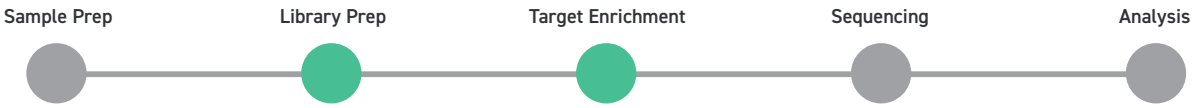
Long-Read Library Preparation and Standard Hyb v2 Enrichment

For use with the Twist NGS Workflow

This Twist long-read library preparation and target enrichment protocol details the steps necessary to prepare genomic DNA (gDNA) libraries using mechanical fragmentation, the Twist Universal Adapter System, and a 16-hour hybridization. The Twist Universal Adapter system consists of Twist Universal Adapters and 10-bp Twist Unique Dual Indexed (UDI) Primers. This long-read protocol is:

- Optimized for use with Twist Mechanical Fragmentation Library Preparation Kit and Universal Adapter System
- Optimized for use with Twist Standard Hyb and Wash Kit v2
- Designed for single or multiplex hybridization reactions using either Twist fixed or custom panels; optional secondary panels (spike-ins) can also be added for additional content



Twist NGS workflow. *The complete workflow takes you from sample preparation to sequencing and data analysis. A component of this workflow—the Twist Long-Read Library Preparation and Standard Hyb v2 Enrichment 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
101280: 16 samples 101281: 96 samples	Twist Mechanical Fragmentation Library Preparation Kit 1	Reagents for library construction • 10x ERA Buffer • 5x ERA Enzyme Mix • DNA Ligation Mix • DNA Ligation Buffer • Amplification Primers	–20°C
	Twist Library Preparation Kit 2	DNA Purification Beads	2–8°C
100401: 16 samples 100573: 96 samples	Twist Library Preparation Kit 2	DNA Purification Beads	2–8°C
101307: 16 samples 101308 (Plate A), 101309 (Plate B), 101310 (Plate C), 101311 (Plate D): 96 samples	Twist Universal Adapter System	Twist Universal Adapters and Twist UDI Primers, provides unique dual-indexed combinations with 1 reaction per index pair	–20°C
104445: 2 reactions 104446: 12 reactions 104447: 96 reactions	Twist Standard Hyb and Wash Kit v2	Twist Hybridization Reagents [Box 1: 101263, 100930, 100982] • Hybridization Mix • Hybridization Enhancer • Amplification Primers Twist Standard Wash Buffers v2 [Box 2: 104133, 104135, 104137] • Binding Buffer • Standard Wash Buffer 1 • Wash Buffer 2	–20°C 2–8°C
100856: 2 reactions 100578: 12 reactions 100767: 96 reactions	Twist Universal Blockers	For the prevention of nonspecific capture • Universal Blockers • Blocker Solution	–20°C

TWIST PROBE PANELS (Ordered separately)			
Choice of panel type and reaction size	Twist Fixed Panel	Fixed content enrichment panel for hybridization reactions (for example, Twist Alliance Long-Read PGx Panel or Twist Alliance Dark Genes Panel)	–20°C
	Twist Custom Panel	Custom enrichment panel for hybridization reactions	–20°C
	(Optional) Secondary Twist Probe Panel	Custom or fixed enrichment panel for adding content to a fixed or custom panel	–20°C

LEGAL

This document may contain references to other third-party resources such as sources of information, hardware or software, products, or services and/or web sites owned or licensed by third parties. Twist Bioscience does not control or take responsibility for any third-party resources, including, without limitation, the accuracy, reliability, copyright compliance, compatibility, performance, legality, or any other aspect of third-party resources. The inclusion of such resources in this document does not imply endorsement by Twist Bioscience of any third-party resources.

Certain processes described in this document may be subject to patent rights or licenses in local jurisdictions, including those owned or licensed by parties other than Twist Bioscience. Purchase of this product does not include a license to perform any such processes. Users of this product may, therefore, be required to obtain a patent license depending upon the particular application and country in which the product is used before performing such processes.

Twist Bioscience, the various Twist logos, and the Twist NGS logo used herein are trademarks of Twist Bioscience Corporation. All other trademarks are the property of their respective owners.

