GenXPro small RNA-Seq Library Preparation Kit

User Guide - Kit for 6 Rx

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1. Overview

1.1 Introduction

The GenXPro small RNA Library Preparation Kit can be used to prepare small RNA libraries to be sequenced any Illumina NGS instruments starting from 1 to 200 ng of total RNA or purified small RNA.

The library preparation methodology used in this kit allows accurate identification and quantification of miRNAs, piRNAs and other small RNAs.

The present kit uses adapters with randomized ends to avoid ligation bias between the adapters and the small RNA. The adapters also include the GenXPro TrueQuant technology to avoid PCR bias.

1.2 TrueQuant Technology

The GenXPro small RNA Library Preparation Kit applies the proprietary TrueQuant technology that eliminates PCR-derived copies from the generated gene expression data. Following sequencing of the adaptor-ligated and PCR-amplified libraries, only the sequence with highest quality of each TrueQuant adapter plus template-sequence combination are maintained.

1.3 Principle and Workflow

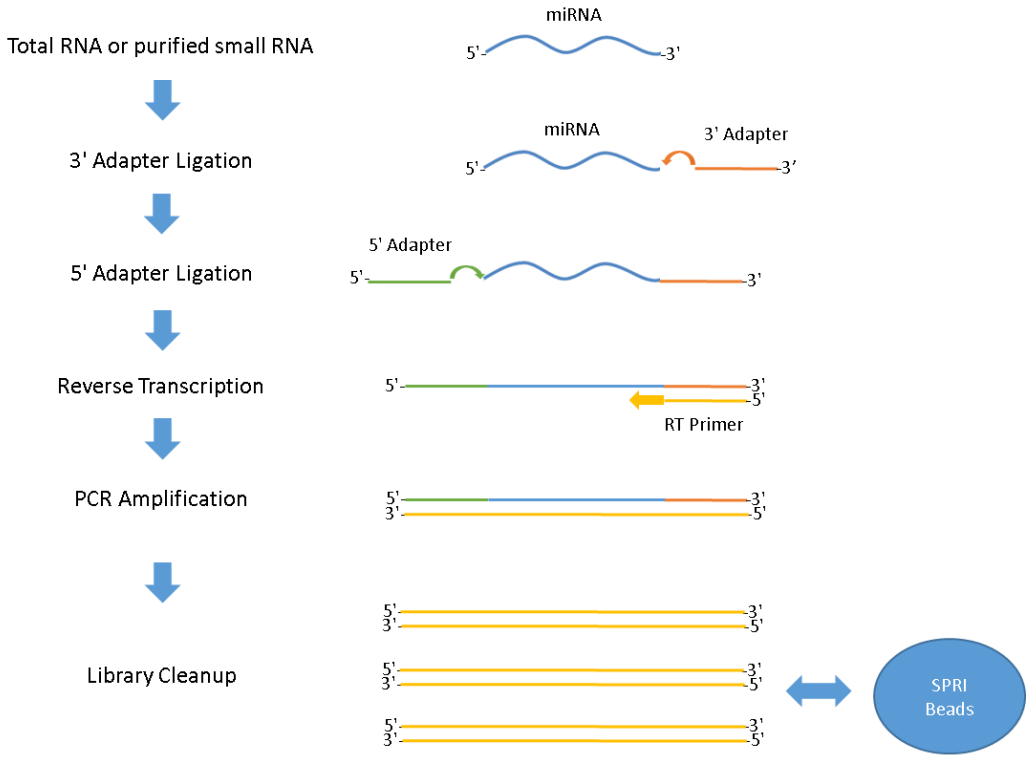


Figure 1: Scheme of the library preparation workflow for small RNA sequencing. Starting with DNase-treated and quality-controlled total RNA, the final libraries are ready for sequencing on any Illumina platform. The present kit allows for multiplexing of up to 4 samples.

The small RNA library preparation procedure is constituted by the following steps:

- 3' Adapter Ligation
- 5' Adapter Ligation
- Reverse Transcription
- PCR Amplification
- Library cleanup

During the library preparation, adapters with randomized ends are ligated to the 3' ends of the small RNA molecules, followed by a 5' end ligation with a second adapter. The synthesis of the first cDNA strand is performed by reverse transcription. The library amplification is performed using a universal forward primer and one of the specific reverse primers that will assign the sample index. A magnetic bead-based cleanup is performed to eliminate the PCR primers (Figure 2-B).

