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Protocol status: Working

We use this protocol and it's working

ddPCR for probes to target eDNA samples : from sample preparation to droplet reading

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ABSTRACT

The aim of this protocol is **the digital droplet PCR (ddPCR) quantification of DNA target(s) using primer and probe sets**. This protocol is optimised for the **analysis of environmental DNA (eDNA) samples** (water, sediment, biofilm and soil matrices) and then rare DNA targets.

The ddPCR can be performed using a QX600 or a QX200 droplet reader system, and this protocol begins **after the DNA extraction step and ends with the droplet reading**.

The **ddPCR for probes** consists of the **absolute quantification of samples using primers and fluorescent dye-labelled probes**. The ddPCR technique used is based on the partitioning of samples into droplets by water-oil emulsion and the reading of the DNA signal in each droplet. **Multiple eDNA targets can be specifically targeted using multiple primer and probe sets** that are compatible.

The advantages of using the ddPCR are: the **absolute quantification**, the **sensitivity** and the **repeatability** of the method.

IMAGE ATTRIBUTION

Image attribution : Bio-Rad

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Keywords: eDNA, DNA, quantification, PCR, ddPCR, rare DNA, absolute quantification, probes, digital droplet, multiplexing, droplet, dPCR

GUIDELINES

The main steps of the protocol are:

- Material preparation
- Plate preparation
- Reaction mix preparation and dispensing
- Droplets generation
- PCR reaction
- Reading of the ddPCR plate

MATERIALS

▪ **Materials:**

- 1000 µL pipet
- 100 µL pipet
- 10 µL pipet
- Multi-channel pipet (40 µL)
- Vortex + benchtop centrifuge (for tubes and PCR strips)
- DG8 cartridge holder (ref. Bio-Rad: 1863051)
- PX1 PCR plate sealer (Bio-Rad)
- Thermal cycler for PCR
- QX200 droplet generator (Bio-Rad)
- QX600 or QX200 droplet reader (Bio-Rad)
- Specific DNA-workstation (sterile area equipped with air filtration and UV systems)

▪ **Consumables:**

All tubes and tips must be sterile

- 1000 µL tips with filter
- 100 µL tips with filter
- 10 µL tips with filter
- PCR strips (one for 8 samples)
- 0.5 mL / 1.5 mL / 2 mL / 5 mL tubes according to the number of samples to be analysed
- DG8 gaskets for ddPCR (one for 8 samples) (ref. Bio-Rad: 1863009)
- DG8 cartridge for ddPCR (one for 8 samples)(ref. Bio-Rad: 1864008)
- ddPCR 96-well plate (ref. Bio-Rad: 12001925)
- Pierceable foil seal (ref. Bio-Rad: 17005225)
- Aluminium foil
- Gloves

For any manipulation in a rare DNA room, provide complete equipment (disposable coat, cap, mask, shoe covers & gloves).

▪ **Reagents:**

- DNase and RNase free water
- 2x ddPCRSupermix for Probes (No dUTP) (ref. Bio-Rad: 1863023 or 1863024)
- Specific forward and reverse primers (50 µM)

- Probe marked with fluorescent dye (50 µM)
- Digestion enzyme and buffer CutSmart (*if applicable*)
- Droplet generation oil (ref. Bio-Rad: 1863004)

- **Samples to be analysed:**

- DNA samples extracted, diluted or not

BEFORE START INSTRUCTIONS

- **Amount of DNA to be used and use of restriction enzyme**

If the target is rare in the DNA sample, do not carry out dilution of the DNA sample (unless PCR inhibitors are abundant) and increase the quantity of DNA (a maximum of 6µL per 20 µL reaction). If the target is abundant in the sample, it is possible to perform a dilution of the DNA sample, for example 1/100.

The use of restriction digestion is recommended above 66 ng DNA per reaction. Ensure that the restriction enzyme selected does not cleave the PCR target. The amount of total digested DNA should not exceed 1000 ng per 20 µL reaction.

- ***Multiplexing***

Multiple sets of primers and probes can be used simultaneously to target multiple targets. In this case, add them to the reaction mix at the same concentrations as indicated in the protocol, reducing the amount of water to be added proportionally. For multiplexing, use probes with different fluorescent dyes and ensure that the primers/probe sets are compatible.

- ***The following precautions must be applied:***

- Wear gloves throughout the extraction process
- Clean the bench with DNA-removing solution (e.g. DNA-off, DNA away).
- Use tips with filters to avoid contaminations
- All steps have to be performed into a specific DNA-work station (sterile area equipped with air filtration and UV systems)

For any manipulation in a rare DNA room, provide complete equipment (disposable coat, cap, mask, shoe covers & gloves).

