Twist Total Nucleic Acids Library Preparation EF Kit 2.0 for Viral Pathogen Detection and Characterization

For use with the Twist NGS Workflow

Viral pathogens come in all four types of genetic material: ssRNA, dsRNA, ssDNA and dsDNA. To address this diversity, Twist developed a single workflow that can be used to generate high quality sequencing libraries from any of these four types in parallel, enabling downstream Next Generation Sequencing (NGS) applications, including metagenomics and target enrichment. The Twist Total Nucleic Acids Library Preparation EF Kit 2.0 for Viral Pathogen Detection and Characterization decreases cost and turn around time by consolidating multiple library preparation methods into a simple one-size-fits-all protocol. The protocol begins with denaturation and reverse transcription using random primers, followed by second strand synthesis with DNA polymerase I and *E. Coli* DNA ligase. The resulting mixture of dsDNA and cDNA is then converted into Illumina TruSeq-compatible libraries using the Twist Library Preparation EF Kit 2.0 with Enzymatic Fragmentation and Unique Dual Indices (UDI). Libraries created with this method are compatible with Twist Target Enrichment Protocols and customizable viral detection panels. Synthetic viral RNA or DNA controls can be spiked into samples to serve as positive controls and to assess sensitivity.

Twist NGS workflow. The complete NGS workflow takes you from sample preparation to NGS sequencing and data analysis. A component of this workflow, works in conjunction with the other component protocols.

*This product is for **research use only**.*

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
104206: 16 rxn 104207: 96 rxn	Twist Library Preparation EF Kit 2.0	Reagents for library construction	
	Twist Library Preparation EF Kit 1, 2.0	Frag/AT Enzymes Frag/AT Buffer Ligation Master Mix Equinox Library Amp Mix (2x) P5/P7 Primers (10x)	–25 to –15°C
	Twist Library Preparation Kit 2	DNA Purification Beads	2 to 8°C
100401: 16 rxn 100573: 96 rxn	Twist Library Preparation Kit 2	DNA Purification Beads (as a Standalone Product, Bead Purification is also needed during cDNA Synthesis)	2 to 8°C
101307: 16 rxn 101308, 101309, 101310, 101311: 96 rxn	Twist Universal Adapter System - TruSeq Compatible	Twist Universal Adapters and Twist UDI Primers, provides unique dual-indexed combinations with 1 reaction per index pair	–25 to –15°C

Optional workflow components like Twist Synthetic Viral Controls can be found in Appendix C.

LEGAL

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MATERIALS SUPPLIED BY USER

The following materials or their equivalent are required to generate libraries using the Twist Library Preparation EF Kit 2.0 for Virus Detection.

PRODUCT	SUGGESTED SUPPLIER
ProtoScript II First Strand cDNA Synthesis Kit	New England Biolabs (E6560L sufficient for 60 samples)
NEBNext Ultra II Non-Directional RNA Second Strand Synthesis Module	New England Biolabs (E6111)
Random Primer 6	New England Biolabs (S1230S)
Gene Expression Universal Reference RNA (Human)	Agilent Technologies (740000-41)
Ethanol (200 proof)	—
Molecular biology grade water	—
10 mM Tris-HCl pH 8 (optional)	—
Buffer EB (optional)	Qiagen
1.5-ml microcentrifuge tubes	VWR
Thin-walled PCR 0.2-ml strip-tubes	Eppendorf
96-well thermal cycling plates	VWR
96-well compatible magnetic plate	Alpaqua, Permagen Labware
Qubit RNA Broad Range Quantitation Assay	Thermo Fisher Scientific
Qubit dsDNA Broad Range Quantitation Assay	Thermo Fisher Scientific
Qubit dsDNA High Sensitivity Quantitation Assay	Thermo Fisher Scientific
Agilent DNA 7500 Kit	Agilent Technologies

EQUIPMENT TO BE SUPPLIED BY USER

The following equipment, or their equivalent, are required to generate libraries using the Twist Library Preparation EF Kit 2.0 for Virus Detection.

