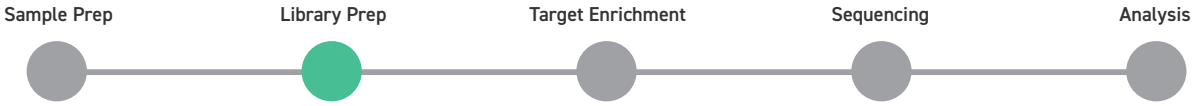
LIBRARY PREPARATION PROTOCOL

Library Preparation EF 2.0 with Enzymatic Fragmentation and Twist UMI Adapter System

For use with the Twist NGS Workflow

This Twist Library Preparation Kit provides the reagents needed to prepare genomic DNA (gDNA) libraries using enzymatic gDNA fragmentation and unique molecular identifiers (UMIs). The Twist UMI Adapter system consists of Twist UMI Adapters and Twist Unique Dual Indexed (UDI) Primers. This manual details the steps for generating the amplified, indexed libraries with UMI tags 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 kit and store components as recommended immediately upon arrival.

CATALOG #	NAME	DESCRIPTION	STORAGE
104206: 16 rxn 104207: 96 rxn	Twist Library Preparation EF Kit 2.0	Reagents for library construction	—
	Twist Library Preparation EF Kit 1, 2.0	• Frag/AT Enzymes • 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
105040: 16 rxn 105041, 105042, 105043, 105044: 96 rxn	Twist UMI Adapter System - TruSeq Compatible	Twist UMI Adapters and Twist UDI Primers provide UMI labeling and unique dual-indexed combinations with 1 reaction per index pair	–20°C

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

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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 2.0 with Enzymatic Fragmentation and Twist UMI Adapter System.

PRODUCT	SUGGESTED SUPPLIER
REAGENTS AND CONSUMABLES	
Ethanol (200 proof)	—
Molecular biology grade water	—
10 mM Tris-HCl pH 8 (optional)	—
Buffer EB (optional)	Qiagen
1.5-ml microcentrifuge tubes	VWR
Thin-walled PCR 0.2-ml strip-tubes	Eppendorf
96-well thermal cycling plates	VWR
96-well compatible magnetic plate	Alpaqua, Permagen Labware
Qubit dsDNA Broad Range Quantitation Assay	Thermo Fisher Scientific
Agilent DNA 7500 Kit	Agilent Technologies
EQUIPMENT	
Pipettes and tips	—
Vortex mixer	—
Benchtop mini centrifuge for 0.2-ml tubes	—
Thermomixer for 1.5-ml tubes	Eppendorf
Thermal cycler (96 well) with heated lid	—
Fluorometer (Qubit 3.0)	Thermo Fisher Scientific
2100 Bioanalyzer	Agilent Technologies

GENERAL NOTES AND PRECAUTIONS

Wear appropriate protective equipment (lab coat, gloves, and protective glasses or goggles) at all times when performing this protocol.

For best results, read this document before performing the protocol, and follow the instructions provided. Twist cannot guarantee the performance of the Twist Library Preparation Kit using Enzymatic Fragmentation and the Twist UMI Adapter System 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

- Use the Thermo Fisher Scientific Qubit dsDNA Broad Range Quantitation Assay to accurately quantify input purified gDNA.
- Measuring DNA concentration by absorbance at 260 nm is not recommended.
- Input DNA should be suspended in Molecular Biology Grade Water, 10 mM Tris-HCl pH 8.0, or Buffer EB.
- It is important to remove all cations and chelators from the starting gDNA sample. The presence of cations and chelators may affect the initial fragmentation reaction.
- The recommended DNA input is 50 ng of high quality gDNA.
- Reagents are compatible with mass input of 1 ng to 500 ng, but may require optimization of the following steps in library preparation to achieve optimal performance:
  - Incubation Time for Fragmentation (Step 1.1, page 7)
  - Amount of Twist UMI Adapter (Step 2.1, page 10)
  - Incubation Time for Ligation Reaction (Step 2.4, page 10)
  - PCR cycles for Amplification (Step 3.1, page 12)
- For additional guidance on library preparation optimization, refer to the Appendix.
- For technical support, contact customersupport@twistbioscience.com.

