Twist Mechanical Fragmentation 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 prepares genomic DNA (gDNA) libraries using mechanically fragmented DNA 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 KIT, MECHANICAL FRAGMENTATION (REAGENTS FOR LIBRARY CONSTRUCTION)			
101280 (16 samples) 101281 (96 samples)	Twist Library Preparation Kit 1, Mechanical Fragmentation	· 10x ERA Buffer · 5x ERA Enzyme Mix · DNA Ligation Mix · DNA Ligation Buffer · Amplification Primers, ILMN*	–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

**Amplification Primers, ILMN are provided with the library preparation kit for applications that require PCR amplification, they are not required for this workflow.*

LEGAL

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MATERIALS SUPPLIED BY USER

The following materials or their equivalent are required to generate libraries using the Twist Library Preparation Kit with Mechanical 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	—
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	—
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 provided instructions. Twist cannot guarantee the performance of the workflow if modifications are made to the protocol.

Do NOT mix or combine the same reagents from different lots.

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

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

FRAGMENTED SAMPLES

- Use any desired mechanical shearing method to prepare the fragmented gDNA sample for use with this protocol.
- For optimum performance, analyze the size distribution of the fragmented input gDNA with an Agilent High Sensitivity DNA Kit before proceeding with end repair and dA-tailing reactions. Ensure the mode of the fragment size distribution is 300–350 bp.
- To optimize insert size, refer to Appendix A: Obtaining Larger Insert Sizes and Handling Alternative Mass Inputs.
- Suspend fragmented input DNA in molecular biology grade water, 10 mM Tris-HCl pH 8.0, or Buffer EB.
- The recommended DNA input for this workflow is 100 ng to 1 µg. Using under 100 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.
- 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.

FOR TECHNICAL SUPPORT, CONTACT CUSTOMERSUPPORT@TWISTBIOSCIENCE.COM

PROTOCOL OVERVIEW

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

	LIBRARY PREP FOR MECHANICAL FRAGMENTATION WITH FULL-LENGTH UDI ADAPTERS	TIME
STEP 1	**Perform 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 END REPAIR AND DA-TAILING

Use mechanically fragmented input genomic DNA (gDNA) to perform end repair and subsequent dA-tailing to generate dA-tailed DNA fragments.

Use any desired mechanical shearing method to prepare the fragmented gDNA sample. For optimum performance, analyze the size distribution of the fragmented input gDNA with an Agilent High Sensitivity DNA Kit before proceeding with end repair and dA-tailing reactions. Ensure the mode of the fragment size distribution is 300–350 bp.

Reagents Required

- Fragmented genomic DNA (gDNA): 100 ng – 1 µg per sample (prepared using any desired mechanical fragmentation method)
- Molecular biology grade water (chilled)
- Qubit dsDNA Broad Range Quantitation Assay (or equivalent)
- From the Twist Library Preparation Kit 1, Mechanical Fragmentation:
  - 5x ERA Enzyme Mix
  - 10x ERA Buffer

Before You Begin

- Thaw 5x ERA Enzyme Mix and gDNA samples on ice, then mix by flicking the tube with a finger.
- Thaw 10x ERA Buffer on ice, then mix by pulse vortexing for 2 seconds. If the buffer contains a white precipitate, vigorously vortex the buffer until the precipitate dissolves.

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.

STEP	TEMPERATURE	TIME
1	4°C	HOLD
2	20°C	30 min
3	65°C	30 min
4	4°C	HOLD

1.2 Use the Qubit dsDNA Broad Range Quantitation Assay to determine the concentration of your gDNA samples.

1.3 Dilute the fragmented gDNA samples (100 ng – 1 µg target mass input) to a volume of 35 µl in water, 10 mM Tris-HCl pH 8.0, or Buffer EB.

1.4 Add 35 µl of each diluted gDNA sample into a 0.2-ml thin-walled PCR 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 END REPAIR AND DA-TAILING (ERA)

1.6 Prepare an ERA reaction master mix in a 1.5-ml microcentrifuge tube on ice. Use the volumes listed below. Mix thoroughly by gentle pipetting.

REAGENT	VOLUME PER REACTION*
10x ERA Buffer	5 µl
5x ERA Enzyme Mix	10 µl
Total	**15 µl**

**Prepare a master mix for multiple reactions.*

1.7 Add 15 µl ERA reaction master mix (from Step 1.6) to each 35 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 4 of the thermal cycler (20°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, Purify, and Perform QC (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 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 1.10)
- Ethanol
- Molecular biology grade water (chilled)
- From the Twist Library Preparation Kit 1, Mechanical Fragmentation:
  - DNA Ligation Mix
  - DNA Ligation Buffer
- 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)
  - DNA Ligation Mix
  - DNA Ligation Buffer
- 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 is at 20°C when the samples are 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 Inputs.

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 (chilled)	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.

PURIFY

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

2.7 Add 50 µl of homogenized (0.5x) 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 or until the supernatant is clear.

2.10 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.

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.

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.

2.14 Air-dry the bead pellet on the magnetic plate for 5 minutes or until the bead pellet is dry.

2.15 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.
NOTE: If performing optional second purification, add 52 µl water, 10 mM Tris-HCl pH 8, or Buffer EB to each sample.

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 20 µl of the clear supernatant containing the ligated and indexed libraries to a clean 0.2-ml thin-walled PCR 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.

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

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

2.21 Incubate the samples for 5 minutes at room temperature.

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

2.23 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.

2.24 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.

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

2.26 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.

2.27 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.

2.28 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.

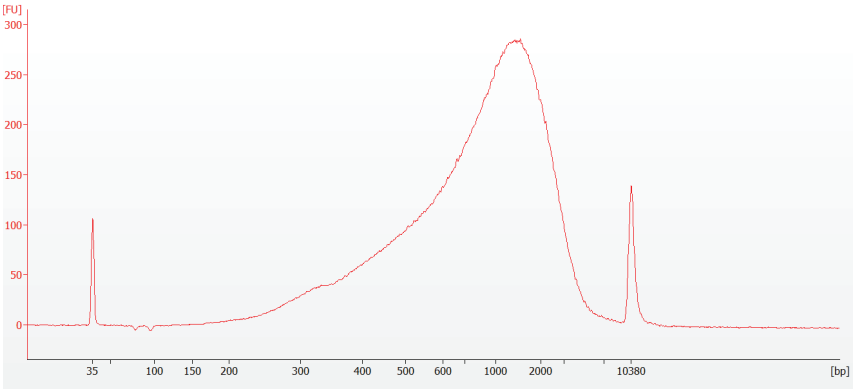
2.29 Incubate at room temperature for 2 minutes.

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

2.31 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

2.32 Quantify each library using an appropriate qPCR-based method. A capillary electrophoresis assay can be used to assess the quality of 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 sheared to a mode of 350 bp using a Covaris ML230. 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