Polymerase Chain Reaction (PCR) and Droplet Digital PCR (ddPCR) Protocol

Katie Jing Kay Lam[1], Olubankole Arogundade[1], Claire Clelland[1,2]

Affiliations:

[1] Weill Institute for Neurosciences, University of California, San Francisco, USA

[2] Department of Neurology, University of California, San Francisco, CA, USA

*Contact: claire.clelland@ucsf.edu

Polymerase Chain Reaction PCR

DNA preparation by QuickExtract™ (Figure 1) – 96w plate

1. Wash cells with 100 µL PBS & aspirate
2. Add 30 µL QuickExtract™
3. Scrape well bottom with pipette tip to detach cells
4. Transfer cells to labelled PCR tubes
5. Vortex 15 secs
6. Incubate at 65°C for 6 mins
7. Vortex for 15 secs
8. Incubate at 98°C for 2 mins
9. Store at -20°C

Figure 1. *Procedure for using QuickExtract DNA Extraction Solution [1]*

Primer preparation

1. Resuspend IDT primers with H2O to 100 µM
    - Example: 20 nmol – Add 200 µL H2O
2. Dilute primer to 10 µM in a new microcentrifuge tube
    - Example: 20 µL + 180 µL H2O
3. Store at -20°C

Reaction setup

1. Prepare Master Mix – Number of reactions + 1 (as extra)
2. For each PCR tube: 1 µL DNA sample + 19 µL Master Mix

3. Bio-Rad Thermal Cycler – Select/Edit Protocol (see PCR Cycle below)

Master Mix – for one 20 µL reaction

Component	Volume
BioMix™ Red	10 µL
DMSO	0.6 µL
Forward primer (10 µM)	1 µL
Reverse primer (10 µM)	1 µL
RNase/DNase-free H2O	6.4 µL

PCR Cycle

#	Step	Temperature	Time
1	Enzyme Activation	94°C	3 mins
2	Denaturation	94°C	30 secs
3	Annealing	50-65°C	30 secs
4	Primary Extension	72°C	1 min/kb
5	Repeat from step 2 (34x)		
6	Secondary Extension	72°C	5 mins
7	Hold	4°C	∞

Note: Annealing temperature should be 5-10°C lower than Tm of primers

Gel electrophoresis

1X TAE buffer

1. Add 36 mL 50X TAE Buffer to 2L bottle
2. Add diH2O up to 1800 mL

Gel preparation

1. Volume: Big tray: ~ 300 mL; Small tray: ~ 150 mL
2. Gel percentage: 1.5-2%
   - For 2% gel: 2 g agarose + 100 mL 1X TAE buffer
3. Heat in microwave until completely dissolve
4. Add 15 µL SYBR™ Safe DNA Gel Stain (for 150 mL)
5. Pour onto the tray
6. Remove big bubbles
7. Put in the comb (15/20 well)
8. Let it cool until solidify

Important note:

** Remember to clean & rinse beaker after making gel every time!

** DO NOT discard excessive gel solution in the sink – Solidified gel will block the drain!

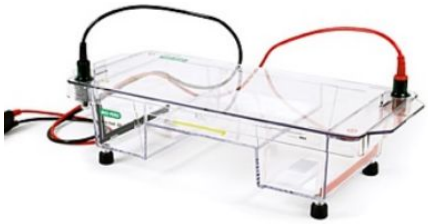

Figure 2. *Bio-Rad Sub-Cell GT Cell [2]*

Running a gel

1. Make sure the gel is solidified completely
2. Carefully remove comb from gel
3. Put tray into Bio-Rad Sub-Cell GT Cell (Figure 2)
   *** Make sure the wells are placed at the end of the negative (black) terminal*
4. Add 1X TAE buffer to the Sub-Cell GT Cell if needed
   - Make sure the gel is submerged in TAE buffer completely
5. Carefully load ~12 µL DNA ladder (Quick-Load® Purple 1 kb Plus DNA Ladder) & PCR products
   - Submerge pipette tips into the well before dispensing PCR product (Figure 3)
   - Prevent touching the wall of wells, which might break the gel
     *** Recommend dispensing liquid by pressing to the first stop only to prevent creating air bubbles, which could lead to loss of PCR product*
6. Set 100-140V on the Bio-Rad Power Supply
   - Voltage depends on gel size
   - Make sure the gel is connected to the power supply properly (Figure 4)
     - Black to Black; Red to Red
7. START

