RNA Library Preparation with Twist UMI Adapter System

For use with the Twist NGS Workflow

This Twist RNA Library Preparation Protocol details all the necessary steps in converting RNA to double-stranded cDNA and preparing cDNA libraries using the Twist UMI Adapter System. The Twist UMI Adapter System consists of Twist UMI Adapters and Twist Unique Dual Indexed (UDI) Primers. This manual details the steps for generating amplified indexed libraries needed for downstream target enrichment and sequencing on Illumina next-generation sequencing (NGS) systems. This protocol is optimized for use with Twist Target Enrichment Kits and should only be performed with reagents specified or their equivalents.

Twist NGS Workflow. *The Twist Library Preparation Workflow is part of a complete NGS workflow that takes you from sample preparation to NGS sequencing and data analysis.*

For Research Use Only. *Not intended for use in diagnostic procedures.*

PROTOCOL COMPONENTS

Please read the product packaging and storage recommendations carefully for each kit and store components as recommended immediately upon arrival.

CATALOG #	NAME	DESCRIPTION	STORAGE
TWIST RNA LIBRARY PREP KIT (FOR RNA LIBRARY PREPARATION)			
107060 (16 rxn) 107061 (96 rxn)	Twist RNA Library Prep Kit	· FFPE Repair Buffer · Fragmentation/Prime Buffer · RT-Enzyme · RT-Buffer · Second Strand Enzyme · Second Strand Buffer · Ligation Enzyme · Ligation Buffer · P5/P7 Primer Mix (10x) · Equinox Amplification Master Mix (2x)	–20°C
107241 (16 rxn)	Twist Purification Beads, 16 Samples	DNA Purification Beads	2-8°C
107242 (96 rxn)	Twist Purification Beads, 96 Samples		
TWIST ADAPTERS (ORDERED SEPARATELY)			
105040 (16 rxn)	Twist UMI Adapter System TruSeq Compatible, 16 samples	· Twist UMI Adapters · Twist UDI Primers	–20°C
105041 / 105042 / 105043 / 105044 (96 rxn)	Twist UMI Adapter System TruSeq Compatible, 96 Samples Plate A/B/C/D		

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MATERIALS SUPPLIED BY USER

The following materials or their equivalent are required to generate libraries using the Twist RNA Library Preparation Kit and the Twist UMI Adapter System.

PRODUCT	SUGGESTED SUPPLIER
REAGENTS AND CONSUMABLES	
RNase-free water	—
Ethanol (200 Proof)	—
Molecular biology grade water	—
96-well compatible magnetic plate	Alpaqua, Permagen Labware
10 mM Tris-HCl pH 8	—
Thin-walled PCR 0.2-ml strip-tubes	Eppendorf
1 ml, 2 ml, 5 ml tubes (RNase-free)	Eppendorf
1.5-ml microcentrifuge tubes	VWR
96-well thermal cycling plates	Eppendorf
Qubit dsDNA Broad Range Quantitation Assay	Thermo Fisher Scientific
Qubit RNA Broad Range Assay Kit	Thermo Fisher Scientific
Agilent DNA 7500 Kit	Agilent Technologies
Agilent RNA 6000 Nano	Agilent Technologies
EQUIPMENT	
Pipettes and tips	—
Vortex mixer	—
Benchtop mini centrifuge for 0.2-ml tubes	—
Thermal cycler (96 well) with heated lid	—
Fluorometer (Qubit 3.0)	Thermo Fisher Scientific
2100 Bioanalyzer	Agilent Technologies

GENERAL NOTES AND PRECAUTIONS

Wear appropriate protective equipment (lab coat, gloves, and protective glasses or goggles) at all times when performing this protocol.

For best results, read this document before performing the protocol, and follow the provided instructions. Twist cannot guarantee the performance of the workflow if modifications are made to the protocol.

Test the compatibility of your thermal cycler and PCR tubes by incubating them at 95°C for up to 5 minutes to ensure the PCR tubes do not crack under heat and pressure. Adjust the tightness of the thermal cycler lid and/or use a spacer specific to the thermal cycler model.

