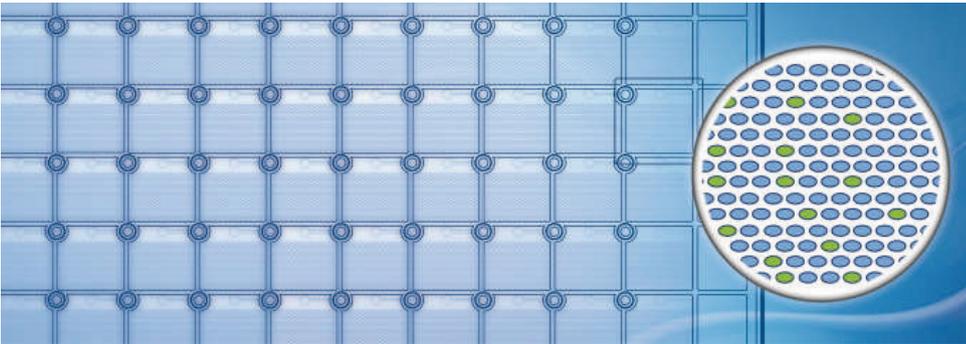


June 2021

QIAcuity[®] User Manual Extension

QIAcuity Application Guide



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Nanoplate Digital PCR

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 which 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.

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.

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 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. Subsequently, the connecting channels between the partitions are being closed by a pressure controlled rolling process.

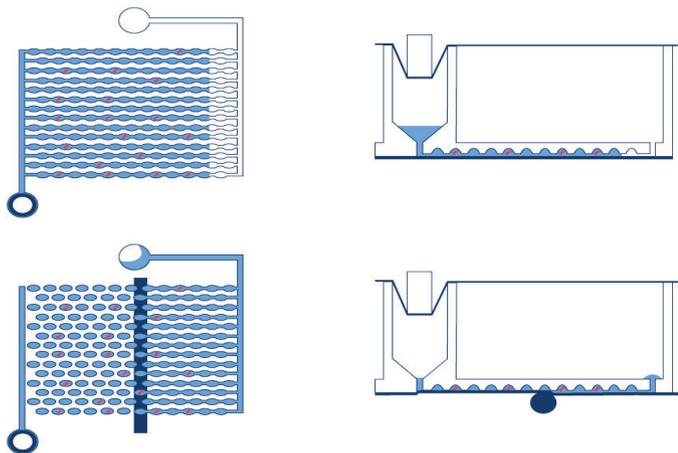


Figure 3. Scheme of filling and partitioning of a well.

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 the 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.

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.

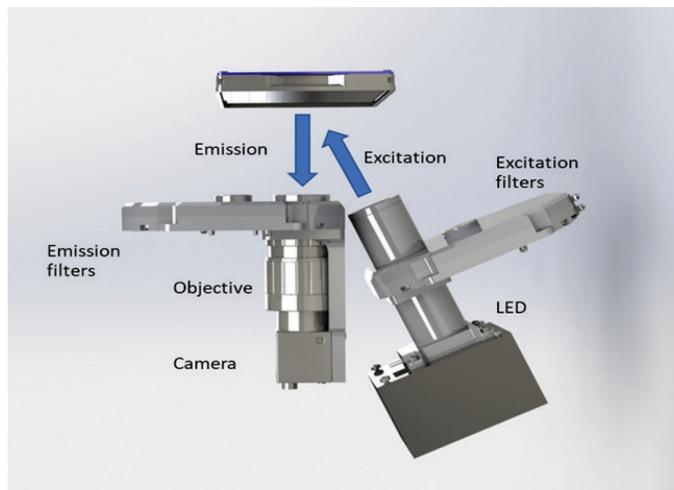


Figure 4. Scheme of the imaging module.

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, JOE
Orange	543–565	580–606	TAMRA, ATTO 550
Red	570–596	611–653	ROX, Texas Red
Crimson	590–640	654–692	Cy5

Statistics of nanoplate dPCR

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 $k = 0, 1, 2, \dots$

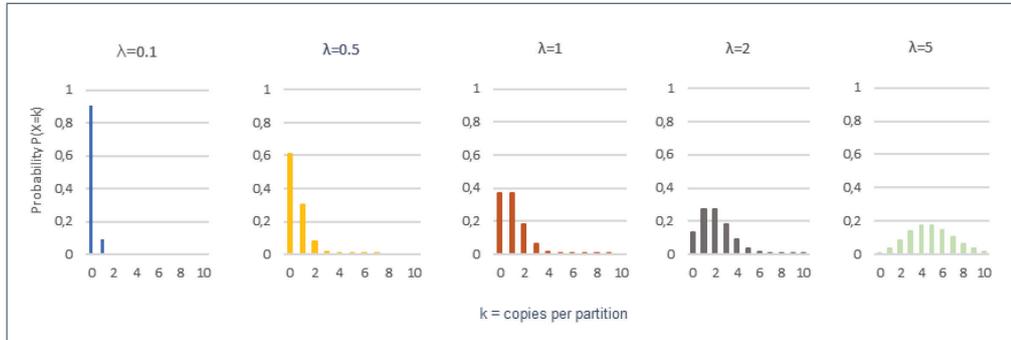


Figure 5. Poisson distribution for different expected values (λ).

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. The calculation of the target concentration is determined by referring to the volume of a valid partition.

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

Example:

For simplicity, the assumption is that 8000 partitions are valid partitions and 4000 partitions are positive. The empirical probability of a negative partition is, therefore, 0.5. The estimated number of copies of the target molecule per positive partition is:

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

$$\lambda = 0.693$$

The total number of copies of the target molecule in all valid partitions is:

$$8000 \times 0.693 = 5544$$

Adding the 95% confidence interval, λ_{low} and λ_{high} are obtained.

$$\lambda_{\text{low}} = 0.693 \cdot + \frac{1.96^2}{2 \cdot 8000} - \sqrt{0.693 \cdot \frac{1.96^2}{8000} + \frac{1.96^4}{4 \cdot 8000^2}} = 0.675142$$

$$\lambda_{\text{high}} = 0.693 \cdot + \frac{1.96^2}{2 \cdot 8000} + \sqrt{0.693 \cdot \frac{1.96^2}{8000} + \frac{1.96^4}{4 \cdot 8000^2}} = 0.711633$$

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.

$$\lambda_{\text{volume}} = \frac{\lambda}{V[\mu\text{l}]}$$

Example:

The number of copies of the target molecule is $\lambda = 0.693$.

The estimated partition volume is $V = 0.34$ nl.

The copies per microliter is:

$$\lambda_{\text{volume}} = \frac{0.693}{0.34} \times 1000 = 2038 \text{ copies}/\mu\text{l}$$

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 $2038 \times 12 = 24,456$ copies.

The following example shows how the copies/ μl can be converted to copies/ μl sample or copies/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 $(12/5) \times 2038 = 4891$ copies/ μl . Therefore, the copies in the 5 μl sample are $5 \times 4891 = 24,456$ 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, $\lambda = 0.693$, 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:

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

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

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 $\lambda = 0.1$, 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 $\lambda = 0.5$, some positive partitions will likely contain more than one copy of the target molecule. For higher concentrations like $\lambda = 5$, 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	23526	2353	118	4	0	0	0	0	0	0	0
	0.5	15770	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

Intended Use of the QIAcuity

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

Requirements for QIAcuity Users

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

Safety Information

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

Experimental Setup and Absolute Quantification

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.

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 μ M). 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 and Cy5.5 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 QIAcuity Probe PCR Kit and QIAcuity EG PCR Kit).

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.

EvaGreen detection

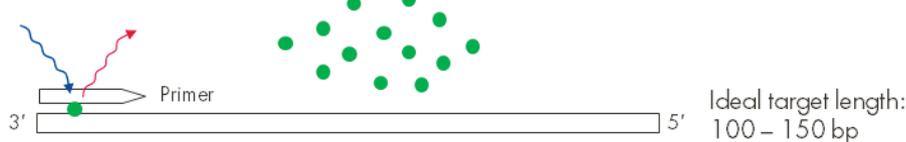
The fluorescent dye EvaGreen binds all double-stranded DNA molecules, emitting a fluorescent signal of a defined wavelength on binding (Figure 6). The excitation and emission peak of EvaGreen I are at 495 and 530 nm, respectively, allowing the detection of the dye on the green channel of the QIAcuity 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.

Principle

1. Denaturation



2. Annealing



3. Extension

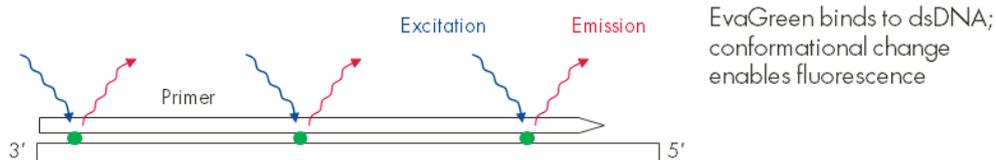


Figure 6. Principle of EvaGreen detection. The PCR primers anneal to the target sequence during the PCR annealing step. During the PCR extension step, Taq DNA polymerase extends the primer and EvaGreen binds to the newly formed double-stranded DNA. Upon DNA binding, a conformational change of the dye 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.

Hydrolysis probe detection

Hydrolysis probes, also known as TaqMan probes, are sequence-specific oligonucleotides with a fluorophore and a quencher moiety attached (Figure 7). 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.

Principle

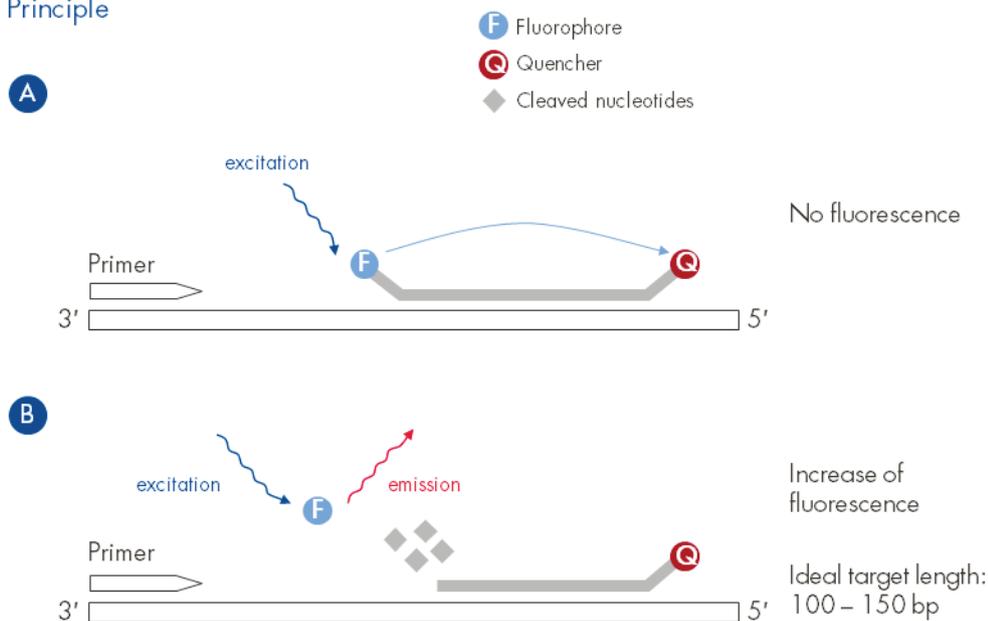


Figure 7. Principle of hydrolysis probe detection. Both the hydrolysis probe and the PCR primers anneal to the target sequence during the combined PCR annealing/extension step. The proximity of the fluorophore with the quencher prevents the fluorophore from fluorescing. During the PCR extension step, 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.

