Heidelberg Biolabs

LABORATORY MANUAL

BioLiqX cf-RNA Isolation QC kit

(based on RT-qPCR)

Catalog Number: HB213A

Table of Contents

1. OVERVIEW

Cell-free mature miRNAs are present in almost all mammalian biological fluids as nuclease resistant entities protected mainly by proteins of Argonautes family as well as by encapsulation into various membrane vesicles. Normally, miRNAs enable posttranscriptional regulation of gene expression, however, they remain in the extracellular milieu upon the death of the parental cells for prolonged time and can be used to monitor the ongoing pathological processes in certain tissues and organs. In addition, miRNAs in biofluids can serve as quality check indicators for the cell-free RNA isolation efficiency.

The BioLiqX cf-RNA Isolation QC Kit contains all reagents and primers required for the detection of three "house-keeping" miRNAs including **hsa-miR-16**, **hsa-miR-21** and **hsa-miR-451a**, as well as a spike-in control **cel-miR-39** in any RNA sample using RT-qPCR. The key features of the method are highly efficient polynucleotide tailing and simultaneous reverse transcription of all short RNA species in a sample which are followed by real-time PCR amplification. The tailing and reverse transcription (**TaRT**) mix included in the kit is an optimized composition of enzymes, nucleotides, mineral salts, and RT primers that secures an efficient conversion of highly diluted miRNA molecules into cDNA in a single reaction. In the second step, the cDNAs serve as templates for ultra-sensitive and low-background real-time PCR reactions with Green DNA Dye (a SYBR® Green analog) and miRNA-specific primers. Due to its simplicity, the procedure secures low variation between technical replicates, short hands-on time, and easiness of automation. High specificity and sensitivity of qPCR are achieved by optimal concentration of nucleotides, mineral salts, Green DNA Dye and Hot Start Taq DNA Polymerase in the reaction. All reactions can be set up at the room temperature without the risk of non-specific amplification. The BioLiqX cf-RNA Isolation QC kit is ideal for monitoring the RNA isolation efficiency between samples and has a similar sensitivity as compared to analogous preamplification-free miRNA assays.

[IMPORTANT NOTE]. Regardless of the supplier, any preamplification-free protocol for miRNA detection with RT-qPCR has a fundamental sensitivity limit due to a dilution factor. Thus, an initial RNA sample suffers typically 2-fold dilution in a cDNA reaction, and further 5-fold dilution in a qPCR reaction. In addition, every protocol requires a final dilution of the cDNA reaction before the qPCR (usually 6-30x depending on the research kit). To negate the dilution effect, the preamplification-based highly sensitive BioLiqX HS miRNA Assays (cat # HB214A) are available as a separate kit.

2. THE METHOD WORKFLOW

On the first step, a polyA-tails are added to 3'-OH termini of all RNAs and cDNA is synthesized simultaneously using an anchored dT-rich primer with custom 5'-terminal sequence. Finally, cDNA is amplified using miRNA-specific forward and reverse primers during qPCR reaction, while Green DNA Dye (a SYBR® Green analog) is used for the detection and quantification of dsDNA amplicons in real-time (Figure 1).