GUIDELINES FOR SAMPLES

RNA SAMPLES

- This kit is compatible with both high- and low-quality samples, including those derived from FFPE.
- Use the Qubit RNA Broad Range Assay to accurately quantify input RNA.
- Assess total RNA quality via an electrophoretic method, such as Agilent BioAnalyzer.
- Reagents are compatible with total RNA mass inputs ranging from 1 ng to 1000 ng.
- Workflow performance can vary when using degraded samples. If using RNA derived from FFPE samples, an FFPE repair (Steps 1.1.1.A to 1.1.5.A) should be performed in order to enhance library quality.
- For FFPE-derived RNA, reduced adapter concentration and/or a second post-ligation purification is recommended to mitigate adapter-dimer carryover.

INPUT RNA PURITY

- RNA inputs should be free from contaminating DNA that may be carried over from extraction. If the total RNA contains DNA, remove the contamination by incubating with DNase I (not supplied with kit). Residual DNase I may interfere with library preparation, so it is important to ensure no residual enzyme remains in the sample.
- RNA should be suspended in RNase-free water and be free of salts (e.g. Mg^{2+} or guanidinium salts), chelating agents (e.g. EDTA or EGTA), and organics (e.g. phenol or ethanol).

RNA HANDLING

- To avoid RNase contamination, work in a laminar flow hood, if available, and keep all sample and reagent tubes closed unless in use. Wear gloves when handling reagents and preparing libraries. Change gloves and pipette tips if they come into contact with non-sterile surfaces.
- To avoid RNA degradation, store RNA in an RNase-free diluent and limit the number of sample freeze-thaw cycles.
- For technical support, contact customersupport@twistbioscience.com.

PROTOCOL OVERVIEW

This protocol begins with RNA and generates amplified, indexed libraries for subsequent target enrichment. Indexed libraries are compatible with Twist Standard Hybridization v2 or Twist Fast Hybridization target enrichment protocols. This protocol allows you to perform RNA library preparation (Steps 1-3) in 4 hours.

	RNA LIBRARY PREP WITH UMI ADAPTERS	TIME
STEP 1	**FFPE Repair** Heat-treated sample(s)	**35 minutes**
	Fragmentation, cDNA Synthesis, End Repair, and dA-Tailing dA-tailed cDNA fragments	**1 hour 25 minutes**
STEP 2	**Ligate Adapters and Purify** cDNA libraries	**55 minutes to 1 hour 25 minutes**
STEP 3	**Library Amplification and Strand Selection** Amplified indexed libraries	**1 hour**

STEP 1

FRAGMENTATION, cDNA SYNTHESIS, END REPAIR, AND dA-TAILING

Perform fragmentation of input RNA, cDNA synthesis, and subsequent end repair and dA-tailing to generate dA-tailed cDNA fragments.

NOTE: Perform FFPE Repair (Step 1.1.1.A to 1.1.5.A) if using FFPE-derived RNA, otherwise skip to Step 1.1.1.B.

Reagents Required

- RNA Sample
- RNase-free water
- From the Twist RNA Library Prep Kit:
  - FFPE Repair Buffer (for FFPE-derived RNA samples only)
  - Fragmentation/Prime Buffer
  - RT-Buffer
  - RT-Enzyme
  - Second Strand Buffer
  - Second Strand Enzyme

Before You Begin

- Thaw by placing on ice, vortex to ensure the reagent is fully mixed:
  - FFPE Repair Buffer (for FFPE-derived RNA samples only)
  - Fragmentation/Prime Buffer
  - RT-Buffer (protect from direct sunlight)
  - Second Strand Buffer
- Place on ice:
  - RT-Enzyme
  - Second Strand Enzyme

FFPE REPAIR

1.1.1.A Dilute input FFPE-derived RNA on ice to 11 µl using RNase-free water and add 4 µl of FFPE Repair Buffer.

1.1.2.A Pipette a minimum of half the total volume up and down 10 times to ensure complete mixing. Pulse-spin to ensure all of the solution is at the bottom of the sample plate or tube(s) and place on ice.

1.1.3.A Program a thermal cycler with the following conditions. Set the temperature of the heated lid to 105°C.

STEP	TEMPERATURE	TIME
1	70°C	30 min
2	4°C	HOLD

1.1.4.A Place tubes in the thermal cycler and initiate the program.

1.1.5.A After the thermal cycler program is complete, remove tubes from the thermal cycler, and place on ice. Proceed immediately to Fragmentation (Step 1.2).

