



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

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## 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 <sup>[1]</sup>

### Primer preparation

1. Resuspend IDT primers with H<sub>2</sub>O to 100 µM
  - Example: 20 nmol – Add 200 µL H<sub>2</sub>O
2. Dilute primer to 10 µM in a new microcentrifuge tube
  - Example: 20 µL + 180 µL H<sub>2</sub>O
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 <sub>2</sub> O	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 T<sub>m</sub> of primers

## Gel electrophoresis

### 1X TAE buffer

1. Add 36 mL 50X TAE Buffer to 2L bottle
2. Add diH<sub>2</sub>O 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

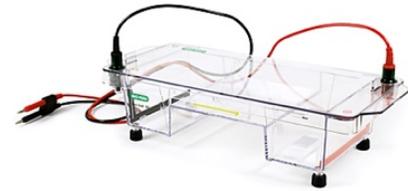


Figure 2. Bio-Rad Sub-Cell GT Cell <sup>[2]</sup>

### 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

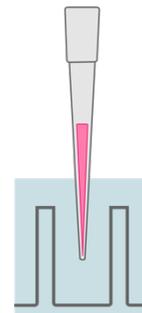


Figure 3. Step 5 – submerging pipette tips in the well <sup>[3]</sup>

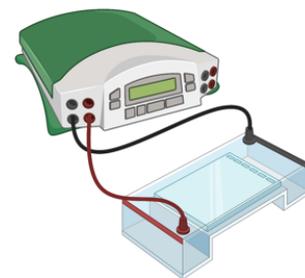


Figure 4. Gel connected to power supply <sup>[3]</sup>

**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 <sup>[4]</sup>

**Note:**

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

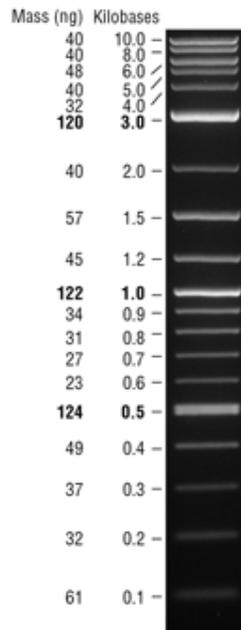


Figure 6. New England BioLabs Quick-Load® Purple 1 kb Plus DNA Ladder on a 1.0% TBE agarose gel <sup>[5]</sup>



### **PCR for sequencing (with gel extraction)**

Run a 50  $\mu\text{L}$  reaction instead of 20  $\mu\text{L}$

#### **Reaction setup**

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

#### **Master Mix – for one 50 $\mu\text{L}$ reaction**

BioMix™ Red	25 $\mu\text{L}$
DMSO	1.5 $\mu\text{L}$
Forward primer (10 $\mu\text{M}$ )	2.5 $\mu\text{L}$
Reverse primer (10 $\mu\text{M}$ )	2.5 $\mu\text{L}$
RNase/DNase-free $\text{H}_2\text{O}$	16 $\mu\text{L}$

#### **PCR Cycle**

	<b>Step</b>	<b>Temperature</b>	<b>Time</b>
1	Enzyme Activation	94°C	3.5 mins
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	$\infty$

**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  $\mu$ L 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  $\mu$ L 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  $\mu$ L 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

