

## dPCR LNA<sup>®</sup> Mutation Assays

This protocol is optimized for the detection of mutant sequences in wild-type background DNA using the dPCR LNA Mutation Assays Kit (cat. nos. 250200, 250201) with the QIAcuity™ Probe PCR Kit (cat. nos. 250101, 250102, 250103) in a duplex reaction using the QIAcuity digital PCR (dPCR) instrument.

The dPCR LNA Mutation Assays are available in 2 different dye combinations (FAM/HEX or Atto550/ROX) to detect the mutant/wild-type sequences. Alternatively, 2 assays detecting 2 mutation targets could be used in a 4-plex reaction. However, cross-reactivity of the different dPCR LNA Mutation Assays was not tested for all assays.

dPCR LNA Mutation Assays are shipped on dry ice and should upon receipt be stored protected from light at  $-30$  to  $-15^{\circ}\text{C}$  in a constant-temperature freezer for long-term storage (e.g., 12 months) or at  $-2$ – $-8^{\circ}\text{C}$  for short-term storage (e.g., 6 months). Under these conditions QIAcuity Mutation Detection digital PCR Assay Kit components are stable, without showing any reduction in performance and quality, until the date indicated on the label.

### Further information

- *QIAcuity Application Guide*: [www.qiagen.com/PROM-16196](http://www.qiagen.com/PROM-16196)
- *QIAcuity User Manual*: [www.qiagen.com/HB-2717](http://www.qiagen.com/HB-2717)
- Safety Data Sheets: [www.qiagen.com/safety](http://www.qiagen.com/safety)
- Technical assistance: [support.qiagen.com](http://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, for reliable detection of proper partition filling in the dPCR plates.
- Pipetting accuracy and precision affect the consistency of results. Make sure that no bubbles are introduced into the wells of the dPCR plate during pipetting.

### Template DNA digestion

- Before partitioning, use restriction digestion to fragment DNA samples with average length  $\geq 20$  kb (e.g., genomic DNA purified via spin



column with silica membrane), to ensure even distribution of template throughout the QIAcuity Nanoplate, for accurate and precise quantification.

- DNA fragmentation via restriction digestion is particularly important when copy number variation (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) or cDNA.
- To perform restriction digestion directly in reaction mix, add the selected restriction enzyme during reaction setup. Use restriction enzymes that will not cut within the amplified sequence.

We recommend using *EcoRI*-HF, *PvuII*, *XbaI* (6-cutters), *AluI*, *CviQI*, *HaeIII* (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, please go to [geneglobe.qiagen.com](https://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	
<i>EcoRI</i>	0.25 U/μl <i>EcoRI</i> -HF®, NEB® 0.025 U/μl Anza™ 11 <i>EcoRI</i> , Thermo Fisher Scientific (TFS)	<i>AluI</i>	0.025 U/μl <i>AluI</i> , NEB 0.025 U/μl Anza 44 <i>AluI</i> , TFS
<i>PvuII</i>	0.025 U/μl <i>PvuII</i> , NEB 0.025 U/μl Anza 52 <i>PvuII</i> , TFS	<i>CviQI</i>	0.025 U/μl <i>CviQI</i> , NEB 0.025 U/μl <i>Csp6I</i> ( <i>CviQI</i> ), TFS
<i>XbaI</i>	0.025 U/μl Anza 12 <i>XbaI</i> , TFS	<i>HaeIII</i>	0.025 U/μl <i>BsuRI</i> ( <i>HaeIII</i> ), TFS

## Procedure

### Reaction setup

1. Thaw the QIAcuity Probe PCR Master Mix, QIAcuity Mutation Detection digital PCR Assay, template DNA, and RNase-free water. Mix the individual solutions.
2. Prepare a reaction mix according to Table 2. Due to the hot-start, it is not necessary to keep samples on ice during reaction setup or while programming the real-time cycler.
3. Vortex the reaction mix.
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:** Template gDNA amount should be  $\geq 20$  ng/reaction and should not exceed 450 ng/reaction for both 26k 24-well and 8.5k 96-well plates.

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

**Table 2. Reaction setup**

Component	Volume/reaction		
	Recommended: Nanoplate 26k (24-well)	Optional: Nanoplate 8.5k (24-well, 96-well)	Final concentration
4x Probe PCR Master Mix	10 µl	3 µl	1x
30x dPCR LNA Mutation Assay-1 (FAM/HEX) Optional (Atto550/ROX)	1.3 µl	0.4 µl	1x
<b>Optional 2nd assay for 4-plex rxn:</b> 30x dPCR LNA Mutation Assay-2* (ROX/Atto550) Optional (FAM/HEX)	1.3 µl	0.4 µl	1x
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	
<b>Total reaction volume</b>	<b>40 µl</b>	<b>12 µl</b>	

\* Add the second 30x dPCR LNA Mutation Assay-2 for a 4-plex reaction to detect 2 mutation targets.

**Important:** Dye combination must be different from what was used in 30x dPCR LNA Mutation Assay-1. Fourplex reactions cannot be detected on QIAcuity One 2-Plex. Not all assays have been tested for cross-reactivity with 2 dPCR LNA Mutation Assays in a 4-plex reaction.

† For selection of restriction enzymes, please refer to assay specifications in GeneGlobe.

5. Transfer the contents of each well of the standard PCR plate to the wells of a 26k 24-well nanoplate.

**Note:** The 8.5k nanoplate can also be used, but the 26k nanoplate is recommended for detecting the lowest fractional abundance.

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 at the QIAcuity instrument according to Table 3.

**Table 3. Cycling conditions**

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

- For duplex 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.
- Place the nanoplate into the QIAcuity instrument and start the dPCR program.

### Data analysis

- 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.

- After the nanoplate run, the raw data is automatically sent to the QIAcuity Software suite.
- 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.

### Document Revision History

Date	Changes
07/2020	Initial release

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