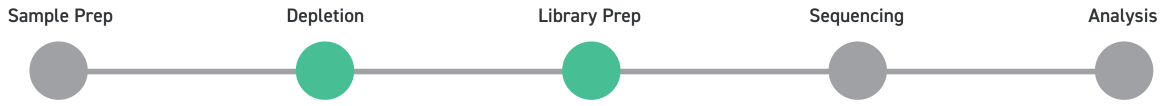
PROTOCOL

RNA Depletion and RNA Library Preparation with Twist Universal Adapter System

For use with the Twist NGS Workflow

This Twist RNA Depletion and RNA Library Preparation Protocol details all the necessary steps for RNA depletion, converting RNA to double-stranded cDNA, and preparing cDNA libraries using the Twist Universal Adapter System. The Twist Universal Adapter System consists of Twist Universal Adapters and Twist Unique Dual Indexed (UDI) Primers. This kit is also compatible with the Twist HT Universal Adapter System, which consists of Twist Universal Adapters and Twist HT UDI Primers. This manual details steps to remove ribosomal RNA and globin from total RNA in order to generate amplified indexed cDNA libraries that can undergo whole transcriptome sequencing on Illumina next-generation sequencing (NGS) systems. If desired, whole transcriptome libraries are compatible with Twist target enrichment workflows. This protocol has been optimized for use with the reagents specified and should only be performed with them or their equivalents.



Twist NGS Workflow. *The complete NGS workflow takes you from sample preparation to NGS sequencing and data analysis.*

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)			
107147 (16 rxn) 107148 (96 rxn)	Twist RNA Library Prep with Depletion	Reagents for depletion and RNA library construction	—
	Twist rRNA and Globin Depletion Kit	· Depletion Targets — rRNA & Globin for HMR · Depletion Master Mix · Oligo Digestion Master Mix	–20°C
	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 boxes of these need to be ordered for this protocol)	2-8°C
107242 (96 rxn)	Twist Purification Beads, 96 Samples		
TWIST ADAPTERS (ORDERED SEPARATELY)			
101307 (16 rxn)	Twist Universal Adapter System TruSeq Compatible, 16 samples	· Twist Universal Adapters · Twist UDI Primers	–20°C
101308 / 101309 / 101310 / 101311 (96 rxn)	Twist Universal Adapter System TruSeq Compatible, 96 Samples Plate A/B/C/D		
106390-106393, 106396, 106398-106400: 384 rxn, 96-well plate 106401-106408: 384 rxn, 384-well plate	Twist HT Universal Adapter System TruSeq Compatible	· Twist Universal Adapters · Twist HT UDI Primers	–20°C

LEGAL

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INTENDED USE

This product is for research use only. This product is not intended for the diagnosis, prevention, or treatment of a disease or condition. Twist Bioscience assumes no liability regarding use of the product for applications in which it is not intended.

TABLE OF CONTENTS

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 (HT) Universal 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
96-well thermal cycling plates	Eppendorf
1.5-ml microcentrifuge tubes	VWR
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 and blood.
- 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 be low when using degraded samples. If using RNA derived from FFPE samples, an FFPE repair (Step 2.1.1.A to 2.1.14.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 an RNA depletion step and generates amplified indexed libraries. This protocol allows you to perform RNA library preparation (Steps 1-4) in 5.5 hours.

	RNA LIBRARY PREP WITH UNIVERSAL ADAPTERS	TIME
STEP 1	**Depletion** Ribodepleted RNA	**1 hour 20 minutes**
STEP 2	**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 3	**Ligate Adapters and Purify** cDNA libraries	**55 minutes to 1 hour 25 minutes**
STEP 4	**Library Amplification and Strand Selection** Amplified indexed libraries	**1 hour**

STEP 1 DEPLETION

Perform ribodepletion to remove rRNA and globin from RNA sample.

