Twist FlexPrep UHT Library Preparation Kit with Enzymatic Fragmentation and Twist UDI Primers

For use with the Twist NGS Workflow

The Twist FlexPrep Ultra High Throughput (UHT) Library Preparation Kit provides the reagents needed to prepare genomic DNA (gDNA) libraries using enzymatic fragmentation and the Twist FlexPrep Normalization Adapters in a high throughput environment. This system utilizes novel adapters that allow users to vary gDNA input so that no normalization is needed to generate sufficient libraries for whole genome sequencing or target enrichment. These normalization adapters also contain unique barcodes that allow for pooling post-ligation, maximizing throughput throughout the workflow. The ligated material is then amplified using the Twist Unique Dual Index (UDI) Primers, generating 12 individual libraries in each library pool. Not only does this system enable a high-throughput environment, but it also involves decreased reaction volumes to enable full-scale automation. 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 the 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 FlexPrep UHT 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
TWIST FLEXPREP UHT LIBRARY PREPARATION KIT (REAGENTS FOR LIBRARY CONSTRUCTION)			
109220 (192 samples) 109224 (1152 samples)	Twist FlexPrep UHT Library Preparation Module	· 10x Twist FlexPrep Fragmentation Enzyme Mix · 5x Twist FlexPrep Fragmentation Buffer · 20x Twist FlexPrep Ligation Mix · 4x Twist FlexPrep Ligation Buffer · 2x Twist Library Amp Mix · Twist FlexPrep Normalization Adapters	–20°C
	Twist FlexPrep Purification Beads	DNA Purification Beads	2-8°C
	Twist UDI Primers - Truseq Compatible	Twist UDI Primers	–20°C

For kits that come with FlexPrep Enrichment components, use the following catalog numbers:

109223: Twist FlexPrep UHT Library Prep and Enrichment Kit, 192 Samples

109226: Twist FlexPrep UHT Library Prep and Enrichment Kit, 1152 Samples

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 FlexPrep UHT Library Preparation Kit.

PRODUCT	SUGGESTED SUPPLIER
REAGENTS AND CONSUMABLES	
Ethanol (200 proof)	—
Molecular biology grade water	—
10 mM Tris-HCl pH 8 (optional)	—
Buffer EB (optional)	Qiagen
15-ml Falcon tubes	VWR
Thin-walled PCR 0.2-ml strip-tubes	Eppendorf
96-well plate	VWR
PCR plate seal	Thermo Fisher Scientific
96-well deep-well plate	Eppendorf
Qubit dsDNA Broad Range Quantification Assay	Thermo Fisher Scientific
Agilent DNA 7500 Kit	Agilent Technologies
EQUIPMENT	
Pipettes and tips	—
Vortex mixer	—
Plate vortexer	Mixmate, Eppendorf
96-well compatible magnetic plate	Alpaqua, Permagen Labware
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 FlexPrep UHT 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 the tubes 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

- Input gDNA can range between 30 ng and 300 ng. Normalization of starting gDNA concentration is not required.
- If unsure of gDNA concentration range, it is recommended to determine the range prior to library preparation by random sampling. If needed, use the Thermo Fisher Scientific dsDNA Broad Range Quantification Assay to quantify the input range of your purified gDNA. Measuring DNA concentration by 260 nm absorbance 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.
- Optimization of the following steps may be required to achieve optimal performance.
  For more information, please see Appendix E:
  - Fragmentation time (Step 1.1, page 9)
  - Number of PCR cycles (Step 3.1, page 16)