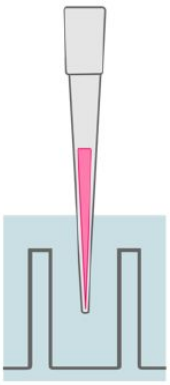

Figure 3. *Step 5 – submerging pipette tips in the well [3]*

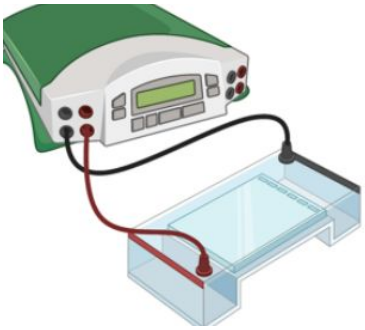

Figure 4. *Gel connected to power supply [3]*

Gel imaging – LI-COR Odyssey M Imager (Figure 5)

1. Clean the imager glass with EtOH and Kimwipe
2. Position the gel
3. Open LI-COR Acquisition Software
4. Select:
    - Scan
    - Username: Clelland Lab; Imager: Odyssey M → Connect
    - Gel → Connect
    - Draw Scan Area → Next
    - 488 SYBR Safe → Save
    - Focus offset (mm): 2.00 → Scan
5. Wait for scan to finish
6. Export image to desired destination

Note:
**Remember to clean the imager glass with EtOH and Kimwipe before and after each use

Figure 5. *LI-COR Odyssey M Imager [4]*

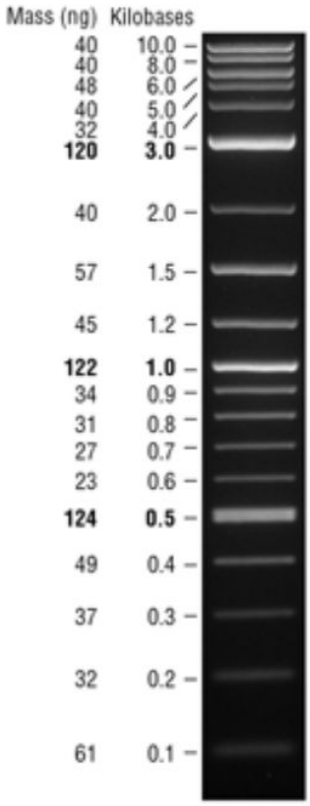

Figure 6. *New England BioLabs Quick-Load® Purple 1 kb Plus DNA Ladder on a 1.0% TBE agarose gel [5]*

PCR for sequencing (with gel extraction)

Run a 50 µL reaction instead of 20 µL

Reaction setup

1. Prepare Master Mix – Number of reactions + 1 (as extra)
2. For each PCR tube: 2.5 µL DNA sample + 47 µL Master Mix
3. Bio-Rad Thermal Cycler – Select/Edit Protocol (see PCR Cycle below)

Master Mix – for one 50 µL reaction

Component	Volume
BioMix™ Red	25 µL
DMSO	1.5 µL
Forward primer (10 µM)	2.5 µL
Reverse primer (10 µM)	2.5 µL
RNase/DNase-free H2O	16 µL

PCR Cycle

#	Step	Temperature	Time
1	Enzyme Activation	94°C	3.5 mins

#	Step	Temperature	Time
2	Denaturation	94°C	45 sec
3	Annealing	50-65°C	45 sec
4	Primary Extension	72°C	1 min/kb
5	Repeat from step 2 (39x)		
6	Secondary Extension	72°C	5 mins
7	Hold	4°C	∞

Note: Annealing temperature should be 5-10°C lower than Tm of primers

Gel extraction

Source: Qiagen MinElute Gel Extraction Kit (modified)

