Quick-Start Protocol February 2021

dPCR Copy Number Assays

This protocol is optimized for the quantification of gene-specific regions in human DNA using the dPCR Copy Number Assays (cat. nos. 250205, 250206) with the QIAcuity® EG PCR Kit (cat. nos. 250111, 250112, 250113), in an EvaGreen®-based reaction using the QIAcuity digital PCR (dPCR) instrument, to detect copy number variation (CNV) in the human genome.

The dPCR Copy Number Assays are shipped on dry ice and should upon receipt be immediately stored protected from light at –30 to –15°C in a constant-temperature freezer for up to 12 months or at 2–8°C for up to 6 months. Under these conditions, the components are stable, without showing any reduction in performance and quality, until the date indicated on the label.

Further information

- *QIAcuity User Manual Extension: QIAcuity Application Guide*: **www.qiagen.com/HB-2839**
- *QIAcuity User Manual*: **www.qiagen.com/HB-2717**
- Safety Data Sheets: **www.qiagen.com/safety**
- Technical assistance: **support.qiagen.com**

Notes before starting

- A reference assay with known copy numbers/genome can be used to identify CNVs in test samples, given that the same DNA sample is used across the reference and test assays. Alternatively, a DNA sample with known copy number for gene of interest (or region of interest) can be used as reference sample. We recommend that the quality and source of reference sample(s) be comparable to that of test samples, if possible.
- Reference assays and samples must be included in the same QIAcuity® Nanoplate, in addition to test assays and samples. We recommend using multiple reference samples and/or reference assays.
- dPCR Copy Number Assays come as a 25x primer mix in a single tube.
- Always start with the dPCR cycling conditions and primer concentrations specified in this protocol.

- Pipetting accuracy and precision affect the consistency of results. Make sure that no bubbles are introduced into the wells of the QIAcuity Nanoplate during pipetting.
- Due to the hot-start, it is not necessary to keep samples on ice during reaction setup or while programming the QIAcuity instrument.

Template DNA digestion

- Before partitioning, use restriction digestion to fragment DNA samples with average length ≥20 kb, to ensure even distribution of template throughout the QIAcuity Nanoplate, for accurate and precise quantification.
- DNA fragmentation via restriction digestion is critical for accurate CNV analysis when 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) or cDNA.
- To perform restriction digestion directly in reaction mix, add recommended restriction enzyme during reaction setup. Use restriction enzymes that do not cut within target amplicon regions.
- We recommend using *Eco*RI-HF, *Pvu*II, *Xba*I (6-cutters), *Alu*I, *Cvi*QI, *Hae*III (4-cutters), which are validated to digest template DNA in 10 min at RT in QIAcuity EG PCR Master Mix without impairing the subsequent PCR amplification (Table 1). For additional assay-specific enzyme recommendations that do not cut in the amplicon, go to **geneglobe.qiagen.com** or refer to the product data sheet (printout sent with the product).

Table 1. Validated restriction enzymes

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

Procedure

Reaction setup

1. Thaw the QIAcuity EG PCR Master Mix, template DNA, dPCR Copy Number Assay, and RNase-Free Water. Mix the individual solutions.
2. Prepare a reaction mix according to Table 2.

3. Vortex the reaction mix.

Table 2. Reaction setup

Component	Volume/reaction		
	Recommended: Nanoplate 8.5k (24-well, 96-well)	**Optional: Nanoplate 26k (24-well)**	**Final concentration**
3x EvaGreen PCR Master Mix (green channel)	4 µl	13.3 µl	1x
25x dPCR Copy Number Assays	0.48 µl	1.6 µl	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 selection of restriction enzymes, please refer to assay specifications in GeneGlobe.

† For gDNA > 20 kb.

‡ Template gDNA amounts should lie within 30–100 ng/reaction and should not exceed 450 ng/reaction when detecting CNV assays 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 24-well or 96-well 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 at room temperature (15–25°C) for 10 min.

Thermal cycling and imaging conditions

1. Set the cycling conditions under the dPCR parameters in the QIAcuity Software Suite or on the QIAcuity instrument according to Table 3.
2. For EvaGreen-based detection, activate the green channel and deactivate the other channels in **Imaging**, under the dPCR parameters in the QIAcuity Software Suite or on the QIAcuity instrument.

Table 3. Cycling conditions

Step	Time	Temperature
PCR initial heat activation	2 min	95°C
3-step cycling (40 cycles)		
Denaturation	15 s	95°C
Annealing	15 s	60°C
Extension	15 s	72°C
Cooling down	5 min	40°C

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. 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 are 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 *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.

Document Revision History

Date	Changes
07/2020	Initial release
02/2021	Corrected the volume of "25x dPCR Copy Number Assays" for the recommended Nanoplate in Table 2. Editorial and layout changes.

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