siTOOLs BIOTECH

RNA-Seq Library Preparation

ARGO Kit:

User Guide

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1. Product Overview

The **Argo RNA-Seq Library Prep Kit** provides a robust, efficient, and rapid solution for generating Illumina-compatible RNA sequencing libraries. The kit is fully compatible with all siTOOLs Biotech riboPOOL™ ribodepletion kits, enabling a seamless transition from rRNA removal to library generation. Alternatively, libraries can also be prepared directly from total RNA or poly(A)-selected RNA samples.

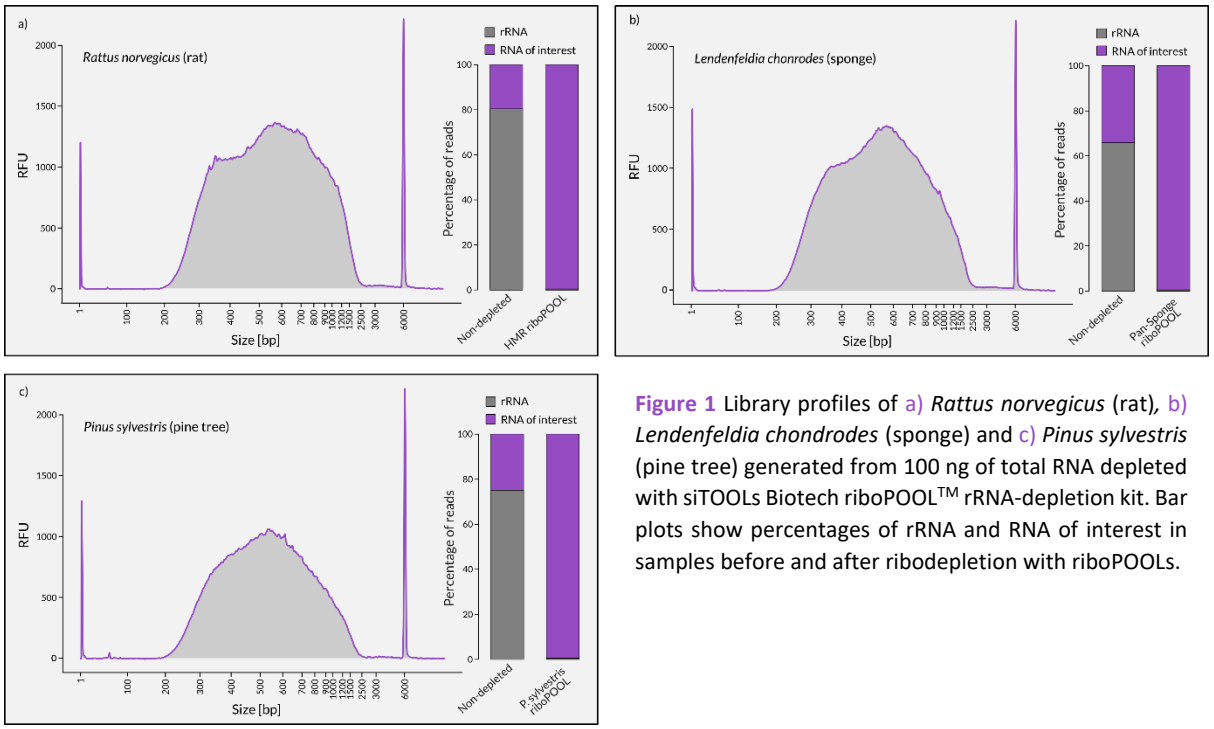


Figure 1 Library profiles of a) *Rattus norvegicus* (rat), b) *Lendenfeldia chondrodes* (sponge) and c) *Pinus sylvestris* (pine tree) generated from 100 ng of total RNA depleted with siTOOLs Biotech riboPOOL™ rRNA-depletion kit. Bar plots show percentages of rRNA and RNA of interest in samples before and after ribodepletion with riboPOOLs.

The recommended input is **1–2 ng of rRNA-depleted or poly(A)-selected RNA**. Quantification should be performed *after* ribodepletion or poly(A) selection to ensure accurate measurement of the usable fraction. For low-input samples where post-depletion quantification is not feasible, recovery can be approximated at ~1% of the starting total RNA when using siTOOLs Biotech riboPOOL™ ribodepletion kit. This value should be considered an estimate, as actual recovery may vary depending on RNA composition, sample type, and the purification method employed (e.g., bead- or column-based cleanup, precipitation). To optimize amplification and preserve library complexity, we recommend performing a qPCR assay with the **Argo RNA-Seq Library Prep Kit - PCR module** (Cat. SB-PCR-96). to determine the appropriate number of PCR cycles rather than relying on a fixed cycle number.

