

# 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
12005579	ddPCR Mutation Detection Assay (Cy5+Cy5.5), 200 x 20 µL reactions
12005580	ddPCR Mutation Detection Assay (Cy5+Cy5.5), 1,000 x 20 µL reactions
12005581	ddPCR Mutation Detection Assay (Cy5+Cy5.5), 2,500 x 20 µL reactions
12016606	ddPCR Mutation Detection Assay (ROX+ATTO 590), 200 x 20 µL reactions
12016633	ddPCR Mutation Detection Assay (ROX+ATTO 590), 1,000 x 20 µL reactions
12016616	ddPCR Mutation Detection Assay (ROX+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. Mutant target and wild-type assays are mixed together and provided in a single tube, with the combination of either FAM and HEX, Cy5 and Cy5.5, or ROX and ATTO 590 targeting the mutant and wild-type alleles, respectively.

## Ordering Information

Visit [bio-rad.com/digital-assays](http://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 (18 µM primers and 5 µM each probe), sufficient for 200, 1,000, or 2,500 x 20 µL reactions, respectively. For assays performed on QX700™ ddPCR Systems, this would correspond to four times the number of reactions listed above, as based on a final sample input volume of 5 µL in RDG16 Cartridges.

## 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)

- C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module (1851197) or PTC Tempo Deepwell Thermal Cycler (12015392)

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

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

For assays using QX700 Droplet Digital PCR Systems (#17011036, #17010638, #17010628):

- naica™ 5X Multiplex ddPCR Mix (#12025253, #12025254)
- naica 10X Multiplex ddPCR Mix (#12025255, #12025256, #12025258)
- PX1 PCR Plate Sealer (#1814000)

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

**Table 1. Mutation detection assay fluorophore compatibility.**

QX200 Droplet Reader	QX ONE ddPCR System	QX600 Droplet Reader	QX700 ddPCR Systems
FAM	FAM	FAM	FAM
HEX	HEX	HEX	HEX
	Cy5	Cy5	Cy5
	Cy5.5	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.



## QX200 and QX600 Workflows

- Using a sample containing both mutant and wild-type alleles as template, prepare reaction mix for at least 8 wells (1 column) plus no template control (NTC) according to the guidelines in the Reaction Setup section
- For optimal performance, follow the recommendations in the Restriction Enzyme Digestion of Sample DNA and Reaction Setup sections
- For QX200 and QX600 System workflows, after droplet generation, proceed to thermal cycling using a C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module or PTC Tempo Deepwell Thermal Cycler. Use an annealing temperature gradient as described in Table 2

## QX700 Workflows

- Prepare a reaction mix for multiple replicates of a positive sample containing both mutant and wild-type alleles and the NTC following the recommendations in the Restriction Enzyme Digestion and Reaction Setup sections
- Define QX700 protocols with different thermal cycling programs, each specifying a unique annealing temperature from the range provided in Table 2 (refer to QX700 ddPCR System Instrument Guide, [10000171493](#))
- Test the NTC and sample replicates across multiple RDG16 Cartridges, with each cartridge assigned to a different protocol

## Additional Reference (QX200, QX600, QX600, QX ONE Systems)

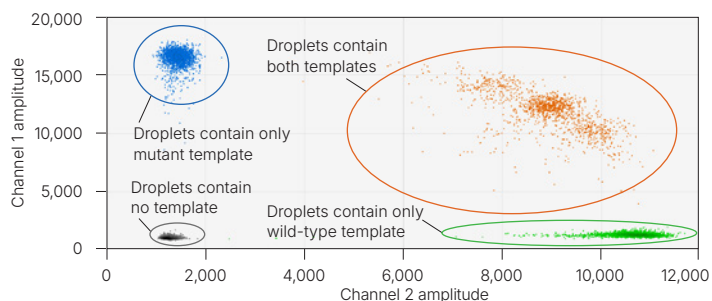
- Optimum annealing temperature range is determined based on the separation between 4 clusters (Figure 1)
- For more information, see Rare Mutation Detection Best Practices Guidelines ([bulletin 6628](#))

**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 <sup>†</sup>	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.

<sup>†</sup> Check/adjust ramp rate settings to ~2°C/sec.



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

## 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 during setup (recommended) or conventional digestion prior to Droplet Digital PCR.

### Digestion Directly in Droplet Digital PCR

- Efficient digestion of sample DNA can be achieved by direct addition of restriction enzyme to the reaction
- 2–5 units of restriction enzyme per 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 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 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

## Protocol: QX200, QX600, and QX ONE ddPCR Systems

### 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  $-20^{\circ}\text{C}$  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 unknown 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, $\mu\text{L}$	Final concentration
2x ddPCR Supermix for Probes (No dUTP)	11	1x
20x target (FAM, Cy5, or ROX) and wild-type (HEX, Cy5.5, or ATTO 590) primers/probe	1.1	1x <sup>†</sup>
Restriction enzyme, diluted*	1.1	2–5 U/reaction
DNA sample or water	Variable	50 fg–100 ng <sup>‡</sup>
Total volume	22	–

\* This component should be replaced by water if digestion is performed prior to Droplet Digital PCR.