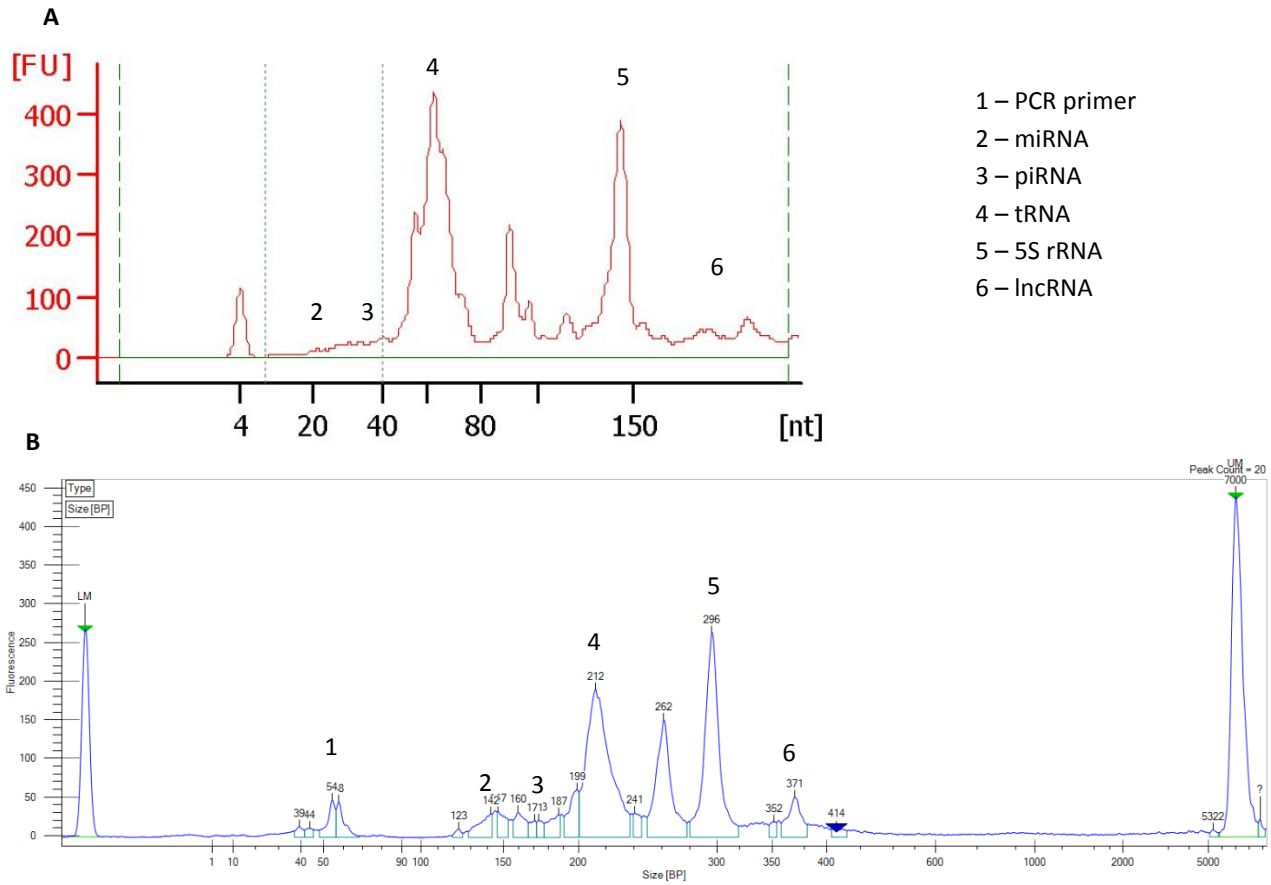


Figure 2: Bioanalyzer Small RNA assay result from Placenta total RNA (1-A). Labchip GXII DNA HS assay result from a small RNA-seq library prepared (before step 7 - Final library cleanup) with 10 ng of RNA from the samples presented in the figure 1-A (1-B).

