# Custom dPCR LNA® Assays designed by QIAGEN® Genomic Services

The Custom dPCR LNA Assays (cat. nos. 338300, 338301, 338302, 338303, and 338304) can be stored at –30 to –15°C. Please refer to the expiry date on the tube label.

## Further information

- Safety Data Sheets: **www.qiagen.com/safety**
- Technical assistance: **support.qiagen.com**

## Notes before starting

- Always start with the cycling conditions and primer concentrations specified in this protocol.
- A fluorescent reference dye is provided as a component of the QIAcuity® Probe PCR Master Mix (cat. nos. 250101, 250102, 250103) and the QIAcuity EG PCR Master Mix (cat. nos. 250111, 250112, 250113) for reliable detection of proper partition filling in the dPCR plates.
- Pipetting accuracy and precision affect the consistency of results. Make sure that no air is introduced into the wells of the dPCR plate during pipetting.
- dPCR LNA Assays are required to be diluted as a 10x primer–probe mix in a single tube format.

## Template DNA fragmentation (optional)

- Restriction digestion is not required for highly fragmented DNA (e.g., FFPE DNA or circulating DNA), cfDNA, or genomes < 20 kb.
- To ensure even distribution and precise quantification of template DNA of average lengths of ≥ 20 kb (e.g., genomics DNA purified via spin columns with silica membrane) across the QIAcuity nanoplate, we recommend an additional step of restriction digestion.

- Fragmentation of DNA via restriction digestion is particularly important in copy number variation (CNV) analyses, where multiple copies of gene might be linked in tandem. Ensure the restriction enzymes do not have cut sites within the amplicon. Selected restriction enzymes can be added directly into the reaction mix during the reaction setup.

Please check Table 3 of the *Custom dPCR LNA Assays Product Sheet* (**www.qiagen.com/HB-2938**) for assay-specific enzyme recommendations that do not cut in the amplicon. We recommend using EcoRI-HF, PvuII, XbaI (6-cutters), AluI, CviQI, HaeIII (4-cutters), which are validated to digest template DNA in 10 minutes at room temperature in the QIAcuity PCR Master Mix without impairing the subsequent PCR amplification (Table 1).

**Table 1. Validated restriction enzymes**

| 6-cutter restriction enzymes | | 4-cutter restriction enzymes | |
|---|---|---|---|
| **EcoRI** | 0.25 U/µl EcoRI-HFR, NEB<br>0.025 U/µl Anza™ 11 EcoRI, Thermo Fisher Scientific (TFS) | **AluI** | 0.025 U/µl AluI, NEB<br>0.025 U/µl Anza 44 AluI, TFS |
| **PvuII** | 0.025 U/µl PvuII, NEB<br>0.025 U/µl Anza 52 PvuII, TFS | **CviQI** | 0.025 U/µl AluI, NEB<br>0.025 U/µl Anza 44 AluI, TFS |
| **XbaI** | 0.025 U/µl Anza 12 XbaI, TFS | **HaeIII** | 0.025 U/µl BsuRI (HaeIII), TFS |

## Reaction setup

1. Thaw the QIAcuity PCR Master Mix, Custom dPCR LNA Assay, template DNA, and RNase-free water. Mix the individual vials thoroughly and do a quick spin.
2. Prepare 10x primer probe mixes according to Table 2. For EvaGreen based detection, prepare a 10x primer mix with 4 µl of each primer.

