February 2025

Quick-Start Protocol

dPCR CNV Probe Assays

This protocol is optimized for the quantification of gene-specific regions in human, mouse, or rat DNA using the dPCR CNV Probe Assays (cat. nos. 250210, 250212, 250213, and 250214) with the QIAcuity® Probe PCR Kit (cat. nos. 250101, 250102, and 250103) or QIAcuity High Multiplex Probe PCR Kit (cat. nos. 250133 and 250134) in a singleplex or multiplex reaction using the QIAcuity digital PCR (dPCR) instrument, to detect copy number variations (CNV). The dPCR CNV Probe Assays are available in 6 different dyes: FAM, HEX, ATTO 550, ROX, Cy5, and ATTO 700. For further information, please refer to the dPCR CNV Probe Assays product page at **www.qiagen.com**

Wet-lab tested dPCR CNV Probe Assays

The wet-lab tested dPCR CNV Probe Assays (cat. no. 250210, 250212, and 250213) are shipped ready-to-use on dry ice. Upon receipt, the assays should be stored protected from light in a constant-temperature freezer at –30 to –15°C for long-term storage (12 months) or at 2–8°C for short-term storage (6 months). Repeated freeze–thaw cycles should be avoided. If possible, store the assay in aliquots. Under these conditions, the components are stable, without showing any reduction in performance and quality.

Custom dPCR CNV Probe Assays

The custom dPCR CNV Probe Assays (cat. no. 250214) are shipped lyophilized at ambient temperature. Upon receipt, the lyophilized custom dPCR CNV Probe Assay should be stored

protected from light between at 2–8°C for short term storage and between –30 to –15°C in a constant-temperature freezer for long term storage. After reconstitution, it is recommended to store the dPCR CNV Probe Assay at –30 to –15°C. Repeated freeze–thaw cycles should be avoided. If possible, store the assay in aliquots. Under these conditions, the dPCR CNV Probe Assay is stable until the expiry date listed on the vial.

Further information

- *dPCR CNV Probe Assay Handbook*:**www.qiagen.com/HB-3542**
- *QIAcuity User Manual*: **www.qiagen.com/HB-2717**
- *QIAcuity User Manual Extension*: **www.qiagen.com/HB-2839**
- Safety Data Sheets: **www.qiagen.com/safety**
- Technical assistance: **support.qiagen.com**
- Application Note: **www.qiagen.com/QPRO-9346**

Important points before starting

- A reference target assay with a known copy number per genome can be used to identify CNVs for target(s) of interest in test samples. For consistency of CNV analysis, the same reference target assay(s) and target-of-interest assay(s) should be used across test samples.
- We recommend using multiple reference target assays depending on experimental conditions. Refer to the *QIAcuity User Manual Extension: QIAcuity Application Guide* for more information on reference target assay selection.
- dPCR CNV Probe Assays are used as a 20x primer/probe mix. Always start with the 20x assay concentration and dPCR cycling conditions in this protocol.
- Pipetting accuracy and precision affect the consistency of results. Ensure that no bubbles are introduced into Nanoplate wells while pipetting.

- Pipetting accuracy and precision affects the consistency of results. Be sure that all pipets and instruments have been checked and calibrated according to the manufacturer's recommendations. Also make sure that no bubbles are introduced into the wells of the QIAcuity Nanoplate during pipetting.
- The QIAcuity High Multiplex Probe PCR Kit requires the QIAcuity Software Suite version 3.0 or later.

Things to do before starting

- Reconstitute custom dPCR CNV Probe Assays to a 20x concentration with nuclease-free water according to their product datasheets, which can be retrieved from each user's GeneGlobe account.
- Thaw genomic DNA and QIAcuity Probe Master Mix or QIAcuity High Multiplex Probe PCR Master Mix on ice (4°C). After thawing, mix gently by repeated pipetting or quick vortex, then quick spin.

Template DNA Digestion

- Before partitioning, DNA samples with an average length of ≥20 kb should be digested. This ensures accurate and precise quantification. DNA fragmentation via restriction digestion is particularly important when CNV analyses are performed where multiple copies of a gene might be linked in tandem. Restriction digestion is not required for highly fragmented DNA (e.g., FFPE DNA or circulating DNA).
- The restriction enzymes in Table 1 are validated to digest template DNA in 10 min at RT in QIAcuity Probe PCR Master Mix and QIAcuity High Multiplex Probe PCR Master Mix without impairing the subsequent PCR amplification. For assay-specific restriction enzyme compatibility, please go to **geneglobe.qiagen.com** or refer to the product data sheet that is provided with each assay.

