# ddPCR™ HDR Genome Edit Detection Assays

| Catalog # | Description |
|---|---|
| 12002312 | **ddPCR™ HDR Genome Edit Detection Assay (FAM)**, 100 x 20 µl reactions |
| 12002313 | **ddPCR™ HDR Genome Edit Detection Assay (FAM)**, 500 x 20 µl reactions |
| 12003796 | **ddPCR™ HDR Genome Edit Detection Package (FAM)**, 1,000 x 20 µl reactions |

For research purposes only.

## Description

ddPCR Homology Directed Repair Genome Edit Detection (HDR GED) Assays are expertly designed specifically for Droplet Digital™ PCR (ddPCR) using proprietary computational algorithms. ddPCR HDR GED Assays contain a FAM probe and must be run duplexed with an amplicon length-matched ddPCR HDR Genome Edit Detection Reference (HDR REF) Assay that contains a HEX probe. ddPCR HDR REF, Predesigned, Genome Edit Detection Assays are available for human, mouse, and rat. These assays have not been wet-lab validated by Bio-Rad. Performance of these assays should be validated prior to use.

## 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 HDR GED 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 100, 500, or 1,000 µl of the 20x assay mix (18 µM primers, 5 µM FAM probe, and 15 µM non-fluorescent probe), sufficient for 100, 500, or 1,000 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.

## Initial Evaluation of ddPCR HDR GED and ddPCR HDR REF Assays

Newly designed ddPCR HDR GED Assays should be run using different assay/template combinations, as shown in Table 1, and the thermal cycling protocol described in Table 2. These experiments are necessary to determine the extension time that optimizes separation between positive and negative droplets while minimizing rain (droplets that fall between the major positive and negative populations). Each assay/template combination should be run in a minimum of two wells. If possible, an extension time that optimizes performance of both the HDR GED Assay and the HDR REF Assay should be selected. If optimal conditions for running the duplexed assays cannot be found, consider using a different reference assay or running assays separately as singleplexes.

**Table 1. Recommended Assay, DNA Template combinations for evaluation of ddPCR HDR GED and ddPCR HDR REF assays**

| Assay | DNA Template | Desired Phenotype | Optimization, If Necessary |
|---|---|---|---|
| HDR GED Only | Wild-type + edited | Single FAM+ droplet cluster separated from FAM- droplet cluster | If clusters are not well separated, increase extension time |
| HDR GED + HDR REF | Wild-type + edited | FAM-HEX-, FAM+, HEX+ and FAM+HEX+ droplets separated as 4 distinct clusters | If the double positive (FAM+HEX+) cluster is missing or merged with HEX+ cluster, try a different or longer HDR REF assay, or run the HDR GED and HDR REF assays as singleplexes, in separate wells |
| HDR GED Only | Wild-type | Single cluster of FAM-HEX- droplets when threshold is set above any low-amplitude FAM+ cluster | High amplitude FAM+ droplets indicate false positives and cross-reactivity of FAM probe with WT DNA. Compare with NTC wells to confirm |
| HDR GED + HDR REF | Wild-type | Two clusters - FAM-HEX+ droplets and FAM-HEX- droplets | High amplitude FAM+ or FAM+HEX+ droplets indicate false positives, and cross-reactivity of FAM probe with WT DNA. Compare with NTC wells to confirm |
| HDR GED Only | No template control (NTC) | Single cluster of FAM-HEX- droplets | Positive droplets indicate contamination. Clean lab space and repeat with fresh reagents |
| HDR GED + HDR REF | No template control (NTC)) | Single cluster of FAM-HEX- droplets | Positive droplets indicate contamination. Clean lab space and repeat with fresh reagents |

**Table 2. Thermal cycling conditions for determination of optimal extension time 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 | 1 |
| Denaturation | 94 | 30 sec | | 40 |
| Annealing/extension | 55 | 1–6** 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.
** Set up initial experiment with 3 min annealing/extension time. If desired results are not achieved, try annealing/extension time of 6 min. If desired results are achieved, run experiments decreasing annealing/extension time in 1 min increments to pick shortest annealing/extension time that yields desired results.

