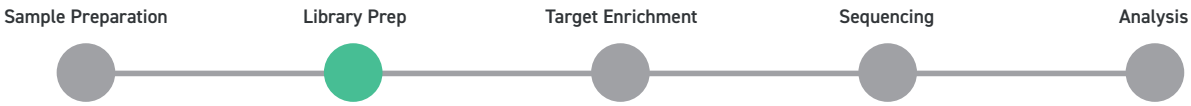
TWIST LIBRARY PREPARATION PROTOCOL

Mechanical 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 mechanically fragmented DNA 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
104176: 16 rxn 104177: 96 rxn	Twist Library Preparation Kit with Amp Mix, Mechanical Fragmentation	Reagents for library construction	
	Twist Library Preparation Kit 1	· 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
	Equinox Library Amp Mix	Equinox Library Amp Mix (2x)	–20°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**.*

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 Using Mechanical Fragmentation and Combinatorial Dual Indices.

PRODUCT	SUGGESTED SUPPLIER
REAGENTS AND CONSUMABLES	
Ethanol (200 proof)	—
Molecular biology grade water	—
10 mM Tris-HCl pH8	—
1.5-ml Microcentrifuge tubes	VWR
0.2-ml Thin-walled PCR strip-tubes	Eppendorf
96-Well thermal cycling plates	VWR
1.5-ml Compatible magnetic stand	Beckman Coulter
96-Well compatible magnetic plate	Alpaqua, Permagen Labware
Qubit dsDNA Broad Range Quantitation Assay	Thermo Fisher Scientific
Agilent DNA 7500 Kit	Agilent Technologies
Agilent High Sensitivity 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 provided instructions. Twist cannot guarantee the performance of this Twist Library Preparation Kit if modifications are made to the protocol.

The 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 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 200–250 bp.
- Suspend fragmented input DNA in Molecular Biology Grade Water, 10 mM Tris-HCl pH 8.0, or Buffer EB.
- The recommended mass input is 50 ng of fragmented gDNA.
- Using higher or lower mass input may require optimization of the following steps in library preparation to achieve optimal performance.
  - Amount of Twist Universal Adapter (Step 2.1, page 10)
  - Incubation time for ligation reaction (Step 2.5, page 11)
- 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

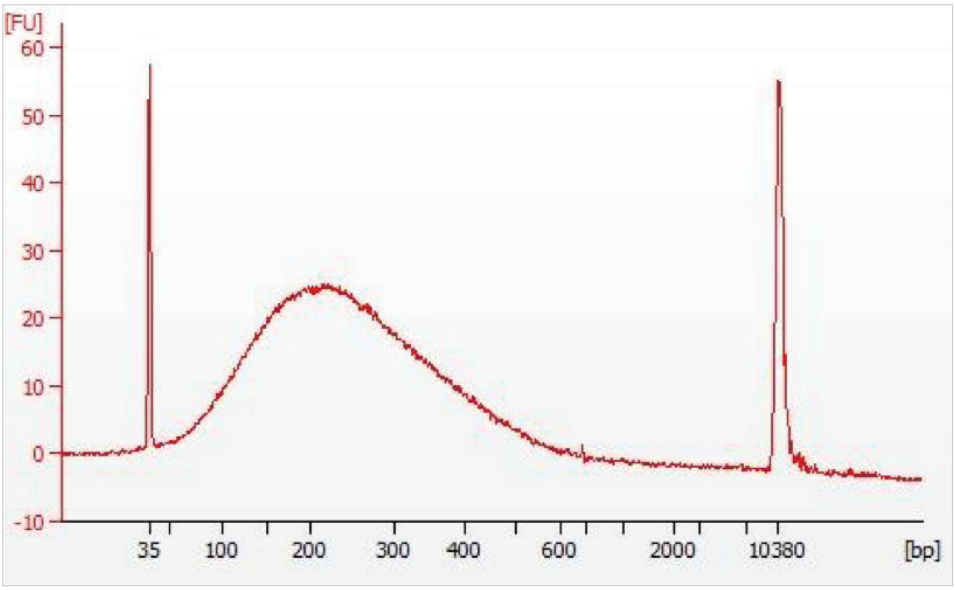
This protocol begins with fragmented input genomic DNA (gDNA) and generates amplified, indexed libraries for subsequent target enrichment. It features combinatorial dual index adapters. This protocol allows you to perform gDNA library preparation (Steps 1–3) in less than 3 hours.

LIBRARY PREPARATION WORKFLOW	TIME
MECHANICAL FRAGMENTATION WITH COMBINATORIAL INDICES (FRAGMENTED DNA, 50 NG STARTING DNA MATERIAL)	
STEP 1 **Perform 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 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 200–250 bp.



DNA fragment size distribution of a gDNA fragment library immediately after mechanical shearing, as analyzed using an Agilent High Sensitivity DNA Assay.

Reagents Required

- Fragmented genomic DNA (gDNA): 50 ng 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, Mechanical Fragmentation:
  - 5x ERA Enzyme Mix
  - 10x ERA Buffer

Before You Begin

- Thaw 5x ERA Enzyme Mix and 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. Start the program to pre-chill the thermal cycler.

	TEMPERATURE	TIME
STEP 1	4°C	HOLD
STEP 2	20°C	30 minutes
STEP 3	65°C	30 minutes
STEP 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 gDNA samples to 5 ng/µl with water.