PROTOCOL OVERVIEW

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

	ENZYMATIC FRAGMENTATION WITH UMI ADAPTERS AND UDI PRIMERS (GENOMIC DNA, 50 NG STARTING DNA MATERIAL)	TIME
STEP 1	**Perform DNA Fragmentation, End Repair, and dA-tailing** dA-tailed DNA fragments	**1 hour**
STEP 2	**Ligate Twist UMI Adapters and Purify** gDNA libraries ready for indexing	**1 hour**
STEP 3	**PCR Amplify Using Twist UDI Primers, 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, 2.0:
  - Frag/AT Enzymes
  - Frag/AT Buffer

Before You Begin

- Thaw or place on ice:
  - Molecular biology grade water
  - gDNA
  - Frag/AT Buffer
  - Frag/AT Enzymes

PREPARE THE THERMAL CYCLER, SAMPLES, AND REAGENTS

1.1

Program the thermal cycler with the following conditions. Use the Step 2 Incubation Time table below to select conditions for fragmentation to achieve the desired insert size. Set the temperature of the heated lid to 105°C. Start the program to pre-chill the thermal cycler.

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

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

STEP 2 INCUBATION TIME*		
DESIRED INSERT SIZE (BP)	@30°C	@37°C
145–175	–	30 min
180–220	–	20 min
250–300	–	10 min
275–350	15 min	–
350–425	10 min	–

**20 min at 37°C is recommended for Twist target enrichment applications utilizing 50 ng of high quality gDNA. Additional conditions in the Step 2 Incubation Time table were also derived using high quality gDNA and should be optimized for each sample type/application. See the Appendix for additional guidance.*

1.2 Mix gDNA by flicking the tube with a finger. Use the Qubit dsDNA Broad Range Quantitation Assay to determine the concentration of your genomic DNA (gDNA) samples.

NOTE: Measuring DNA concentration by absorbance at 260 nm is not recommended.

1.3 Dilute the gDNA sample(s) to a final concentration of 1.25 ng/µl with chilled water. Mix well with gentle pipetting.

NOTE: If a mass input other than 50 ng is desired, dilute the target mass to a volume of 40 µl.

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

1.5 Pulse-spin to ensure all of the solution is at the bottom of the tube and place on ice.

PERFORM FRAGMENTATION, END REPAIR, AND dA-TAILING

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

1.7 Invert Frag/AT Enzymes a minimum of 10 times to homogenize or briefly vortex to ensure complete mixing. Pulse-spin to collect all liquid in the bottom of the tube.

1.8 Prepare an enzymatic fragmentation mix in a 1.5 ml microfuge tube on ice. Use the volumes listed below. Homogenize the mastermix with moderate vortexing for 5 seconds or pipetting a minimum of half the total volume up and down 10 times (avoid formation of bubbles).

REAGENT	VOLUME PER REACTION*
Frag/AT Buffer	4 µl
Frag/AT Enzymes	6 µl
Total	10 µl

**Prepare a master mix for multiple reactions.*

1.9 Add 10 µl enzymatic fragmentation mix (from Step 1.8) to each 40 µl gDNA sample tube or well. Homogenize with moderate vortexing for 5 seconds or by pipetting a minimum of half the total volume up and down 10 times (avoid formation of bubbles). Cap the tube(s) or seal the plate and keep the reaction on ice.

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

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

1.11 Initiate steps 2 to 4 of the thermal cycler program (refer to the table in Step 1.1 above).

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

1.12 When the thermal cycler program is complete and the sample block has returned to 4°C, remove the samples from the block and place them on ice.

PROCEED IMMEDIATELY TO STEP 2: LIGATE TWIST UMI ADAPTERS AND PURIFY

STEP 2 LIGATE TWIST UMI ADAPTERS AND PURIFY

Ligate Twist UMI Adapters to the dA-tailed DNA fragments from Step 1 and purify to generate gDNA libraries ready for index introduction through amplification in Step 3.

Reagents Required

- dA-tailed DNA fragments (from Step 1.12)
- Ethanol
- Molecular biology grade water
- 10 mM Tris-HCl pH 8 or Buffer EB (optional, for elution)
- From the Twist Library Preparation EF Kit 1, 2.0:
  - Ligation Master Mix
- From the Twist UMI Adapter System:
  - Twist UMI Adapters
- From the Twist Library Preparation Kit 2:
  - DNA Purification Beads

Before You Begin

- Thaw or place on ice:
  - Twist UMI Adapters (tube; utilized for all samples)
  - Ligation Master Mix
- Prepare 1 ml 80% ethanol for each sample (for use in both Steps 2 and 3 of the protocol).
- Equilibrate DNA Purification Beads to room temperature for at least 30 minutes (for use in both Steps 2 and 3 of the protocol).
- Program a thermal cycler to incubate samples at 20°C with the heated lid set to minimum temperature or turned off. Start the program so that the cycler has reached 20°C when the samples are done being prepared.

