Heidelberg Biolabs

LABORATORY MANUAL

BioLiqX Small RNA-seq Kit

(for Illumina platforms)

Catalog Number: HB202A

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1. OVERVIEW

BioLiqX Small RNA-seq Kit includes all the components required to generate DNA libraries for massive parallel sequencing of small RNA (or long RNA after fragmentation) on Illumina platforms. The kit utilizes a single-tube protocol implying the addition of certain reagents and enzymes to the RNA sample in a sequential manner. The DNA library preparation workflow was originally developed for 10 ng – 1 pg inputs of highly fragmented RNA present in biofluids such as blood plasma and serum (typically between 3-100 nt). However, the kit can be also used for other inputs including total RNA purified from cells or enriched small RNA fractions of cellular RNAs.

Biological fluids (like any other sources) consist of distinct populations of RNAs carrying either hydroxyl (-OH) or phosphate (-P) groups at their 5'- and 3'- termini. Those include: (1) 5'- and 3'-phosphorylated RNAs (**PP**); (2) 5'-OH/3'-OH carrying RNAs (**OO**); (3) 5'-OH and 3'-phosphorylated RNAs (**OP**) and (4) 5'-phosphorylated/3'-OH RNAs (**PO**). For instance, the **PO** category includes predominantly mature miRNAs and snoRNAs. Therefore, the BioLiqX Small RNA-seq Kit was designed to capture RNA carrying distinct 5'- and 3'-terminal groups by including slight modifications of the protocol depending on the task and the scientific question to be addressed. The libraries generated by BioLiqX Small RNA-seq Kit are stranded, with Read1 and Read2 corresponding to the sense and antisense strand of the input RNA, respectively. The whole procedure can be completed within approximately 6 hours and requires a hands-on time between 20-60 minutes depending on the number of samples.

The kit is delivered in a standard cardboard microtube box that includes reagents for polynucleotide tailing and optional end-repair (**Tailing Buffer**, **PdP Enzyme**, **Tailing Nucleotides**, **Tailing Enzyme**), ligation reagents (**Ligation Buffer**, **Ligation Enzyme**), reverse transcription reagents (**RT Buffer**, **RT Enzyme**, **RT primers**) as well as **PreAmp Mix** for the final cDNA preamplification. By default, the kit is supplied with 24 standard P7 **Index primers** for multiplexing. Synthetic **cel-miR-39** ssRNA (included) is recommended as positive control. The volume and exact composition of the kit (along with the primer types required for multiplexing) can be customized depending on number of samples, type of sequencing and research goals. However, the minimal size of the kit is for 24 samples.

2. THE METHOD WORKFLOW

The BioLiqX Small RNA-seq Kit is based on Capture and Amplification by Tailing and Ligation (CATL) approach to generate sequencing libraries for Illumina platforms. On the first step, single-stranded RNA is subjected to a highly efficient polyadenylation reaction. The optional steps including phosphorylation of the 5'-termini and/or removal of 3'-phosphate/cyclophosphate groups can be included depending on the experimental goals and the desired populations of RNAs to be sequenced. The polyadenylation reaction is 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. Multiplexing i7 indexes are introduced during the final PCR amplification with primers carrying P5 and P7 terminal sequences required for cluster generation on Illumina machines.