PRODUCT	SUGGESTED SUPPLIER
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	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 instructions provided. Twist cannot guarantee performance if modifications are made to the protocol.

This library preparation method may yield more material than needed for target enrichment. Excess product can be stored at –20°C for later use.

Test the compatibility of your thermal cycler and PCR tubes by incubating 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.

IMPORTANT NOTES

Store all RNA samples at –80°C when not in use. Keep on ice at all times when in use.

Input RNA or DNA should be suspended in Molecular Biology Grade Water, 10 mM Tris-HCl pH 8.0, or Buffer EB.

The recommended RNA input is 50 ng.

If starting from cDNA, it is important to remove all cations and chelators as these may affect the initial fragmentation reaction.

For complementary DNA (cDNA) samples, correct input quantity is critical for achieving optimal yield and library fragment length.

To accomodate research samples with low viral titer, we recommend using 2.5X reactions of ProtoScript II First Strand cDNA Synthesis Kit compared to what NEB recommends using.

The recommended DNA input is 25 ng of purified cDNA.

Using higher cDNA input will increase library diversity and reduce duplication rate, but optimization of the following steps in library preparation may still be required to achieve optimal performance.

1. Incubation time for fragmentation (Step 3.1, page 13)
2. Amount of Twist Universal Adapter (Step 4.1, page 15)
3. Incubation time for ligation reaction (Step 4.5, page 16)

Measuring DNA concentration by absorbance at 260 nm is not recommended. Use the Thermo Fisher Scientific Qubit dsDNA Broad Range Quantitation Assay to accurately quantify input purified cDNA.

For technical support, contact customersupport@twistbioscience.com.

PROTOCOL OVERVIEW

This protocol can be applied to ssRNA, dsRNA, ssDNA or dsDNA, and generates double stranded cDNA from RNA and dsDNA from ssDNA. This mixture can then be used to make libraries using enzymatic fragmentation and Twist Universal Adapters with UDI primers. This protocol allows you to perform cDNA synthesis and library preparation (Steps 1–5) in 5 hours.

	HUMAN GENOMIC SAMPLES, HUMAN GENOMIC REFERENCE RNAS, AND TWIST SYNTHETIC CONTROLS	TIME
STEP 1	**Dilution of Nucleic Acid Samples and optional Synthetic Controls** Diluted Experimental Samples and Synthetic Controls	**10 minutes**
STEP 2	**Execute cDNA Synthesis and Purify** cDNA material for Library Preparation	**2.75 hours**
	cDNA	
STEP 3	**Perform DNA Fragmentation, End Repair, and dA-Tailing** dA-Tailed DNA Fragments	**1 hour**
STEP 4	**Ligate Twist Universal Adapters and Purify** cDNA Libraries with Universal Adapters	**1 hour**
STEP 5	**PCR Amplify Using Twist UDI Primers, Purify, and Perform QC** Amplified Indexed Libraries STOPPING POINT	**1 hour**

USER NOTE: This protocol is compatible with all starting nucleic acid types: ssRNA, dsRNA, ssDNA and dsDNA. Prior to first strand cDNA synthesis this protocol excludes DNAse treatment and contains a denaturation step to be inclusive of all double stranded or single stranded nucleic acid species.

STEP 1 DILUTION OF EXPERIMENTAL SAMPLES AND SYNTHETIC CONTROLS

Reagents Required

- Experimental Samples: 50 ng RNA per sample
- Gene Expression Universal Reference RNA (Human)
- Twist Synthetic Controls
- Molecular Biology Grade Water

Before You Begin

Thaw on ice:

- Experimental Samples
- Gene Expression Universal Reference RNA (Human)
- Twist Synthetic Controls

DILUTE THE SAMPLES

1.1 Dilute the Experimental Samples to 3.3 ng/µl in Molecular Biology Grade Water.

1.2 Dilute the Gene Expression Universal Reference RNA (Human) to 3.85 ng/µl.

OPTIONAL: If using Twist Synthetic Controls, we recommend diluting to the following concentrations:

- $0.5x10^6$ copies/µl
- $0.5x10^3$ copies/µl
- $0.5x10^2$ copies/µl
- $0.5x10^1$ copies/µl
- Negative Control (Molecular Biology Grade Water only)

Add 2 µl of the diluted Twist Synthetic Control to 13 µL of diluted Gene Expression Universal Reference RNA (Human) from step 1.2.