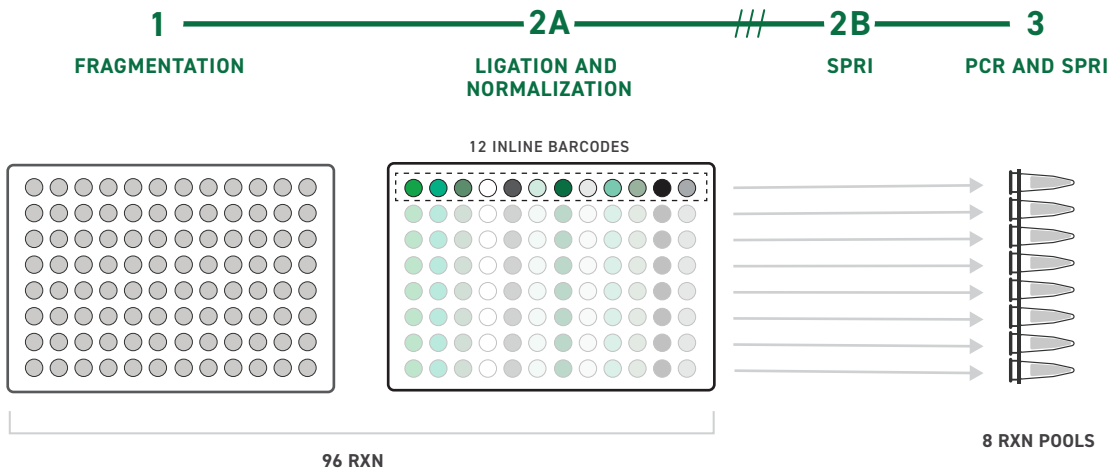
FOR TECHNICAL SUPPORT, CONTACT CUSTOMERSUPPORT@TWISTBIOSCIENCE.COM.

PROTOCOL OVERVIEW

This protocol begins with genomic DNA (gDNA) mass input ranging from 30 ng to 300 ng and generates amplified, indexed libraries for subsequent target enrichment or whole genome sequencing. It features enzymatic fragmentation, pooling post-ligation with inline barcodes found on the normalization adapters, and read index addition by PCR using UDI Primers.

	TWIST FLEXPREP UHT WITH TWIST NORMALIZATION ADAPTERS AND UDI PRIMERS (GENOMIC DNA, 30 NG TO 300 NG STARTING DNA MATERIAL)	TIME
STEP 1	**Perform DNA Fragmentation, End Repair, and dA-tailing** dA-tailed DNA fragments	**2 hours**
STEP 2	**Ligate Twist FlexPrep Normalization Adapters, Pool, and Purify** gDNA library pools ready for indexing	**2 hours**
STEP 3	**PCR Amplify Using Twist UDI Primers, Purify, and Perform QC** Amplified indexed libraries	**1 hour**

This protocol allows you to perform gDNA library preparation for 384 samples in 5 hours. Not only are reactions in this protocol miniaturized, but a pooling step that is designated by "///" in the figure below also allows for reactions to be combined into pools of 12 post-ligation by utilizing the Twist FlexPrep Normalization Adapters. This contributes to an overall decrease in end-to-end workflow time.



Twist FlexPrep UHT Library Preparation Workflow. *The additional pooling step found in the middle of the protocol (designated as ///) provides higher throughput and an overall decrease in end-to-end workflow time.*

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

- A total yield of 30 ng to 300 ng gDNA per sample in a total volume of 5 µl
- Molecular biology grade water
- From the Twist FlexPrep UHT Library Preparation Module:
  - 10x Twist FlexPrep Fragmentation Enzyme Mix
  - 5x Twist FlexPrep Fragmentation Buffer

Before You Begin

- Thaw or place on ice:
  - Molecular biology grade water
  - gDNA samples
  - 10x Twist FlexPrep Fragmentation Enzyme Mix
  - 5x Twist FlexPrep Fragmentation Buffer

PREPARE THE THERMAL CYCLER, SAMPLES, AND REAGENTS

1.1

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

NOTES:

- Depending on the number of libraries being generated, multiple thermal cyclers may be needed for this protocol.
- This protocol generates libraries that are approximately 400 bp to 450 bp in length. To achieve different fragment sizes, optimization will be needed (refer to Appendix C).
- If inhibitors are present or gDNA is not fully intact, optimization may be needed to generate libraries with appropriate lengths. Please see Appendix E for more information.