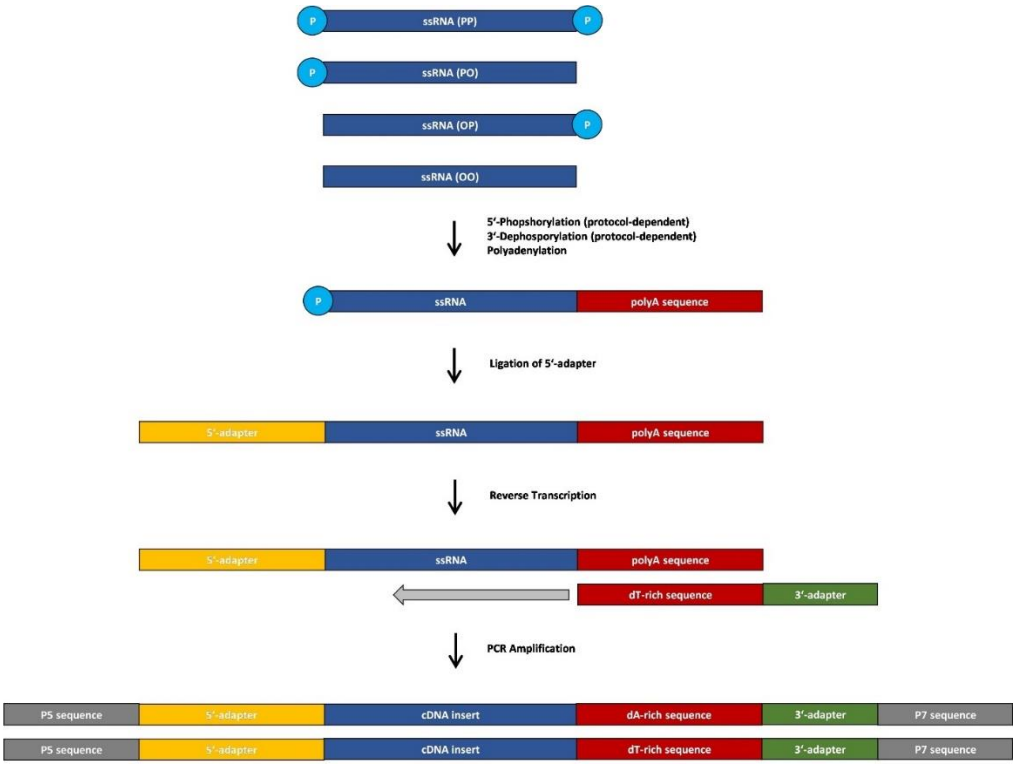

Figure 1. Schematic of Capture and Amplification by Tailing and Ligation (CATL) used by BioLiqX small RNA-seq kit to generate libraries for Illumina sequencing platforms.

In most cases, the prepared library can be used directly for loading onto an Illumina sequencer after clean-up from the excess of PCR primers with magnetic SPRI beads or column-based purification. However, certain samples may require additional size-selection with either SPRI beads or the BluePippin system what secures a greater control over final library insert sizes. From our experience, the total libraries generated from cell- and cell debris-free blood plasma or serum do not require additional size-selection.

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
PdP Enzyme	Red	HBPP20A	Custom volume
Ligation Buffer	Yellow	HBLB20A	Custom volume
Ligation Enzyme	Yellow	HBLE20A	Custom volume
RT Buffer	Green	HBRTB21A	Custom volume
RT Enzyme	Green	HBRTE21A	Custom volume
RT primers	Green	HBRP20A	Custom volume
PreAmp Mix	White	HBPA20A	Custom volume
Index 1 primer	Transparent	HBI120A	Custom volume
Index 2 primer	Transparent	HBI220A	Custom volume
Index 3 primer	Transparent	HBI320A	Custom volume
Index 4 primer	Transparent	HBI420A	Custom volume
Index 5 primer	Transparent	HBI520A	Custom volume
Index 6 primer	Transparent	HBI620A	Custom volume
Index 7 primer	Transparent	HBI720A	Custom volume
Index 8 primer	Transparent	HBI820A	Custom volume
Index 9 primer	Transparent	HBI920A	Custom volume
Index 10 primer	Transparent	HBI1020A	Custom volume
Index 11 primer	Transparent	HBI1120A	Custom volume
Index 12 primer	Transparent	HBI1220A	Custom volume
Index 13 primer	Transparent	HBI1320A	Custom volume
Index 14 primer	Transparent	HBI1420A	Custom volume
Index 15 primer	Transparent	HBI1520A	Custom volume
Index 16 primer	Transparent	HBI1620A	Custom volume
Index 18 primer	Transparent	HBI1820A	Custom volume
Index 19 primer	Transparent	HBI1920A	Custom volume
Index 20 primer	Transparent	HBI2020A	Custom volume
Index 21 primer	Transparent	HBI2120A	Custom volume
Index 22 primer	Transparent	HBI2220A	Custom volume
Index 23 primer	Transparent	HBI2320A	Custom volume
Index 25 primer	Transparent	HBI2520A	Custom volume
Index 27 primer	Transparent	HBI2720A	Custom volume
cel-miR-39	Blue	HBC39121A	100 µL (1 ng/µL)

All components of the BioLiqX Small RNA-seq Kit 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 0.5 ml and 1.5 ml tubes).
- Calibrated single-channel pipettes for handling 0.5 µL to 1,000 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.

Reagents and equipment for final purification step and QC

- AMPure® XP Beads (Beckman Coulter), or MagSi-NGS^{prep} Plus beads (Steinbrenner Laborsysteme GmbH).
- Absolute ethanol (molecular biology grade).
- Magnetic Stand for 6-96 samples depending on the experimental setup.
- 1X TE buffer (e.g. Sigma, 93283-100ML).
- Nuclease-free 1.5 ml tubes.
- Agilent 2100 Bioanalyzer® and Agilent High Sensitivity DNA Kit (Agilent, 5067-4626); or Agilent 2200 TapeStation system and High Sensitivity D1000 ScreenTape (5067- 5584) with High Sensitivity D1000 Reagents (5067- 5585).
- Qubit® Fluorometer (Thermo Fisher Scientific); Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific, Q32851).

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 at all steps to 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**, **PdP 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.
- RNA sample quality may vary between preparations. It is the user's responsibility to optimize the initial RNA input amount to obtain desired PCR bands for purification and sequencing. Refer to the Table 1 below for additional information.
- Please read the complete manual before first time use.