LIGATE TWIST UMI ADAPTERS

2.1 Add 3 µl Twist UMI Adapters into each sample well or tube containing the dA-tailed DNA fragments from Step 1. Mix gently by pipetting and keep on ice.

NOTE: For mass inputs other than 50 ng, refer to the Appendix for guidance on adapter volume.

2.2 Invert the Ligation Master Mix a minimum of 10 times until homogenized and place on ice.

NOTE: Do not vortex the Ligation Master Mix.

2.3 Add 20 µl of Ligation Master Mix to each sample from Step 2.1. Pipette a minimum of half the total volume up and down 10 times to ensure complete mixing. Seal or cap the sample plate or tube(s) and pulse-spin to ensure all solution is at the bottom of the tube.

2.4 Incubate the ligation reaction at 20°C for 15 minutes in the thermal cycler, then move the samples to the bench top. Proceed to the Purify step.

⚠ IMPORTANT: Turn off the heated lid or set to minimum temperature.

NOTE: While the thermal cycler program is running, prepare the reagents for Step 3: PCR Amplify Using Twist UDI Primers, Purify, and Perform QC (see Before You Begin).

PURIFY

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

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

2.7 Incubate the samples for 5 minutes at room temperature.

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

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

2.10 Wash the bead pellet by gently adding 200 µl freshly prepared 80% ethanol (do not disturb the pellet). Incubate for 1 minute, then remove and discard the ethanol.

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

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

NOTE: Before pipetting, the bead pellet may be briefly spun to collect ethanol at the bottom of the plate or tube and returned to the magnetic plate.

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

2.14 Remove the plate or tube(s) from the magnetic plate and add 17 µl water to each sample. Mix by pipetting until homogenized.

NOTE: 10 mM Tris-HCl pH 8 or Buffer EB may also be utilized for elution.

2.15 Incubate at room temperature for 2 minutes.

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

2.17 Transfer 15 µl of the clear supernatant containing the ligated libraries to a clean thin- walled PCR 0.2-ml strip-tube or well of a 96-well thermal cycling plate, making sure not to disturb the bead pellet.

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

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

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

Reagents Required

- Ligated libraries (from Step 2.17)
- 80% Ethanol (from Step 2)
- Equilibrated DNA Purification Beads (from Step 2)
- Molecular biology grade water
- 10 mM Tris-HCl pH 8 or Buffer EB (optional, for elution)
- From the Twist Library Preparation EF Kit 1, 2.0
  - Equinox Library Amp Mix (2x)
- From the Twist UMI Adapter System:
  - Twist UDI Primers

⚠ IMPORTANT: Use of P5/P7 Primers (10x) tubes 104127, 103842 contained in the Twist Library Preparation EF Kit 1, 2.0 is not required. Using these primers with the Twist UMI Adapter System will result in a failed PCR amplification.

Before You Begin

- Thaw or place on ice:
  - Equinox Library Amp Mix (2x)
  - Twist UDI Primers (plate with single-use primers)

PREPARE THE THERMAL CYCLER

3.1

Program a thermal cycler with the following conditions. Set the temperature of the heated lid to 105°C.

STEP		TEMPERATURE	TIME	NUMBER CYCLES
1	Initialization	98°C	45 seconds	1
2	Denaturation	98°C	15 seconds	6–8*
	Annealing	60°C	30 seconds	
	Extension	72°C	30 seconds	
3	Final Extension	72°C	1 minute	1
4	Final Hold	4°C	HOLD	–

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

PERFORM PCR

3.2 Add 10 µl of Twist UDI Primer from the provided 96-well plate to each of the gDNA libraries from Step 2.17 and mix well by gentle pipetting.

NOTE: For index selection and pooling guidelines for downstream target enrichment and sequencing, refer to the Appendix.

3.3 Add 25 µl of Equinox Library Amp Mix (2x) to the gDNA libraries from Step 3.2 and mix well by gentle pipetting.

NOTE: Invert Equinox Library Amp Mix (2x) 5 times before use. Do not vortex.

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

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

PURIFY

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

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

3.8 Incubate the samples for 5 minutes at room temperature.

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

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

3.11 Wash the bead pellet by gently adding 200 µl freshly prepared 80% ethanol (do not disturb the pellet), incubate for 1 minute, then remove and discard the ethanol.

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

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

NOTE: Before pipetting, the bead pellet may be briefly spun to collect ethanol at the bottom of the plate or tube and returned to the magnetic plate.

