Heidelberg Biolabs

LABORATORY MANUAL

BioLiqX HS miRNA Assays

(based on RT-qPCR)

Catalog Number: HB214A

Table of Contents

1. OVERVIEW

BioLiqX HS miRNA Assays include all reagents and primers required for the simultaneous qPCR detection of "house-keeping" miRNAs **hsa-miR-16**, **hsa-miR-21** and **hsa-miR-451a**, a spike-in synthetic control **cel-miR-39**, as well as miRNAs which proved themselves as promising extracellular circulating biomarkers in multiple peer-review research publications. Currently, the latter list includes: **hsa-miR-122** (liver-specific), **hsa-miR-208a** (myocard-specific), **hsa-miR-208b** (myocard-specific), **hsa-miR-499** (myocard-specific), **hsa-miR-133a**, **hsa-miR-206**, **hsa-miR-9**, **hsa-miR-124**, **hsa-miR-150**, **hsa-miR-216**, **hsa-miR-375**, **hsa-miR-192**, **hsa-miR-30**, **hsa-miR-200a**, **hsa-miR-200b**, **hsa-miR-200c**, **hsa-miR-141**, and **hsa-miR-429**. Besides, we offer assays for custom miRNAs (including truncated miRNAs and isomiRs) as well as any other short RNA fragment.

The kit utilizes a single-tube protocol for the prior conversion of all short RNA molecules into a pre-amplified cDNA library by adding certain reagents and enzymes to the RNA sample in a sequential manner. Afterward, the pre-amplified cDNA serves as a template for ultra-sensitive and low-background real-time PCR reactions and miRNA-specific primers. The BioLiqX HS miRNA Assays allow detection of very short cDNA fragments including truncated microRNAs and isomiRs. In addition, pre-amplified libraries generated by the protocol can be used for the simultaneous qPCR analysis of various other cell-free RNA fragments using custom primer pairs.

Apart from the preamplification reaction, high-specificity and sensitivity of BioLiqX HS miRNA Assays are achieved by the optimal concentration of nucleotides, mineral salts, Green DNA Dye (a SYBR® Green analog) and Hot Start Taq DNA Polymerase in the final qPCR reaction. BioLiqX HS miRNA Assays are ideal for detecting low copy number circulating miRNAs in the biological fluids but can be also applied to any RNA sample. The whole procedure can be completed within approximately 8 hours and requires typically a hands-on time between 30-60 minutes depending on the number of samples.

The kit is delivered in a standard cardboard microtube box that includes: (1) reagents for cDNA synthesis and preamplification (**Tailing Buffer**, **Tailing Nucleotides**, **Tailing Enzyme**, **Ligation Buffer**, **Ligation Enzyme**, **RT Mix**, **RT primers**, **PreAmp Mix** and **PreAmp primers**; (2) **qPCR Mix** for Real-Time PCR reaction and vials with miRNA-specific primers (**hsa-miR-XXX PM**). By default, the kit also includes synthetic **cel-miR-39** spike-in control. The supplied volume and the exact composition of BioLiqX HS miRNA Assays can be fully customized depending on your research goals and the miRNA species to be analyzed. However, the minimal volumes of the reagents used for generating initial pre-amplified libraries are for 24 samples. Besides, all miRNA-specific primers are compatible with the NGS libraries prepared by BioLiqX Small RNA-seq Kit (cat # HB202A). Therefore, small RNA sequencing results can be validated directly on the same libraries using **qPCR Mix** and miRNA primer pairs from BioLiqX HS miRNA Assays.

2. THE METHOD WORKFLOW

The BioLiqX HS miRNA Assays are based on the so-called “Capture and Amplification by Tailing and Ligation (CATL)” approach for cDNA synthesis and pre-amplification of short or fragmented RNA in the original sample before the final Real-Time PCR step. Briefly, miRNA is subjected to a polyadenylation reaction followed by the ligation of 5’-adapter. The input RNA flanked by 5’-adapter and 3’-poly(A) tails is then converted into cDNA using anchored RT primer carrying poly(T)-rich sequence and custom 3’-adapter sequence. The cDNA is then PCR amplified (typically for 10-14 cycles) using primers carrying terminal sequences matching the 5'- and 3'-adapters. Finally, the pre-amplified cDNA is used as input for Real-Time PCR reaction with miRNA-specific forward and reverse primers. The Green DNA Dye (a SYBR® Green analog) is used for DNA quantification during Real-Time PCR (Figure 1).