NOTE: Dilute and add the Twist Synthetic Controls regardless of its starting nucleic acid species. All nucleic acids can be used as starting input.

PROCEED IMMEDIATELY TO STEP 2: EXECUTE cDNA SYNTHESIS AND PURIFY

STEP 2 EXECUTE cDNA SYNTHESIS AND PURIFY

Generate cDNA from diluted Experimental Samples and (optional) Synthetic Controls.

Reagents Required

- Diluted Experimental Samples and Synthetic Controls (from Steps 1.1 and 1.2 above)
- Random Primer 6 (resuspended in water at 50uM)
- ProtoScript II First Strand cDNA Synthesis Kit
- NEBNext Ultra II Non-Directional RNA Second Strand Synthesis Module
- Molecular Biology Grade Water
- From the Twist Library Preparation Kit 2:
  - DNA Purification Beads

Before You Begin

- Thaw on ice:
  - ProtoScript II First Strand cDNA Synthesis Buffers and Reagents
  - NEBNext Ultra II Non-Directional RNA Second Strand Synthesis Buffers and Reagents
- Prepare 2 ml 80% ethanol for each sample (for use in Steps 2, 4 and 5 of the protocol).
- Equilibrate DNA Purification Beads to room temperature for at least 30 minutes (for use in Steps 2, 4 and 5 of the protocol).
- Resuspend the Random Primer 6 reagent, with Molecular Biology Grade Water, to 50 µM.

PREPARE PRIMER ANNEALING SOLUTION

2.1 Add the following volumes of reagents to each reaction tube (either a 0.2-ml thin walled PCR strip-tube or 96-well plate). Mix by flicking the tube(s).

REAGENT	VOLUME
Random Primer 6	5 µl
Diluted Experimental Samples or Synthetic Controls	15 µl
Total	20 µl

2.2 Pulse-spin the tube(s) and ensure there are minimal bubbles present.

2.3 Heat the Primer Annealing Solution to 95°C for 5 minutes in a thermal cycler with the lid at 105°C, then ramp down to 20°C. Immediately place on ice. Proceed to the next step.

PREPARE FIRST STRAND SYNTHESIS SOLUTION

2.4 Add the following volumes of reagents to each tube of Primer Annealing Solution. Mix by flicking the tube(s).

REAGENT	VOLUME
ProtoScript II Reaction Mix	25 µl
ProtoScript II Enzyme Mix	5 µl

**Prepare a master mix for multiple reactions.*

2.5 Incubate the First Strand Synthesis Solution at the following conditions: 25°C for 5 minutes, 42°C for 1 hour, 80°C for 5 minutes, 4°C hold. Set the temperature of the heated lid to 105°C. When incubation is finished, proceed to the next step.

PREPARE SECOND STRAND SYNTHESIS SOLUTION

2.6 Add the following volumes of reagents to each tube of First Strand Synthesis Solution. Mix by flicking the tube(s).

REAGENT	VOLUME
Molecular Biology Grade Water	18 µl
NEBNext Second Strand Synthesis Reaction Buffer	8 µl
NEBNext Second Strand Synthesis Enzyme Mix	4 µl

2.7 Incubate the Second Strand Synthesis Solution at 16°C for 1 hour in the thermal cycler, then move the samples to the bench top. Proceed to the Purify step.

⚠ IMPORTANT: Turn off the heated lid or set to minimum temperature.

PURIFY

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

2.9 Add 96 µl of homogenized (1.2x) DNA Purification Beads to each Second Strand Synthesis Solution from Step 2.7. Mix well by vortexing.

2.10 Incubate the samples for 5 minutes at room temperature.

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

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

2.13 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.

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

2.15 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.

2.16 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.

