ddPCR™ Mutation Detection Assays

Catalog #	Description
10042964	**ddPCR Mutation Detection Assay (FAM)**, 200 x 20 µl reactions
10042965	**ddPCR Mutation Detection Assay (FAM)**, 1,000 x 20 µl reactions
10042966	**ddPCR Mutation Detection Assay (FAM)**, 2,500 x 20 µl reactions
10042967	**ddPCR Mutation Detection Assay (HEX)**, 200 x 20 µl reactions
10042968	**ddPCR Mutation Detection Assay (HEX)**, 1,000 x 20 µl reactions
10042969	**ddPCR Mutation Detection Assay (HEX)**, 2,500 x 20 µl reactions
12008220	**ddPCR Mutation Detection Assay (Cy5)**, 200 x 20 µl reactions
12008221	**ddPCR Mutation Detection Assay (Cy5)**, 1,000 x 20 µl reactions
12008222	**ddPCR Mutation Detection Assay (Cy5)**, 2,500 x 20 µl reactions
12008223	**ddPCR Mutation Detection Assay (Cy5.5)**, 200 x 20 µl reactions
12008224	**ddPCR Mutation Detection Assay (Cy5.5)**, 1,000 x 20 µl reactions
12008225	**ddPCR Mutation Detection Assay (Cy5.5)**, 2,500 x 20 µl reactions
12016622	**ddPCR Mutation Detection Assay (ROX)**, 200 x 20 µl reactions
12016623	**ddPCR Mutation Detection Assay (ROX)**, 1,000 x 20 µl reactions
12016631	**ddPCR Mutation Detection Assay (ROX)**, 2,500 x 20 µl reactions
12016592	**ddPCR Mutation Detection Assay (ATTO 590)**, 200 x 20 µl reactions
12016632	**ddPCR Mutation Detection Assay (ATTO 590)**, 1,000 x 20 µl reactions
12016613	**ddPCR Mutation Detection Assay (ATTO 590)**, 2,500 x 20 µl reactions

For research purposes only.

Description

ddPCR Mutation Detection Assays are expertly designed specifically for Droplet Digital™ PCR (ddPCR) using proprietary computational algorithms. These assays have not been wet-lab validated by Bio-Rad™ Laboratories, Inc. Performance of these assays should be validated prior to use. Reference assays with the complementary fluorophore can be purchased separately.

Ordering Information

Visit bio-rad.com/digital-assays to order ddPCR Mutation Detection Assays.

Storage and Stability

ddPCR Mutation Detection Assays are stable for 12 months when stored at 4°C protected from light. The 20x assay mix can be kept at –20°C for long-term storage.

Kit Contents

The ddPCR Mutation Detection Assay is a 20x concentrated, ready-to-use primer-probe mix. Each kit comes with 200, 1,000, or 2,500 µl of the 20x assay mix (9 µM primers and 5 µM probe), sufficient for 200, 1,000, or 2,500 x 20 µl reactions, respectively.

Reagents and Equipment

For assays using the QX200™ Droplet Generator (catalog #1864002) or Automated Droplet Generator (#1864101):

- For 1–2 targets, ddPCR Supermix for Probes (No dUTP) (#1863023, 1863024, 1863025) is recommended
- For >2 targets, ddPCR Multiplex Supermix (#12005909, 12005910, 12005911) is recommended
- QX200 Droplet Reader (#1864003) or QX600™ Droplet Reader (#12013328)
- PX1 PCR Plate Sealer (#1814000)

For assays using the QX ONE™ Droplet Digital PCR System (#12006536):

- ddPCR Multiplex Supermix (#12005909, 12005910, 12005911)
- PX1 PCR Plate Sealer (#1814000)

Refer to the QX200 Droplet Generator Instruction Manual (10031907), QX ONE Droplet Digital PCR System and QX ONE Software User Guide (10000116512), or Automated Droplet Generator Instruction Manual (10043138) for ordering information on consumables such as oils, cartridges, gaskets, plates, and seals. See Table 1 for fluorophore compatibility with instruments.

Table 1. Fluorophore compatibility.

QX200 Droplet Reader	QX ONE ddPCR System	QX600 Droplet Reader
FAM	FAM	FAM
HEX	HEX	HEX
	Cy5	Cy5
	Cy5.5	Cy5.5
		ROX
		ATTO 590

Determination of Optimal Annealing Temperature

Newly designed ddPCR Mutation Detection Assays should be run across a thermal gradient (50–60°C), to determine the annealing/extension temperature that optimizes separation between positive and negative droplets while minimizing rain (droplets that fall between the major positive and negative populations). If possible, an annealing/extension temperature that optimizes performance of both target and reference assays should be selected.

