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One step RT-ddPCR for probes to target eRNA samples : from sample preparation to droplet reading

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We use this protocol and it's working

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Abstract

The aim of this protocol is **the digital droplet PCR (ddPCR) quantification of RNA target(s) using primer and probe sets. Reverse transcription and amplification are carried out in a single step**, using the One Step RT-ddPCR Advanced kit for probes (Bio-Rad).

This protocol is optimised for the **analysis of environmental RNA (eRNA) 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 (Bio-Rad), and this protocol begins **after the RNA extraction step and ends with the droplet reading.**

Multiple eRNA 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,** the **efficiency** and the **specificity** of the method for **RNA target quantification**.

Image Attribution

Image attribution : Bio-Rad

Guidelines

The main steps of the protocol are:

- Material preparation
- Reaction mix preparation
- Reaction mix, RNA samples and controls 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/RNA-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:**

One-Step RT-ddPCR advanced kit for Probes containing:
- 2x ddPCRSupermix for Probes (No dUTP) (ref. Bio-Rad: 1863023 or 1863024)
- Reverse transcriptase (RT)
- Dithiothreitol solution (DTT)

All components of the One-Step RT-ddPCR advanced kit for Probes are stable for 12 months when stored in a constant temperature freezer at -20°C. Repeated freezing and thawing of the supermix is not recommended. DTT should be aliquoted to multiple tubes and stored at -20°C to minimize freezing and thawing.

- DNase and RNase free water
- Specific forward and reverse primers (50 µM)
- Probe marked with fluorescent dye (50 µM)
- Droplet generation oil (ref. Bio-Rad: 1863004)

- **Samples to be analysed:**

- RNA samples extracted, diluted or not

Troubleshooting

Before start

- **Amount of RNA to be used**

The suggested input quantities of total RNA are 100 fg - 100 ng per reaction.
To minimize freezing and thawing, prepare aliquots of the extracted RNA samples in 0.5 mL tubes after the RNA extraction step. Prepare the RNA sample before setting up the transcription reaction mix and keep both of them on ice.

- **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 RNA-removing solution (e.g. RNA-off, RNA away).
- Use tips with filters to avoid contaminations
- All steps have to be performed into a specific DNA/RNA-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

- *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.

Reaction mix & PCR strips preparation

2 Before starting, calculate the reagent requirements according to the number of samples to be analysed (Table 2). 50m 40s

Reagents	Initial concentration	Final concentration	Unit	Volume (µL) for 1 sample
RT ddPCRSupermix for Probes	4	1	X	5.5
Reverse transcriptase	200	20	units/µL	2.2
300 mM DTT	300	15	mM	1.1
Forward primer	50	0.9	µM	0.396
Reverse primer	50	0.9	µM	0.396
Probes	50	0.25	µM	0.11
RNA	-	-	ng/µL	2
Water	-	-	-	10.298
	Final volume / sample (µL) = 22			

Table 1: Preparation of reaction mix (calculated for one RNA sample with 2 µL added)

Note: The suggested input quantities of total RNA are 100 fg - 100 ng per reaction.

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: Before manipulation, all reagents should be thawed on ice for 30min. Prepare the RNA sample before setting up the transcription reaction mix and keep both of them on ice.

During these steps, manipulate under specific DNA/RNA-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.

The reactions should be done on ice before droplet generation to prevent a nonspecific reverse transcription reaction from occuring.

- **Reaction mix preparation**

- Collect the reagents and defrost them On ice for 00:30:00 .

- Vortex each tube thoroughly to ensure homogeneity for 00:00:30 , as a concentration gradient may form during -20°C storage. And benchtop centrifuge briefly all the reagents.

- 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 for immediate use and On ice for later use.

- **Distribution of Reaction mix, RNA samples and controls into the PCR strips**

- Collect the negative and positive controls from the freezer and defrost them 00:10:00 at On ice , and take DNase and RNase free water for the NTC.

- Collect the *RNA samples and defrost them* 00:10:00 On ice

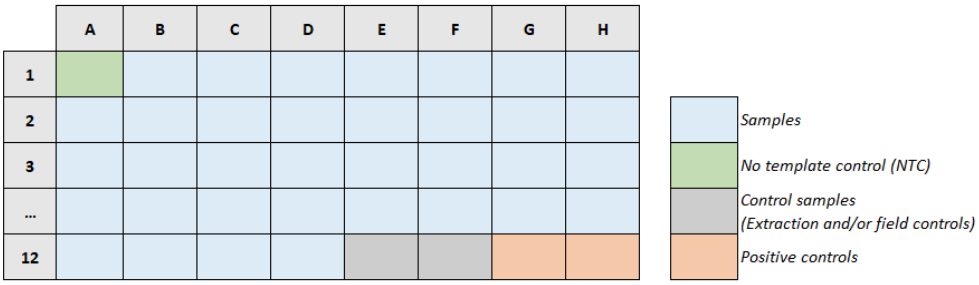


Table 2: Extracted RNA samples and controls distribution example

- Pipet the reaction mix into each well of the PCR strip. The volume of reaction mix to be added depends on the volume of RNA selected. In our example, there is 2 µL of RNA, so 20 µL of reaction mix must be added into each PCR strip well.

