

# Droplet Digital PCR (ddPCR) Copy Number Variation Assay for GFP-Edited Cells Using Manual Droplet Generation

## Required Reagent List:

- DG8 Cartridges (catalog # 1864108, Bio-Rad)
- PureLink® Pro 96 Genomic DNA Purification Kit (catalog # K182104A, Invitrogen) Primers mEGFP-(FWD-GCCGACAAGCAGAAGAACG; REV-GGGTGTCTGCTGGTAGTGG)
- Probe mEGFP-(/56-FAM/AGATCCGCC/ZEN/ACAACATCGAGG/3IABkFQ/)
- Primers AmpR-(FWD-TTTCCTGTGCGCCCTTATTCC; REV-ATGTAACCCACTCGTGCACCC)
- Probe AmpR -(/5HEX/TGGGTGAGC/ZEN/AAAAACAGGAAGGC/3IABkFQ/)
- ddPCR 2X Master Mix for Probes, no dUTP (catalog # 1863024, Bio-Rad)
- HindIII-HF (20,000 U/mL) (catalog # R3104S- NEB )
- 20X PrimePCR RPP30 Reference Assay (catalog # 1863024, Bio-Rad)
- Gaskets (catalog # 186-3009, Bio-Rad)
- 96 well Plates (catalog #12001925, Bio-Rad)
- Pierceable Foil Heat Seals (catalog# 1814040, Bio-Rad)
- Droplet Generator Oil for Probes (catalog 1863005, Bio-Rad)
- Droplet Reader Oil (catalog 186-3004, Bio-Rad)
- DG8™ Cartridge Holder (catalog 186-3051, Bio-Rad)
- Molecular Grade Water
- RNase Away (catalog, #10328011, Fisher)

## Required Instruments or Equipment:

- Thermal Cycler with Deep Well Block for 96-well plates
- 96 Well Cold Block
- X200 Droplet Generator
- X200 Droplet Reader, with Plate Holder
- Thermal Plate Sealer

## Steps Before Starting

1. All Reactions should be set up in a dedicated, decontaminated PCR hood to avoid contamination of reaction with aerosolized plasmid and/or gDNA.
  - a. Decontaminate PCR hood by applying RNase Away as per recommended instructions to all surfaces and pipettes.
  - b. Following RNase Away treatment clean all surfaces and pipettes with 70% EtOH and allow to dry.
  - c. Finally, treat hood with UV light for 15 min.
2. In cleaned PCR hood prepare 20X Primer Probe Assay Master Mix for the GFP and AmpR assays. 20X Primer Probe Assay Master Mix can be premade up to six months prior to experiment and stored at 4°C.

*Note: RPP30 20X Primer Probe Assay Master Mix is purchased directly from BioRad, see Reagent List.*

- a. Prepare 20X GFP Primer Probe Assay Master Mix with final concentrations as follows: 18  $\mu\text{M}$  GFP FWD Primer, 18  $\mu\text{M}$  GFP REV Primer, and 5  $\mu\text{M}$  GFP Probe

21.6 $\mu\text{L}$	100 $\mu\text{M}$ GFP FWD Primer
21.6 $\mu\text{L}$	100 $\mu\text{M}$ GFP REV Primer
6 $\mu\text{L}$	100 $\mu\text{M}$ GFP Probe
Bring final volume to 120 $\mu\text{L}$	Molecular Grade H <sub>2</sub> O

- b. Prepare 20X AmpR Primer Probe Assay Master Mix with final concentrations as follows: 18  $\mu\text{M}$  AmpR FWD Primer, 18  $\mu\text{M}$  AmpR REV Primer, and 5  $\mu\text{M}$  AmpR Probe

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21.6 $\mu\text{L}$	100 $\mu\text{M}$ AmpR FWD Primer
21.6 $\mu\text{L}$	100 $\mu\text{M}$ AmpR REV Primer
6 $\mu\text{L}$	100 $\mu\text{M}$ AmpR Probe
Bring final volume to 120 $\mu\text{L}$	Molecular Grade H <sub>2</sub> O

- Bring all reagents to room temperature (RT) and vortex well before use.
- Prepare gDNA using Purelink gDNA kit (Invitrogen) at final concentration of 2-20 ng/ $\mu\text{L}$ , eluted in Molecular Grade H<sub>2</sub>O.
- Recommended Controls:
  - GFP+/AmpR- (gDNA from a known GFP+ cell line with no AmpR integration)
  - GFP+/AmpR+ (gDNA from a known GFP+ and AmpR+ cell Line)
  - WT gDNA with Donor plasmid added
    - To avoid the risk of plasmid contamination mix control in separate PCR hood
  - Wild type or untreated gDNA
  - No Template Control (NTC)
- Generate an experimental template using the QuantaSoft Software provided with the Droplet Reader that is specific to your experiment.