- Using a sample containing both mutant and wild-type alleles as template, prepare reaction mix for at least 8 wells (1 column) according to the guidelines in the Reaction Setup section
- For optimal performance, follow recommendations in the Restriction Enzyme Digestion of Sample DNA and Reaction Setup sections
- After droplet generation, proceed to thermal cycling on a C1000 Touch Thermal Cycler with 96–Deep Well Reaction Module (1851197). Use an annealing temperature gradient as described in Table 2
- Optimum annealing temperature range is determined based on the separation between four clusters (Figure 1)
- For more information, see Rare Mutation Detection Best Practices Guidelines (bulletin 6628)
- Run several no template control (NTC) wells

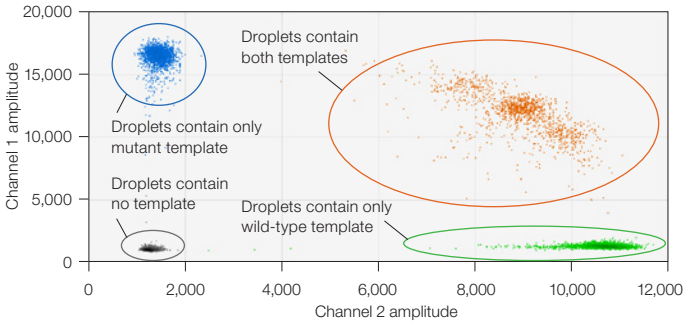

Fig. 1. Two-dimensional scatter plot demonstrating the four clusters obtained with a mutant and wild-type allele.

Table 2. Thermal gradient cycling conditions for determination of optimal annealing/extension temperature.*

Cycling Step		Temperature, °C	Time	Number of Cycles
Hold (QX ONE ddPCR System only)		25	3 min	1
Enzyme activation		95	10 min	1
Denaturation		94	30 sec	40
Annealing/extension		~50–60	1 min**	40
Enzyme deactivation		98	10 min	1
Hold	QX200 or QX600 ddPCR System (optional)	4	Infinite	1
	QX ONE ddPCR System (required)	25	1 min	1

* For the C1000 Touch Thermal Cycler, use a heated lid set to 105°C and set the sample volume to 40 µl.

** Check/adjust ramp rate settings to ~2°C/sec

Restriction Enzyme Digestion of Sample DNA

DNA fragmentation by restriction digestion prior to droplet generation enables optimal accuracy by separating tandem gene copies, reducing sample viscosity, and improving template accessibility. Two strategies may be used to perform restriction digestion of DNA samples: digestion directly in the ddPCR reaction during setup (recommended) or conventional digestion prior to Droplet Digital PCR.

Digestion Directly in ddPCR Reaction

- Efficient digestion of sample DNA can be achieved by direct addition of restriction enzyme to the ddPCR reaction
- 2–5 units of restriction enzyme per ddPCR reaction are recommended
- Dilute the restriction enzyme using the recommended diluent buffer according to the manufacturer's instructions, and then add 1 µl to the ddPCR reaction according to the guidelines in Table 3
- Reactions can be set up at room temperature; no additional incubation time is required
- The addition of restriction enzyme buffers with high salt content can inhibit Droplet Digital PCR and should be avoided

Digestion Prior to Droplet Digital PCR

- Restriction enzyme digestion can be carried out as a separate reaction before ddPCR reaction setup
- Use 10–20 units of restriction enzyme per microgram of genomic DNA
- Incubate the reaction for 1 hr at the temperature recommended for the restriction enzyme
- Heat inactivation is not required but can be considered if long-term storage is required; do not heat inactivate above 65°C
- DNA purification is not necessary after restriction digestion
- Use a minimum tenfold dilution of the digest to reduce the salt content of the sample in Droplet Digital PCR
- Store digested DNA at –20°C or below

Reaction Setup

1. Thaw all components to room temperature. Mix thoroughly by vortexing each tube to ensure homogeneity because a concentration gradient may form during –20°C storage. Centrifuge briefly to collect contents at the bottom of each tube and store protected from light.
2. Prepare samples at the desired concentration before setting up the reaction mix according to the guidelines in Table 3. If multiple samples are to be assayed using the same target and reference duplex, prepare a master reaction mix without sample template, dispense equal aliquots into the reaction tubes, and add the sample template to each reaction tube as the final step.
3. Prepare a negative control: at least one well containing only wild-type template at a concentration similar to the concentration of unkown samples.
4. Prepare a positive control: at least one well with a mix of 7 ng of mutant DNA in a background of 130 ng of wild-type DNA.

