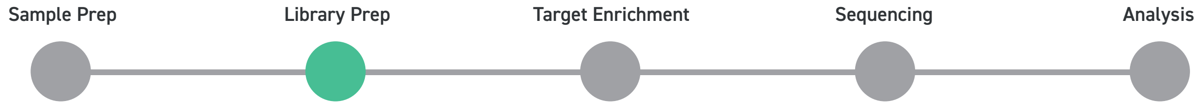
Twist Library Preparation Protocol — Enzymatic Fragmentation and Combinatorial Dual Indices

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 Y-shaped adapters. 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.

PROTOCOL COMPONENTS

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

CATALOG #	NAME	DESCRIPTION	STORAGE
100253: 16 rxn 100572: 96 rxn	Twist Library Preparation EF Kit 1	Reagents for enzymatic DNA fragmentation and adapter ligation: • 5x Fragmentation Enzyme • 10x Fragmentation Buffer • DNA Ligation Mix • DNA Ligation Buffer • Amplification Primers, ILMN	−20°C
100255: 16 rxn 100577: 96 rxn	Twist CD Index Adapter Set	Adapter set, provides dual-indexed combinations, 1 reaction per index	−20°C
100401: 16 rxn 100573: 96 rxn	Twist Library Preparation Kit 2	DNA Purification Beads	2–8°C

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

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TABLE OF CONTENTS

MATERIALS SUPPLIED BY USER

The following materials or their equivalents are required to generate libraries with the Twist Library Preparation Kit Using Enzymatic Fragmentation and Combinatorial Dual Indices.

PRODUCT	SUGGESTED SUPPLIER
REAGENTS AND CONSUMABLES	
Ethanol (200 proof)	—
Molecular biology grade water	—
KAPA HiFi HotStart ReadyMix	Kapa Biosystems
1.5-ml microcentrifuge tubes	VWR
Thin-walled PCR 0.2-ml strip-tubes	Eppendorf
96-well thermal cycling plates	VWR
1.5-ml compatible magnetic stand	Beckman Coulter
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	—
Lab shaker, rocker, rotator	—
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 this Twist Library Preparation Kit 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.

GUIDELINES FOR gDNA SAMPLES

- For genomic (gDNA) samples, correct input quantity is critical for achieving optimal yield and library fragment length.
- The recommended DNA input is 50 ng of purified gDNA, but more gDNA can be used if available.
- Using higher gDNA input will increase library diversity and reduce duplication rate but optimization of the following steps in library preparation may be required to achieve optimal performance.
    - Incubation time for fragmentation at 32°C (Step 1.1, page 7)
    - Amount of Indexed Adapter (Step 2.1, page 9)
    - Incubation time for ligation reaction (Step 2.5, page 10).
- 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.
- 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.
- Input DNA should be suspended in Molecular Biology Grade Water, 10 mM Tris-HCl pH 8.0, or Buffer EB.
- For technical support, contact NGSsupport@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 combinatorial dual index adapters. This protocol allows you to perform gDNA library preparation (Steps 1–3) in less than 3 hours.

	LIBRARY PREPARATION WORKFLOW	TIME
	ENZYMATIC FRAGMENTATION WITH COMBINATORIAL INDICES (GENOMIC DNA, 50 NG STARTING DNA MATERIAL)	
STEP 1	**Perform DNA fragmentation, end repair, and dA-tailing** dA-tailed DNA fragments	**1 hour**
STEP 2	**Ligate combinatorial dual indexed adapters and purify** Indexed gDNA libraries	**1 hour**
STEP 3	**Pre-capture PCR amplify, purify, and perform QC** Amplified indexed libraries	**1 hour**

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:
    - 5x Fragmentation Enzyme
    - 10x Fragmentation Buffer

Before You Begin

- Thaw 5x Fragmentation Enzyme and gDNA samples on ice, then mix by flicking the tube with a finger.
- Thaw 10x Fragmentation Buffer on ice, then mix by pulse vortexing for 2 seconds.

PREPARE THE THERMAL CYCLER, SAMPLES, AND REAGENTS

1.1

Program the thermal cycler with the following conditions. Set the temperature of the heated lid to 70°C. Start the program to pre-chill the thermal cycler.

	TEMPERATURE	TIME
STEP 1	4°C	HOLD
STEP 2	32°C	22 minutes
STEP 3	65°C	30 minutes
STEP 4	4°C	HOLD

1.2

Use the Qubit dsDNA Broad Range Quantitation Assay to determine the concentration of your genomic DNA (gDNA) samples.

1.3

Dilute the gDNA samples to 5 ng/µl with water.