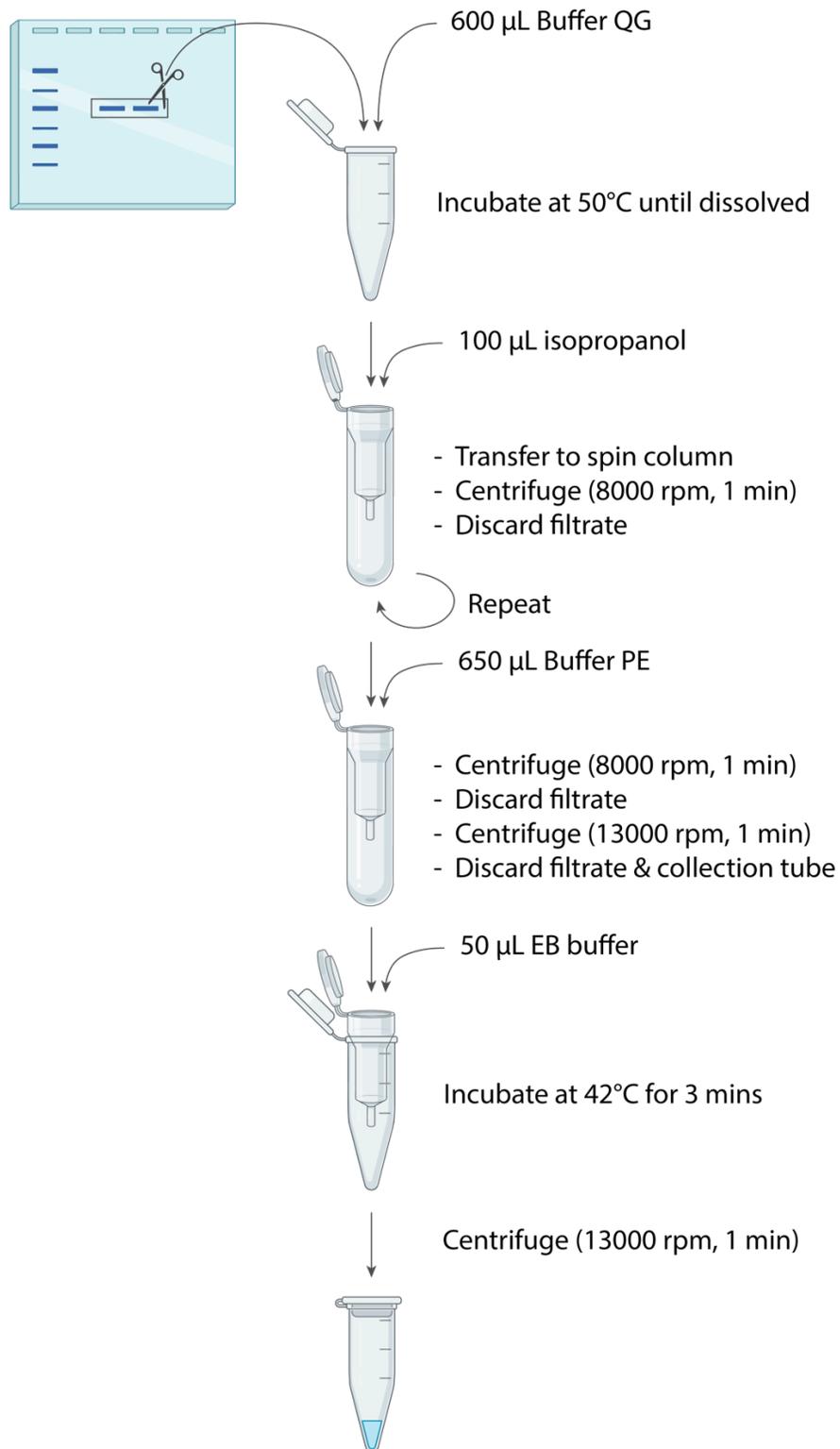


Figure 7. Gel extraction workflow <sup>[3]</sup>



**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  $\mu$ L

**20X target primers/probe**

Forward primer ( <u>100 <math>\mu</math>M</u> )	18 $\mu$ L
Reverse primer ( <u>100 <math>\mu</math>M</u> )	18 $\mu$ L
Target probe ( <b>FAM</b> ) *light sensitive	5 $\mu$ L
RNase/DNase-free H <sub>2</sub> O	59 $\mu$ L

**Note:** Store at -20°C

**Master Mix – 22  $\mu$ L**

2X ddPCR Supermix for Probes (No dUTPs)	11 $\mu$ L
20X target primers/probe ( <b>FAM</b> )	1.1 $\mu$ L
20X reference primers/probe ( <b>HEX/VIC</b> ) – <b>RPP30</b>	1.1 $\mu$ L
RNase/DNase-free H <sub>2</sub> O	Up to 22 $\mu$ L (including DNA sample)

## Droplet generation

1. Insert DG8™ Cartridge into cartridge holder (Figure 8)
2. **FIRST:** Load >20  $\mu\text{L}$  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  $\mu\text{L}$  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)

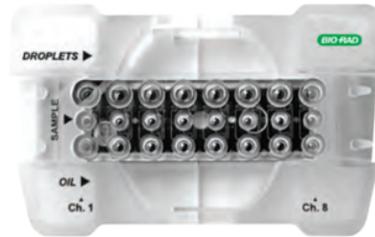


Figure 8. Loaded DG8 Cartridge [7]



