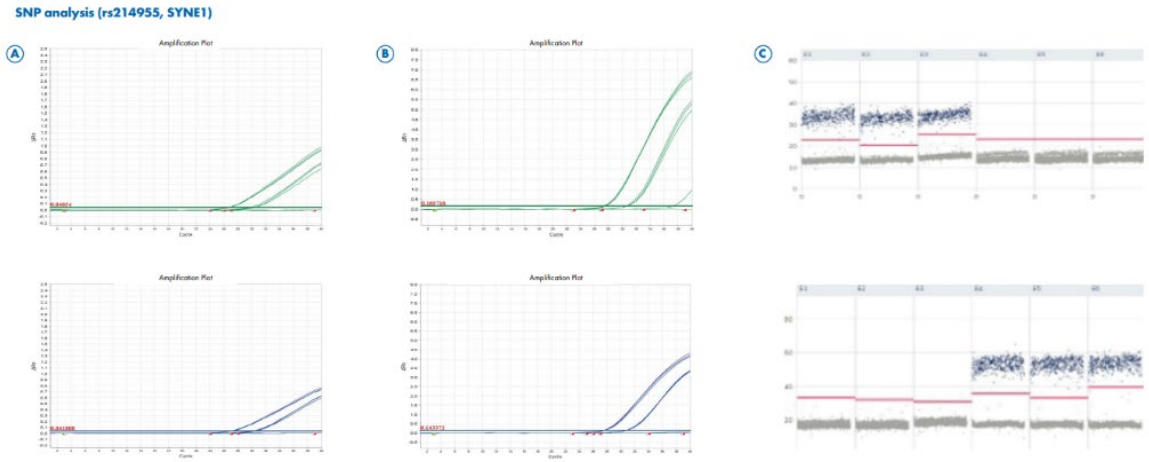


Figure 59. SNP analysis. qPCR assays shown were tested **(A)** using qPCR and assay supplier's recommended master mix, **(B)** using qPCR and QIAcuity Probe PCR master mix, and **(C)** using dPCR on the QIAcuity Four System using the QIAcuity Probe PCR master mix. Upper panels: SNP1 (FAM); lower panels: SNP2 (VIC).

14.4 Correlation of qPCR and dPCR

When transferring from q to dPCR it is important to be aware how some variables correlate between the two types of PCR. Therefore this chapter provides some considerations to me made when transiting.

First of all some commonly accepted facts about qPCR important to keep in mind. A qPCR with 1000 starting copies of template corresponds to a C_q value of 27 (+/–2) for probe-based and 22 (+/–2) for Eva/Cyber Green-based detection. Doubling the starting material results in one C_q change, 10 times starting material results in a 3.3 C_q shift (assuming a high amplification efficiency assay). More extreme, a 1000-fold difference leads to a difference of ~10 cycles (Cqs). This inherent correlation results in a relative low resolution (one C_q shift requires twice the amount of DNA) and high dynamic range. Table 24 summarize this correlation.

Table 24. Exemplary calculation from Cq to copies/µL

Copies	Probe Cq	SYBR/EvaGreen Cq
1000	27	22
1,000,000	17	12

When transitioning to dPCR, 1,000,000 copies would correspond to 2,350,000 analyzed templates on the 8.5K plate and 5,330,000 analyzed templates on the 26K plate. This means an average copies per partition of 27.6 on the 8.5K plate and 20.2 on the 26K plate, respectively (Table 25). This also means, that there won't be enough empty partitions needed to perform Poisson statistics. The recommendation here would be to use a 1/10 dilution (100,000 templates) instead, which results in 2–3 copies/partition on average.

Table 25. Results of the distribution of 1,000,000 templates on the QIAcuity Nanoplates

Plate	Reaction volume	Copies/µL	Analyzed volume (µL)	Total copies analyzed	Number of partitions	Copies/partitions
96-Well plate (12 µL)	12	83,333	2.82	235,000	8510	27.6
24-Well plate (40 µL)	40	25,000	21.32	533,000	26,384	20.2

Upper row: Distribution of a dPCR reaction containing 1,000,000 templates on our two plate types. On the 96-well plate, 8.5K plate 1,000,000 distributed templates lead to 27.6 copies/partition on average with likely no empty partitions.
Lower row: The same PCR reaction distributed on our 26K plate. Due to the higher amount of partitions, the average copies/partition is lower; however, still no empty partitions are likely to be found.

14.5 Alternative fluorophores for QIAcuity

In addition to recommended dyes on page 103, the optical system and filters of the QIAcuity system allows the use of various alternative dyes in all channels. When a non-standard dye is required, first to check would be what the extinction and emission wavelength are and if these parameters are within the limits of the QIAcuity system. For example, the green channel excitation (in nm) ranges from 463–503 and the corresponding emission from 518–548 nm. This means a potential dye for the green channel has to fall in these two ranges from excitation and emission. When first testing, the assay with this dye it is recommended to try in single plex and importantly to also image the neighboring channels. In our example here, testing alternative dyes for the green channel, the neighboring yellow channel should be checked for potential crosstalk. 60 shows a test with the same assay (ERBB2), labelled with each of our recommended alternative dyes (left panel). The right panel shows the results from the same experiments imaged in the yellow channel. None of the alternative dyes displayed crosstalk into the yellow channel. When testing alternative dyes, major crosstalk prevents accurate thresholding. The general recommendation is to lower the fluorescence of the assay in the channel causing the crosstalk. For example, when major crosstalk into the red channel causes a problem, reducing the fluorescence in the neighboring channels (Crimson and orange). For example, by reducing the final probe concentration to 0.5x, reducing the cycle number to 30 or limiting the primer concentrations lead to a lower fluorescence level and therefore less possible crosstalk into the neighboring channels.