Table 1. Guidelines for different RNA inputs and types. Some optimizations may be required.

RNA input	RNA types	RT primer dilution	PCR cycles
1 pg - 10 pg small RNA	PP, PO, OP and OO	10-fold dilution	19
	PO only	10-fold dilution	21
	PP and PO	10-fold dilution	19
	PO and OO	10-fold dilution	21
20 pg - 100 pg small RNA	PP, PO, OP and OO	Undiluted	16
	PO only	10-fold dilution	19
	PP and PO	Undiluted	16
	PO and OO	10-fold dilution	19
200 pg – 1 ng small RNA	PP, PO, OP and OO	Undiluted	13
	PO only	Undiluted	16
	PP and PO	Undiluted	13
	PO and OO	Undiluted	16
2 ng – 10 ng small RNA	PP, PO, OP and OO	Undiluted	10
	PO only	Undiluted	13
	PP and PO	Undiluted	10
	PO and OO	Undiluted	13

The quality and quantity of input RNA could be estimated using various assays; however, it is frequent that amounts of RNA isolated from biofluids are below the detection limit even with most sensitive methods and may range from 1 pg to 10 pg per microliter of eluate. For such samples we recommend using a maximal possible volume of RNA sample. In addition, we recommend using the syntetic 22 nt cel-miR-39 control (included in the kit) to monitor the efficiency of the procedure. Thus, when using 80 pg of cel-miR-39 as input, the user should expect to observe a strong 156-163 bp product following 15 pre-amplification cycles.

6. SHORT PROTOCOL FOR ALL RNAs (PP, OP, PO and OO)

1. 8 µL **RNA sample** + [2.5 µL **Tailing Buffer** + 0.5 µL **PdP Enzyme**].
2. Incubate 15 min at 37°C.
3. + [1 µL **Tailing Nucleotides** + 0.5 µL **Tailing Enzyme**].
4. Centrifuge the tube(s)/plate(s) for 5 sec at maximal speed.
5. Incubate 25 min at 37°C and 20 min at 65°C, hold at 4°C.
6. + [11 µL **Ligation Buffer** + 0.5 µL **PdP Enzyme** + 1 µL **Ligation Enzyme**].
7. Incubate 15 min at 37°C, 60 min at 25°C and 15 min at 65°C, hold at 4°C.
8. + [8.5 µL **RT Buffer** + 1.5 µL **RT Enzyme** + 1 µL **RT Primers** (undiluted or diluted depending on the RNA input, please see Table 1)].
9. Incubate 30 min at 42°C and 5 min at 85°C, hold at 4°C.
10. + 50 µL **PreAmp Mix** + 10 µL of the **Index primer**.
11. Perform PCR amplification:
    - Initial denaturation: 95°C for 30 sec

    10 - 19 cycles (please see Table 1):
    - 95°C for 10 sec
    - 62°C for 30 sec
    - 72°C for 30 sec
12. [OPTIONAL] Run 20 µL of PCR reaction on a 3-4% agarose gel.
13. [OPTIONAL] If the library is poorly visible on an agarose gel - add 2-3 more amplification cycles.
14. Purify the remaining final PCR reaction with AMPure® XP Beads, MagSi-NGS^{prep} Plus beads or similar. Add **1.6 volumes** of beads to final PCR reaction during beads purification step.
15. Perform quality check of the library with Qubit and Bioanalyser.

7. SHORT PROTOCOL FOR 5'-P/3'-OH RNAs (PO) ONLY

1. 8 µL **RNA sample** + [2.5 µL **Tailing Buffer** + 1 µL **Tailing Nucleotides** + 0.5 µL **Tailing Enzyme**].
2. Centrifuge the tube(s)/plate(s) for 5 sec at maximal speed.
3. Incubate 25 min at 37°C and 20 min at 65°C, hold at 4°C.
4. + [11 µL **Ligation Buffer** + 1 µL **Ligation Enzyme**].
5. Incubate 15 min at 37°C, 60 min at 25°C and 15 min at 65°C, hold at 4°C.
6. + [8.5 µL **RT Buffer** + 1.5 µL **RT Enzyme** + 1 µL **RT Primers** (undiluted or diluted depending on the RNA input, please see Table 1)].
7. Incubate 30 min at 42°C and 5 min at 85°C, hold at 4°C.
8. + 50 µL **PreAmp Mix** + 10 µL of the **Index primer**.
9. Perform PCR amplification:
    - Initial denaturation: 95°C for 30 sec

    13 - 21 cycles (please see Table 1):
    - 95°C for 10 sec
    - 62°C for 30 sec
    - 72°C for 30 sec
10. [OPTIONAL] Run 20 µL of PCR reaction on a 3-4% agarose gel.
11. [OPTIONAL] If the library is poorly visible on an agarose gel - add 2-3 more amplification cycles.
12. Purify the remaining final PCR reaction with AMPure® XP Beads, MagSi-NGS^{prep} Plus beads or similar. Add **1.6 volumes** of beads to final PCR reaction during beads purification step.
13. Perform quality check of the library with Qubit and Bioanalyser.