Note: siTOOLs Biotech Argo RNA-Seq Library Prep kit and its underlying technology is covered by issued or pending patents

2. Contents of the kit

2.1 List of Reagents

Code	Component	24 reaction kit	96 reaction kit	Storage
RTM 🔵	Reverse Transcription Mix	1 x 476 µl	2 x 951 µl	-20°C
RTL 🔵	Reverse Transcription Mix Long	1 x 476 µl	2 x 951 µl	-20°C
RTP 🔵	Reverse Transcription Primer	1 x 27 µl	1x 106 µl	-20°C
RTE 🔵	Reverse Transcription Enzyme	1x 27 µl	1x 106 µl	-20°C
LM 🟢	Ligation Mix	1x 951 µl	3x 1268 µl	-20°C
DTT 🟢	Dithiothreitol	1x 27 µl	1x 106 µl	-20°C
LO 🟢	Linker Oligo	1x 27 µl	1x 106 µl	-20°C
LE 🟢	Ligation Enzyme	1x 53 µl	1x 106 µl	-20°C
BB ○	Beads Buffer	1x 2086 µl	4x 8343 µl	-20°C / +4°C
LAM ○	Library Amplification Mix	185 µl	4x 740 µl	-20°C
LAE ○	Library Amplification Enzyme	27 µl	4x 106 µl	-20°C
PB	Purification Beads	1x 1307 µl	4x 5228 µl	+4°C
PS	Purification Solution	1x 1901 µl	4x 7604 µl	+4°C
EB	Elution Buffer	1x 3221 µl	4x 12884	+4°C
12 nt UDI-24	12 nt Unique Dual Indexes	10 µl / well		-20°C
12 nt UDI-96	12 nt Unique Dual Indexes		10 µl / well	-20°C

2.2 Storage Instruction

The kit is shipped under cold conditions to maintain reagent stability during transit. Upon receipt, all reagents should be stored at the temperatures indicated in the instructions listed in the above table. Reagents for beads purification – which needs to be stored at 4°C - are supplied in a separate bag within the kit box.

Note: If the kit was purchased in combination with siTOOLs Biotech riboPOOL ribodepletion kits, refer to the riboPOOL user guide for storage conditions of ribodepletion reagents.

3. Additional Material (not supplied in the kit)

3.1 Reagents

- 80% ethanol (prepared fresh)
- Argo RNA-Seq Library Prep Kit - PCR module (Cat. SB-PCR-96) for estimating number of PCR cycles. Recommended SYBR Green I products are Sigma-Aldrich Cat. No. S9430 and ThermoFisher, Cat. No. S7585

3.2 Consumables and Equipment

- Magnetic rack (e.g., 200 µl or 1.5 ml) or plate.
- Single and/or multichannel pipettes (1 µl – 1000 µl) and sterile, low-retention pipette tips for minimal surface binding of RNA and beads.
- RNase-free low-binding reaction tubes (1.5 ml and 200 µl) and/or plate and sealing foil or caps.
- Benchtop centrifuge for 1.5 ml tubes (12,000g) or 96 well plates (3,000g).
- Vortexer
- Thermal cycler
- UV-spectrophotometer for RNA and quantification.
- Benchtop fluorometer (e.g., Invitrogen Qubit) and reagents for RNA quality control and library quantification.
- Personal protection equipment – lab coat, gloves

3.3 Optional

- Automated electrophoresis platform for RNA and library QC (e.g., Agilent Bioanalyzer 2100, Agilent TapeStation or Agilent Fragment Analyzer.

- qPCR machine

4. Protocol for Short Insert Sizes (RTM)

This protocol describes how to prepare RNA-Seq libraries with an average insert size of ca. 150 nt. The protocol is compatible with both high-quality and degraded (FFPE) RNA.

4.1 Reverse Transcription

Scope: generate cDNA first-strand fragments with partial adapter sequence at the 5'-end.

Reagent	Storage	Preparation
Reverse Transcription Primer (**RTP**)	-20°C	Thaw at room temperature
Reverse Transcription Mix (**RTM**)	-20°C	Thaw at 30°C and 1250 rpm on Thermomixer. Ensure the reagent is completely dissolved before use
Reverse Transcription Enzyme (**RTE**)	-20°C	Keep on ice or at -20° C

Note: The Reverse Transcription Mix (**RTM**) is a viscous reagent. Follow the instructions provided in the above table and ensure the reagent is completely dissolved and mixed before using it.

Note: add 10% extra volume per reaction when preparing the mastermix for reverse transcription (Step 4.1b). Example: use 154 µl of RTM (14 µl x 10 reactions x 1.1) and 11 µl of RTP (1 µl x 10 reactions x 1.1).

a. Equilibrate the reagents for post-ligation purification (see Section 4.2) to room temperature for at least 30 minutes before use.

b. Prepare a mastermix – for each sample – consisting of 14 µl x of Reverse Transcription Mix (**RTM**) and 1 µl of Reverse Transcription Primer (**RTP**). Mix well and spin down.

c. Add 15 µl of the mastermix to 10 µl of RNA sample. If using < 10 µl, add RNase-free H2O to a final volume of 25 µl. Mix well and spin down.

d. Incubate the samples for 3 minutes at 94°C followed by 15 minutes at 16°C.

e. Prepare a mastermix – for each sample – consisting of 4 µl Reverse Transcription Mix (**RTM**) and 1 µl of Reverse Transcription Enzyme (**RTE**).

f. Spin down the samples from step d. and add 5 µl of the mastermix from step e.

g. Incubate the samples using the following program:

10 min 25°C → 40 min 37°C → 10 min 42°C → cool to 25°C and hold 1 min

h. Proceed directly to post-reverse transcription purification

4.2 Post-Reverse Transcription Purification

Scope: after first-strand synthesis, cDNA samples are purified to remove reaction components.