Reagents Required

- RNA sample
- RNase-free water
- Ethanol
- Molecular biology grade water
- From the Twist rRNA and Globin Depletion Kit:
    - Depletion Master Mix
    - Depletion Targets - rRNA & Globin for HMR
    - Oligo Digestion Master Mix
- From the Twist RNA Library Prep Kit:
    - Fragmentation/Prime Buffer
    - FFPE Repair Buffer (for FFPE-derived RNA samples only)
- From the Twist Purification Beads, 16 Samples or Twist Purification Beads, 96 Samples kit
    - DNA Purification Beads

Before You Begin

- Thaw by placing on ice, vortex to ensure the reagent is fully mixed:
    - Depletion Master Mix
    - Depletion Targets - rRNA & Globin for HMR
    - Oligo Digestion Master Mix
    - Fragmentation/Prime Buffer
    - FFPE Repair Buffer (for FFPE-derived RNA samples only)
- Prepare 2 ml 80% ethanol for each sample (for use in Steps 1, 3, and 4 of the protocol)
- Equilibrate DNA Purification Beads to room temperature for at least 30 minutes (for use in Steps 1, 3, and 4 of the protocol)

rRNA AND GLOBIN DEPLETION

1.1

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

STEP	TEMPERATURE	TIME
1	77°C	HOLD
2	77°C	2 min
3	65°C	15 min
4	4°C	HOLD

1.2

Prepare the Depletion Reaction Mix on ice as indicated below.

REAGENT	VOLUME PER REACTION*
Depletion Master Mix	4.5 µl
Depletion Targets — rRNA & Globin for HMR	2.5 µl

**Prepare a master mix for multiple reactions.*

1.3

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.

1.4

Prepare input RNA on ice in a total volume of 18 µl using RNase-free water and add to labeled 0.2 ml PCR tubes or PCR plate.

1.5

Add 7 µl of the Depletion Reaction Mix to each sample from Step 1.4. Pipette a minimum of half the total volume up and down 10 times to ensure complete mixing. Pulse-spin to ensure all the solution is at the bottom of the sample plate or tube(s). Immediately return to ice.

NOTE: Do not leave samples on ice for more than 10 minutes.

1.6

Place the tube into the heated thermal cycler. Initiate steps 2 to 4 of the thermal cycler program (see table in Step 1.1).

1.7

When the thermal cycler program is complete, place samples on ice and proceed immediately to Oligo Digestion.

OLIGO DIGESTION

1.8

Program a thermal cycler with the following conditions. Set the temperature of the heated lid to 50°C. Start the program to pre-chill the thermal cycler.

STEP	TEMPERATURE	TIME
1	4°C	HOLD
2	37°C	10 min
3	4°C	HOLD

1.9

Add 35 µl of the Oligo Digestion Master Mix to each sample from Step 1.7 on ice and pipette a minimum of half the total volume up and down 10 times to ensure complete mixing.

1.10

Pulse-spin to ensure all solution is at the bottom of the plate or tube(s) and immediately transfer to the pre-chilled thermal cycler.

1.11 Initiate steps 2 to 3 of the thermal cycler program (see table in Step 1.8).

NOTE: While the thermal cycler program is running, prepare the reagents for Step 2: Fragmentation, cDNA Synthesis, End Repair, and dA-Tailing (See Before You Begin).

⚠ IMPORTANT: While the thermal cycler program is running prepare the master mix listed in Step 2.1.1.A (for FFPE-derived RNA samples) or Step 2.1.1.B (for all other RNA). This master mix is used to elute dry beads from Step 1.21. Allowing beads to overdry will impact kit performance.

1.12 When the thermal cycler program is complete, proceed immediately to Purify.

PURIFY

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

NOTE: DNA purification beads are compatible with RNA.

1.14 Add 108 µl (1.8X) of homogenized DNA Purification Beads to each sample from Step 1.12. Mix well by vortexing.

1.15 Incubate the samples for 5 minutes at room temperature.

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

1.17 The DNA Purification Beads form a pellet, leaving a clear supernatant. Without removing the plate or tube(s) from the magnetic plate, remove and discard the supernatant.