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.

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.

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.

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. 2.9	12	42,500 (Probe) 17,000 (EG)	170,000 (Probe) 68,000 (EG)
26k Nanoplate	Approx. 26,000	5 (Probe) 2 (EvaGreen)	Approx. 24.0	40	130,000 (Probe) 52,000 (EG)	217,000 (Probe) 69,000 (EG)

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. 3.3×10^9 bp, the calculation is as follows:

$$3.3 \times 10^9 \text{ bp} \times 1.096 \times 10^{-21} \text{ g/bp} = 3.3 \times 10^{-12} \text{ g} = 3.3 \text{ pg}$$

The genome sizes of many organisms have been published.

A few examples are depicted in the image below.

	<i>Homo sapiens</i> :	3.3×10^9 bp	3,300,000,000	http://www.ensembl.org/Homo_sapiens/Info/Index
	Rat:	3.0×10^9 bp	3,000,000,000	http://www.ensembl.org/Rattus_norvegicus/Info/Annotation
	Mouse:	3.5×10^9 bp	3,500,000,000	http://www.ensembl.org/Mus_musculus/Info/Annotation
	Zebrafish:	1.7×10^9 bp	1,700,000,000	http://www.ensembl.org/Danio_rerio/Info/Annotation
	<i>Saccharomyces cerevisiae</i> :	1.2×10^7 bp	12,000,000	http://www.ensembl.org/Saccharomyces_cerevisiae/Info/Annotation
	<i>Escherichia coli</i> :	4.6×10^6 bp	4,600,000	http://bacteria.ensembl.org/Escherichia_coli_str_k_12_substr_mg1655/Info/Annotation/#about
	Standard plasmid DNA:	3.5×10^3 bp	3500	

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

- Human: ca. 3,000 copies
- Zebrafish: ca. 5,400 copies
- Yeast: ca. 760,500 copies
- E. coli: ca. 2,000,000 copies
- Plasmid DNA (3.5 kb): 2,600,000,000 copies

Not to forget:

- In dPCR, the average number of copies/partitions must not exceed 5, ideally in the range of 0.5–3.

- This means ideally:
 - 8.5K Nanoplate: ca. 17,000–100,000 copies per 12 μ l reaction, 3 μ l analyzed (ca. 56–330 ng of human gDNA for single-copy gene)
 - 26K Nanoplate: ca. 22,000–130,000 copies per 40 μ l reaction, 24.3 μ l analyzed (ca. 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.

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.

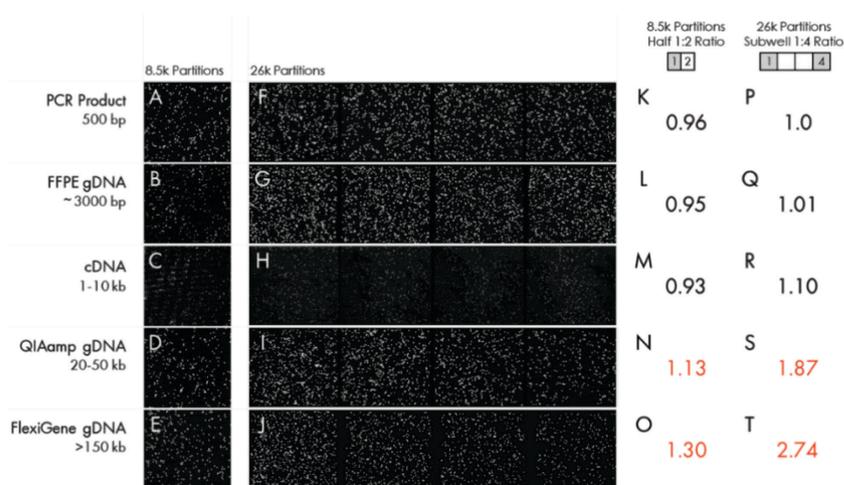


Figure 8. Uniformity of DNA distribution in QIAcuity Nanoplates is size dependent. Typical QIAcuity samples ranging from 500 to >150 kb in size served as template in 8.5k and 26k QIAcuity dPCR reactions. Images of PCR signal from 8.5k (A–C) and 26k (F–H) Nanoplates indicate that smaller DNA templates 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.

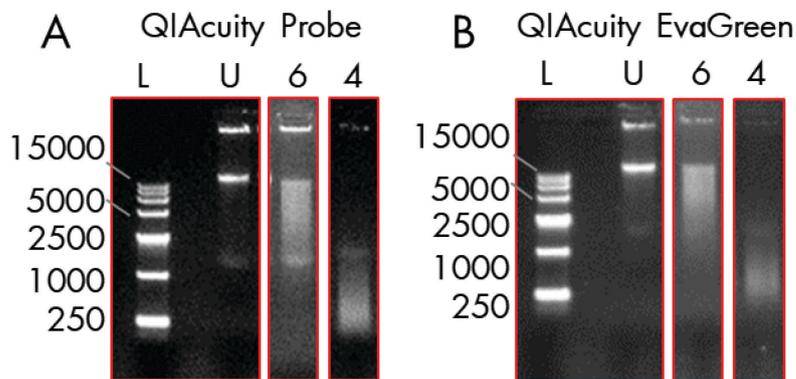


Figure 9. Restriction enzymes fragment DNA directly in QIAcuity Probe and QIAcuity EvaGreen mixes. The activity of two restriction enzymes was assayed in 12 μ l QIAcuity Probe (A) or QIAcuity EvaGreen (B) reaction mixes containing 210 ng of FlexiGene[®] gDNA. Untreated DNA (U) appears as two high molecular 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 and 0.025 U/ μ l, respectively. GelPilot High Range Ladder (100) was used as size ladder (L).

Absolute quantification and analysis

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.

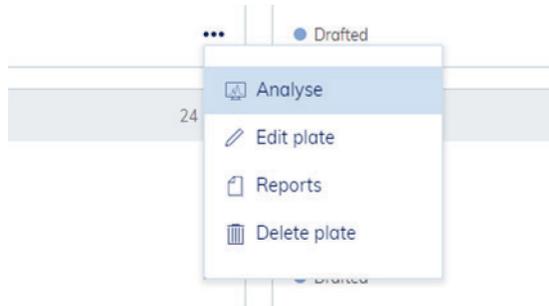
Accessing the analysis environment

To access the Plate Analysis environment:

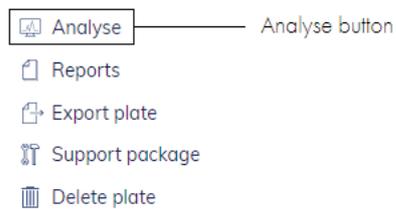
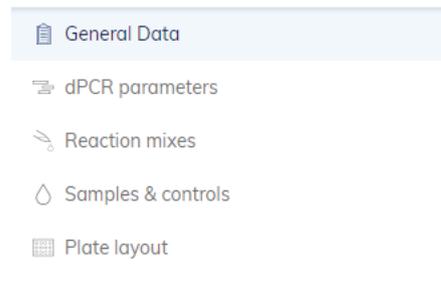
1. On the **Plates Overview** page of the Plates environment, search for the plate that you want to analyze.

Note: The plate run must be completed.

2. Click the **More** icon .
3. Select **Analyse** from the context menu.

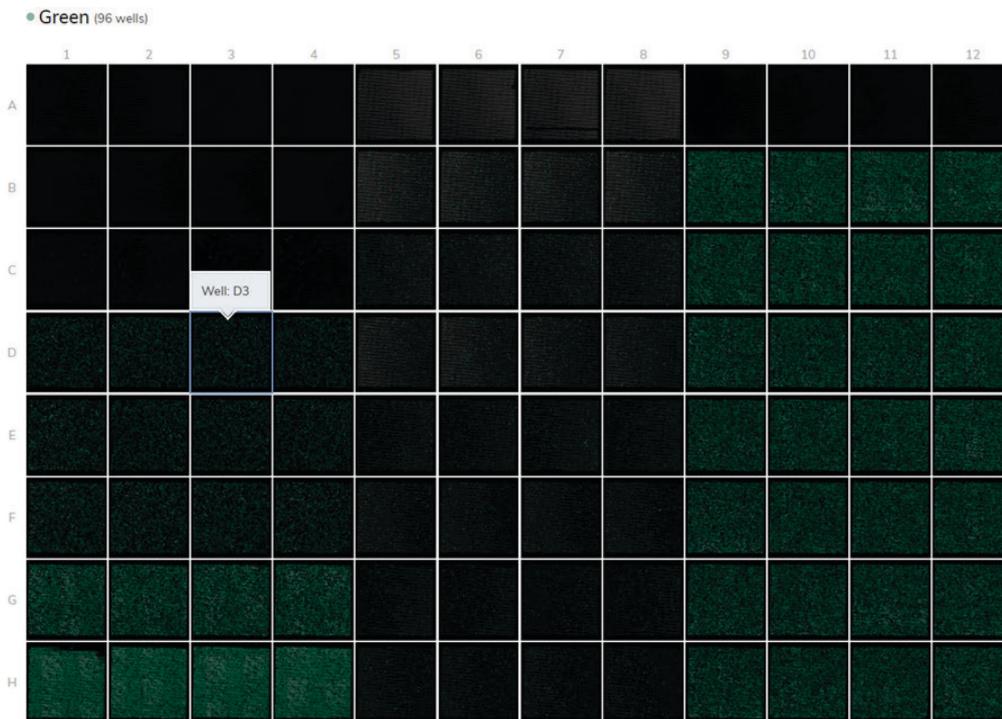


4. Alternatively, you can open the **Plate Analysis** environment. Click the plate name in the **Plate Overview**, then select **Analyse** from the context menu.