1.4

Add 10 µl of each diluted gDNA sample (50 ng total gDNA) into a thin-walled PCR 0.2-ml strip-tube or well of a 96-well thermal cycling plate, and place on ice.

1.5 Mix the diluted gDNA sample by flicking with a finger, then pulse-spin to ensure all of the solution is at the bottom of the tube.

PERFORM FRAGMENTATION, END REPAIR, AND dA-TAILING

1.6 Prepare an enzymatic fragmentation mix in a 1.5-ml microcentrifuge tube on ice. Use the volumes listed below. Mix well by gentle pipetting.

REAGENT	VOLUME PER REACTION*
Water	25 µl
10x Fragmentation Buffer	5 µl
5x Fragmentation Enzyme	10 µl
Total	40 µl

**Prepare a master mix for multiple reactions.*

1.7 Add 40 µl enzymatic fragmentation mix (from Step 1.6) to each 10 µl gDNA sample well or tube, and mix well by gentle pipetting. Cap the tube and keep the reaction on ice.

1.8 Pulse-spin the sample plate or tubes and immediately transfer to the pre-chilled thermal cycler.

1.9 Initiate steps 2 to 3 of the thermal cycler program (32°C step of the thermocycler program in Step 1.1 above).

NOTE: While the thermal cycler program is running, prepare the reagents for Step 2 Ligate Indexed Adapters and Purify (see Before You Begin).

1.10 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 TO STEP 2: LIGATE INDEXED ADAPTERS AND PURIFY

STEP 2 LIGATE INDEXED ADAPTERS AND PURIFY

Ligate adapters to the dA-tailed DNA fragments from Step 1 and purify to generate indexed gDNA libraries.

Reagents Required

- dA-tailed DNA fragments (from Step 1.10)
- Ethanol
- Molecular biology grade water
- From the Twist Library Preparation EF Kit 1:
  - DNA Ligation Mix
  - DNA Ligation Buffer
- From the Twist CD Index Adapter Set:
  - Indexed Adapters
- From the Twist Library Preparation Kit 2:
  - DNA Purification Beads

Before You Begin

- Thaw on ice:
  - Indexed Adapters
  - DNA Ligation Mix
  - DNA Ligation Buffer
- 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 is at 20°C when the samples are prepared.

LIGATE ADAPTERS

2.1 Add 5.5 µl Indexed Adapters into each sample well or tube containing the dA-tailed DNA fragments from Step 1. Mix gently by pipetting and keep on ice.

NOTES:

- If you are multiplexing eight samples, use the index combinations recommended in the pooling guidelines in the Appendix. To avoid index read failure, do not mix Indexed Adapters from different sets.
- If using other adapter sources, use 5.5 µl of an adapter pair, where each member of the pair is at a concentration of 10 µM (e.g., 5.5 pmol each individual member of the adapter pair; 11 pmol total).

2.2 Prepare a ligation mix in a 1.5-ml microcentrifuge tube on ice as indicated below. Mix well by gentle pipetting.

REAGENT	VOLUME PER REACTION*
Water	14.5 µl
DNA Ligation Buffer	20 µl
DNA LIgation Mix	10 µl
Total	44.5 µl

**Prepare a master mix for multiple reactions.*

2.3 Add 44.5 µl ligation mix to the sample from Step 2.1 and mix well by gentle pipetting.

2.4 Seal or cap the tubes and pulse-spin to ensure all solution is at the bottom of the tube.

2.5 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 (see Step 3: Pre-Capture Amplify, Purify, and Perform QC, Before You Begin).

PURIFY

2.6 Vortex the pre-equilibrated DNA Purification Beads until well mixed.

2.7 Add 80 µl (0.8x) DNA Purification Beads to each ligation sample from Step 2.5. Mix well by vortexing.

2.8 Incubate the samples for 5 minutes at room temperature.

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

2.10 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.

2.11 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.

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

2.13 Carefully remove all remaining ethanol with a 10-µl pipette, making sure not to disturb the bead pellet.

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

2.15 Remove the plate or tubes from the magnetic plate and add 17 µl water to each sample. Mix by pipetting.

2.16 Incubate at room temperature for 2 minutes.

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

2.18 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 3: PRE-CAPTURE PCR AMPLIFY, PURIFY, AND PERFORM QC

STEP 3 PRE-CAPTURE PCR AMPLIFY, PURIFY, AND PERFORM QC

Amplify the indexed gDNA libraries, purify them, and perform quality control (QC) analysis to complete the protocol.