PREPARE HIGH-QUALITY OR PARTIALLY-DEGRADED RNA

1.1.1.B Prepare input RNA on ice in a total volume of 15 µl using RNase-free water and add to labeled 0.2 ml PCR tubes or PCR plate. Proceed immediately to Fragmentation (Step 1.2).

FRAGMENTATION

1.2 Program a thermal cycler with the following conditions. Use the Step 2 Incubation table below to select the conditions for fragmentation based on the quality of your RNA and target insert size. Set the temperature of the heated lid to 105°C. Start the program to pre-chill the thermal cycler.

STEP	TEMPERATURE	TIME
1	4°C	HOLD
2	*Use the table to the right to select the time and temperature for the RNA quality used and target insert size*	
3	12°C	HOLD

**Expected insert size guidelines are based on libraries generated with 100 ng of high-quality RNA, optimization may be required for other configurations. Lower fragmentation times and temperatures may facilitate longer insert sizes for partially degraded samples and higher mass inputs. See Appendix B for additional guidance.*

STEP 2 INCUBATION			
RNA QUALITY	RIN	EXPECTED INSERT SIZE	FRAGMENTATION CONDITION*
Intact	> 7	175 - 195 bp	80°C for 10 min
		160 - 180 bp	85°C for 10 min
		145 - 165 bp	90°C for 10 min
		120 - 140 bp	95°C for 10 min
Partially degraded	2 - 7	100 - 300 bp	85°C for 2-5 min
Degraded, non-FFPE	1 - 2	Dictated by RNA Quality	65°C for 1 min
FFPE	—	Dictated by RNA Quality	65°C for 1 min

1.3 To each sample from Step 1.1.5.A or 1.1.1.B, add 10 µl of the Fragmentation/Prime Buffer on ice.

1.4 Pipette a minimum of half the total volume up and down 10 times to ensure complete mixing. Pulse-spin to ensure all of the solution is at the bottom the sample plate or tube(s) and immediately transfer to the pre-chilled thermal cycler.

1.5 Initiate steps 2 to 3 of the thermal cycler program (see table in Step 1.2).

1.6 When the thermal cycler program is complete and the sample block has returned to 12°C, proceed immediately to 1st Strand Synthesis.

1ST STRAND SYNTHESIS

NOTE: The RT-Buffer is photosensitive. Protect from direct sunlight while thawing and in use.

1.7 Program a thermal cycler with the following conditions. Set the temperature of the heated lid to 105°C. Start the program to pre-chill the thermal cycler.

STEP	TEMPERATURE	TIME
1	4°C	HOLD
2	25°C	10 min
3	42°C	15 min
4	70°C	15 min
5	4°C	HOLD

1.8 Prepare the 1st Strand Master Mix on ice as indicated below.

REAGENT	VOLUME PER REACTION*
RT-Buffer	9 µl
RT-Enzyme	1 µl

**Prepare a master mix for multiple reactions.*

1.9 Vortex for 4 seconds. Pulse-spin to ensure all of the solution is at the bottom of the sample plate or tube(s) and place on ice.

1.10 Add 10 µl of the 1st Strand Master Mix to each sample from Step 1.6 on ice.

1.11 Pipette a minimum of half the total volume up and down 10 times to ensure complete mixing. Pulse-spin to ensure all of the solution is at the bottom the sample plate or tube(s) and immediately transfer to the pre-chilled thermal cycler.

1.12 Initiate steps 2 to 5 of the thermal cycler program (see table in Step 1.7).

1.13 When the thermal cycler program is complete, place the samples on ice or leave in the thermal cycler at 4°C. Proceed immediately to 2nd Strand Synthesis and dA-Tailing.

2ND STRAND SYNTHESIS & dA-TAILING

1.14 Program a thermal cycler with the following conditions. Set the temperature of the heated lid to 80°C. Start the program to pre-chill the thermal cycler.

STEP	TEMPERATURE	TIME
1	4°C	HOLD
2	42°C	5 min
3	62°C	10 min
4	4°C	HOLD

1.15 Prepare the 2nd Strand Master Mix on ice as indicated below.

REAGENT	VOLUME PER REACTION*
Second Strand Buffer	14 µl
Second Strand Enzyme	1 µl

**Prepare a master mix for multiple reactions.*

1.16 Vortex for 4 seconds. Pulse-spin to ensure all of the solution is at the bottom the sample plate or tube(s) and place on ice.