Note : As RNA degrades quickly, gradually thaw the tubes corresponding to the filling of the current strip and after use, store them quickly again at -80 °C *. Repeat this operation for each PCR strip.*

- Vortex and benchtop centrifuge briefly each RNA and control samples.

- According to the plate layout, insert the quantity of each RNA sample and controls into the corresponding PCR strip well.

- Once each PCR strip is complete, close it and annotate it.

- Cover the samples strips with aluminium foil

-Mix thoroughly by vortexing the PCR strips at maximum speed for 00:00:10 , and centrifuge briefly. Allow the PCR strips to equilibrate to Room temperature for no more than 10 minutes before droplet generation. If a longer waiting time is estimated, place them o On ice .

Droplets generation

3 *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 **RNA 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.

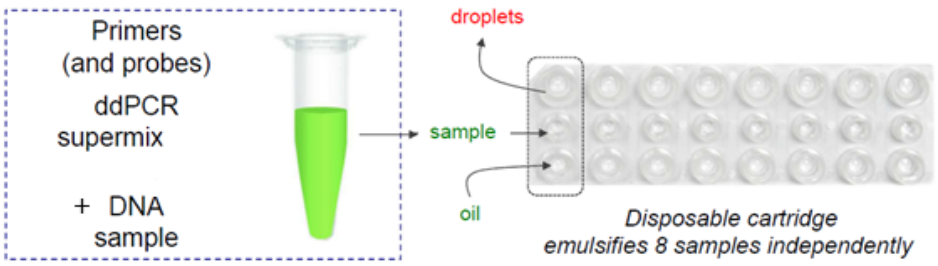


Figure 1: DG8 cartridge filling

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

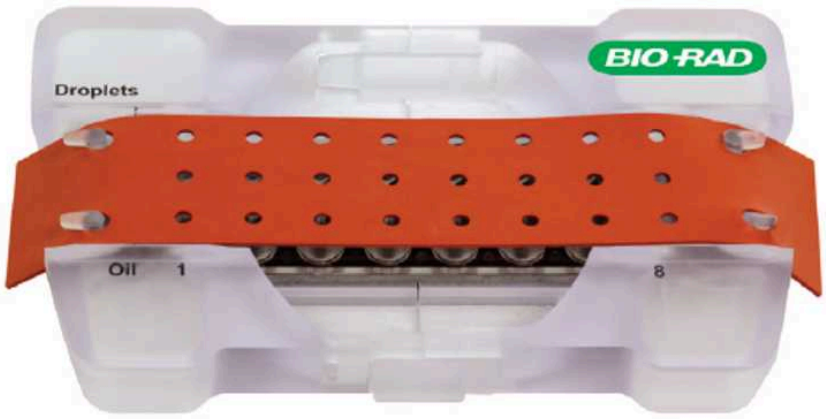


Figure 2: Correct placement of the gasket over the cartridge holder

- 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 40 µL 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 40µL 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 On ice 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 180 °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

4 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	Number of Cycles
Hold (QX ONE ddPCR System only)		25	3 min	1
Reverse transcription		42–50	60 min	1
Enzyme activation		95	10 min	1
Denaturation		95	30 sec	40
Annealing/extension		55–65	1 min**	40
Enzyme deactivation		98	10 min	1
Hold	QX600 or QX200 ddPCR System	4	30 min	1
	QX ONE ddPCR System	25	1 min	1

* For the PTC Tempo Deepwell Thermal Cycler or C1000 Touch Thermal Cycler with 96–Deep Well Reaction Module, use a heated lid set to 105°C and set the sample volume to 40 µl.
** Check/adjust ramp rate settings to ~2°C/sec.

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.

