

Droplet Digital PCR guide

INSTRUMENTS: QX200™ Droplet Generator, QX200™ Droplet Reader, and PX1™ PCR Plate Sealer

Manufacturer: Bio-Rad Laboratories

Instrument specialist: Pelle Larshammar,
email: per_larshammar@bio-rad.com,
tel: +46 (0)8-555 127 00, mobile: +46 (0)709-27 97 22

Technical support: techsupport.nordic@bio-rad.com

Owner: Tampere University / Laboratory Services (LAS)

Location: ARVO E216 / E241

Responsible persons:

Technical: Ulla Kala, tel. 050 326 5988, email: ulla.kala@tuni.fi

Marjo Salminen, tel. 040 190 1507, email: marjo.salminen@tuni.fi

Academic: Annika Kohvakka, email: annika.kohvakka@tuni.fi

(Heini Kallio, email: heini.kallio@tuni.fi)

Reservations for QX200 Droplet Reader: (*billing according to reservations*) in AGENDO:

<https://next.cirklo.org/biomeditech/>. Allow at least 10 min for set up and 1.5 min per sample for Droplet Reading.

REAGENTS:

- QX200™ ddPCR™ EvaGreen Supermix (Bio-Rad)
- ddPCR™ Supermix for Probes (No UTP) (Bio-Rad)

Supermixes are stable through the expiration date printed on the label when stored in a freezer at a constant temperature of -20°C and protected from light. Once thawed, it can be stored at 4°C for up to 2 weeks. Repeated freezing and thawing of the supermix is not recommended.

Location: ARVO E242 freezer

MANUALS:

All the manuals for instruments and reagents can be found in the device folder situated next to the QX200 Droplet Reader (E216), in *arvolab* folder (N:\arvolab\Laitemanuaaleja ja ohjeita\ddPCR), or on Bio-Rad's website (www.bio-rad.com/en-fi/category/digital-pcr).

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REAGENTS AND INSTRUMENTS

1. Materials provided

Name	Catalog #	Step	Location
QX200™ ddPCR™ EvaGreen Supermix	1864033/4/5/6	Sample preparation	-20°C, E242
ddPCR™ Supermix for Probes (No UTP)	1863023/4/5	Sample preparation	-20°C, E242
QX200™ Droplet Generation Oil for EvaGreen	1864005/6	Droplet generation	RT, E241
Droplet Generation Oil for Probes	1863005	Droplet generation	RT, E241
DG8™ Cartridges	1864008	Droplet generation	RT, E241
DG8™ Gaskets	1863009	Droplet generation	RT, E241
ddPCR™ 96-Well Plates	12001925	Preparation for PCR	RT, E241
Finntip Filter 100 µl tips	94052200	Preparation for PCR	RT, E241
PCR Plate Heat Seal, foil, pierceable	1814040	Preparation for PCR	RT, E241
ddPCR™ Droplet Reader Oil	1863004	Droplet reading	RT, E241

2. Materials required but not provided

Name	Supplier	Step	Notes
RNase/DNase-free tubes/plates	any	Sample preparation	Available in PCR rooms
RNase/DNase-free water	any	Sample preparation	e.g. Milli-Q water
Primers	any	Sample preparation	
Probes	many	Sample preparation	Non-fluorescent quenchers preferred. Not for EvaGreen.
DNA sample		Sample preparation	e.g. genomic DNA (gDNA) or complementary DNA (cDNA)
Pipettes and tips	many	Sample preparation	Do not use ART pipet tips (Thermo Fisher)!
Vortex	any	Sample preparation	Available in PCR rooms
Table centrifuge/spinner	any	Sample preparation	Available in PCR rooms

3. Instruments

Name	Catalog #	Step	Location
QX200™ Droplet Generator	1864002	Droplet generation	E241
Droplet generator cartridge holders	1863051	Droplet generation	E241
Finnpipette F2, 8-channel pipette, 5-50 µl	4662010	Preparation for PCR	E241
PX1™ PCR Plate Sealer	1814000	Preparation for PCR	E241
Plate support block	1814085	Preparation for PCR	E241
Thermal Cycler (Specifications required: ○ Accuracy: +/- 0.2°C ○ Uniformity: +/- 0.4°C well-to-well within 10 sec ○ Adjustable ramp capability with required ramp rate: up to 2°C/sec ○ Temperature range: 0-100°C)	various	PCR	e.g. T100™ Thermal Cyclers in E208
QX200™ Droplet Reader	12001045	Droplet reading	E216
Droplet reader plate holders	51010608	Droplet reading	E216

DESIGNING EXPERIMENTS

1. Sample design

- **The minimum number of samples is 8**, and the total number of samples should be divisible by 8 (for example 8-16-24-...-96). If you have less than 8 samples, add more negative controls.
- **Do not load too much DNA**, as empty droplets are essential for quantification.
 - Absolute quantification and gene expression:
 - Genomic DNA (**gDNA**): 50 fg to 100 ng; 0.1-100 ng optimal with EvaGreen.
 - Complementary DNA (**cDNA**): up to 50 ng of reverse transcribed RNA.
 - If you have measured the samples with qPCR, Ct values should be >21.
 - CNV analysis: If diploid target copy number is expected to be ≤ 10 , add ≤ 50 ng of gDNA; 10-50 copies: ≤ 15 ng of gDNA; >50 copies: strategies using multiple wells can be used.
 - Rare mutation detection: for most routine mutation detection applications, up to 130 ng of human gDNA can be added per reaction for a final concentration of 2,000 copies/ μ l.
- **DNA fragmentation** by restriction digestion prior to droplet generation enables optimal accuracy by separating tandem gene copies, reducing sample viscosity, and improving template accessibility.
 - Especially suggested when loading >66 ng of human gDNA per reaction.
 - CNV and absolute quantification: always do digestion at all DNA concentrations unless you want to access proximal replicate sequences.
 - Fragmentation not needed for cDNA.
 - Already fragmented DNA (FFPE samples, cfDNA, liquid biopsies etc.) may not need digestion.
- **Add at least one negative control** sample without template per 8 samples.
 - No template control (NTC) for all experiments; add water instead of DNA
→ controls cleanliness of reagents and process.
 - Rare mutation detection: a negative control containing only wild-type DNA at a concentration similar to the unknown samples
→ estimates false positives and purity of controls.
- When possible, **add a positive control sample** at a concentration similar to the known samples.
 - Rare mutation detection: run a mutation-positive control with a wild-type DNA background (for example, a mix of 7 ng of mutant DNA in a background of 130 ng wild-type DNA).
→ expected cluster locations
- **No need for technical replicates**. Technical replicates might be useful to do, when the expression of your target is known to be extremely low (e.g. >32 Ct values in qPCR).
- For gene expression studies,
 - there is **no need for a standard curve** or a calibrator sample.
 - run at least one **reference gene** (e.g. a housekeeping gene) or add a consistent amount of synthetic DNA to normalize the variations in DNA loading.