### Method: ddPCR Reaction Set Up

- Prepare Reaction Master Mixes: prepare all reactions at RT in PCR hood
  - GFP-FAM/RPP30-HEX Duplexed Master Mix

12.5 $\mu\text{L}$	2X ddPCR Supermix for Probes, No UTP
1.25 $\mu\text{L}$	20X Primer Probe Assay Master Mix for GFP
1.25 $\mu\text{L}$	20X PrimerPCR® RPP30 Reference Assay
0.30 $\mu\text{L}$	HindIII HF (20,000U/mL)
30 ng	Template gDNA
Up to 25 $\mu\text{L}$	Molecular Grade H <sub>2</sub> O

- AmpR-HEX Master Mix

12.5 $\mu\text{L}$	2X ddPCR Supermix for Probes, No UTP
1.25 $\mu\text{L}$	20X Primer Probe Assay Master Mix for AmpR
0.30 $\mu\text{L}$	HindIII HF (20,000U/mL)
30 ng	Template gDNA
Up to 25 $\mu\text{L}$	Molecular Grade H <sub>2</sub> O

- Vortex mixes to ensure homogenous distribution of reagents.
- Pipette 22  $\mu\text{L}$  of GFP-FAM/RPP30-HEX Duplexed Master Mix into a standard 96 well plate in only the odd numbered columns (1,3,5,7,9,11- or a subset if running fewer than 48 samples/plate).
- Pipette 22  $\mu\text{L}$  of AmpR-HEX Master Mix into the even numbered columns (2,4,6,8,10,12- or a subset if running fewer than 48 samples/plate).

Note: a multi-channel pipette or electronic pipette is recommended for pipetting master mixes.

- Pipette 3  $\mu$ L of template Gdna into each reaction well: 1 for GFP-FAM/RPP30-HEX and 1 for AmpR-HEX. Be sure to change tips between wells.

Note: 8 wells should be dedicated for controls (4 for GFP and 4 for AmpR, see “Steps Before Starting” for recommended controls). Each 96-well reaction plate can analyze 44 samples and 4 controls.

- Mix reaction plate by gently pipetting 20  $\mu$ L up and down 5 times or vortexing plate, avoiding bubbles as they drastically inhibit droplet formation. Spin down reaction plate.