1.4 Add 10 µl of each diluted gDNA sample (50 ng total gDNA) 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*
Water (chilled)	25 µl
10x ERA Buffer	5 µl
5x ERA Enzyme Mix	10 µl
Total	40 µl

**Prepare a master mix for multiple reactions.*

1.7 Add 40 µl ERA reaction master mix (from Step 1.6) to each 10 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 3 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 and Purify (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 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 1.10)
- Ethanol
- Molecular biology grade water (chilled)
- From the Twist Library Preparation Kit 1, Mechanical Fragmentation:
  - DNA Ligation Mix
  - DNA Ligation Buffer
- From the Twist CD Index Adapter Set:
  - Indexed Adapters
- From the Twist Library Preparation Kit 2:
  - DNA Purification Beads

Before You Begin

- Thaw on ice:
  - Indexed Adapters (plate)
  - DNA Ligation Mix
  - DNA Ligation Buffer
- Prepare 1 ml 80% ethanol for each sample (for use in 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 is at 20°C when the samples are 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 eight samples, 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 you are using other adapter sources, use 5.5 µl of an adapter pair where each member of the pair is present at a concentration of 10 µM (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.

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 the minimum temperature.

NOTE: While the thermal cycler program is running, prepare the reagents for Step 3 (see Step 3: Pre-Capture Amplify, Purify, and Perform QC, Before You Begin).

PURIFY

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

2.7 Add 80 µl of homogenized (0.8x) 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. Do not overdry the bead pellet.

2.15 Remove the plate or tubes from the magnetic plate and add 17 µl water, 10mM Tris-HCl pH 8, or Buffer EB to each sample. Mix by pipetting until homogenized.

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 15 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.

PROCEED TO STEP 3: PRE-CAPTURE AMPLIFY, PURIFY, AND PERFORM QC

STEP 3 PRE-CAPTURE 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 2.18)
- 80% Ethanol (from Step 2)
- Equilibrated DNA Purification Beads (from Step 2)
- Molecular biology grade water
- Equinox Library Amp Mix (2x)
- From the Twist Library Preparation Kit:
  - Amplification Primers, ILMN

Before You Begin

Thaw on ice:

- Amplification Primers, ILMN (tube)
- Equinox Library Amp Mix (2x)

PREPARE THE THERMAL CYCLER AND PCR MIX

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	8–10*
	Annealing	60°C	30 seconds	
	Extension	72°C	30 seconds	
3	Final Extension	72°C	1 minute	1
4	Final Hold	4°C	HOLD	–

**8–10 cycles is recommended when starting with 50–100 ng of high quality gDNA.*

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*
Amplification Primers, ILMN	10 µl
Equinox Library Amp Mix (2x)	25 µl
Total	35 µl

**Prepare a master mix for multiple reactions.*

PERFORM PCR

3.3 Add 35 µl PCR mix to the ligated, indexed libraries from Step 2.18 and mix well by gentle pipetting.

3.4 Pulse-spin the sample plate or tube and immediately transfer it 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 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 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.

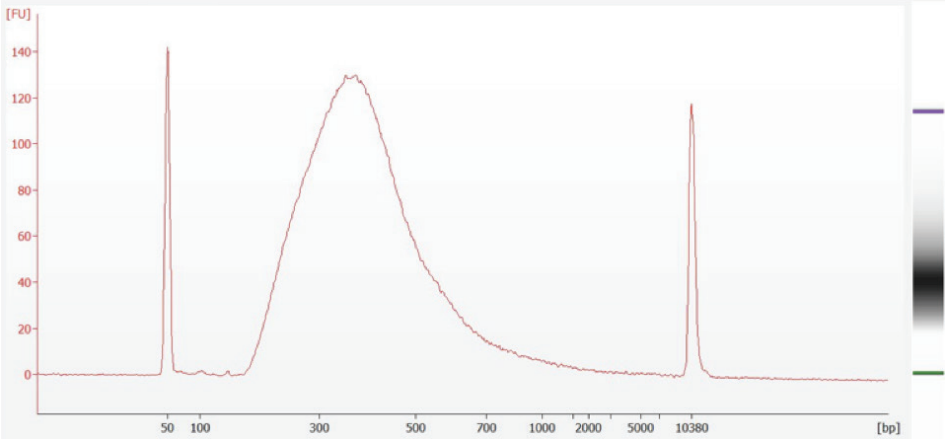
3.16 Incubate at room temperature for 2 minutes.

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 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.

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. Final concentration values should be ≥80 ng/µl, and average fragment length should be 375–425 bp using a range setting of 150–1,000 bp.



Electropherogram generated by an Agilent 7500 DNA analysis of gDNA library samples that were prepared as described. Note the single prominent peak at ~400 bp.

You can proceed with concentrations lower than 80 ng/µl, but low concentrations may reflect inefficient sample preparation and can result in low library diversity after hybridization.

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												

END OF APPENDIX

DOC-001086 REV 2.0 | LAST REVISED: April 27, 2021

REVISION	DATE	DESCRIPTION
2.0	Apr 27, 2021	• Library Amplification Mix is now included with the Library Prep kit. • Kit catalog numbers, kit component list, and workflow steps are updated to include Library Amplification Mix. • Minor workflow steps updated for more clarity.