November 2024

Quick-Start Protocol

dPCR LNA® 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 MasterMix (cat. no. 1133251) or QIAcuity High Multiplex Probe PCR Kit (cat. nos. 250133, 250134) using the QIAcuity digital PCR (dPCR) instrument.

The dPCR LNA Mutation Assays are available in 3 different dye combinations (FAM/HEX, Atto 550/ROX, and Cy5/ATTO 700) to detect mutant/wild- type sequences. Use of Cy5/ATTO 700 assays is only possible in combination with the QIAcuity High Multiplex Probe PCR Kit and QIAcuity Software Suite 3.0 or newer. Multiple assays can be combined in a multiplex reaction. However, cross-reactivity has not been tested for all assay combinations.

The dPCR LNA Mutation Assays should be stored immediately upon receipt at –30°C to –15°C in a constant- temperature freezer and protected from light. Under these conditions, the components are stable for 12 months without showing any reduction in performance and quality, unless otherwise indicated on the label.

Further information

- *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**

Things to do before starting

- Always start with the 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 dPCR plate during pipetting.
- Refer to the *QIAcuity User Manual* and *QIAcuity User Manual Extension* for guidance on assay design and experimental setup for the QIAcuity platform.
- Use of Cy5/ATTO 700 assays is only possible in combination with the QIAcuity High Multiplex Probe PCR Kit and QIAcuity Software Suite 3.0 or newer.

Template DNA digestion

- DNA samples with ≥20 kb average length (e.g., genomic DNA purified via spin column with silica membrane or salting out method) should be fragmented by restriction digestion before partitioning. Enzymatic fragmentation of larger DNA ensures even distribution of template throughout the QIAcuity Nanoplate for accurate and precise quantification.
- Restriction digestion is not required for highly fragmented DNA (e.g., FFPE DNA or circulating DNA) or cDNA.
- The following validated enzymes will digest DNA in 10 min at room temperature (15–25°C) when added directly to the QIAcuity reaction mix at the indicated concentrations:

  We recommend using *EcoR*I-HF, *Pvu*II, *Xba*I (6-cutters), *Alu*I, *CviQ*I, *Hae*III (4-cutters), which are validated to digest template DNA in 10 min at RT in the QIAcuity MasterMix and QIAcuity High Multiplex Probe PCR Master Mix without impairing the subsequent PCR

amplification (Table 1). For assay-specific recommendations for enzymes that do not cut in the amplicon, please 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	
*EcoR*I	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	*CviQ*I	0.025 U/µL CviQI, NEB 0.025 U/µL Csp6I (CviQI), TFS
*Xba*I	0.025 U/µL Anza 12 XbaI, TFS	*HaeIII*	0.025 U/µL BsuRI (HaeIII), TFS

IMPORTANT: To ensure that your DNA samples are fully digested, the particular versions of the restriction enzymes from the indicated suppliers must be used.

Procedure

Reaction setup

1. Thaw the selected master mix, template, dPCR LNA Mutation Assay, and RNase- Free Water. Vigorously mix the master mix and the individual solutions. Centrifuge the tubes briefly to settle the liquids.
2. Prepare a master mix according to Table 2 or Table 3, depending on master mix. Due to the hot-start, it is not necessary to keep samples on ice during reaction setup or while programming the real-time cycler.

Table 2. Reaction setup for QIAcuity MasterMix

	Volume/reaction		
Component	**Recommended: Nanoplate 26k (8-well, 24-well)**	**Optional: Nanoplate 8.5k (24-well, 96-well)**	**Final concentration**
4x QIAcuity MasterMix	10 µL	3 µL	1x
30x dPCR LNA Mutation Assay-1 (FAM/HEX) Optional (Atto550/ROX)	1.3 µL	0.4 µL	1x
Optional 2nd assay for 4-plex rxn: 30x dPCR LNA Mutation Assay-2 (ATTO 550/ROX) 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 gDNA (added at step 4)	Variable	Variable	
Total reaction volume	**12 µL**	**40 µL**	

* 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 l dPCR LNA Mutation Assays have been tested for cross-reactivity with used in a 4-plex reaction.

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

Table 3. Reaction setup for High Multiplex Probe PCR Kit

	Volume/reaction		
Component	**Recommended: Nanoplate 26k (8-well, 24-well)**	**Optional: Nanoplate 8.5k (24-well, 96-well)**	**Final concentration**
4x QIAcuity High Multiplex Probe PCR MasterMix	10 µL	3 µL	1x
30x dPCR LNA Mutation Assay-1 (FAM/HEX) Optional: (ATTO 550/ROX or Cy5/ATTO 700)	1.3 µL	0.4 µL	1x
Optional 2nd assay for 4-plex rxn: 30x dPCR LNA Mutation Assay-2 (ATTO 550/ROX) Optional (FAM/HEX or Cy5/ATTO 700)*	1.3 µL	0.4 µL	1x
Optional 3rd assay for 6-plex rxn: 30x dPCR LNA Mutation Assay-2 (Cy5/ATTO 700) Optional: (FAM/HEX or ATTO 550/ROX)*	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 gDNA (added at step 4)	Variable	Variable	
Total reaction volume	**12 µL**	**40 µL**	

* Add the second and third 30x dPCR LNA Mutation Assays for a 4-plex or 6-plex reaction to detect 2 or 3 mutation targets, respectively.

Important: Dye combinations must be different from what was used in 30x dPCR LNA Mutation Assay-1 and 30x dPCR LNA Mutation Assay-2. Fourplex and sixplex reactions cannot be detected on QIAcuity One 2-Plex. Not all dPCR LNA Mutation Assays have been tested for cross-reactivity when used in a 4-plex reaction. dPCR LNA Mutation Assays have not been tested for cross-reactivity when used in a 6-plex reaction.

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

3. Vortex the reaction.
4. Dispense appropriate volumes of the reaction mix, which contains all components except the template, into the wells of a standard PCR plate (pre-plate). Then, add template DNA into each well that contains the reaction mix. Thoroughly mix the template DNA with the reaction mix by pipetting up and down.

   Note: Template gDNA amount should be ≥20 ng/reaction and should not exceed 450 ng/reaction for both 26k and 8.5k plates. The appropriate amounts of reaction mix and template DNA depends on various parameters. Please refer to the *QIAcuity Application Guide* for details. The pre-plate may be assembled at room temperature.
5. Transfer the contents of each well in the pre-plate to the wells of a Nanoplate.
6. Seal the Nanoplate properly using the QIAcuity Nanoplate Seal provided in the QIAcuity Nanoplate Kits.
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 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

5. Activate the required channels in Imaging, under the dPCR parameters in the QIAcuity Software Suite or at the QIAcuity instrument.
6. 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 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 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.
11/2024	- Added additional dyes (Cy5/ATTO 700) to the protocol - Added the protocol for multiplexing with the dPCR High Multiplex Probe PCR Kit and SW 3.0 - Streamlined with the dPCR CNV Probe Assy QSP