Reagent	Storage	Preparation
Purification Beads (**PB**)	4°C	Equilibrate at room temperature for 30 min Mix well by pipetting or vortexing
Elution Buffer (**EB**)	4°C	Equilibrate at room temperature for 30 min
Beads Buffer (**BB**)	-20°C / 4°C	Equilibrate at room temperature for 30 min
Ethanol 80%	User-supplied	Prepare fresh

a. Prepare a mastermix of 9 µl Purification Beads (**PB**) and 29 µl of Beads Buffer (**BB**) for each sample. Add 38 µl of the mastermix to the cDNA samples from the Step 4.1h. Mix well and incubate for 5 minutes at room temperature.

a. Place the samples on a magnet and wait for the beads to adhere completely to the magnet and the solution to become clear (approximately 2-5 minutes).

b. With the samples still on the magnet, slowly remove and discard the supernatant without disturbing the beads.

c. With the samples still on the magnet, add 120 µl of 80 % ethanol and incubate the beads for 30 seconds. Remove and discard the supernatant.

d. Repeat the previous wash step for a total of two washes. Be sure to remove all ethanol from the tube, since traces of ethanol can interfere with linker ligation.

e. Let the beads air-dry for 5-10 minutes on the magnet. The tube should air-dry until the last visible traces of ethanol evaporate. Be careful to not over dry the beads.

f. Add 20 µl of Elution Buffer (**EB**) to each sample. Remove the samples from the magnet, and resuspend the beads fully in **EB**. Incubate for 2 minutes at room temperature.

g. Place the samples onto a magnet and wait for the solution to become clear (approximately 2-5 minutes).

h. Transfer 20 µl of the samples into new PCR tubes/plate. Samples can be stored at -20 °C (**safe stopping point**).

4.3 Linker Oligo Ligation

Scope: partial Illumina-compatible adapters are added to the 3' end of cDNA fragments.

Reagent	Storage	Preparation
Linker Oligo (**LO**)	-20°C	Thaw at room temperature for 30 min Mix well by pipetting or vortexing
Ligation Enzyme (**LE**)	-20°C	Keep on ice or at -20°C
Ligation Mix (**LM**)	-20°C	Thaw at 30°C and 1250 rpm on Thermomixer. Ensure the reagent is completely dissolved before use
DTT	-20°C	Thaw at room temperature and mix gently
Purified reverse transcription samples		Equilibrate at room temperature if stored at -20°C

Note: Ligation Mix (**LM**) and Enzyme (**LE**) Are viscous reagent. Follow the instructions provided in the above table and ensure the reagent is completely dissolved and mixed before using it.

a. Equilibrate the reagents for post-ligation purification (see Section 4.2) to room temperature for at least 30 minutes before use.

b. Prepare a mastermix – for each reverse transcription sample – consisting of 36 µl of Ligation Mix (**LM**), 1 µl DTT, 1 µl Linker Oligo (**LO**) and 2 µl of Ligation Enzyme (**LE**). Mix well and spin down. Keep the mastermix at room temperature and proceed immediately to the next step.

c. Add 40 µl of the mastermix to each purified reverse transcription sample. Mix well and spin down.

d. Incubate the samples at 37°C for 30 minutes. After, allow samples to cool down to room temperature. Proceed directly with post-ligation purification.

4.4 Post-Ligation Purification

Scope: after linker oligo ligation, ligated samples are purified to remove reaction components.

Reagent	Storage	Preparation
Purification Beads (**PB**)	4°C	Equilibrate at room temperature for 30 min Mix well by pipetting or vortexing
Purification Solution (**PS**)	4°C	Equilibrate at room temperature for 30 min
Elution Buffer (**EB**)	4°C	Equilibrate at room temperature for 30 min
Beads Buffer (**BB**)	4°C	Equilibrate at room temperature for 30 min
Ethanol 80%	User-supplied	Prepare fresh

a. Prepare a mastermix of 9 µl Purification Beads (**PB**) and 50 µl of Beads Buffer (**BB**) for each sample. Add 59 µl of the mastermix to the ligation reaction from the step Step 4.3d. Mix well, and incubate for 5 minutes at room temperature.

b. Place the samples on a magnet and wait for the beads to adhere completely to the magnet and the solution to become clear (approximately 2-5 minutes).

c. With the samples still on the magnet, slowly remove and discard the supernatant without disturbing the beads.

d. Add 30 µl of Elution Buffer (**EB**) and remove the samples from the magnet. Resuspend the beads in EB by pipetting up and down several times. Incubate for 2 minutes at room temperature.

e. Add 42 µl of Purification Solution (**PS**) to the beads to reprecipitate the library. Mix well and incubate for 5 minutes at room temperature.

f. Place the samples on a magnet and wait for the beads to adhere completely to the magnet and the solution to become clear (approximately 2-5 minutes).

g. With the samples still on the magnet, slowly remove and discard the supernatant without disturbing the beads.

h. Add 120 µl of 80 % EtOH and incubate the beads for 30 seconds. Leave the samples on the magnet (do not be resuspended during this washing step). Remove and discard the supernatant.

i. Repeat the previous wash step (h.) for a total of two washes. Be sure to remove all ethanol from the tube, since traces of ethanol can interfere with library PCR amplification.

j. Let the beads air-dry for 5-10 minutes on the magnet. The tubes should air-dry until the last visible traces of ethanol evaporate. Be careful to not over dry the beads.

k. Add 20 µl of Elution Buffer (**EB**) to each sample. Remove the samples from the magnet, and resuspend the beads in **EB**. Incubate for 2 minutes at room temperature.

l. Place the samples on a magnet and wait for the beads to adhere completely to the magnet and the solution to become clear (approximately 2-5 minutes).

m. Transfer 17 µl of the samples into new PCR tubes/plate. Add an additional 2 µl of Elution Buffer (**EB**) when using Argo RNA-Seq Library Prep Kit - PCR module (Cat. SB-PCR-96) to determine the PCR cycle number for library amplification. Samples can be stored at -20 °C (**safe stopping point**).