2. Assay design

- QX200 System is compatible only with hydrolysis probes and EvaGreen dsDNA binding dye.
NOTE: no SYBR Green.
 - Two fluorescence detection channels, channel 1: FAM and channel 2: HEX or VIC.
- Primer/probes
 - length: 15-35 nucleotides, optimal at 17-25 nucleotides.
 - GC content of 50-60%.
 - melting temperature (T_m):
 - primers: 50-70°C, optimal at 55-65°C .
 - probes: 3-10°C higher than that of the primers.
 - avoid secondary structure, G/C repeats longer than 3 nt, 3' end complimentary b/w primer pairs.
 - non-fluorescent quenchers preferred (e.g. Black Hole Quencher or Iowa Black)
 - for TAMRA quenchers, duplex assay is not possible as only FAM channel can be used for detection
- Amplicon length: 50-250+, optimal at 55-100 nucleotides. Shorter length improves quantification of fragmented or damaged DNA samples.
 - GC content of 40-60%.
- Single nucleotide polymorphism (SNP) detection or detecting single nucleotide differences: T_m at 55°C, short amplicon length <90 bp, and a shorter probe prevents cross-reactivity. Always adjust the WT and mutant probe T_m s to match. Poor amplitude will improve with stronger primers.
- Copy number variation assays benefit from matching unknown and reference amplicon lengths, especially with fragmented DNA.

3. Timetable

Step	Page	Description	Estimated hands on time	Estimated instrument time
1	5-9	Sample preparation	~0.5-2 hours (8 to 96 samples)	N/A
2	10-11	Droplet generation	<5 min for 8 samples	~2 min per cartridge
3	12	Preparation for PCR	<5 min for 8 samples	5 sec
4	13	PCR	<5 min	~2 hours
5	14-15	Droplet reading	<15 min	~1.5 min per sample
		In total	~3 hours for 8 samples, ~8 hours for 96 samples	

More info on designing experiments, see “Droplet Digital PCR Applications Guide” (Chapter 2).

QUICK GUIDE

1. Sample preparation (preferably in PCR rooms: E241/242 and E316/317)

- Combine ddPCR Supermix (no SYBR Green), primers/probes, and DNA sample. Vortex thoroughly and spin down.
- Prepare ddPCR reaction mix for at least 8 samples (8-16-24- ... -96), and for 1.25x as many wells as you plan to run.
- Prepare at least 1 negative control per 8 samples.
- The total volume of the reaction mixture in each tube should be at least 22 µl.

2. Droplet generation (in E241)

- a. Insert droplet generator cartridge into the holder.
- b. Transfer 20 µl of each prepared sample to middle row of the cartridge.
- c. Transfer 70 µl of droplet generator oil to bottom row of the cartridge. **NOTE THAT THERE ARE DIFFERENT OILS FOR EVAGREEN AND PROBES!**
- d. Hook the gasket over the cartridge holder.
- e. Place the cartridge holder into the instrument and push the button. When droplet generation is complete, all 3 indicator lights are solid green.

3. Preparation for PCR (in E241)

- a. Pipette 40 µl of the contents of the top wells of cartridge into a single column of a 96-well PCR plate.
 - Place the PCR plate on top of the PX1 plate support block.
 - Draw droplets slowly with an 8-channel pipette at a ~30-40° angle.
 - To dispense droplets, position the pipet tip along the side of the well and push slowly.
 - Do multiple rounds if necessary. Discard the empty cartridge.
- b. Cover each row on the PCR plate with a tape, lid or similar after each cartridge.
- c. Turn on the PX1 PCR plate sealer. Check that there is no support block inside the sealer.
- d. Seal the PCR plate with one pierceable foil in PX1 PCR plate sealer (180°C, 5 s) on the support block. The stripe on the foil should face up.
- e. Begin PCR within 30 min or store the plate at 4°C for <4 h. **DO NOT SPIN THE PLATE!**

4. PCR

- Select a thermal cycler with an adjustable ramp rate, e.g. T100 Thermal Cycler (in E208).
- Follow the cycling conditions in Supermix manuals (annealing can be changed).
- **DO NOT SPIN THE PLATE BEFORE OR AFTER PCR!**

5. Droplet reading (in E216; reservation in **Agendo**)

- a. Switch on the QX200 Droplet Reader and open the QuantaSoft software. Check that the indicator lights are green, not amber. Check the logbook; if used last time >1 week ago, prime the reader.
- b. Place the PCR plate into the plate holder (A1 top left), press the **release tabs down**, and load the holder into the droplet reader.
- c. In QuantaSoft, click **Setup**. Load Template or create a new. → Double-click samples to open well editor. → Adjust Sample and Target panels of each well you plan to read. **Supermix selection cannot be modified after data collection!**
- d. Click **Run**. Select the correct channel and click OK.
- e. When droplet reading is complete, remove the plate holder and discard the PCR plate.
- f. Fill in the logbook and switch off all the instruments.

PROTOCOL

1. Sample preparation

To limit workspace contamination, work in PCR rooms (E241/242 and E316/317). Follow the room instructions (can be found inside the rooms).

1.1. Absolute quantification and gene expression

IMPORTANT: Use only Bio-Rad's ddPCR Supermixes (core is providing Supermixes for **EvaGreen** and **Probes**), **no SYBR Green!** SYBR Green will break the reader!