1. Run gel electrophoresis as instructed above
2. Excise the desired gel band under UV light
3. Transfer the gel slice into a 1.5 mL tube
4. Add 0.6 mL Qiagen Buffer QG
5. Incubate at 50°C until gel has completely dissolved
    - Mix the tube every 1-2 mins to help dissolve the gel
6. Add 100 µL 100% isopropanol & mix by inverting
7. Transfer sample to a MinElute spin column (stored at 4°C)
    - Centrifuge at 8000 rpm for 1 min
    - Discard the filtrate
    - Put back the spin column to the same collection tube
8. Repeat step 7 until all sample has passed through the column
9. Add 650 µL Buffer PE to spin column
    - Centrifuge at 8000 rpm for 1 min
    - Discard the filtrate
    - Put back the spin column to the same collection tube
10. Centrifuge again at 13000 rpm for 1 min
    - Discard the filtrate & collection tube
11. Place the column in a new 1.5 mL tube
12. Add 35-50 µL Buffer EB
    - Make sure it is added to the center of the membrane
13. Incubate at 42°C for 3 mins
14. Centrifuge at 13000 rpm for 1 min
15. Repeat step 12-14 to increase product yield
    - Reload with the DNA product at step 14 instead of adding new Buffer EB

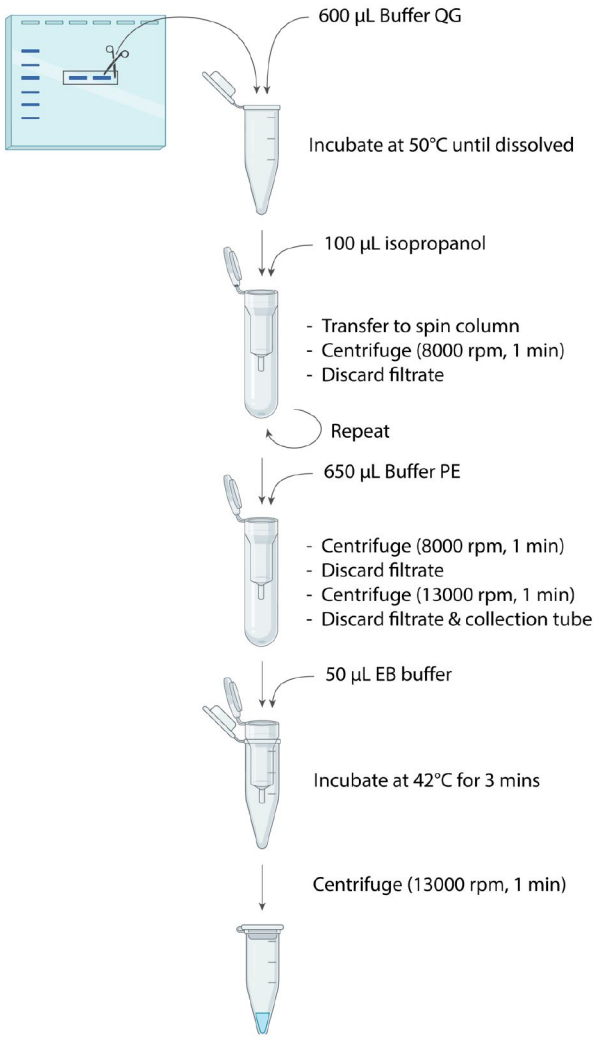

Figure 7. *Gel extraction workflow [3]*

Droplet Digital PCR ddPCR – QX200 Droplet Generator

Source: Bio-Rad (modified)

Note: Remember to schedule for equipment at CAT on iLab

Reaction setup

Vortex for 15 secs on full speed when mixing

1. Thaw all components to room temperature
2. Prepare 20X target primers/probe
3. Prepare Master Mix – Number of reactions + 1
4. For each PCR tube: DNA sample (Up to 350 ng) + Master Mix = 22 µL

20X target primers/probe

Component	Volume
Forward primer (100 µM)	18 µL

Component	Volume
Reverse primer (100 µM)	18 µL
Target probe (FAM) **light sensitive*	5 µL
RNase/DNase-free H2O	59 µL