2.17 Remove the plate or tube(s) from the magnetic plate and add 27 µl water to each sample. Mix by pipetting until homogenized.

2.18 Incubate at room temperature for 2 minutes.

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

2.20 Transfer 25 µl of the clear supernatant containing the DNA and/or cDNA Pool 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.

OPTIONAL: To perform a QC step, use the Thermo Fisher Scientific Qubit dsDNA High Sensitivity Quantification Assay.

NOTE: Final concentrations below 1 ng/µl may reflect inefficient sample preparation and can result in low library diversity after hybridization.

PROCEED TO STEP 3: PERFORM DNA FRAGMENTATION, END REPAIR, AND dA-TAILING

STOPPING POINT: If not immediately proceeding to Step 3, store the DNA and/or cDNA Pool at –20°C.

STEP 3 PERFORM DNA FRAGMENTATION, END REPAIR, AND dA-TAILING

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

Reagents Required

- DNA and/or cDNA Pool (from Steps 2.20 above)
- Molecular biology grade water
- Qubit dsDNA Broad Range Quantitation Assay (or equivalent)
- From the Twist Library Preparation EF Kit 1, 2.0:
  - Frag/AT Enzymes
  - Frag/AT Buffer

Before You Begin

- Thaw or place on ice:
  - Molecular biology grade water
  - DNA and/or cDNA Pool
  - Frag/AT Buffer
  - Frag/AT Enzymes

PREPARE THE THERMAL CYCLER, SAMPLES, AND REAGENTS

3.1

Program the thermal cycler with the following conditions. Use the Step 2 Incubation Time table to select conditions for fragmentation to achieve the desired insert size. Set the temperature of the heated lid to 105°C. Start the program to pre-chill the thermal cycler.

	TEMPERATURE	TIME
STEP 1	4°C	HOLD
STEP 2	*Use the table to the right to select the time and temperature for desired insert size*	
STEP 3	65°C	30 minutes
STEP 4	4°C	HOLD

STEP 2 INCUBATION TIME*		
DESIRED INSERT SIZE (BP)	@30°C	@37°C
145–175	–	30 min
180–220	–	20 min
250–300	–	10 min
275–350	15 min	–
350–425	10 min	–

**20 min at 37°C is recommended for Twist target enrichment applications. All recommendations are a starting point and should be optimized for each application.*

3.2

Add 25 µl of each DNA and/or cDNA Pool into either a thin-walled PCR 0.2-ml striptube or a well of a 96-well thermal cycling plate.

OPTIONAL: If QC step is performed after Step 2.20, dilute the DNA and/or cDNA Pools to 1 ng/µl with water before aliquoting.

NOTE: Further fragmentation time optimization may be needed if a QC step is not performed.

3.3

Pulse-spin to ensure all of the solution is at the bottom of the tube and place on ice.

PERFORM FRAGMENTATION, END REPAIR, AND dA-TAILING

3.4 Vortex the Frag/AT Buffer for 5 seconds. Pulse-spin to collect all liquid in the bottom of the tube.

3.5 Invert Frag/AT Enzymes a minimum of 10 times to homogenize or briefly vortex to ensure complete mixing. Pulse-spin to collect all liquid in the bottom of the tube.

3.6 Prepare an enzymatic fragmentation mix in a 1.5 ml microfuge tube on ice. Use the volumes listed below. Homogenize the mastermix with moderate vortexing for 5 seconds or pipetting a minimum of half the total volume up and down 10 times (avoid formation of bubbles).

REAGENT	VOLUME PER REACTION*
Molecular Biology Grade Water	15 µl
Frag/AT Buffer	4 µl
Frag/AT Enzymes	6 µl
Total	25 µl

**Prepare a master mix for multiple reactions.*

3.7 Add 25 µl enzymatic fragmentation mix (from Step 3.3) to each 25 µl sample well or tube. 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). Cap the tube(s) or seal the plate and keep the reaction on ice.

NOTE: Complete mixing is critical to achieve consistent fragment lengths.