IMPORTANT: If you use other ddPCR Supermixes than those that the ddPCR core is providing, use the manufacturer's protocol for the sample preparation.

1. Thaw all components at room temperature. If necessary, prepare DNA samples on ice, but equilibrate the final prepared sample to room temperature. Mix thoroughly by inverting the tube several times to ensure homogeneity, and centrifuge briefly.
2. Prepare ddPCR reaction mix for at least 8 samples. Calculate the amounts of components you need (**Table 1** for **EvaGreen** and **Table 2** for **Probes**). *Note: you can use ready-made ddPCR template for calculation (found in arvolab-folder).*
 - a. When running different samples, assemble all required components except the DNA sample in the same tube.
 - i. Prepare enough reaction mixture for **1.25x as many wells as you plan to run.**
 - ii. Mix thoroughly by vortexing or pipetting up and down 5-10 times, and centrifuge briefly.
 - iii. Dispense equal aliquots into reaction tubes and add the DNA sample to each reaction tube.
 - iv. **The total volume of the reaction mixture in each tube should be at least 22 μ l.**
 - b. When running identical samples, assemble a common reaction mix with all required components and DNA sample in the same tube. Prepare enough reaction mixture for **1.25x as many wells as you plan to run.**
 - c. Prepare at least one negative control, where you add RNase/DNase-free water instead of DNA sample (called NTC = no template control). If you have less than 7 DNA samples to run, you can include more NTCs.
3. Allow reaction tubes to equilibrate at room temperature for about 3 min, vortex thoroughly, and spin down assembled reaction.

→ Next, see **page 10** for "Droplet generation".

1.2. Copy number variation (CNV)

1. Thaw all components at room temperature. If necessary, prepare DNA samples on ice, but equilibrate the final prepared sample to room temperature. Mix thoroughly by inverting the tube several times to ensure homogeneity, and centrifuge briefly.
2. Prepare ddPCR reaction mix for at least 8 samples. Calculate the amounts of components you need (**Table 3**).
 - a. When running different samples, assemble all required components except the DNA sample in the same tube. *Note: you can use ready-made ddPCR template for calculation (found in arvolab-folder).*
 - i. Prepare enough reaction mixture for **1.25x as many wells as you plan to run.**
 - ii. Mix thoroughly by vortexing or pipetting up and down 5-10 times, and centrifuge briefly.
 - iii. Dispense equal aliquots into reaction tubes and add the DNA sample to each reaction tube.
 - iv. **The total volume of the reaction mixture in each tube should be at least 22 µl.**
 - b. When running identical samples, assemble a common reaction mix with all required components and DNA sample in the same tube. Prepare enough reaction mixture for **1.25x as many wells as you plan to run.**
 - c. Prepare at least one negative control, where you add RNase/DNase-free water instead of DNA sample (called NTC = no template control). If you have less than 7 DNA samples to run, you can include more NTCs.
3. Allow reaction tubes to equilibrate at room temperature for about 3 min, vortex thoroughly, and spin down assembled reaction.

→ Next, see **page 10** for “Droplet generation”.

1.3. Rare mutation detection

4. Thaw all components at room temperature. If necessary, prepare DNA samples on ice, but equilibrate the final prepared sample to room temperature. Mix thoroughly by inverting the tube several times to ensure homogeneity, and centrifuge briefly.
5. Prepare ddPCR reaction mix for at least 8 samples. Calculate the amounts of components you need (**Table 4**).
 - a. When running different samples, assemble all required components except the DNA sample in the same tube. *Note: you can use ready-made ddPCR template for calculation (found in arvolab-folder).*
 - i. Prepare enough reaction mixture for **1.25x as many wells as you plan to run.**
 - ii. Mix thoroughly by vortexing or pipetting up and down 5-10 times, and centrifuge briefly.
 - iii. Dispense equal aliquots into reaction tubes and add the DNA sample to each reaction tube.
 - iv. **The total volume of the reaction mixture in each tube should be at least 22 μ l.**
 - b. When running identical samples, assemble a common reaction mix with all required components and DNA sample in the same tube. Prepare enough reaction mixture for **1.25x as many wells as you plan to run.**
 - c. Prepare at least one mutation-negative control containing only wild-type DNA at a concentration similar to the unknown samples.
 - d. Run a mutation-positive control with a wild-type DNA background at a concentration similar to the unknown samples (for example, a mix of 7 ng of mutant DNA in a background of 130 ng wild-type DNA).
6. Allow reaction tubes to equilibrate at room temperature for about 3 min, vortex thoroughly, and spin down assembled reaction.

→ Next, see **page 10** for “Droplet generation”.

Table 1. EvaGreen reaction mix components

Component	Volume per Reaction, μ l	Final Concentration
2x QX200 ddPCR EvaGreen supermix	11	1x
Forward primer	Variable	100–250 nM
Reverse primer	Variable	100 nM
<i>Optional:</i> Restriction enzyme, diluted (see DNA digestion below)	1.1	Variable
DNA template* and RNase/DNase-free water	Variable	50 fg to 100 ng*
Total volume	22	--

***gDNA** amount: <66 ng per 20 μ l; if higher, digest DNA. **cDNA** amount: up to 50 ng of initial RNA, and <10% of the PCR reaction volume; generally 1 ng of RNA is sufficient.

Table 2. Probe reaction mix components

Component	Volume per Reaction, μ l	Final Concentration
2x ddPCR supermix for probes (no dUTP)	11	1x
20x target primers/probe (FAM)	1.1	900 nM / 250 nM
<i>Optional:</i> 20x reference primers/probe (HEX/VIC)	1.1	900 nM / 250 nM
<i>Optional:</i> Restriction enzyme, diluted (see DNA digestion below)	1.1	Variable
DNA template* and RNase/DNase-free water	Variable	50 fg to 100 ng*
Total volume	22	--

***gDNA** amount: <66 ng per 20 μ l; if higher, digest DNA. **cDNA** amount: up to 50 ng of initial RNA, and <10% of the PCR reaction volume; generally 1 ng of RNA is sufficient.