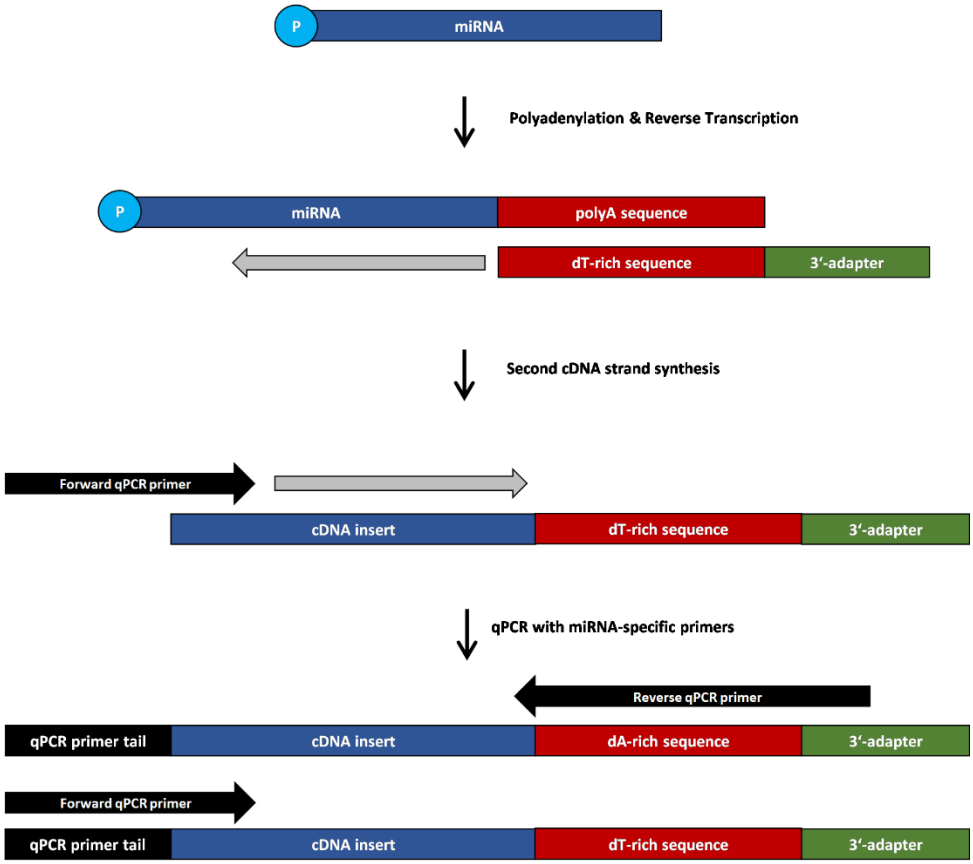

Figure 1. Principle used by BioLiqX cf-RNA Isolation QC kit for miRNA detection.

3. KIT COMPONENTS AND STORAGE

Tube Name	Cap color	Catalog No	Volume
TaRT Mix	Red	HBTRM21A	Custom volume (50 µL per aliquot)
qPCR Mix	White	HBQM21A	Custom volume
hsa-miR-16 PM	Colorless	HBH16P21A	Custom volume
hsa-miR-21 PM	Colorless	HBH21P21A	Custom volume
hsa-miR-451a PM	Colorless	HBH45121A	Custom volume
cel-miR-39 PM	Colorless	HBC39P21A	Custom volume
cel-miR-39	Blue	HBC39121A	100 µL (1 ng/µL)

All components of the BioLiqX cf-RNA Isolation QC kit should be stored in a -20 °C or -80 °C freezer immediately upon receipt. Avoid repeated freezing thawing. The **TaRT mix** should **not** be freeze-thawed more than 5 times.

4. REQUIRED MATERIALS NOT INCLUDED

Check to ensure that all necessary materials and equipment are available before starting with library preparation. All reagents, equipment, and labware must be free of nucleases and nucleic acid contamination.

General laboratory equipment and consumables

- Benchtop centrifuge (3,000 x g, rotor compatible with 96-well plates).
- Benchtop centrifuge (14,000 x g, rotor compatible with 0.2 mL or 0.5 ml tubes).
- Calibrated single-channel pipettes for handling 0.5 µL to 100 µL volumes.
- PCR Thermocycler.
- Vortex mixer.
- Ribonuclease-free pipette tips (pipette tips with aerosol barriers recommended).
- Ribonuclease-free 0.2 mL or 0.5 mL PCR tubes.
- Ribonuclease-free 96-well PCR plates with caps or sealing foil.
- Real Time PCR machine and related consumables including 96/384-sell plates and sealing foil.
- Nuclease-free water (e.g. provided with BioLiqX RNA Isolation kit, cat # HB205A).