Images

- The QIAcuity Software Suite also enables you to view the images. After a run is completed, you can view the images in the Plate Analysis environment. To access the images, click **Check source images**.
- To select a channel, click the corresponding button next to the relevant channel.
- To zoom in and out, click the image.
- To download, click the image to open it in a new window. Then, click the  icon located at the top left corner of the image.



Selecting wells for analysis

- To select multiple wells at the same time, click the individual wells or click one well then drag your mouse until all wells are selected.
- To select all wells, click **Select all**.
- To remove a selected well, click the well.
- To remove all selected wells, click **Unselect all**.

Select source of analysis (2 wells)



To view more information about an individual well, double-click the well in the plate layout. The **Well Information** dialog box appears. Click **OK** to close the dialog box.

Well Information (A10) ✕

04
Reaction mix name EGFR_COSM6254
Sample ID 04
Sample name Sample 4 0.5%

Index	Target	IC	Dye	Channel
1	Mutation	-	FAM	● Green
2	Wildtype	-	HEX	● Yellow
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-

OK

Multiple imaging steps

If the plate is configured with multiple imaging steps, you can select the one used for analysis.

To select the imaging step, click one of the available boxes indicated by this icon . The channels that were activated in the selected imaging step are shown below the imaging step buttons, along with their duration and gain.

Note: If an imaging step failed during the run, a warning message is shown to indicate that the results might be incorrect.

Select imaging step



Green (700ms / 8)

Yellow (700ms / 8)

Orange (600ms / 6)

Red (400ms / 4)

Crimson (500ms / 8)

Figure 10. Select imaging step.

Thresholding in QIAcuity Software Suite

The **Threshold** field shows the threshold value of the fluorescence intensity that is used to distinguish between positive and negative calls. If only one source well is selected, the value of the threshold is shown in the **Threshold** field and on the graph as a red line. If multiple source wells are defined and their automatically calculated threshold values are different, a threshold value is initially not shown in the histogram.

There are two ways to change the threshold. The first one involves pointing to the chart, which triggers the appearance of a dotted line. Once the dotted line is in the appropriate spot, click the chart. The line becomes solid, and the threshold value is updated and displayed in the **Threshold** field. To change the value again using this method, click the red line and drag it to the appropriate spot. In addition to moving the line, you can also directly edit the value in the **Threshold** box. To apply the new value on that data, click **Recalculate** to trigger the recalculation of results.

Note: For a multiwell selection the user cannot change the thresholds individually. The new threshold becomes valid for all selected source wells. To change thresholds individually, change the source selection to a single well selection.

To reset the software to automatically recalculate the threshold, click **Automatic threshold** . Again, click **Recalculate** to apply the auto-threshold to the results.

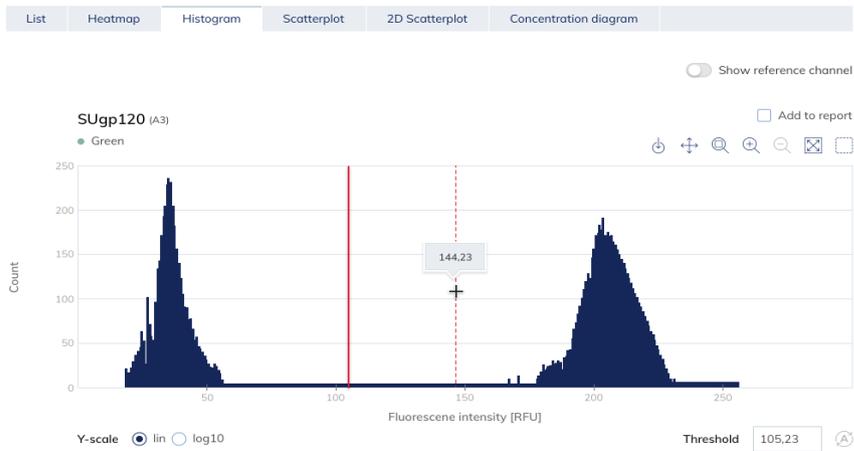


Figure 11. Threshold setting in histogram view.

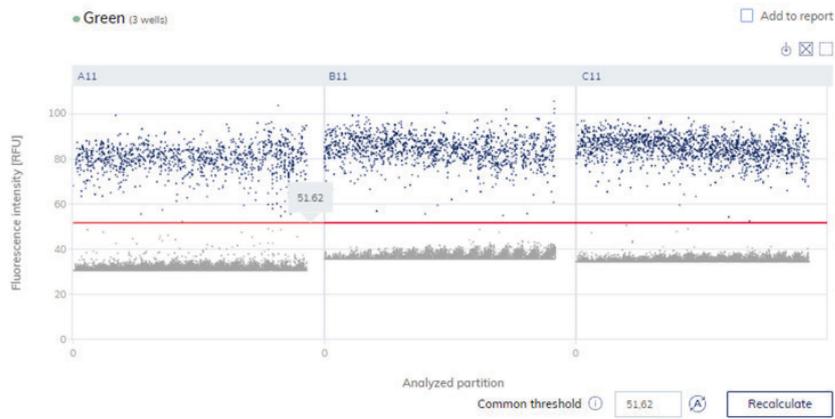


Figure 12. Thresholding in 1D scatterplot view.

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.

Using dPCR benefits, one of the most popular applications that benefit from high sensitivity and precision of dPCR is the detection of rare genetic mutations in sample mixtures, including wild-type (WT) sequences. Another popular application which benefits from the greater precision than qPCR is copy number variation (CNV) analysis. The higher precision with absolute quantification allows more precise copy number estimates.

Absolute quantification

The readout of dPCR is target copies per microliter. The calculation of the target copies per microliter is based on a binary positive/negative calling of a partition, which allows absolute quantification without using a standard curve. Absolute quantification provides, for example, more precise viral load analysis or microbial quantification.

Rare mutation detection

dPCR allows for increased sensitivity in the detection and absolute quantification of rare events/sequence variants in a WT background because the partitioning enables an increase in the specific concentration of the target. Besides, the end-point amplification and detection over noise, with a binary positive/negative target calling, allow precise quantification more independent from amplification performance (e.g., by PCR inhibitors).

Emerging uses of dPCR for rare mutation detection include:

- Identifying residual cancer below current detection levels
- Detecting new mutations and duplications in cancer
- Monitoring rare drug-resistance mutations

Copy number variation

dPCR can easily detect small fold-change differences such as less than a 1.2-fold change in CNV from four to five copies, without requiring a standard curve. The precision and sensitivity of the technology allow the system to distinguish subtle changes in copy number. The accuracy is less sensitive to changes in amplification efficiency and the presence of inhibitors. Emerging uses of dPCR for CNV lie in the field of cancer research (screening and monitoring of cancer biomarkers), research into neurological conditions, and population studies.

Gene expression and lncRNA or miRNA analysis

dPCR provides absolute quantification of gene expression and lncRNA or miRNA expression levels, especially of low abundant targets. The increased precision of dPCR can offer higher resolution gene expression analysis, enabling the monitoring of finer changes in expression levels.

Other emerging applications benefiting from the precision and absolute quantification are:

- Next-generation sequencing library quantification
- Genome edit detection
- Microbial pathogen and viral detection
- GMO detection

CNV Analysis

Introduction

CNVs (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.

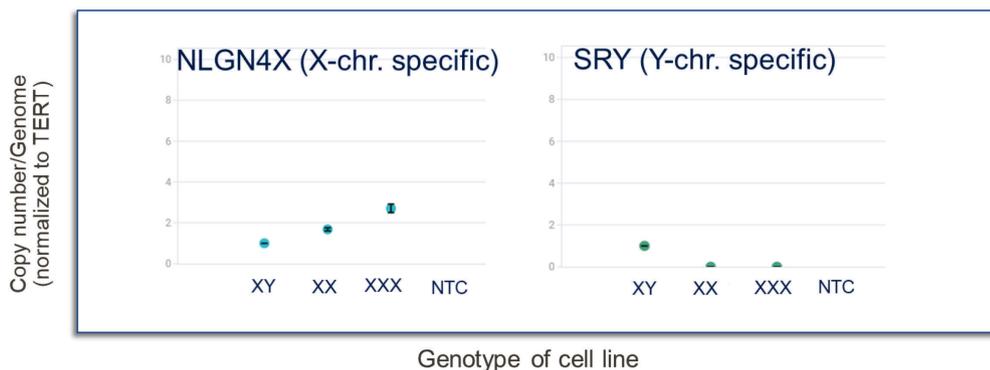


Figure 13. dPCR Copy Number Assays accurately identify aneuploidy. dPCR Copy Number Assays designed to target NLGN4X and SRY genes, which are on X and Y chromosomes, were tested using 3 Coriell templates with XY, XX, and XXX genotypes. TERT assay was used as reference assay, with stable copy numbers 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.