- 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

5

- 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 & Configurate plate "

- Load a plate template or create a new plate > Name the plate and select the appropriate Supermix

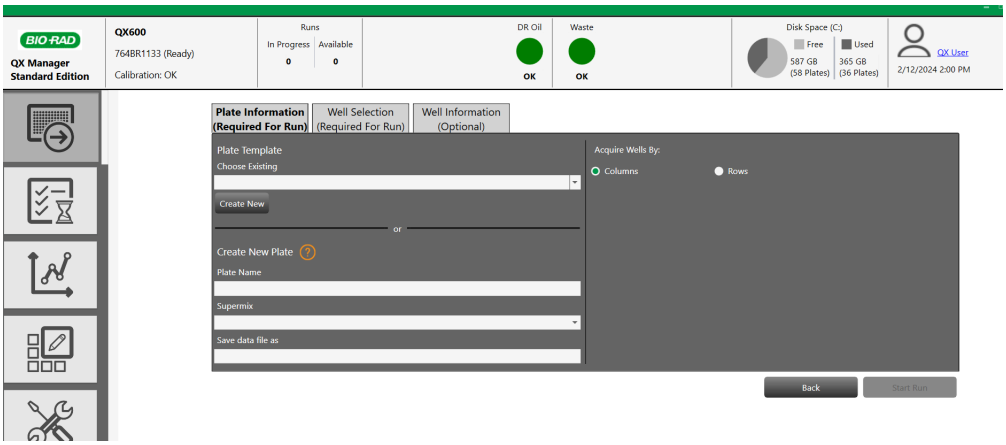
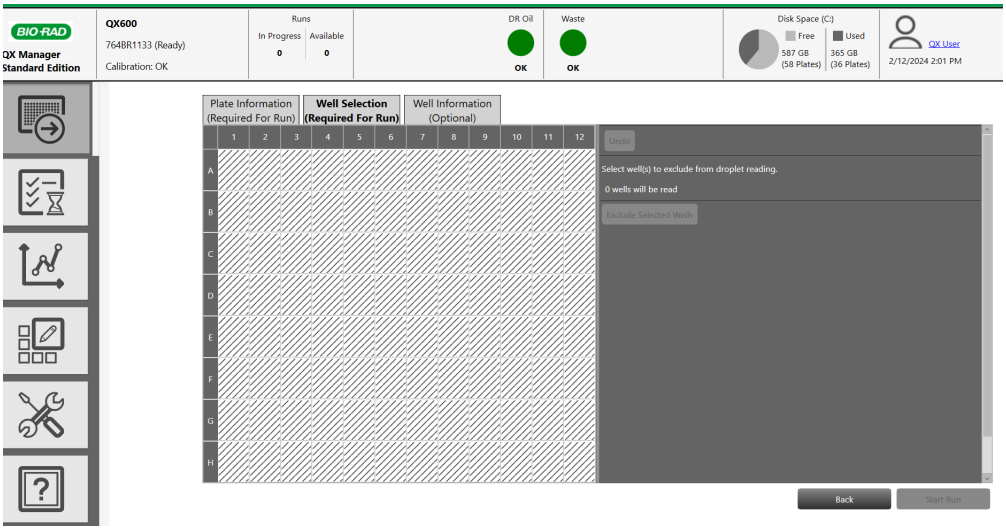


Plate information (required for run)

2) Well selection (*required for run*)

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

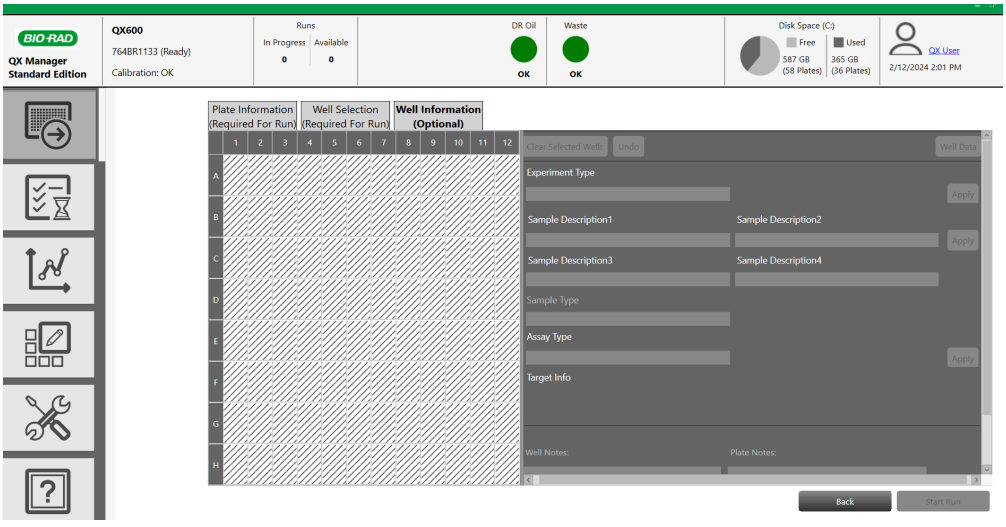


Well selection (*required for run*)

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).

Protocol references

QX200 droplet generator instruction manual:

10031907-manuel ddgenerator.pdf

QX200 droplet reader and QX Manager software standard edition user guide:

QX600.pdf

QX200 droplet reader and QuantaSoft software instruction manual:

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