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

**22 min at 32°C generates library sizes that are approximately 400 bp to 450 bp in length. To produce different library sizes, refer to Appendix C.*

***The 65°C step is necessary to stop the reaction from fragmenting.*

1.2 Mix gDNA by flicking the tube with a finger.

1.3 Add 5 µl of gDNA ranging between 6 ng/µl and 60 ng/µl in as many wells as needed in a 96-well plate giving total gDNA mass between 30 ng and 300 ng. The number of samples in the plate must be divisible by 12, as there are 12 unique inline barcoded adapters in each row of the inline barcoded adapter plate.

NOTE: The inline barcoded adapters are organized by row in the adapter plate. Every well in a column of the adapter plate contains identical adapter sequences. Therefore, any set of 12 samples processed through this protocol must be pooled together by row in a 96-well plate. Please see Appendix A for additional details.

1.4 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

1.5 Vortex the 5x Twist FlexPrep Fragmentation Buffer for 5 seconds. Pulse-spin to collect all liquid in the bottom of the tube.

1.6 Invert the 10x Twist FlexPrep Fragmentation Enzyme Mix 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.

1.7 Prepare an enzymatic fragmentation mix in a 15-ml Falcon 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). The number of reactions will depend on the number of samples used to generate libraries. Total number of samples must be divisible by 12 for proper inline barcode usage.

REAGENT	VOLUME PER 1 SAMPLE*	VOLUME PER 12 SAMPLES*	VOLUME PER 96 SAMPLES*
5x Twist FlexPrep Fragmentation Buffer	2 µl	24 µl	192 µl
10x Twist FlexPrep Fragmentation Enzyme Mix	1 µl	12 µl	96 µl
Water**	2 µl	24 µl	192 µl
Total	**5 µl**	**60 µl**	**480 µl**

**Always prepare a master mix. Volumes found on the right are for sets of 12 and 96 samples, scale the amount accordingly based on the number of samples. Overage is not included in this calculation. An overage of 25% is recommended when using automation.*

***Water can be excluded from the master mix if 7 µl of gDNA sample is needed to meet the mass input requirement.*

1.8 Add 5 µl enzymatic fragmentation mix (from Step 1.7) to each 5 µl gDNA sample 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). Seal the plate and keep the reaction on ice.

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

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

1.10 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 FlexPrep Normalization Adapters, Pool, and Purify (see Before You Begin)

1.11 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 FLEXPREP NORMALIZATION ADAPTERS, POOL, AND PURIFY

STEP 2 LIGATE TWIST FLEXPREP NORMALIZATION ADAPTERS, POOL, AND PURIFY

Ligate Twist FlexPrep Normalization Adapters to the dA-tailed DNA fragments from Step 1, pool row-wise, and purify to generate gDNA libraries ready for UDI index introduction through amplification in Step 3.

Reagents Required

- dA-tailed DNA fragments (from Step 1.11)
- Ethanol
- Molecular biology grade water
- 10 mM Tris-HCl pH 8 or Buffer EB (optional, for elution)
- From the Twist FlexPrep UHT Library Preparation Module:
  - 20x Twist FlexPrep Ligation Mix
  - 4x Twist FlexPrep Ligation Buffer
  - Twist FlexPrep Normalization Adapters
- From the Twist FlexPrep Purification Beads:
  - DNA Purification Beads

Before You Begin

- Thaw or place on ice:
  - Twist FlexPrep Normalization Adapters (96-well plate; multiple reactions in each well)
  - 20x Twist FlexPrep Ligation Mix
  - 4x Twist FlexPrep Ligation Buffer
- Prepare 2 ml 80% ethanol for every 12 libraries generated (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 FLEXPREP NORMALIZATION ADAPTERS

2.1

Program thermal cyclers with the following conditions. The number of thermal cyclers needed will depend on the number of plates being prepared at once. Set the temperature of the heated lid to 70°C. Start the program to pre-chill the thermal cycler.