1.4 Input material

The GenXPro small RNA sequencing kit was designed for total RNA or purified small RNA. Best results are obtained with high quality total RNA. The use of degraded RNA may result in increased background. We recommend assessing the RNA quality of each purified and DNase I treated sample with an automated microfluidic electrophoresis station and quantify the concentration by a fluorometric measurement.

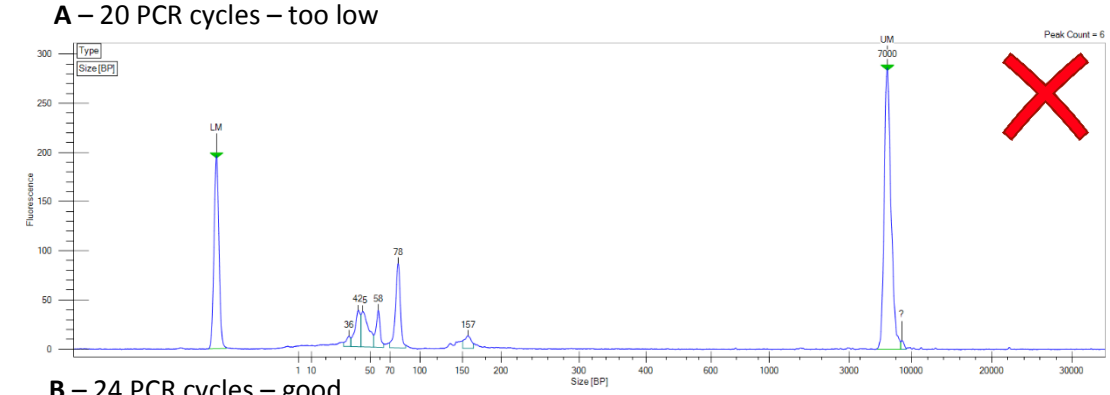
The 3' Adapter, 5' Adapter and RT Primer have to be diluted by the user according to the amount of input RNA (Table 1). The amount of adapters supplied in the kit is sufficient for 4 reactions. The adapters should be aliquoted according to the dilutions and samples to be processed. In case of questions, please contact GenXPro customer service (customerservices@genxpro.de).

Template (total RNA)	Adapter	RT Primer	PCR
200 ng – 100 ng	undiluted	undiluted	8 – 14
100 ng – 20 ng	1:2 – 1:5	1:2 – 1:5	12 – 18
20 ng – 5 ng	1:5 – 1:10	1:5 – 1:10	18 – 22
5 ng – 1 ng	1:10 – 1:40	1:10 – 1:40	22 – 26

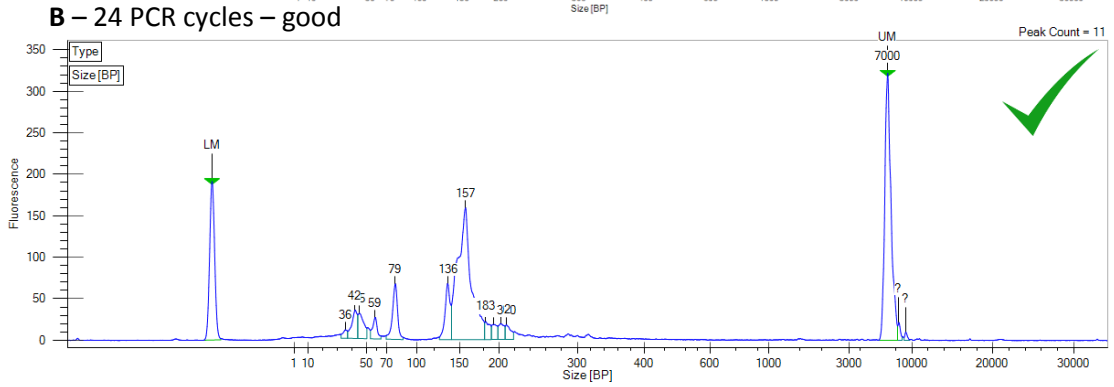
Table 1

The number of PCR cycles needed for optimal yield may vary depending on the amount of cDNA. We recommend assessing the optimal cycle number based on a PCR test amplification. The number of PCR cycles can be defined using the results from an automated microfluidic electrophoresis station, as demonstrated in Figure 2.