Table 3. CNV ddPCR reaction mix components

Component	Volume per Reaction, μ l	Final Concentration
2x ddPCR supermix for probes (no dUTP)	11	1x
20x target primers/probe (FAM)	1.1	900 nM / 250 nM
20x wild type primers/probe (HEX/VIC)	1.1	900 nM / 250 nM
<i>Optional:</i> Restriction enzyme, diluted (see DNA digestion below)	1.1	Variable
DNA template* and RNase/DNase-free water	Variable	\leq 50 ng/well*
Total volume	22	--

* If diploid target copy number is expected to be \leq 10: add \leq 50 ng of gDNA; 10-50 copies: add \leq 15 ng of gDNA; >50 copies: strategies using multiple wells can be used.

Table 4. Mutation detection ddPCR reaction mix components

Component	Volume per Reaction, μ l	Final Concentration
2x ddPCR supermix for probes (no dUTP)	11	1x
20x target primers/probe (FAM)	1.1	450 nM / 250 nM
20x wild type primers/probe (HEX)	1.1	450 nM / 250 nM
<i>Optional:</i> Restriction enzyme, diluted (see DNA digestion below)	1.1	2-5 U per reaction
DNA template* and RNase/DNase-free water	Variable	50 fg to 100 ng*
Total volume	22	--

*For most routine mutation detection applications, up to 130 ng of human gDNA can be added per ddPCR well for a final concentration of 2000 copies/ μ l.

Optional steps:

- DNA digestion in ddPCR reaction
 - Efficient digestion of sample DNA can be achieved by direct addition of restriction enzymes to the ddPCR reaction
 - The concentration of intact human genomic DNA (gDNA) should be <66 ng per 20 μ l reaction. If using higher concentrations, digest DNA with a restriction endonuclease that does not cut target or reference amplicons.
 - Dilute the restriction enzyme using the recommended diluent buffer according to manufacturer instructions and add 1 μ l to the ddPCR reaction; ~2–5 units of restriction enzyme per 20 μ l ddPCR reaction are recommended.
 - The reaction can be set up at room temperature and no additional incubation time is required.
 - The addition of restriction enzyme buffers with high salt can inhibit ddPCR and should be avoided.
 - HaeIII, MseI, AluI, HindIII, and CviQI have been observed to work well to digest in ddPCR reactions.

- DNA digestion prior to ddPCR
 - Restriction enzyme digestion can be carried out as a separate reaction before ddPCR reaction setup.
 - When loading >130 ng of DNA per well, digestion prior to ddPCR is recommended.
 - Use 5–10 enzyme units per microgram of DNA, and 10–20 enzyme units per microgram of genomic DNA.
 - Incubate the reaction for 1 h at the temperature recommended for the restriction enzyme
 - Heat inactivation is not required, but can be considered if long-term storage is required; do not heat inactivate above 65°C.
 - DNA purification is not necessary after restriction digestion.
 - Use a minimum tenfold dilution of the digest to reduce the salt content of the sample in the ddPCR reaction.
 - Store digested DNA at –20°C or below.

NOTE: no digestion for complementary DNA (cDNA). Additionally, already fragmented DNA (FFPE samples, cfDNA, liquid biopsies etc.) may not need digestion.

- Uracyl N-glycosylase (UNG)
 - Can be used to prevent carryover contamination.
 - Compatible with “QX200 ddPCR EvaGreen supermix”, but not with “ddPCR Supermix for Probes (no dUTP)”; UNG compatible “ddPCR Supermix for Probes” (Cat.# 1863026) can be purchased from Bio-Rad.
 - UNG is not provided by the ddPCR core service and can be purchased from a licensed supplier.

2. Droplet generation

The QX200 Droplet Generator can be found in one of the PCR cabinets in E214. Clean the cabinet before and after use as advised, but **DO NOT USE UV LIGHT**, as it will deteriorate the instrument. UV light will be used periodically by the ddPCR core personnel.

1. Power on the droplet generator by plugging in the power chord. There is no ON/OFF switch.
2. Insert the DG8 droplet generator cartridge into the holder with the notch in the cartridge at the upper left of the holder (**Figure 1**).
 - a. Open the cartridge holder by pressing the latches in the middle.
 - b. Slide the cartridge into the right half of the holder, then drop it down.
 - c. Press the halves of the holder together to snap it closed.
3. Transfer 20 µl of each prepared sample to the wells (middle row) of the cartridge. Avoid creating air bubbles by
 - a. gently sliding the pipet tip down the side of the wall at a ~15° angle until it passes over the ridge near the bottom (**Figure 2**). Holding the angle, ground the pipet top against the bottom edge of the sample well while slowly dispensing a small portion of the sample; do not pipet directly onto the side (wall) of the well.
 - b. after dispensing about half of the sample, slowly draw the tip up the wall while dispensing the rest of the sample; do not push the pipet plunger past the first stop.
4. Transfer 70 µl of droplet generator oil to the oil wells (bottom row) of the cartridge. **NOTE: there are different oils for EvaGreen and Probes!**
5. Hook the gasket over the cartridge holder using the holes on both sides. The gasket must be securely hooked on both ends of the holder (**Figure 3**).
6. Open the QX200 droplet generator by pressing the button on the green top and place the cartridge holder into the instrument. When the holder is in the correct position, both the power (left light) and holder (middle light) indicator lights are green.
7. Press the button on the top again to close the door. This initiates droplet generation. The droplet indicator light (at right) flashes after 10 sec to indicate droplet generation is in progress.
8. When droplet generation is complete, all three indicator lights are solid green. Open the door by pressing the button and remove the holder from the unit. Remove the disposable gasket from the holder and discard it. **The top wells of the cartridge contain droplets.**
9. Unplug the droplet generator. There is no ON/OFF switch.

IMPORTANT:

- ✓ All 8 sample wells in the cartridge must contain sample, and all 8 oil wells must contain droplet generator oil!
- ✓ Do not load sample or oil into the cartridge unless it is inserted in the holder!

→ Next, see **page 12** for “Preparation for PCR”.

