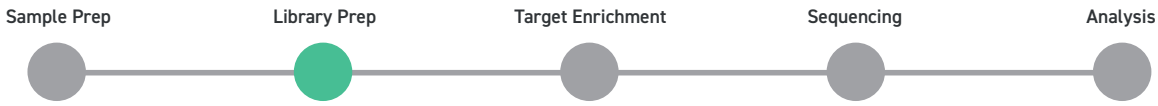
# RNA Library Preparation with Twist Universal Adapter System

## 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 fragmentation and 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 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**<br>(FOR RNA LIBRARY PREPARATION) | | | |
| 107060 (16 rxn)<br>107061 (96 rxn) | Twist RNA Library Prep Kit | · FFPE Repair Buffer<br>· Fragmentation/Prime Buffer<br>· RT-Enzyme<br>· RT-Buffer<br>· Second Strand Enzyme<br>· Second Strand Buffer<br>· Ligation Enzyme<br>· Ligation Buffer<br>· P5/P7 Primer Mix (10x)<br>· 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**<br>(ORDERED SEPARATELY) | | | |
| 101307 (16 rxn) | Twist Universal Adapter System TruSeq Compatible, 16 samples | · Twist Universal Adapters<br>· 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<br><br>106401-106408: 384 rxn, 384-well plate | Twist HT Universal Adapter System TruSeq Compatible | · Twist Universal Adapters<br>· Twist HT UDI Primers | –20°C |

# LEGAL

This document may contain references to other third-party resources such as sources of information, hardware or software, products, or services and/or web sites owned or licensed by third parties. Twist Bioscience does not control or take responsibility for any third-party resources, including, without limitation, the accuracy, reliability, copyright compliance, compatibility, performance, legality, or any other aspect of third-party resources. The inclusion of such resources in this document does not imply endorsement by Twist Bioscience of any third-party resources.

Certain processes described in this document may be subject to patent rights or licenses in local jurisdictions, including those owned or licensed by parties other than Twist Bioscience. Purchase of this product does not include a license to perform any such processes. Users of this product may, therefore, be required to obtain a patent license depending upon the particular application and country in which the product is used before performing such processes.

Twist Bioscience, the various Twist logos, and the Twist NGS logo used herein are trademarks of Twist Bioscience Corporation. All other trademarks are the property of their respective owners.



# 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 |
| 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 UNIVERSAL ADAPTERS | TIME |
|---|---|---|
| STEP 1 | **FFPE Repair**<br>Heat-treated sample(s) | **35 minutes** |
| | **Fragmentation, cDNA Synthesis, End Repair, and dA-Tailing**<br>dA-tailed cDNA fragments | **1 hour 25 minutes** |
| STEP 2 | **Ligate Adapters and Purify**<br>cDNA libraries | **55 minutes to 1 hour 25 minutes** |
| STEP 3 | **Library Amplification and Strand Selection**<br>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 DNA 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 C 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 Universal 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 UNIVERSAL ADAPTERS AND PURIFY**

# STEP 2 LIGATE TWIST UNIVERSAL ADAPTERS AND PURIFY

Ligate Twist Universal 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 (HT) Universal Adapter System:
  - Twist Universal Adapters (tube; utilized for all samples)

**Before You Begin**

- Thaw or place on ice:
  - Ligation Buffer
  - Ligation Enzyme
  - Twist Universal 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 UNIVERSAL 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 | |
|---|---|---|---|---|
| | UNIVERSAL ADAPTER VOLUME | 10 mM TRIS-HCL, pH 8.0 VOLUME | UNIVERSAL ADAPTER VOLUME | 10 mM TRIS-HCL, pH 8.0 VOLUME |
| 1 ng | 0.25 µl | 4.75 µl | 0.18 µl | 4.82 µl |
| 10 ng | 2 µl | 3 µl | 1 µl | 4 µl |
| 100 ng | 5 µl | — | 1 µl | 4 µl |
| 1000 ng | 5 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)
- 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 (HT) 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 Primers or Twist HT 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 | 10 - 11 | 12 - 13 |
| 1 ng | 14 - 15 | 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 or Twist HT UDI Primer from the provided 96-well or 384-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 or B depending on the adapter system being used.

**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.

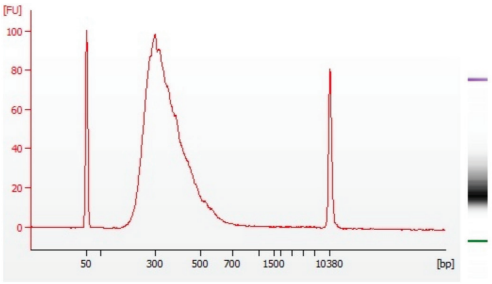
**3.17** Incubate at room temperature for 2 minutes.

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

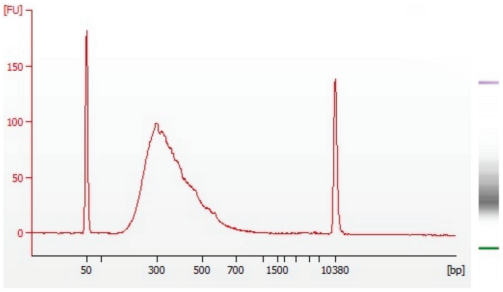
**3.19** Transfer 20 µl of the clear supernatant containing the Amplified Indexed Libraries to a clean thin-walled PCR 0.2-ml strip-tube or well of a 96-well thermal cycling plate, making sure not to disturb the bead pellet.

## 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 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 |

# APPENDIX C: 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 |

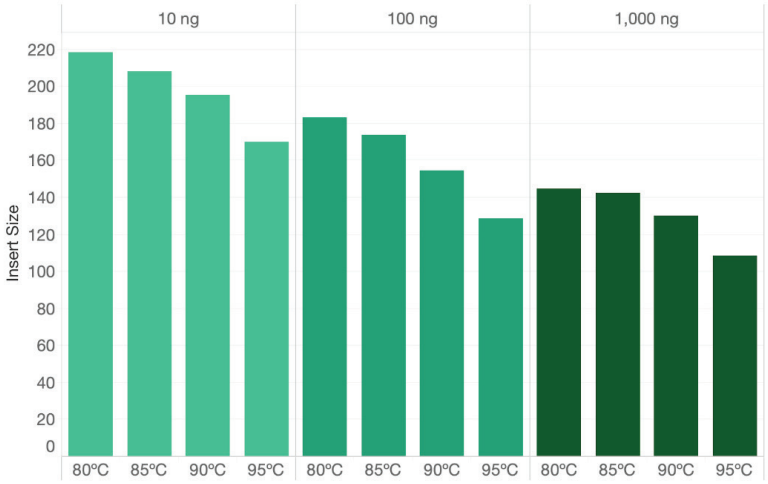



***Figure 1. Insert Size of RNA Libraries.*** *10, 100, or 1000 ng of high-quality RNA were taken through the the Twist RNA Library Preparation with Twist Universal Adapter System Protocol. The fragmentation incubation temperature in Step 1.2 was set between 80°C and 95°C to modulate RNA library insert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