<sup>†</sup> 900 nM primers/250 nM each probe.

<sup>‡</sup> 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 min.
6. Transfer the reaction mix from the reaction tubes to the appropriate ddPCR Cartridge as follows.
  - For the QX200 Droplet Generator, load 20  $\mu\text{L}$  of each reaction mix into a sample well of DG8 Cartridges. 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  $\mu\text{L}$  of each reaction mix into a sample well of GCR96 Cartridges. Follow subsequent instructions as specified in the QX ONE Droplet Digital PCR System and QX ONE Software Instrument 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  $180^{\circ}\text{C}$  for 5 sec. Proceed to thermal cycling (see Table 4)
- For the Automated Droplet Generator, remove the plate containing ddPCR droplets from the Automated Droplet Generator. Seal the plate using the PX1 PCR Plate Sealer at  $180^{\circ}\text{C}$  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 Instrument Guide (10000116512) for plate setup instructions. Use appropriate thermal cycling conditions as specified in Table 4

**Table 4. Thermal cycling conditions.\***

Cycling step	Temperature, $^{\circ}\text{C}$	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 <sup>†</sup>	1 min <sup>†</sup>	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  $105^{\circ}\text{C}$  and set the sample volume to 40  $\mu\text{L}$ .

<sup>†</sup> Use optimal annealing temperature determined.

<sup>‡</sup> Check/adjust ramp rate settings to  $\sim 2^{\circ}\text{C}/\text{sec}$ .

## Protocol: QX700 ddPCR Systems

### Reaction Setup

1. Thaw Buffers A and B completely before each use. Vortex thoroughly (suggested three times for 5–10 sec each at maximum speed) and briefly centrifuge to collect the liquid at the bottom of the tube. For Buffer B, it is recommended to start with a final concentration of 4% and not to exceed 5% during assay optimization. Typical final concentrations range from 2 to 5%. Vortex the primers and probes thoroughly before use.

- Prepare samples at the desired concentration before setting up the reaction mix according to the guidelines in Table 5. 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.
- Prepare a negative control: at least one well containing only wild-type template at a concentration similar to the concentration of unknown samples.
- 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.
- After combining all reagents, vortex thoroughly (suggested 5–10 sec at maximum speed) to mix the contents. Centrifuge briefly to collect the liquid at the bottom of the tube before loading the reaction mix onto consumable chips; immediately load the reaction on the respective chip. It is not recommended to freeze the combined reagent solution. For RDG16 Cartridges, the final well reaction volume is 5  $\mu$ L.

**Table 5. Reaction assembly for QX700 Droplet Digital PCR Systems.**

Component	Final concentration	Volume per reaction, $\mu$ L	
		5X Buffer A	10X Buffer A
naica Multiplex ddPCR Mix Buffer A	1x	1.4	0.7
naica Multiplex ddPCR Mix Buffer B	4%*	0.28	0.28
20x target (FAM) and wild-type (HEX) primers/probe	1x†	0.35	0.35
Restriction enzyme†	2–5 U/reaction	Variable	Variable
DNA sample	50 fg–100 ng/reaction§	Variable	Variable
RNase-/DNase-free water	—	Variable	Variable
Total volume		7	7

\* Suggested final concentration not to exceed 5%. Buffer B is provided at an initial concentration of 100%.

† 900 nM primers/250 nM each probe.

‡ This component should be replaced by water if digestion is performed prior to Droplet Digital PCR.

§ 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.

## Thermal Cycling

See Table 6 for thermal cycling conditions using QX700 Droplet Digital PCR Systems.

**Table 6. Thermal cycling conditions for QX700 Droplet Digital PCR Systems.**

Steps*	Temperature, °C	Time
Step 1	95	180 sec
Step 2	Begin loop for 45 iterations	
Step 3	95	10 sec
Step 4	50–60†	15 sec

\* Use a ramp of 1°C/sec for each step.

† The optimal annealing temperature should be determined following the guidelines in this insert.

Use QX700 ddPCR System Analysis Software to edit/modify the thermal cycling conditions.

## Data Acquisition and Analysis (QX200, QX600, QX700, QX ONE Systems)

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 Instrument Guide for Standard Edition ([10000116655](#)) or Regulatory Edition ([10000116656](#)).
- For the QX700 ddPCR System, refer to the QX700 System Analysis Software User Guide ([10000171494](#)).



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