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

**The 65°C step is necessary to stop the reaction from ligating and prevent crosstalk during the pooling step.*

2.2 Vortex the Twist FlexPrep Normalization Adapter plate for 5 seconds. Pulse-spin to collect all liquid in the bottom of each well.

2.3 Add 4 µl of Twist FlexPrep Normalization Adapters into each sample well containing the dA-tailed DNA fragments from Step 1.11. Mix gently by pipetting and keep on ice.
NOTE: Each row of Twist FlexPrep Normalization Adapters coincides with a pool post-ligation. When adding adapters, make sure to plan accordingly. For inline barcode sequences and pooling guidelines, refer to Appendix A.

2.4 Vortex the 4x Twist FlexPrep Ligation Buffer for 5 seconds. Pulse-spin to collect all liquid in the bottom of the tube.

2.5 Invert the 20x Twist FlexPrep Ligation Mix 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.

2.6 Prepare a ligation mix in a 15-ml Falcon tube on ice. Use the volumes listed below. Homogenize the master mix with moderate vortexing for 5 seconds or pipetting a minimum of half the total volume up and down 10 times (avoid formation of bubbles). The number of reactions will depend on the number of samples generating libraries for.

REAGENT	VOLUME PER 1 SAMPLE*	VOLUME PER 12 SAMPLES*	VOLUME PER 96 SAMPLES*
4x Twist FlexPrep Ligation Buffer	5 µl	60 µl	480 µl
20x Twist FlexPrep Ligation Mix	1 µl	12 µl	96 µl
Total	**6 µl**	**72 µl**	**576 µl**

**Always prepare a master mix. Volumes found on the right are for sets of 12 and 96 samples, scale the amount accordingly based on the number of samples. Overage is not included in this calculation. An overage of 25% is recommended when using automation.*

2.7 Add 6 µl ligation mix (from Step 2.6) to each 14 µl enzymatic fragmentation and normalization adapter well (from Step 2.3). 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.

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

2.9 Initiate steps 2 to 4 of the thermal cycler program (refer to the table in Step 2.1).

2.10 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.
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).

POOL

2.11 Each row of a plate coincides with a pool that will be purified together. Add 16 µl from all 12 wells found in a single full row to one well of a deep-well plate to prepare for purification. Perform this for each row as applicable. Cover the deep-well plate using a PCR plate seal.
NOTES: Each row of a plate represents up to 12 samples that will be pooled and purified together. For inline barcode sequences and pooling guidelines, refer to Appendix A. After pooling, there will be approximately 4 µl of reaction left in the well.

2.12 Vortex the deep-well plate containing the pooled samples for 30 seconds at 2000 rpm. Pulse-spin to collect all liquid in the bottom of each well.

PURIFY

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

2.14 Add 134.4 µl of homogenized (0.7x) DNA Purification Beads to each ligation pool from Step 2.12. Cover the deep-well plate using a PCR plate seal. Mix well by vortexing. Pulse-spin to collect all liquid in the bottom of the tube.

2.15 Incubate the samples for 10 minutes at room temperature.

2.16 Place the samples on a magnetic plate for 5 minutes or until the supernatant is clear.
NOTE: The supernatant may take longer to become clear due to the large reaction volume.

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

2.18 Wash the bead pellet by gently adding 500 µl freshly prepared 80% ethanol (do not disturb the pellet). Incubate for 1 minute, then remove and discard the ethanol.

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

2.20 Carefully remove all remaining ethanol with a 10-µl pipette, making sure not to disturb the bead pellet.
NOTE: Before pipetting, the bead pellet may be briefly spun to collect ethanol at the bottom of the plate or tube and returned to the magnetic plate.

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

2.22 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 be used as a substitute for water in this step.

2.23 Incubate at room temperature for 5 minutes.
NOTE: This incubation time is required to ensure full elution of samples off beads.

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

2.25 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.
NOTE: Minimal bead carryover is common at Step 2.25. This will not impact future steps.