5. GENERAL GUIDLINES

We strongly recommend that you read the following warnings and precautions. If you need further assistance, you may contact us at info@heidelbergbiolabs.com

- Wear gloves and lab coat at all steps to minimize contamination and protect samples from degradation by nucleases.
- Wear a protective mask which prevents breathing into the test tubes.
- All containers and storage areas must be free of contaminants and nucleases.
- The use of ice is not required during the entire procedure. All steps should be performed at room temperature (preferably 20°–25°C).
- Vortex and centrifuge each component prior to use. To ensure material has not lodged in the cap or side of the tube, centrifuge in a microcentrifuge at >12,000 x g for 5 seconds.
- Please read the complete manual before first time use.

5. IMPORTANT NOTES

The **qPCR mix** contains an optimized concentration of the Green DNA Dye (a SYBR® Green analog), which binds all dsDNA molecules and emits a fluorescent signal upon binding. The Green DNA Dye has excitation and emission maxima at **495 nm** and **520 nm**, respectively and is compatible with any Real-Time PCR cycler.

The presence of ROX passive reference dye may be necessary in the final qPCR reaction for Real-Time PCR instruments from Applied Biosystems. In this case, ROX dye can be purchased from a corresponding supplier and added to the **qPCR mix**.

- Use 300-600 nM final ROX concentration for the following instruments from Applied Biosystems: PRISM, 7000, 7300, 7700, 7900HT, 7900HTFast, StepOne®, StepOne Plus.
- Use 30-150 nM final ROX concentration for the following instruments from Applied Biosystems: 7500, 7500 Fast, ViiA® 7, QuantStudio® Systems.

6. PROTOCOL

(A) CDNA SYNTHESIS

1. Refreeze **RNA sample** and **TaRT mix** at room temperature. Mix by vortexing and centrifuge for 5 sec.
2. Start cDNA synthesis reaction by adding 5 µL **TaRT mix** to 5 µL **RNA sample**.
3. Mix by pipetting up and down 5 times with the same pipette tip.
4. Incubate 60 min at 37^{o}C and 5 min at 85^{o}C.
5. Dilute cDNA from previous step 1:7 by adding 60 µL of nuclease-free water to 10 µL of cDNA.
6. At this stage diluted cDNA can be stored at +4^{o}C for several hours. For long-term storage, the cDNA should be kept on -20°C or -80°C.

(B) REAL-TIME QPCR

7. Refreeze **qPCR mix**, **hsa-miR-16 PM, hsa-miR-21 PM, hsa-miR-451 PM**, **cel-miR-39 PM** and **diluted cDNA** (if it was kept on -20°C or -80°C) at room temperature. Mix by vortexing and centrifuge for 5 sec.
8. Set-up Real Time PCR reactions for each miRNA by mixing 7.5 µL **qPCR mix**, 0.5 µL corresponding primer mix (**hsa-miR-16 PM, hsa-miR-21 PM, hsa-miR-451 PM** or **cel-miR-39 PM**). Calculate the number of required reactions and include 10% overage for each component when scaling for multiple reactions. Mix by vortexing and centrifuge briefly.
9. At least two technical replicates should be prepared for each miRNA per sample.
10. Aliquot 8 µL of each qPCR reaction in 96- or 384-well qPCR plate.
11. Add 2 µL **diluted cDNA** to each reaction well of the plate.
12. Perform amplification and detection in a Real Time PCR instrument.
    - Initial denaturation: 95^{o}C for 10 min

    40-50 cycles:
    - 95^{o}C for 10 sec
    - 65^{o}C for 30 sec
    - 72^{o}C for 30 sec
13. Perform a melting curve analysis immediately after qPCR cycling according to the recommendations of your Real Time PCR instrument manual. Example: temperature range: 60^{o}C - 95^{o}C, heating rate: 0.5^{o} C/time, constant temperature: 6 sec/time.
14. Obtain raw Cp or Ct values (depending on Real Time PCR instrument) using data analysis software supplied with your Real Time PCR instrument.

7. CONTACT INFORMATION

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TRADEMARKS

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