1.18 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.

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

1.20 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.

1.21 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.

PROCEED IMMEDIATELY TO STEP 2: FRAGMENTATION, cDNA SYNTHESIS, END REPAIR, AND dA-TAILING

STEP 2 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 2.1.1.A to 2.1.14.A) if using FFPE-derived RNA, otherwise skip to Fragmentation for High-quality and Partially-degraded RNA (Step 2.1.1.B).

Reagents Required

- Dry beads (from Step 1.21)
- Thawed in Step 1
    - FFPE Repair Buffer (for FFPE-derived RNA samples only)
    - Fragmentation/Prime Buffer
- RNase-free water
- From the Twist RNA Library Prep Kit:
    - RT-Buffer
    - RT-Enzyme
    - Second Strand Buffer
    - Second Strand Enzyme

Before You Begin

- Thaw by placing on ice, vortex to ensure reagent is fully mixed:
    - RT-Buffer (Make sure to protect from direct sunlight)
    - Second Strand Buffer
- Take out the following reagents from -20°C right before use and return back immediately after use:
    - RT-Enzyme
    - Second Strand Enzyme

FFPE REPAIR

2.1.1.A Prepare the FFPE Repair Master Mix on ice as indicated below.

REAGENT	VOLUME PER REACTION*
FFPE Repair Buffer	4.5 µl
RNase-Free Water	12.5 µl

**Prepare a master mix for multiple reactions.*

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

2.1.3.A Add 17 µl of FFPE Repair Master Mix to the dry beads from Step 1.21.

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

2.1.5.A Place the samples on a magnetic plate for at least 1 minute or until the supernatant is clear.

2.1.6.A Carefully remove 15 µl of the supernatant and transfer 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.

2.1.7.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

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

2.1.9.A When the thermal cycler program is complete, proceed immediately to Fragmentation for FFPE-derived RNA.

FRAGMENTATION FOR FFPE-DERIVED RNA

2.1.10.A 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	65°C	1 min
3	12°C	HOLD

2.1.11.A To each reaction, add 10 µl of the Fragmentation/Prime Buffer and pipette a minimum of half the total volume up and down 10 times to ensure complete mixing.

2.1.12.A 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.

2.1.13.A Initiate steps 2 to 3 of the thermal cycler program (see table in Step 2.1.10.A).

2.1.14.A When the thermal cycler program is complete, proceed immediately to 1st Strand Synthesis (Step 2.2 on page 15).

FRAGMENTATION FOR HIGH-QUALITY AND PARTIALLY-DEGRADED RNA

2.1.1.B Prepare the Fragmentation/Prime Master Mix on ice as indicated below.

REAGENT	VOLUME PER REACTION*
Fragmentation/Prime Buffer	10.8 µl
RNase-Free Water	16.2 µl

**Prepare a master mix for multiple reactions.*

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

2.1.3.B Add 27 µl of the Fragmentation/Prime Master Mix to the dry beads from Step 1.21.

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

2.1.5.B 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. 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

STEP 2 INCUBATION			
RNA QUALITY	**RIN**	**EXPECTED INSERT SIZE**	**FRAGMENTATION CONDITION***
Intact	> 7	175 - 250 bp	85°C for 5 min
Partially degraded	2 - 7	100 - 300 bp	85°C for 1-5 min
Degraded, non-FFPE	1 - 2	Dictated by RNA Quality	65°C for 1 min

**Expected insert size guidelines are based on libraries generated with 100 ng of 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.*

2.1.6.B Place the tubes into the pre-chilled thermal cycler. Initiate steps 2 to 3 of the thermal cycler program.

2.1.7.B When the thermal cycler program is complete and the sample block has returned to 12°C, place tubes on a magnetic plate for at least 1 minute or until the supernatant is clear.

2.1.8.B Carefully remove 25 µl of the supernatant and transfer 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. Proceed immediately to 1st Strand Synthesis (Step 2.2).