1.17 Add 15 µl of the 2nd Strand Master Mix to each sample from Step 1.13 on ice.

1.18 Pipette a minimum of half the total volume up and down 10 times to ensure complete mixing. Pulse-spin to ensure all of the solution is at the bottom the sample plate or tube(s) and immediately transfer to the pre-chilled thermal cycler.

1.19 Initiate steps 2 to 4 of the thermal cycler program (see table in Step 1.14).
NOTE: While the thermal cycler program is running, prepare the reagents for Step 2: Ligate Twist UMI Adapters and Purify (See Before You Begin).

1.20 When the thermal cycler program is complete and the sample block has returned to 4°C, remove the samples from the block and place them on ice.

PROCEED IMMEDIATELY TO STEP 2: LIGATE TWIST UMI ADAPTERS AND PURIFY

STEP 2 LIGATE TWIST UMI ADAPTERS AND PURIFY

Ligate Twist UMI Adapters to the dA-tailed cDNA fragments from Step 1 and purify to generate cDNA libraries ready for index introduction through amplification in Step 3.

Reagents Required

- dA-tailed cDNA fragments (from Step 1.20)
- Ethanol
- Molecular biology grade water
- 10 mM Tris-HCl pH 8.0
- From the Twist RNA Library Preparation Kit:
  - Ligation Buffer
  - Ligation Enzyme
- From the Twist Purification Beads, 16 Samples or Twist Purification Beads, 96 Samples kit:
  - DNA Purification Beads
- From the Twist UMI Adapter System:
  - Twist UMI Adapters (tube; utilized for all samples)

Before You Begin

- Thaw or place on ice:
  - Ligation Buffer
  - Ligation Enzyme
  - Twist UMI Adapters
- Freshly prepare 1.5 ml of 80% Ethanol per sample (for use in both Steps 2 and 3 of the protocol)
- Equilibrate DNA Purification Beads to room temperature for at least 30 minutes (for use in both Steps 2 and 3 of the protocol)

LIGATE TWIST UMI ADAPTERS

2.1

Program a thermal cycler with the following conditions. Set the temperature of the heated lid to OFF. Start the program to pre-chill the thermal cycler.

STEP	TEMPERATURE	TIME
1	4°C	HOLD
2	20°C	15 min
3	4°C	HOLD*

**Place on ice following ligation to reduce adapter-dimer formation prior to purification.*

2.2 Vortex the thawed Ligation Buffer for 20 seconds to fully homogenize the solution, pulse-spin to ensure all solution is at the bottom of the tube and place on ice.

2.3 Prepare 5 µl of adapter solution per sample using the volumes specified in the table below.
NOTE: Storing diluted adapter solutions for extended periods of time is not recommended.
⚠IMPORTANT: DO NOT use an unbuffered solution (e.g. molecular biology grade water) as a diluent.

RNA INPUT	HIGH-QUALITY AND PARTIALLY-DEGRADED RNA		FFPE-DERIVED RNA	
	UMI ADAPTER VOLUME	10 mM TRIS-HCL, pH 8.0 VOLUME	UMI ADAPTER VOLUME	10 mM TRIS-HCL, pH 8.0 VOLUME
1 ng	0.25 µl	4.75 µl	0.18 µl	4.82 µl
10 ng	2 µl	3 µl	1 µl	4 µl
100 ng	3 µl	2 µl	1 µl	4 µl
1000 ng	3 µl	2 µl	1 µl	4 µl

2.4 Add 5 µl of appropriate adapter solution to each sample from Step 1.20.

2.5 Prepare the Ligation Master Mix on ice as indicated below.

REAGENT	VOLUME PER REACTION*
Ligation Buffer	40 µl
Ligation Enzyme	5 µl

**Prepare a master mix for multiple reactions.*

2.6 Vortex for 4 seconds. Pulse-spin to ensure all of the solution is at the bottom the sample plate or tube(s) and place on ice.

2.7 Add 45 µl of the Ligation Master Mix to each sample from Step 2.4 on ice.

2.8 Homogenize with moderate vortexing for 5 seconds or by pipetting a minimum of half the total volume up and down 10 times (avoid formation of bubbles). Pulse-spin to ensure all of the solution is at the bottom the sample plate or tube(s).

2.9 Place the sample plate or tube(s) in the thermal cycler. Initiate steps 2 to 3 of the thermal cycler program (see table in Step 2.1).
NOTE: While the thermal cycler program is running, prepare the reagents for Step 3: PCR Amplify and Strand Select Using UDI Primers, Purify, and Perform QC (see Before You Begin).