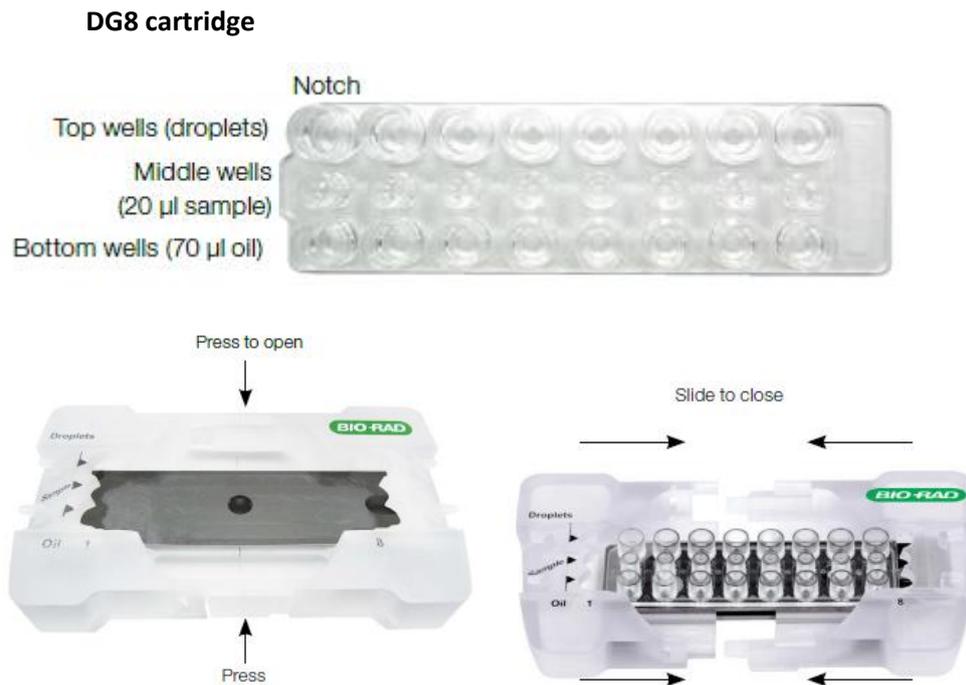


Figure 1. Inserting the DG8 cartridge into the cartridge holder.

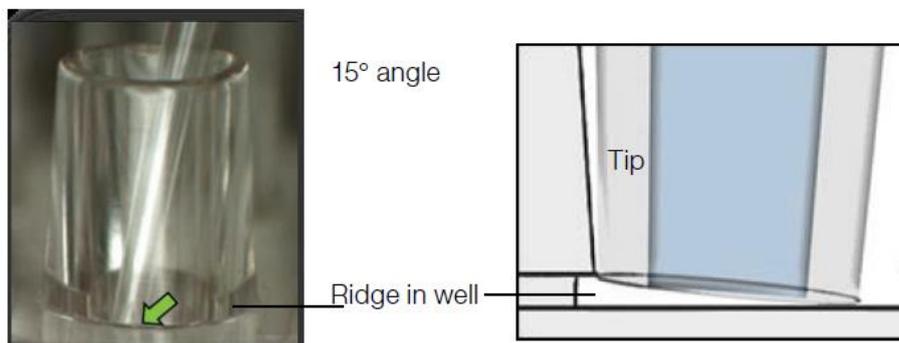


Figure 2. Pipetting sample to the wells of the DG8 cartridge.

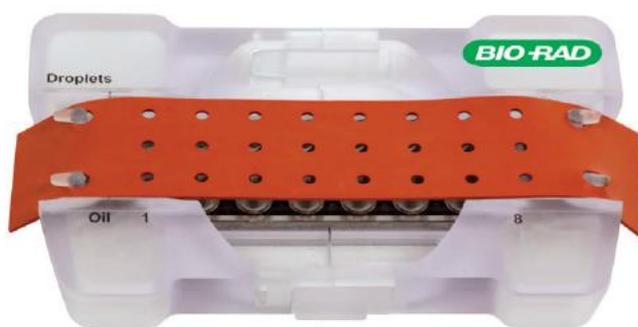


Figure 3. Correct placement of the gasket over the cartridge holder.

3. Preparation for PCR

1. Pipette 40 µl of the contents of the top wells of DG8 cartridge (the droplets) into a single column of a 96-well PCR plate. Use a manual 8-channel Finnpiquette F2 (5-50 µl) pipette with 100 µl Finntip Filter tips. **If using multiple supermixes, separate them on different PCR plates.**
 - a. Plate the PCR plate on top of the PX1 plate support block.
 - b. Place the cartridge holder on a flat surface and position the pipet tips in each of the 8 top wells at a ~30-40° angle, vertical into the junction where the side wall meets the bottom of the well. Do not position the pipet tip in a vertical orientation (90°) or against any flat surface of the well; do not allow the tips to be flat against the bottoms of the wells
 - c. Slowly draw 40 µl of the droplets into the pipet tip (should take ~5 sec, and ~5 µl air is expected); do not aspire >40 µl, as this causes air to percolate through the droplets.
 - d. To dispense droplet into the 96-well plate, position the pipet tip along the side of the well – near, but not at, the bottom of the well – and slowly dispense the droplets (~5 sec).
 - e. Do multiple rounds if necessary. Do not pipette the droplets back into the cartridge if you did not succeed to draw all the droplets at one go. Discard the empty cartridge.
 - f. To prevent evaporation and contamination with particulates, cover the plate (e.g. with tape, 8-cap strip, or the lid from a pipet tip box) as you work.
2. Seal the PCR plate with foil immediately after transferring droplets to avoid evaporation.
 - a. Set the PX1 PCR plate sealer temperature to 180°C and time to 5 sec. Check that there is no plate support block inside the sealer.
 - b. Touch the arrow to open the PX1 tray door. Position the support block on the tray with the 96-well side facing up. Place the 96-well plate onto the support block.
 - c. Cover the 96-well plate with one sheet of pierceable foil seal.
 - i. The stripe on the foil should face up. Additionally, the yellow label on the heat seal bag identifies the sealing surface.
 - ii. Do not attempt to place the frame over the foil-covered plate. The frame is only for use with other seals.
 - d. Touch the seal button. The tray will close, and heat sealing will initiate.
 - e. The PX1 door opens automatically, when heat sealing is complete. Remove the plate from the block for thermal cycling. Remove the block from the PX1 and switch off the sealer.

