ddPCR™ NHEJ Genome Edit Detection Assays

Catalog #	Description
12002314	**ddPCR™ NHEJ Genome Edit Detection Assay (FAM+HEX)**, 100 x 20 µl reactions
12002315	**ddPCR™ NHEJ Genome Edit Detection Assay (FAM+HEX)**, 500 x 20 µl reactions
12003794	**ddPCR™ NHEJ Genome Edit Detection Package (FAM+HEX)**, 1,000 x 20 µl reactions

For research purposes only.

Description

ddPCR Non-Homologous End Joining Genome Edit Detection (NHEJ GED) Assays are expertly designed specifically for Droplet Digital™ PCR (ddPCR) using proprietary computational algorithms. ddPCR NHEJ GED Assays contain a pair of primers, a FAM probe, and a HEX probe. The FAM probe binds distant from the site of potential NHEJ, and is designed to count all copies of the amplicon. A HEX-labeled drop-off probe is designed to bind WT DNA and targets the predicted cut site. The HEX probe will not bind, or will "drop off," when the allele has an insertion or deletion at this site. It is the loss of this HEX signal (while maintaining FAM signal) that indicates presence of an NHEJ allele. An automated analysis mode for NHEJ is available in QuantaSoft™ Analysis Pro Software. 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 5 µM HEX 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 NHEJ GED Assays

Newly designed ddPCR NHEJ 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 and template combination should be run in a minimum of two wells. The desired phenotype of an NHEJ assay is shown in Figure1.

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

Assay	DNA Template	Desired Phenotype of 2-D Fluorescence Intensity Plots	Optimization, If Necessary
NHEJ GED	Wild-type + edited	Three clusters – FAM+, FAM+HEX+, and FAM-HEX-	If three clusters are not visible or not well separated, increase extension time
NHEJ GED	Wild-type	Two clusters – FAM+HEX+ and FAM-HEX-	High amplitude FAM+ droplets indicate false positives and cross-reactivity of FAM probe with WT DNA. Compare with NTC wells to confirm
NHEJ GED	NTC (No template control)	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 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 1000-2000 copies/ul 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 NHEJ GED 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.

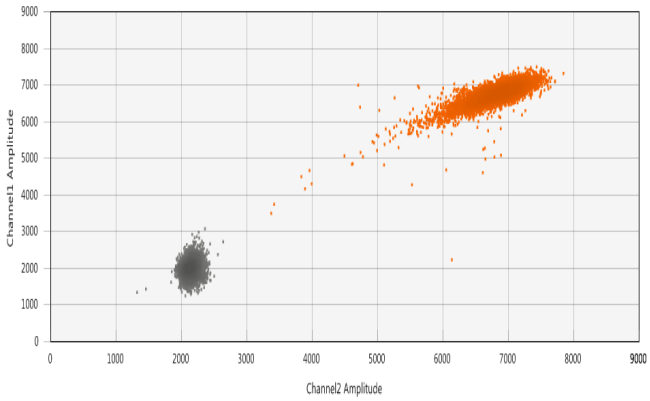
Data Acquisition

1. Data acquisition must be performed using QuantaSoft Software. Data analysis must be performed using 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 the sample name, **ABS** as experiment type, **ddPCR Supermix for Probes (No dUTP)** as the supermix type, target name(s), and target type(s): **Ch1** for FAM and **Ch2** for HEX.
6. Select **Apply** to load the wells and, when finished, select **OK**. Once the plate layout is complete, select **Run** to begin the droplet reading process. Select the appropriate dye set and run options when prompted.

Data Analysis

1. Open the qlp file in QuantaSoft Analysis Pro Software as described in the instruction manual (bulletin 6827).
2. Designate the experiment type as **Drop Off (DOF)**. For target name associated with wild type set target type as **REF**. For target name associated with the NHEJ Edit set target type as **Unkn**. Apply changes.
3. In the 2D Amplitude tab annotate the FAM+ only cluster as unknown (blue), the FAM+HEX+ cluster as reference (orange) and the FAM-HEX- cluster gray.
4. Select the Ratio tab. Ratio of NHEJ edit to wild type is displayed.
5. To view fractional abundance, select the chart options symbol on the top right corner of the plot window and check the Fractional Abundance box. Fractional abundance (%) is calculated as NHEJ edited alleles/(wild-type + NHEJ edited alleles), in other words, edited alleles/total alleles (edited + unedited).
6. For a clonal population that is heterozygous fractional abundance is 50%.
7. When attempting to quantify NHEJ edits in a sample that contains WT, HDR edited DNA, and NHEJ edited DNA, the HDR edited DNA may appear in any of three clusters (FAM+HEX-, FAM+HEX+, FAM-HEX-). This affects interpretation of concentration measurements.
   - If the HDR edit does not affect binding of either the FAM probe or the HEX probe, the HDR edited alleles appear in the FAM+HEX+ cluster. The FAM+HEX+ cluster quantifies both WT and HDR edited alleles.
   - If the HDR edit causes the HEX probe to drop off, the HDR-edited DNA will be in the FAM+HEX- cluster. The FAM+HEX- cluster quantifies NHEJ and HDR edited alleles.
   - In rare cases, the HDR edit prevents binding of the FAM reference probe and the HDR edited DNA appears in the FAM-HEX+ cluster. This cluster maybe grouped with the double negative cluster for quantification. If the HDR edit also prevents binding of the HEX drop off probe, the HDR edited DNA appears in the FAM-HEX- cluster.
8. To assess which cluster contains the HDR-edited DNA, run a 100% HDR positive control template with the NHEJ assay.
   - A FAM+HEX+ cluster indicates that the FAM reference probe and the HEX drop-off probe both bind to HDR edited DNA.
   - A FAM+HEX- cluster indicates that only the FAM reference probe binds to HDR edited DNA. In other words, the HDR edit has caused the HEX probe to drop off.
   - The absence of both a FAM+HEX- and a FAM+HEX+ cluster indicates that the NHEJ assay does not detect HDR edits.
   - In rare cases, only a FAM-HEX+ cluster may be observed, indicating that the FAM reference probe does not bind to HDR edited alleles.
9. The concentration reported is copies/µl of the final 1x ddPCR reaction.

A)



B)

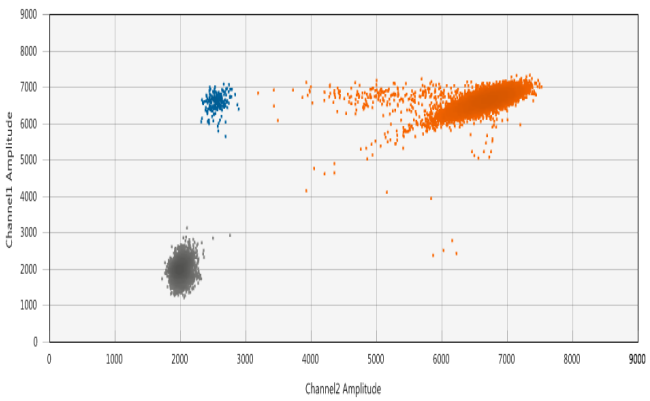


Fig. 1. 2-D droplet fluorescence intensity plots of an NHEJ drop-off assay A, WT-only DNA; **B)** NHEJ mutation-positive control (WT + 1% gblocks gene fragment containing a one base pair deletion at the predicted cut site). The WT cluster is positive for both FAM and HEX, while the NHEJ mutant single-positive droplets are positive for FAM only.

1077026

Bio-Rad Laboratories, Inc.
2000 Alfred Nobel Drive, Hercules, CA 94547
1-800-424-6723