8. SHORT PROTOCOL FOR 5'-P RNAs (PP and PO) ONLY

1. 8 µL **RNA sample** + [2.5 µL **Tailing Buffer** + 0.5 µL **PdP Enzyme**].
2. Incubate 15 min at 37°C and 20 min at 65°C, hold at 4°C.
3. + [1 µL **Tailing Nucleotides** + 0.5 µL **Tailing Enzyme**].
4. Centrifuge the tube(s)/plate(s) for 5 sec at maximal speed.
5. Incubate 25 min at 37°C and 20 min at 65°C, hold at 4°C.
6. + [11 µL **Ligation Buffer** + 1 µL **Ligation Enzyme**].
7. Incubate 15 min at 37°C, 60 min at 25°C and 15 min at 65°C, hold at 4°C.
8. + 8.5 µL **RT Buffer** + 1.5 µL **RT Enzyme** + 1 µL **RT Primers** (undiluted or diluted depending on the RNA input, please see Table 1).
9. Incubate 30 min at 42°C and 5 min at 85°C, hold at 4°C.
10. + 50 µL **PreAmp Mix** + 10 µL of the **Index primer**.
11. Perform PCR amplification:
    - Initial denaturation: 95°C for 30 sec

    10 - 19 cycles (please see Table 1):
    - 95°C for 10 sec
    - 62°C for 30 sec
    - 72°C for 30 sec
12. [OPTIONAL] Run 20 µL of PCR reaction on a 3-4% agarose gel.
13. [OPTIONAL] If the library is poorly visible on an agarose gel - add 2-3 more amplification cycles.
14. Purify the remaining final PCR reaction with AMPure® XP Beads, MagSi-NGS^{prep} Plus beads or similar. Add **1.6 volumes** of beads to final PCR reaction during beads purification step.
15. Perform quality check of the library with Qubit and Bioanalyser.

9. SHORT PROTOCOL FOR 3'-OH RNAs (PO and OO) ONLY

1. 8 µL **RNA sample** + [2.5 µL **Tailing Buffer** + 1 µL **Tailing Nucleotides** + 0.5 µL **Tailing Enzyme**].
2. Centrifuge the tube(s)/plate(s) for 5 sec at maximal speed.
3. Incubate 25 min at 37°C and 20 min at 65°C, hold at 4°C.
4. + [11 µL **Ligation Buffer** + 0.5 µL **PdP Enzyme** + 1 µL **Ligation Enzyme**].
5. Incubate 15 min at 37°C, 60 min at 25°C and 15 min at 65°C, hold at 4°C.
6. + [8.5 µL **RT Buffer** + 1.5 µL **RT Enzyme** + 1 µL **RT Primers** (undiluted or diluted depending on the RNA input, please see Table 1)].
7. Incubate 30 min at 42°C and 5 min at 85°C, hold at 4°C.
8. + 50 µL **PreAmp Mix** + 10 µL of the **Index primer**.
9. Perform PCR amplification:
    - Initial denaturation: 95°C for 30 sec

    13 - 21 cycles (please see Table 1):
    - 95°C for 10 sec
    - 62°C for 30 sec
    - 72°C for 30 sec
10. [OPTIONAL] Run 20 µL of PCR reaction on a 3-4% agarose gel.
11. [OPTIONAL] If the library is poorly visible on an agarose gel - add 2-3 more amplification cycles.
12. Purify the remaining final PCR reaction with AMPure® XP Beads, MagSi-NGS^{prep} Plus beads or similar. Add **1.6 volumes** of beads to final PCR reaction during beads purification step.
13. Perform quality check of the library with Qubit and Bioanalyser.

10. DETAILED PROTOCOL FOR ALL RNA (PP, OP, PO and OO)

(A) END-REPAIR AND POLYADENYLATION

1. Refreeze **Tailing Buffer** and **Tailing Nucleotides** at room temperature. Mix by vortexing and centrifuge for 5 sec.
2. Prepare end-repair master mix by mixing 2.5 µL **Tailing Buffer** and 0.5 µL **PdP Enzyme** per each sample [include 10% overage for each component when scaling for multiple reactions]. Mix by vortexing and centrifuge briefly.
3. Add 3 µL end-repair master mix to 8 µL of RNA sample. Mix by pipetting up and down 5 times.
4. Incubate 15 min at 37°C on PCR Thermocycler.
5. Prepare tailing master mix by mixing 1 µL **Tailing Nucleotides** and 0.5 µL **Tailing Enzyme** per each sample [include 10% overage for each component when scaling for multiple reactions]. Mix by vortexing and centrifuge briefly.
6. Add 1.5 µL tailing master mix to 11 µL of end-repair reaction. Mix by pipetting up and down 5 times.
7. **[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.
8. Incubate 25 min at 37°C and 20 min at 65°C on PCR Thermocycler. Cool to 4°C.