Figure 9. Loaded DG8 Cartridge in QX200™ Droplet Generator [8]

## Transfer Droplets (within 5 mins of generation)

1. Transfer 40  $\mu\text{L}$  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

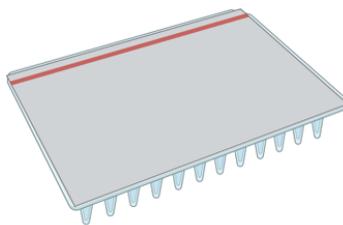


Figure 10. 96-well plate covered with foil seal sheet [3]



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°C/sec
2	Denaturation	94°C	30 sec	
3	Annealing/Extension	*50-65°C	1 min	
4	Repeat from step 2 (39x)			
5	Enzyme Deactivation	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<sup>[10]</sup>

### 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<sub>m</sub>): between 50-65°C, within 5°C of each other
  - o T<sub>m</sub> 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<sub>m</sub>): 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

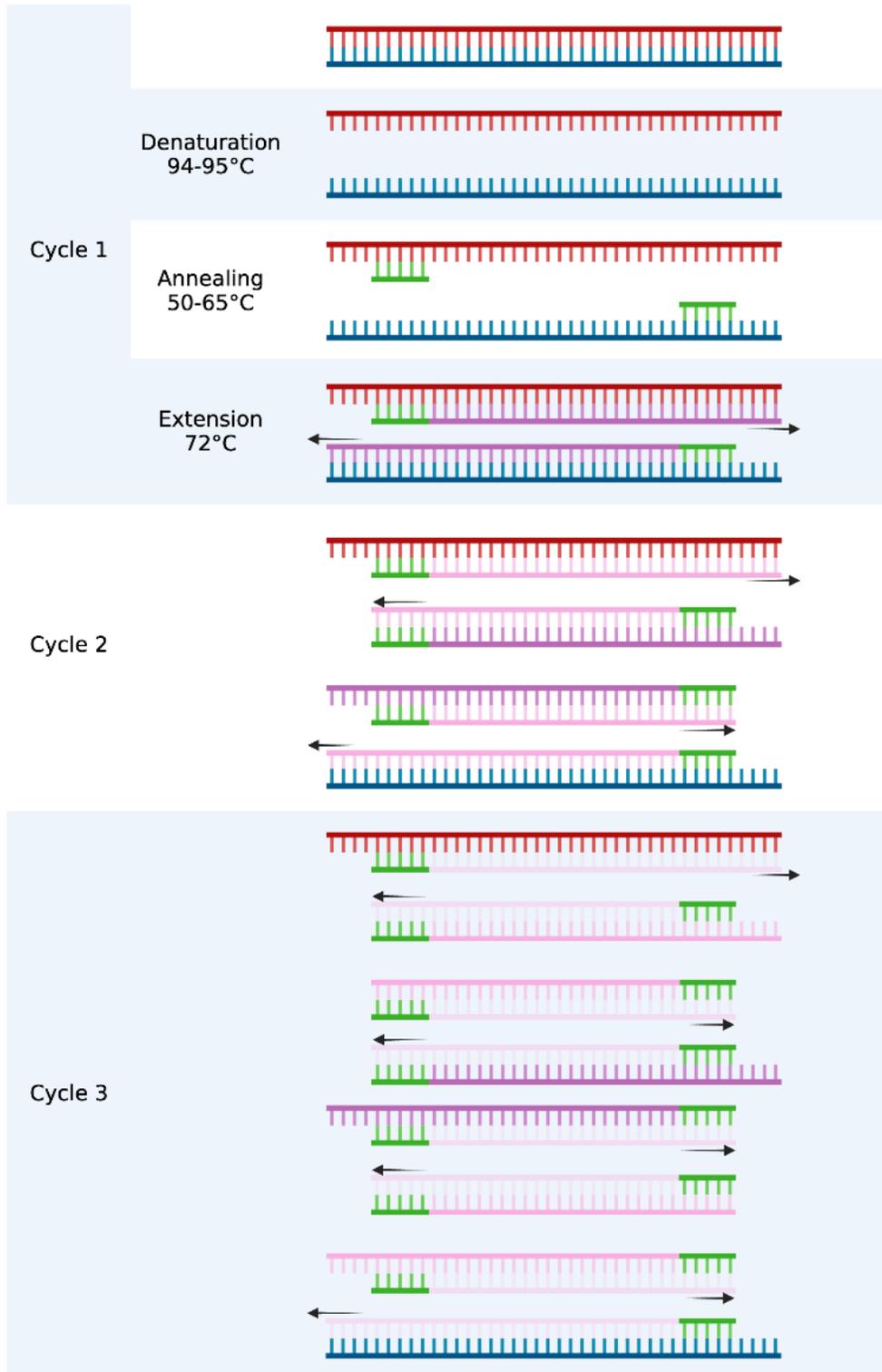
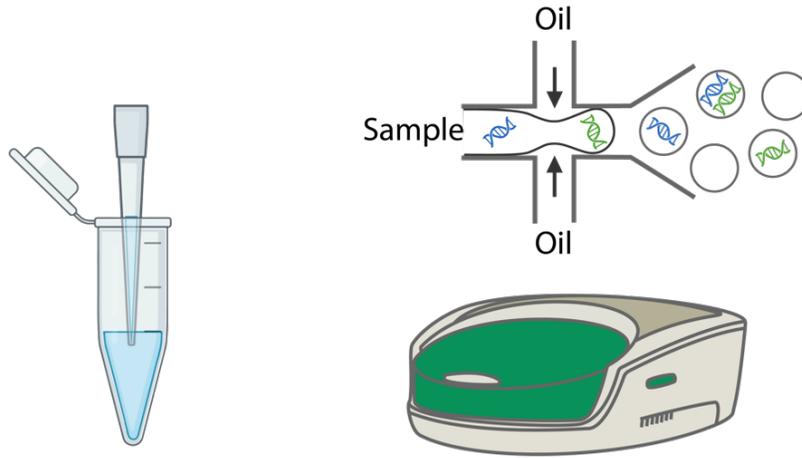