Material preparation

- 1
 - *Pre and post extraction equipment decontamination:*
 - *Specific DNA-workstation: UV decontamination.*
 - *Turn on the following equipments:*
 - *the QX200 droplet generator*
 - *the PX1 PCR plate sealer at 180 °C*
 - *the thermal cycler for PCR*
 - *the QX600 or QX200 droplet reader for ddPCR*
 - *Tubes annotation*
 - *One 0.5 mL / 1.5 mL / 2 mL or 5 mL tube for the reaction mix according to the number of samples to be analysed.*
 - *PCR strips: 8 samples per PCR strip.*

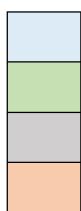
Plate preparation

- 2
 - ***Preparation of the plate layout with the samples to be analysed.***

10m

Note: It is possible to prepare the PCR strips with the DNA samples and the controls before the day of the ddPCR analysis by freezing them.

	A	B	C	D	E	F	G	H
1								
2								
3								
...								
12								

Samples


- Samples
- No template control (NTC)
- Control samples
(Extraction and/or field controls)
- Positive controls

Table 1: Extracted DNA samples and controls distribution example

▪ Distribution of DNA samples and controls into the PCR strips

-Collect the DNA samples and the negative and positive controls from the freezer and defrost them

 00:10:00 at  4 °C, and take DNase and RNase free water for the NTC.

- Vortex and benchtop centrifuge briefly each sample.
- According to the plate layout, insert the quantity of each DNA sample and controls into the corresponding PCR strip well.

Note: If the target is rare in the DNA sample, do not carry out dilution of the DNA sample and increase the quantity of DNA (a maximum of 6 μ L per 20 μ L reaction). If the target is abundant in the sample, it is possible to perform a dilution of the DNA sample, for example 1/100.

- Once each PCR strip is complete, close it and annotate it.
- When all the PCR strips are filled in, store them at $\text{–}20\text{ }^{\circ}\text{C}$ if the ddPCR is to be performed the next day or at $4\text{ }^{\circ}\text{C}$ if the ddPCR is to be performed immediately.

Reaction mix preparation and dispensing

- 3 Before starting, calculate the reagent requirements according to the number of samples to be analysed (Table 2). 13m

Reagents	Initial concentration	Final concentration	Unit	Volume (μ L) for 1 sample
CutSmart buffer	10	1	X	0,11
Restriction enzyme	20	3,5	units/ μ L	0,19
2x ddPCRSupermix for Probes (No dUTP)	2	1	-	11
Forward primer	50	0,9	μ M	0,396
Reverse primer	50	0,9	μ M	0,396
Probes	50	0,25	μ M	0,11
Water	-	-	-	5,445
DNA	-	-	ng/ μ L	3
Final volume / sample (μL) = 22				

Table 2: Preparation of reaction mix (calculated for one DNA sample with a DNA volume added of 3 μ L)

Note: The total volume prepared is 22 μ L for a final ddPCR reaction of 20 μ L, the 2 μ L being the pipetting margin for transferring the mix.

Note: Primers can be multiplexed by using probes with different fluorescent labels and ensuring that they are compatible. With the addition of one or more primer and probe sets, the amount of water in the reaction mix needs to be adjusted.

Note: The use of a restriction enzyme is recommended above 66 ng of DNA per reaction.

During these steps, manipulate under specific DNA-workstation and cover the tubes and PCR strips containing the photosensitive reaction mix, the probe tube and the supermix tube with aluminium foil to prevent any damage to the fluorescence reaction.

▪ Reaction mix preparation

- Collect the reagents and defrost them at Room temperature (supermix) or at 4 °C (primers, probe, water and buffer), but leave the restriction enzyme in the freezer until you need it.
- Vortex and benchtop centrifuge briefly all the reagents. Vortex the supermix thoroughly to ensure homogeneity, as a concentration gradient may form during -20°C storage.
- Prepare the reaction mix by adding all the reagents in the tube one by one.
- Vortex and benchtop centrifuge briefly the reaction mix.
- Cover the reaction mix tube with aluminium foil and store at Room temperature before immediately dispensing it into the PCR strips, otherwise keep it at 4 °C for few hours.

