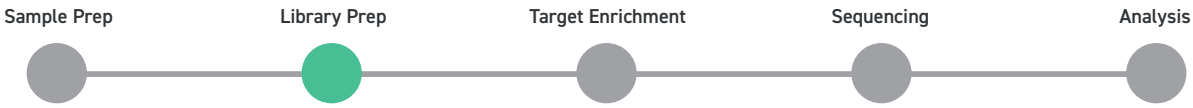
LIBRARY PREPARATION PROTOCOL

Library Preparation EF 2.0 with Enzymatic Fragmentation and Combinatorial Dual Indices

For use with the Twist NGS Workflow

This Twist Library Preparation Kit provides the reagents needed to prepare genomic DNA (gDNA) libraries using enzymatic gDNA fragmentation and Y-shaped adapters. This manual details the steps for generating the amplified, indexed libraries needed for downstream target enrichment and sequencing on Illumina next-generation sequencing (NGS) systems. This library preparation protocol is optimized for use with Twist Target Enrichment Kits and should only be performed with reagents specified or their equivalents.



Twist NGS workflow. The complete NGS workflow takes you from sample preparation to NGS sequencing and data analysis. A component of this workflow, the Twist Library Preparation Protocol, works in conjunction with the other component protocols.

PROTOCOL COMPONENTS

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

CATALOG #	NAME	DESCRIPTION	STORAGE
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
100577: 96 rxn	Twist CD Index Adapter Set	Adapter set, provides dual-indexed combinations, 1 reaction per index	–20°C

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

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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 Combinatorial Dual Indices.

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 this Twist Library Preparation Kit if modifications are made to the protocol.

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

Test the compatibility of your thermal cycler and PCR tubes by incubating at 95°C for up to 5 minutes to ensure the PCR tubes do not crack under heat and pressure. Adjust the tightness of the thermal cycler lid and/or use a spacer specific to the thermal cycler model.

GUIDELINES FOR gDNA SAMPLES

- 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 Indexed Adapter (Step 2.1, page 10)
  - Incubation time for Ligation Reaction (Step 2.4, page 11)
  - PCR cycles for Amplification (Step 3.1, page 13)
- 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 combinatorial dual index adapters. This protocol allows you to perform gDNA library preparation (Steps 1–3) in less than 3 hours.

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

STEP 1 PERFORM DNA FRAGMENTATION, END REPAIR, AND dA-TAILING

Perform enzymatic fragmentation of input gDNA and subsequent end repair and dA-tailing to generate dA-tailed DNA fragments.

Reagents Required

- Genomic DNA (gDNA): 50 ng per sample
- Molecular biology grade water
- Qubit dsDNA Broad Range Quantitation Assay (or equivalent)
- From the Twist Library Preparation EF Kit 1, 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 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 Indexed 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 INDEXED ADAPTERS AND PURIFY

STEP 2 LIGATE INDEXED ADAPTERS AND PURIFY

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

Reagents Required

- dA-tailed DNA fragments (from Step 1.12)
- 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 CD Index Adapter Set:
  - Indexed Adapters
- From the Twist Library Preparation Kit 2:
  - DNA Purification Beads

Before You Begin

- Thaw or place on ice:
  - Indexed Adapters (plate)
  - 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 ADAPTERS

2.1

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

NOTES:

- If you are multiplexing samples for a downstream target enrichment workflow, use the index combinations recommended in the pooling guidelines in the Appendix. To avoid index read failure, do not mix Indexed Adapters from different sets.
- If using other adapter sources, use 5.5 µl of an adapter pair, where each member of the pair is at a concentration of 10 µM (e.g., 5.5 pmol each individual member of the adapter pair; 11 pmol total).
- Adjustment of adapter loading may be required for optimum performance and is dependent on the mass input and/or quality of DNA into fragmentation (Step 1).

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, 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 22 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 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.

PROCEED TO STEP 3: PCR AMPLIFY, PURIFY, AND PERFORM QC

STEP 3 PCR AMPLIFY, PURIFY, AND PERFORM QC

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

Reagents Required

- Ligated, indexed libraries (from Step 2.17)
- 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)
  - P5/P7 Primer Mix (10x)

Before You Begin

- Thaw or place on ice:
  - Equinox Library Amp Mix (2x)
  - P5/P7 Primer Mix (10x)

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

REAGENT	VOLUME PER REACTION*
Equinox Library Amp Mix (2x)	25 µl
P5/P7 Primer Mix (10x)	5 µl
Total	30 µl

**Prepare a master mix for multiple reactions.*

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

3.3 Add 30 µl PCR mix to the ligated, indexed libraries from Step 2.17 and mix well by gentle pipetting.

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 the Purify step.

PURIFY

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

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

3.8 Incubate the samples for 5 minutes at room temperature.

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

3.10 The DNA Purification Beads form a pellet, leaving a clear supernatant. 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.

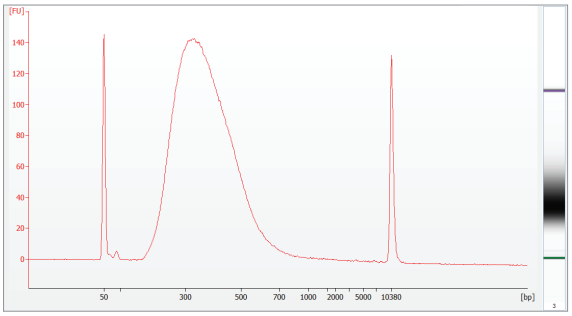
3.17 Place the plate or tubes on a magnetic plate and let stand for 3 minutes or until the beads form a pellet.

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: ADAPTER SEQUENCES AND POOLING GUIDELINES

CD INDEX SEQUENCES

Table 1. Sequences of Compatible Barcodes.

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

Table 2. Adapter Plate Combinatorial Dual Index Layout.

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

APPENDIX: ADAPTER SEQUENCES AND POOLING GUIDELINES

POOLING GUIDELINES

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

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

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

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

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

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.

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

LAST REVISED: August 15, 2022

REVISION	DATE	DESCRIPTION
2.0	Aug 15, 2022	• Language changes to clarify reagent handling • Updated insert size optimization guidelines • Added guidelines for broad DNA input range and fragment insert sizes in Appendix