ddPCR Cell and Gene Therapy Assays

Catalog #	Description
12014589	**ddPCR CGT Assay (FAM)**, 200 x 20 µl reactions
12014590	**ddPCR CGT Assay (HEX)**, 200 x 20 µl reactions
12014641	**ddPCR CGT Assay (FAM + HEX)**, 200 x 20 µl reactions
12014642	**ddPCR CGT Assay (Cy5)**, 200 x 20 µl reactions
12014643	**ddPCR CGT Assay (Cy5.5)**, 200 x 20 µl reactions
12014644	**ddPCR CGT Assay (Cy5 + Cy5.5)**, 200 x 20 µl reactions
12014645	**ddPCR CGT Assay (FAM)**, 1,000 x 20 µl reactions
12014646	**ddPCR CGT Assay (HEX)**, 1,000 x 20 µl reactions
12014647	**ddPCR CGT Assay (FAM + HEX)**, 1,000 x 20 µl reactions
12014648	**ddPCR CGT Assay (Cy5)**, 1,000 x 20 µl reactions
12014649	**ddPCR CGT Assay (Cy5.5)**, 1,000 x 20 µl reactions
12014650	**ddPCR CGT Assay (Cy5 + Cy5.5)**, 1,000 x 20 µl reactions
12014651	**ddPCR CGT Assay (FAM)**, 1,000 x 20 µl reactions*
12014652	**ddPCR CGT Assay (HEX)**, 1,000 x 20 µl reactions*
12014653	**ddPCR CGT Assay (FAM + HEX)**, 1,000 x 20 µl reactions*
12014654	**ddPCR CGT Assay (Cy5)**, 1,000 x 20 µl reactions*
12014655	**ddPCR CGT Assay (Cy5.5)**, 1,000 x 20 µl reactions*
12014656	**ddPCR CGT Assay (Cy5 + Cy5.5)**, 1,000 x 20 µl reactions*

* Primer and probe sequences provided.

For research purposes only.

Description

ddPCR Cell and Gene Therapy (CGT) 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. Positive and negative controls should be run along with samples when using these assays. ddPCR CGT Assays can be ordered with FAM, HEX, Cy5, and Cy5.5 fluorophores or FAM + HEX and Cy5 + Cy5.5 fluorophore blends.

ddPCR Expert Design Assays for common viral vector backbone or plasmid elements (**Vector Backbone Assays**) are available for multiplexing. Please refer to bulletin 7282 for a full list of available ddPCR Expert Design Assays.

Note: Cy5, Cy5.5, and Cy5 + Cy5.5 Assays are for use with the QX ONE Droplet Digital PCR System only. The FAM + HEX Assay and Cy5 + Cy5.5 Assay are recommended to be used when multiplexing (3-plex) with FAM and HEX Assays and Cy5 and Cy5.5 Assays, respectively.

Ordering Information

Go to **bio-rad.com/digital-assays** to order ddPCR Assays.

Storage and Stability

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

Kit Contents

The ddPCR CGT Assay is a 20x concentrated, ready-to-use primer-probe mix optimized for use with ddPCR Supermix for Probes (No dUTP) when using FAM and/or HEX Assays, or ddPCR Multiplex Supermix when using Cy5 and/or Cy5.5 Assays on the QX ONE ddPCR System or when running three or more assays on any system. Each kit comes with 200 or 1,000 µl of the 20x assay mix (18 µM primers and 5 µM probe), sufficient for 200 or 1,000 µl x 20–22 µl reactions, respectively.

Reagents and Equipment

- QX200 Droplet Digital PCR System (catalog #1864001), QX200 AutoDG Droplet Digital PCR System (#1864100), or QX ONE ddPCR System (#12006536)
- C1000 Touch Thermal Cycler with 96–Deep Well Reaction Module (#1851197) (for use with the QX200 or QX200 AutoDG ddPCR System only)
- PX1 PCR Plate Sealer (#1814000)
- ddPCR Supermix for Probes (No dUTP) (#1863023, 1863024, or 1863025) or ddPCR Multiplex Supermix (#12005909, 12005910, or 12005911)
- **Optional:** Vector Backbone Assays for multiplexed viral titer or vector copy number measurement

Note: For optimal adeno-associated virus (AAV) vector genome titer measurements, use the recommended protocol detailed in bulletin 7407.