▪ Reaction mix dispensing

- Collect the filled PCR strips and defrost them 00:10:00 at 4 °C if they were frozen.
- Vortex and benchtop centrifuge briefly the PCR strips. Vortex the reaction mix thoroughly to homogenise the reagents and benchtop centrifuge briefly.
- Pipet the reaction mix into each well of the PCR strip. The volume of reaction mix to be added depends on the volume of DNA selected. In our example, there is 3 µL of DNA, so 19 µL of reaction mix must be added into each PCR strip well.
- Cover the samples strips with aluminium foil and place them at 4 °C until droplet generation if the samples are prone to thermal degradation, or leave them at Room temperature. In any case, allow the tubes to stand at Room temperature for at least 00:03:00 before transferring them into the cartridge.

Droplets generation

4 Steps 4 and 5 must follow each other quickly (less than one hour between these two steps).

15s



- Place a DG8 cartridge into the cartridge holder.
- Vortex and benchtop centrifuge all the PCR strips.
- Dispense the following volumes into the corresponding cartridge compartments (Figure 1):

- Middle line

20 µL of the **DNA sample + reaction mix** previously prepared into the PCR strips.

- Bottom line

70 µL of **droplet generation oil**.

Note: One cartridge corresponds to one sample strip and the 8 samples must be filled (no empty well).

Note: Avoid the formation of bubbles in the cartridge well containing the samples by tilting the pipette at 15° and gently dispense the sample without pushing the pipet plunger after the first stop.

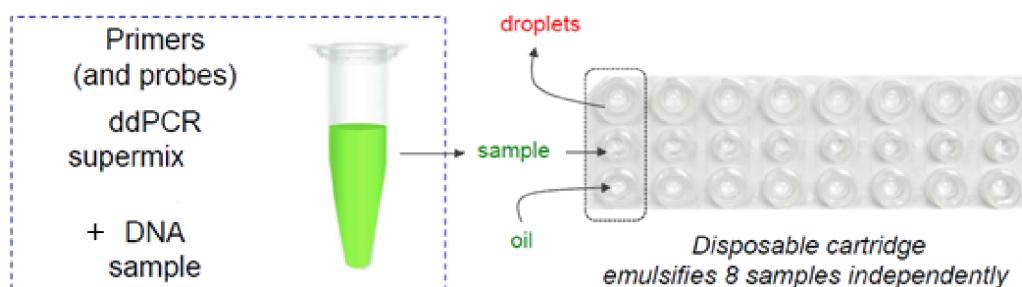


Figure 1 : DG8 cartridge filling

- Place a DG8 gasket for ddPCR on the DG8 cartridge (Figure 2)

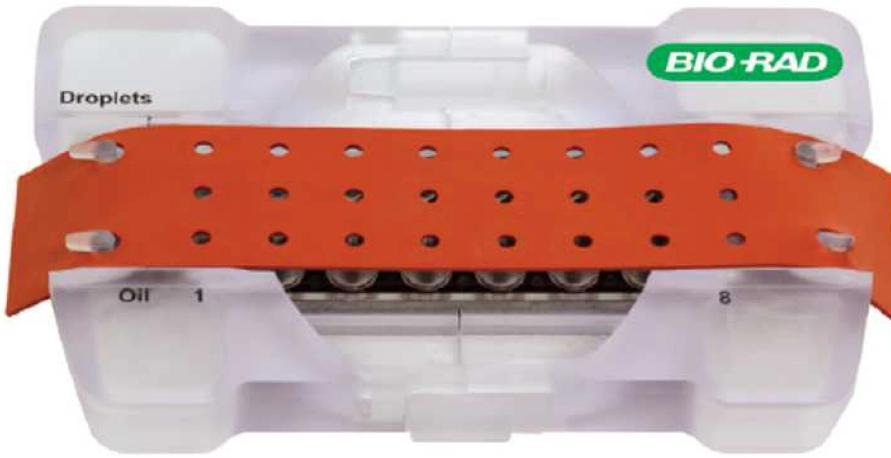


Figure 2 : Correct placement of the gasket over the cartridge holder.

- Open the droplet generator by pressing the green button on the front of the machine and insert the cartridge. Press the button again to close the machine and start the droplet generation. When the reaction is complete, the 3 indicator lights will turn green.
- Take out the cartridge from the droplet generator and remove the gasket from it.
- With the multi-channel pipet, transfer slowly $\text{40 } \mu\text{L}$ of droplets generated from the top line of the cartridge (Figure 1) into the ddPCR 96-well plate.
 - **Intake:** place the tips at the bottom of the well, hold the pipet with a 30-45° angle and slowly draw 40 μL of droplets into the pipet tip (it should take around 00:00:05).
 - **Dispense:** position the pipet tip along the side of the well, near, but not at, the bottom of the well, and slowly dispense the droplets (it should take around 00:00:05).
- Cover and reserve the plate at Room temperature or On ice (if there are many cartridges to process) until all the samples have been loaded into the ddPCR 96-well plate.
- Once the ddPCR 96-well plate is filled, place one sheet of pierceable foil seal (with the coloured line on top) onto the plate, and seal the plate at $\text{180 } ^\circ\text{C}$ for 00:00:05 using the PCR plate sealer. Check that all wells in the plate are sealed. Once sealed, the plate is ready for thermal cycling.

