December 2025

Supplementary Protocol

dPCR CNV Probe Assays – MLH1 Methylation Assay

For quantification of MLH1-specific methylation in human DNA using dPCR CNV Probe Methylation Assay MLH1 on the QIAcuity® platform in combination with methylation-sensitive restriction enzymes. The methylation-sensitive restriction enzymes cannot cut methylated DNA. By comparing the DNA copy number remaining after digestion with that in an undigested sample, the relative amount of methylated versus unmethylated MLH1 DNA can be determined for specific methylation sites.

The wet-lab-tested dPCR CNV Probe Methylation Assay MLH1 (cat. no. 250210; GeneGlobe-ID: DCG0000619) is shipped ready-to-use on dry ice. Upon receipt, the assay should be stored protected from light in a constant-temperature freezer at –30°C to –15°C for long-term storage (12 months) or at 2–8°C for short-term storage (6 months).

Wet-lab testing was performed using QIAcuity Nanoplate 26k (cat. no. 250001) and the QIAcuity Probe PCR Kit (cat. nos. 250101, 250102, or 250103) with 20 ng of FFPE-derived DNA per reaction, with detection in the FAM channel.

For more information, refer to the "Shipping and Storage" chapter in *dPCR CNV Number Variation (CNV) Probe Assays Handbook* (**www.qiagen.com/HB-3545**).

Equipment and reagents to be supplied by the user

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

- Genomic DNA isolation kit
- QIAcuity Digital PCR instrument*
- QIAcuity dPCR Nanoplates*
- QIAcuity Probe PCR Kit or QIAcuity High Multiplex Probe PCR Kit*
- Multichannel pipettor
- Nuclease-free pipette tips and tubes
- HinP1I restriction enzyme (e.g., HinP1I, New England Biolabs®)
- **Optional**: *EcoR*I restriction enzyme (e.g., EcoRI-HF®, New England Biolabs)

For more information, refer to the "Equipment and Reagents to be Supplied by User" chapter in *dPCR CNV Number Variation (CNV) Probe Assays Handbook* (**www.qiagen.com/HB-3545**).

*Refer to "Ordering Information" on page 7.

Protocol: MLH1 Methylation analysis

Template preparation

1. Extract DNA from samples (e.g., FFPE sections of endometrial carcinomas).
2. Measure DNA concentration by a fluorometric method (i.e., Qubit™).
3. Add 10–100 ng (FFPE recommended 20 ng) DNA in the reaction setup. For ease of use, we recommend to use the same volume for every DNA sample (recommended: at least 2 µL).

   Note: FFPE-derived DNA is often difficult to quantify reliably, which can result in large variations in detected copy numbers even at the same nominal DNA input.

Reaction setup

4. Thaw the QIAcuity (High Multiplex) Probe PCR Master Mix, template DNA, RNase-free water, and primers. Thoroughly vortex all components before use. Centrifuge briefly to collect liquids at the bottom of the tubes.
5. Prepare a reaction mix for the number of reactions needed according to Table 1 and Table 2. Add 10% extra to prevent pipetting errors. Due to the hot-start, it is not necessary to keep samples on ice during setup.
6. Set two reaction mixes per sample; one with the methylation-sensitive restriction enzyme (RE), and one without RE, as indicated in Table 1 and Table 2.

Table 1. Reaction mix without RE

	Volume/reaction		
Reagents	**Nanoplate 26k (8-well, 24-well)**	**Nanoplate 8.5k (24-well, 96-well)**	**Final concentration**
QIAcuity (High Multiplex) Probe PCR Master Mix (4x)	10.0 µL	3.0 µL	1x
MLH1 assay (20x)	2.0 µL	0.6 µL	1x
Q-solution	4.0 µL	1.2 µL	0.5x
*EcoR*I	0.5 µL	0.15 µL	–
RNase-free Water	Variable	Variable	–
DNA	Variable	Variable	10–100 ng
Total reaction volume	**40.0 µL**	**12.0 µL**	