4.5 Library Amplification with 12 nt Dual Indices

Scope: the library is amplified to add adapter sequences required for cluster generation and unique indices for multiplexing.

Reagent	Storage	Preparation
PCR Mix (**PM**)	-20°C	Thaw at room temperature. Spin down.
PCR Enzyme (**PE**)	-20°C	Thaw at room temperature. Spin down. Keep on ice.
UDI 12 nt Sets	-20°C	Thaw at room temperature. Spin down.
Libraries		Equilibrate at room temperature if stored at -20°C

Note: The number of PCR cycles has to be adjusted depending on RNA input amount, quality and type. We recommend performing a qPCR assay to determine the PCR cycle number required for optimal library amplification, using Argo RNA-Seq Library Prep Kit **-** PCR module (Cat. SB-PCR-96).

Note: If proceeding directly to Post-PCR Purification after Library Amplification, equilibrate the purification reagents (PB, PS, and EB) to room temperature for at least 30 minutes before use.

a. Prepare a mastermix containing 7 µl of PCR Mix (**PM**) and 1 µl PCR Enzyme (**PE**) for each reaction.

b. Add 8 µl of the PM/PE mastermix to 17 µl of the eluted library.

c. Spin down the plate containing the Dual Indices before opening. Pierce the foil of the wells containing the desired indices. Add 10 µl of the respective Dual Index Primer pair to each reaction. Use only one Dual Index per sample.

d. Reseal the pierced wells of the Dual Indices plate after use with new foil to avoid cross contamination.

e. Conduct 11 – 25 cycles of PCR (as determined with the Argo RNA-Seq Library Prep Kit **-** PCR module (Cat. SB-PCR-96) using the following program:

$$\text{60 sec 95°C} \rightarrow \underset{11 - 25x}{\boxed{\text{15 sec 95°C} \rightarrow \text{15 sec 60°C} \rightarrow \text{60 sec 72°C}}} \rightarrow \text{6 min 72°C} \rightarrow \infty\ \text{10°C}$$

f. Proceed with Post-PCR Library Purification. Alternatively, libraries can be stored at -20 °C (**safe stopping point**.

4.6 Post-PCR Purification

Scope: the final library is purified to remove PCR components that can interfere with quantification.

List of reagents required for library post-PCR purification

Reagent	Storage	Preparation
Purification Beads (PB)	4°C	Equilibrate at room temperature for 30 min Mix well by pipetting or vortexing
Purification Solution (PS)	4°C	Equilibrate at room temperature for 30 min
Elution Buffer (EB)	4°C	Equilibrate at room temperature for 30 min
Libraries		Equilibrate at room temperature if stored at -20°C
Ethanol 80%	User-supplied	Prepare fresh

a. Add 31.5 µl of Purification Beads (PB) to each sample. **Note:** for libraries generated from FFPE RNA samples, add 42 µl of PB. Mix well, and incubate for 5 minutes at room temperature.

b. Place the samples on a magnet and wait for the beads to adhere completely to the magnet and the solution to become clear (approximately 2-5 minutes).

c. With the samples still on the magnet, slowly remove and discard the supernatant without disturbing the beads.

d. Add 30 µl of Elution Buffer (EB) to each sample. Remove the samples from the magnet, and resuspend the beads in EB. Incubate for 2 minutes at room temperature.

e. Add 30 µl of Purification Solution (PS) to the samples to reprecipitate the library. Mix thoroughly and incubate for 5 minutes at room temperature.

f. Place the samples onto a magnet and wait for the beads to adhere completely to the magnet and the solution to become clear (approximately 2-5 minutes).

g. With the samples still on the magnet, slowly remove and discard the supernatant without disturbing the beads.

h. Add 120 µl of 80 % EtOH and incubate the beads for 30 seconds. Leave the samples on the magnet (do not be resuspended during this washing step). Remove and discard the supernatant.

i. Repeat this washing step once for a total of two washes. **Note:** ensure to remove the supernatant completely.

j. Let the beads air-dry for 5-10 minutes on the magnet. The tubes should air-dry until the last visible traces of ethanol evaporate. Be careful to not over dry the beads.

k. Add 20 µl of Elution Buffer (EB) to each sample. Remove the samples from the magnet, and resuspend the beads by mixing well. Incubate for 2 minutes at room temperature.

a. Place the samples on a magnet and wait for the beads to adhere completely to the magnet and the solution to become clear (approximately 2-5 minutes).

l. Transfer 17 µl of the supernatant into a fresh PCR plate. **Note:** do not to transfer any beads.

m. The libraries are finished and ready for quality control (see Section 6), pooling, and cluster generation. **Safe stopping point**: libraries can be stored at -20 °C.

5. Protocol for Long Insert Sizes (RTL)

This protocol describes how to prepare RNA-Seq libraries with an average insert size of > 150 base pairs (library average size is ca. 550 base pairs. **This protocol is not compatible with low-quality and degraded (FFPE) RNA samples.** Use the RTM protocol (see Section 4) when working with low-quality/FFPE RNA samples.