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

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

3.16 Incubate at room temperature for 2 minutes.

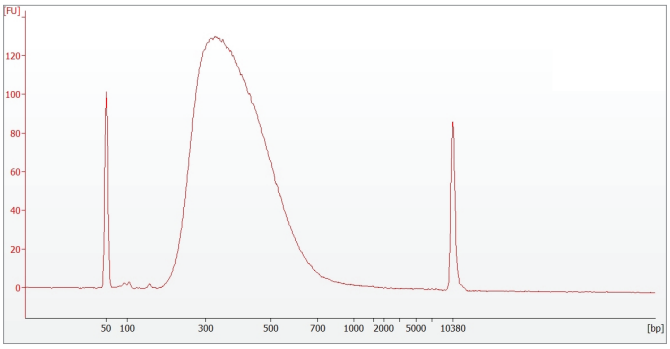
3.17 Place the plate or tubes on a magnetic plate and let stand for 3 minutes or until the beads form a pellet.

3.18 Transfer 20 µl of the clear supernatant containing the Amplified Indexed Libraries to a clean thin-walled PCR 0.2-ml strip-tube or well of a 96-well thermal cycling plate, making sure not to disturb the bead pellet.

PERFORM QC

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

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



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

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

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

END OF WORKFLOW

APPENDIX

UDI SEQUENCES

For a complete guide of the Twist UDI sequences please refer to PDF document DOC-001129 or Excel file DOC-001130. Both files are available for download at twistbioscience.com/resources.

POOLING GUIDELINES

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

Table 1. Twist UDI primer plate layouts and pooling guidelines.
Twist UMI Adapter System: TruSeq Compatible, 16 Samples (105040)

	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										

*PLEASE NOTE: The indexes in the 16 sample plate are not the same in 96 samples, Plate A.

APPENDIX

Twist UMI Adapter System: TruSeq Compatible, 96 Samples, Plates A to D (105041, 105042, 105043, 105044)

Plate A.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	49	57	65	73	81	89
B	2	10	18	26	34	42	50	58	66	74	82	90
C	3	11	19	27	35	43	51	59	67	75	83	91
D	4	12	20	28	36	44	52	60	68	76	84	92
E	5	13	21	29	37	45	53	61	69	77	85	93
F	6	14	22	30	38	46	54	62	70	78	86	94
G	7	15	23	31	39	47	55	63	71	79	87	95
H	8	16	24	32	40	48	56	64	72	80	88	96

Plate B.

	1	2	3	4	5	6	7	8	9	10	11	12
A	97	105	113	121	129	137	145	153	161	169	177	185
B	98	106	114	122	130	138	146	154	162	170	178	186
C	99	107	115	123	131	139	147	155	163	171	179	187
D	100	108	116	124	132	140	148	156	164	172	180	188
E	101	109	117	125	133	141	149	157	165	173	181	189
F	102	110	118	126	134	142	150	158	166	174	182	190
G	103	111	119	127	135	143	151	159	167	175	183	191
H	104	112	120	128	136	144	152	160	168	176	184	192

Plate C.

	1	2	3	4	5	6	7	8	9	10	11	12
A	193	201	209	217	225	233	241	249	257	265	273	281
B	194	202	210	218	226	234	242	250	258	266	274	282
C	195	203	211	219	227	235	243	251	259	267	275	283
D	196	204	212	220	228	236	244	252	260	268	276	284
E	197	205	213	221	229	237	245	253	261	269	277	285
F	198	206	214	222	230	238	246	254	262	270	278	286
G	199	207	215	223	231	239	247	255	263	271	279	287
H	200	208	216	224	232	240	248	256	264	272	280	288

Plate D.

	1	2	3	4	5	6	7	8	9	10	11	12
A	289	297	305	313	321	329	337	345	353	361	369	377
B	290	298	306	314	322	330	338	346	354	362	370	378
C	291	299	307	315	323	331	339	347	355	363	371	379
D	292	300	308	316	324	332	340	348	356	364	372	380
E	293	301	309	317	325	333	341	349	357	365	373	381
F	294	302	310	318	326	334	342	350	358	366	374	382
G	295	303	311	319	327	335	343	351	359	367	375	383
H	296	304	312	320	328	336	344	352	360	368	376	384

APPENDIX

FRAGMENTATION OPTIMIZATION

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

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

ADAPTER LOADING

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

MASS INPUT	VOLUME OF TWIST UMI ADAPTERS (10 µM)
⩾ 30 ng	3 µl
10 ng - 30 ng	2 µl
⩽ 10 ng	1 µl

PCR CYCLE NUMBER

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

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

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

END OF APPENDIX