3.8 Pulse-spin the sample plate or tube(s) and immediately transfer to the pre-chilled thermal cycler.

3.9 Initiate steps 2 to 4 of the thermal cycler program (refer to the table in Step 3.1 above).

NOTE: While the thermal cycler program is running, prepare the reagents for Step 4: Ligate Twist Universal Adapters and Purify (see Before You Begin).

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

PROCEED IMMEDIATELY TO STEP 4: LIGATE TWIST UNIVERSAL ADAPTERS AND PURIFY

STEP 4 LIGATE TWIST UNIVERSAL ADAPTERS AND PURIFY

Ligate Twist Universal Adapters to the dA-tailed DNA fragments from Step 3 and purify to generate DNA libraries ready for index introduction through amplification in Step 5.

Reagents Required

- dA-tailed DNA fragments (from Step 3.10)
- 80% Ethanol (from Step 2)
- Equilibrated DNA Purification Beads (from Step 2)
- Molecular biology grade water
- 10 mM Tris-HCl pH 8 or Buffer EB (optional, for elution)
- From the Twist Library Preparation EF Kit 1, 2.0:
  - Ligation Master Mix
- From the Twist Universal Adapter System:
  - Twist Universal Adapters
- From the Twist Library Preparation Kit 2:
  - DNA Purification Beads

Before You Begin

- Thaw or place on ice:
  - Twist Universal Adapters (tube; utilized for all samples)
  - Ligation Master Mix
- Program a thermal cycler to incubate samples at 20°C with the heated lid set to minimum temperature or turned off. Start the program so that the cycler has reached 20°C when the samples are done being prepared.

LIGATE TWIST UNIVERSAL ADAPTERS

4.1 Add 2.5 µl of Twist Universal Adapters into each sample well or tube containing the dA-tailed DNA fragments from Step 3.10. Mix gently by pipetting and keep on ice.

4.2 Invert the Ligation Master Mix a minimum of 10 times until homogenized and place on ice.

NOTE: Do not vortex the Ligation Master Mix.

4.3 Add 2.5 µl of Molecular Biology Grade Water and 20 µl of Ligation Master Mix to each sample from Step 4.1. Pipette a minimum of half the total volume up and down 10 times to ensure complete mixing. Seal or cap the sample plate or tube(s) and pulse-spin to ensure all solution is at the bottom of the tube.

4.4 Incubate the ligation reaction at 20°C for 15 minutes in the thermal cycler, then move the samples to the bench top. Proceed to the Purify step.

⚠ IMPORTANT: Turn off the heated lid or set to minimum temperature.

NOTE: While the thermal cycler program is running, prepare the reagents for Step 5: PCR Amplify Using Twist UDI Primers, Purify, and Perform QC (see Before You Begin).

PURIFY

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

4.6 Add 60 µl of homogenized (0.8x) DNA Purification Beads to each ligation sample from Step 4.4. Mix well by vortexing.

4.7 Incubate the samples for 5 minutes at room temperature.

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

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

4.10 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.

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

4.12 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.

4.13 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.

4.14 Remove the plate or tube(s) from the magnetic plate and add 17 µl water to each sample. Mix by pipetting until homogenized.

NOTE: 10 mM Tris-HCl pH 8 or Buffer EB may also be utilized for elution.

4.15 Incubate at room temperature for 2 minutes.

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

4.17 Transfer 15 µl of the clear supernatant containing the ligated and 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.

PROCEED TO STEP 5: PCR AMPLIFY USING TWIST UDI PRIMERS, PURIFY, AND PERFORM QC

STEP 5 PCR AMPLIFY USING TWIST UDI PRIMERS, PURIFY, AND PERFORM QC

Complete the protocol by amplifying the adapted libraries with Twist UDI Primers, purifying them, and performing QC analysis.

Reagents Required

- Ligated, adapted libraries (from Step 4.17)
- 80% Ethanol (from Step 4)
- Equilibrated DNA Purification Beads (from Step 4)
- Molecular biology grade water
- 10 mM Tris-HCl pH 8 or Buffer EB (optional, for elution)
- From the Twist Library Preparation EF Kit 1, 2.0
  - Equinox Library Amp Mix (2x)
- From the Twist Universal Adapter System:
  - Twist UDI Primers

⚠ IMPORTANT: Use of P5/P7 Primers (10x) tubes 104127, 103842 contained in the Twist Library Preparation EF Kit 1, 2.0 is not required. Using these primers with the Twist Universal Adapter System will result in a failed PCR amplification.