5.1 Reverse Transcription

Scope: generate cDNA first-strand fragments with partial adapter sequence at the 5'-end.

Reagent	Storage	Preparation
Reverse Transcription Primer (**RTP**)	-20°C	Thaw at room temperature
Reverse Transcription Mix Long (**RTL**)	-20°C	Thaw at 30°C and 1250 rpm on Thermomixer. Ensure the reagent is completely dissolved before use
Reverse Transcription Enzyme (**RTE**)	-20°C	Keep on ice or at -20° C

Note: The Reverse Transcription Mix (**RTM**) is a viscous reagent. Follow the instructions provided in the above table and ensure the reagent is completely dissolved and mixed before using it.

Note: add 10% extra volume per reaction when preparing the mastermix for reverse transcription (Step 5.1b). Example: use 154 µl of RTM (14 µl x 10 reactions x 1.1) and 11 µl of RTP (1 µl x 10 reactions x 1.1).

a. Equilibrate the reagents for post-ligation purification (see Section 5.2) to room temperature for at least 30 minutes before use.

b. Prepare a mastermix – for each sample – consisting of 14 µl x of Reverse Transcription Mix Long (**RTL**) and 1 µl of Reverse Transcription Primer (**RTP**). Mix well and spin down.

c. Add 15 µl of the mastermix to 10 µl of RNA sample. If using < 10 µl, add RNase-free H2O to a final volume of 25 µl. Mix well and spin down.

d. Incubate the samples for 1 minutes at 94°C followed by 15 minutes at 16°C. If library with an average size of 700 base pairs are needed, skip the 1-minute incubation at 94°C.

e. Prepare a mastermix – for each sample – consisting of 4 µl Reverse Transcription Mix Long (**RTL**) and 1 µl of Reverse Transcription Enzyme (**RTE**).

f. Spin down the samples from step d. and add 5 µl of the mastermix from step 5.1e.

g. Incubate the samples using the following program:

10 min 25°C → 40 min 37°C → 10 min 42°C → cool to 25°C and hold 1 min

h. Proceed directly to post-reverse transcription purification

5.2 Post-Reverse Transcription Purification

Scope: after first-strand synthesis, cDNA samples are purified to remove reaction components.

Reagent	Storage	Preparation
Purification Beads (**PB**)	4°C	Equilibrate at room temperature for 30 min Mix well by pipetting or vortexing
Purification Solution (**PS**)	4°C	Equilibrate at room temperature for 30 min
Elution Buffer (**EB**)	4°C	Equilibrate at room temperature for 30 min
Ethanol 80%	User-supplied	Prepare fresh

a. Prepare a mastermix of 9 µl Purification Beads (**PB**) and 6 µl of Purification Solution (**PS**) for each sample. Add 15 µl of the mastermix to the cDNA samples from the Step 5.1h. Mix well and incubate for 5 minutes at room temperature.

b. Place the samples on a magnet and wait for the beads to adhere completely to the magnet and the solution to become clear (approximately 2-5 minutes).

c. With the samples still on the magnet, slowly remove and discard the supernatant without disturbing the beads.

d. With the samples still on the magnet, add 120 µl of 80 % ethanol and incubate the beads for 30 seconds. Remove and discard the supernatant.

e. Repeat the previous wash step for a total of two washes. Be sure to remove all ethanol from the tube, since traces of ethanol can interfere with linker ligation.

f. Let the beads air-dry for 5-10 minutes on the magnet. The tube should air-dry until the last visible traces of ethanol evaporate. Be careful to not over dry the beads.

g. Add 20 µl of Elution Buffer (**EB**) to each sample. Remove the samples from the magnet, and resuspend the beads fully in **EB**. Incubate for 2 minutes at room temperature.

h. Place the samples on a magnet and wait for the beads to adhere completely to the magnet and the solution to become clear (approximately 2-5 minutes).

i. Transfer 20 µl of the samples into new PCR tubes/plate. Samples can be stored at -20 °C (**safe stopping point**).

5.3 Linker Oligo Ligation

Scope: partial Illumina-compatible adapters are added to the 3' end of cDNA fragments.

Reagent	Storage	Preparation
Linker Oligo (**LO**)	-20°C	Thaw at room temperature for 30 min Mix well by pipetting or vortexing
Ligation Enzyme (**LE**)	-20°C	Keep on ice or at -20°C
Ligation Mix (**LM**)	-20°C	Thaw at 30°C and 1250 rpm on Thermomixer. Ensure the reagent is completely dissolved before use
DTT	-20°C	Thaw at room temperature and mix gently
Purified reverse transcription samples		Equilibrate at room temperature if stored at -20°C

Note: Ligation Mix (**LM**) and Enzyme (**LE**) Are viscous reagent. Follow the instructions provided in the above table and ensure the reagent is completely dissolved and mixed before using it.

a. Equilibrate the reagents for post-ligation purification (see Section 4.4) to room temperature for at least 30 minutes before use.

b. Prepare a mastermix – for each reverse transcription sample – consisting of 36 µl of Ligation Mix (**LM**), 1 µl DTT, 1 µl Linker Oligo (**LO**) and 2 µl of Ligation Enzyme (**LE**). Mix well and spin down. Keep the mastermix at room temperature and proceed immediately to the next step.

c. Add 40 µl of the mastermix to each purified reverse transcription sample. Mix well and spin down.

d. Incubate the samples at 37°C for 30 minutes. After, allow samples to cool down to room temperature. Proceed directly with post-ligation purification.