	01	02	03	04	05	06	07	08	09	10	11	12
A	COLUMN 1 A ABS N GFP U RPP30	COLUMN 1 A ABS N AMP	COLUMN 2 A ABS U GFP U RPP30	COLUMN 2 A ABS U AMP	COLUMN 3 A ABS U GFP U RPP30	COLUMN 3 A ABS U AMP	COLUMN 4 A ABS U GFP U RPP30	COLUMN 4 A ABS U AMP	COLUMN 5 A ABS U GFP U RPP30	COLUMN 5 A ABS U AMP	COLUMN 6 A ABS U GFP U RPP30	COLUMN 6 A ABS U AMP
B	COLUMN 1 A ABS N GFP U RPP30	COLUMN 1 A ABS N AMP	COLUMN 2 A ABS U GFP U RPP30	COLUMN 2 A ABS U AMP	COLUMN 3 A ABS U GFP U RPP30	COLUMN 3 A ABS U AMP	COLUMN 4 A ABS U GFP U RPP30	COLUMN 4 A ABS U AMP	COLUMN 5 A ABS U GFP U RPP30	COLUMN 5 A ABS U AMP	COLUMN 6 A ABS U GFP U RPP30	COLUMN 6 A ABS U AMP
C	COLUMN 1 A ABS N GFP U RPP30	COLUMN 1 A ABS N AMP	COLUMN 2 A ABS U GFP U RPP30	COLUMN 2 A ABS U AMP	COLUMN 3 A ABS U RPP30	COLUMN 3 A ABS U AMP	COLUMN 4 A ABS U GFP U RPP30	COLUMN 4 A ABS U AMP	COLUMN 5 A ABS U RPP30	COLUMN 5 A ABS U AMP	COLUMN 6 A ABS U RPP30	COLUMN 6 A ABS U AMP
D	COLUMN 1 A ABS N GFP U RPP30	COLUMN 1 A ABS N AMP	COLUMN 2 A ABS U GFP U RPP30	COLUMN 2 A ABS U AMP	COLUMN 3 A ABS U RPP30	COLUMN 3 A ABS U AMP	COLUMN 4 A ABS U GFP U RPP30	COLUMN 4 A ABS U AMP	COLUMN 5 A ABS U RPP30	COLUMN 5 A ABS U AMP	COLUMN 6 A ABS U RPP30	COLUMN 6 A ABS U AMP
E	COLUMN 1 A ABS N GFP U RPP30	COLUMN 1 A ABS N AMP	COLUMN 2 A ABS U GFP U RPP30	COLUMN 2 A ABS U AMP	COLUMN 3 A ABS U GFP U RPP30	COLUMN 3 A ABS U AMP	COLUMN 4 A ABS U GFP U RPP30	COLUMN 4 A ABS U AMP	COLUMN 5 A ABS U GFP U RPP30	COLUMN 5 A ABS U AMP	GFP+/RPP30+... A ABS + RPP30	GFP+/RPP30+... A ABS - AMP
F	COLUMN 1 A ABS N GFP U RPP30	COLUMN 1 A ABS N AMP	COLUMN 2 A ABS U GFP U RPP30	COLUMN 2 A ABS U AMP	COLUMN 3 A ABS U RPP30	COLUMN 3 A ABS U AMP	COLUMN 4 A ABS U GFP U RPP30	COLUMN 4 A ABS U AMP	COLUMN 5 A ABS U RPP30	COLUMN 5 A ABS U AMP	GFP+/RPP30/A... A ABS + RPP30	GFP+/RPP30/A... A ABS + AMP
G	COLUMN 1 A ABS N GFP U RPP30	COLUMN 1 A ABS N AMP	COLUMN 2 A ABS U GFP U RPP30	COLUMN 2 A ABS U AMP	COLUMN 3 A ABS U RPP30	COLUMN 3 A ABS U AMP	COLUMN 4 A ABS U GFP U RPP30	COLUMN 4 A ABS U AMP	COLUMN 5 A ABS U RPP30	COLUMN 5 A ABS U AMP	NTC A ABS N RPP30	NTC A ABS N AMP
H	COLUMN 1 A ABS N GFP U RPP30	COLUMN 1 A ABS N AMP	COLUMN 2 A ABS U GFP U RPP30	COLUMN 2 A ABS U AMP	COLUMN 3 A ABS U RPP30	COLUMN 3 A ABS U AMP	COLUMN 4 A ABS U GFP U RPP30	COLUMN 4 A ABS U AMP	COLUMN 5 A ABS U RPP30	COLUMN 5 A ABS U AMP	NTC A ABS N RPP30	NTC A ABS N AMP

Figure 1. Example Plate Layout

## Method: ddPCR Droplet Generation

**\*Before starting!** Be sure to have all reactions set up before starting droplet generation as droplets are not stabilized before cycling and should not be left at room temperature for more than 15 min. If using multiple cartridges, droplets can be left on ice for up to 45 min.

- Insert a DG8 Cartridge into a DG8 Cartridge Holder.
- For droplet generation, transfer 20  $\mu$ L of the 25  $\mu$ L reaction prepared in the above steps into the middle row of a DG8 Cartridge, labeled “Sample”. Each DG8 Cartridge (as shown below) can



hold 8 samples. For 96 samples, 12 DG8 cartridges can be pre-loaded with sample and oil prior to droplet generation in step 4, below.

3. Load 70  $\mu\text{L}$  of Droplet Generation Oil for Probes into the bottom wells of the DG8 Cartridge, labeled "Oil".



4. Once both Sample and Oil are loaded, attach a Gasket across the top of the cartridge and place into the Q200 Droplet Generator and close lid. Droplet formation will start automatically and will take  $\sim 90$  s per cartridge. Only one cartridge can be loaded at a time, therefore 12 cartridges will take approximately 35-45 min.



5. After droplet formation is complete, the resulting droplets will be available in the top row of the cartridge, labeled "Droplets", and should appear cloudy. Using a multi-channel P200 pipette set to 40  $\mu\text{L}$ , slowly aspirate up the droplets and deposit into a fresh Bio-Rad 96 well plate kept in a

96 well cold block, or ice bucket.



6. Once all droplets have been generated, and the 96 well plate has been loaded with droplets, place pierceable foil seal on top of plate with the red band facing up. Seal the plate in a thermal plate sealer at 180°C for 5 s.
7. Once the plate has been sealed, immediately place the plate into the deep well thermal cycler and run the PCR cycling program. **Never spin droplet plate.**
  - a. Cycling Conditions for ddPCR Assays is carried at with a ramp rate of 2°C/s.