IMPORTANT: DO NOT SPIN THE PLATE!

→ Begin thermal cycling (see “PCR” on **page 13**) within 30 min of sealing the plate or store the plate at 4°C for up to 4 hours prior to thermal cycling.

4. PCR

IMPORTANT: If you use other ddPCR Supermixes than those that the ddPCR core is providing, use the manufacturer's cycling protocol for the PCR.

1. Program thermal cycling protocol on the thermal cycler (e.g. T100™ in room E208). Protocols for absolute quantification and gene expression with **EvaGreen** in **Table 5**, and with **Probes** in **Table 6**. Protocols for **Rare mutation detection** and for **CNV analysis** in **Table 6**.
 - a. Use only thermal cyclers where you can adjust the ramp rate to max 2.0°C/sec for every step.
 - b. Use a heated lid set to 105°C and set the sample volume to 40 µl.
2. Load the PCR plate onto the thermal cycler and start the PCR run. After PCR, remove the plate from the thermal cycler. **IMPORTANT: DO NOT SPIN THE PLATE!**

→ Next, see **page 14** for “Droplet reading”, or store the plate for up to 24 hours (**EvaGreen**) / 48 hours (**Probes**) at 4°C.

Table 5. Cycling protocol for **EvaGreen**.

Cycling Step	Temperature, °C	Time	Ramp Rate	# Cycles
Enzyme activation	95	5 min	~2°C/sec	1
Denaturation	95	30 sec		40
Annealing/extension*	60*	1 min		1
Signal stabilization	4	5 min		1
	90	5 min		1
Hold (optional)	4	Infinite		1

*Annealing/extension temperature can be adjusted for the optimal temperature of your assays.

Table 6. Cycling protocol for **Probes**, **Rare mutation detection**, and **CNV analysis**.

Cycling Step	Temperature, °C	Time	Ramp Rate	# Cycles
Enzyme activation	95	10 min	~2°C/sec	1
Denaturation	94	30 sec		40
Annealing/extension*	60*	1 min		1
Enzyme deactivation	98	10 min		1
Hold (optional)	4	Infinite		1

*Annealing/extension temperature can be adjusted for the optimal temperature of your assays.

5. Droplet reading

IMPORTANT: Reserve the Droplet Reader in Agendo before use!

1. Power on the QX200 Droplet Reader using switch at the back. Switch on the PC and launch QuantaSoft software.
2. Check the indicator lights on the front of the droplet reader (**Table 5**). The first two lights at left should be solid green. If the second light is flashing green, you can start the run. **If the lights are flashing amber, the run cannot be started**; clean out the waste bottle or replace the oil (see p. 18).
3. Check the logbook if the reader has been used within 1 week. If not, prime the reader (see p. 18).
4. Place the 96-well PCR plate into the plate holder (Figure 4).
 - a. Place the 96-well PCR plate containing the amplified droplets into the base of the plate holder. **Well A1** of the PCR plate (the only non-cut corner) must be in the **top left** position.
 - b. Move the release tabs of the top of the plate holder into the “up” position and place the top on the PCR plate. **Firmly press both release tabs down** to secure the PCR plate in the holder.
5. Press the button on the green lid to open the droplet reader. Load the plate holder into the droplet reader and press the button on the lid again to close the cover. Confirm the first two indicator lights are green (**Table 5**).
6. In QuantaSoft software, click Setup in the left navigation bar to define your experiment. For detailed instructions, see the manual for QX200 Droplet Reader (Section 3).
 - a. Open a saved template (**Template > Load**) or create a new one (**Template > New** or if you already have a blank template, move to the next step). If you want to modify an old template but keep the original, load the old template, make modifications, and save the modified template (**Template > Save As**).
 - b. **Double-click on the well you wish to edit** to open the well editor. Use Shift or Ctrl to select multiple wells; to select whole columns/rows, click the column number/row letter. Selected wells are highlighted in grey, and the well editor appears across the top of the interface.
 - c. In the Sample panel, select
 - i. Name: define the name of your sample.
 - ii. Experiment: select **ABS** for absolute quantification and gene expression analysis, **CNV** for CNV analysis; selection can be changed after data collection.
 - iii. Supermix: choose the one you used: **QX200 ddPCR EvaGreen Supermix** for **EvaGreen**, and **ddPCR Supermix for Probes (no dUTP)** for **Probes**. If you used Supermix that was not provided by the ddPCR core, select the correct one from the list. **NOTE**: Selection **cannot** be modified after data collection.
 - d. In the Target 1 and 2 panels, select
 - i. Name: define the name of your assay/target gene.

- ii. Type: select your sample type; with **EvaGreen**, use Target 1 and leave Target 2 unused; with **Probes**, use Target 1 for FAM and Target 2 for VIC/HEX.
 - e. Save the changes in the well editor with Apply or OK. Exit the well editor. Save the template (**Template > Save as**).
7. Click **Run** in the left navigation bar to start the run.
 - a. In the Run Options window, select the detection chemistry. **EvaGreen**: EVA; **Probes**: FAM/VIC or FAM/HEX. Click **OK**.
 - b. A green circle appears next to the abort button and flashed periodically, and the run indicator light (**Table 5**) flashes green to indicate droplet reading is in progress.
8. When droplet reading is complete, all 4 indicator lights are solid green. Open the door and remove the plate holder from the unit. Remove the 96-well PCR plate from the holder and discard it. Switch off the reader and **fill in the logbook**.

Table 5. Status indicator lights on the QX200 droplet reader.

				
Solid green	Power on	Bottle levels OK*	Plate in place	Run complete
Flashing green	—	Oil <30% or waste >70%**	—	Run in progress
Flashing amber	—	Oil <10% or waste >90%***	—	Error during run
Off	Power off	—	No plate	Idle

* There is sufficient oil and room in the waste bottle to run 96 wells.