5.4 Post-Ligation Purification

Scope: after linker oligo ligation, ligated samples are purified to remove reaction components.

Reagent	Storage	Preparation
Purification Beads (**PB**)	4°C	Equilibrate at room temperature for 30 min Mix well by pipetting or vortexing
Purification Solution (**PS**)	4°C	Equilibrate at room temperature for 30 min
Elution Buffer (**EB**)	4°C	Equilibrate at room temperature for 30 min
Beads Buffer (**BB**)	4°C	Equilibrate at room temperature for 30 min
Ethanol 80%	User-supplied	Prepare fresh

a. Prepare a mastermix of 9 µl Purification Beads (**PB**) and 76 µl of Beads Buffer (**BB**) for each sample. Add 85 µl of the mastermix to the ligation reaction from the Step 5.3d. Mix well, and incubate for 5 minutes at room temperature.

b. Place the samples on a magnet and wait for the beads to adhere completely to the magnet and the solution to become clear (approximately 2-5 minutes).

c. With the samples still on the magnet, slowly remove and discard the supernatant without disturbing the beads.

d. Add 30 µl of Elution Buffer (**EB**) and remove the samples from the magnet. Resuspend the beads in EB by pipetting up and down several times. Incubate for 2 minutes at room temperature.

e. Add 42 µl of Purification Solution (**PS**) to the beads to reprecipitate the library. Mix well and incubate for 5 minutes at room temperature.

f. Place the samples on a magnet and wait for the beads to adhere completely to the magnet and the solution to become clear (approximately 2-5 minutes).

g. With the samples still on the magnet, slowly remove and discard the supernatant without disturbing the beads.

h. Add 120 µl of 80 % EtOH and incubate the beads for 30 seconds. Leave the sample on the magnet (do not be resuspended during this washing step). Remove and discard the supernatant.

i. Repeat the previous wash step (h.) for a total of two washes. Be sure to remove all ethanol from the tube, since traces of ethanol can interfere with library PCR amplification.

j. Let the beads air-dry for 5-10 minutes on the magnet. The tube should air-dry until the last visible traces of ethanol evaporate. Be careful to not over dry the beads.

k. Add 20 µl of Elution Buffer (**EB**) to each sample. Remove the samples from the magnet, and resuspend the beads fully in **EB**. Incubate for 2 minutes at room temperature.

l. Place the samples on a magnet and wait for the beads to adhere completely to the magnet and the solution to become clear (approximately 2-5 minutes).

m. Transfer 17 µl of the samples into new PCR tubes/plate. Add an additional 2 µl of Elution Buffer (**EB**) when using Argo RNA-Seq Library Prep Kit - PCR module (Cat. SB-PCR-96) to determine the PCR cycle number for library amplification. Samples can be stored at -20 °C (**safe stopping point**).

5.5 Library Amplification with 12 nt Dual Indices

Scope: the library is amplified to add adapter sequences required for cluster generation and unique indices for multiplexing.

Reagent	Storage	Preparation
PCR Mix (**PM**)	-20°C	Thaw at room temperature. Spin down.
PCR Enzyme (**PE**)	-20°C	Thaw at room temperature. Spin down. Keep on ice.
UDI 12 nt Sets	-20°C	Thaw at room temperature. Spin down.
Libraries		Equilibrate at room temperature if stored at -20°C

Note: The number of PCR cycles has to be adjusted depending on RNA input amount, quality and type. We recommend performing a qPCR assay to determine the PCR cycle number required for optimal library amplification, using Argo RNA-Seq Library Prep Kit - PCR module (Cat. SB-PCR-96).

Note: If proceeding directly to Post-PCR Purification (p. X) after Library Amplification, equilibrate the purification reagents (PB, PS, and EB) to room temperature for at least 30 minutes before use.

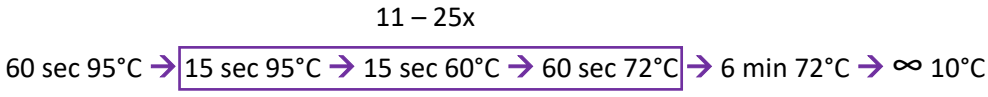
a. Prepare a mastermix containing 7 µl of PCR Mix (**PM**) and 1 µl PCR Enzyme (**PE**) for each reaction.

b. Add 8 µl of the PM/PE mastermix to 17 µl of the eluted library.

c. Spin down the plate containing the Dual Indices before opening. Pierce the foil of the wells containing the desired indices. Add 10 µl of the respective Dual Index Primer pair to each reaction. Use only one Dual Index per sample.

d. Reseal the pierced wells of the Dual Indices plate after use with new foil to avoid cross contamination.

e. Conduct 11 – 25 cycles of PCR (as determined with the Argo RNA-Seq Library Prep Kit - PCR module) using the following program:



f. Proceed with Post-PCR Library Purification. Alternatively, libraries can be stored at -20 °C (**safe stopping point**.

5.6 Post-PCR Purification

Scope: the final library is purified to remove PCR components that can interfere with quantification.

Reagent	Storage	Preparation
Purification Beads (**PB**)	4°C	Equilibrate at room temperature for 30 min Mix well by pipetting or vortexing
Purification Solution (**PS**)	4°C	Equilibrate at room temperature for 30 min
Elution Buffer (**EB**)	4°C	Equilibrate at room temperature for 30 min
Libraries		Equilibrate at room temperature if stored at -20°C
Ethanol 80%	User-supplied	Prepare fresh

a. A. Add 31.5 µl of Purification Beads (**PB**) to each sample. **Note:** for libraries generated from FFPE RNA samples, add 42 µl of PB. Mix well, and incubate for 5 minutes at room temperature.

b. Place the samples on a magnet and wait for the beads to adhere completely to the magnet and the solution to become clear (approximately 2-5 minutes).

c. With the samples still on the magnet, slowly remove and discard the supernatant without disturbing the beads.

d. Add 30 µl of Elution Buffer (**EB**) to each sample. Remove the samples from the magnet, and resuspend the beads in **EB**. Incubate for 2 minutes at room temperature.

e. Add 30 µl of Purification Solution (**PS**) to the samples to reprecipitate the library. Mix thoroughly and incubate for 5 minutes at room temperature.

f. Place the samples onto a magnet and wait for the beads to adhere completely to the magnet and the solution to become clear (approximately 2-5 minutes).

g. With the samples still on the magnet, slowly remove and discard the supernatant without disturbing the beads.

h. Add 120 µl of 80 % EtOH and incubate the beads for 30 seconds. Leave the samples on the magnet (do not be resuspended during this washing step). Remove and discard the supernatant.

i. Repeat this washing step once for a total of two washes. **Note:** ensure to remove the supernatant completely.

j. Let the beads air-dry for 5-10 minutes on the magnet. The tubes should air-dry until the last visible traces of ethanol evaporate. Be careful to not over dry the beads.

k. Add 20 µl of Elution Buffer (**EB**) to each sample. Remove the samples from the magnet, and resuspend the beads by mixing well. Incubate for 2 minutes at room temperature.

j. Place the samples on a magnet and wait for the beads to adhere completely to the magnet and the solution to become clear (approximately 2-5 minutes).

l. Transfer 17 µl of the supernatant into a fresh PCR plate. **Note:** do not to transfer any beads.

m. The libraries are finished and ready for quality control (see Section 6), multiplexing and pooling (see Section 7), and cluster generation. **Safe stopping point**: libraries can be stored at -20 °C.

6. Library QC and Quantification

Analysis of a small portion of the amplified library by microcapillary electrophoresis can provide information on both library concentration and fragment size distribution. Several electrophoresis platforms are available from different manufacturers. For low- to medium-throughput workflows, Agilent Bioanalyzer 2100 with High Sensitivity DNA chips is recommended. For higher-throughput applications, instruments such as the Agilent Fragment Analyzer**,** Agilent 2200 TapeStation, or PerkinElmer LabChip GX II are suitable alternatives. The required library volume can vary. Usually**,** 1 µL of Argo library samples is sufficient for the analysis. Depending on the sample loading requirements of each instrument, this volume may be diluted as needed (e.g., to 2 µL for the TapeStation or 10 µL for the LabChip GX II).

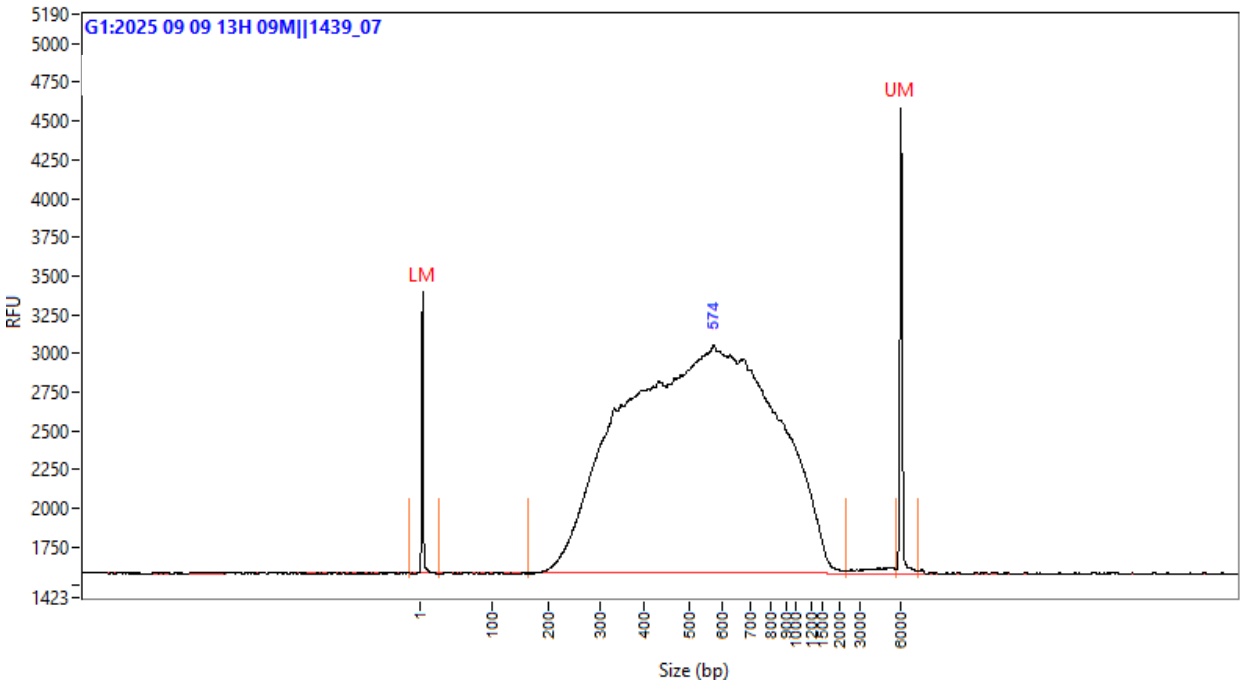


Figure 2 Fragment Analyzer profile of Argo RNA-Seq library generated from 100 ng of rat total RNA after depletion with Human-Mouse-Rat riboPOOL.