2.10 When the program is complete, proceed immediately to Purify.

PURIFY

2.11 Vortex the pre-equilibrated DNA Purification Beads until mixed.

2.12 Add 70 µl (0.7X) of homogenized DNA Purification Beads to each sample from Step 2.10. Mix well by vortexing.

2.13 Incubate the samples for 5 minutes at room temperature.

2.14 Place the samples on a magnetic plate for 1 minute or until the supernatant is clear.

2.15 The DNA Purification Beads form a pellet, leaving a clear supernatant. Without removing the plate or tubes from the magnetic plate, remove and discard the supernatant.

2.16 Wash the bead pellet by gently adding 200 µl of freshly prepared 80% ethanol (do not disturb the pellet). Incubate for 1 minute, then remove and discard the ethanol.

2.17 Repeat this wash once, for a total of two washes, while keeping the samples on the magnetic plate.

2.18 Carefully remove all remaining ethanol with a 10-µl pipette, making sure not to disturb the bead pellet.
NOTE: Before pipetting, the bead pellet may be briefly spun to collect ethanol at the bottom of the plate or tube and returned to the magnetic plate.

2.19 Air-dry the bead pellet on the magnetic plate for 5 minutes or until the bead pellet is dry. Do not overdry the bead pellet.

2.20 Remove the plate or tubes from the magnetic plate and add 22 µl 10 mM Tris-HCl pH 8 to each sample. Mix by pipetting until homogenized.

2.21 Incubate at room temperature for 2 minutes.

2.22 Place the plate or tubes on a magnetic plate and let stand for 3 minutes or until the beads form a pellet.

2.23 Transfer 20 µl of the clear supernatant containing the ligated libraries to a clean thin-walled PCR 0.2-ml strip-tube or well of a 96-well thermal cycling plate, making sure not to disturb the bead pellet.

SAFE STOPPING POINT: Samples can be frozen at -20˚C.

2ND PURIFY (OPTIONAL)

NOTE: This is an optional step and only recommended when working with:
Low RNA mass input (e.g. 10 ng or less)

2.24 Vortex the pre-equilibrated room temperature DNA Purification Beads until mixed.

2.25 Add 20 µl (1X) of homogenized DNA Purification Beads to each sample from Step 2.23. Mix well by vortexing.

2.26 Incubate the samples for 5 minutes at room temperature.

2.27 Place the samples on a magnetic plate for 1 minute or until the supernatant is clear.

2.28 The DNA Purification Beads form a pellet, leaving a clear supernatant. Without removing the plate or tubes from the magnetic plate, remove and discard the supernatant.

2.29 Wash the bead pellet by gently adding 200 µl of freshly prepared 80% ethanol (do not disturb the pellet). Incubate for 1 minute, then remove and discard the ethanol.

2.30 Repeat this wash once, for a total of two washes, while keeping the sample(s) on the magnetic plate.

2.31 Carefully remove all remaining ethanol with a 10-µl pipette, making sure not to disturb the bead pellet.
NOTE: Before pipetting, the bead pellet may be briefly spun to collect ethanol at the bottom of the plate or tube and returned to the magnetic plate.

2.32 Air-dry the bead pellet on the magnetic plate for 5 minutes or until the bead pellet is dry. Do not overdry the bead pellet.

2.33 Remove the plate or tubes from the magnetic plate and add 22 µl 10 mM Tris-HCl pH 8 to each sample. Mix by pipetting until homogenized.

2.34 Incubate at room temperature for 2 minutes.

2.35 Place the plate or tubes on a magnetic plate and let stand for 3 minutes or until the beads form a pellet.

2.36 Transfer 20 µl of the clear supernatant containing the ligated libraries to a clean thin-walled PCR 0.2-ml strip-tube or well of a 96-well thermal cycling plate, making sure not to disturb the bead pellet.

SAFE STOPPING POINT: Samples can be frozen at -20˚C.

PROCEED TO STEP 3: PCR AMPLIFY AND STRAND SELECT USING UDI PRIMERS, PURIFY, AND PERFORM QC

STEP 3 PCR AMPLIFY AND STRAND SELECT USING UDI PRIMERS, PURIFY, AND PERFORM QC

Amplify the adapted cDNA libraries with Twist UDI Primers, purify them, and perform quality control (QC) analysis to complete the protocol.

