

## miRCURY LNA™ Universal RT microRNA PCR

# Protocol for microRNA qPCR profiling using microRNA LNA™ PCR primers sets with Fluidigm® BioMark system

Supplement for Fluidigm® ADP 14: Gene Expression using EvaGreen® DNA Binding Dye with 48.48 and 96.96 Dynamic Array™ IFCs

## Introduction

This protocol describes overall steps in how to run a 48.48 or 96.96 Dynamic Array™ IFC from Fluidigm® using the Exiqon miRCURY LNA™ Universal RT microRNA PCR system. Sample input amount and dilution level of the Specific Target Amplification (STA) are meant as a guide. Empirical determination of sample input amount and STA dilution factor may be required. The microRNA LNA™ PCR primers are optimized to work with miRCURY LNA™ Universal RT microRNA PCR, SYBR® Green master mix. Some changes in sensitivity and specificity may be observed when using other master mixes.

The present protocol should be regarded as a recommendation for a good starting point for experiments combining the miRCURY LNA™ Universal RT microRNA PCR system with the Fluidigm® BioMark microfluidic system. It is meant as a supplement to the Fluidigm® protocol for Gene Expression using EvaGreen® DNA Binding Dye with 48.48 and 96.96 Dynamic Array™ IFCs. Exiqon strongly advise reading the Fluidigm® protocol and the Instruction Manual for miRCURY LNA™ Universal RT microRNA PCR before starting the experiment.

## Required reagents from Exiqon

For a setup of 10 Dynamic Array™ IFC chips, the following reagents from Exiqon are needed:

	48.48 (480 samples)	96.96 (960 samples)
Universal cDNA Synthesis Kit*	15	30
Primer pairs ordered in 96 well plate**	48 microRNA LNA™ PCR primer sets (standard size)	96 microRNA LNA™ PCR primer sets (standard size)

\*Excess for pipetting loss not included.

\*\*Exiqon recommend including an RNA spike-in control per sample. The RNA spike-in control primer set (UniSp6 CPI) is available from Exiqon upon request. The RNA spike-in template is provided with the Universal cDNA Synthesis kit. Read more about use of the RNA spike-in control in the Instruction Manual, miRCURY LNA™ Universal RT microRNA PCR.

Please consult with the Fluidigm® protocol for Gene Expression using EvaGreen® DNA Binding Dye with 48.48 and 96.96 Dynamic Array™ IFCs (PN100-1208 B1) for additional required reagents.



## Protocol

Overview of all steps of the protocol:

- Universal cDNA synthesis (Step 1-5)
- Preparation of primers for STA (Step 6-7)
- Perform STA (Step 8-10)
- Prepare pre-sample mix (Step 11-12)
- Prepare assay mix (primers) (Step 13)
- Run Fluidigm® chip (Step 14)

### Universal cDNA synthesis:

#### Step 1

Dilute template RNA

Adjust each of the template RNA samples to a concentration of 5 ng/μL using nuclease free water (optimization might be necessary depending on the origin and quality of the RNA sample).

#### Step 2

Prepare reagents

Gently thaw the 5x Reaction buffer and nuclease-free water, and immediately place on ice. Mix by vortexing. 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

Prepare an RT master mix of the 5x Reaction buffer, water and Enzyme mix, and RNA spike-in. Mix according to Table 1.

**Table 1 – Reverse transcription master mix**

Reagent	Volume (μL) for each sample	48 samples*	96 samples*
5x Reaction buffer	4	204	408
Nuclease-free water	9	459	918
Enzyme mix	2	102	204
Synthetic spike in, optional replace with H <sub>2</sub> O if omitted	1	51	102
Total volume	16	816	1632

\*6% Excess included for loss during pipetting.

The following procedure is recommended:

1. Prepare the required amount of RT master mix and place it on ice.
2. Dispense 16 μL RT master mix into nuclease free tubes.
3. Dispense 4 μL template RNA (5 ng/μL) in each tube.