Note: Store at -20℃

Master Mix – 22 µL

Component	Volume
2X ddPCR Supermix for Probes (No dUTPs)	11 µL
20X target primers/probe (FAM)	1.1 µL
20X reference primers/probe (HEX/VIC) – **RPP30**	1.1 µL
RNase/DNase-free H2O	Up to 22 µL (including DNA sample)

Droplet generation

1. Insert DG8™ Cartridge into cartridge holder (Figure 8)
2. FIRST: Load >20 µL sample into wells
   - Empty wells: Add a 50:50 mix of Supermix & water
   - All 8 wells must be loaded with samples or supermix + water
   - No bubbles!!!
3. THEN: Load 70 µL of generation oil into all 8 wells
   - Generation oil is one-time use/can only be used within a day once opened
4. Apply a DG8™ Gasket on cartridge
5. Load cartridge into QX200™ Droplet Generator (Figure 9)
6. START (~1 min)

Figure 8. *Loaded DG8 Cartridge [7]*

Figure 9. *Loaded GD8 Cartridge in QX200™ Droplet Generator [8]*

Transfer Droplets (within 5 mins of generation)

1. Transfer 40 µL droplets to a ddPCR™ 96-Well Plates
    - Pipette **SLOWLY** for droplet suction and dispensing (>7 secs)
2. Cover 96w plate with a foil seal sheet – Red line facing **up** (Figure 10)
3. Seal plate in a PX1 PCR Plate Sealer (Figure 11)
    - 180°C for 5 secs

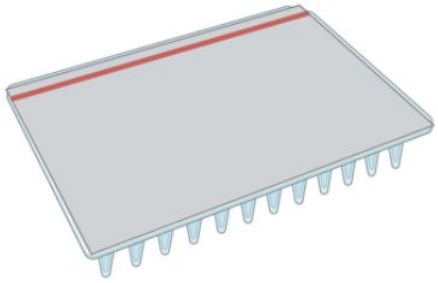

Figure 10. *96-well plate covered with foil seal sheet [3]*

Figure 11. *Bio-Rad PX1 PCR Plate Sealer [9]*

ddPCR Cycle (within 40 mins after sealing)

1. Place plate into Bio-Rad Thermal Cycler with 96-Deep Well C1000 block

#	Step	Temperature	Time	Ramp rate
1	Enzyme Activation	94°C	10 mins	
2	Denaturation	94°C	30 sec	2°C/sec
3	Annealing/Extension	*50-65°C	1 min	2°C/sec
4	Repeat from step 2 (39x)			
5	Enzyme Deactivation	98°C	10 mins	

#	Step	Temperature	Time	Ramp rate
6	Hold	4°C	∞	

Note: Optimized annealing temperature when using primers/probe set for the first time by using gradient temperatures

Reading the plate

1. Set plate in room temperature for 1-2mins
    - The plate can be stored at 4°C for ≤3 days before reading
2. Place plate in Bio-Rad QX200™ Droplet Reader (Figure 12)
3. Open QuantaSoft™ & set up
    - Select a well
      Example:
    - Sample
        - Enter name
        - Experiment: CNV2
        - Supermix: ddPCR Supermix for Probes (no dUTP)
    - Target 1 (FAM)
        - Enter name
        - Type: Ch 1 Unknown
    - Target 2 (HEX/VIC)
        - Enter name (e.g. RPP30)
        - Type: Ch 2 Reference

      Note: Copy & paste for every well used in the plate
4. Save template
5. RUN

Figure 12. *Bio-Rad QX200™ Droplet Reader [10]*

Data analysis

1. Set threshold manually (pink line)
    - Make sure the positive droplets (blue/green) are separated from the negative droplets (grey) (Figure 15-Figure 16)

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Experimental/Assay design

Designing PCR primers

- Length: 18-30 bases (~20 is good)
- GC content: 40-60%
- 3’ ending in G or C
- Avoid (>3) repeats of Gs and Cs
    - Balanced base distribution
- Melting temperature (Tm): between 50-65℃, within 5℃ of each other
    - Tm of primer pairs within 5℃
- Avoid complementary regions within/between primers

Product length:

- **PCR**: 200-1000 base pairs (can be as long as 2-3k bp if needed)
- **ddPCR**: 50-250 base pairs (optimal 80-120 bp)