** The run can be started if <96 wells are run (for example, only 19% oil is required for 24 wells); if there is not enough oil for the run, the software will not allow you to start the run.

*** The software will not allow you to start the run.

**Figure 4.** Placing the 96-well plate into the plate holder.

DATA ANALYSIS

1. In the **Setup** window, load plate (*filename.qlp*), if not already loaded.
2. Click **Analyze** in the left navigation bar. The data analysis interface is separated into three windows:
 - a. *Results table* – summarizes results for wells selected in the well selector.
 - b. *Well selector* – enables selection of wells for targeted analysis.
 - c. *Processed data/graphical display* – allows visualization of graphical data from selected wells.
3. Click **1D Amplitude** to visualize the data collected from each channel of selected wells.
 - a. Use the radio buttons to select the channels to be displayed.
 - b. Adjust the thresholds to assign the positive and negative droplets.
 - i. For single wells, use single-well threshold tool () , or enter threshold values in the Set Threshold field. The assigned threshold appears as a horizontal pink line.
 - ii. For multiple wells, use multi-well threshold tool () .
 - iii. To revert to automatic threshold settings and calculations, click Auto Analyze.
 - iv. Adjustments can also be made in the 2D Amplitude clustering plots (when available).
4. Click **2D Amplitude** to view the channel 1 vs. channel 2 clustering plot and enable options for adjusting the thresholds.
 - a. To reset automatic thresholds for positives and negatives, click Auto Analyze.
 - b. To manually assign thresholds,
 - i. use the thresholds crosshair to assign classification regions for the whole plot
 - ii. use the ellipse, rectangle, or lasso threshold adjustment tool to classify a region of the plot. Click the tool, then click the region type in the working cluster selector. Use the tool to select the region within the plot.
 - c. Tip: mouse over any well in the well selector to preview data from that well in the clustering plot.
5. Click **Concentration** to visualize data in concentration plots.
 - a. Use the radio buttons (top) to select the channels displayed.
 - b. Use radio buttons (bottom) to display data from single wells, merged data, or both.
 - c. Y2 Axis options:
 - i. None – no Y2 axis
 - ii. Ch2 – display Ch2 concentration
 - iii. CNV – display copy number (CNV analysis only)
 - iv. Ratio(a/b) – ratio (a/b) of unknown:reference
 - v. fractional Abundance(a/a+b) – ratio (a/a+b) of unknown:reference; abundance
 - vi. Inverse – inverse of ratio or fractional abundance
 - d. Hover over data points to reveal well identity, concentration, and Poisson confidence limits. Solid data points indicate merged data; open data points indicate data from single wells.
 - e. Select y log to convert the y-axis to logarithmic scale.
 - f. Error bars reflect total error or Poisson 95% confidence limits.
 - g. **To export the data for analysis** in spreadsheet applications (e.g. Excel), click **Export CSV** underneath the *Results table*.

6. Click **Copy Number** to view copy number for selected wells/samples.
 - a. Use radio buttons (bottom) to display data from single wells, merged data, or both.
 - b. Hover over data points to reveal well identity, concentration, and Poisson confidence limits. Solid data points indicate merged data; open data points indicate data from single wells.
 - c. Select y log to convert the y-axis to logarithmic scale.
7. Click **Ratio** to view ratio data for selected wells/samples.
 - a. Use radio buttons (top) to select a plot of the Ratio (unknown:reference) or Fractional Abundance (% of sample); select Inverse to apply the inverse of either.
 - b. Hover over data points to reveal well identity, concentration, and Poisson confidence limits. Solid data points indicate merged data; open data points indicate data from single wells.
 - c. Select y log to convert the y-axis to logarithmic scale.
8. Click **Events** to view the number of droplet events counted for selected wells/samples.
 - a. Use the radio buttons (top) to select the channels displayed.
 - b. View positive, negative, or total droplet counts, or any combination of these (bottom right).

IMPORTANT:

- ✓ All the data will be automatically saved in **C:\QuantaLife\Data**. The name of your data folder will be the same as the name of the template you were using. The file type used for data analysis in QuantaSoft software is **QuantaSoft Plate** (.qlp).
- ✓ To transfer the data to your own computer, **map your computer to QuantaLife network drive**. It allows you to access the Data file on the ddPCR computer. You can find the instructions for mapping from the arvolab-folder (*QuantaLife_network-drive_mapping*). Username and password can be found by the ddPCR computer.
- ✓ ddPCR core will **store the files at least 12 months**, after which the files will be erased. **Save your files** (at least *QuantaSoft Plate* file, .qlp) **to your own computer**.
- ✓ If you want to analyze the data on your own computer, you can get the **QuantaSoft software free of charge** from N:\arvolab\Laitemanuaaleja ja ohjeita\ddPCR, or by contacting people from the ddPCR core.

More info on software usage, see *“QX200 Droplet Reader and QuantaSoft Software” manual (Bio-Rad)*.

More info on data analysis, see *“Droplet Digital PCR Applications Guide” (Bio-Rad)*.

TROUBLESHOOTING: see *“Droplet Digital PCR Applications Guide” (Section 10)*.

MAINTENANCE

1. Replacing droplet reader oil and removing waste

When the second indicator light on the left (**Table 5**) on the front of the droplet reader flashes amber, either the droplet reader oil is finishing, or the waste bottle is full.

- ➔ Use the handle built into the side compartment to slide out the carriage (left side of the reader) containing both reader oil and waste bottles to see, which one is the problem.
- ➔ Waste:
 - Add 50 ml 10% Na-hypochlorite (can be found under the sink in E216) to a new waste bottle to prevent microbial growth; empty oil bottles can be used as new waste bottles (the storage is next to the reader).
 - Place a “WASTE” label on the new waste bottle (ready-made labels can be found inside oil boxes).
 - Screw the cap of the old waste bottle open and replace the old bottle with a new one. Screw the old cap into the new bottle.
 - Pour the waste into the drain and discard the old waste bottle.
- ➔ Oil:
 - Screw the cap of the old oil bottle open and replace the old bottle with a new one (the storage is next to the reader). Screw the old cap into the new bottle. **Do not throw away the residue oil**, as it can be used until the expiration date. If there are two residue oil bottles, combine the oils and mark the older expiration date to the bottle. Storage the empty bottle as a waste bottle next to the reader.
 - **Prime** the instrument (see instructions below).

