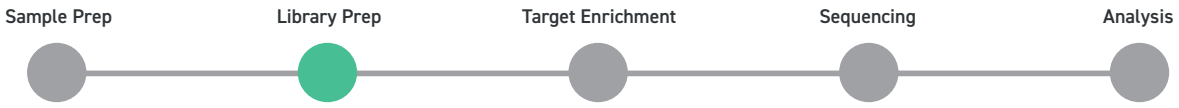
# Library Preparation EF 2.0 with Enzymatic Fragmentation and Twist Universal Adapter System

## For use with the Twist NGS Workflow

This Twist Library Preparation Kit provides the reagents needed to prepare genomic DNA (gDNA) libraries using enzymatic gDNA 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 Twist HT Universal Adapter System, which consists of Twist Universal Adapters and Twist HT UDI Primers. This manual details the steps for generating the amplified, indexed libraries needed for downstream target enrichment and sequencing on Illumina next-generation sequencing (NGS) systems. This library preparation 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 complete NGS workflow takes you from sample preparation to NGS sequencing and data analysis. A component of this workflow, the Twist Library Preparation Protocol works in conjunction with the other component protocols.

***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 |
|---|---|---|---|
| 104206: 16 rxn<br>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<br>· Frag/AT Buffer<br>· Ligation Master Mix<br>· Equinox Library Amp Mix (2x)<br>· P5/P7 Primers (10x) | –20°C |
| | Twist Library Preparation Kit 2 | DNA Purification Beads | 2–8°C |
| 101307: 16 rxn<br>101308, 101309, 101310, 101311: 96 rxn | Twist Universal Adapter System - TruSeq Compatible | Twist Universal Adapters and Twist UDI Primers | –20°C |
| 106390-106393, 106396, 106398-106400: 384 rxn, 96-well plate | Twist HT Universal Adapter System - TruSeq Compatible | Twist Universal Adapters and Twist HT UDI Primers, provide dual-indexed combinations with 1 reaction per index pair | –20°C |
| 106401-106408: 384 rxn, 384-well plate | | | |

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# TABLE OF CONTENTS

# MATERIALS SUPPLIED BY USER

The following materials or their equivalent are required to generate libraries using the Twist Library Preparation Kit 2.0 with Enzymatic Fragmentation and Twist Universal Adapter System or Twist HT Universal Adapter System.

| PRODUCT | SUGGESTED SUPPLIER |
|---|---|
| **REAGENTS AND CONSUMABLES** | |
| 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 dsDNA Broad Range Quantitation Assay | Thermo Fisher Scientific |
| Agilent DNA 7500 Kit | Agilent Technologies |
| **EQUIPMENT** | |
| Pipettes and tips | — |
| Vortex mixer | — |
| Benchtop mini centrifuge for 0.2-ml tubes | — |
| Thermomixer for 1.5-ml tubes | Eppendorf |
| 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 instructions provided. Twist cannot guarantee the performance of the Twist Library Preparation Kit using Enzymatic Fragmentation with either the Twist Universal Adapter System or the Twist HT Universal Adapter System if modifications are made to the protocol.

The Twist HT Universal Adapter System can be directly substituted for the Twist Universal Adapter System without any protocol modification. In all instances where UDI Primers are mentioned in this protocol, the HT UDI Primers can be used instead.

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.

# GUIDELINES FOR SAMPLES

## gDNA SAMPLES

- Use the Thermo Fisher Scientific Qubit dsDNA Broad Range Quantitation Assay to accurately quantify input purified gDNA.
- Measuring DNA concentration by absorbance at 260 nm is not recommended.
- Input DNA should be suspended in Molecular Biology Grade Water, 10 mM Tris-HCl pH 8.0, or Buffer EB.
- It is important to remove all cations and chelators from the starting gDNA sample. The presence of cations and chelators may affect the initial fragmentation reaction.
- The recommended DNA input is 50 ng of high quality gDNA.
- Reagents are compatible with mass input of 1 ng to 500 ng, but may require optimization of the following steps in library preparation to achieve optimal performance:
  - Incubation Time for Fragmentation (Step 1.1, page 9)
  - Amount of Twist Universal Adapter (Step 2.1, page 12)
  - Incubation Time for Ligation Reaction (Step 2.4, page 12)
  - PCR cycles for Amplification (Step 3.1, page 14)
- For additional guidance on library preparation optimization, refer to Appendix C.
- For technical support, contact customersupport@twistbioscience.com.

# PROTOCOL OVERVIEW

This protocol begins with genomic DNA (gDNA) and generates amplified, indexed libraries for subsequent target enrichment. It features enzymatic fragmentation and Twist Universal Adapters with UDI or HT UDI Primers. This protocol allows you to perform gDNA library preparation (Steps 1–3) in 3 hours.

| | **ENZYMATIC FRAGMENTATION WITH UNIVERSAL ADAPTERS AND UDI PRIMERS** (GENOMIC DNA, 50 NG STARTING DNA MATERIAL) | **TIME** |
|---|---|---|
| STEP 1 | **Perform DNA Fragmentation, End Repair, and dA-tailing**<br>dA-tailed DNA fragments | **1 hr** |
| STEP 2 | **Ligate Twist Universal Adapters and Purify**<br>gDNA libraries ready for indexing | **1 hr** |
| STEP 3 | **PCR Amplify Using Twist UDI Primers, Purify, and Perform QC**<br>Amplified indexed libraries | **1 hr** |

# STEP 1 PERFORM DNA FRAGMENTATION, END REPAIR, AND dA-TAILING

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

**Reagents Required**

- Genomic DNA (gDNA): 50 ng per sample
- 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
  - gDNA
  - Frag/AT Buffer
  - Frag/AT Enzymes

## PREPARE THE THERMAL CYCLER, SAMPLES, AND REAGENTS

**1.1**

Program the thermal cycler with the following conditions. Use the Step 2 Incubation Time table below 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.

NOTE: Fragmentation temperature (Step 2) does not impact library performance.

| STEP | TEMPERATURE | TIME |
|---|---|---|
| 1 | 4°C | HOLD |
| 2 | *Use the table to the right to select the time and temperature for the desired insert size* | |
| 3 | 65°C | 30 min |
| 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 utilizing 50 ng of high quality gDNA. Additional conditions in the Step 2 Incubation Time table were also derived using high quality gDNA and should be optimized for each sample type/application. See Appendix C for additional guidance.*

**1.2** Mix gDNA by flicking the tube with a finger. Use the Qubit dsDNA Broad Range Quantitation Assay to determine the concentration of your genomic DNA (gDNA) samples.
NOTE: Measuring DNA concentration by absorbance at 260 nm is not recommended.

**1.3** Bring 50 ng of each gDNA sample to a total volume of 40 µl with water, 10 mM Tris-HCl pH 8, or buffer EB. Mix well with gentle pipetting.
NOTE: If a mass input other than 50 ng is used, bring the target mass to a volume of 40 µl.

**1.4** Add 40 µl of each diluted gDNA sample (50 ng total gDNA) into either a thin-walled PCR 0.2-ml strip-tube or a well of a 96-well thermal cycling plate.

**1.5** 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

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

**1.7** 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.

**1.8** 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* |
|---|---|
| Frag/AT Buffer | 4 µl |
| Frag/AT Enzymes | 6 µl |
| **Total** | **10 µl** |

**Prepare a master mix for multiple reactions.*

**1.9** Add 10 µl enzymatic fragmentation mix (from Step 1.8) to each 40 µl gDNA sample tube or well. 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.

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

**1.11** Initiate steps 2 to 4 of the thermal cycler program (refer to the table in Step 1.1).
NOTE: While the thermal cycler program is running, prepare the reagents for Step 2: Ligate Twist Universal Adapters and Purify (see Before You Begin).

**1.12** 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 DNA fragments from Step 1 and purify to generate gDNA libraries ready for index introduction through amplification in Step 3.

**Reagents Required**

- dA-tailed DNA fragments (from Step 1.12)
- Ethanol
- 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 (HT) 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
- Prepare 1 ml 80% ethanol for each 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).
- 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

**2.1** Add 5 µl Twist Universal Adapters into each sample well or tube containing the dA-tailed DNA fragments from Step 1. Mix gently by pipetting and keep on ice.
NOTE: For mass inputs other than 50 ng, refer to Appendix C for guidance on adapter volume.

**2.2** Invert the Ligation Master Mix a minimum of 10 times until homogenized and place on ice.
NOTE: Do not vortex the Ligation Master Mix.

**2.3** Add 20 µl of Ligation Master Mix to each sample from Step 2.1. 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.

**2.4** 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 3: PCR Amplify Using Twist UDI Primers, Purify, and Perform QC (see Before You Begin).

## PURIFY

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

**2.6** Add 60 µl of homogenized (0.8x) DNA Purification Beads to each ligation sample from Step 2.4. Mix well by vortexing.

**2.7** Incubate the samples for 5 minutes at room temperature.

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

**2.9** 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.

**2.10** 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.

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

**2.12** 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.

**2.13** 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.

**2.14** 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.

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

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

**2.17** Transfer 15 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.

**PROCEED TO STEP 3: PCR AMPLIFY USING TWIST UDI PRIMERS, PURIFY, AND PERFORM QC**

# STEP 3 PCR AMPLIFY USING TWIST UDI PRIMERS, PURIFY, AND PERFORM QC

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

**Reagents Required**

- Ligated libraries (from Step 2.17)
- 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
  - Equinox Library Amp 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 104127, 103842 contained in the Twist Library Preparation EF Kit 1, 2.0 is not required. Using these primers with the Twist (HT) 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 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 | 6-8* |
| | Annealing | 60°C | 30 seconds | |
| | Extension | 72°C | 30 seconds | |
| 3 | Final Extension | 72°C | 1 minute | 1 |
| 4 | Final Hold | 4°C | HOLD | – |

**6–8 cycles is recommended for Twist target enrichment workflows when starting with 50 ng high quality gDNA. For mass inputs other than 50 ng, refer to Appendix C for guidance on PCR cycles.*

## PERFORM PCR

**3.2** Add 10 µl of Twist UDI or Twist HT UDI Primer from the provided 96-well or 384-well plate to each of the gDNA libraries from Step 2.17 and mix well by gentle 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** Add 25 µl of Equinox Library Amp Mix (2x) to the gDNA libraries from Step 3.2 and mix well by gentle pipetting.
NOTE: Invert Equinox Library Amp Mix (2x) 5 times before use. Do not vortex.

**3.4** Pulse-spin sample plate or tube(s) and immediately transfer to the thermal cycler. Start the program.

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

## PURIFY

**3.6** Vortex the pre-equilibrated DNA Purification Beads until mixed.

**3.7** Add 50 µl (1x) of homogenized DNA Purification Beads to each ligation sample from Step 3.5. Mix well by vortexing.

**3.8** Incubate the samples for 5 minutes at room temperature.

**3.9** Place the samples on a magnetic plate for 1 minute.

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

**3.11** 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.

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

**3.13** Carefully remove all remaining ethanol with a 10-µl pipet, 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.

**3.14** 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.

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

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

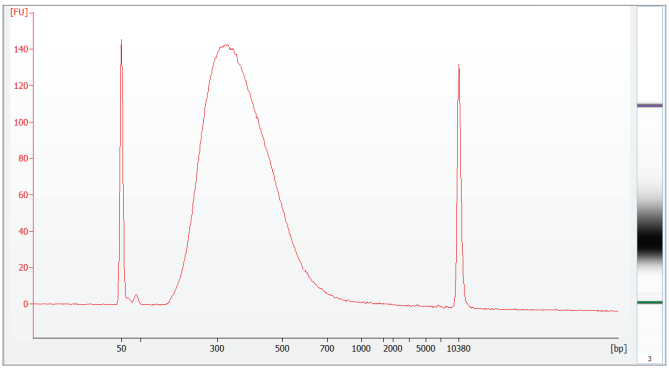
**3.17** Place the plate or tubes on a magnetic plate and let stand for 3 minutes or until the beads form a pellet.