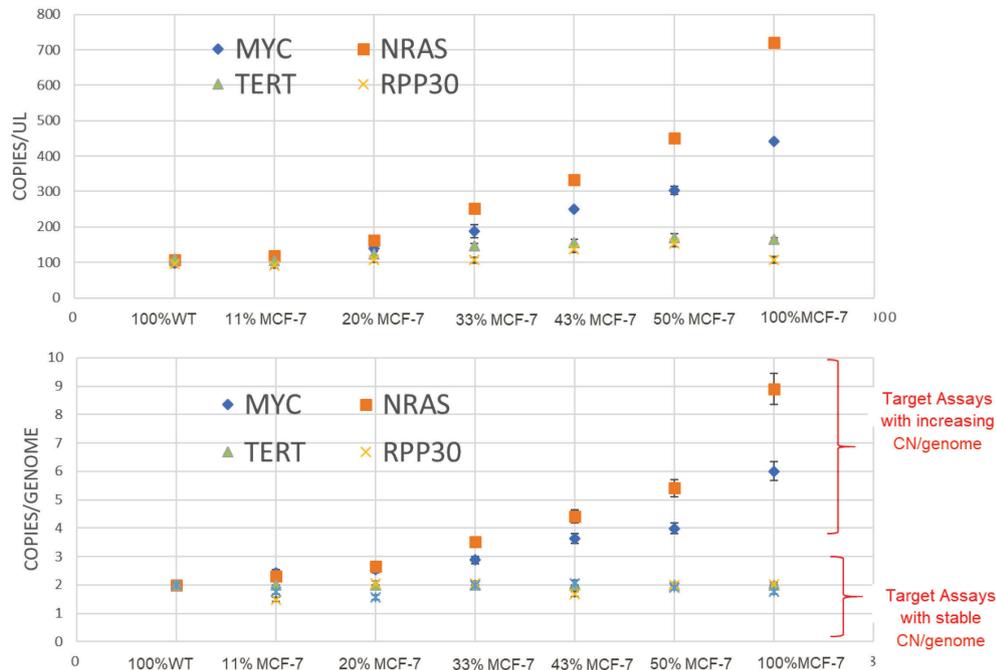
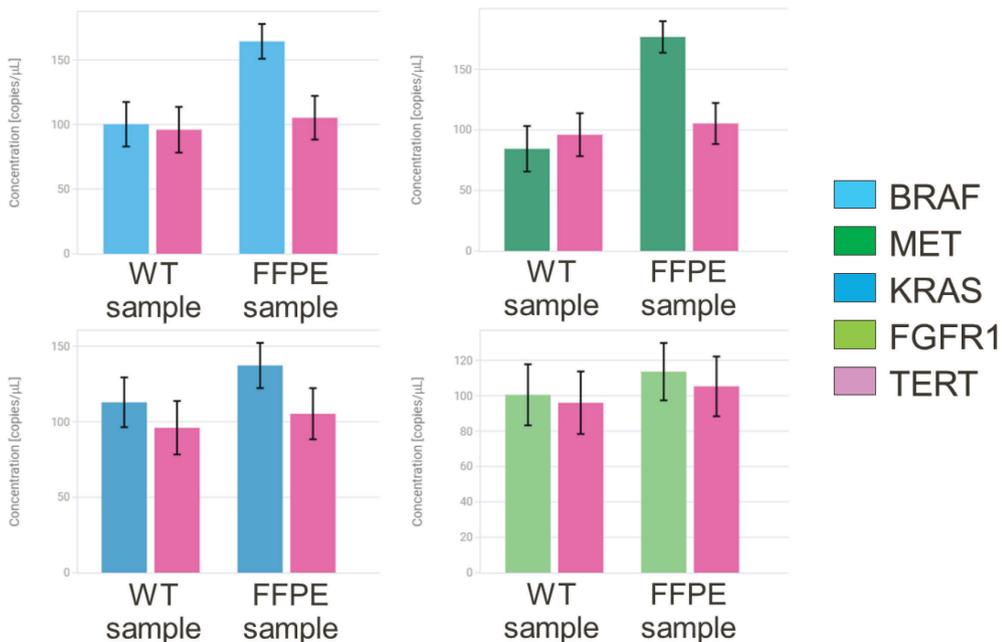


Figure 14. dPCR Copy Number Assays accurately detect small changes in copy number. dPCR Copy Number Assays targeting MYC and NRAS were tested using heterogeneous samples. Samples were prepared by mixing MCF-7 cell line genomic DNA (100% MCF7) and WT genomic DNA from healthy donor (100% WT) in different 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 heterogeneous 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.



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

Figure 15. 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. WT 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.

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 (a) absolute quantification of copy amount of GOI (or TOI) present in the sample and (b) calculation of copy numbers of GOI (or TOI) per genome with either reference sample(s) or reference assay(s).

Reference 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/ μ l) will be compared to the concentration of reference assay (copies/ μ l) 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: $(\text{Conc}^{\text{TARGET}}/\text{Conc}^{\text{REF. ASSAY}}) * \text{Copy no. of Ref. Assay}$

$\text{Conc}^{\text{TARGET}}$: concentration of GOI (or TOI) (copies/ μ l)

$\text{Conc}^{\text{REF. ASSAY}}$: concentration of Ref. Assay (copies/ μ l)

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

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

Copy number of Ref. Assay^{REF. SAMPLE}: not needed

$\text{Conc}^{\text{TARGET}}$: concentration of GOI (or TOI) (copies/ μ l)

$\text{Conc}^{\text{REF. ASSAY}}$: concentration of Ref. Assay (copies/ μ l)

$\text{Conc Ratio}^{\text{TARGET SAMPLE}}$: $\text{Conc}^{\text{TARGET ASSAY}}/\text{Conc}^{\text{REF. ASSAY}}$ in target sample

$\text{Conc Ratio}^{\text{REF. SAMPLE}}$: $\text{Conc}^{\text{TARGET ASSAY}}/\text{Conc}^{\text{REF. ASSAY}}$ in reference sample

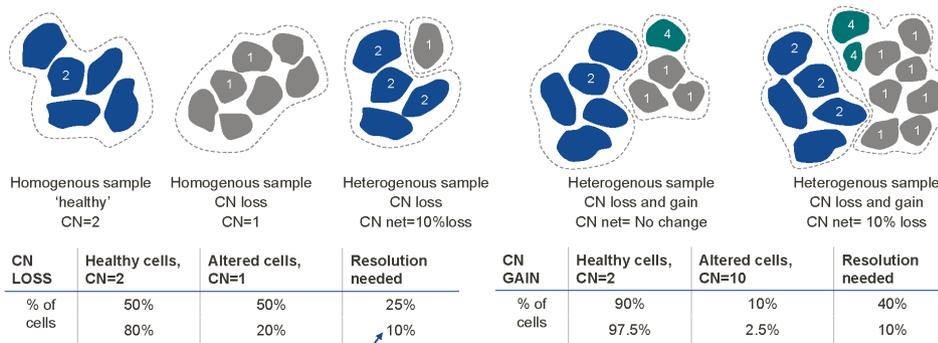
QIAcuity Software Suite 1.1.3 allows normalization by reference sample(s).

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 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 16). 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 5).

TUMOR SAMPLE HETEROGENEITY



Fine resolution and high quantitative power is needed!

BMC Genomics volume 15, Article number: 84 (2014)

Figure 16. Sample heterogeneity affects copy number analysis.

Table 5. Copy number analysis in heterogeneous samples

CN of CNV-harboring cells	Expected CNV of total sample				
	% 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 5 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.

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 assays 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 assays are present in 2 copies per genome; however, reference assays present in multiple copies must be taken into consideration when target assays are expected in high copy numbers (Table 6). 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 assays that are unrelated to the biological phenotype in question, thus most likely to stay unaltered in target samples, can also be used as alternative reference assays.

Table 6. 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 (minimum 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 assays increase. 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 17).

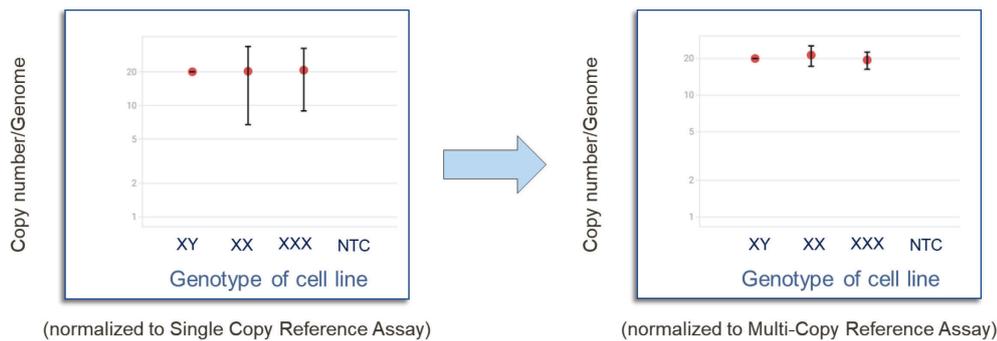


Figure 17. When target assays are expected to have high copy number/genome, normalization using multicopy reference assays provide more accurate results. dPCR Copy Number Assay targeting ACTB gene, which has 20 copies in genome, was tested using a single copy reference assay (left) and multicopy reference assay (right) under exact same experimental conditions.

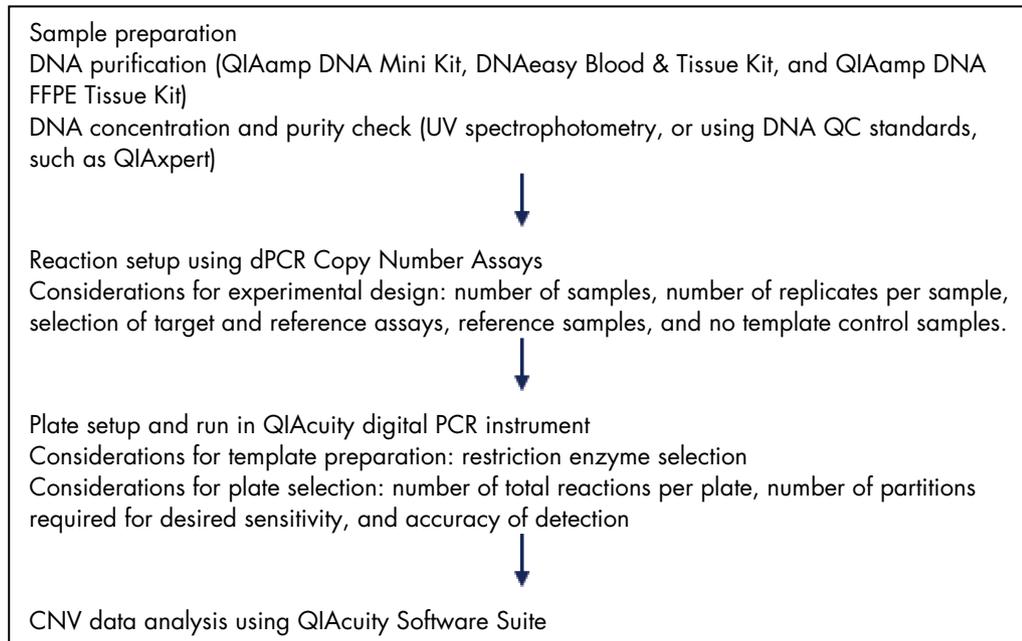
When reference samples are used for normalization of copy numbers, it is recommended that, if possible, the reference samples should come from the same type of tissue as the target samples. Thus, the DNA quality of both the reference and target samples matches. For fresh or frozen tissue samples, the 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.

Experiment planning

QIAcuity dPCR platform provides easy-to-interpret, reproducible, and accurate CNV analysis results for individual GOIs or TOIs using dPCR Copy Number Assays and QIAcuity EG PCR Kit. Simplicity of 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 next page).

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 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 5), CNVs in target samples are calculated based on absolute copy numbers detected in samples using QIAcuity Software Suite.

QIAcuity CNV dPCR workflow overview



Considerations

We recommend the following precautions:

- Use of sterile pipet tips with filters
- Decontamination of workspace and labware to avoid any foreign DNA contamination
- Storage of DNA-containing material separate from all other reagents
- Preparation of DNA-containing reaction mixes in spatially separated rooms or facilities
- Separation of 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 pipets 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

Sample isolation and preparation

Sample DNA quantity and purity can affect the performance of QIAcuity CNV dPCR Assays. 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 employed 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 dPCR Copy Number Assays (Table 7). DNA purification can be carried out according to the kit instructions.