**Table 2. Setup for dilution of primers and probes (x) as a 10-fold working stock solution (to be scaled up if needed), for each individual assay**

| Mix 1 (10x primer-probe) | | Mix 2 (10x primer-probe) | | Mix ... (10x primer-probe) | |
|---|---|---|---|---|---|
| Forward Primer 1 | 8 µl | Forward Primer 2 | 8 µl | Forward Primer … | 8 µl |
| Reverse Primer 1 | 8 µl | Reverse Primer 2 | 8 µl | Reverse Primer … | 8 µl |
| Probe 1 | 4 µl | Probe 2 | 4 µl | Probe … | 4 µl |
| RNase-free water | 80 µl | RNase-free water | 80 µl | RNase-free water | 80 µl |
| Total stock solution volume | 100 µl | Total stock solution volume | 100 µl | Total stock solution volume | 100 µl |

3. Prepare a reaction mix which contains all components except the sample/template according to Table 3. Adjust volumes for the number of samples to be analyzed. Due to the hot-start enzyme it is not necessary to keep samples on ice during reaction setup or while programming the real-time cycler.

**Table 3. dPCR reaction setup for a multiplex assay. Alternatively, dPCR reaction can be set up for individual assays in the framework of a singleplex setup**

| | Volume/reaction | | |
|---|---|---|---|
| **Step** | **Recommended: Nanoplate 26k (24-well)** | **Alternative: Nanoplate 8.5k (24-well, 96-well)** | **Final concentration** |
| 4x Probe PCR Master Mix | 10 µl | 3 µl | 1x |
| 10x primer probe Mix 1 | 4 µl | 1.2 µl | 0.8 µM primer (each)<br>0.4 µM probe |
| 10x primer probe Mix 2 | 4 µl | 1.2 µl | 0.8 µM primer (each)<br>0.4 µM probe |
| 10x primer probe Mix … | 4 µl | 1.2 µl | 0.8 µM primer (each)<br>0.4 µM probe |
| Restriction Enzyme* (optional) | Up to 1 µl | Up to 1 µl | 1–10 units/reaction |
| RNase-free water | variable | variable | |
| Template DNA (added at step 4) | variable | variable | |
| Total reaction volume | 40 µl | 12 µl | |

*For optional restriction enzyme use, refer to the amounts shown in Table 1.

4. Vortex briefly the reaction mix.
5. 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/cDNA into each well that contains the reaction mix, and mix thoroughly by pipetting.

   **Note**: The appropriate amounts of the template can vary based on various parameters. Please refer to the *QIAcuity Application Guide* to determine the appropriate template concentrations. As a general recommendation, template gDNA amount should be ≥20 ng/reaction and should not exceed 450 ng/reaction for both 26k 24-well and 8.5k 96-well plates. For RNA samples and cDNA synthesis, the QuantiTect Reverse Transcription Kit is recommended for best signal-to-noise ratio. As a starting point for

highly expressed targets, use 1–3 ng reverse transcribed RNA per microliter of dPCR reaction; for low abundant targets, increase the cDNA input to 3–9 ng/µl reaction. The total volume of QuantiTect cDNA reaction in the PCR reaction should not exceed 10%.

6. Transfer the contents from the standard PCR plate to the wells of the appropriate nanoplate.

   **Note**: We recommend a 26k nanoplate for detecting the lowest fractional abundance, although 8.5k nanoplate could be used.

7. Seal the nanoplate properly using the QIAcuity Nanoplate Seal provided in the QIAcuity Nanoplate Kits. For exact sealing procedure, see the *QIAcuity User Manual*.
8. 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 at the QIAcuity instrument according to Table 4.

**Table 4. Cycling conditions**

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

2. For multiplex probe detection, activate the appropriate channel and deactivate the other channels in Imaging, under the dPCR parameters in the QIAcuity Software Suite or at the QIAcuity instrument.
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 uploaded 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 Application Guide* and *QIAcuity User Manual* for details on how to analyze the data to get absolute quantification and mutation analysis (fractional abundance) data.

## General Remarks

Custom assays are designed to function under the conditions mentioned above. Adjustment of primer concentration or cycling conditions (number of cycles, annealing temperature, or introduction of specific elongation or extension steps at 72°C) may enhance the performance of the individual assays.

## Document Revision History

| Date | Changes |
|---|---|
| 11/2021 | Initial release |

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