Protocol for microRNA PCR profiling using microRNA LNA™ PCR primer sets with the QX200™ Droplet Digital™ PCR System

Introduction

This protocol describes overall steps in how to run the QX200™ Droplet Digital™ PCR System from Bio-Rad using the Exiqon miRCURY LNA™ Universal RT microRNA PCR system. Sample input amount and dilution level of cDNA reactions are meant as a guide. Empirical determination of sample input amount and dilution factor may be required. The microRNA LNA™ PCR primers are optimized to work with ExiLENT SYBR® Green PCR Master Mix. Some changes in sensitivity and specificity may be observed when using EvaGreen® supermix.

The present protocol should be regarded as a recommendation for a good starting point for experiments combing the miRCURY LNA™ Universal RT microRNA PCR system with the QX200™ Droplet Digital™ PCR System. It is meant as a supplement to the QX200™ Droplet Digital™ PCR System and QX200™ Droplet Generator protocols. Exiqon strongly advise reading the QX200™ protocols and the Instruction Manual for miRCURY LNA™ Universal RT microRNA PCR before starting the experiment.

Required reagents from Exiqon

To set up miRCURY LNA™ Universal RT microRNA PCR experiments on the QX200™ Droplet Digital™ PCR System it is recommended to use the following reagents from Exiqon:

- Universal cDNA synthesis kit II, 8-64 rxns (product # 203301)
- MicroRNA LNA™ PCR primer set (product # 204000-206xxx and 2100000-21xxxxx)
- RNA Spike-ins and associated control primer sets (optional)

Additional required materials:

- 96 well thermocycler for first-strand cDNA synthesis and PCR reactions
- Micro centrifuge
- QX200™ Droplet Generator
- QX200™ Droplet Reader
- QX200™ ddPCR™ EvaGreen® Supermix, 186-4033, 186-4034, 186-4035, 186-4036
- Droplet generation oil for EvaGreen®, 186-4005, 186-4006
- Pierceable foil plate seals, 181-4040
- PX1™ PCR plate sealer, 181-4000

Protocol

Overview of all steps of the protocol:

- Universal cDNA synthesis (Step 1-5)
- Setup PCR reaction (step 6-9)
- Droplet generation (step 10)
- PCR reaction (step 11-12)
- Droplet counting on QX200™ (step 13)

Step 1
RNA input volume

Biofluid samples:
Since the RNA concentration in extractions from serum and plasma cannot be determined accurately we recommend using the amount of starting material as a measure for input amount.
Cells and tissue samples:
Adjust each of the template RNA samples to a concentration of 5 ng/µL using nuclease free water.

Step 2
Prepare reagents

Gently thaw the 5x Reaction buffer and nuclease-free water, and immediately place on ice. Mix by vortexing. Re-suspend the RNA spike-ins according to the appropriate RNA Spike-ins protocol, leave on ice for 15-20 minutes. Immediately before use, remove the Enzyme mix from the freezer, mix by flicking the tubes and place on ice. Spin down all reagents.

Step 3
Combine reagents according to Table 1

Note: remember to calculate necessary excess volume for pipetting and robotic dead volume.

If performing first-strand cDNA synthesis on multiple RNA samples, it is recommended to prepare an RT working solution of the 5x Reaction buffer, water, Enzyme mix and RNA spike ins (in the proportion indicated in the first four lines of Table 1).

The following procedure is recommended:
1. Prepare the required amount of RT working solution and place it on ice.
2. Dispense RT working solution into nuclease free tubes.
3. Dispense template RNA in each tube.

Table 1 – Reverse transcription reaction set-up

Reagent	Volume (µL) RT reaction
5x Reaction buffer	4
Nuclease-free water	9
Enzyme mix	1
Synthetic RNA spike ins, optional replace with H_2O if omitted	2
Template total RNA	4
Total volume	20

Step 4
Mix and spin reagents

Mix the reaction by very gentle vortexing or pipetting to ensure that all reagents are thoroughly mixed. After mixing, spin down.

Step 5
Incubate and heat inactivate[1]

- Incubate for 60 min at 42°C.
- Heat-inactivate the reverse transcriptase for 5 min at 95°C.
- Immediately cool to 4°C.
- Store at 4°C or freeze.