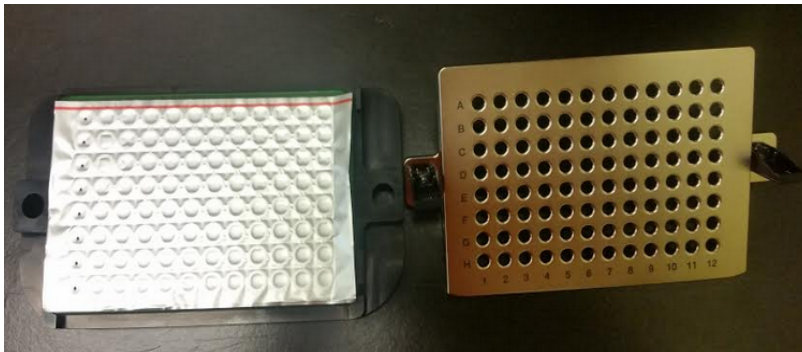
98°C	10 min	
94°C	30 s	X 39 cycles
60°C	20 s	
72°C	15 s	
98°C	10 min	

\*After PCR cycling, droplets are stable at 4°C for up to 3 days

### Method: ddPCR Droplet Reading

*(A full 96 well plate will take ~ 2.5hrs to read on the QX200 Droplet Reader)*

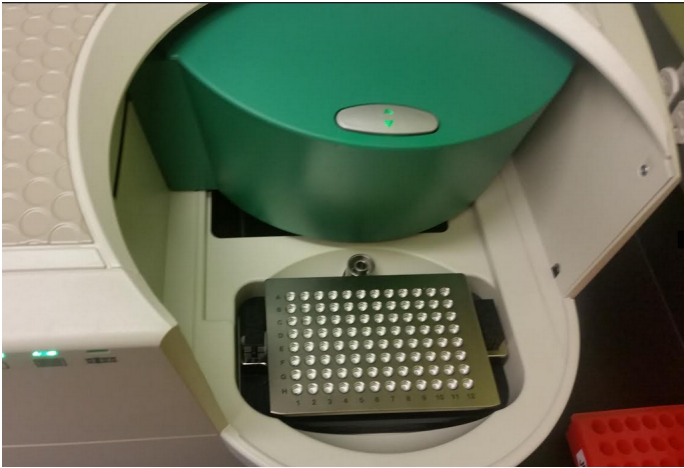
1. After cycling is complete, load plate in proper orientation into the QX200 Droplet Reader Plate Holder, as shown below.



2. Securely snap the metal lid shut over the top of the plate, as shown below.



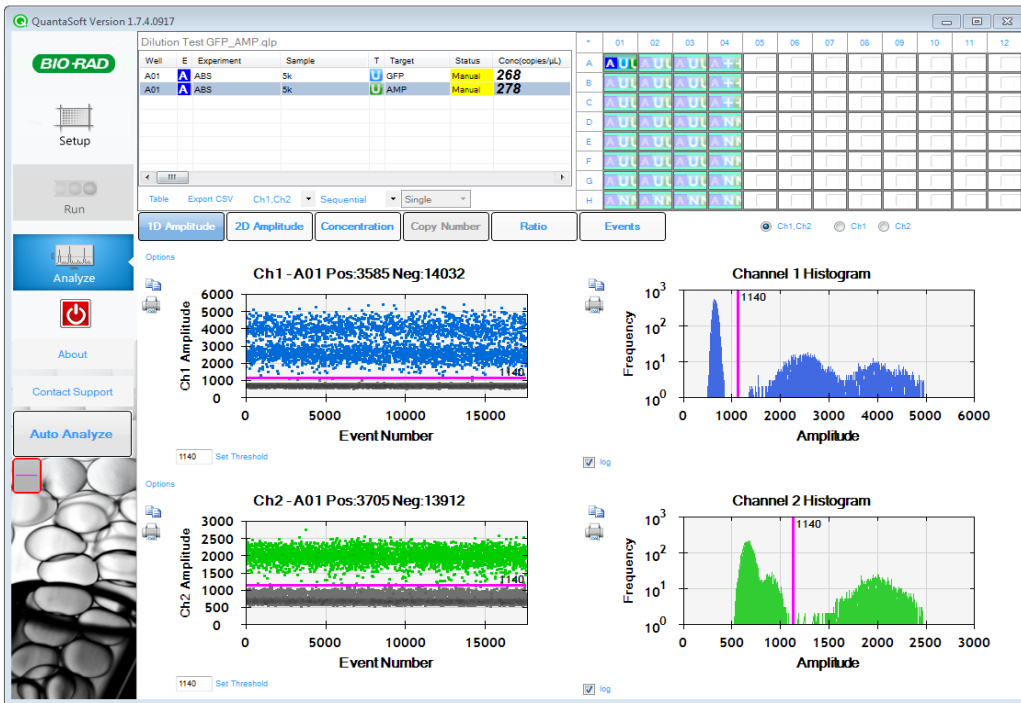
3. Load the entire Plate Holder into the QX200 Droplet Reader.