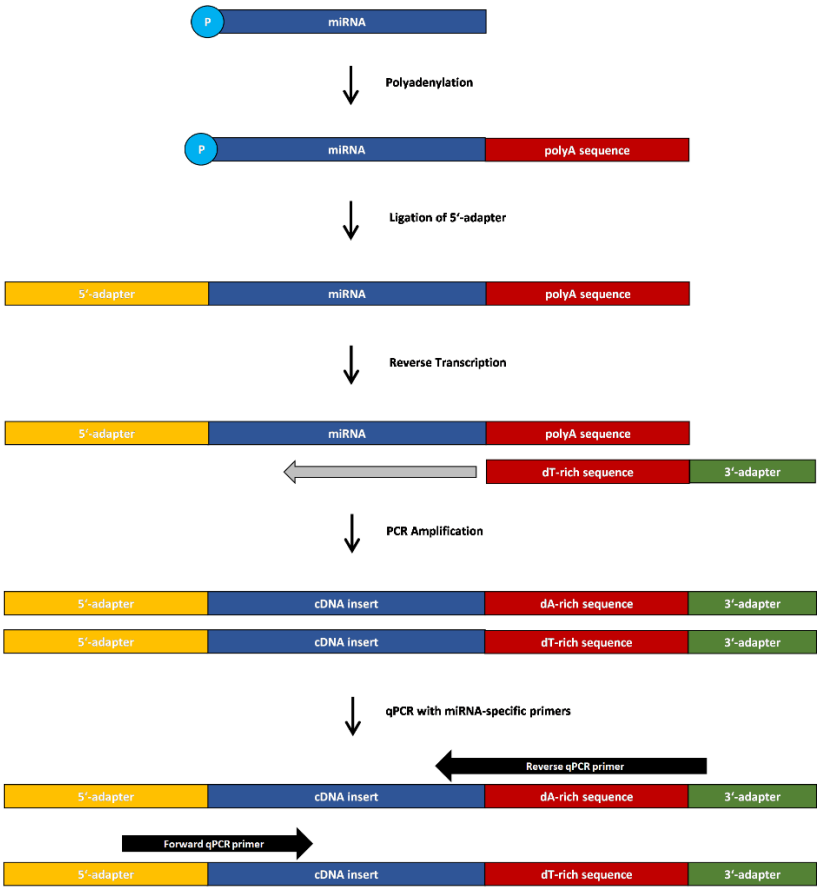


Figure 1. Schematic of Capture and Amplification by Tailing and Ligation (CATL) used by BioLiqX HS miRNA Assays to generate pre-amplified cDNA and subsequent Real-Time PCR.

3. KIT COMPONENTS AND STORAGE

Tube Name	Cap color	Catalog No	Volume
Tailing Buffer	Red	HBTB20A	Custom volume
Tailing Nucleotides	Red	HBTN20A	Custom volume
Tailing Enzyme	Red	HBTE20A	Custom volume
Ligation Buffer	Yellow	HBLB20A	Custom volume
Ligation Enzyme	Yellow	HBLE20A	Custom volume
RT Mix	Green	HBRT20A	Custom volume
RT primers	Green	HBRP20A	Custom volume
PreAmp Mix	White	HBPA20A	Custom volume
PreAmp Primers	White	HBPAP20A	Custom volume
qPCR Mix	White	HBQM21A	Custom volume
hsa-miR-16 PM	Transparent	HBH16P21A	Custom volume
hsa-miR-21 PM	Transparent	HBH21P21A	Custom volume
hsa-miR-451a PM	Transparent	HBH45121A	Custom volume
hsa-miR-24 PM	Transparent	HBH24P21A	Custom volume
hsa-miR-133a PM	Transparent	HBH133AP22	Custom volume
hsa-miR-206 PM	Transparent	HBH206P22	Custom volume
hsa-miR-208a PM	Transparent	HBH208AP22	Custom volume
hsa-miR-208b PM	Transparent	HBH208BP22	Custom volume
hsa-miR-499 PM	Transparent	HBH499P22	Custom volume
hsa-miR-122 PM	Transparent	HBH122P22	Custom volume
hsa-miR-150 PM	Transparent	HBH150P22	Custom volume
hsa-miR-216 PM	Transparent	HBH216P22	Custom volume
hsa-miR-192 PM	Transparent	HBH192P22	Custom volume
hsa-miR-9 PM	Transparent	HBH9P22	Custom volume
hsa-miR-124 PM	Transparent	HBH124P22	Custom volume
hsa-miR-30 PM	Transparent	HBH30P22	Custom volume
hsa-miR-375 PM	Transparent	HBH375P22	Custom volume
hsa-miR-200a PM	Transparent	HBH200aP22	Custom volume
hsa-miR-200b PM	Transparent	HBH200bP22	Custom volume
hsa-miR-200c PM	Transparent	HBH200cP22	Custom volume
hsa-miR-141 PM	Transparent	HBH141P22	Custom volume
hsa-miR-429 PM	Transparent	HBH429P22	Custom volume
cel-miR-39 PM	Transparent	HBC39P21A	Custom volume
cel-miR-39	Blue	HBC39121A	100 µL (1 ng/µL)