Table 7. 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 $>10 \mu\text{g/ml}$
- $A_{260}:A_{230}$ 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 $\mu\text{g}/\mu\text{l}$.

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 450 ng of human genomic DNA should be used per dPCR reaction when detecting CNV assays present in 2 copies/diploid genome. DNA samples should be digested according to recommendations in the assay product sheet.

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.

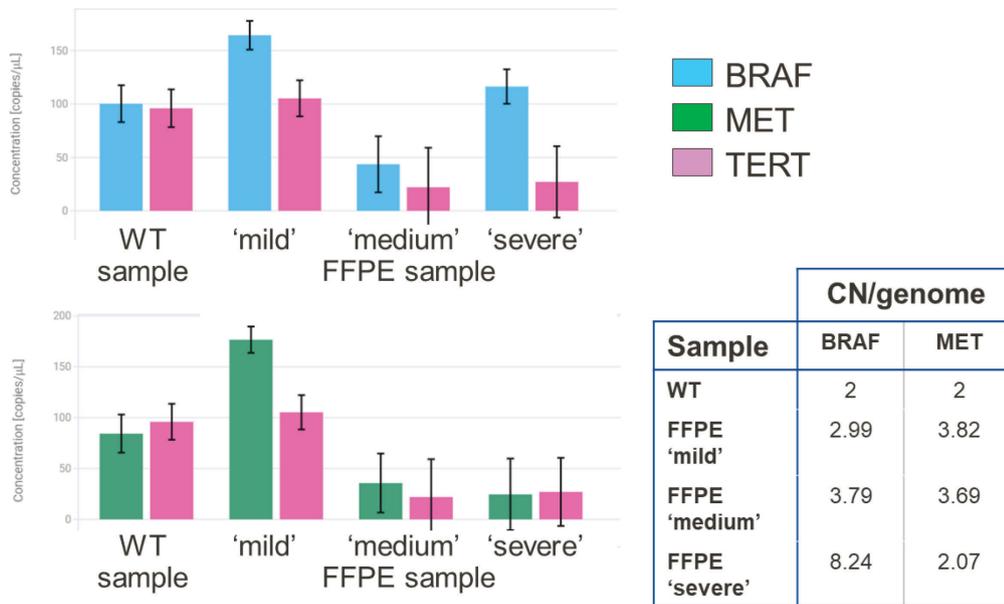


Figure 18. Use of DNA templates with compromised quality (here, fragmented FFPE material) might result in inaccurate CNV detection. Examples given here are target assays BRAF and MET, which were tested using genomic DNA extracted from FFPE tissue material with varying levels of fragmentation and formalin 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 will be performed during the dPCR run.
- DNA digested prior to the experiment can be stored at –20°C.

Table 8. List of recommended enzymes

6-Cutter restriction enzymes		4-Cutter restriction enzymes	
<i>EcoRI</i>	0.25 U/μl EcoRI-HF®, NEB® 0.025 U/μl Anza™ 11 EcoRI, TFS	<i>AluI</i>	0.025 U/μl AluI, NEB 0.025 U/μl Anza 44 AluI, TFS
<i>PvuII</i>	0.025 U/μl PvuII, NEB 0.025 U/μl Anza 52 PvuII, TFS	<i>CviQI</i>	0.025 U/μl CviQI, NEB 0.025 U/μl Csp6I (CviQI), TFS
<i>XbaI</i>	0.025 U/μl Anza 12 XbaI, TFS	<i>HaeIII</i>	0.025 U/μl BsuRI (HaeIII), TFS

NEB, New England Biolabs; TFS, Thermo Fisher Scientific.

Reference assays selection

dPCR 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 (a) variation in the quality and quantity of DNA from sample to sample, especially when the origin of samples is unknown, and (b) 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. Reference assays RPP30, TERT, and APB3B are present in 2 copies per genome. Reference assays R6 and R10 are present in multiple copies per genome. 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 9).

Table 9. List of recommended multiple-copy reference assays

Assay name	Copy number (diploid genome)	GeneGlobe cat. no.
RPP30	2	DCH110-0463302A
TERT	2	DCH105-0006472A
AP3B1	2	DCH105-0386794A
R6	12	DCH101-0003124A
R10	20	DCH101-0003135A

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 QIAcuity dPCR instrument (see section “Sample input amount”, page 17). 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 lie within 0.5–2 copies/partitions. In addition, template amounts should lie within 30–100 ng/μl and should not exceed 300 ng for optimal loading of a single-copy target in a single dPCR reaction. Table 10 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 10. Copy number effect on loading amounts of samples in nanoplates

Assay Type	Copy number/diploid genome	Loading amount (0.005 copies/partitions)	Loading amount (2 copies/partitions)
Reference	2	0.3 copies/ μ l	120 copies/ μ l
Target 1	10	1.5 copies/ μ l	600 copies/ μ l
Target 2	20	3 copies/ μ l	1200 copies/ μ l
Target 3	50	15 copies/ μ l	6000 copies/ μ l

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 dPCR. More than 200 assays targeting key CNVs are validated experimentally on the QIAcuity 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.

Using QIAcuity EG PCR Kit and dPCR Copy Number Assays for Copy Number Analysis on QIAcuity dPCR platform

For optimal performance, QIAcuity EG PCR Kit is highly recommended for setting up reactions using dPCR Copy Number Assays. 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 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 multiple dPCR platforms and can be ordered through geneglobe.qiagen.com/.

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

Recommendation of a QIAcuity Nanoplate

In addition to dPCR Copy Number Assays and QIAcuity EG PCR Kit, selection of appropriate QIAcuity Nanoplates is crucial for reaching desired sensitivity and range of copy number detection. QIAcuity Nanoplates come in two different sizes, 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.

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.

Setting up CNV analysis

The first step is absolute quantification of samples to determine absolute copies/ul 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 19).

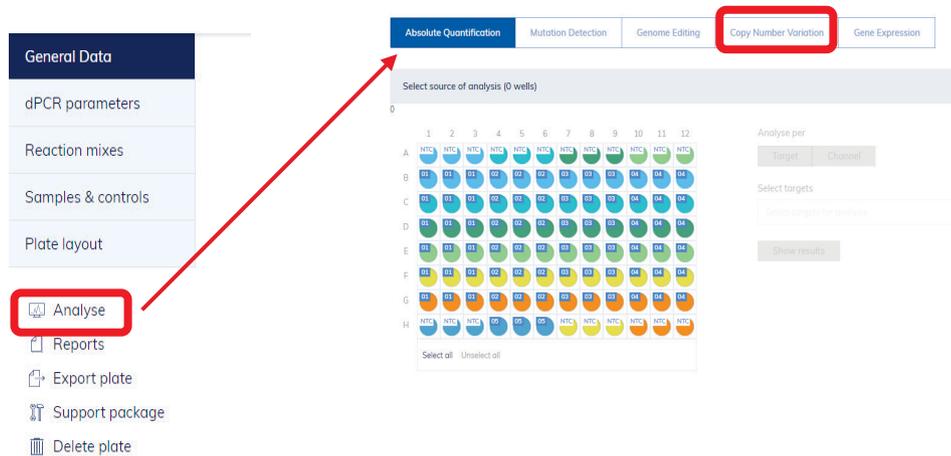


Figure 19. QIAcuity Software Suite has integrated workflow for absolute quantification as well as CNV analysis.

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".

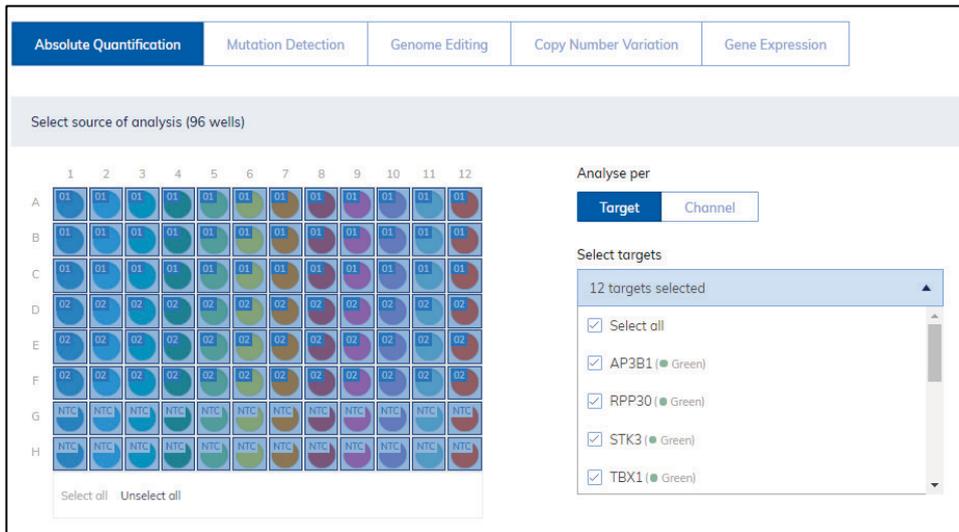
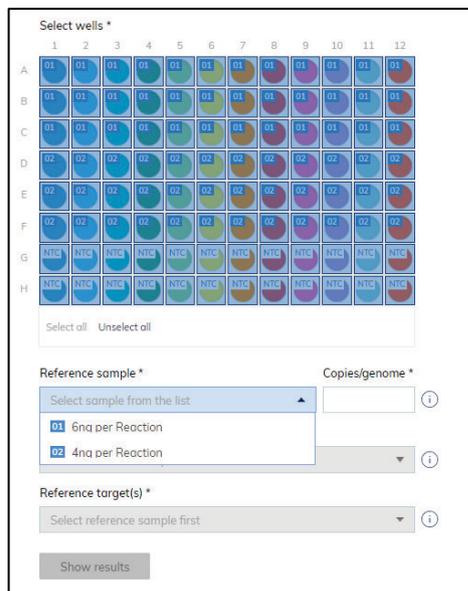


Figure 20. Absolute quantification of multiple targets can be done by selecting multiple wells and relevant TOIs.