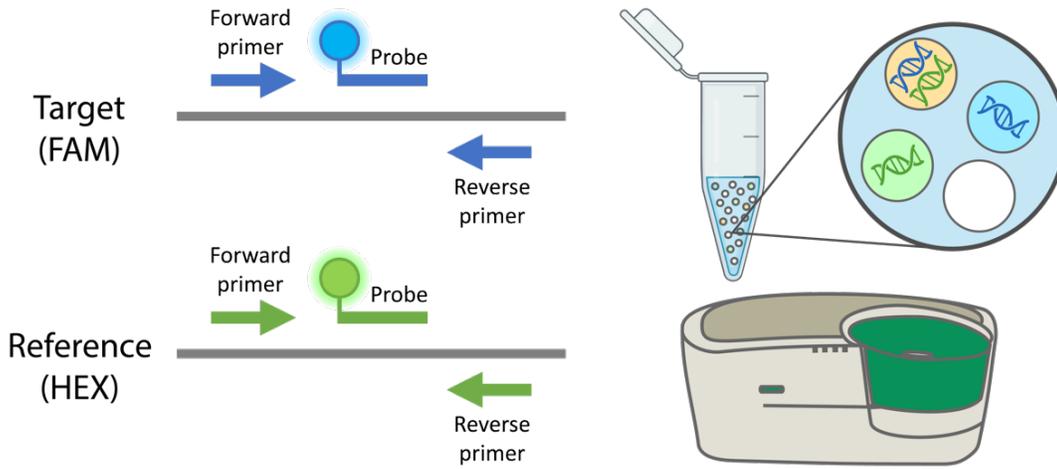
Figure 13. PCR cycles <sup>[3]</sup>

**More about ddPCR...**



1. Sample preparation

2. Droplet generation



3. PCR

4. Read droplets

Figure 14. ddPCR workflow <sup>[3]</sup>

## Data analysis

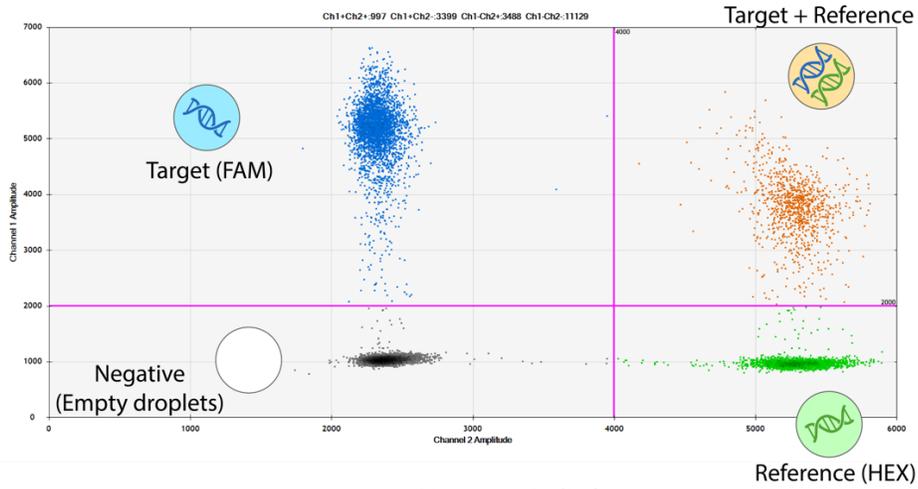
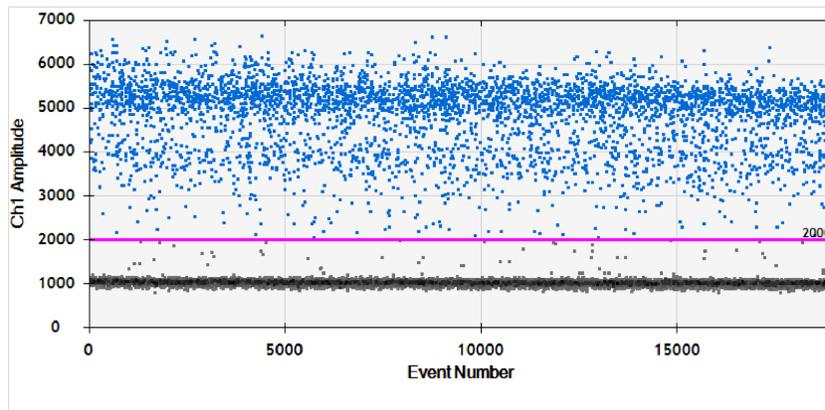


Figure 15. ddPCR 2-D plot [3,11]

### Target (FAM)



### Reference (HEX)

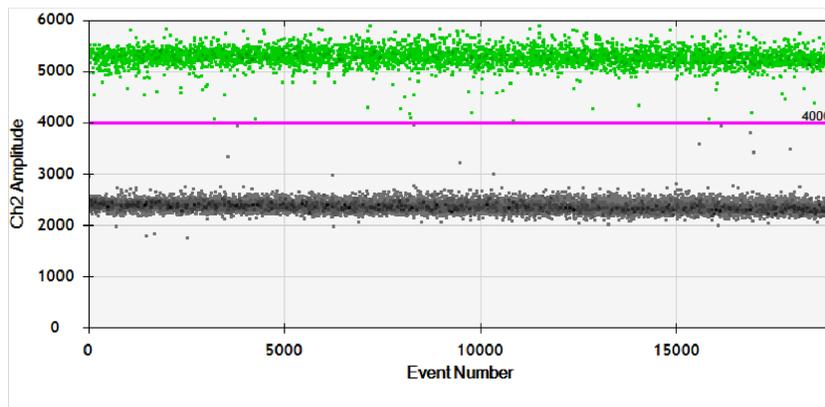


Figure 16. ddPCR 1-D plot [11]



Reagent	Manufacturer	Catalog No.
<b>DNA preparation</b>		
QuickExtract™ DNA Extraction Solution	Biosearch Technologies	76081-768
<b>PCR</b>		
BioMix™ Red	Meridian Life Sciences	C755F25
Dimethyl sulfoxide (DMSO)	Sigma Aldrich Fine Chemicals Biosciences	D8418
<b>Gel electrophoresis</b>		
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
<b>ddPCR</b>		
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.
<b>PCR</b>		
Thermal Cycler	Bio-Rad	
<b>Gel electrophoresis</b>		
LI-COR Odyssey M Imager	LI-COR	
PowerPac™ HC Power Supply	Bio-Rad	
Sub-Cell GT Horizontal Electrophoresis System	Bio-Rad	
<b>ddPCR</b>		
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 <sup>[3]</sup>, Figure 14 are created with [BioRender.com](https://www.biorender.com)
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11. Data from Figures Figure 15-Figure 16 are generated from Bio-Rad QuantaSoft™ Analysis Pro Software