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 normalized and adapted gDNA library pools with Twist UDI Primers, purify them, and perform quality control (QC) analysis to complete the protocol.

Reagents Required

- Ligated and pooled libraries (from Step 2.25)
- 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 FlexPrep UHT Library Preparation Module:
  - 2x Twist Library Amp Mix
- Twist UDI Primers - TruSeq Compatible:
  - Twist UDI Primers

Before You Begin

- Thaw or place on ice:
  - 2x Twist Library Amp Mix
  - Twist UDI 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 OF CYCLES
1	Initialization	98°C	45 seconds	1
2	Denaturation	98°C	15 seconds	6*
	Annealing	60°C	30 seconds	
	Extension	72°C	30 seconds	
3	Final Extension	72°C	1 minute	1
4	Final Hold	4°C	HOLD	–

**Depending on the sample input and purity of gDNA, the number of cycles may need to be increased to get the yield of the library needed for future protocols. To achieve amplification yields specific to your sample, optimization will be needed. For more information, please see Appendix E.*

PERFORM THE PCR

3.2 Add 10 µl of Twist UDI Primers from the provided 96-well plate to each of the ligated library pools from Step 2.25 and mix well by gentle pipetting.
NOTES: For UDI index selection and pooling guidelines for downstream target enrichment and sequencing, refer to Appendix B. If generating less than a full 96-well plate of libraries, color balancing of the Twist UDI Primer should be carefully evaluated before picking which primers to use.

3.3 Add 25 µl of 2x Twist Library Amp Mix to the ligated library pools with UDI primers from Step 3.2 and mix well by gentle pipetting.
NOTE: Invert 2x Twist Library Amp Mix 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 of the amplified library pools 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 the 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 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.

3.14 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.

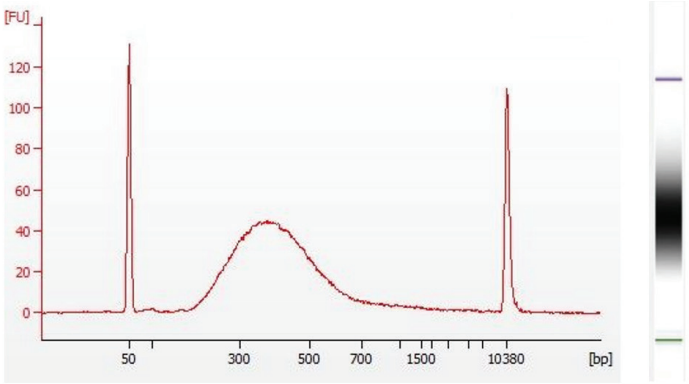
3.18 Transfer 20 µl of the clear supernatant containing the purified and amplified library pools to a clean thin-walled PCR 0.2-ml strip-tube or well of a 96-well plate, making sure not to disturb the bead pellet.

PERFORM QC

3.19 Quantify and validate the size range of each library pool using the Thermo Fisher Scientific Qubit dsDNA Broad Range Quantification Assay and Agilent DNA 7500 Assay.

Under the protocol conditions, the average fragment length is typically between 400 bp to 450 bp using a range setting of 150 bp to 1,000 bp.

This protocol is compatible with the Twist FlexPrep Target Enrichment Protocol. For more information, refer to Appendix D.



Representative electropherogram of a purified and 12-sample pooled library generated with input between 30 ng and 300 ng of high-quality gDNA.

NOTE: Leftover DNA Purification Beads and 2x Twist Library Amp Mix from this protocol should be used in the Twist FlexPrep Target Enrichment Protocol

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

END OF WORKFLOW

APPENDIX A: INLINE BARCODE SEQUENCES AND POOLING GUIDELINES

INLINE BARCODE SEQUENCES FOUND IN THE TWIST FLEXPREP NORMALIZATION ADAPTER PLATE

Twist FlexPrep Normalization Adapters include inline barcode sequences for ultra-high-throughput sequencing. The inline barcode sequences are base balanced within a pool of 12 barcoded samples, with all wells found in a column of Twist FlexPrep Normalization Adapters containing the same inline barcode sequence.