- **Important**: To ensure complete DNA digestion, it is essential that the specific versions of the restriction enzymes from the indicated suppliers are used.

Table 1. List of recommended enzymes

6-Cutter restriction enzymes		4-Cutter restriction enzymes	
EcoRI	0.25 U/µL EcoRI-HF®, NEB* 0.025 U/µL Anza™ 11 EcoRI, TFS†	AluI	0.025 U/µL AluI, NEB 0.025 U/µL Anza 44 AluI, TFS
PvuII	0.025 U/µL PvuII, NEB 0.025 U/µL Anza 52 PvuII, TFS	CviQI	0.025 U/µL CviQI, NEB 0.025 U/µL Csp6I (CviQI), TFS
XbaI	0.025 U/µL Anza 12 XbaI, TFS	HaeII	0.025 U/µL BsuRI (HaeIII), TFS

* NEB, New England Biolabs.

†TFS, Thermo Fisher Scientific.

Procedure

Reaction setup

1. Thaw the selected master mix, template DNA, dPCR CNV Probe Assays, and RNase-free water. Mix the individual solutions.
2. Prepare a reaction mix according to Table 2, 3, or 4 (excluding the DNA template), depending on the master mix and application. Due to the hot-start, it is not necessary to keep samples on ice during reaction setup or while programming the QIAcuity instrument.
3. Vortex the reaction mix.

Table 2. Reaction setup for QIAcuity Probe PCR Master Mix

	Volume/reaction		
Component	**Recommended: Nanoplate 8.5k (24-well, 96-well)**	**Optional: Nanoplate 26k (8-well, 24-well)**	**Final concentration**
4x Probe PCR Master Mix	3 µL	10 µL	1x
20x dPCR CNV Probe Assay 1*	0.6 µL	2 µL	1x
20x dPCR CNV Probe Assay 2,3,4,5*	0.6 µL (each)	2 µL (each)	1x
Restriction Enzyme† (highly recommended‡)	0.3–3 units	1–10 units	0.025–0.25 U/µL
RNase-free water	Variable	Variable	
Template gDNA (added at step 4)	Variable§	Variable§	
Total reaction volume	**12 µL**	**40 µL**	

* For respective dye recommendations for the CNV Probe Assays and available channels on QIAcuity, as well as for multiplexing, please see the *QIAcuity User Manual* or the *QIAcuity User Manual Extension: QIAcuity Application Guide*.

† For selection of restriction enzymes, please refer to assay specifications and the *QIAcuity User Manual Extension: QIAcuity Application Guide*.

‡ For gDNA with an average length ≥20 kb.

§ Template gDNA amounts ideally should lie within 30–100 ng/reaction and should not exceed 450 ng/reaction when detecting CNV targets present in 2 copies/diploid genome.

Table 3. Reaction setup for QIAcuity High Multiplex Probe PCR Master Mix for detecting up to 6 targets per reaction (one target per channel in up to 6 channels)

	Volume/reaction		
Component	**Nanoplate 8.5k (24-well, 96-well)**	**Nanoplate 26k (8-well, 24-well)**	**Final concentration**
4x QIAcuity High Multiplex Probe PCR Master Mix	3 µL	10 µL	1x
20x Primer–probe mix 1–6*	0.6 µL (each)	2 µL (each)	1x
Restriction Enzyme† (optional‡)	Up to 1 µL	Up to 1 µL	0.025–0.25 U/µL
RNase-free water	Variable	Variable	–
Template DNA (added at step 4)	Variable§	Variable§	–
Total reaction volume	**12 µL**	**40 µL**	**–**

* For respective dye recommendations, refer to the *QIAcuity User Manual* or the *QIAcuity User Manual Extension: QIAcuity Application Guide*.

† For selection of restriction enzymes, please refer to assay specifications and the *QIAcuity User Manual Extension: QIAcuity Application Guide*.

‡ For gDNA with an average length ≥20 kb.