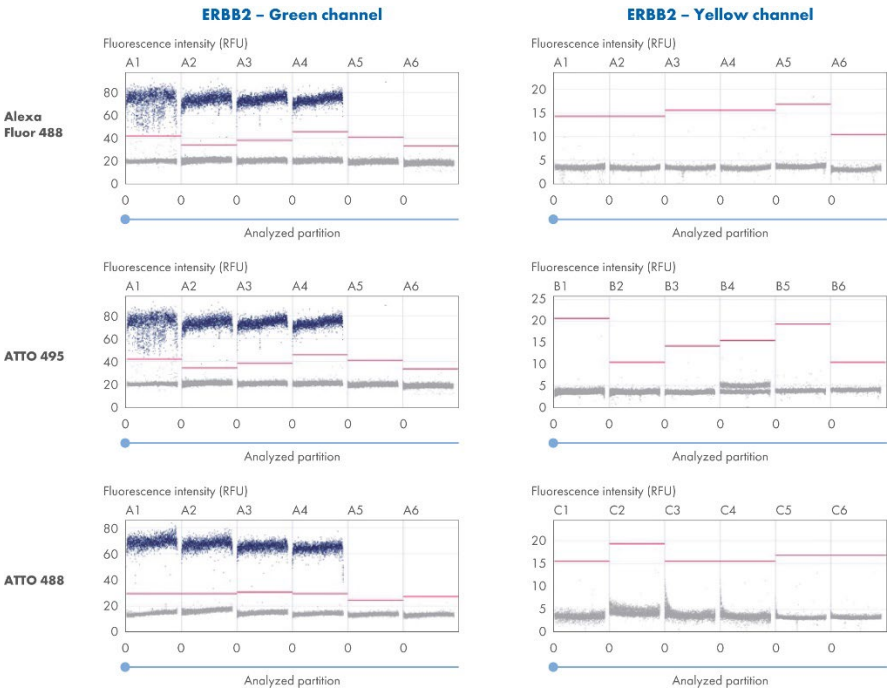


Figure 60. Left panel: An assay detecting ERRB2 labelled with different fluorophore in the FAM channel. **Right panel:** The same experiment imaged in the yellow channel.

On Table 26 is a selection of dyes that have been tested on the QIAcuity system and are available from at least two suppliers.

Table 26. Wetlab-tested alternative dyes available from at least 2 suppliers

Channel	Dye	A max (mm)	E max (nm)
Green	Alexa Fluor 488	495	519
Green	ATTO 495	495	527
Green	ATTO 488	501	523
Yellow	Alexa Fluor 532	532	524
Yellow	ATTO 532	532	553
Orange	BDP TMR	545	570
Orange	NED	553	575
Orange	Alexa Fluor 546	556	573
Red	Alexa Fluor 568	578	603
Crimson	ATTO 633	629	657
Crimson	Quasar 670	247	667

15 References

1. Bustin SA, et al. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clin Chem*. 2009;*55*(4):611–622.
2. Caballero-Sollares A, Hall JR, Xue X, Rise ML. Reverse Transcription-Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR) for Gene Expression Analyses. *Meth Mol Biol*. 2022;2508:319–340.
3. Hugget JF, et al. The Digital MIQE Guidelines: Minimum Information for Publication of Quantitative Digital PCR Experiments. *Clin Chem*. 2013;59(6): 892–902.
4. Whale AS, et al. The Digital MIQE Guidelines: Minimum Information for Publication of Quantitative Digital PCR Experiments. *Clin Chem*. 2020;66(8): 1012–1029.

16 Example Publications

Oncology

- Wells J, Patel A, Torres MM, Costello J, Jensen K, Vasko V. Clinical and molecular characterization of thyroid cancer when seen as a second malignant neoplasm. *Adv Endocrinol Metab*. 2021;12:1–13.
- Sun N, et al. Coupling lipid labeling and click chemistry enables isolation of extracellular vesicles for noninvasive detection of oncogenic gene alterations. *Adv Sci*. 2022;9(14):2105853.

Copy Number Variation

- Wang J, et al. Preliminary study of noninvasive prenatal screening for 22q11.2 deletion/duplication syndrome using multiplex dPCR assay. *Orphanet J. Rare Dis*. 2023;18:278.