Tools: Primer-BLAST (NCBI), Primer3web, OligoCalc (Properties Calculator)
Ordering in IDT: Custom DNA oligos

Designing ddPCR probes

- Between primer pair; no overlapping
- Length: <30 bases
- Melting temperature (Tm): 3-10℃ higher than primers
- More Cs than Gs in probe
- No G at 5’ end

Tools: Primer-BLAST (NCBI), Primer3web
Ordering in IDT: PrimeTime™ qPCR Probes (5’ FAM/ZEN/3’ Iowa Black FQ)

About PCR

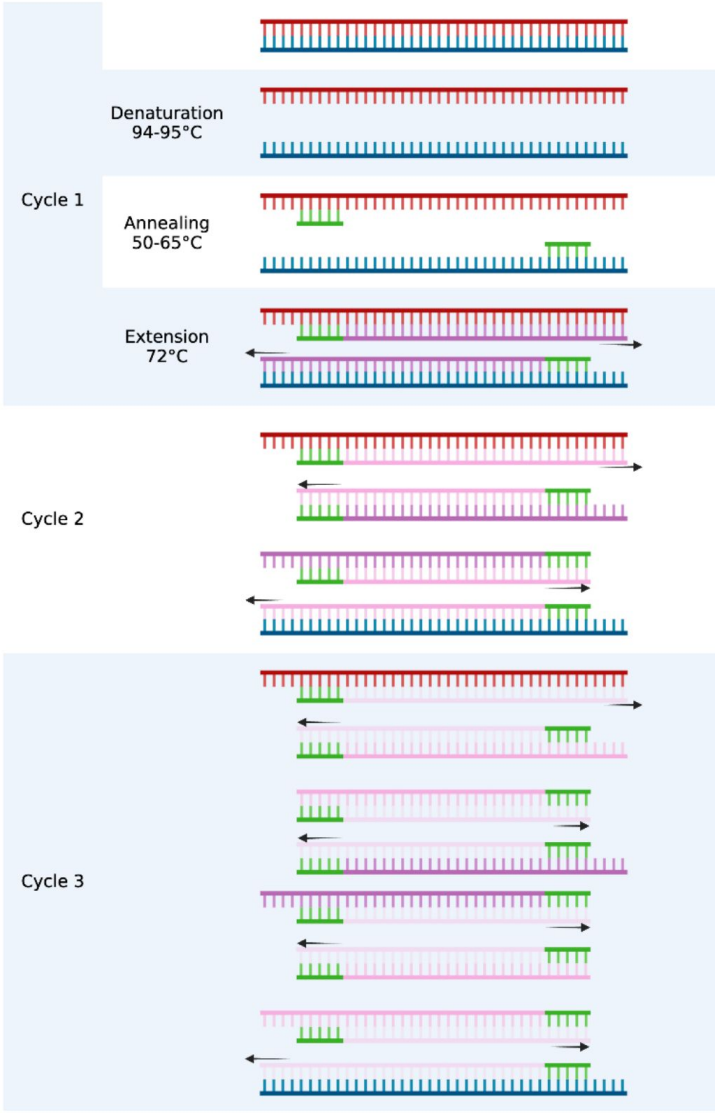

Figure 13. *PCR cycles [3]*

More about ddPCR...