Reagents Required

- Ligated, purified libraries (from Step 2.23 or 2.36)
- 80% Ethanol (from Step 2)
- Equilibrated DNA Purification Beads (from Step 2)
- 10 mM Tris-HCl pH 8.0
- From the Twist RNA Library Preparation Kit:
  - Equinox Amplification Master Mix (2X)
- Twist UMI Adapter System:
  - Twist UDI Primers

⚠ IMPORTANT: Use of P5/P7 Primers (10X) tubes 107075, 107085 contained in the Twist RNA Library Prep Kit is not required. Using these primers with the Twist UMI Adapter System will result in a failed PCR amplification.

Before You Begin

- Thaw by placing on ice:
  - Equinox Amplification Master Mix (2X)
  - Twist UDI Primers (plate with single-use primers)

PREPARE THE THERMAL CYCLER

3.1

Program a thermal cycler with the following conditions. Set the temperature of the heated lid to 105°C.

	STEP	TEMPERATURE	TIME	NUMBER CYCLES
1	Initialization	98°C	45 seconds	1
2	Denaturation	98°C	15 seconds	*Use the table on the next page to select the number of cycles needed based on the mass input and RNA quality*
	Annealing	55°C	30 seconds	
	Extension	72°C	30 seconds	
3	Final Extension	72°C	1 minute	1
4	Final Hold	12°C	HOLD	–

RNA INPUT INTO LIBRARY PREPARATION	PCR CYCLE RECOMMENDATION*	
	HIGH-QUALITY AND PARTIALLY-DEGRADED RNA	FFPE-DERIVED RNA
1000 ng	7 - 8	7 - 8
100 ng	9 - 10	9 - 10
10 ng	11 - 12	12 - 13
1 ng	15 - 16	20 - 21

**Cycle number recommendations are a starting point for Twist target enrichment workflows and provide sufficient yield for use in 1-plex target enrichment. Cycle number should be modified for each sample type/application.*

PERFORM THE PCR

3.2 Add 5 µl of Twist UDI Primer from the provided 96-well plate to each of the cDNA libraries from Step 2.23 or Step 2.36 and mix well by pipetting.
NOTE: For index selection and pooling guidelines for downstream target enrichment and sequencing, refer to Appendix A.

3.3 Invert Equinox Library Master Mix (2X) 5 times before use.
NOTE: DO NOT VORTEX.

3.4 Add 25 µl of Equinox Amplification Master Mix (2X) to the cDNA libraries from Step 3.2 and mix well by pipetting or vortex for 4 seconds.

3.5 Pulse-spin the sample plate or tube(s) and immediately transfer to the thermal cycler. Start the program (see table in Step 3.1).

3.6 Remove the sample(s) from the block when the thermal cycler program is complete. Proceed to Purify.

PURIFY

3.7 Vortex the pre-equilibrated DNA Purification Beads until mixed.

3.8 Add 50 µl (1X) of homogenized DNA Purification Beads to each sample from Step 3.6. Mix well by vortexing.

3.9 Incubate the samples for 5 minutes at room temperature.

3.10 Place the samples on a magnetic plate for 1 minute or until the supernatant is clear.

3.11 The DNA Purification Beads form a pellet, leaving a clear supernatant. Without removing the plate or tubes from the magnetic plate, remove and discard the supernatant.

3.12 Wash the bead pellet by gently adding 200 µl freshly prepared 80% ethanol (do not disturb the pellet), incubate for 1 minute, then remove and discard the ethanol.

3.13 Repeat this wash once, for a total of two washes, while keeping the samples on the magnetic plate.

3.14 Carefully remove all remaining ethanol with a 10-µl pipette, making sure not to disturb the bead pellet.
NOTE: Before pipetting, the bead pellet may be briefly spun to collect ethanol at the bottom of the plate or tube and returned to the magnetic plate.

3.15 Air-dry the bead pellet on the magnetic plate for 5 minutes or until the bead pellet is dry. Do not overdry the bead pellet.

3.16 Remove the plate or tubes from the magnetic plate and add 22 µl 10 mM Tris-HCl pH 8 to each sample. Mix by pipetting until homogenized.