Reagents Required

- Ligated, indexed libraries (from Step 2.18)
- 80% Ethanol (from Step 2)
- Equilibrated DNA Purification Beads (from Step 2)
- Molecular biology grade water
- KAPA HiFi HotStart ReadyMix (or equivalent)
- From the Twist Library Preparation EF Kit 1:
  - Amplification Primers, ILMN

Before You Begin

Thaw on ice:

- Amplification Primers, ILMN
- KAPA HiFi HotStart ReadyMix

PREPARE THE THERMAL CYCLER AND PCR MIX

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	10
	Annealing	60°C	30 seconds	
	Extension	72°C	30 seconds	
3	Final Extension	72°C	1 minute	1
4	Final Hold	4°C	HOLD	–

3.2 Prepare a PCR mix in a 1.5-ml microcentrifuge tube on ice as indicated below. Mix well by gentle pipetting.

REAGENT	VOLUME PER REACTION*
Amplification Primers, ILMN	10 µl
KAPA HiFi HotStart ReadyMix	25 µl
Total	35 µl

**Prepare a master mix for multiple reactions.*

PERFORM PCR

3.3 Add 35 µl PCR mix to the ligated, indexed libraries from Step 2.18 and mix well by gentle pipetting.

3.4 Pulse-spin sample plate or tube 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 the Purify step.

PURIFY

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

3.7 Add 50 µl (1x) 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. Remove and discard the clear supernatant. Do not remove the plate or tubes from the magnetic plate.

3.11 Wash the bead pellet with 200 µl freshly prepared 80% ethanol, 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.

3.14 Air-dry the bead pellet on the magnetic plate for 5–10 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 to each sample. Mix by pipetting until homogenized.

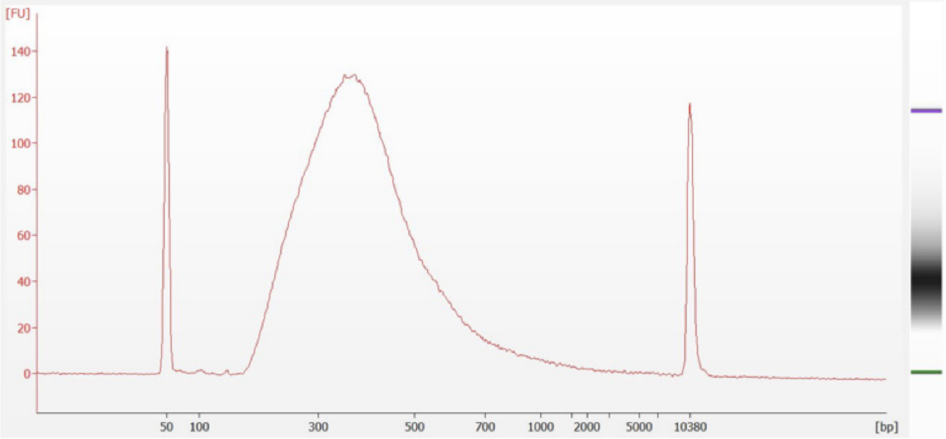
3.16 Incubate at room temperature for 2 minutes.

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. Final concentration values should be ≥80 ng/µl, and average fragment length should be 375–425 bp using a range setting of 150–1,000 bp.



Electropherogram generated by an Agilent 7500 DNA analysis of gDNA library samples that were prepared as described. Note the single prominent peak at ~400 bp.

NOTES: If the average fragment length is not in the 375–425 bp range:

- Input DNA concentration may be inaccurate. Typically, using too much DNA leads to larger fragments, while not using enough DNA leads to shorter fragments.
- The presence of cations and chelators may also affect the average fragment length.
- If neither of the above factors apply optimize the 32°C fragmentation in Step 1.1 by changing the time in 3 minute increments—increase time to produce shorter fragments, and decrease time to produce longer fragments.

You can proceed with concentrations lower than 80 ng/µl, but low concentrations may reflect inefficient sample preparation and can result in low library diversity after hybridization.

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

END OF WORKFLOW

APPENDIX: ADAPTER SEQUENCES AND POOLING GUIDELINES

CD INDEX SEQUENCES

Table 1. Sequences of Compatible Barcodes.

D50X SERIES	i5 BASES FOR SAMPLE SHEET ENTRY (NOVASEQ, MISEQ, HISEQ 2000/2500)	i5 BASES FOR SAMPLE SHEET ENTRY (MINISEQ, NEXTSEQ, HISEQ 3000/4000)	D70X SERIES	i7 BASES FOR SAMPLE SHEET ENTRY
D501	TATAGCCT	AGGCTATA	**D701**	ATTACTCG
D502	ATAGAGGC	GCCTCTAT	**D702**	TCCGGAGA
D503	CCTATCCT	AGGATAGG	**D703**	CGCTCATT
D504	GGCTCTGA	TCAGAGCC	**D704**	GAGATTCC
D505	AGGCGAAG	CTTCGCCT	**D705**	ATTCAGAA
D506	TAATCTTA	TAAGATTA	**D706**	GAATTCGT
D507	CAGGACGT	ACGTCCTG	**D707**	CTGAAGCT
D508	GTACTGAC	GTCAGTAC	**D708**	TAATGCGC
			D709	CGGCTATG
			D710	TCCGCGAA
			D711	TCTCGCGC
			D712	AGCGATAG

Table 2. Adapter Plate Combinatorial Dual Index Layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	501/701	501/702	501/703	501/704	501/705	501/706	501/707	501/708	501/709	501/710	501/711	501/712
B	502/701	502/702	502/703	502/704	502/705	502/706	502/707	502/708	502/709	502/710	502/711	502/712
C	503/701	503/702	503/703	503/704	503/705	503/706	503/707	503/708	503/709	503/710	503/711	503/712
D	504/701	504/702	504/703	504/704	504/705	504/706	504/707	504/708	504/709	504/710	504/711	504/712
E	505/701	505/702	505/703	505/704	505/705	505/706	505/707	505/708	505/709	505/710	505/711	505/712
F	506/701	506/702	506/703	506/704	506/705	506/706	506/707	506/708	506/709	506/710	506/711	506/712
G	507/701	507/702	507/703	507/704	507/705	507/706	507/707	507/708	507/709	507/710	507/711	507/712
H	508/701	508/702	508/703	508/704	508/705	508/706	508/707	508/708	508/709	508/710	508/711	508/712

APPENDIX: ADAPTER SEQUENCES AND POOLING GUIDELINES

POOLING GUIDELINES

When pooling dual-indexed libraries, refer to the Illumina TruSeq pooling guidelines to avoid index read failure during sequencing. Two options for dual-index 8-plex pooling are provided below. For additional multiplexing options, please refer to the Illumina Index Adapters Pooling Guide at support.illumina.com.

Table 3. Dual-Indexed-8-plex, Option 1.

	D701	D702	D703	D704	D705	D706	D707	D708	D709	D710	D711	D712
D501	🟩	🟩	🟩	🟩	🟦	🟦	🟦	🟦	🟩	🟩	🟩	🟩
D502	🟩	🟩	🟩	🟩	🟦	🟦	🟦	🟦	🟩	🟩	🟩	🟩
D503	🟦	🟦	🟦	🟦	🟩	🟩	🟩	🟩	🟦	🟦	🟦	🟦
D504	🟦	🟦	🟦	🟦	🟩	🟩	🟩	🟩	🟦	🟦	🟦	🟦
D505	🟩	🟩	🟩	🟩	🟦	🟦	🟦	🟦	🟩	🟩	🟩	🟩
D506	🟩	🟩	🟩	🟩	🟦	🟦	🟦	🟦	🟩	🟩	🟩	🟩
D507	🟦	🟦	🟦	🟦	🟩	🟩	🟩	🟩	🟦	🟦	🟦	🟦
D508	🟦	🟦	🟦	🟦	🟩	🟩	🟩	🟩	🟦	🟦	🟦	🟦

Table 4. Dual-Indexed-8-plex, Option 2.

	D701	D702	D703	D704	D705	D706	D707	D708	D709	D710	D711	D712
D501	🟩	🟩	🟦	🟦	🟩	🟩	🟦	🟦	🟩	🟩	🟦	🟦
D502	🟩	🟩	🟦	🟦	🟩	🟩	🟦	🟦	🟩	🟩	🟦	🟦
D503	🟩	🟩	🟦	🟦	🟩	🟩	🟦	🟦	🟩	🟩	🟦	🟦
D504	🟩	🟩	🟦	🟦	🟩	🟩	🟦	🟦	🟩	🟩	🟦	🟦
D505	🟦	🟦	🟩	🟩	🟦	🟦	🟩	🟩	🟦	🟦	🟩	🟩
D506	🟦	🟦	🟩	🟩	🟦	🟦	🟩	🟩	🟦	🟦	🟩	🟩
D507	🟦	🟦	🟩	🟩	🟦	🟦	🟩	🟩	🟦	🟦	🟩	🟩
D508	🟦	🟦	🟩	🟩	🟦	🟦	🟩	🟩	🟦	🟦	🟩	🟩

END OF APPENDIX