If a peak around 175 bp is present when analysing dual-indexed libraries, this indicates the presence of linker-linker dimers in the sample. If the proportion of linker-linker dimers is above 0-3% of the library, it is recommended to re-purify the library before sequencing. This can be done using the Purification Module of the Argo RNA-Seq Library Prep Kit by following the steps listed below:

a. Adjust the total volume of the library to 20 µl using Elution Buffer (**EB**) or molecular biology-grade water.

b. Add 0.9 volumes of Purification Beads (**PB**). Mix well and incubate for 5 minutes at room temperature.

c. Follow the protocol from Step 4.6b onwards to purify the library.

The appearance of a second peak at high molecular weights (1,000–9,000 bp) indicates PCR over-cycling. To avoid this, determine the optimal endpoint cycle number using Argo RNA-Seq Library Prep Kit - PCR module. Although over-cycled libraries can still be sequenced, they may produce biased gene expression values**.**

7. Multiplexing and Pooling

Libraries prepared using the siTOOLs Biotech 12 nt UDI Barcode Set are compatible with all Illumina sequencing instruments listed below. The Barcode Set support adjustable index read-out lengths of 8, 10, or 12 nucleotides, while maintaining optimal error-correction performance. A longer index read-out increases error-correction capacity.

Depending on the instrument workflow, flow cell type (paired-end, PE; or single-read, SR), and sequencing chemistry, i5 indices may be sequenced in either the forward (Illumina Workflow A) or reverse complement (Illumina Workflow B) orientation. However, some instruments using workflow B still require the i5 sequence to be entered in forward orientation, as the sequencing software automatically applies the reverse-complement conversion during processing.

Complete lists of i5 (both forward and reverse orientations) and i7 index sequences for are available at www.sitoolsbiotech.com. We recommend checking your instrument manual or confirming with your sequencing provider which i5 index orientation is required for your specific workflow. For further assistance, please contact us at info@sitools.de.

Instruments	i5 Index Orientation To Use	Description
MiSeq, HiSeq 2000/2500, MiniSeq (Rapid kits), NovaSeq 6000 (v1.0)	Forward	Instrument reads the i5 index in the forward orientation
NextSeq 1000/2000, NovaSeq X / X Plus, NovaSeq 6000 (v1.5)	Forward	Instrument reads reverse complement, but software corrects it automatically.
NextSeq 500/550, HiSeq 3000/4000/X, iSeq 100, older MiniSeq	Reverse Complement	Instrument reads reverse complement without automatic correction.

For multiplexed sequencing, libraries should ideally be pooled at equimolar concentrations. Accurate quantification of both the individual libraries and the final pooled library (lane mix) is essential to ensure balanced sequencing coverage.

To quantify your libraries:

a. Measure library concentration using a quantitative PCR (qPCR) assay or a fluorescence-based method (e.g., Qubit).

b. Determine the average library size using microcapillary electrophoresis (e.g., Bioanalyzer, Agilent Technologies). Set the analysis range to 175–1,000 bp to capture the complete library size distribution, and exclude linker–linker artefacts (~175 bp) and any over-cycling peaks (>1,000 bp).

The library molarity (nM) can then be calculated from the measured concentration and average library size using the equation below:

$$\text{Molarity (nM)} = \frac{\text{Library concentration (ng/µl)} \times 10^6}{660 \times \text{Average library size (bp)}}$$

8. Manual Version and Appropriate Use

This manual and its contents are proprietary to siTOOLs Biotech GmbH and is solely intended for use by its customers for the purpose described herein. The manual and its contents shall not be used, distributed, communicated, or reproduced in any way for any other purpose whatsoever without the prior written consent of siTOOLs Biotech GmbH. The instructions within this manual should be strictly followed by qualified personnel for safe and proper use of the product(s) described herein. Failure to completely read and perform the protocol in an adequate test environment may result in damage to the product(s), injury to persons, including to users or others, and damage to other property. siTOOLs Biotech does not assume any liability arising out of the improper use of the product(s) in any form or environment. The Argo RNA-Seq library kit is developed, designed, produced, and sold FOR RESEARCH PURPOSES only. No claim or representations is intended for clinical use (included, but not limited to diagnostic, prognostic, therapeutic purposes). It is rather the responsibility of the user to inspect and assure the use of the Argo RNA-Seq library kit for a well-defined and specific application. For other general terms of business and safety documentation, please refer to the siTOOLs Biotech website (www.sitoolsbiotech.com) under Resources > Other Downloads. This manual, referred to hereby as Argo_Library_PCRModule_Manual_v1, was first created on 1st October 2025 It may be subject to future revisions. Please refer to our website for the latest updates.