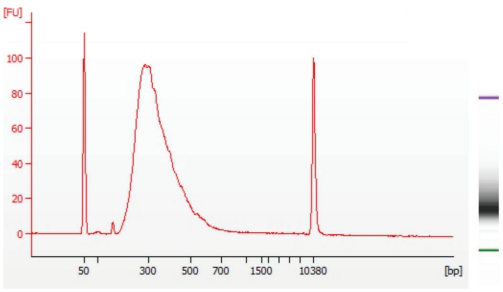
3.17 Incubate at room temperature for 2 minutes.

3.18 Place the plate or tubes on a magnetic plate and let stand for 3 minutes or until the beads form a pellet.

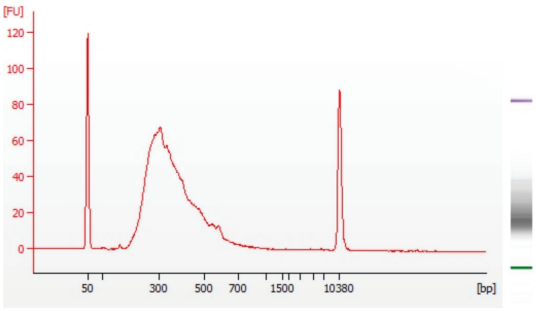
3.19 Transfer 20 µl of the clear supernatant containing the Amplified Indexed Libraries to a clean thin-walled PCR 0.2-ml strip-tube or well of a 96-well thermal cycling plate, making sure not to disturb the bead pellet.

PERFORM QC

3.20 Quantify and validate the size range of each library using the Thermo Fisher Scientific Qubit dsDNA Broad Range Quantitation Assay and Agilent DNA 7500 Assay. Final concentration values should be ≥ 25 ng/µl. The average fragment length will vary based on the quality of input RNA and the fragmentation condition selected.



Representative electropherogram of a library generated from 100 ng of high-quality RNA, using 10 minute incubation at 90°C for fragmentation, only one post-ligation purification, and 9 cycles of PCR.



Representative electropherogram of a library generated from 100 ng of FFPE-derived RNA, using 1 minute incubation at 65°C for fragmentation, only one post-ligation purification, and 9 cycles of PCR.

STOPPING POINT: If not proceeding immediately to a Twist Target Enrichment System, store the amplified indexed libraries at –20°C.

END OF WORKFLOW

APPENDIX A: UDI SEQUENCES AND POOLING GUIDELINES

UDI SEQUENCES

For a complete guide of the Twist UDI sequences used in the Twist UMI Adapter System, please refer to the UDI Sequences Reference Spreadsheet and the Sample Sheet Template. All files are available for download at twistbioscience.com/resources/protocol/unique-dual-index-sequences-protocol-reference-document-spreadsheet-and-sample.

POOLING GUIDELINES

Twist UDI primers are base balanced for next-generation sequencing on a column basis. When pooling unique dual-indexed libraries for 8-plex hybridization, it is recommended that libraries be selected from a single column. Multiple columns may be selected in any desired combination across a single plate or multiple plates for sequencing.

Table 1. Twist UDI primer plate layouts and pooling guidelines.
Twist UMI Adapter System: TruSeq Compatible, 16 Samples (105040)

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9										
B	2	10										
C	3	11										
D	4	12										
E	5	13										
F	6	14										
G	7	15										
H	8	16										

NOTE: The indexes in the 16 sample plate are not the same in 96 samples, Plate A.

APPENDIX A: UDI SEQUENCES AND POOLING GUIDELINES

Twist UMI Adapter System: TruSeq Compatible, 96 Samples, Plates A to D (105041, 105042, 105043, 105044

Plate A.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	49	57	65	73	81	89
B	2	10	18	26	34	42	50	58	66	74	82	90
C	3	11	19	27	35	43	51	59	67	75	83	91
D	4	12	20	28	36	44	52	60	68	76	84	92
E	5	13	21	29	37	45	53	61	69	77	85	93
F	6	14	22	30	38	46	54	62	70	78	86	94
G	7	15	23	31	39	47	55	63	71	79	87	95
H	8	16	24	32	40	48	56	64	72	80	88	96

Plate B.

	1	2	3	4	5	6	7	8	9	10	11	12
A	97	105	113	121	129	137	145	153	161	169	177	185
B	98	106	114	122	130	138	146	154	162	170	178	186
C	99	107	115	123	131	139	147	155	163	171	179	187
D	100	108	116	124	132	140	148	156	164	172	180	188
E	101	109	117	125	133	141	149	157	165	173	181	189
F	102	110	118	126	134	142	150	158	166	174	182	190
G	103	111	119	127	135	143	151	159	167	175	183	191
H	104	112	120	128	136	144	152	160	168	176	184	192

Plate C.