All components of the BioLiqX HS miRNA Assays should be stored in a -20 °C or -80 °C freezer immediately upon receipt. Avoid repeated freezing thawing.

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.
- Do not remove **Tailing Enzyme** or **Ligation Enzyme** from -20°C or -80 °C until immediately before use and return to -20°C or -80 °C immediately after use.
- Please read the complete manual before first time use.

6. 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.

7. PROTOCOL

(A) POLYADENYLATION

1. Refreeze **Tailing Buffer** and **Tailing Nucleotides** at room temperature. Mix by vortexing and centrifuge for 5 sec.
2. Prepare polyadenylation master mix by combining 1.25 µL **Tailing Buffer**, 0.5 µL **Tailing Nucleotides** and 0.25 µL **Tailing Enzyme** per reaction [include 10% overage for each component when scaling for multiple reactions]. Mix by vortexing and centrifuge briefly.
3. Add 2 µL polyadenylation master mix to 4 µL of RNA sample. Mix by pipetting up and down 5 times.
4. **[IMPORTANT]**. Centrifuge the tube(s) or 96-well plate(s) for 5 sec at maximal speed to ensure that material has not lodged on the sides of the tubes/wells.
5. Incubate 25 min at 37°C and 20 min at 65°C on PCR Thermocycler. Cool to 4°C.

(B) LIGATION OF 5'-ADAPTER

6. Refreeze **Ligation Buffer** at room temperature. Mix by vortexing and centrifuge for 5 sec.
7. Prepare ligation master mix by combining 5.5 µL **Ligation Buffer** and 0.5 µL **Ligation Enzyme** per reaction [include 10% overage for each component when scaling for multiple reactions]. Mix slowly by pipetting up and down 5 times.

Aspirate and dispense viscous Ligation Buffer and ligation master mix solutions slowly.

8. Add 6 µL ligation master mix to each sample. Mix by pipetting up and down 5 times.
9. Incubate 2 hours at 25°C and 15 min at 65°C on PCR Thermocycler. Cool to 4°C.

(C) REVERSE TRANSCRIPTION

10. Refreeze **RT Mix** and **RT Primers** at room temperature. Mix by vortexing and centrifuge for 5 sec.
11. Prepare reverse transcription master mix by combining 5 µL **RT Mix** and 0.5 µL **RT Primers** per reaction [include 10% overage for each component when scaling for multiple reactions]. Mix by vortexing and centrifuge briefly.
12. Add 5.5 µL reverse transcription master mix to each sample. Mix by pipetting up and down 5 times.
13. Incubate 1 hour at 42°C and 5 min at 85°C on PCR Thermocycler. Cool to 4°C.

The procedure can be stopped here with samples stored at -20°C before proceeding to next step.

(D) PCR AMPLIFICATION

14. Refreeze **PreAmp Mix** and **PreAmp primers** at room temperature. Mix by vortexing and centrifuge for 5 sec.
15. Prepare preamplification master mix by combining 25 µL **PreAmp Mix** and 5 µL **PreAmp primers** per reaction [include 10% overage for each component when scaling for multiple reactions]. Mix by vortexing and centrifuge briefly.
16. Add 30 µL preamplification master mix to each sample.
17. Perform amplification on PCR Thermocycler.
    - Initial denaturation: 95°C for 30 sec

    14 cycles:
    - 95°C for 10 sec
    - 62°C for 30 sec
    - 72°C for 30 sec
18. Dilute pre-amplified cDNA from previous step 1:10 (e.g. by adding 450 µL of nuclease-free water to 50 µL of cDNA).

The procedure can be stopped here with samples stored at -20°C before proceeding to next step.