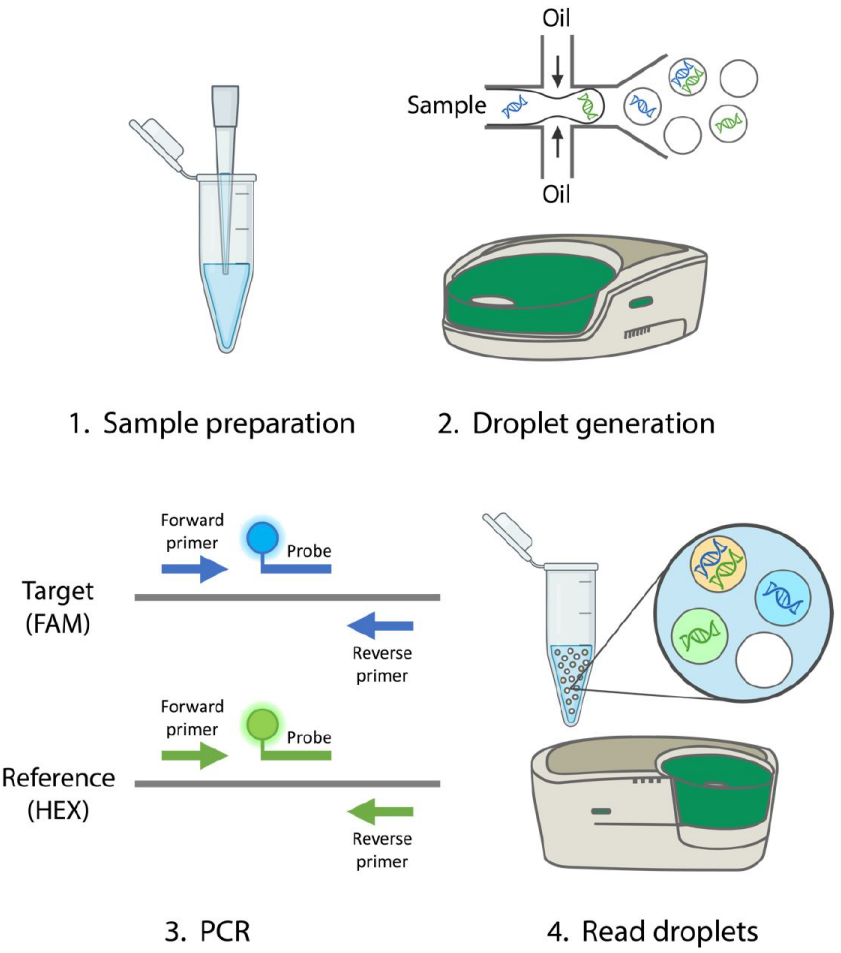

Figure 14. *ddPCR workflow [3]*

Data analysis

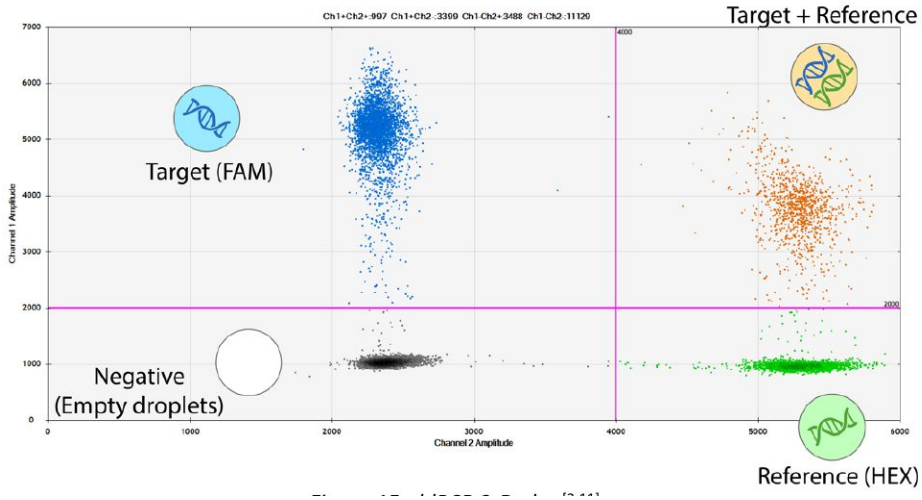

Figure 15. *ddPCR 2-D plot [3,11]*

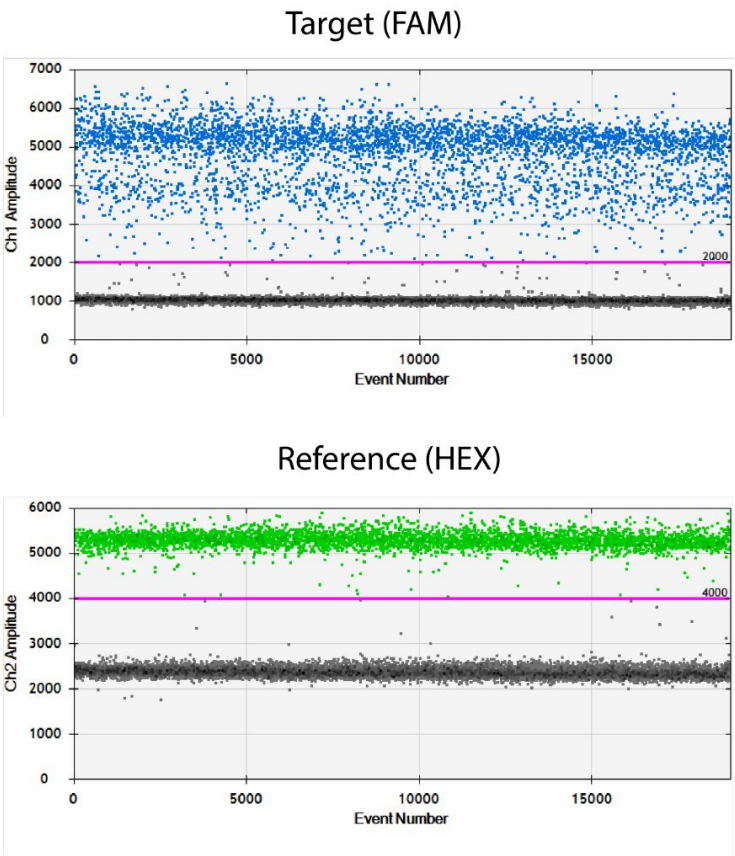

Figure 16. *ddPCR 1-D plot [11]*

Materials

Reagents

Reagent	Manufacturer	Catalog No.
DNA preparation		
QuickExtract™ DNA Extraction Solution	Biosearch Technologies	76081-768
PCR		
BioMix™ Red	Meridian Life Sceiences	C755F25
Dimethyl sulfoxide (DMSO)	Sigma Aldrich Fine Chemicals Biosciences	D8418
Gel electrophoresis		
Quick-Load® Purple 1 kb Plus DNA Ladder	New England Biolabs Inc.	N0550S
SYBR™ Safe DNA Gel Stain	Invitrogen	S33102
TAE Buffer (Tris-acetate-EDTA) (50X)	Thermo Scientific	B49
ddPCR		
ddPCR™ Copy Number Assay: RPP30, Human, Homo sapiens	Bio-Rad	dHsaCP1000485

Reagent	Manufacturer	Catalog No.
ddPCR Supermix for Probes (No dUTP)	Bio-Rad	1863024
Droplet Generation Oil for Probes	Bio-Rad	1863005

Equipment/Consumables

Equipment/Consumable	Manufacturer	Catalog No.
PCR		
Thermal Cycler	Bio-Rad	
Gel electrophoresis		
