Twist Enzymatic Fragmentation 2.0 for PCR Free Whole Genome Sequencing

For use with the Twist NGS Workflow

This Twist Whole Genome Sequencing Workflow details all the necessary steps for generating PCR-free indexed libraries for sequencing on Illumina next-generation sequencing (NGS) systems. The workflow uses reagents to prepare genomic DNA (gDNA) libraries using enzymatic fragmentation and full-length Y-shaped adapters. This library preparation workflow has been optimized for use with the reagents specified and should only be performed with them 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 component, and store components as recommended immediately upon arrival.

CATALOG #	NAME	DESCRIPTION	STORAGE
TWIST LIBRARY PREPARATION EF KIT 2.0 (REAGENTS FOR LIBRARY CONSTRUCTION)			
104206: 16 rxn 104207: 96 rxn	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)*	–20°C
	Twist Library Preparation Kit 2	DNA Purification Beads	2–8°C
TWIST ADAPTERS (ORDERED SEPARATELY)			
107381, 107469, 107470, 107471: 384 samples, 96-well plate	Twist Full Length UDI Adapters, Plate 1-16	Full Length Adapter Set, 1 reaction per index	–20°C

**Equinox Library Amp Mix (2x) and P5/P7 Primers (10X) are provided with this library preparation kit for applications that require PCR amplification, they are not required for this workflow.*

LEGAL

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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 2.0 with Enzymatic Fragmentation and Twist Full Length UDI Adapters.

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
qPCR Library Quantification Kit	—
Agilent High Sensitivity DNA Kit (Optional)	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
Real-Time PCR Instrument	—

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 sequencing. 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 75 - 500 ng of high-quality gDNA. Using under 75 ng starting mass may not generate sufficient material for sequencing and may impact library quality.
- When using degraded DNA or low mass inputs, modify adapter input following Appendix A: Obtaining Larger Insert Sizes and Handling Alternative Mass Inputs.

FOR TECHNICAL SUPPORT, CONTACT CUSTOMERSUPPORT@TWISTBIOSCIENCE.COM

PROTOCOL OVERVIEW

This protocol begins with genomic DNA (gDNA) and generates indexed libraries for whole genome sequencing applications. It features enzymatic fragmentation and full-length unique dual index (UDI) adapters. This protocol allows you to perform gDNA library preparation (Steps 1–2) in 2.5 hours.

	LIBRARY PREP FOR ENZYMATIC FRAGMENTATION WITH FULL-LENGTH UDI ADAPTERS	TIME
STEP 1	**Perform DNA fragmentation, end repair, and dA-tailing** dA-tailed DNA fragments	**1 hour**
STEP 2	**Ligate full-length UDI adapters, purify, and perform QC** Indexed gDNA libraries	**1.5 hours**

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)
- Molecular biology grade water
- Qubit dsDNA Broad Range Quantitation Assay (or equivalent)
- From the Twist Library Preparation EF Kit 1, 2.0:
  - Frag/AT Buffer
  - Frag/AT Enzymes

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 to determine the fragmentation time needed 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.

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

STEP 2 INCUBATION TIME*	
DESIRED INSERT SIZE (bp)	TIME
350 – 400	10 Minutes
375 – 425	5 Minutes
400 – 450	3 Minutes

**5 minutes at 30°C is the recommended starting condition for whole genome sequencing applications utilizing 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. Optimize fragmentation by adjusting the incubation time in 2-3 minute increments.*

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 Dilute the gDNA sample(s) (75 ng to 500 ng target mass input) to a volume of 40 µl in water, 10 mM Tris-HCl pH 8.0, or Buffer EB.

1.4 Add 40 µl of each diluted gDNA sample 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 at 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 at 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 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).

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 above).
NOTE: While the thermal cycler program is running, prepare the reagents for Step 2: Ligate Indexed Adapters, Purify, and Perform QC (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 INDEXED ADAPTERS, PURIFY, AND PERFORM QC

STEP 2 LIGATE INDEXED ADAPTERS, PURIFY, AND PERFORM QC

Ligate adapters to the dA-tailed DNA fragments from Step 1 and purify them to generate indexed gDNA libraries. Perform QC to complete the protocol.

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 Full Length UDI Adapter Set:
  - Twist Full Length UDI Adapters (plate)
- From the Twist Library Preparation Kit 2:
  - DNA Purification Beads

Before You Begin

- Thaw on ice:
  - Twist Full Length UDI Adapters (plate)
  - Ligation Master Mix
- Prepare 0.5 ml 80% ethanol for each sample.
- Equilibrate DNA Purification Beads to room temperature for at least 30 minutes.
- 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 ADAPTERS

2.1 Add 5.5 µl Full Length UDI Adapter into each sample well or tube containing the dA-tailed DNA fragments from Step 1. Mix gently by pipetting and keep on ice.
NOTE: When using degraded DNA or low mass inputs, modify adapter input following Appendix A: Obtaining Larger Insert Sizes and Handling Alternative Mass Input.

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.

PURIFY

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

2.6 Add 37.5 µl of homogenized (0.5x) 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 the 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 22 µl water, 10 mM Tris-HCl pH 8, or Buffer EB to each sample. Mix by pipetting until homogenized.
NOTE: If performing optional second purification, add 52 µl water, 10 mM Tris-HCl pH 8, or Buffer EB to each sample.

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 20 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.
NOTE: If performing optional second purification, transfer 50 µl of clear supernatant containing the ligated and indexed libraries to a clean tube or plate.

2ND PURIFY (OPTIONAL)

NOTE: 2nd purification is recommended when performing library preparation on degraded gDNA or mass inputs ≤100 ng.

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

2.19 Add 50 µl (1x) homogenized DNA Purification Beads to each ligation sample from Step 2.18. Mix well by vortexing.

2.20 Incubate the samples for 5 minutes at room temperature.

2.21 Place the samples on a magnetic plate for 1 minute or until the supernatant clears.

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

2.23 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.

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

2.25 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.

2.26 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.

2.27 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.

2.28 Incubate at room temperature for 2 minutes.

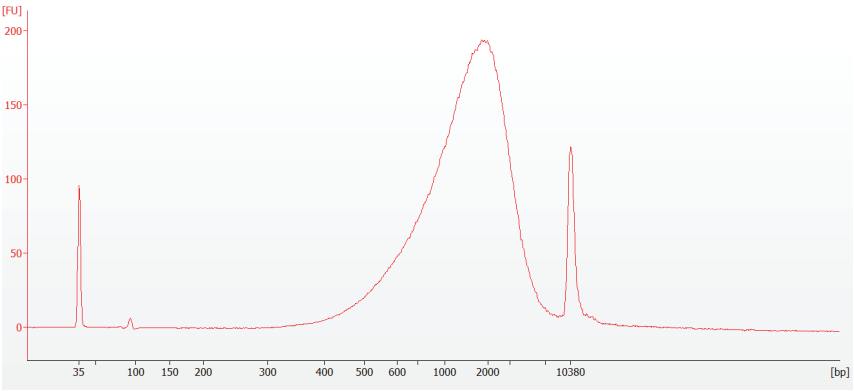
2.29 Place the plate or tubes on a magnetic plate and let stand for 3 minutes or until the beads form a pellet.

2.30 Transfer 20 µl of the clear supernatant containing the 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

2.31

Quantify each library using an appropriate qPCR-based method. A capillary electrophoresis assay can be used to assess the quality of the libraries. Note that full-length Y-adapters contain single-stranded regions that slow the migration of molecules in electrophoretic assays leading to larger than expected fragment size profiles. Check for the absence of low molecular weight contamination like adapter dimers.



Representative electropherogram of a library generated with input of 500 ng of high-quality gDNA using a 5-minute fragmentation incubation at 30°C. For analysis, the library was diluted 1:10 and run using an Agilent High Sensitivity DNA Kit.

STOPPING POINT: If not proceeding immediately to sequencing, store the indexed libraries at –20°C.

END OF WORKFLOW

APPENDIX A: OBTAINING LARGER INSERT SIZES AND HANDLING ALTERNATIVE MASS INPUTS

OPTIMIZING LIBRARY SIZE

For applications that require longer insert sizes, the library length can be increased by adjusting fragmentation conditions and the post-ligation SPRI bead ratio. Reducing fragmentation increases overall library size, but can generate broad libraries where shorter fragments preferentially cluster on flow cells during sequencing. Adjusting the post-ligation SPRI bead ratio can remove shorter fragments prior to sequencing. A 0.5X bead ratio is a recommended starting point for generating average library insert sizes >250 bp. To optimize for other insert sizes, test a range of bead ratios between 0.4X and 0.8X. Increase the ratio to retain smaller fragments and reduce the ratio to remove smaller fragments. Note that reducing the bead ratio will reduce library yield.

ADAPTER LOADING

In PCR-free applications, a small amount of full-length adapters can carry over into sequencing and cluster on the flow cell, which reduces the desired library reads. To minimize residual adapters in the final libraries, the volume of full-length adapters can be adjusted. Refer to the table below for guidance, add additional buffer as needed to reach a total volume of 5.5 µl. In addition, performing an optional second 1X SPRI ratio purification after ligation on low mass input or degraded samples can remove residual adapter dimers.

DNA INPUT	VOLUME OF FL ADAPTER (µl)	VOLUME OF TRIS-HCL PH 8.0 (µl)
≤100	1	4.5
200	2	3.5
300	3	2.5
≥500	5.5	—

APPENDIX B: FULL LENGTH UDI ADAPTER SEQUENCES AND PLATE MAP

The Twist Full Length UDI Adapter set contains indexes of 10 nucleotides. To avoid potential index clashes, do not sequence in combination with other adapter sets.

For a complete guide to the Twist Full Length UDI Adapter sequences, please refer to the Full Length UDI Adapter Sequences Reference Spreadsheet and Full Length UDI Adapter Sample Sheet Templates. These files are available for download here: www.twistbioscience.com/resources/data-files/full-length-unique-dual-index-sequences-reference-spreadsheets-and-sample

Representative Plate Layouts

Twist Full Length UDI Adapter, Plates 1 - 16 (107381, 107469, 107470, 107471)

Plate 1.

	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 2.

	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 3.

	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 4.

	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