- Prepare reaction mix for several wells according to the guidelines in Table 3 to avoid pipetting small volumes of reagents
- 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. Use thermal cycling conditions as described in Table 2
- Optimum extension time is determined based on separation between four clusters (Figure 1)
- For more information, see Droplet Digital PCR Applications Guide (bulletin 6407)
- Run several NTC wells with each experiment

## 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 a restriction enzyme buffer 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 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 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, with 100-500 copies/µl of edited DNA and 1,000–2,000 copies/µl 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 HDR GED Assay | 1 | 1x* |
| 20x HDR REF Assay | 1 | 1x* |
| Restriction enzyme, diluted** | 1 | 2–5 U/reaction |
| DNA sample or water | Variable | Up to 130ng*** |
| **Total Volume** | **20**§ | **–** |

* 900 nM primers/250 nM each probe.
**This component should be replaced by water if digestion is performed prior to Droplet Digital PCR.
***Input may be lowered if cluster separation is not adequate.
§ For the Automated Droplet Generator, prepare 22 µl per well.

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. For the Automated Droplet Generator, follow instructions in the Automated Droplet Generator Instruction Manual.

## 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 4) and subsequent reading of droplets in the QX100 or QX200 Droplet Reader.

**Table 4. 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 | 55 | Optimum** | | |
| 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 extension time determined per Table 2.

### Data Acquisition and Analysis

1. Data acquisition must be performed using QuantaSoft Software. Data analysis may be performed using QuantaSoft or QuantaSoft Analysis Pro Software.
2. After thermal cycling, place the sealed 96-well plate in the QX100 or QX200 Droplet Reader.
3. Set up a new plate layout according to the experimental design using QuantaSoft Software. Refer to the QX100 or QX200 Droplet Reader and QuantaSoft Software Instruction Manual (bulletin 6827).
4. Under Setup, double click on a well in the plate layout to open the Well Editor dialog box.
5. Designate sample names.
6. To determine ratio of edited/reference genes designate experiment type as **ABS** in QuantaSoft Software, or **Direct Quantification** in QuantaSoft Analysis Pro Software.
7. To determine whether a clonal population is homozygous or heterozygous, designate the experiment type as **CNV2** in QuantaSoft Software, or as **CNV, and Ref Copies: 2** in QuantaSoft Analysis Pro Software.
8. Designate the supermix type as **ddPCR Supermix for Probes (No dUTP)**. Designate target name(s), target type(s), and reference as **Ch1** for FAM and **Ch2** for HEX.
9. Select **Apply** to load the wells and, when finished, select **OK**.
10. Once the plate layout is complete, select **Run** to begin the droplet reading process. Select the appropriate dye set and run options when prompted.
11. After data acquisition, select samples in the well selector under Analyze.
12. Set appropriate threshold for the ddPCR HDR GED Assay. For more detailed information about setting thresholds, please refer to the Droplet Digital PCR Applications Guide.
13. If ABS or Direct Quantification was used for experiment type, the ratio provided is the number of edited copies/total number of copies of the reference genome. Percentage of edited cells is obtained by multiplying ratio by 100.
14. If CNV or CNV2 was selected as experiment type, a copy number of 2 indicates that the clones are homozygous, a copy number of 1 indicates that the clones are heterozygous.
15. The concentration reported is copies/µl of the final 1x ddPCR reaction.