Please refer to the QX200 Droplet Generator or Automated Droplet Generator Instruction Manual (10031907 or 10043138, respectively), or the QX ONE Droplet Digital PCR System and QX ONE Software User Guide (10000116512) for ordering information about consumables, such as oils, cartridges, gaskets, plates, and seals.

Determination of Optimal Annealing Temperature

Newly designed ddPCR CGT 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 the performance of all the assays being multiplexed should be selected.

- Using a test sample 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 the 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. Use an annealing temperature gradient as described in Table 1
- Optimum annealing temperature range is determined based on the separation between 4 clusters
  - See Figure 1 for the QX200 ddPCR System
  - See Figure 2 for the QX ONE ddPCR System

Table 1. Thermal gradient cycling conditions for determination of optimal annealing/extension temperature.*

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		40
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.
** QX ONE thermal cycling protocols do not require a hold step and the ramp rate is preset.

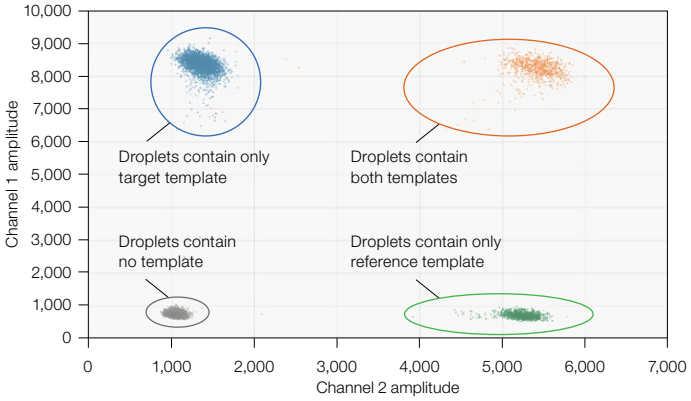

Fig. 1. Two-dimensional scatter plot from a QX200 ddPCR System. The scatter plot shows the four clusters obtained with a target (FAM) and reference assay (HEX).

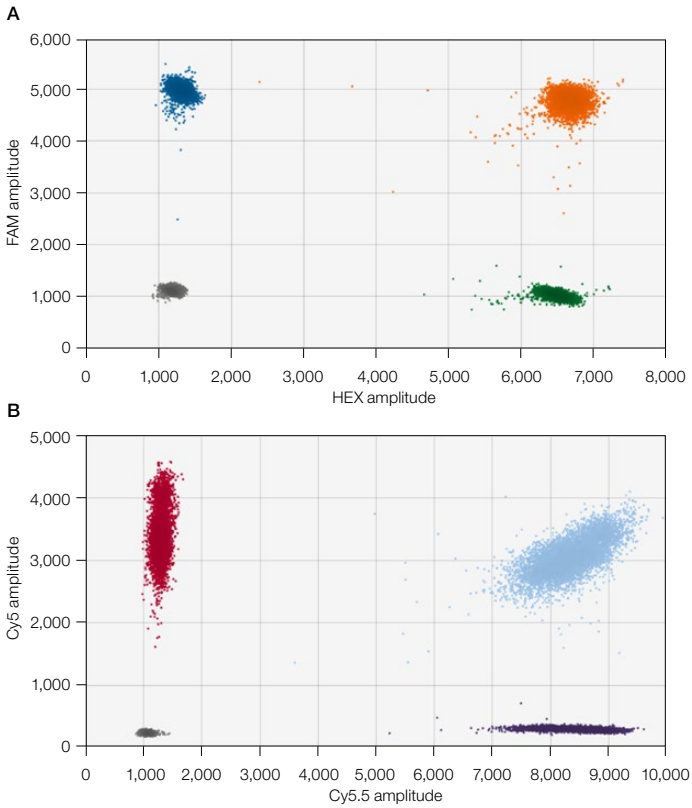

Fig. 2. Two-dimensional scatter plots from a QX ONE ddPCR System. The scatter plots show eight clusters obtained with a target and a reference assay in the same well. **A**, target (FAM) and reference (HEX) assays; **B**, target (Cy5) and reference (Cy5.5) assays.