Before You Begin

- Thaw or place on ice:
  - Equinox Library Amp Mix (2x)
  - Twist UDI Primers (plate with single-use primers)

PREPARE THE THERMAL CYCLER AND PCR MIX

5.1

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	8–12*
	Annealing	60°C	30 seconds	
	Extension	72°C	30 seconds	
3	Final Extension	72°C	1 minute	1
4	Final Hold	4°C	HOLD	–

**Number of cycles will depend on the integrity and purity of starting cDNA.*

PERFORM PCR

5.2 Add 10 µl of Twist UDI Primer from the provided 96-well plate to each of the cDNA libraries from Step 4.17 and mix well by gentle pipetting.

NOTE: For index selection and pooling guidelines for downstream target enrichment and sequencing, refer to the Appendix.

5.3 Add 25 µl of Equinox Library Amp Mix (2x) to the cDNA libraries from Step 5.2 and mix well by gentle pipetting.

NOTE: Invert Equinox Library Amp Mix (2x) 5 times before use. Do not vortex.

5.4 Pulse-spin sample plate or tube and immediately transfer to the thermal cycler. Start the program.

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

PURIFY

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

5.7 Add 50 µl (1x) of homogenized DNA Purification Beads to each ligation sample from Step 5.5. Mix well by vortexing.

5.8 Incubate the samples for 5 minutes at room temperature.

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

5.10 The DNA Purification Beads form a pellet, leaving a clear supernatant. With plate or tubes still on the magnetic plate, remove and discard the supernatant.

5.11 Gently wash the bead pellet by adding 200 µl of freshly prepared 80% ethanol (do not disturb the pellet), incubate for 1 minute, then remove and discard the ethanol.

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

5.13 Carefully remove all remaining ethanol with a 10 µl pipet, making sure not to disturb the pellet.

5.14 Air-dry the pellet on the magnetic plate for 5–10 minutes or until it is dry. Do not overdry.

5.15 Remove the plate or tubes from the magnetic plate and add 22 µl of water, 10 mM Tris-HCl pH 8, or Buffer EB to each sample. Mix by pipetting until homogenized.

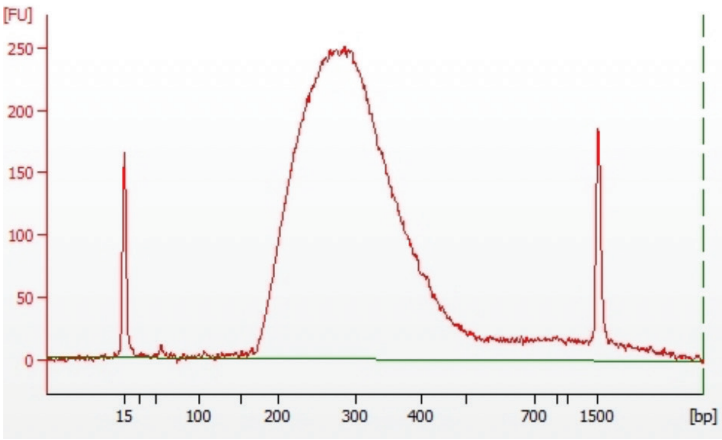
5.16 Incubate at room temperature for 2 minutes.

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

5.18 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 pellet.

PERFORM QC

5.19 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. Average fragment length should be 250–450 bp using a range setting of 150–1000 bp.



Electropherogram generated by an Agilent 7500 DNA analysis of cDNA library samples that were prepared as described. Note the single prominent peak.

NOTES: If the average fragment length is not within the desired range, optimize the fragmentation conditions used in Step 3.1. Test a range of fragmentation times in 3-5 minute increments around the target size from the Step 2 Incubation Time table. Increase time to produce shorter fragments and decrease time to produce longer fragments.