Table 1. Twist inline barcode sequences found in each column of a Twist FlexPrep Normalization Adapter plate.

COLUMN NUMBER	SEQUENCE
1	GCCATA
2	TCTGGT
3	TGGCTT
4	AACACT
5	TCAGGA
6	TGGTCC
7	CAACTG
8	CGGACC
9	ATCGAG
10	ATGGTG
11	CTTAAG
12	GAGTGC

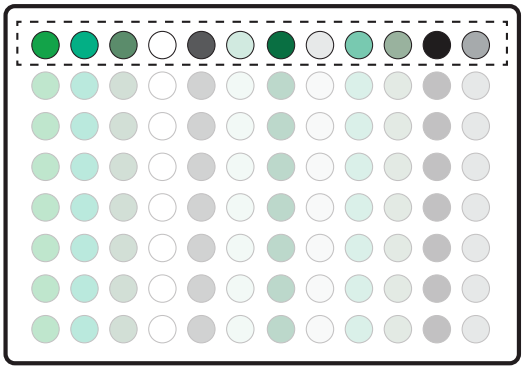
COLOR BALANCING AND PHIX

The inline barcodes sequences that make up the first 6 bases found in the Twist FlexPrep Normalization Adapters are of lower diversity than typical library content. Anywhere between 5% and 10% of PhiX should be added to each sequencing run to provide diverse signals at each cycle and to maintain sequencing run quality.

POOLING RECOMMENDATIONS FOR THE TWIST FLEXPREP NORMALIZATION ADAPTER PLATE

The Twist FlexPrep Normalization Adapters are arranged in a 96-well format as shown below, where 12 unique barcodes are replicated across rows A through H. When pooling individual ligated libraries, each row is collapsed into a single well for ease of processing downstream.

Figure 1. Twist FlexPrep Normalization Adapter layout. Twelve unique barcodes can be found in every row across the 96-well plate.



APPENDIX B: UDI ADAPTER SEQUENCES AND POOLING GUIDELINES

UDI SEQUENCES

For a complete guide of the Twist UDI primer sequences used with the Twist FlexPrep UHT Library Preparation Kit, please refer to the UDI Sequences Reference Spreadsheet. 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 2. Twist UDI primer plate layouts and pooling guidelines for 192-sample kit.
Twist UDI Primers: TruSeq Compatible, 16 Samples

	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										

Table 3. Twist UDI primer plate layouts and pooling guidelines for 1152-sample kit.
Twist UDI Primers: TruSeq Compatible, 96 Samples, Plate B

	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

NOTE: The indexes in the 16-library pool plate are not the same as those in the 96-library pool plate.

APPENDIX C: OBTAINING LARGER LIBRARY SIZE RANGES

FRAGMENTATION SIZE 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 table below. The current protocol fragmentation temperature and time (32°C for 22 minutes) results in library sizes that are roughly between 400 bp and 450 bp long. By decreasing the amount of time the reaction is held at 32°C, the library size will become longer, producing larger libraries. The table below can be used to determine if the reaction time should be shortened based on the library size needed.

Table 4. Library length based on fragmentation time. Modifications can be made in the fragmentation step to change the size of the final library.

LIBRARY SIZE	FRAGMENTATION TIME
550 bp to 600 bp	5 minutes
470 bp to 510 bp	10 minutes
445 bp to 470 bp	15 minutes
400 bp to 450 bp	22 minutes
350 bp to 400 bp	30 minutes

ADAPTER LOADING AND PCR CYCLE NUMBER

We do not recommending adjusting adapter volume input for optimal normalization performance. However, depending on the sample input and purity of gDNA, the number of cycles may need to be increased to get the yield of library needed for future protocols. To achieve amplification yields specific to your sample, optimization will be needed.

