ddPCR™ Protocol

Required Materials
(Bio-Rad) Droplet Generation Oil for Probes (186-3005)
(Bio-Rad) DG8 Gaskets for QX200 Droplet Generator (186-3009)
(Bio-Rad) DG8 Cartridges fro Droplet Generator (186-4008)
(Bio-Rad) ddPCR Supermix for Probes (No dUTP) (186-3023/3024 depending on size)
(Bio-Rad) Pierceable Foil Heat Seal (181-4040)
(Fisher) Eppendorf twin.tec 96-well plates, semi-skirted (951020346)

I. **Temperature Gradient Set-Up**

II. **HEK 293 Set-Up**

III. **Sib-selection Set-Up**

IV. **Droplet Generation**

I. Temperature Gradient Set-Up

1. Prepare the reaction master mix with water, ddPCR™ Supermix for Probes, Taqman FAM/VIC or FAM/HEX probes, and 1:1 plasmid mixture (0.5 pg for plasmid 0.05 pg for DNA fragment/uL).

	Per Reaction(1x)	Reaction Master Mix (8x)
Water	9 uL	72 ul
2x Supermix	12.5 uL	100 uL
20x FAM probe	1.25 uL	10 uL
20x VIC/HEX probe	1.25 uL	10 uL
1:1 Plasmid Mixture	1uL	8 uL
	Total = 25 uL	200 uL

2. Aliquot 24.5 uL reaction master mix into each tube of an 8-tube PCR strip compatible with a multi-channel micropipettor.
3. Spin the PCR strip down.
4. See **IV. Droplet Generation** Section.
5. Run foil-sealed PCR plate on thermocycler with the following program:
   95°C 10 min
   (94°C 30 sec, 50°C-60°C gradient 1 min) x 40
   98°C 10 min
   12°C forever
6. Load plate into QX100™ Droplet Reader and determine optimal temperature for separation of distinct negative, FAM-positive, VIC-positive, and double-positive populations.

II. HEK293 Set-Up

1. Dilute HEK293 gDNA samples to 100-150 ng.
2. Prepare the reaction master mix with water, ddPCR™ Supermix for Probes, and Taqman FAM/VIC or FAM/HEX probes. Prepare enough to analyze your gDNA samples in addition to a water only negative control and 1:1 plasmid mixture positive control.

	Per Reaction	Reaction Master Mix for N Samples
Water	9 uL	
2x Supermix	12.5 uL	x N
20x FAM probe	1.25 uL	
20x VIC/HEX probe	1.25 uL	
Total =	24 uL	

3. Aliquot 23.5 uL reaction master mix into 8-tube PCR strips compatible with a multi-channel micropipettor.
4. Add 1 uL water to the first tube of the strip to use as a negative control.
5. Add 1 uL of each HEK293 gDNA sample (100 ng/uL) to the subsequent tubes.
6. Add 1 uL 1:1 plasmid mixture (0.5 pg/uL) or 1:1 DNA fragment mixture (0.05 pg/uL) to the last tube to use as a positive control.
7. Since Droplet Generation must be done in an 8-chamber format, if the total number of samples to be analyzed is not a multiple of eight, the remaining empty tubes of the PCR strip must be filled. Add 12 uL ddPCR™ Buffer Control Kit (2x) and 12 uL water to each empty tube.
8. Spin the PCR strip down.
9. See **IV. Droplet Generation** Section.
10. Run foil sealed plate on thermocycler with the following program:
    95°C 10 min
    (94°C 30 sec, XX*°C 1 min) x 40
    98°C 10 min
    12°C forever
    *XX is the optimized temperature found with the temperature gradient
11. Load plate into QX100™ Droplet Reader and analyze.

III. Sib-selection Set-Up

1. Spec a couple wells of the 96-well plate to ensure that there is sufficient DNA concentration for PCR. The 96-well plate format gDNA extraction typically yields concentrations from 5 ng/uL – 50 ng/uL in our hands, so 5 uL per sample is the standard

volume used in this analysis. The system recommends 100 ng DNA but only requires that total DNA amount be between 25 ng and 350 ng.