**3.18** 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 bead pellet.

## PERFORM QC

**3.19** 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.

50 ng of high quality gDNA into a 20 minute fragmentation at 37°C and 6 cycles of PCR should result in final concentration values of ≥50 ng/µl. Concentrations below 50 ng/µl may reflect inefficient sample preparation and can result in low library diversity after hybridization. Under these conditions, the average fragment length is typically observed between 350–425 bp using a range setting of 150–1,000 bp.




*Representative electropherogram of a purified library generated with input of 50 ng of high quality gDNA into a 20 minute fragmentation at 37°C and 6 cycles of PCR.*

NOTE: If the average fragment length is not in the desired range, refer to Appendix C for size optimization guidelines.

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 ADAPTER 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-dual-index-sequences-protocol-reference-document-spreadsheet-and-sample.

## POOLING GUIDELINES

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

**Table 1. Twist UDI primer plate layouts and pooling guidelines.**
Twist 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 ADAPTER 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/protocol/high-throughput-unique-dual-index-sequences-datafiles-reference-spreadsheetand-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 LARGER INSERT SIZES AND HANDLING ALTERNATIVE MASS INPUTS

## FRAGMENTATION OPTIMIZATION

Fragmentation rates may vary depending on the quality and type of starting material. The presence of contaminants like cations and chelators in DNA samples can also inhibit the fragmentation reaction. Buffers containing >0.1 mM EDTA will significantly slow the enzymatic fragmentation reaction. In order to achieve optimum performance of the fragmentation reaction, a DNA clean up step may be used to remove contaminants before library preparation.

Begin optimization by selecting the desired size range condition from the tables in Step 1.1. Test a range of fragmentation times around this initial condition by adjusting the incubation time in 3-5 minute increments. Increase time to produce shorter fragments and decrease time to produce longer fragments. Greater control for long insert sizes can be achieved using the 30°C fragmentation temperature. For insert sizes >400 bp, fragmentation time can be reduced below 10 minutes. Additional, finer optimizations for insert size may be carried out if necessary.

## ADAPTER LOADING

For mass inputs other than 50 ng into fragmentation, the volume of Twist Universal Adapters added to ligation in Step 2.1 can be adjusted. Refer to the table below for guidance and if required, add additional chilled water to reach a total volume of 5 µL.

| MASS INPUT | VOLUME OF TWIST UNIVERSAL ADAPTERS (10 µM) |
|---|---|
| >10 ng | 5 µl |
| 10 ng | 2 µl |
| 1 ng | 1 µl |

## PCR CYCLE NUMBER

For mass inputs into fragmentation other than 50 ng, the number of PCR cycles in Step 3.1 can be adjusted.

| MASS INPUT | PCR CYCLE RECOMMENDATION* |
|---|---|
| 500 ng | 3 cycles |
| 100 ng | 4–6 cycles |
| 50 ng | 6–8 cycles |
| 25 ng | 7–9 cycles |
| 10 ng | 8–10 cycles |
| 1 ng | 11–12 cycles |

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

## END OF APPENDIX

**LAST REVISED:** April 8, 2024

| REVISION | DATE | DESCRIPTION |
|---|---|---|
| 6.0 | April 8, 2024 | • Clarified wording in Step 1.3 |
| 5.0 | Apr 25, 2023 | • Fixed storage temperature typo for the Twist HT Universal Adapter System |
| 4.0 | Feb 24, 2023 | • Updated for compatibility with Twist HT Universal Adapter System |
| 3.0 | Aug 15, 2022 | • Language changes to clarify reagent handling<br>• Update insert size optimization guidelines |
| 2.0 | Sep 27, 2021 | • Minor language change<br>• Added guidelines for broad DNA input range and fragment insert sizes in Appendix |