**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\*

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

Store at 4°C or freeze or proceed immediately to STA. Before starting with STA, the cDNA should be diluted 1:10 in nuclease free water (should be empirically determined).

\*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.

**Preparation of primers for STA:**

**Step 6**  
Re-suspend primers

For LNA™ primer pairs ordered as standard size LNA™ primer set pre-mixed in a 96-well plate, dissolve all wells (48 or 96) in 33µL 1xTE buffer/well to a final primer concentration of 20 µM each. Leave primers at 4°C for 30 min. to ensure that all primers are properly dissolved.

**Step 7**  
Pool primers for STA according to Table 2

Pool primers according to Table 2 for each chip.

**Table 2 – Primer pair preparation**

Reagent	Volume (µL) for 48 sample	Volume (µL) for 96 sample
Volume of each primer pair	1	1.4
1xTE Buffer	52	5.6
Total volume	100	140

The remaining volume of primers in the 96-well plate are used in Step 13 for the PCR reaction.



**Perform Specific Target Amplification (STA):****Step 8**

Combine reagents according to Table 3

For each sample mix according to Table 3.

**Table 3 – STA reaction mix**

Reagent	Volume (µL) for each sample
TaqMan® PreAmp Master Mix (Applied Biosystems PN 4361128)	2.5
Pooled primer mix from Step 7	1.25
Diluted cDNA from Step 5	1.25
Total volume	5

**Step 9**

Perform 15 cycles of pre-amplification

PreAmp 15 cycles using the following thermal protocol as a guide:

**Table 4 – PreAmp (STA)**

Process step	Settings
Polymerase Activation/Denaturation	95°C for 10 minutes
Amplification	15 cycles at 95°C for 15 seconds, 60°C for 4 minutes*

\* This time can be optimized.

**Step 10**

Dilute STA 1:10

Dilute STA reaction 1:10 in nuclease free water (should be empirically determined).



**Prepare Pre-Sample Mix:****Step 11**

Combine reagents according to Table 5

Prepare the Pre-Sample Mix according to Table 5.

**Table 5 – Pre-Sample Mix**

Reagent	Volume (μL) for each sample	48 samples*	96 samples*
2X TaqMan® Gene Expression Master Mix (Applied Biosystems PN 4369016)	2.5	132.5	275
20X DNA Binding Dye Sample Loading Reagent (Fluidigm®, PN 100-0388)	0.25	13.25	27.5
20X EvaGreen™ (Biotium, catalog # 31000)	0.25	13.25	27.5
1x TE Buffer	0.75	39.75	82.5
Sample/cDNA, from Step 10	1.25		
Total volume	5		

\*10% Excess included for loss during pipetting

**Step 12**

Prepare the Pre-Sample Mix with the sample

Combine 3.75 μL of Pre-Sample Mix from Table 5 with 1.25 μL of sample (diluted STA from Step 10) and vortex gently, but thoroughly. Spin to collect contents. Keep on ice.



**Prepare Assay Mix (primers):****Step 13**

Prepare primers according to Table 6

For each assay (primer pair) mix according to Table 6.

**Table 6 – Preparing Assay Mix**

Reagent	Volume (µl) for each assay (48 and 96)
Forward and Reverse primer mix from Step 6	1.25
2X Assay Loading Reagent	2.5
1X TE Buffer	1.25
Total volume	5.0

Vortex and spin to collect volume.

**Run Fluidigm® chip:****Step 14**

Perform 30 PCR cycles on the Fluidigm® platform

Pipette samples and assays on to chip according to Fluidigm® recommendations and set up PCR cycles according to Table 7:

**Table 7 – PCR steps**

Process step	Settings
Polymerase Activation/Denaturation	95°C for 10 minutes
Amplification	30 cycles at 95°C for 10 seconds, 60°C for 1 minute

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