A – 20 PCR cycles – too low



B – 24 PCR cycles – good



C – 28 PCR cycles – too much

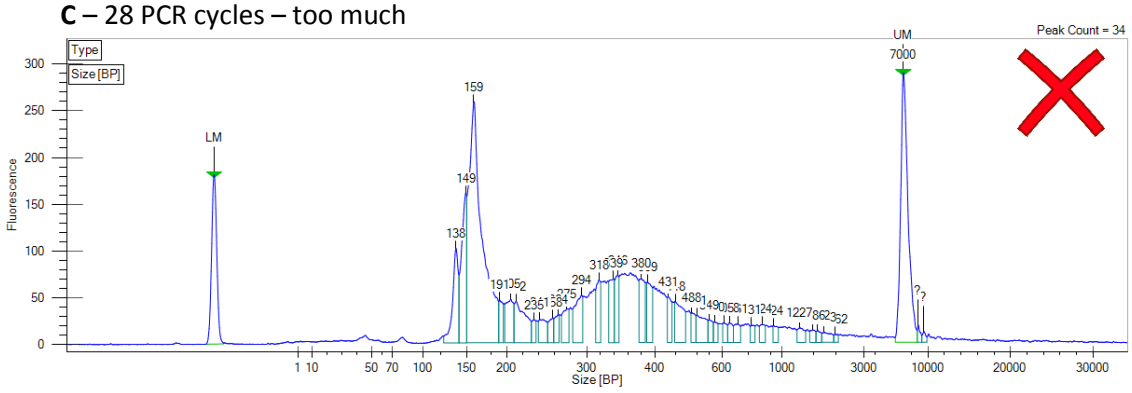


Figure 3: Test PCR from library preparation using 200 ng of small RNA with 20 (A), 24 (B) and 28 (C) PCR cycles. In figure 2-A, the PCR product (157 bp) is under amplified. In figure 2-B, the PCR product is correctly amplified. After the consumption of the PCR primers, the product concatemerize and create longer DNA fragments, figure 2-C. The massive PCR from this sample should be performed with 24 cycles.

2. Equipment and Consumables

Required equipment
Bioanalyzer or TapeStation (Agilent) LabChip GX or GXII (PerkinElmer) or similar
Qubit (Thermo Fisher Scientific) or similar Fluorometer
Magnetic stand (suitable for 1.5 ml tubes)
Thermocycler (suitable for 0.2 ml tubes)
Benchtop microcentrifuge for 0.2-1.5 ml tubes
Vortex mixer
Calibrated single-channel pipettes

Required consumables and reagents	
SPRI Beads	Agencourt AMPure XP or Agencourt SPRIselect
Fluorometric RNA Quantitation	RNA High Sensitivity Assay (Qubit)
Fluorometric DNA Quantitation	dsDNA High Sensitivity Assay (Qubit)
Pipette tips (with aerosol barriers, nuclease-free)	General laboratory supplier
1.5 ml tubes (low binding, nuclease-free)	General laboratory supplier
0.2 ml tubes (nuclease-free)	General laboratory supplier
85% Ethanol (freshly prepared)	General laboratory supplier

small RNA-Seq Library Preparation Kit supplied Reagents*	
All Reactions	Nuclease-free water
3' Adapter Ligation	3' Adapter
	Ligase Buffer
	Ligase Enhancer
	RI Enzyme Mix
	3' Ligase Mix
	AI Enzyme Mix 1
	AI Enzyme Mix 2
5' Adapter Ligation	5' Adapter
	5' Ligase Mix
Reverse Transcription	RT Primer Mix
	RT Buffer Mix
	RT Enzyme Mix
	PI Enzyme Mix
PCR Amplification	Index Primer ID 2, 4, 6, 12
	PCR Buffer Mix
	PCR Enzyme Mix

*Store all reagents at -20 °C.

3. Protocol

3.1 3' Adapter Ligation

Assess the RNA quality of each purified and DNase I treated sample using a Microfluidic Capillary Electrophoresis System or alike and quantify the concentration by fluorometric measurement.

For low amount of RNA, dilute the 3' Adapter with nuclease-free water according to Table 2. Mix by vortexing and spin down briefly.