Note: Begin the thermal cycling within 30 min after the sealing of the plate, or store at 4°C for up to 4 hours prior to thermal cycling.

PCR reaction

5

Place the sealed ddPCR 96-well plate into the PCR thermal cycler – *see instrument manual*

- Program the following cycling conditions (Table 3) :

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

* Use a heated lid set to 105°C and set the sample volume to 40 µl.

Table 3 : Recommended cycling conditions

Note: The temperature and duration of annealing/extension step, as well as the number of cycles can be adjusted if necessary. Do not exceed 50 cycles. Use a 2.5°C/s ramp rate to ensure each droplet reaches the correct temperature for each step during the cycling.

- Start the PCR run.

Note: Once the PCR is complete, the plate containing the droplets can be stored for up to 24 hours before the reading of the ddPCR plate.

Reading of the ddPCR plate

30m

- 6
- Switch on the droplet reader (QX200 or QX600).
 - Place the ddPCR 96-well plate into the droplet reader support - *see instrument manual*

- Open the droplet reader by pressing the green button on the front of the machine and insert the droplet reader support. Press the button again to close the machine.
- Open the ddPCR software associated with the droplet reader in use (QX200 or QX600) and check the oil and waste bin levels (an error message will appear if one of the bottles needs to be changed).

Note: If necessary, place a new oil bottle and use the old bottle as a waste container. If a new oil bottle has been installed, you must press "prime". If the machine has not been used for 1 month or more, press "flush system" to clean the system.

- Define the experiment:

Note: The information given below applies to the QX600 reader. For the QX200, please refer to the user manual.

1) Plate information (required for run)

- Click on "Add plate & Configure plate "
- Load a plate template or create a new plate > Name the plate and select the appropriate Supermix

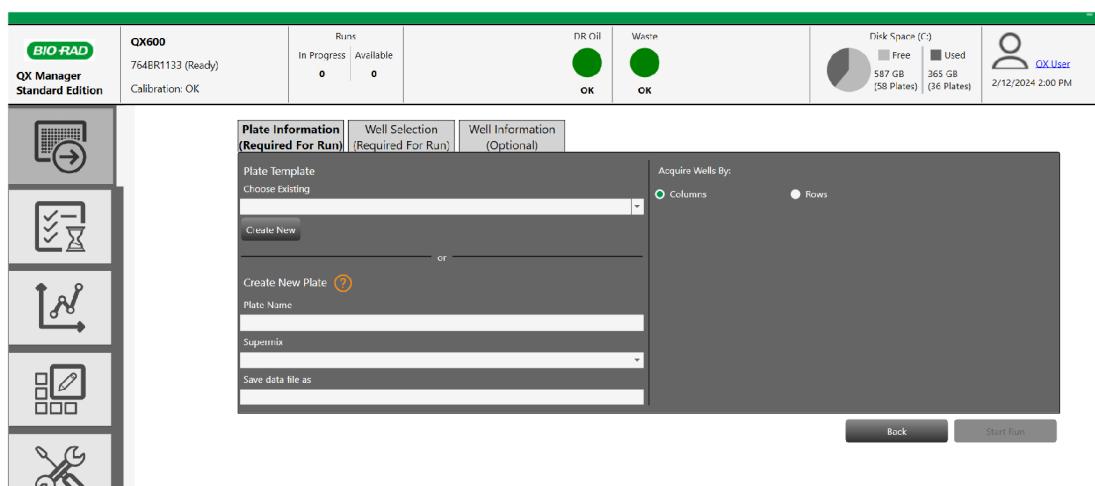
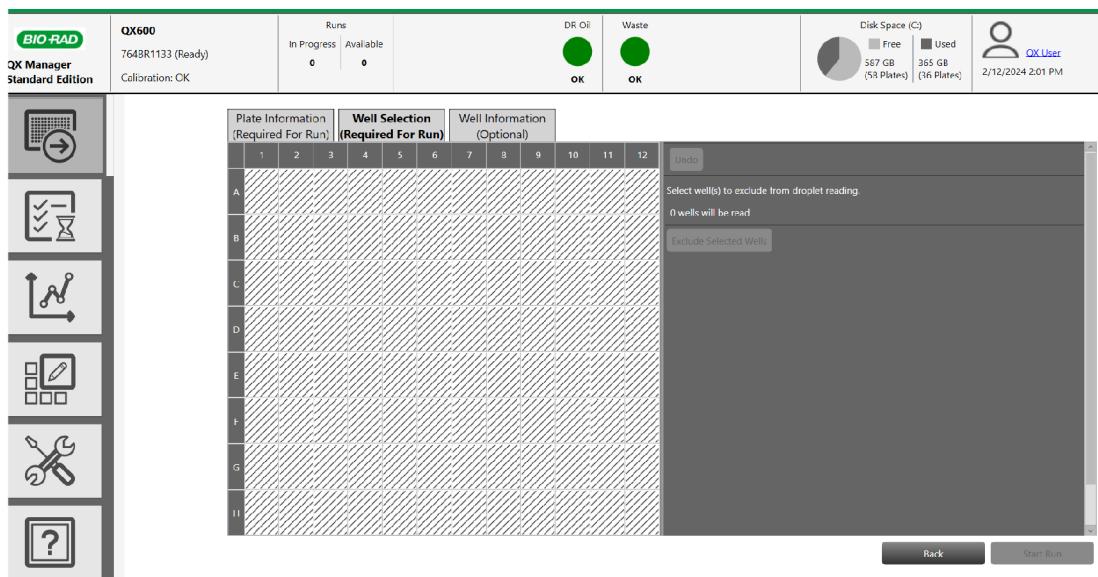


