ddPCR™ Mutation Detection Assays

Catalog #	Description
10049047	**ddPCR™ Mutation Detection Assay (FAM+HEX)**, 200 x 20 µl reactions
10049048	**ddPCR™ Mutation Detection Assay (FAM+HEX)**, 1,000 x 20 µl reactions
10049049	**ddPCR™ Mutation Detection Assay (FAM+HEX)**, 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. Performance of these assays should be validated prior to use. Mutant target and wild-type assays are mixed together and provided in a single tube, with FAM targeting the mutant allele and HEX targeting the wild-type allele.

Ordering Information

The ddPCR assays can be ordered online at bio-rad.com/digital-assays

Storage and Stability

The ddPCR 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 optimized for use with ddPCR supermix for probes (no dUTP). Each kit comes with 200, 1,000, or 2,500 µl of the 20x assay mix (18 µM primers and 5 µM each probe), sufficient for 200, 1,000, or 2,500 x 20 µl reactions, respectively.

Required Reagents and Equipment

- ddPCR supermix for probes (no dUTP) (catalog #1863023, 1863024, 1863025)
- QX100™ or QX200™ Droplet Generator (catalog #1863002 or 1864002, respectively) or Automated Droplet Generator (catalog #1864101)
- QX100 or QX200 Droplet Reader (catalog #1863003 or 1864003, respectively)
- C1000 Touch™ Thermal Cycler with 96–Deep Well Reaction Module (catalog #1851197)
- PX1™ PCR Plate Sealer (catalog #1814000)

Please refer to the QX100 or QX200 Instruction Manuals (#10026321 and 10026322 or 10031906 and 10031907, respectively) or the Automated Droplet Generator Instruction Manual (#10043138) for ordering information on consumables such as oils, cartridges, gaskets, plates, and seals.

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 eight wells (one column) according to the guidelines in Table 2
- For optimal performance, follow recommendations below for restriction digestion and ddPCR reaction setup
- After droplet generation, proceed to thermal cycling on a C1000 Touch™ Thermal Cycler with 96–Deep Well Reaction Module (catalog #1861197). Use an annealing temperature gradient as described in Table 1
- Optimum annealing temperature range is determined based on the separation between four clusters (Figure 1)
- For more information, see Rare Mutation Detection Best Practices Guide (bulletin 6628)
- Run several no template control (NTC) wells

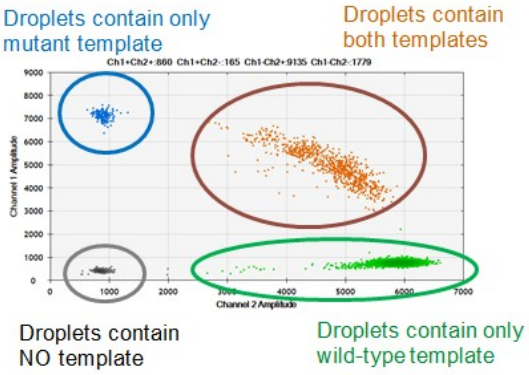

Fig. 1. Two-dimensional scatter plot demonstrating the four clusters obtained with a mutant and wild-type allele.

Table 1. Thermal gradient cycling conditions for determination of optimal annealing/extension temperature on Bio-Rad's C1000 Touch Thermal Cycler.*

Cycling Step	Temperature, °C	Time	Ramp Rate	Number of Cycles
Enzyme activation	95	10 min	2°C/sec (Note: Ramp rate option not available for gradient step)	1
Denaturation	94	30 sec		40
Annealing/extension	~50–60	1 min		
Enzyme deactivation	98	10 min		1
Hold (optional)	4	Infinite		1

* Use a heated lid set to 105°C and set the sample volume to 40 µl.

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 2
- 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 10-fold 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 the 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 2. 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 2. Preparation of the reaction mix.

Component	Volume per Reaction, µl	Final Concentration
2x ddPCR Supermix for Probes (No dUTP)	10	1x
20x target (FAM) and wild-type (HEX) primers/probe	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.
*** 900 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 the tube. Centrifuge briefly to ensure that all components are at the bottom of the reaction tube. Allow reaction tubes to equilibrate at room temperature for about 3 min.
6. Once the reaction mixtures are ready, load 20 µl of each reaction mix into a sample well of a DG8™ Cartridge (catalog #1864008) followed by 70 µl of Droplet Generation Oil for Probes (catalog #1863005) into the oil wells, according to the QX100 or QX200 Droplet Generator Instruction Manual (#10026322 or 10031907, respectively). For the Automated Droplet Generator, follow instructions in the Automated Droplet Generator Instruction Manual (#10043138).

Thermal Cycling Conditions

1. After droplet generation with the QX100 or QX200 Droplet Generator, carefully transfer droplets into a clean 96-well plate or remove the plate containing ddPCR droplets from the Automated Droplet Generator. Seal the plate with the PX1 PCR Plate Sealer.

2. Proceed to thermal cycling (see protocol in Table 3) and subsequent reading of droplets in the QX100 or QX200 Droplet Reader.

Table 3. Cycling conditions for Bio-Rad's C1000 Touch Thermal Cycler.*

Cycling Step	Temperature, °C	Time	Ramp Rate	Number of Cycles
Enzyme activation	95	10 min	2°C/sec	1
Denaturation	94	30 sec		40
Annealing/extension	Optimum**	1 min		
Enzyme deactivation	98	10 min		1
Hold (optional)	4	Infinite	1°C/sec	1

* Use a heated lid set to 105°C and set the sample volume to 40 µl.

** Use optimal annealing temperature determined.

Data Acquisition and Analysis

1. After thermal cycling, place the sealed 96-well plate in the QX100 or QX200 Droplet Reader.
2. Open QuantaSoft™ Software to set up a new plate layout according to the experimental design. Refer to the QX100 or QX200 Droplet Reader and QuantaSoft Software Instruction Manual (catalog #10026321 or 10031906, respectively).
3. Under Setup, double click on a well in the plate layout to open the Well Editor dialog box.
4. Designate the sample name, experiment type, **ddPCR Supermix for Probes (No dUTP)** as the supermix type, target name(s), target type(s), and reference: **Ch1** for FAM and **Ch2** for HEX.
5. Select **Apply** to load the wells and, when finished, select **OK**.
6. Once the plate layout is complete, select **Run** to begin the droplet reading process. Select the appropriate dye set and run options when prompted.
7. After data acquisition, select samples in the well selector under Analyze. Set appropriate threshold for the ddPCR Mutation Detection Assay. For more detailed information about setting thresholds, please refer to the Rare Mutation Detection Best Practices Guide (bulletin 6628).
8. The concentration reported is copies/µl of the final 1x ddPCR reaction.

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