2. Select 10-20 wells of the mutagenesis 96-well plate to analyze. For convenience sake, the total number of samples to be analyzed should be a multiple of 8 (including three controls) and easily transferable via multichannel pipettor (in rows or columns). The first two wells in analysis should contain the water only negative control and the transfection with unrelated TALENs/pX335 negative control. The last well in analysis should be the 1:1 plasmid mixture positive control.
3. Prepare the reaction master mix with water, ddPCR™ Supermix for Probes, and Taqman FAM/VIC or FAM/HEX probes.

	Per Reaction	Reaction Master Mix for N Samples
Water	4 uL*	
2x Supermix	12.5 uL	x N
20x FAM probe	1.25 uL	
20x VIC/HEX probe	1.25 uL	
	Total = 20* uL	

*These numbers will vary depending on how much DNA is used for analysis. The total volume of reaction mixture plus gDNA should be 25 uL so adjust accordingly.

3. Aliquot 19.5uL reaction master mix into 8-tube PCR strips compatible with a multi-channel micropipettor.
4. Add 5 uL water to the first tube to use as a negative control.
5. In the second tube, add 5 uL gDNA prep from 96-well plate with negative control transfection using unrelated TALENs/pX335.
6. Using a multichannel pipettor, transfer 5 uL gDNA prep from wells of mutagenesis plates to appropriate tubes.
7. Add 4 uL water + 1 uL 1:1 plasmid/DNA fragment mixture (0.5 pg/uL/0.05 pg/uL) to the last tube use as a positive control.
8. Since Droplet Generation must be done in an 8-chamber format, if the total number of samples to be analyzed is not a multiple of eight, the remaining empty tubes of the PCR strip must be filled. Add 12 uL ddPCR™ Buffer Control Kit (2x) and 12 uL water to each empty tube.
9. Spin the PCR strip down.
10. See **IV. Droplet Generation** Section.
11. Run foil-sealed plate on thermocycler with the following program:
    95°C 10 min
    (94°C 30 sec, XX*°C 1 min) x 40

98°C 10 min

12°C forever

*XX is the optimized temperature found with the temperature gradient

12. Load plate into QX100™ Droplet Reader and analyze.

IV. Droplet Generation

1. Set aside an Eppendorf twin.tec 96-well PCR plate and cover with a piece of Pierceable Foil Heat Seal. Make sure the red line on the foil faces upward.
2. Load a DG80™ Cartridge into the Cartridge Holder and close the holder.
3. Using a multi-channel pipettor set to 20 uL, gently pipette the reaction mixture in the 8-tube PCR strip up and down to mix. Take care not to create any bubbles. Transfer 20 uL reaction mixture from the 8-tube PCR strip into the middle row of chambers on the Cartridge designated for “Sample”. Remove any bubbles in the chambers with a P20 micropipettor. The instance of bubbles drastically reduces droplet count in the generation step. Take note of orientation of the cartridge with respect to sample order to ensure correct loading of the final 96-well PCR plate.
4. Add 70 uL Droplet Generator Oil to the bottom row of the cartridge designed for “Oil.” Do not load oil before samples as this will reduce droplet number.
5. Fit rubber DG80™ Gasket onto Cartridge by catching two holes on either end of the Gasket with coordinated hooks on the edges of the Cartridge Holder.
6. Gently place Cartridge holder into the QX1000™ Droplet Generator. Droplet generation should take about 1 minute.
7. Remove the Cartridge Holder from the machine and discard Gasket. Droplets are held in the top row of the Cartridge and slightly opaque. Use a multi-channel pipettor set to 45uL to transfer droplets into a column of the Eppendorf twin.tec 96-well PCR plate. Do not press the pipettor tightly to the bottom of the cartridge or pipette to vigorously as this will shear the droplets.
8. Cover the PCR plate with the foil sheet immediately after to reduce the risk of contamination.
9. Repeat steps 2-8 to generate droplets for all your samples.
10. Load the 96-well plate and foil seal into the PX1™ PCR Plate Sealer set to 180°C and seal plate.