Template Input Amount (total RNA)	3' Adapter Dilution
200 ng – 100 ng	undiluted
100 ng – 20 ng	1:2 – 1:5
20 ng – 5 ng	1:5 – 1:10
5 ng – 1 ng	1:10 – 1:40

Table 2

Reagent	Volume per Reaction (µl)
RNA Template	4
3' Adapter	1
Total volume after this step	5

Table 3

- Add the diluted 3' adapter to the RNA template in a 200 µl microcentrifuge tube, mix by vortexing and spin down briefly.
- Incubate at 70 °C for 2 minutes (with heated lid at 90 °C).
- Immediately place on ice for 5 minutes.

Reagent	Volume per Reaction (µl)
Ligase Buffer	1
Ligase Enhancer	2.5
RI Enzyme Mix	0.5
3' Ligase Mix	1
Total volume after this step	10

Table 4

- Add all the reagents according to Table 4. Mix by vortexing and spin down briefly.
- Incubate at 22 °C for 2 hours in a thermocycler. For higher yield or difficult ligation (2'-O-methylated small RNA) incubation at 16 °C for 18 hours is highly recommended.
- Incubate 10 minutes at 75 °C.
- Hold at 4 °C.

Reagent	Volume per Reaction (µl)
AI Enzyme Mix 1	1.25
Total volume after this step	11.25

Table 5

- Add the reagent according to Table 5. Mix by vortexing and spin down briefly.
- Incubate 30 min at 30 °C.
- Incubate 30 min at 37 °C.
- Hold at 37 °C.

Reagent	Volume per Reaction (µl)
AI Enzyme Mix 2	1
Total volume after this step	12.25

Table 6

- Add the reagent according to Table 6. Mix by vortexing and spin down briefly.
- Incubate 1 hour at 37 °C.
- Incubate 10 min at 75 °C.
- Hold at 4 °C.

3.2 5' Adapter Ligation

For low RNA input, dilute 5' Adapter with nuclease-free water according to Table 6. Mix by vortexing and spin down briefly.

Template Input Amount (total RNA)	5' Adapter Dilution
200 ng – 100 ng	undiluted
100 ng – 20 ng	1:2 – 1:5
20 ng – 5 ng	1:5 – 1:10
5 ng – 1 ng	1:10 – 1:40

Table 7

- Add 1µl of the diluted 3' adapter in a 200 µl microcentrifuge tube.
- Incubate at 70 °C for 2 minutes (with heated lid at 90 °C).
- Immediately place on ice for 5 minutes.

Reagent	Volume per Reaction (µl)
5' Adapter	1
Ligase Activator	1
5' Ligase Mix	1
Total volume after this step	15.25

Table 8

- Add all the reagents according to Table 7. Mix by vortexing and spin down briefly.
- Incubate 2 hour at 20 °C.
- Incubate 20 min at 70 °C (with heated lid at 90 °C).
- Hold at 4 °C.

3.3 Reverse Transcription

For low RNA input, dilute the RT Primer Mix using nuclease-free water according to table 9. Mix by vortexing and spin down briefly.

Template Input Amount (total RNA)	RT Primer Dilution
200 ng – 100 ng	undiluted
100 ng – 20 ng	1:2 – 1:5
20 ng – 5 ng	1:5 – 1:10
5 ng – 1 ng	1:10 – 1:40

Table 9

Reagent	Volume per Reaction (µl)
RT Primer Mix	1.5
RT Buffer Mix	7.5
Total volume after this step	23.25

Table 10

- Add all the reagents according to Table 10. Mix by vortexing and spin down briefly.
- Incubate for 5 minutes at 75 °C.
- Slowly cool down to 50 °C (with 0.1 °C/s).
- Hold at 50 °C.

Reagent	Volume per Reaction (µl)
RT Enzyme Mix	1.75
Total volume after this step	25

Table 11

- Add the reagents according to Table 11. Mix by vortexing and spin down briefly.
- Incubate at 50 °C for 1 hour (with heated lid at 70 °C).
- Incubate at 70 °C for 15 minutes.
- Store at 4 °C.

Reagent	Volume per Reaction (µl)
PI Enzyme Mix	1.5
Total volume after Step 8	26.5

Table 12

- Add the reagents according to Table 12. Mix by vortexing and spin down briefly.
- Incubate at 37 °C for 2 hours.
- Incubate at 65 °C for 20 minutes.
- Store at 4 °C (The procedure could be safely stopped at this point and samples stored at -20°C).