§ Template gDNA amounts ideally should lie within 30–100 ng/reaction and should not exceed 450 ng/reaction when detecting CNV targets present in 2 copies/diploid genome.

Table 4. Reaction setup for QIAcuity High Multiplex Probe PCR Master Mix for detecting up to 12 targets per reaction with amplitude-based multiplexing

	Volume/reaction		
Component	**Nanoplate 8.5k (24-well, 96-well)**	**Nanoplate 26k (8-well, 24-well)**	**Final concentration**
4x QIAcuity High Multiplex Probe PCR Master Mix	3 µL	10 µL	1x
20x Primer–probe mix 1–12* (for multiplex)	Variable	Variable	Variable†
Restriction Enzyme‡ (optional§)	Up to 1 µL	Up to 1 µL	0.025–0.25 U/µL
RNase-free water	Variable	Variable	–
Template DNA (added at step 4)	Variable¶	Variable¶	–
Total reaction volume	**12 µL**	**40 µL**	**–**

* For respective dye recommendations, refer to the *QIAcuity User Manual* or the *QIAcuity User Manual Extension: QIAcuity Application Guide*.

† For reactions that employ amplitude-based multiplexing, variable assay concentrations are required. Refer to the *QIAcuity High Multiplex Probe PCR Kit Handbook* for more details.

‡ For selection of restriction enzymes, please refer to assay specifications and the *QIAcuity User Manual Extension: QIAcuity Application Guide*.

§ For gDNA with an average length ≥20 kb.

¶ Template gDNA amounts ideally should lie within 30–100 ng/reaction and should not exceed 450 ng/reaction when detecting CNV targets present in 2 copies/diploid genome.

4. Dispense appropriate volumes of the reaction mix, which contains all components except the template, into the wells of a standard PCR plate. Then, add template DNA into each well that contains the reaction mix.

   Note: The appropriate amounts of reaction mix and template DNA depends on various parameters. Please refer to the *QIAcuity User Manual Extension: QIAcuity Application Guide* for details.

5. Transfer the contents of each well of the standard PCR plate to the wells of a 26k or 8.5k nanoplate.

6. Seal the nanoplate properly using the QIAcuity Nanoplate Seal provided in the QIAcuity Nanoplate Kits. For exact sealing procedure, see the *QIAcuity User Manual*.

7. If a restriction enzyme for DNA digestion has been included in the reaction, leave the plate for 10 min at room temperature (15–25°C).

Thermal cycling and imaging conditions

1. Set the cycling conditions under the dPCR parameters in the QIAcuity Software Suite or at the QIAcuity instrument according to Cycling conditions.

2. Activate the required channels in Imaging, under the dPCR parameters in the QIAcuity Software Suite or at the QIAcuity instrument. Imaging settings should be set to the default values.

   Note: If using the QIAcuity High Multiplex Probe PCR Kit, the "Enable High-Multiplexing-Reference channel" toggle in the Imaging tab must be activated. Otherwise, NO valid wells will appear during analysis. Refer to the *QIAcuity High Multiplex Probe PCR Kit Handbook* for recommendations on the maximum RFU levels for each channel when performing higher-order multiplexing.

Table 5. Cycling conditions

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

3. Place the nanoplate into the QIAcuity instrument and start the dPCR program.

Data analysis

1. To set up a plate layout according to the experimental design, open the QIAcuity Software Suite and define the reaction mixes, samples, and controls. The plate layout can be defined before or after the nanoplate run.

   Note: Refer to the *QIAcuity User Manual* for details on setting up the plate layout.

2. After the nanoplate run, the raw data is automatically sent to the QIAcuity Software Suite.

3. For data analysis, open the QIAcuity Software Suite and select the individual nanoplate for the analysis in **Plate Overview** of the software suite.

   Note: Refer to the *QIAcuity User Manual Extension: QIAcuity Application Guide* and the *QIAcuity User Manual* for details on how to analyze the data and calculate copy numbers per human genome based on the reference(s) you have added to the nanoplate.

   Note: The "Copy Number Variation" analysis option in the QIAcuity Software Suite is not available when amplitude-based multiplexing is used.

Document Revision History

Date	Changes
03/2024	Initial release
02/2025	Inclusion of QIAcuity High Multiplex Probe Kit.

Scan QR code for *QIAcuity User Manual*.

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual.