



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

Katie Jing Kay Lam¹, Olubankole Arogundade¹, 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

BioMix™ Red	10 μL
DMSO	0.6 μL
Forward primer (10 μM)	1 μL
Reverse primer (10 μM)	1 μL
RNase/DNase-free H ₂ O	6.4 μL

PCR Cycle

	Step	Temperature	Time
1	Enzyme Activation	94°C	3 mins
2	Denaturation	94°C	30 sec
3	Annealing	50-65°C	1 min
4	Primary Extension	72°C	1 min/kb
5	Repeat from step 2 (34x)		
6	Secondary Extension	72°C	15 mins
7	Hold	4°C	∞

Note: Annealing temperature should be 5-10°C lower than T_m 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

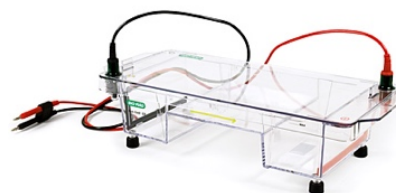


Figure 2. Bio-Rad Sub-Cell GT Cell ^[2]

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!

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

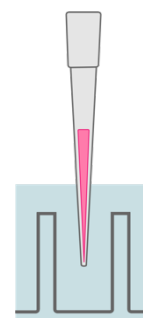


Figure 3. Step 5 – submerging pipette tips in the well ^[3]

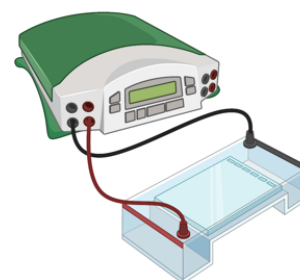


Figure 4. Gel connected to power supply ^[3]

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



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

Note:

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

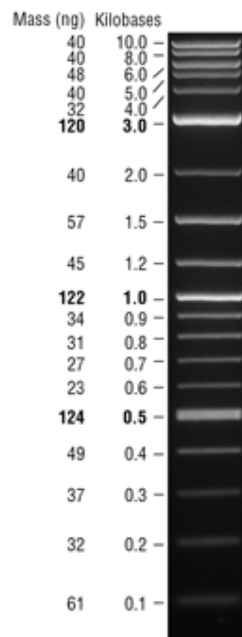


Figure 6. New England BioLabs Quick-Load® Purple 1 kb Plus DNA Ladder on a 1.0% TBE agarose gel [5]



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

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 H_2O	16 μL

PCR Cycle

	Step	Temperature	Time
1	Enzyme Activation	94°C	3 mins
2	Denaturation	94°C	30 sec
3	Annealing	50-65°C	1 min
4	Primary Extension	72°C	1 min/kb
5	Repeat from step 2 (39x)		
6	Secondary Extension	72°C	15 mins
7	Hold	4°C	∞

Note: Annealing temperature should be 5-10°C lower than T_m 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

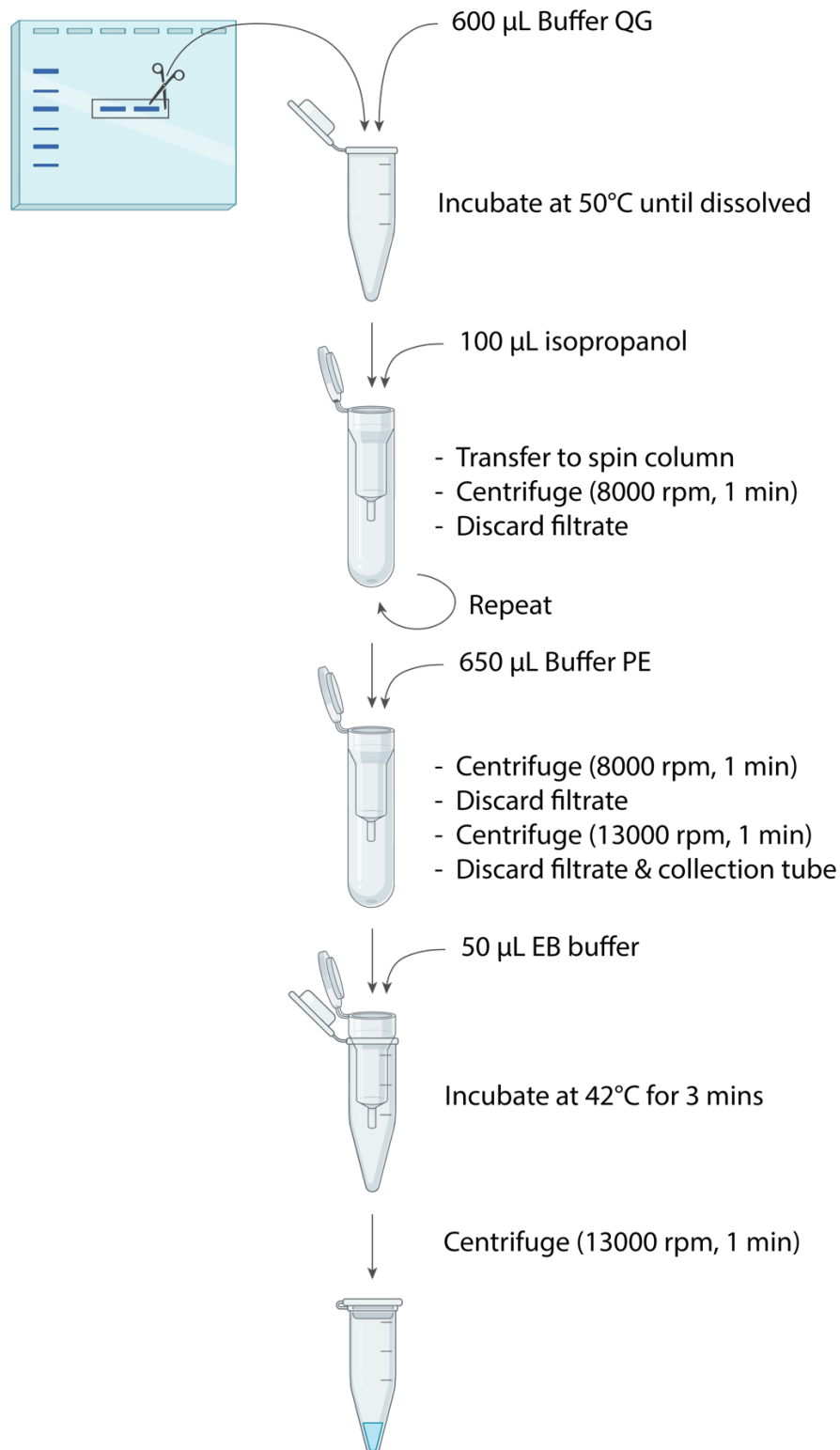


Figure 7. Gel extraction workflow ^[3]



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 μ L

20X target primers/probe

Forward primer (<u>100 μM</u>)	18 μ L
Reverse primer (<u>100 μM</u>)	18 μ L
Target probe (FAM) *light sensitive	5 μ L
RNase/DNase-free H ₂ O	59 μ L

Note: Store at -20°C

Master Mix – 22 μ L

2X ddPCR Supermix for Probes (No dUTPs)	11 μ L
20X target primers/probe (FAM)	1.1 μ L
20X reference primers/probe (HEX/VIC) – RPP30	1.1 μ L
RNase/DNase-free H ₂ O	Up to 22 μ L (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)

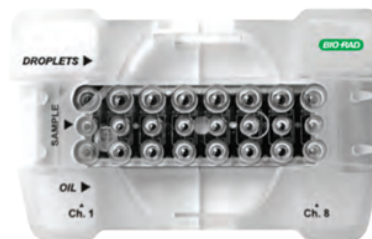


Figure 8. Loaded DG8 Cartridge [7]



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

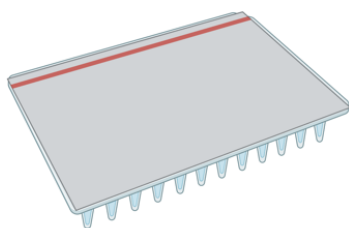


Figure 10. 96-well plate covered with foil seal sheet [3]



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	
1	Enzyme Activation	94°C	10 mins	Ramp rate
2	Denaturation	94°C	30 sec	2°C/sec
3	Annealing/Extension	*50-65°C	1 min	
4	Repeat from step 2 (39x)			
5	Enzyme Deativation	98°C	10 mins	
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:
 - Supermix:
- Target 1 (FAM)
 - Enter name
 - Type:
- Target 2 (HEX/VIC)
 - Enter name (e.g. RPP30)
 - Type:

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)



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
 - o Balanced base distribution
- Melting temperature (T_m): between 50-65°C, within 5°C of each other
 - o T_m of primer pairs within 5°C
- 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 (T_m): 3-10°C 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

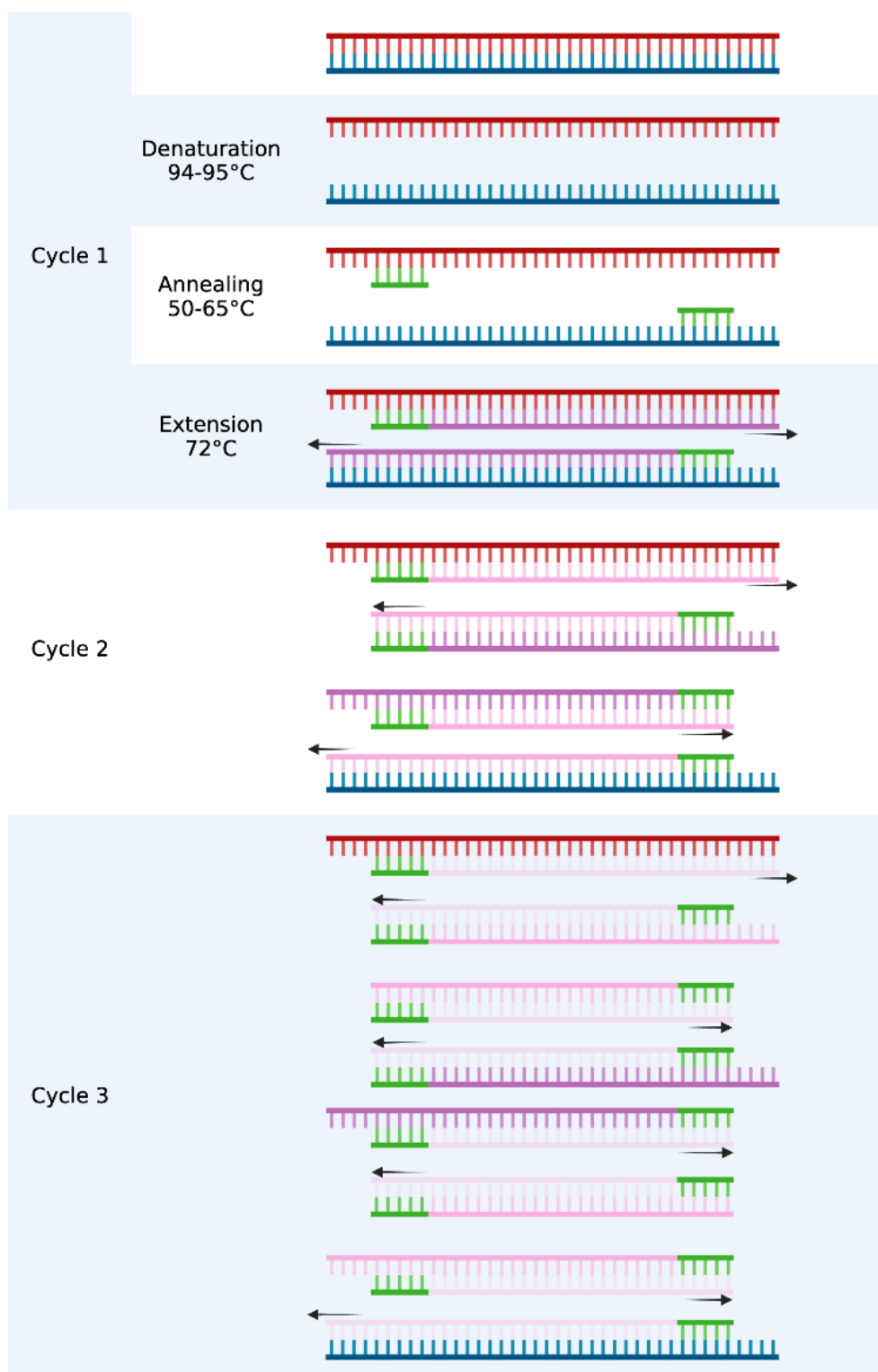
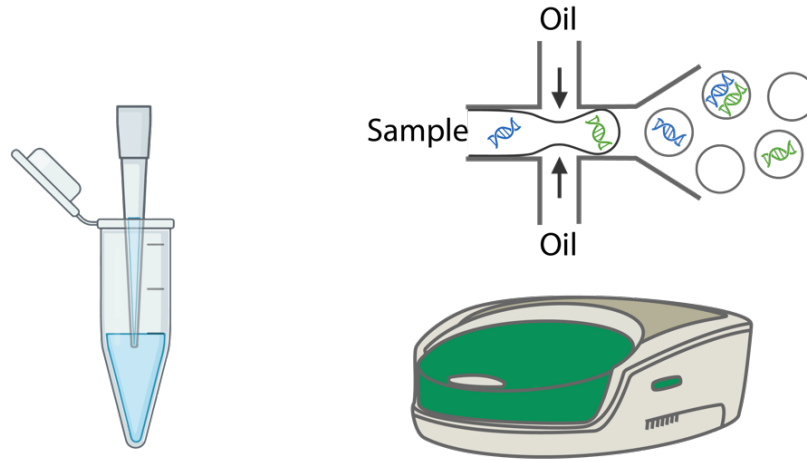


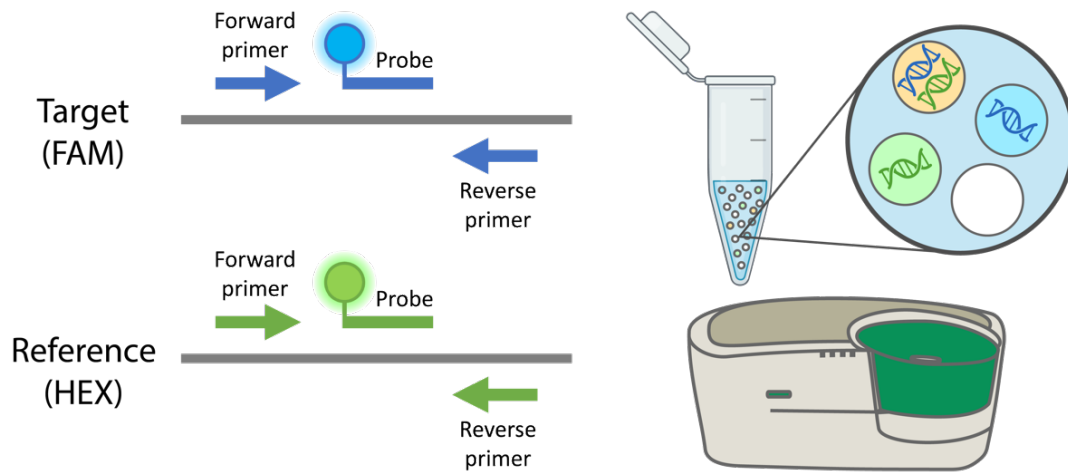
Figure 13. PCR cycles ^[3]

More about ddPCR...



1. Sample preparation

2. Droplet generation



3. PCR

4. Read droplets

Figure 14. ddPCR workflow ^[3]

Data analysis

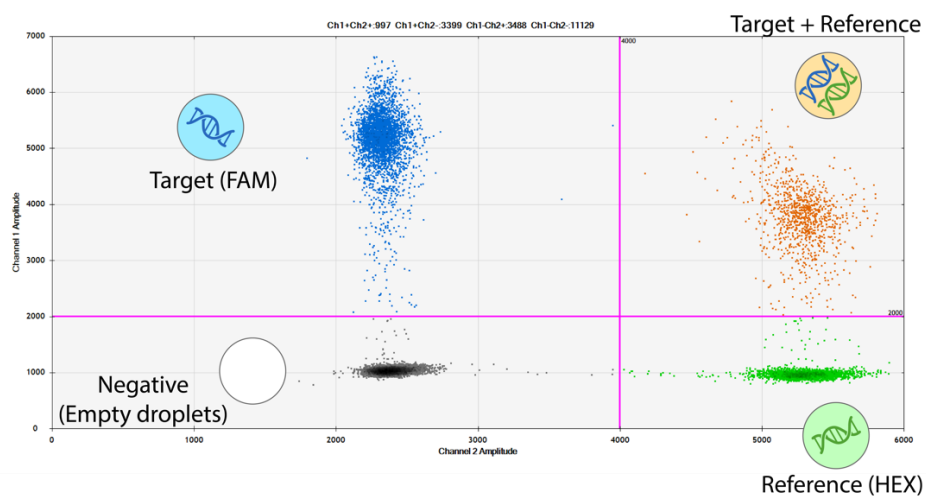


Figure 15. ddPCR 2-D plot [3,11]