3.4 PCR Test Amplification

Use 5 µl template and prepare the PCR reaction mix according to Table 13.

Reagent	Volume per Reaction (µl)
Template	5
Index Primer	2.5
PCR Buffer Mix	21.5
PCR Enzyme Mix	1
Nuclease-free water	20
Total volume of the PCR reaction	50

Table 13

- Incubate the PCR reaction as described in Table 14.

Temperature	Time	Cycles
98 °C	45 s	1
98 °C	20 s	depending on RNA input*
65 °C	30 s	
72 °C	30 s	
72 °C	2 min	1
4 °C	store	1

Table 14

* During the test PCR, collect 5 µl of the reaction mixture after the elongation step of the suggested PCR cycles (Table 15).

Template Input Amount (total RNA)	PCR Cycles
200 ng – 100 ng	8 – 14
100 ng – 20 ng	12 – 18
20 ng – 5 ng	18 – 22
5 ng – 1 ng	22 – 26

Table 15

Assess the PCR products using an automated microfluidic electrophoresis station. The number of PCR cycles can be defined using the electropherograms, as demonstrated in Figure 2.

3.5 PCR Amplification

Use 20 µl template and prepare the PCR reaction mix on ice according to Table 16.

Reagent	Volume per Reaction (µl)
Template	20
Index Primer	2.5
PCR Buffer Mix	21.5
PCR Enzyme Mix	1
Nuclease-free water	5
Total volume of the PCR reaction	50

Table 16

- Incubate the PCR reaction as described in Table 17.

Temperature	Time	Cycles
98 °C	45 s	1
98 °C	20 s	depending on the PCR test**
65 °C	30 s	
72 °C	30 s	
72 °C	2 min	1
4 °C	store	1

Table 17

** Define the number of PCR cycles that should be used with the PCR test amplification (Figure 2). We recommend using the same number of cycles minus 2 for the massive PCR amplification with 20 µl of template (4 times more template than in the PCR test amplification).

3.6 Library Cleanup

Consumables and reagents needed for magnetic beads size selection:

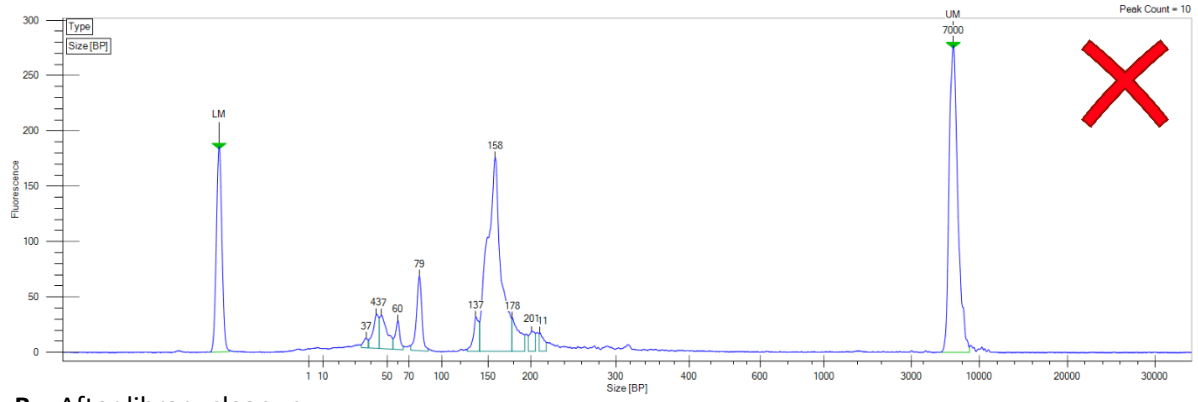
- SPRI beads
- 85 % ethanol (freshly prepared)
- Nuclease-free water
- 1.5 ml low binding tube
- Magnetic stand

SPRI Beads: use a ratio of 1.0 volumes of your sample to 1.0 volumes of the beads