Table 2. Reaction mix with RE

	Volume/reaction		
Reagents	**Nanoplate 26k (8-well, 24-well)**	**Nanoplate 8.5k (24-well, 96-well)**	**Final concentration**
QIAcuity (High Multiplex) Probe PCR Master Mix (4x)	10.0 µL	3.0 µL	1x
MLH1 assay (20x)	2.0 µL	0.6 µL	1x
Hinp1I (10,000 U/mL)	1.0 µL	0.3 µL	0.25 U/µL
Q-solution	4.0 µL	1.2 µL	0.5x
*EcoR*I*	0.5 µL	0.15 µL	–
RNase-free Water	Variable	Variable	–
DNA	Variable	Variable	10–100 ng
Total reaction volume	**40 µL**	**12.0 µL**	

7. Vortex each reaction mix at low speed.
8. Dispense appropriate volumes of the reaction mix into the wells of a standard PCR plate. This reaction contains all components except the template DNA.

   Important: Do not use the nanoplate at this step.
9. Add the needed template DNA volume into each well that contains the reaction mix.
10. Seal the plate, vortex briefly, and spin to ensure thorough mixing.
11. Transfer the content of each well from the standard PCR plate to the wells of the nanoplate. To ensure bubble-free pipetting, we recommend to pipette 39 µL for Nanoplate 26k, and 11 µL for Nanoplate 8.5k.
12. Seal the nanoplate using the QIAcuity Nanoplate Seal provided in the QIAcuity Nanoplate Kits following the recommendations of the *QIAcuity User Manual* (refer to the user manual of the corresponding format, **www.qiagen.com/QIAcuitydPCRSystem**).

Thermal cycling conditions

13. Program the cycler of the QIAcuity instrument according to Table 3.

Table 3. Cycling conditions

Step	Time	Temperature (°C)	No. of cycles
DNA digestion with restriction enzyme	60 min	37	–
PCR initial heat activation	2 min	95	–
Denaturation	15 s	95	40 cycles
Combined annealing/extension	30 s	60	40 cycles

14. For multiplex probe detection, activate the appropriate fluorescence channel corresponding to the fluorophore of the probe used, and deactivate the other channels in **Imaging** under the dPCR parameters in the QIAcuity Software Suite or at the QIAcuity instrument.
15. Place the nanoplate into the QIAcuity instrument and start the dPCR program.

Ordering Information

Product	Contents	Cat. no.
dPCR CNV Probe Methylation Assay MLH1	20x concentrated, ready-to-use wet-lab tested dPCR CNV Probe Gene of Interest Assay	250210 GeneGlobe-ID: DCG0000619
QIAcuity Probe PCR Kit	5 x 5 mL 4x concentrated QIAcuity Probe Mastermix, 4 x 20 mL Water	250101, 250102, 250103
QIAcuity High Multiplex Probe PCR Kit	1mL or 5mL 4x concentrated QIAcuity High Multiplex Probe PCR Master Mix, 2 x 1.9 mL RNAse-free Water, QN Internal Control DNA	250133, 250134
QIAcuity Nanoplate 26k 24-well	Microfluidic digital PCR plates for 24 samples with up to 26,000 partitions each	250001
QIAcuity Nanoplate 8.5k 24-well	Microfluidic digital PCR plates for 24 samples with up to 8500 partitions each	250011
QIAcuity Nanoplate 8.5k 96-well	Microfluidic digital PCR plates for 96 samples with up to 8500 partitions each	250021
QIAcuity Nanoplate 26k 8-well	Microfluidic digital PCR plates for 8 samples with up to 26,000 partitions each	250031
Nanoplate Seals (11)	11 top seals for the nanoplates	250099
QIAcuity One, 2plex	One-plate digital PCR instrument for detecting up to 2 fluorescent dyes	911001
QIAcuity One, 5plex	One-plate digital PCR instrument for detecting up to 5 fluorescent dyes	911021
QIAcuity Four, 5plex	Four-plate digital PCR instrument for detecting up to 5 fluorescent dyes	911042
QIAcuity Eight, 5plex	Eight-plate digital PCR instrument for detecting up to 5 fluorescent dyes	911052

Document Revision History

Date	Changes
12/2025	Initial release.

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services or your local distributor.

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