(B) LIGATION OF 5'-ADAPTER

9. Refreeze **Ligation Buffer** at room temperature. Mix by vortexing and centrifuge for 5 sec.
10. Prepare ligation master mix by mixing 11 µL **Ligation Buffer** and 0.5 µL **PdP Enzyme** and 1 µL **Ligation Enzyme** per each sample [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.

11. Add 12.5 µL ligation master mix to 12.5 µL of tailing reaction from previous step. Mix by pipetting up and down 5 times.
12. Incubate 15 min at 37°C, 60 min at 25°C and 15 min at 65°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.

(C) REVERSE TRANSCRIPTION

13. Refreeze **RT Buffer** and **RT Primers** at room temperature. Mix by vortexing and centrifuge for 5 sec.
14. Prepare reverse transcription master mix by combining 8.5 µL **RT Buffer**, 1.5 µL **RT Enzyme** and 1 µL **RT Primers** (undiluted or diluted depending on the RNA input, please see Table 1) per each sample. Include 10% overage for each component when scaling for multiple reactions]. Mix slowly by pipetting up and down 5 times.
15. Add 11 µL reverse transcription master mix to 25 µL of ligation reaction from previous step. Mix by pipetting up and down 5 times.
16. Incubate 30 min 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

17. Refreeze **PreAmp Mix** and **Index primers** at room temperature. Mix by vortexing and centrifuge for 5 sec.
18. Add 50 µL **PreAmp Mix** and 10 µL of the corresponding **Index primer** to each sample.
19. Perform final amplification on PCR Thermocycler.
    - Initial denaturation: 95°C for 30 sec

    10 - 19 cycles (please see Table 1):
    - 95°C for 10 sec
    - 62°C for 30 sec
    - 72°C for 30 sec
20. [OPTIONAL]. Run 20 µL of PCR reaction on a 3-4% Agarose gel.
21. [OPTIONAL]. If the library is poorly visible on Agarose gel, add 2-3 more amplification cycles.
22. Purify the remaining final PCR reaction with AMPure® XP Beads, MagSi-NGS^{prep} Plus beads or similar. Add **1.6 volumes** of beads to final PCR reaction during beads purification step.
23. Perform quality check of the library with Qubit and Agilent Bioanalyser (or Tapestation).

11. EXAMPLE OF DNA LIBRARY FROM PLASMA RNA

The typical NGS library preparation workflow with BioLiqX Small RNA-seq Kit includes three general QC steps (Figure 1). Primarily, the quantity of input RNA could be estimated using Qubit™ RNA HS Assays. However, the amounts of RNA isolated from adequate volumes of blood plasma is usually below the detection limit even with most sensitive methods and may range from 1 pg to 10 pg per microliter of eluate (despite a nearly complete recovery of cell-free RNA during isolation). As a results, it is frequently not possible to estimate optimal number of PCR cycles to be used during cDNA amplification step.