Waste Water Analysis

- Warish A, et al. Comparison of RT-qPCR and RT-dPCR platforms for the trace detection of SARS-CoV-2 RNA in wastewater. *CS ES T Water*. 2022;2(11);1871–1880.
- Amman F, et al. Viral variant-resolved wastewater surveillance of SARS-CoV-2 at national scale. *Nat Biotechnol*. 2022;40:1814–1822.
- Whale AS, et al. Digital PCR can augment the interpretation of RT-qPCR Cq values for SARS-CoV-2 diagnostics. *Methods*. (2022);201:5–14.

Gene Expression

- Feliciello I, et al. Regulation of ssb gene expression in *Escherichia coli*. *Int J Mol Sci*. 2022;23(18):10917.
- Jurek B, Denk L, Schäfer N, Salehi MS, Pandamooz S, Harteis S. Oxytocin accelerates tight junction formation and impairs cellular migration in 3D spheroids: evidence from Gapmer-induced exon skipping. *Front Cell Neurosci*. 2022;16:000538.

Cell and Gene Therapy

- Meierrieks F, Kour A, Pätz M, Pflanz K, Wolff MW, Pickl A. Unveiling the secrets of adeno-associated virus: novel high-throughput approaches for the quantification of multiple serotypes. *Mol Ther Methods Clin Dev*. 2023;31:101118.
- Murphy LA, et al. Digital polymerase chain reaction strategies for accurate and precise detection of vector copy number in CAR T cell products. *Cytotherapy*. 2023;25(1):94–102.

Virus Detection

- Specchiarello E, et al. Development and validation of a nanoplate-based digital PCR assay for absolute MPXV quantification. *J Virol Methods*. 2023;321:114802.
- Tumpach C, et al. Adaptation of the intact proviral DNA assay to a nanowell-based digital PCR platform, *J Virus Erad*. 2023;9(2):100335.
- Portanti O, et al. Development and validation of an RT-qPCR for detection and quantitation of emerging epizootic hemorrhagic disease virus serotype 8 RNA from field samples. *J Virol Methods*. 2023;321:114808.

Methylation Analysis

- Torres Silva FL, et al. 11p15 Epimutations in pediatric embryonic tumors: insights from a methylome analysis. *Cancers*. 2023;15(17):4256.

Plant Research

- Passera A, et al. Bacterial communities in the embryo of maize landraces: relation with susceptibility to fusarium ear rot. *Microorganisms*. 2021;9(11):2388.
- Tergemina E, et al. A two-step adaptive walk rewires nutrient transport in a challenging edaphic environment. *Sci Adv*. 2022;8(20):1–14.

Food analysis

- Travadi T, Sharma S, Pandit R, Nakrani M, Joshi C, Madhvi Joshi M. A duplex PCR assay for authentication of *Ocimum basilicum* L. and *Ocimum tenuiflorum* L. in Tulsi churna. *Food Control*. 2022;137:108790.

Microbiology

- Yu H, Kim J, Rhee C, Shin J, Shin SG, Lee C.. Effects of different pH control strategies on microalgae cultivation and nutrient removal from anaerobic digestion effluent. *Microorganisms*. (2022);210(2):357.
- Jung H, Yu H, Lee C. Direct interspecies electron transfer enables anaerobic oxidation of sulfide to elemental sulfur coupled with CO2-reducing methanogenesis. *iScience*. 2023;26(9):107504.

Transplantation

- Christophe Picard C, et al. New methods for the quantification of mixed chimerism in transplantation. *Front Immunol*. 2023;14:1023116.

Forensics

- Ghemrawi M, McCord B. Development of a nanoplate-based digital PCR assay for species identification with mixture deconvolution. *Forensic Sci Int Genet Suppl Ser*. 2022;8:193–195.

Abbreviations

cDNA	Complementary DNA
cfDNA	Circulating cell-free DNA
CN	Copy number
CNA	Copy number alteration
CNV	Copy number variation
cp	Copy
ctDNA	Circulating tumor DNA
Cy5	Cyanine5
DEPC	Diethyl pyrocarbonate
dPCR	Digital PCR
FAM	Carboxyfluorescein
FFPE	Formalin-fixed, paraffin-embedded
gDNA	Genomic DNA
GMO	Genetically modified organism
GOI	Gene of interest
HEX	Hexachlorofluorescein
LNA	Locked nucleic acid
NEB	New England Biolabs
NTC	No template control sample
QN IC	QuantiNova Internal Control
ROX	Carboxyrhodamine
RT	Reverse transcription
RT-qPCR	qPCR using cDNA template after reverse transcription
TAMRA	Carboxytetramethylrhodamine
TFS	Thermo Fisher Scientific
Tm	Melting temperature
TOI	Target of interest
UV	Ultraviolet
WT	Wild type

Document Revision History

Revision	Description
April 2021	Updated the “Statics of nanoplate dPCR” section. Updated Table 14. Removed the section “QuantiNova Internal Control (QN IC) RNA”.
June 2021	Corrected the equation for λ_{low} on page 9. Corrected Table 8.
November 2023	Updated “Statistics of nanoplate dPCR”, “Absolute quantification”, “Mutation detection”, and “Gene Expression” Sections. Added sections for multiplexing, cell and gene therapy, wastewater analysis, miRNA quantification, transferring assays from q to dPCR, and alternative dyes. Updated and refined the references section.

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