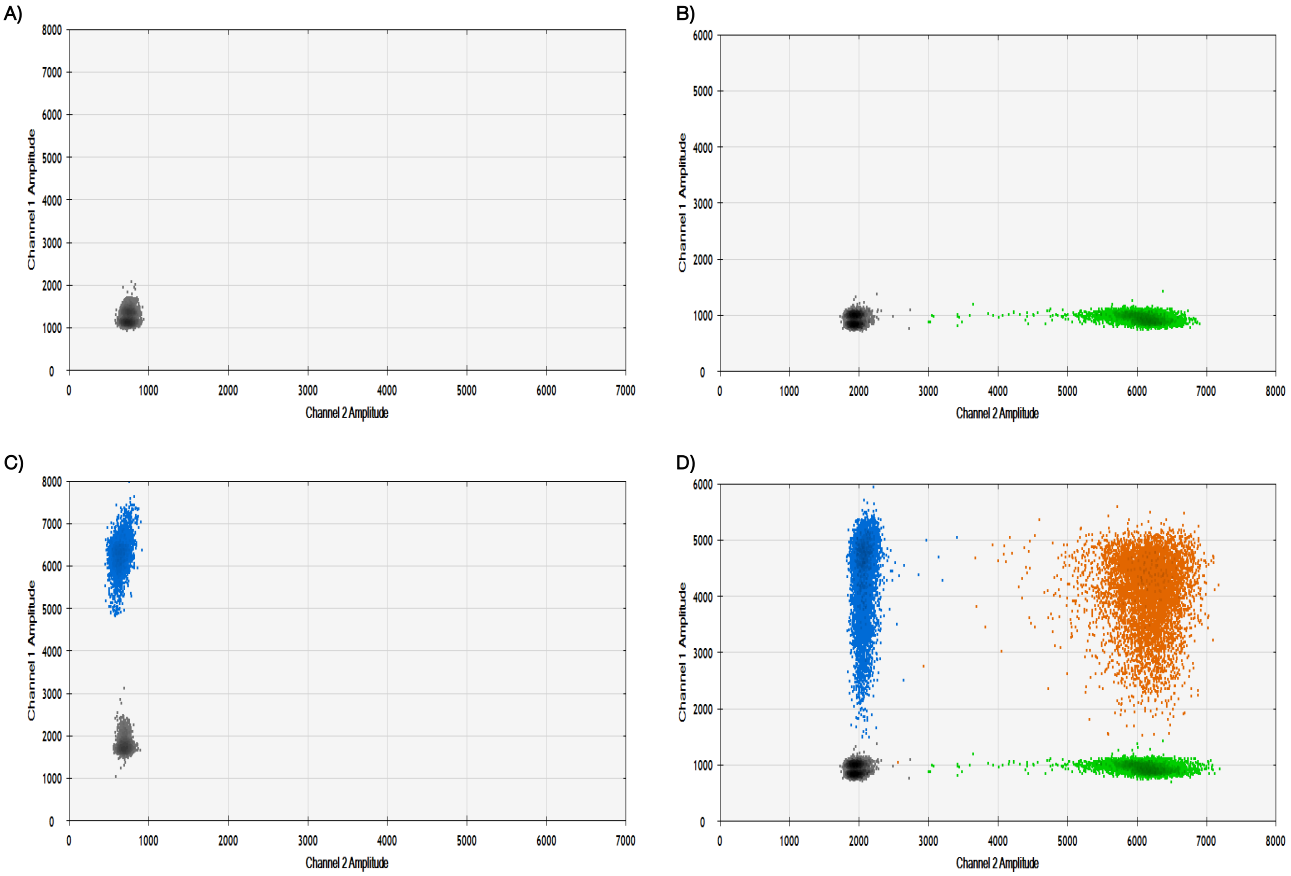


**Fig. 1. 2-D droplet fluorescence intensity plots. A**, WT-only DNA with HDR GED Assay only; **B,** WT-only DNA with HDR GED + HDR REF Assay; **C**, HDR mutation positive control (WT + 20% gblocks gene fragment containing a single base pair substitution) with HDR GED Assay only; **D**, HDR mutation positive control (WT + 20% gblocks gene fragment containing a single base pair substitution) with HDR GED + HDR REF Assay.

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