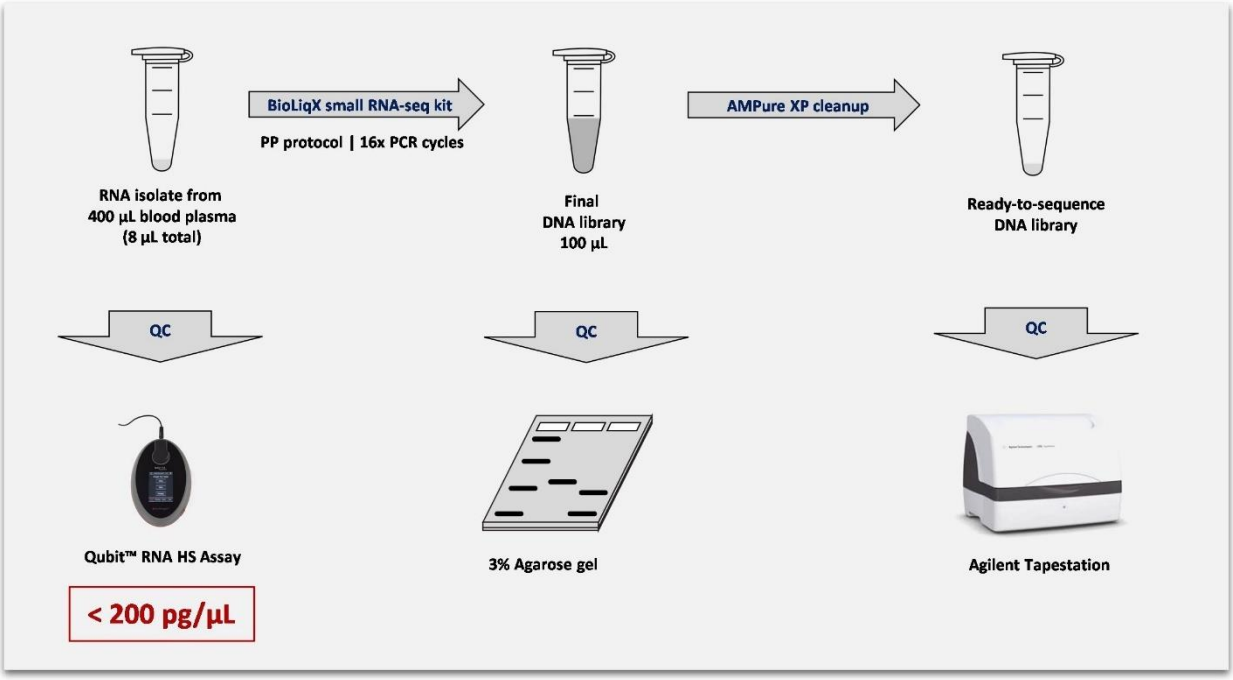

Figure 1. The typical experimental workflow for generating NGS libraries from plasma RNA with BioLiqX Small RNA-seq Kit including recommended QC steps.

We, therefore, highly recommend running aliquots of DNA libraries immediately after final amplification step on the 3-4% Agarose gel to assess whether additional PCR cycles are required. In this example, strong PCR product was evident after 16x amplification cycles (Figure 2A). If the library is hardly visible on a gel, 3 – 4x PCR cycles should be added before the final purification procedure. In most cases, BioLiqX libraries can be used for sequencing directly after a single SPRI beads clean-up to remove the excess of PCR primers, and the final quality check on Agilent 2200 Tapestation (Figure 2B), or similar device. However, certain libraries may require additional size-selection steps.

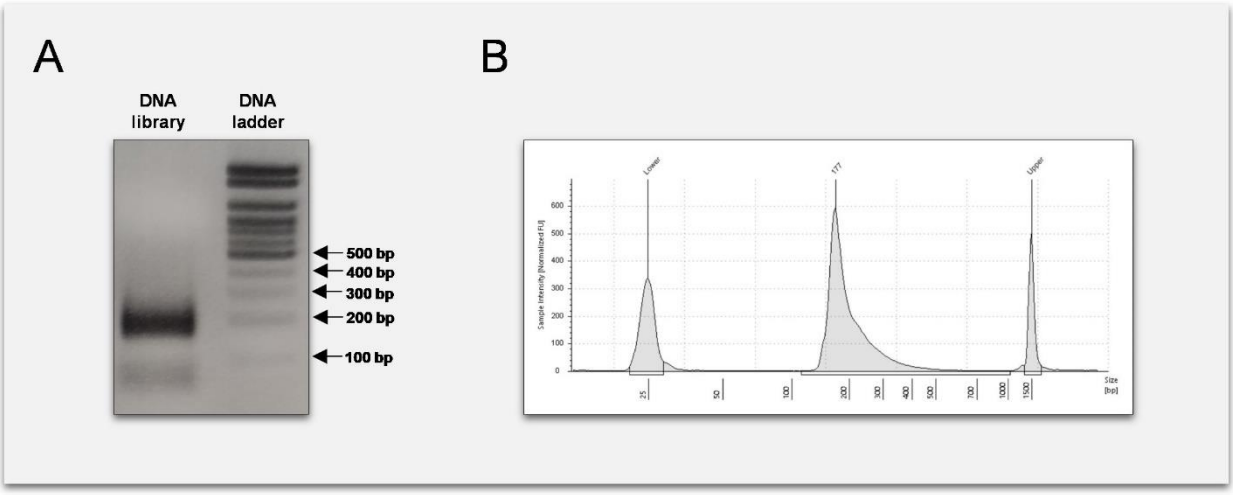

Figure 2. Example of final DNA library prepared by BioLiqX Small RNA-seq (PP, PO, OP and OO protocol). The RNA was isolated from 400 µL of cell debris-free human blood plasma, eluted in 50 µL of RNAse-free water, and 8 µL of eluate were taken as input for library preparation. The concentration of RNA in the eluate was below the detection threshold. **(A)** 3% Agarose electropherogram of a 20 µL (1/5 total) aliquot of DNA library after the final 16 cycles PCR amplification. The remaining 80 µL of DNA library were purified from contaminating primers using MagSi-NGS^{prep} Plus beads and further analyzed on Agilent 2200 TapeStation using High Sensitivity D1000 ScreenTape **(B)**. The lower and upper peaks correspond to DNA reference markers. The peak at 177 bp corresponds to combined size of processed plasma RNA plus adapters.