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

1. Select wells. Select reference sample.



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

Reference sample * Copies/genome *

02 4nq per Reaction 2 (i)

Target of interest *

Select target from the list (i)

Reference target(s) *

Select at least one target from the list (i)

Show results

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

Reference sample * Copies/genome *

02 4nq per Reaction 2 (i)

Target of interest *

STK3 (● Green) (i)

Reference target(s) *

Select at least one target from the list (i)

Show results

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

Reference sample * Copies/genome *

02 4nq per Reaction 2 (i)

Target of interest *

STK3 (● Green) (i)

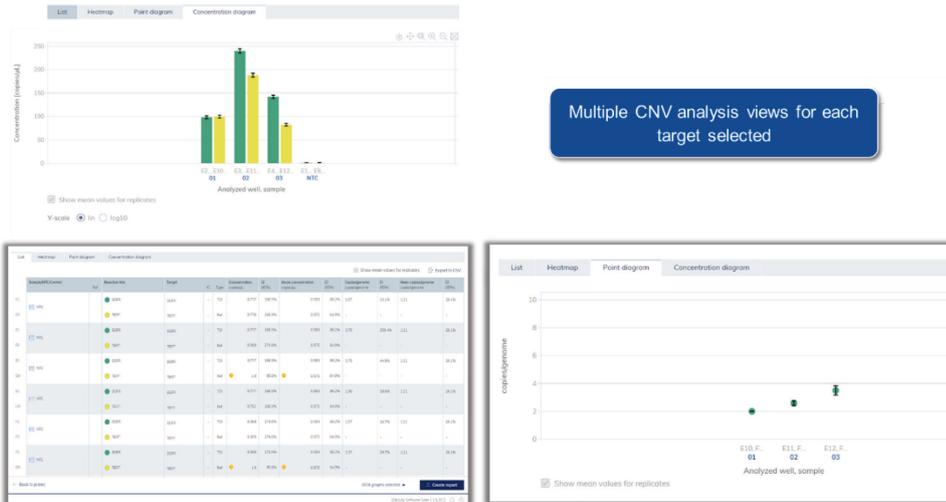
Reference target(s) *

3 targets selected (i)

- Ref Assay 11
 - TERT
- Ref Assay 3
 - AP3B1
- Ref Assay 6
 - RPP30

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.



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 coefficient of variation for the copy numbers/genome.

Show mean values for replicates Export to CSV

Sample/NTC/Control	Ref	Reaction Mix	Target	IC	Type	Concentration (copies/μL)	CI (95%)	Mean concentration (copies/μL)	CI (95%)	Copies/genome (copies/genome)	CI (95%)	Mean copies/genome (copies/genome)	CI (95%)
A1 NTC		ERBB2	ERBB2	-	TOI	0.0	-	0.204	168.6%	0.00	-	0.51	36.1%
E9		TERT	TERT	-	Ref	0.389	274.9%	0.878	68.7%	-	-	-	-
A1 NTC		ERBB2	ERBB2	-	TOI	0.0	-	0.204	168.6%	0.00	-	0.51	36.1%
F9		TERT	TERT	-	Ref	0.389	274.9%	0.878	68.7%	-	-	-	-
A1 NTC		ERBB2	ERBB2	-	TOI	0.0	-	0.204	168.6%	0.00	-	0.51	36.1%
G9		TERT	TERT	-	Ref	1.9	95.8%	0.878	68.7%	-	-	-	-
A1 NTC		ERBB2	ERBB2	-	TOI	0.0	-	0.204	168.6%	0.00	-	0.51	36.1%
H9		TERT	TERT	-	Ref	0.781	168.9%	0.878	68.7%	-	-	-	-

Figure 21. 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”.

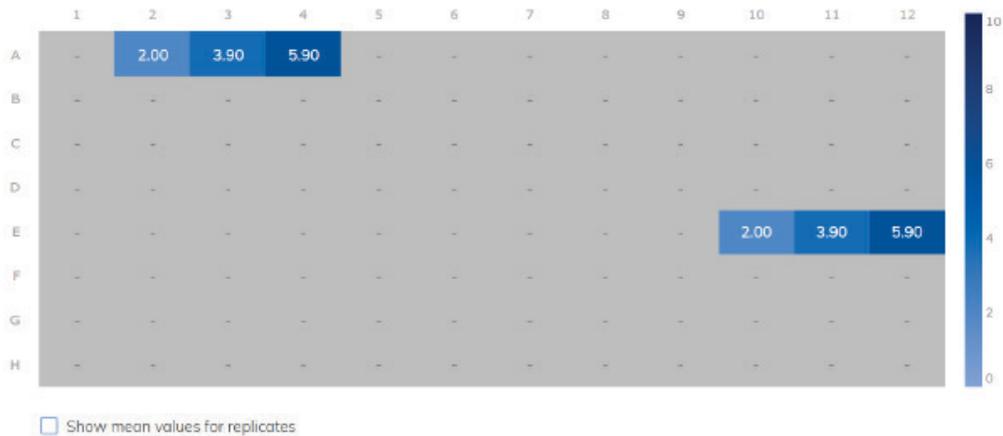


Figure 22. Heatmap tab for copy number analysis.

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 *QIAcuity User Manual*, section “Reports”.

Point diagram

The Point diagram tab shows a diagram which 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 23. 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 *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".

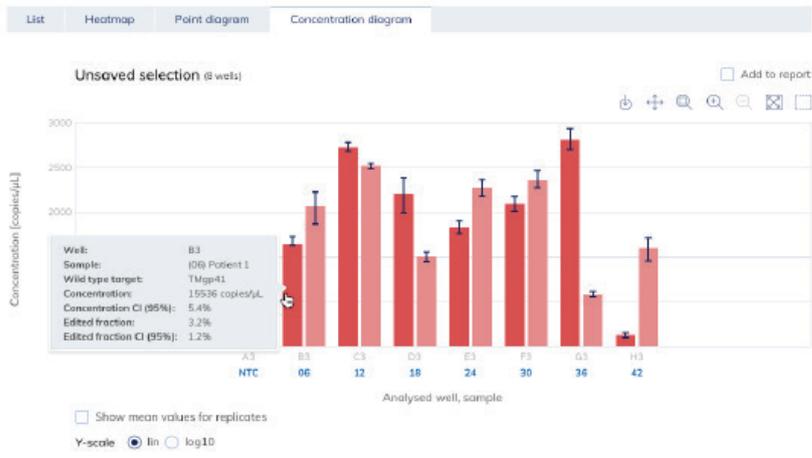


Figure 24. Concentration diagram tab for copy number analysis.

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

Mutation Detection

Introduction

A main area of interest in many biological and pharmacological studies is the detection of mutations for diagnosis of genetic disorders and diseases, such as cancer. The majority of genomic alterations leading to cancer or other diseases are acquired in somatic cells and are not inherited in contrast to germline mutations. This results in cells or tissues or tumors harboring unique sets of mutations in contrast to surrounding healthy cells or other types of altered tissues. Once mutations in tumors are characterized, their underlying molecular mechanisms and potential new drug targets can be identified.

A major challenge of mutation detection is the reliable and efficient detection of mutations present at low quantities in samples. Patient samples, patient-derived cell lines, and tumor biopsies are often heterogenous mixture of cells. The number of cells carrying the mutation of interest can be very small, further decreasing the quantity of mutation in the samples. Thus, it is essential to have an accurate and sensitive detection of mutations even at very low frequencies.

The dPCR LNA Mutation Assays combined with QIAcuity dPCR platform provide sensitive detection of rare mutations in high amounts of WT background. Partitioning of both WT and mutant sequences in a dPCR reaction allows for separation of mutant sequences from their WT counterparts, leading to positive partitions and PCR amplification signal in dPCR and accurate concentration estimation based on the presence or absence of signal. This is in striking contrast to traditional PCR reactions, where a single reaction mix contains highly abundant WT sequences with the advantage of reaching high amplification amounts at the end of PCR cycles, resulting in further reduction of the mutant signal.

Frequency of a rare mutation is calculated based on the absolute number of mutant copies detected in relation to total number of copies in a given dPCR reaction. No additional references or endogenous controls are needed for calculating mutation frequency.

$\text{Conc}^{\text{MUTANT}}$: concentration of mutant sequence (copies/ μl)

$\text{Conc}^{\text{WILD TYPE}}$: concentration of WT sequence (copies/ μl)

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

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

Experiment planning

Simplicity of workflow and large number of assays available in dPCR LNA Mutation Assays portfolio allow fast, accurate, and highly sensitive detection of target mutation frequencies in a wide variety of samples. QIAcuity mutation detection dPCR workflow 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 dPCR Probe Master Mix and dPCR LNA Mutation Assays according to the reaction setup. The reaction mixtures are then aliquoted into each well of QIAcuity Nanoplates. After the dPCR run (see section “QIAcuity system and workflow”, on page 5), mutation frequencies in target samples are calculated based on the relation of absolute copy numbers of WT and mutant sequences detected in samples using QIAcuity Software Suite.

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 of sterile pipet tips with filters.
- Decontamination of workspace and labware to avoid any foreign DNA contamination.
- Storage of DNA-containing material separate from all other reagents.
- Preparation of DNA-containing reaction mixes in spatially separated rooms or facilities.
- Separation of 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.

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.

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 11). DNA purification can be carried out according to the kit instructions.

Table 11 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.

LoD and amount of starting material

dPCR LNA Mutation Assays can be used to identify rare mutations in a wide variety of samples. These sample types include fresh frozen cell lines and tissues, FFPE samples, cfDNA samples, and tissue biopsies. These samples are often quality compromised due to crosslinking as well as fragmentation and can contain very low amounts of genomic DNA. Although restriction digestion of templates is not necessary, we recommend checking the DNA quality and quantity of samples using UV spectrophotometry before setting up the reactions (for details, see section “Sample preparation”, page 17).

For obtaining accurate results using QIAcuity Mutation Detection dPCR Assays, see section “Sample input amount” on page 17 for more information.

Using QIAGEN’s dPCR LNA Mutation Assays for rare mutation detection

dPCR LNA Mutation Assays portfolio offers sensitive and accurate detection of 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 provide multiple advantages compared to standard nucleic acids, such as (a) specific detection of shorter DNA targets, (b) higher affinity of binding to complementary DNA sequence, (c) superior single nucleotide discrimination, and (d) increased target specificity and discrimination.

dPCR LNA Mutation Assays are available in easy-to-handle format, a mixture of primers targeting the mutation harboring genomic region and two probes for detecting either the mutant or WT sequence, labelled with FAM and HEX fluorophores, respectively. Single mutations can be detected using duplexing assays. For detection of two mutations simultaneously, multiplexing analysis of two unique mutations in one reaction can be achieved by using compatible dye combinations: FAM + HEX dye combination for the first and Atto550 + ROX dye combination for the second mutation of interest.