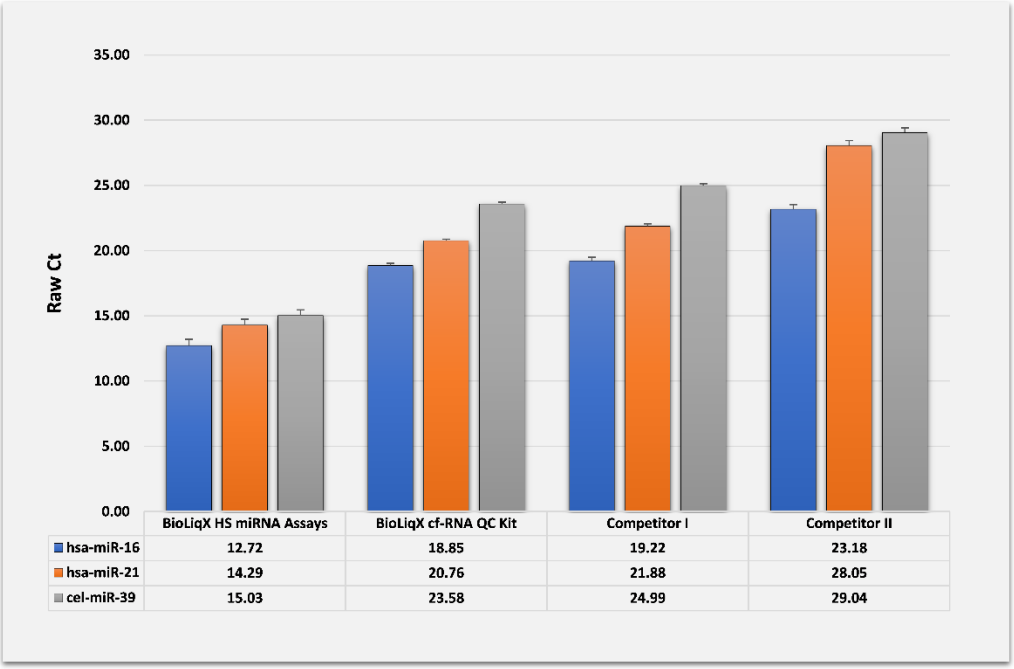
(E) REAL-TIME QPCR

19. Refreeze **qPCR mix**, **corresponding miRNA-specific primer mix** and **diluted cDNA** (if it was kept on -20°C or -80°C) at room temperature. Mix by vortexing and centrifuge for 5 sec.
20. 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**, **cel-miR-39 PM,** etc.). Calculate the number of required reactions and include 10% overage for each component when scaling for multiple reactions. Mix by vortexing and centrifuge briefly.
21. At least two technical replicates should be prepared for each miRNA per sample.
22. Aliquot 8 µL of each qPCR reaction in 96- or 384-well qPCR plate.
23. Add 2 µL **diluted cDNA** to each reaction well of the plate.
24. Centrifuge the reaction plate/tubes briefly to spin down the contents.
25. Perform amplification and detection in a Real Time PCR instrument.
    - Initial denaturation: 95°C for 10 min

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

8. EXAMPLE OF ASSAYS PERFORMANCE

BioLiqX HS miRNA Assays have superior sensitivity as compared to preamplification-free protocols mainly due to negating RNA and cDNA dilution effect during RT-qPCR. The figure below shows the benchmarking of BioLiqX HS miRNA hsa-miR-16, hsa-miR-21 and cel-miR-39 assays with the corresponding preamplification-free assays from either BioLiqX cf-RNA Isolation QC Kit or two competing research kits.



	BioLiqX HS miRNA Assays	BioLiqX cf-RNA QC Kit	Competitor I	Competitor II
hsa-miR-16	12.72	18.85	19.22	23.18
hsa-miR-21	14.29	20.76	21.88	28.05
cel-miR-39	15.03	23.58	24.99	29.04

Figure 1. Benchmarking of BioLiqX HS miRNA Assays with preamplification-free protocols. Specifically, total RNA was isolated from 0.4 mL of human blood plasma with 0.5 pg 22 nt cel-miR-39 control premixed after the initial lysis. The RNA was eluted in the total volume of 50 µL, and 5 µL of eluates were taken as inputs for each reaction (in triplicates). The BioLiqX HS miRNA libraries were subject to 12x pre-amplification cycles and diluted 10-fold in nuclease-free water. The cDNA generated by BioLiqX cf-RNA Isolation QC kit and the competing kits was diluted 6-fold. The proportions of the diluted cDNA in the final qPCR reactions were equal for each protocol. The Real-Time PCR was performed using LightCycler 480 Real-Time PCR System (Roche) according to the recommendations of each kits' manuals. Raw threshold cycle (Ct) values were determined by the second derivative max method. Each bar represents the mean (SD) of three technical replicates.

9. CONTACT INFORMATION

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TRADEMARKS

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