After purification from excess of primers BioLiqX Small RNA-seq (three independent replicates) were sequenced on Illumina HiSeq2000 platform. The obtained raw FASTQ files were trimmed from polyA-tails and size-selected using cutadapt software. (Figure 3)

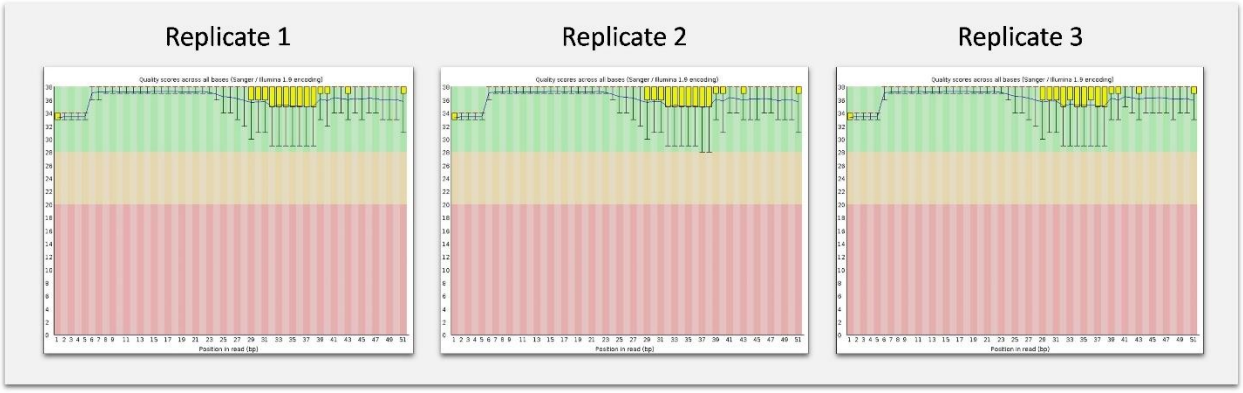

Figure 3. The quality score reports obtained by *fastqc* software after HiSeq2000 sequencing of three independent BioLiqX Small RNA-seq libraries generated from human plasma RNA sample. The raw FASTQ files were trimmed using *cutadapt*. The reads shorter than 15 nt were discarded.

Finally, trimmed and size-selected reads were aligned to the custom curated hg38 reference transcriptomes in a sequential manner. First, all reads were mapped to RNA species with low sequence complexity and/or high number of repeats: rRNA, tRNA, RN7S, snRNA, snoRNA/scaRNA, vault RNA, RNY as well as mitochondrial chromosome (mtRNA). All reads that did not map to the above RNAs were aligned sequentially to mature miRNA, pre-miRNA , protein-coding mRNA transcripts (mRNA) and long non-coding RNAs (lncRNAs). The reads which did not map to the above RNAs were aligned to the remaining transcriptome [other ncRNAs containing mostly pseudogenes and non-protein coding parts of mRNAs].

Finally, all reads which did not map to human transcriptome were aligned to the human genome reference (rest hg38) that corresponds to introns and intergenic regions. The combined raw count tables from each replicated were combined and compared for reproducibility using correlation plots (Figure 4)

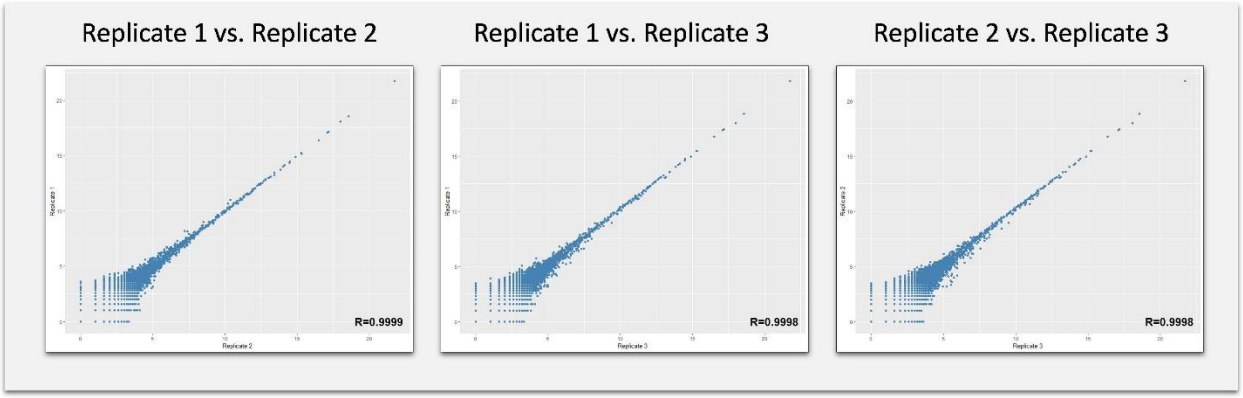

Figure 4. Correlation plots of raw human transcriptome reads obtained from three independent BioLiqX Small RNA-seq libraries generated from human plasma RNA sample and sequenced on HiSeq2000.