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.

dPCR LNA Mutation Assays and dPCR Probe Kit on the QIAcuity

QIAcuity dPCR platform provides a highly sensitive and accurate detection of low frequency mutations. QIAcuity instrument has been tested with a wide range of mutation detection assays from different manufacturers. For best usability and performance, we recommend using dPCR Probe Kit and dPCR LNA Mutation Assays on QIAcuity dPCR instrument.

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 dPCR 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.

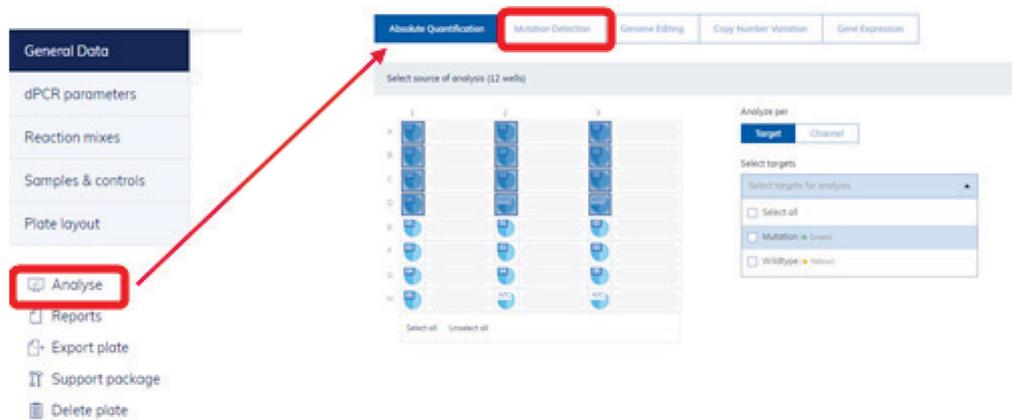
QIAcuity Probe PCR Kit is developed for optimal performance of QIAcuity dPCR LNA Mutation Assays in QIAcuity dPCR platform. The hot-start QuantiNova DNA Polymerase enzyme, along with other proprietary chemical components in QIAcuity Probe PCR Kit, is essential for delivering highest performance in mutation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

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

Recommendation of a QIAcuity Nanoplate

For highly sensitive and accurate detection of mutations using QIAcuity dPCR platform, we recommend using 26K nanoplates. In this type of nanoplate, one reaction mix is distributed over 4 wells and separated into approximately 26,000 partitions, increasing the accuracy of copy number determination. However, 8.5K nanoplates can also be used according to experimental needs such as for 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.

Mutation analysis in QIAcuity Software Suite



The QIAcuity Software Suite analyzes the mutation frequencies in target samples. The analysis consists of absolute quantification of copies of each WT and mutant target present in samples, followed by determination of mutation frequency of each target mutation in samples. Results can be analyzed using QIAcuity Software Suite by selecting **Analyse** option from left section bars. In the top left and right, **Absolute Quantification** and **Mutation Detection** plugins, respectively, can be found. For additional information regarding setting up the plates and samples, see *QIAcuity User Manual*, section "Setting up an experiment".

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** pane.
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**.



Select source of analysis (12 wells)



Analyze per



Select targets

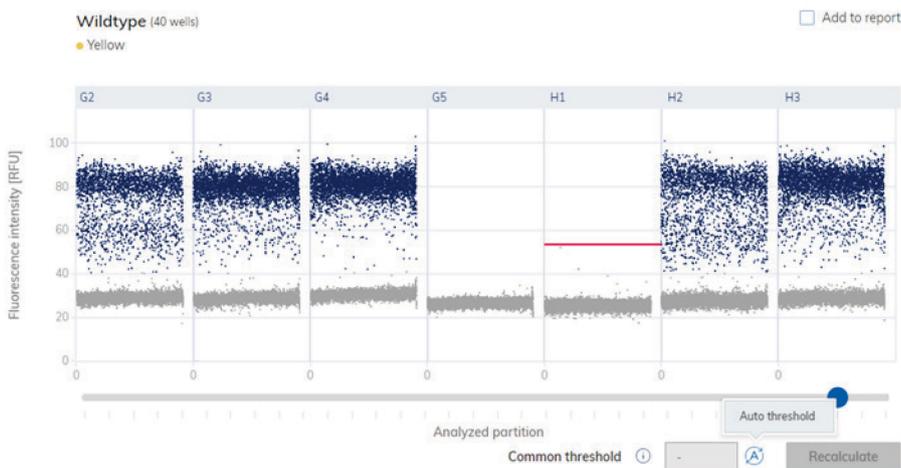


The results are divided into several tabs: List view, Signalmap, Heatmap, Histogram, 1D and 2D Scatterplots, as well as concentration diagram. To view the contents of each tab, click the tab title. For additional information regarding absolute quantification analysis, see *QIAcuity User Manual*, section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

Reaction Mix	Target	Sample/NTC/Control	Concentration (copies/μL)	CI (95%)	Partitions valid	positive	negative
A1	Mutation	100% Mut	1919.3	2.7%	6259	3006	4152
	Wildtype		0.0	-	8254	0	8254
A2	Mutation	50% Mut	871.5	4%	6178	2095	6083
	Wildtype		1011.7	3.7%	6178	2378	5800
A3	Mutation	10% Mut	184.3	8.6%	6259	501	7758
	Wildtype		1926.0	2.7%	6259	3965	4294
A4	Mutation	0% Mut	0.0	-	6239	0	6239
	Wildtype		2136.0	2.5%	6256	4259	3997
A5	Mutation	NTC	0.0	-	8245	0	8245
	Wildtype		0.0	-	8248	0	8248
B1	Mutation	100% Mut	1745.4	2.8%	6276	3701	4575
	Wildtype		0.0	-	6275	0	6275

List view is one of the multiple **Results view** generated in **Absolute Quantification Analysis** tab.

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**.



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 new threshold by pressing **Recalculate**.

List Signalmap Heatmap Histogram 1D Scatterplot 2D Scatterplot Concentration diagram

Add to report

X-axis target Common threshold

Y-axis target Common threshold

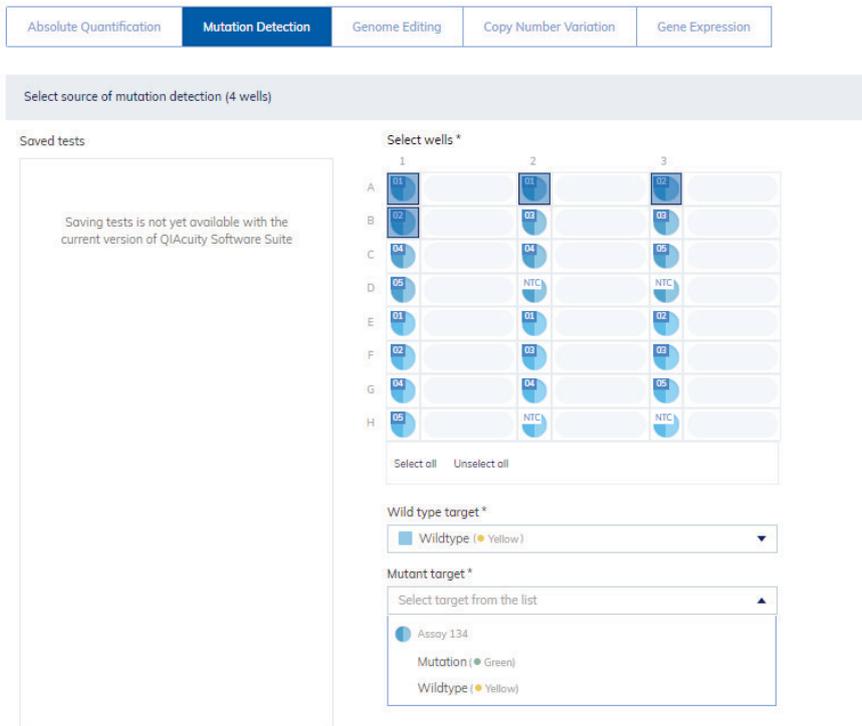
To see the plot, please assign targets to the X and Y axes in the form above.



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.

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/ul)** – 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.

Sample/NT/Control	Reaction rate	Target	IC	Type	Concentration (copies/L)	CI (95%)	Mutant fraction	CI (95%)
B1 1Mut	90	MUT	-	WT	14.9	31.2%	0.70%	40%
		WT	-	WT	1033.4	2.9%	-	-
B6 2Mut	90	MUT	-	WT	6.6	48.9%	0.37%	55%
		WT	-	WT	1795.6	2.9%	-	-
B9 3Mut	90	MUT	-	WT	9.4	40.9%	0.61%	48%
		WT	-	WT	1810.8	2.9%	-	-
C1 3.2Mut	90	MUT	-	WT	0.19%	274.6%	0.02%	100%
		WT	-	WT	1978.3	2.9%	-	-
C5 3.2Mut	90	MUT	-	WT	0.0	-	0%	-
		WT	-	WT	1806.7	2.9%	-	-
C9 2Mut	90	MUT	-	WT	0.0	-	0%	-
		WT	-	WT	1813.5	2.9%	-	-
D1 2Mut	90	MUT	-	WT	0.0	-	0%	-
		WT	-	WT	2013.8	2.7%	-	-
B4 1NT	90	MUT	-	WT	0.0	-	0%	0%
		WT	-	WT	0.0	-	-	-
D8 1NT	90	MUT	-	WT	0.0	-	0%	0%
		WT	-	WT	0.0	-	-	-

Figure 25. List tab for Mutation Detection.

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 *QIAcuity User Manual*, section “Heatmap tab for mutation detection”.

Point to a well to see a more detailed information.

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

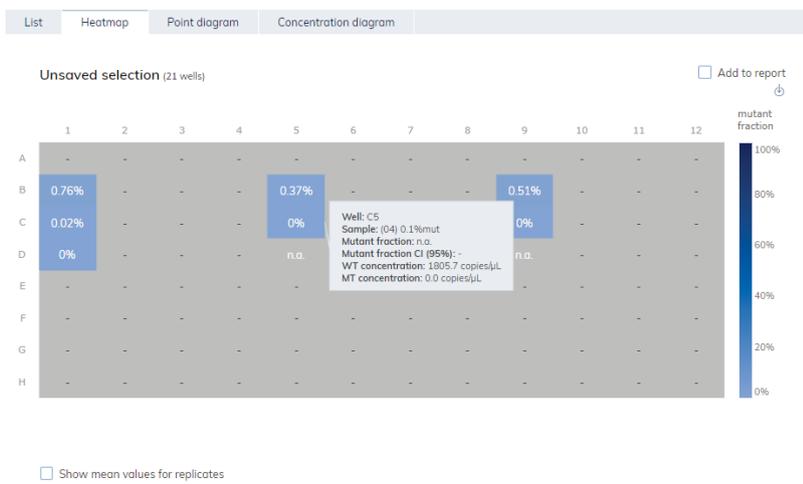


Figure 26. The Heatmap tab.