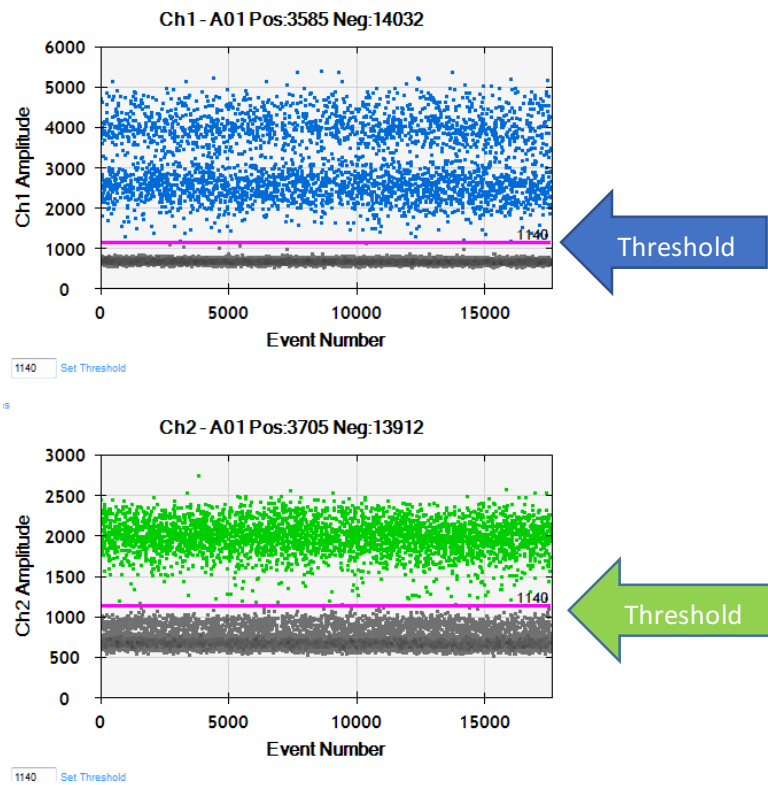
4. Launch the QuantaSoft software and load your prepared template. Click “run” and set the dye set to FAM/HEX to start the droplet reading process.
5. Results can be analyzed using the QuantaSoft program or exported as an Excel worksheet.

## Data Analysis

1. Open data file in Quanta soft and click on the analysis tab.



2. Select a sample well(s) and place your threshold gate. This should be set so as to give a clear separation of positive and negative drops



- Once threshold gates have been set, use the calculated copies/ul to further calculate copy number/genome

Well	E	Experiment	Sample	T	Target	Status	Conc(copies/μL)	Supermix
A01	A	ABS	5k	U	GFP	Manual	<b>268</b>	ddPCR Super
A01	A	ABS	5k	U	AMP	Manual	<b>278</b>	ddPCR Super

- To calculate the final copy number of GFP per genome calculate as the ratio of  $\frac{(\text{copies}/\mu\text{L}_{\text{GFP}}) - (\text{copies}/\mu\text{L}_{\text{nonintegratedAmpR}})}{(\text{copies}/\mu\text{L}_{\text{RPP30}})}$ , where a ratio of 0.5 is equal to 1 integrated copy/genome and a ratio of 1 is equal to 2 integrated copies/genome.
- The AMP signal is determined to be from residual non-integrated/background plasmid when the ratio of AmpR/RPP30 or KAN/RPP30 falls below 0.2 copies/genome, ratios above 0.2 copies/genome could indicate a non-clonal line with at least 1 copy of the backbone stably integrated.
- To calculate copies/genome  $\frac{(\text{copies}/\mu\text{L}_{\text{GFP}})}{(\text{copies}/\mu\text{L}_{\text{RPP30}})} * (\text{copies of RPP30/genome}) = \text{GFP copy\#/genome}$ .