- Remove the SPRI beads from storage (4 °C) and equilibrate to room temperature.
- Mix the SPRI beads thoroughly by vortexing for 30 seconds before pipetting.
- Add 50 µl of SPRI beads to 50 µl of the PCR amplified library.
- Gently pipette the entire volume up and down 10 times to mix thoroughly.
- Incubate for 5 minutes at room temperature.
- Place the tube on the magnetic stand for 5 minutes until the liquid appears completely clear.
- Carefully remove the supernatant (optional: keep the supernatant). Take care not to disturb the beads.
- Add carefully 200 µl of freshly prepared 85 % ethanol without disturbing the beads. Do not remove the tube from the magnetic stand.
- Incubate 1 minute, then remove and discard the supernatant. Take care not to disturb the separated magnetic beads.
- Spin down in microcentrifuge to collect the remaining ethanol at the bottom of the tube and place it back on the magnetic stand.
- Remove the remaining ethanol and air-dry the beads for 3 minutes on the magnetic stand with the cap open.
- Take the tube from the magnetic stand and add 20 µl of nuclease-free water.
- Gently pipette the entire volume up and down 10 times to mix thoroughly. Ensure that the beads are completely rehydrated and resuspended.
- Incubate for 2 minutes at room temperature.
- Place the tube on the magnetic stand for 5 minutes
- Save the supernatant in a fresh 1.5 ml low binding tube.
- Store the library at -20 °C.

Assess the library quality on a microfluidic electrophoresis station. Repeat the SPRI clean-up, if a peak at around 80 bp (primers) is shown in the electropherogram (Figure 4).

A – Before library cleanup



B – After library cleanup

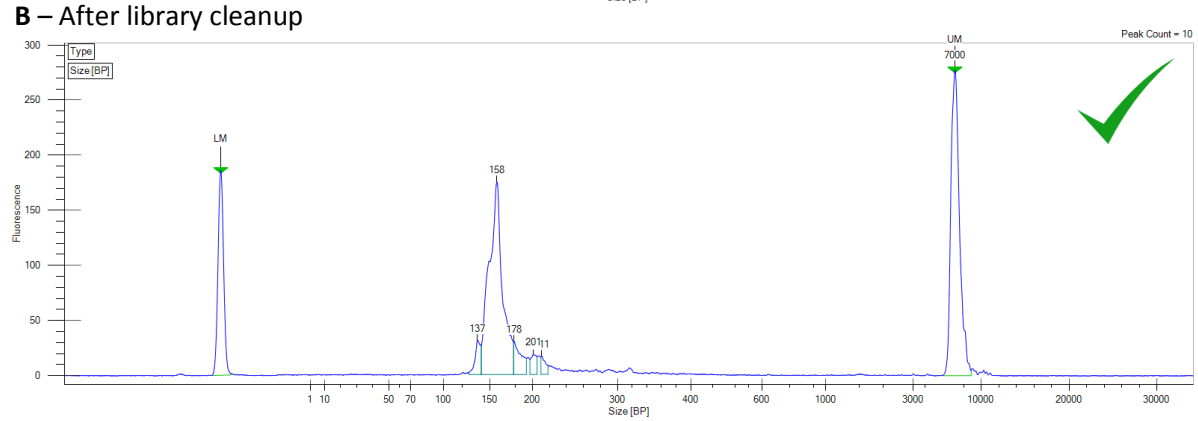


Figure 4: cDNA library prepared using 1 ng of total RNA and amplified for 24 PCR cycles. In figure 4-A, PCR primers can be found with 40-80 nt. In figure 4-B, the PCR were cleaned by magnetic beads size selection during the final library cleanup.

Assess the library concentration by a fluorescence-based quantification assay (Qubit). Dilute the library to the required concentration for Illumina sequencing according.

To process the raw sequencing data with our TrueQuant technology, upload the bcl or fastq files with the Data Uploader after login at http://tools.genxpro.net.

In case of questions, please contact GenXPro customer service (customerservices@genxpro.de).

4. Appendix

Index Primer ID	Index Sequence
2	CGATGT
4	TGACCA
5	ACAGTG
6	GCCAAT
7	CAGATC
12	CTTGTA

Table 10: Illumina TruSeq Index Sequence

GenXPro small RNA-Seq Library Prep Kit (Kit for 6 Rx) · LOT XX-XX-XXXX · User Guide 08-02-2018

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GenXPro GmbH

Altenhöferallee 3

60438 Frankfurt am Main

www.genxpro.de