Table 3. Preparation of the reaction mix.

Component	Volume per Reaction, µl	Final Concentration
2x ddPCR Supermix for Probes (No dUTP)	10	1x
20x target primers/ probe (FAM, Cy5 or ROX)	1	1x***
20x reference primers/ probe (HEX, Cy5.5 or ATTO 590)	1	1x***
Restriction enzyme, diluted*	1	2–5 U/reaction
DNA sample or water	Variable	50 fg–100 ng†
Total Volume	**20****	**–**

* This component should be replaced by water if digestion is performed prior to Droplet Digital PCR.
** For the Automated Droplet Generator, prepare 22 µl per well.
*** 450 nM primers/250 nM each probe.
† For most assays, up to 130 ng of DNA containing mutant DNA at >2% fractional abundance is expected to perform adequately. Input may be lowered if cluster separation is not adequate.

5. Mix thoroughly by vortexing each tube. Centrifuge briefly to ensure that all components are at the bottom of each reaction tube. Allow reaction tubes to equilibrate at room temperature for about 3 minutes.
6. Transfer the reaction mix from the reaction tubes to the appropriate ddPCR Cartridge as follows:
   - For the QX200 Droplet Generator, load 20 µl of each reaction mix into a sample well of a DG8 Cartridge. Follow subsequent instructions as specified in the QX200 Droplet Generator Instruction Manual (10031907)
   - For the Automated Droplet Generator, follow instructions in the Automated Droplet Generator Instruction Manual (10043138)
   - For the QX ONE ddPCR System, load 20 µl of each reaction mix into a sample well of a GCR96 Cartridge. Follow subsequent instructions as specified in the QX ONE Droplet Digital PCR System and QX ONE Software User Guide (10000116512)

Thermal Cycling Conditions

Follow instructions for thermal cycling based on the droplet generator used:

- For the QX200 Droplet Generator, carefully transfer droplets into a clean 96-well plate. Seal the plate using the PX1 PCR Plate Sealer at 180°C for 5 sec. Proceed to thermal cycling (see Table 4)
- For the Automated Droplet Generator, remove the droplet plate containing ddPCR droplets from the Automated Droplet Generator. Seal the plate using the PX1 PCR Plate Sealer at 180°C for 5 sec. Proceed to thermal cycling (see Table 4)
- For the QX ONE ddPCR System, thermal cycling is integrated into and sequentially performed by the system itself. Hence, no additional equipment or sample handling is required for this step. Refer to the QX ONE Droplet Digital PCR System and QX ONE Software User Guide (10000116512) for plate setup instructions. Use appropriate thermal cycling conditions as specified in Table 4

Table 4. Thermal cycling conditions.*

Cycling Step		Temperature, °C	Time	Number of Cycles
Hold (QX ONE ddPCR System only)		25	3 min	1
Enzyme activation		95	10 min	1
Denaturation		94	30 sec	40
Annealing/extension		Optimum**	1 min***	40
Enzyme deactivation		98	10 min	1
Hold	QX200 or QX600 ddPCR System (optional)	4	Infinite	1
	QX ONE ddPCR System (required)	25	1 min	1

* For the C1000 Touch Thermal Cycler, use a heated lid set to 105°C and set the sample volume to 40 µl.
** Use optimal annealing temperature determined.
** Check/adjust ramp rate settings to ~2°C/sec

Data Acquisition and Analysis

Follow instructions for data acquisition and analysis based on the droplet reader in use:

- For the QX200 Droplet Reader, refer to the QX200 Droplet Reader and QX Manager Software Standard Edition User Guide (10000107223) or the QX200 Droplet Reader and QX Manager Software Regulatory Edition User Guide (10000107224)
- For the QX600 Droplet Reader, refer to the QX600 Droplet Reader and QX Manager Software Standard Edition User Guide (10000153877) or the QX600 Droplet Reader and QX Manager Software Premium Edition User Guide (10000153878)
- For the QX ONE ddPCR System, refer to the QX ONE Droplet Digital PCR System and QX ONE Software User Guide (10000116512) and the QX ONE Software Standard Edition or Regulatory Edition User Guide (10000116655 or 10000116656, respectively)

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