2. Priming the droplet reader

After the reader oil has been changed, or when the reader has been in low use and no one has been using it over 1 week (according to logbook), the droplet reader should be primed.

- ➔ Insert the **plate holder** inside the droplet reader. The plate holder does not have to have a plate inside of it.
- ➔ In QuantaSoft software, under Instrument Routines in the Setup window, click **Prime**. Priming takes a couple of minutes.

FREQUENTLY ASKED QUESTIONS

WHAT IS DIGITAL DROPLET PCR?

Digital Droplet PCR uses microfluidics to separate a standard PCR reaction into a water/oil emulsion of thousands of nanodroplets, essentially allowing one to perform thousands of independent PCR reactions in a single tube. Following PCR in a standard thermocycler, the number of positive droplets is determined using flow cytometry and the number of copies of DNA template in the initial reaction can be inferred using Poisson statistics. The Bio-Rad QX200 can read droplets in two different fluorescence channels (FAM and HEX/VIC) allowing for multiplexing of probes. The technology is also compatible with EvaGreen dye-based PCR.

Digital droplet PCR has advantages over real-time quantitative PCR in that it allows for easy, sensitive and accurate quantification of template copy number without the need for standard curves. In addition, due to the large number of droplets in each tube, each of which is an independent assay, there is no need for technical replicates in ddPCR analysis.

IS IT TRUE THAT YOU CAN MULTIPLEX DDPCR USING EVAGREEN DYE?

Yes. Briefly, the fluorescence intensity for each product can be varied by:

- selecting primers that generate products of different lengths – a 60bp PCR product and a 300bp product will bind very different amounts of EvaGreen giving easily distinguishable products.
- varying the PCR reaction conditions to reduce the efficiency of one reaction – adding less of one primer set, or designing a primer set that works better at a lower T_m can be very effective.

Here are some references on the topic:

High Sensitivity Detection and Quantitation of DNA Copy Number and Single Nucleotide Variants with Single Color Droplet Digital PCR. Miotke et al. Anal Chem. 2014; 86(5): 2618–2624.

Multiplexed target detection using DNA-binding dye chemistry in droplet digital PCR. McDermott et al. Anal Chem. 2013; 85(23):11619–27.

CAN I USE DDPCR WITH MY EXISTING PRIMERS?

The short answer is yes. Any primer set that works with SYBR-Green should be easily adapted for EvaGreen ddPCR. In addition, probes that can be detected in the FAM or HEX/VIC channels can also be used with the QX200.

Two things to note are:

- The linear range of ddPCR (10-100,000 copies) is lower than for qPCR (100-10⁹ copies) and it can therefore be easily overloaded. It is recommended that you use 10-50x less cDNA template for gene expression assays than you would use in qPCR assays, especially if your template is abundant.
- Genomic DNA may need to be pre-digested. Often this can be performed in the PCR reaction buffer prior to droplet generation.

HOWEVER, if you are designing a new set of primers specifically for ddPCR there are some differences between primer design for ddPCR and for qPCR.

WHERE CAN I GET THE QUANTASOFT SOFTWARE?

QuantaSoft software is compatible with PCs and is available free of charge. You can download it from N:\arvolab\Laitemanuaaleja ja ohjeita\ddPCR, or request a copy by emailing ddPCR core personnel.

HOW CAN I SCHEDULE TIME ON THE DROPLET GENERATOR AND READER?

There is no reservation for the Droplet Generator. Reservations for the Droplet Reader are made through Agendo (next.cirklo.org/biomeditech/). Allow 10 min for set up (or more if you prepare the template just before the run) plus 1.5 min per sample for Droplet Reading.

To save time, prepare the template beforehand. You can also do this on your own computer with the QuantaSoft software (see page 19 how to get one).

HOW MUCH DOES DDPCR COST?

In some ways, ddPCR is cheaper than qPCR:

- There is no need for a standard curve – as the reaction is assessed at the endpoint it does not matter too much whether it is 100% efficient. This saves you time and money in optimization.
- There is no need for technical replicates – this saves you time in setting up reactions and reduces the cost of reagents.
- You can multiplex to assay your reference gene and gene of interest in the same well, which will halve your expense and pipetting.

However, there are additional costs associated with ddPCR:

- Cost of droplet generation cartridges, gaskets and oil.

ddPCR core will provide two type of supermixes (one for EvaGreen and one for probes), and all the special plastics and oils needed for ddPCR (see page 1). The pricing can be found in Agendo (next.cirklo.org/biomeditech/ – you need to be logged in).

Overall, you can expect ddPCR to be slightly more expensive than qPCR for most applications. However, you should take into consideration that ddPCR is more sensitive and will give more reliable results in many applications.

CAN I USE MY OWN SUPERMIX/MASTER MIX?

Yes, as soon as they are ddPCR compatible (www.bio-rad.com/en-fi/category/digital-pcr-supermixes). Never use SYBR Green dye containing master mixes, as they will break the droplet reader! If you are using your own supermix, remember to select the right number of samples from field “*Samples - own supermix used*” in Agendo. You also have to select “*Own supermix used*” in field “*Samples - core supermix used*”.

CAN I GET AWAY WITH REACTION VOLUMES OF LESS THAN 20 µL?

We have not tried this. You need 10 000 droplets to be able to determine copy number accurately using Poisson statistics. 20 µl is routinely giving us 14-19 000 droplets. So it is possible you can get away with 15 µl reactions once you know what you are doing. Let us know if you try it and it works.

CAN I REUSE THE CARTRIDGES?

Unfortunately, the microfluidic cartridges are not reusable primarily because of the potential for contamination and the difficulty in cleaning out the tiny channels. In addition, it is not recommended to reuse the gaskets, as the potential for contamination of your samples with DNA from previous experiments is still there.