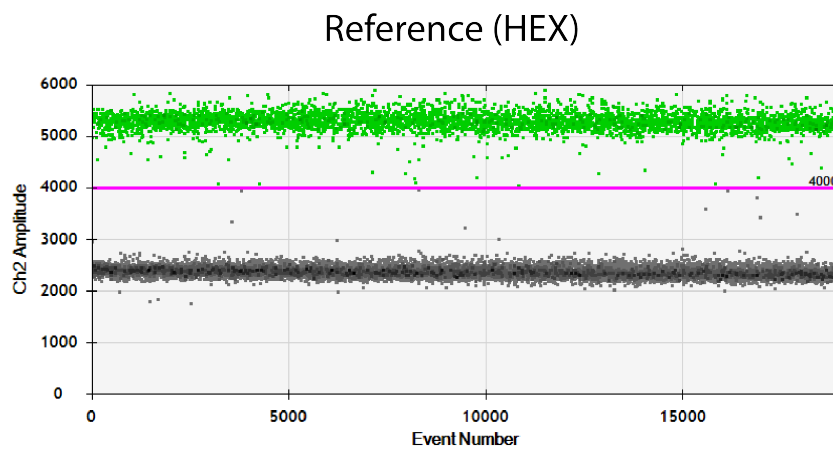
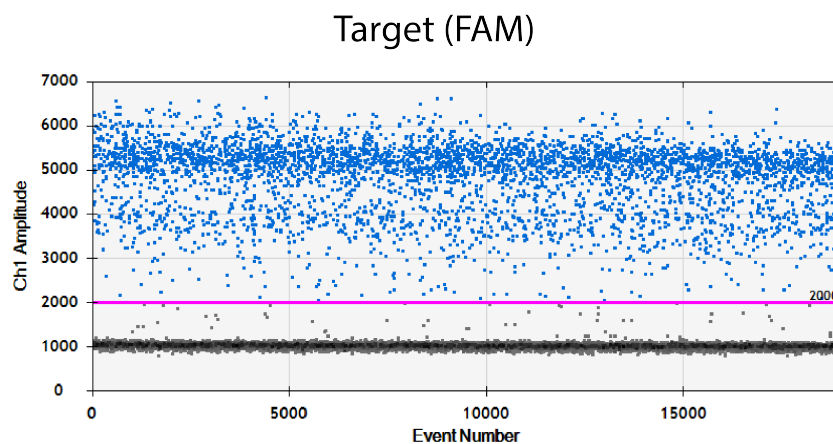


Figure 16. ddPCR 1-D plot [11]

Reagent	Manufacturer	Catalog No.
DNA preparation		
QuickExtract™ DNA Extraction Solution	Biosearch Technologies	76081-768
PCR		
BioMix™ Red	Meridian Life Sciences	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
ddPCR Supermix for Probes (No dUTP)	Bio-Rad	1863024
Droplet Generation Oil for Probes	Bio-Rad	1863005

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

1. Biosearch Technologies. QuickExtract DNA Extraction Solution. Retrieved May 25, 2023, from https://cdn.media.amplience.net/i/biosearchtech/Epicentre-i_gexpccrreadydna.gif?sfvrsn=0.17608973563178687
2. Bio-Rad. Sub-Cell GT Cell. Retrieved May 25, 2023, from https://www.bio-rad.com/sites/default/files/styles/brc_pdp_470x302_/public/webroot/web/images/lsr/products/electrophoresis/product_detail/global/lsr_sub_cell_gt_cell.webp?itok=XEdPGEIY
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](https://www.biorender.com)
4. LI-COR. Odyssey Imagers. Retrieved May 25, 2023, from <https://www.licor.com/images/bio/photos/odyssey-m/odyssey-m-studio-facing-left.png>
5. New England Biolabs. Quick-Load® Purple 1 kb Plus DNA Ladder. Retrieved May 25, 2023, from https://www.neb.com/products/-/media/catalog/gel-photos/n3200_thumb.gif
6. Qiagen. MinElute Gel Extraction Kit. Retrieved May 25, 2023, from <https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/dna-clean-up/minelute-gel-extraction-kit?catno=28604>
7. Bio-Rad. Droplet Digital™ PCR Applications Guide. Retrieved May 25, 2023, from https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6407.pdf
8. Bio-Rad. QX200™ Droplet Generator Instruction Manual. Retrieved May 25, 2023, from <https://www.bio-rad.com/webroot/web/pdf/lsr/literature/10031907.pdf>
9. Bio-Rad. PX1™ PCR Plate Sealer Instruction Manual. Retrieved May 25, 2023, from https://www.bio-rad.com/sites/default/files/webroot/web/pdf/lsr/literature/bulletin_10023997.pdf
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