Plate information (required for run)

2) Well selection (required for run)

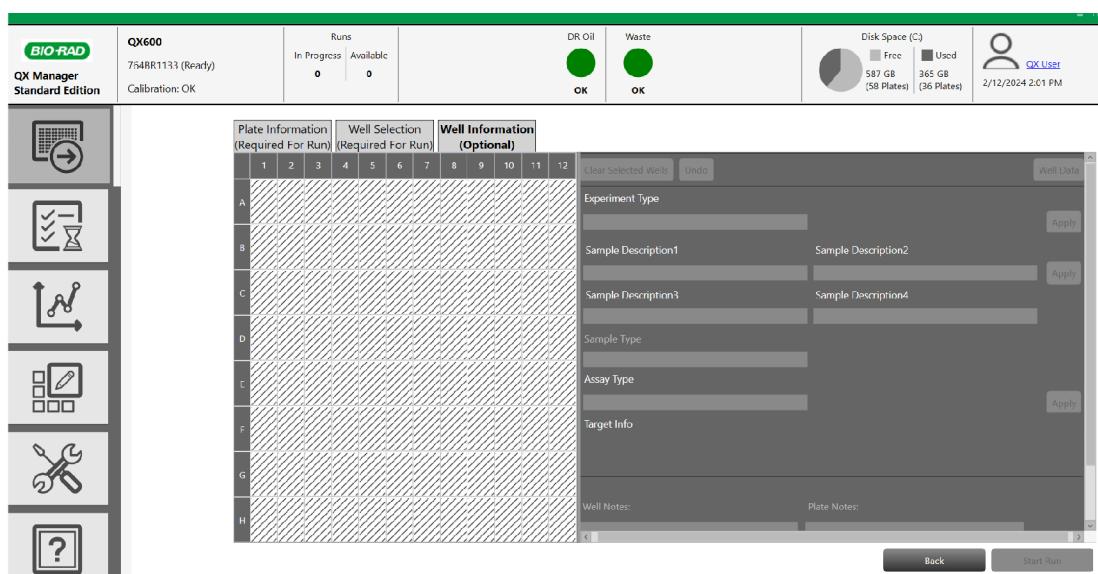
- Select the wells to be analysed on the plate layout and click "Include selected wells".



Well selection (*required for run*)

3) Well information (*optional*)

- If necessary, fill in the different information (name of the target, the channel used and the sample type (by default select "Unknown")).
- Then click 'Apply'. The information entered will now appear in the plate layout.



Well information (*optional*)

- Click "Start Run" at the bottom right of the screen.

After start confirmation, the run starts and the analysis time is displayed (depending on the number of samples). The results can be viewed live as the wells are read. At the end of the run, the results are automatically stored in the dedicated folder and the analyses can be performed (see the software manual of the droplet reader used).