LARGER LIBRARIES AND NORMALIZATION RANGE

Normalization range is dependent on the number of molecules generated by fragmentation. As library sizes increase with shorter fragmentation time, fewer molecules are generated and the successful range of normalization changes. For library sizes ranging between 400 bp to 450 bp, the normalization range for mass input is 30 ng to 300 ng of gDNA. As library sizes increase to 500 bp to 550 bp, the normalization range will increase to 50 ng to 500 ng of gDNA. Some optimization may be needed.

APPENDIX D: TWIST FLEXPREP UHT LIBRARY PREPARATION KIT INTO TARGET ENRICHMENT

TARGET ENRICHMENT

Libraries generated from the Twist FlexPrep UHT Library Preparation Kit are compatible with the Twist FlexPrep Target Enrichment Protocol. This system generates enriched DNA libraries for sequencing using a 16-hour hybridization in a two-day workflow. The protocol for the Twist FlexPrep Target Enrichment workflow is available for download at twistbioscience.com/resources/protocol/twist-target-enrichment-standard-hybridization-v2-protocol.

MULTIPLEXING

An advantage to using the Twist FlexPrep UHT Library Preparation Kit with the Twist FlexPrep Target Enrichment Protocol is that the diversity of the libraries is capped during the library preparation workflow, allowing the system to handle a large number of samples to be multiplexed during target enrichment. The typical target enrichment workflow using the Library Preparation EF 2.0 with Enzymatic Fragmentation and Twist Universal Adapter System supports up to an 8-plex during hybridization. Using the Twist FlexPrep UHT Library Preparation Kit permits for multiplexing up to a 96-plex.

MASS INTO CAPTURE

Panel size may play a role in how much mass into capture is needed. For panels targeting less than 1 Mb, a library mass input ranging between 4 µg and 10 µg is adequate to get desirable coverage and diversity. When using larger panels, diversity may decrease and troubleshooting may be needed.

APPENDIX E: FRAGMENTATION AND CYCLING OPTIMIZATION

Often, gDNA can contain inhibitors or become partially fragmented due to harsh extraction methods. This can cause libraries to be longer or shorter (respectively) than intended. When using the Twist FlexPrep UHT Library Preparation Kit on multiple plates of samples, Twist suggests carrying out an optimization run prior to performing the full-length experiment. This optimization experiment is designed to test the fragmentation time and cycling needed, and does not require any sequencing. Data from the Thermo Fisher Scientific Qubit dsDNA Broad Range Quantification Assay and Agilent DNA 7500 Assay are adequate to determine optimized conditions.

FRAGMENTATION TIME

The initial fragmentation time for this protocol is 22 minutes at 32°C, with an additional 30-minute hold at 65°C for enzyme denaturation. To test if inhibitors are present or if gDNA is not fully intact, Twist recommends performing additional incubations at 12 minutes and 32 minutes for entire rows using six amplification cycles, resulting in three library pools (a total of 36 samples).

PCR CYCLING

Amplification cycling post-ligation starts at six cycles. The final yield is dependent on how well fragmentation worked, if inhibitors are present, and/or whether the gDNA is fully intact. In addition to adding two new incubation times in the optimization run, Twist also suggests performing all three fragmentation incubation times (12 minutes, 22 minutes, and 32 minutes) at eight amplification cycles as well. The final number of optimization library pools should be six in total (a total of 72 individual samples).

FINAL QC

Quantification post-amplification should be performed using the Thermo Fisher Scientific Qubit dsDNA Broad Range Quantification Assay and Agilent DNA 7500 Assay. Yield should be ≥1000 ng, while fragment size should range between 400 bp and 450 bp. Fragmentation time and PCR cycling conditions of best results within the ranges outlined above should be used moving forward with full plates.

END OF APPENDIX

LAST REVISED: DECEMBER 6, 2024

REVISION	DATE	DESCRIPTION
2.0	Dec 6, 2024	· Fixed typos for index spreadsheet link · Changed recommended overages when using automation