12. SEQUENCING GUIDLINES

- Due to the similar 5'-end adapters, BioLiqX Small RNA-seq libraries can be submitted for single-end (read 1) sequencing in the same way as other Illumina-based small RNA-seq kits, such as NEBNext Small RNA Library Prep Set for Illumina (New England Biolabs).
- In case paired-end sequencing is required, Heidelberg Biolabs GmbH will supply the exact guidelines for this procedure as well as suitable custom read 2 primer free-of-charge, depending on the type of Illumina platform used.
- BioLiqX Small RNA-seq libraries are compatible with any Illumina sequencing machine, although technology-specific recommendations can be provided for the particular platform free-of-charge.

13. DATA ANALYSIS GUIDELINES

- Any default automatic adapter trimming procedure included in some Illumina instruments should not be used.
- We recommend *cudatapt* software to remove adapter sequences and performing size-selection of reads before mapping [Martin et al. 2011 / DOI:10.14806/ej.17.1.200].
- Typically, the inserts shorter than 15 nt should be discarded to avoid ambiguous mapping.

BioLiqX Small RNA-Seq library:	5'-AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGACGATC-INSERT-AAAAAAGAAAAAACAAAAAAAGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-i7-ATCTCGTATGCCGTCTTCTGCTTG-3'
Recommended adapter trimming command:	cutadapt -a AAAAAAGAAAAAA input.fastq | cutadapt -a AAAAAAAAAAAAAAAAAAAA - | cutadapt -a AAAAAAN$ -a AAAAAN$ -a AAAAN$ - | cutadapt -a AGAGCACACGTCTG - | cutadapt -O 8 -g GTTCAGAGTTCTACAGTCCGACGATC - | cutadapt -m 15 -o output.fastq -

- Please be aware that it will not be possible to determine if a given RNA fragment ends with an A, since oligo-dT priming cannot differentiate between naturally occurring and artificially added As at RNA termini.
- Heidelberg Biolabs GmbH can suggest the detailed data analysis pipeline tailored for your sample types, sequencing strategy, and research goals.

14. INDEX PRIMERS AND MULTIPLEXING

The BioLiqX Small RNA-seq Kit allows for multiplexing of up to 24 samples on a single flow cell lane and, thus, the 24 index primers (each premixed with the universal forward PCR primer) listed below are supplied by default. However, due to immense complexity of the final DNA libraries generated by the kit, we recommend obtaining at least **20 Mio** reads for each sample. Although, much higher sequencing depth might be required to realize the full dynamic range of RNA species detected by sequencing with BioLiqX Small RNA-seq Kit.

Oligo Name	Oligo Sequence	Index Read
Forward PCR primer*	AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGA	NA
Index 1 primer	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	ATCACG
Index 2 primer	CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	CGATGT
Index 3 primer	CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	TTAGGC
Index 4 primer	CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	TGACCA
Index 5 primer	CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	ACAGTG
Index 6 primer	CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	GCCAAT
Index 7 primer	CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	CAGATC
Index 8 primer	CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	ACTTGA
Index 9 primer	CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	GATCAG
Index 10 primer	CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	TAGCTT
Index 11 primer	CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	GGCTAC
Index 12 primer	CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	CTTGTA
Index 13 primer	CAAGCAGAAGACGGCATACGAGATTTGACTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	AGTCAA
Index 14 primer	CAAGCAGAAGACGGCATACGAGATGGAACTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	AGTTCC
Index 15 primer	CAAGCAGAAGACGGCATACGAGATTGACATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	ATGTCA
Index 16 primer	CAAGCAGAAGACGGCATACGAGATGGACGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	CCGTCC
Index 18 primer	CAAGCAGAAGACGGCATACGAGATGCGGACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	GTCCGC
Index 19 primer	CAAGCAGAAGACGGCATACGAGATTTTCACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	GTGAAA
Index 20 primer	CAAGCAGAAGACGGCATACGAGATGGCCACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	GTGGCC
Index 21 primer	CAAGCAGAAGACGGCATACGAGATCGAAACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	GTTTCG
Index 22 primer	CAAGCAGAAGACGGCATACGAGATCGTACGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	CGTACG
Index 23 primer	CAAGCAGAAGACGGCATACGAGATCCACTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	GAGTGG
Index 25 primer	CAAGCAGAAGACGGCATACGAGATATCAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	ACTGAT
Index 27 primer	CAAGCAGAAGACGGCATACGAGATAGGAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	ATTCCT

* Forward PCR primer is premixed with corresponding index primers to the total concentration of 10 µM each.

- Heidelberg Biolabs can provide recommendations for the exact combination of index primers depending on the number of samples in the multiplex.
- By default, we recommend the following combination of index primers:

Pool of 2 samples	E.g. index primer 2 and 6
Pool of 3 samples	E.g. index primer 4, 6 and 12
Pool of 4 samples	E.g. index primer 2, 4, 6 and 12
Pool of 6 samples	E.g. index primer 2, 4, 5, 6, 7 and 12
Pool of 12 samples and more	Any combination

15. CONTACT INFORMATION

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WARRANTY

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TRADEMARKS

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