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 *QIAcuity User Manual*, section “Reports”.

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.

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

Point to a well to see a more detailed information about 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 27. 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 *QIAcuity User Manual*, section “Reports”.

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.

Point to the diagram to view the additional options. An additional toolbar and the threshold value are shown. For more information about the toolbar, refer to *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, point to one of the bars.

Point to a well to see a 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 28. The Concentration diagram tab.

Gene Expression

Introduction

A common approach for studying gene expression is to convert RNA into cDNA (using reverse transcriptase) followed by quantitative PCR (RT-qPCR). RT-qPCR has a broad linear range and is capable of quantifying low to high abundant transcripts.

One disadvantage of RT-qPCR is that it only provides relative expression levels, that is, the obtained signals have to be determined relative to a reference target (e.g., housekeeping gene). Additionally, small changes in expression like changes in the range of 10–20% cannot be reliably detected with RT-qPCR.

These limitations can be overcome by using dPCR after reverse transcription. When using the correct dilution factor (see section “General considerations for performing a gene expression analysis on the QIAcuity dPCR instrument”, page 70), each transcript of interest will lead to a positive partition in the QIAcuity Nanoplate. As random distribution can lead to more than 1 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 7) without the need for using a reference target. It is nevertheless necessary to use reference targets in case the concentration of the input cDNA differs between samples.

Using the QIAcuity EG PCR Kit and QIAcuity Probe PCR Kit for gene expression analysis on the QIAcuity

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

Using the QuantiNova LNA PCR Assays on the QIAcuity

The QIAcuity instrument has been tested with a wide range of gene expression assays of different manufacturers (EvaGreen-based assays using the QIAcuity EG PCR Kit). For best usability and performance, we recommend using the QuantiNova LNA PCR Assays.

QuantiNova LNA PCR Assay and Panels provide highly sensitive and accurate LNA-enhanced dPCR quantification assays for mRNA and lncRNA targets in an easy-to-handle format. They are designed for use with universal reverse transcription (RT), followed by either real-time PCR or dPCR amplification using EvaGreen for detection.

The QuantiNova LNA PCR Assays therefore provide a versatile, flexible tool for gene expression analysis on multiple platforms.

The forward and reverse PCR amplification primers are LNA enhanced 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 accurate quantification of very low levels of mRNA/lncRNA.

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.

Using probe-based gene expression assays on the QIAcuity

The QIAcuity instrument has been tested with probe-based gene expression assays of different manufacturers using the QIAcuity Probe PCR Kit. Several key assays showed overall compatibility with the QIAcuity Probe PCR Kit.

For custom assay designs, we recommend following the identical rules as for the design of qPCR probe-based gene expression assays. Therefore, the vast majority of existing qPCR assays can be directly used in dPCR on the QIAcuity instrument or just require minor adaptations (see section “Assay design guidance”, page 13).

RNA isolation

The most important prerequisite for any gene expression 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 real-time PCR performance.

Recommended RNA preparation methods

High-quality total RNA for your real-time 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 12. Recommended RNA preparation methods

Sample type	Recommendation
Cultured cells	RNeasy Plus Mini Kit (cat. no. 74134)
Tissue samples	Use 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, RNeasy [®] Reagent)	Purify further using the RNeasy Mini Kit. Important: Perform the on-column DNase digestion step described in the <i>RNeasy Mini Handbook</i> , www.qiagen.com/HB-0435 .
Other biological samples	Contact QIAGEN Technical Service

Storage of RNA

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

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", next page). For small amounts of RNA, however, it may be difficult to determine amounts photometrically. Small amounts of RNA can be accurately quantified using an Agilent® 2100 Bioanalyzer, quantitative RT-PCR, or fluorometric quantification.

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 ($A_{260}=1 \rightarrow 44 \mu\text{g/ml}$). 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 and 280 nm gives an estimate of RNA purity. As discussed below (see "Purity of RNA"), the ratio between absorbance values at 260 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 = $44 \mu\text{g/ml} \times A_{260} \times \text{dilution factor}$
= $44 \mu\text{g/ml} \times 0.2 \times 50$
= $440 \mu\text{g/ml}$

Total amount = concentration \times volume in milliliters
= $440 \mu\text{g/ml} \times 0.1 \text{ ml}$
= $44 \mu\text{g}$ of RNA

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 $\mu\text{g}/\text{ml}$ RNA) is based on an extinction coefficient calculated for RNA at neutral pH (see “Spectrophotometric quantification of RNA”).

Integrity of RNA

Run an aliquot of each RNA sample on a denaturing agarose gel, the Agilent Bioanalyzer using an RNA 6000 Nano LabChip®, the QIAxpert® Instrument (cat. no. 9002340), or the QIAxcel® (cat. no. 9001941 or 9002123). Verify that there are sharp bands/peaks present for both the 18S and 28S ribosomal RNAs. Any smearing of the RNA bands or shoulders on the RNA peaks indicate that degradation has occurred in the RNA sample.

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

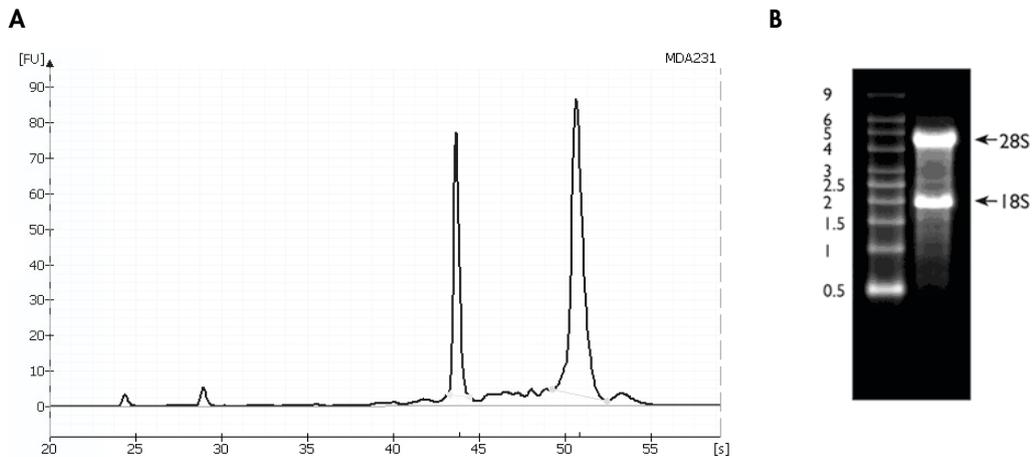


Figure 29. Ribosomal RNA integrity. **A:** Agilent Bioanalyzer electropherogram of high-quality total RNA showing sharp peaks for the 18S (left) and 28S (right) ribosomal RNA. Due to high quality of the RNA, peaks do not have shoulders (especially to the left of each peak). **B:** Agarose gel electrophoresis shows sharp bands (especially at the bottom of each band) for 28S and 18S ribosomal RNA.

Genomic DNA contamination

Unwanted signals generated by contaminating genomic DNA in a sample is limited by the design of 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.

cDNA synthesis

For cDNA synthesis the use of the QuantiTect Reverse Transcription Kit 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*.

General considerations for performing a gene expression analysis on the QIAcuity dPCR instrument

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

Table 13. 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	
			4.1	35.7	351.7	3517	17,586	70,344	Number of input template molecules per reaction well
8.5K	12 µl	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	
			1.7	43.33	433.3	4333	21,667	86,667	Number of input template molecules per reaction well
26K	40 µl	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.

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.

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 13.

Normalization and recommended controls

The efficiency of RNA extraction and cDNA synthesis 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 reference gene that you are using does not change between the different conditions that you are analyzing.

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

Table 14 provides a list of recommended reference genes for different expression ranges.

Table 14. 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).

Conducting a gene expression analysis in the QIAcuity Software Suite

You can open the **Gene Expression Plugin** of the QIAcuity Software Suite 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 30).

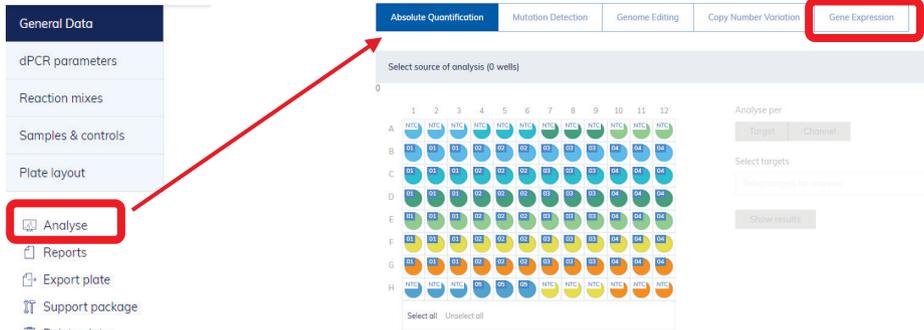


Figure 30. Navigation to second-level analysis for gene expression in the QIAcuity Software Suite.

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 31).

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 one or multiple TOIs have to be selected. TOIs are all specific assays that are not used as reference gene.

Select wells *

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC											
B	01	01	01	02	02	02	03	03	03	04	04	04
C	01	01	01	02	02	02	03	03	03	04	04	04
D	01	01	01	02	02	02	03	03	03	04	04	04
E	01	01	01	02	02	02	03	03	03	04	04	04
F	01	01	01	02	02	02	03	03	03	04	04	04
G	01	01	01	02	02	02	03	03	03	04	04	04
H	NTC	NTC	NTC	05	05	05	NTC	NTC	NTC	NTC	NTC	NTC

Select all Unselect all

Reference sample *

Select sample from the list

Target of interest *

Select reference sample first

Reference target(s) *

Select reference sample first

Show results

Figure 31. 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. **Note:** this heatmap will only work in multiplex experiments where a reference target is multiplexed together with the **Target of interest** in the same well.

Troubleshooting

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

If you need to contact QIAGEN Technical Services about an error, note the steps leading to the error and the information from any dialog boxes. This will help the QIAGEN Technical Service Specialist to resolve the error.

References

1. Wilfinger, W.W., Mackey, M., and Chomczynski, P. (1997) Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. *Biotechniques*. **22**, 474–481.

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
T _m	Melting temperature
TOI	Target of interest
UV	Ultraviolet
WT	Wild type

Document Revision History

Date	Changes
December 2020	Initial release
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.

Notes

Notes

Notes

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