Step 6
Prepare reagents for ddPCR

Place cDNA (from Step 5) and nuclease free water and thaw for 15-20 min. Protect the EvaGreen® Supermix vials from light.
Immediately before use, mix the reagents by vortexing and spin down.

Step 7
Dilute cDNA template in nuclease free water[2]

Immediately before use, dilute only the amount of cDNA template needed for the planned PCR reactions in nuclease free water. It is important that "low-nucleic acid binding" tubes or plates are used.
We recommend diluting the cDNA reaction between 1:50 and 1:500 in water, dependent on target abundance.

It is not recommended to store the diluted cDNA.

Step 8
Combine PCR Supermix, PCR primer mix and cDNA according to Table 2.

Mix thoroughly

Note: remember to calculate necessary excess volume for pipetting and robotic dead volume.

When multiple ddPCR reactions are performed with the same microRNA primer set, it is recommended to prepare a primer master mix working-solution of the PCR primers and the PCR EvaGreen® Supermix (in the proportion indicated in Table 2).

The following procedure is recommended:
1. Prepare the required amount of primer + Supermix working-solution (see Table 2) and place it on ice. It is recommended to include excess of all reagents in the master mix to compensate for pipetting excess material.
2. Place the relevant volume of primer:supermix working-solution in PCR tubes/wells (see Table 2) and spin tubes/plate briefly in a centrifuge (1500g for 1 minute), to remove air bubbles.
3. Add cDNA template to each tube/well.

Table 2 – ddPCR reaction, pr. 20 µL reaction

Reagent	Volume per reaction (µL)
2x EvaGreen® Supermix	10
PCR primer mix	1
Diluted cDNA template	9
Total volume	20

Step 9
Mix and spin reagents

Mix the reaction by pipetting up and down to ensure that all reagents are mixed thoroughly. Centrifuge briefly to collect sample.
Make sure to equilibrate for 3 minutes at room temperature before preparing the droplets.

Step 10
Droplet generation

Insert the DG8 cartridge into the holder, following the Bio-Rad recommendations. Transfer 20 µL of each sample prepared in step 8 to the sample wells (middle row) of the DG8 cartridge while avoiding air bubbles and fill each oil well (bottom row) with 70 µL DG oil for EvaGreen®.
Hook the gasket over the cartridge holder and insert into the QX200™ Droplet Generator.
Close the lid to start droplet generation. When droplet generation has finished, open the lid, remove the disposable gasket. The top wells of the cartridge contain the droplets.

Step 11
Preparation for PCR

Pipet 40 µL of the contents of the top wells (the droplets) into a single column of a 96-well PCR plate, by following the recommended pipetting technique by Bio-Rad, to avoid shearing or coalescing of the droplets.
Seal the PCR plate with foil immediately after transferring droplets to avoid evaporation. Use pierceable foil plate seals that are compatible with the PX1™ PCR plate sealer and the needles in the QX200™ droplet reader (catalog #181-4040).

Begin thermal cycling (PCR) within 30 min of sealing the plate.

Step 12
PCR amplification

Perform PCR amplification according to Table 3.

Table 3 – ddPCR reaction, pr. 20 µL reaction

Process step	Settings
Polymerase Activation/ Denaturation	95°C, 5 min
Amplification	40 amplification cycles at 95°C, 30 s 58°C, 1 min,[3] ramp-rate 1.6°C/s
Dye stabilization	4°C, 5 min 90°C, 5 min 4°C, indefinitte

Step 13
Sample counting on QX200™ Droplet Reader

Power on the QX200™ Droplet Reader and make sure the two first indicator lights are green:

- Place the 96-well PCR plate containing the amplified droplets into the base of the plate holder.
- 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.
- 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 three indicator lights are green.
- Start the run from the QuantaSoft™ software, using absolute quantification mode
- When droplet reading is complete, all four lights are green

1) The protocol can be interrupted at this stage. The undiluted cDNA may be kept at -20°C for up to 5 weeks (optional store at 4°C for up to 4 days). It is recommended that synthesized cDNA is stored in "low-nucleic acid binding" tubes or plates.
2) Adjust volumes to accommodate your in-house liquid handling system volume loss when pipetting.
3) The annealing temperature should be optimized for each assay. Usually 58°C works, but some primers need 56°C or 60°C to get a better separation.

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