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

END OF WORKFLOW

APPENDIX A: ADAPTER SEQUENCES AND POOLING GUIDELINES

UDI SEQUENCES

For a complete guide of the Twist UDI sequences please refer to PDF document DOC-001129 or Excel file DOC-001130. Both files are available for download at twistbioscience.com/resources.

POOLING GUIDELINES

Twist UDI primers are base balanced by column for next generation sequencing. 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										

*PLEASE NOTE: The indexes in the 16-sample plate are not the same for 96 samples (Plate A).

APPENDIX A: ADAPTER 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

END OF APPENDIX A

APPENDIX B: TWIST TARGET ENRICHMENT PROTOCOL

The Twist Target Enrichment Protocol is compatible with this workflow. It generates enriched TruSeq DNA libraries for sequencing on Illumina next-generation sequencing (NGS) systems using a 16-hour hybridization time in a two-day target enrichment workflow. This can be found at twistbioscience.com/sites/default/files/resources/2019-11/Protocol_NGS_HybridizationTE_31OCT19_Rev1.pdf.

When using the Twist SARS-CoV-2 Research Panel or the Twist Respiratory Virus Research Panel in conjunction with this capture workflow, there are no modifications. Both panels are designed for the detection, characterization, and environmental surveillance of viral pathogens. The SARS-CoV-2 Research Panel specifically targets SARS-CoV-2—the virus responsible for the COVID-19 pandemic. The Twist Respiratory Virus Research Panel is more broad, targeting several viral genomes known to cause respiratory illnesses in humans.

CATALOG #	NAME	DESCRIPTION	STORAGE
102016: 2 rxn 102017: 12 rxn 102018: 96 rxn	Twist SARS-CoV-2 Research Panel	Custom DNA Panel for SARS-CoV-2 Viral Detection	–25 to –15°C
103066: 2 rxn 103067: 12 rxn 103068: 96 rxn	Twist Respiratory Virus Research Panel & One Codex Software	Custom DNA Panel for Respiratory Viral Detection & One Codex software analysis credits	–25 to –15°C
More information at twistbioscience.com/products/ngs	Custom Viral Detection Panel	E.g. Specific Viral Genome Research Panel, etc.	–25 to –15°C
100856: 2 rxn 100578: 12 rxn 100767: 96 rxn	Twist Universal Blockers	For the prevention of nonspecific capture: Universal Blockers Blocker Solution	–25 to –15°C
101262: 2 rxn 100983: 12 rxn 100984: 96 rxn	Twist Binding and Purification Beads	For target enrichment and purification: Streptavidin Binding Beads DNA Purification Beads	2 to 8°C
101279: 2 rxn 101025: 12 rxn 101026: 96 rxn	Twist Hybridization and Wash Kit (2 Boxes) Twist Hybridization Reagents (Box 1 of 2) Twist Wash Buffers (Box 2 of 2)	For target enrichment with standard hybridization: Hybridization Mix Hybridization Enhancer Amplification Primers Binding Buffer Wash Buffer 1 Wash Buffer 2	 –25 to –15°C 2 to 8°C

END OF APPENDIX B

APPENDIX C: TWIST VIRAL SYNTHETIC TOOLS

Each viral control is made synthetically with Twist's silicon platform and *in vitro* transcription. Each control is broken up between different lengths, depending on the viral genome. For a full list of available viral controls, please visit: twistbioscience.com/resources/product-sheet/Twist-Synthetic-Infectious-Disease-Controls-Product-List

END OF APPENDIX C

LAST REVISED: November 1, 2022

REVISION	DATE	DESCRIPTION
4.0	Nov 1, 2022	• Language changes to clarify reagent handling, required inputs, and steps • Updated insert size optimization guidelines • Removed table in Appendix C and provided website link with updated synthetic controls list
3.0	Feb 9, 2022	• Minor language update to clarify steps
2.0	Oct 7, 2021	• Updated protocol to use with Twist Library Preparation EF Kit 2.0 • Minor language changes