	1	2	3	4	5	6	7	8	9	10	11	12
A	193	201	209	217	225	233	241	249	257	265	273	281
B	194	202	210	218	226	234	242	250	258	266	274	282
C	195	203	211	219	227	235	243	251	259	267	275	283
D	196	204	212	220	228	236	244	252	260	268	276	284
E	197	205	213	221	229	237	245	253	261	269	277	285
F	198	206	214	222	230	238	246	254	262	270	278	286
G	199	207	215	223	231	239	247	255	263	271	279	287
H	200	208	216	224	232	240	248	256	264	272	280	288

Plate D.

	1	2	3	4	5	6	7	8	9	10	11	12
A	289	297	305	313	321	329	337	345	353	361	369	377
B	290	298	306	314	322	330	338	346	354	362	370	378
C	291	299	307	315	323	331	339	347	355	363	371	379
D	292	300	308	316	324	332	340	348	356	364	372	380
E	293	301	309	317	325	333	341	349	357	365	373	381
F	294	302	310	318	326	334	342	350	358	366	374	382
G	295	303	311	319	327	335	343	351	359	367	375	383
H	296	304	312	320	328	336	344	352	360	368	376	384

APPENDIX B: OBTAINING ALTERNATIVE INSERT SIZES

FRAGMENTATION OPTIMIZATION

Fragmentation rates may vary depending on many factors including RNA quality, RNA mass input, fragmentation temperature, and fragmentation time. For high-quality RNA inputs, begin optimization of Fragmentation (Protocol Step 1.2) by selecting the desired size range condition for the mass input being used from the table below. Test a range of fragmentation temperatures around the initial condition in 5 degree increments. Longer insert sizes may be achieved by lowering the fragmentation time in 3-5 minute increments.

NOTE: degraded samples, including those derived from FFPE, may be difficult to modulate. Use lower temperatures and shorter incubation times for these sample types.

STEP 2 INCUBATION				
RNA QUALITY	FRAGMENTATION CONDITION*	EXPECTED INSERT SIZE		
		≤10 NG	100 NG	1000 NG
Intact RIN >7	80°C for 10 min	210 - 230 bp	175 - 195 bp	140 - 160 bp
	85°C for 10 min	200 - 220 bp	160 - 180 bp	130 - 150 bp
	90°C for 10 min	190 - 210 bp	145 - 165 bp	120 - 140 bp
	95°C for 10 min	160 - 180 bp	120 - 140 bp	100 - 120 bp

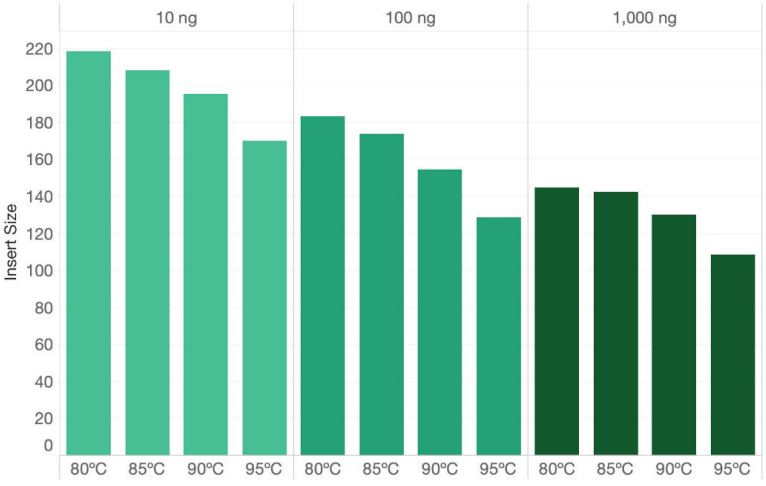


Figure 1. Insert Size of RNA Libraries. *10, 100, or 1000 ng of high-quality RNA were taken through the the Twist RNA Library Preparation with Twist Universal Adapter System Protocol. The fragmentation incubation temperature in Step 1.2 was set between 80°C and 95°C to modulate RNA library insert size.*

END OF APPENDIX