1ST STRAND SYNTHESIS

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

2.2 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

2.3 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.*

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

2.5 Add 10 µl of 1st Strand Master Mix to each sample from Step 2.1.14.A or Step 2.1.8.B on ice. Pipette a minimum of half the total volume up and down 10 times to ensure complete mixing.

2.6 Pulse-spin to ensure all of the solution is at the bottom of the sample plate or tube(s) and immediately transfer to the pre-chilled thermal cycler.

2.7 Initiate steps 2 to 5 of the thermal cycler program (see table in Step 2.2).

2.8 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

2.9

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

2.10

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.*

2.11

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

2.12

Add 15 µl of the 2nd Strand Master Mix to each sample from Step 2.8.

2.13

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

2.14

Initiate steps 2 to 4 of the thermal cycler program (see table in Step 2.9).

NOTE: While the thermal cycler program is running, prepare the reagents for Step 3: Ligate Twist Universal Adapters and Purify (See Before You Begin).

2.15

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 3: LIGATE TWIST UNIVERSAL ADAPTERS AND PURIFY

STEP 3 LIGATE TWIST UNIVERSAL ADAPTERS AND PURIFY

Ligate Twist Universal Adapters to the dA-tailed cDNA fragments from Step 2 and purify to generate cDNA libraries ready for index introduction through amplification in Step 4.

Reagents Required

- dA-tailed cDNA fragments (from Step 2.15)
- 80% Ethanol (from Step 1)
- Equilibrated DNA Purification Beads (from Step 1)
- 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 (HT) Universal Adapter System:
  - Twist Universal Adapters (tube; utilized for all samples)

Before You Begin

- Thaw by placing on ice:
  - Ligation Buffer
  - Ligation Enzyme
  - Twist Universal Adapters

LIGATE TWIST UNIVERSAL ADAPTERS

3.1 Program a thermal cycler with the following conditions. Set 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 samples on ice following ligation to reduce adapter-dimer formation prior to purification.*

3.2 Vortex the thawed Ligation Buffer for 20 seconds to fully homogenize the solution before pulse-spinning to collect all of the solution at the bottom of the tube and placing it on ice.

3.3

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	
	UNIVERSAL ADAPTER VOLUME	10 mM TRIS-HCL, pH 8.0 VOLUME	UNIVERSAL ADAPTER VOLUME	10 mM TRIS-HCL, pH 8.0 VOLUME
1 ng	0.5 µl	4.5 µl	0.2 µl	4.8 µl
10 ng	1 µl	4 µl	0.2 µl	4.8 µl
100 ng	2 µl	3 µl	0.2 µl	4.8 µl
1000 ng	3 µl	2 µl	0.5 µl	4.5 µl

3.4

Add 5 µl of the appropriate adapter solution to each sample from Step 2.15.

3.5

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.*

3.6

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

3.7

Add 45 µl of the Ligation Master Mix to each sample from Step 3.4 on ice.

3.8

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

3.9

Place the sample plate or tube(s) in the pre-chilled thermal cycler. Initiate steps 2 to 3 of the thermal cycler program (see table in Step 3.1).

NOTE: While the thermal cycler program is running, prepare the reagents for Step 4: PCR Amplify and Strand Select Using UDI Primers, Purify, and Perform QC (see Before You Begin).

3.10

When the program is complete, proceed immediately to Purify.

PURIFY

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

3.12 Add 70 µl (0.7X) of homogenized DNA Purification Beads to each ligation sample from Step 3.10. Mix well by vortexing.

3.13 Incubate the samples for 5 minutes at room temperature.

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

3.15 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.

3.16 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.

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

3.18 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.

3.19 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.

3.20 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.

3.21 Incubate at room temperature for 2 minutes.

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

3.23 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:
RNA mass input 50 ng or less

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

3.25 Add 20 µl (1X) of homogenized DNA Purification Beads to each ligation sample from Step 3.23. Mix well by vortexing.

3.26 Incubate the samples for 5 minutes at room temperature.

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

3.28 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.

3.29 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.