LI-COR Odyssey M Imager	LI-COR	
PowerPac™ HC Power Supply	Bio-Rad	
Sub-Cell GT Horizontal Electrophoresis System	Bio-Rad	
ddPCR		
C1000 Touch™ Thermal Cycler with 96–Deep Well Reaction Module	Bio-Rad	1851197
ddPCR™ 96-Well Plates	Bio-Rad	12001925
DG8™ Cartridges	Bio-Rad	1864008
DG8™ Cartridge Holder	Bio-Rad	1863051
DG8™ Gaskets	Bio-Rad	1863009
PCR Plate Heat Seal, foil, pierceable	Bio-Rad	1814040
PX1 PCR Plate Sealer	Bio-Rad	1814000
QX200 Droplet Digital PCR System	Bio-Rad	

References

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3. Figures Figure 4, Figure 10, Figure 13, and part of Figures Figure 3, Figure 7. Gel extraction workflow [3], Figure 14 are created with BioRender.com
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10. Bio-Rad. QX200 Droplet Reader and QuantaSoft Software Instruction Manual. Retrieved May 25, 2023, from https://www.bio-rad.com/webroot/web/pdf/lsr/literature/10031906.pdf
11. Data from Figures Figure 15-Figure 16 are generated from Bio-Rad QuantaSoft™ Analysis Pro Software