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 Tables 2 and 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 1–20 enzyme units per microgram DNA according to the enzyme manufacturer's instructions
- 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 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 the viral vector sample at the desired concentration before setting up the reaction mix. Plasmids or viruses encapsulating a genome containing the DNA of interest should be diluted into the ddPCR detection range (less than 100,000 copies in a 20 µl ddPCR reaction). AAV vector samples should be diluted before heat denaturation of the capsid (10 min at 95°C, then cool to 4°C). Serial dilutions should be done using a DNA or RNA carrier buffer solution, such as 10 mM Tris-HCl, pH 8, 0.1 mM EDTA, 100 ng/µl polyA. Refer to bulletin 7407 for a detailed protocol to measure AAV vector genome titer.
3. Set up the reaction mix according to the guidelines in Table 2 or 3. If multiple samples are to be assayed using the same assay(s), 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.

Table 2. Preparation of the reaction mix for FAM and/or HEX Assays.

Component	Volume per Reaction, µl	Final Concentration
2x ddPCR Supermix for Probes (No dUTP)	10	1x
20x FAM Assay (e.g., ddPCR CGT Assay)	1	1x*
20x HEX Assay (e.g., Vector Backbone Assay)	1	1x*
Restriction enzyme, diluted**	1	2–5 U/reaction
DNA sample or water	Variable	Variable
Total volume	**20**	—

Table 3. Preparation of the reaction mix for Cy5 and/or Cy5.5 Assays.

Component	Volume per Reaction, µl	Final Concentration
4x ddPCR Multiplex Supermix	5	1x
20x FAM Assay (e.g., ddPCR CGT Assay 1)	1	1x*
20x HEX Assay (e.g., Vector Backbone Assay 1)	1	1x*
20x Cy5 Assay (e.g., ddPCR CGT Assay 2)	1	1x*
20x Cy 5.5 Assay (e.g., Vector Backbone Assay 2)	1	1x*
Restriction enzyme, diluted**	1	2–5 U/reaction
DNA sample or water	Variable	Variable
Total volume	**20**	—

* 900 nM primers/250 nM probe.

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

4. Mix thoroughly by vortexing each tube. Centrifuge briefly to ensure that all components are at the bottom of the reaction tubes. Allow reaction tubes to equilibrate at room temperature for about 3 min.
5. Transfer the reaction mix from the reaction tubes to the appropriate cartridge as follows:
   - For the QX200 ddPCR System, 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 QX200 AutoDG ddPCR System, 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 based on the system in use:

- For the QX200 ddPCR System, carefully transfer droplets into a clean 96-well plate. Seal the plate at 180°C for 5 sec using the PX1 PCR Plate Sealer. Proceed to thermal cycling (see Table 4)
- For the QX200 AutoDG ddPCR System, seal the 96-well plate at 180°C for 5 sec using the PX1 PCR Plate Sealer. Proceed to thermal cycling (see Table 4)
- For the QX ONE ddPCR System, use appropriate thermal cycling conditions as specified in Table 4 during plate setup. Refer to the QX ONE ddPCR System and QX ONE Software User Guide (10000116512) for plate setup instructions

Table 4. Thermal cycling conditions.*

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

Note: For data acquisition and analysis using the QX ONE ddPCR System, refer to the QX ONE Software Standard Edition User Guide (10000116655) or the QX ONE Software Regulatory Edition User Guide (10000116656).

For data acquisition and analysis using the QX200 or QX200 AutoDG ddPCR System, perform the following steps.

1. After thermal cycling, place the sealed 96-well plate in the QX200 Droplet Reader.
2. Launch the appropriate software to set up a new plate layout according to the experimental design.
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 as Copy Number Variation (CNV), supermix type, target name(s), target type(s), and reference.
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 the appropriate threshold based on your experimental design. For more detailed information about setting thresholds, please refer to the Droplet Digital PCR Applications Guide (bulletin 6407).
8. The concentration reported is copies/µl of the final 1x ddPCR reaction.

Visit **bio-rad.com/DropletDigitalPCRAssays** for more information.

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