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

3.31 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.

3.32 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.

3.33 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.

3.34 Incubate at room temperature for 2 minutes.

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

3.36 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 4: PCR AMPLIFY AND STRAND SELECT USING UDI PRIMERS, PURIFY, AND PERFORM QC

STEP 4 PCR AMPLIFY AND STRAND SELECT USING UDI PRIMERS, PURIFY, AND PERFORM QC

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

Reagents Required

- Ligated, purified libraries (from Step 3.23 or 3.36)
- 80% Ethanol (from Step 1)
- Equilibrated DNA Purification Beads (from Step 1)
- 10 mM Tris-HCl pH 8.0
- From the Twist RNA Library Preparation Kit:
  - Equinox Amplification Master Mix (2X)
- If using the Twist Universal Adapter System:
  - Twist UDI Primers
- If using the Twist HT Universal Adapter System:
  - Twist HT 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 Universal Adapter System will result in a failed PCR amplification.

Before You Begin

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

PREPARE THE THERMAL CYCLER

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

STEP		TEMPERATURE	TIME	NUMBER CYCLES
1	Initial Denaturation	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	8 - 9	14 - 15
100 ng	11 - 12	18 - 19
10 ng	15 - 16	21 - 22
1 ng	20 - 21	22 - 23

**Cycle number recommendations are a starting point and should be modified for each sample type/application.*

PERFORM THE PCR

4.2 Add 5 µl of Twist UDI or Twist HT UDI Primer from the provided 96-well or 384-well plate to each of the cDNA libraries from Step 3.23 or Step 3.36 and mix well by pipetting.
NOTE: For index selection and pooling guidelines for downstream target enrichment and sequencing, refer to Appendix A or B depending on the adapter system being used.

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

4.4 Add 25 µl of Equinox Amplification Mastermix (2X) to the cDNA libraries from Step 4.2 and mix well by pipetting or vortex for 4 seconds.

4.5 Pulse-spin the sample plate or tube(s) and immediately transfer to the thermal cycler. Start the program (see table in Step 4.1).

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

PURIFY

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

4.8 Add 50 µl (1X) of homogenized DNA Purification Beads to each ligation sample from Step 4.6. Mix well by vortexing.

4.9 Incubate the samples for 5 minutes at room temperature.

4.10 Place the samples on a magnetic plate for 1 minute.

4.11 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.

4.12 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.

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

4.14 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.

4.15 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.

4.16 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.

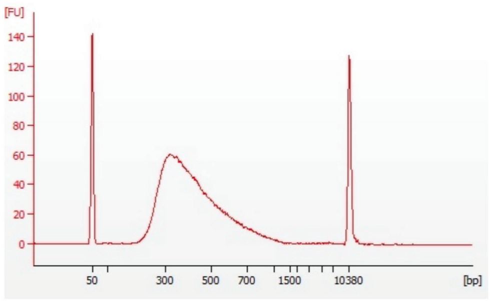
4.17 Incubate at room temperature for 2 minutes.

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

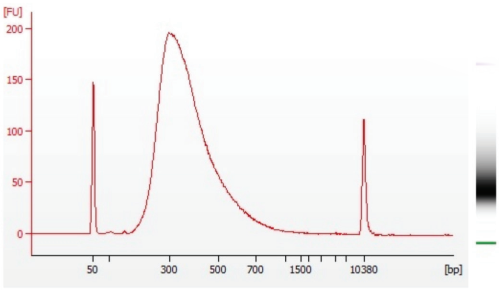
4.19 Transfer 20 µl of the clear supernatant containing the Amplified Indexed Libraries to a clean thin-walled PCR 0.2-ml strip-tube or well of a 96-well thermal cycling plate, making sure not to disturb the bead pellet.

PERFORM QC

4.20 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 ≥ 15 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 5 minute incubation at 85°C for fragmentation, only one post-ligation purification, and 12 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 19 cycles of PCR.

🛑 STOPPING POINT: If not proceeding immediately to sequencing, 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 Universal 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%20Dual-Index-Sequences-reference-spreadsheets-and-sample-sheet-templates.

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 Universal Adapter System: TruSeq Compatible, 16 Samples (101307)

	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 Universal Adapter System: TruSeq Compatible, 96 Samples, Plates A to D (101308, 101309, 101310, 101311)

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: HT UDI SEQUENCES AND POOLING GUIDELINES

HT UDI SEQUENCES

The Twist HT UDI Primers in the Twist HT Universal Adapter System contain indexes of 12 nucleotides that are distinct from the Twist UDI Primers in the Twist Universal Adapter System. All 3,072 indexes are available in either 96-well or 384-well plate format. Representative plate layouts are below.

CAUTION: Sequence only one plate configuration at a time because the 96-well and 384-well plate formats both use the same 3,072 indexes.

For a complete guide of the Twist HT UDI sequences used in the Twist HT Universal Adapter System, please refer to the HT UDI Sequences Reference Spreadsheets and HT UDI Sample Sheet Templates. These files are available for download here: twistbioscience.com/resources/data-files/high-throughput-unique-dual-index-sequences-reference-spreadsheets-and-sample.

POOLING GUIDELINES

The Twist HT UDI Primers are base balanced for next-generation sequencing on a column basis. When pooling unique dual-indexed libraries for multiplex 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 2: Representative 96-Well Plate Layouts

Twist HT Universal Adapter System: TruSeq Compatible, 96-Well, Plates AAA01-AAA16 and BBB01-BBB16 (106390-106393, 106396, 106398-106400)

Plate 96W-AAA01

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

Plate 96W-AAA02

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

Plate 96W-AAA03

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

Plate 96W-AAA04

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

Table 3: Representative 384-Well Plate Layout

Twist HT Universal Adapter System: TruSeq Compatible, 384-Well, Plates AAA01-AAA04 and BBB01-BBB04 (106401-106408)

Plate 384W-AAA01

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1	97	2	98	3	99	4	100	5	101	6	102	7	103	8	104	9	105	10	106	11	107	12	108
B	193	289	194	290	195	291	196	292	197	293	198	294	199	295	200	296	201	297	202	298	203	299	204	300
C	13	109	14	110	15	111	16	112	17	113	18	114	19	115	20	116	21	117	22	118	23	119	24	120
D	205	301	206	302	207	303	208	304	209	305	210	306	211	307	212	308	213	309	214	310	215	311	216	312
E	25	121	26	122	27	123	28	124	29	125	30	126	31	127	32	128	33	129	34	130	35	131	36	132
F	217	313	218	314	219	315	220	316	221	317	222	318	223	319	224	320	225	321	226	322	227	323	228	324
G	37	133	38	134	39	135	40	136	41	137	42	138	43	139	44	140	45	141	46	142	47	143	48	144
H	229	325	230	326	231	327	232	328	233	329	234	330	235	331	236	332	237	333	238	334	239	335	240	336
I	49	145	50	146	51	147	52	148	53	149	54	150	55	151	56	152	57	153	58	154	59	155	60	156
J	241	337	242	338	243	339	244	340	245	341	246	342	247	343	248	344	249	345	250	346	251	347	252	348
K	61	157	62	158	63	159	64	160	65	161	66	162	67	163	68	164	69	165	70	166	71	167	72	168
L	253	349	254	350	255	351	256	352	257	353	258	354	259	355	260	356	261	357	262	358	263	359	264	360
M	73	169	74	170	75	171	76	172	77	173	78	174	79	175	80	176	81	177	82	178	83	179	84	180
N	265	361	266	362	267	363	268	364	269	365	270	366	271	367	272	368	273	369	274	370	275	371	276	372
O	85	181	86	182	87	183	88	184	89	185	90	186	91	187	92	188	93	189	94	190	95	191	96	192
P	277	373	278	374	279	375	280	376	281	377	282	378	283	379	284	380	285	381	286	382	287	383	288	384

END OF APPENDIX