INTENDED USE

This product is for research use only. This product is not intended for the diagnosis, prevention, or treatment of a disease or condition. Twist Bioscience assumes no liability regarding use of the product for applications in which it is not intended. Twist Bioscience has not demonstrated or validated the use of this protocol for customer intended use. As such, the protocol may need to be optimized by the customer to fit specialized needs.

TABLE OF CONTENTS

MATERIALS SUPPLIED BY USER

The following materials or their equivalent are required to generate libraries using the Twist Mechanical Fragmentation Library Preparation Kit with Universal Adapter System, and Twist Standard Hyb and Wash Kit v2.

PRODUCT	SUGGESTED SUPPLIER
REAGENTS AND CONSUMABLES	
KOD Xtreme Hot Start DNA polymerase*	Millipore
PrimeSTAR GXL Polymerase**	Takara
Dynabeads M-270 Streptavidin	Invitrogen (PN 65305)
Ethanol (200 proof)	—
Molecular Biology Grade (nuclease-free) Water	—
10 mM and 200 mM Tris-HCl pH8	—
2 N NaOH	—
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	Permagen Labware (PN T480)
Qubit dsDNA High Sensitivity and/or Broad Range Quantitation Assay	Thermo Fisher Scientific
Femto Pulse gDNA 165 kb Analysis Kit (Alternative: Bioanalyzer High Sensitivity DNA Kit)	Agilent Technologies
EQUIPMENT	
Pipettes and Tips (low-retention and wide-bore)	—
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
Femto Pulse Systems (Alternative: Bioanalyzer)	Agilent Technologies
Vacuum concentrator (if unavailable, see Appendix A)	—

**In Japan this reagent is called "KOD FX" and is supplied by Toyobo (KFX-101)*

***PrimeSTAR GXL DNA Polymerase supplied by Takara can be used as an alternative to KOD Xtreme*

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 Twist Long-read Workflow 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.

This protocol details different methods for mixing reagents (gentle pipetting, flicking or tapping, vortexing), depending on the volume, vessel, and reagents involved.

Ensure input DNA is RNA-free. If RNA is present, it is recommended to treat the input DNA with RNase.

GUIDELINES FOR SAMPLES

- Use any desired mechanical shearing method (i.e. Megaruptor or Covaris g-Tube) 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 Femto Pulse Systems before proceeding with end repair and dA-tailing reactions.
- Suspend fragmented input DNA in Molecular Biology Grade Water, 10 mM Tris-HCl pH 8.0, or Buffer EB.
- The recommended mass input is 200–1000 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 11)
  - Incubation time for ligation reaction (Step 2.5, page 12)
- 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.

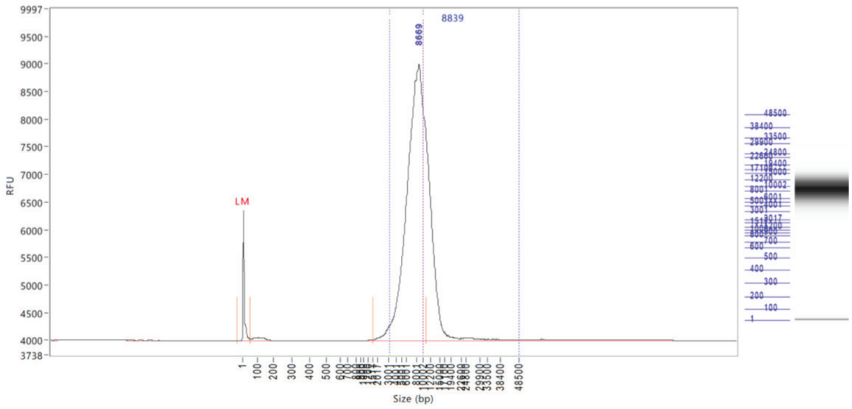
PROTOCOL OVERVIEW

This protocol begins with fragmented input gDNA and generates amplified, indexed libraries for subsequent target enrichment. Please refer to the manufacturer's instructions for the Diagenode Megaruptor, Covaris g-Tube, or other fragmentation instrument for recommendations on fragmentation setting of preferred fragment size. Size selection after shearing is recommended to remove short fragments (<3 kb) before End Repair and A-Tailing. The library preparation workflow features Twist Universal Adapters with UDI primers. The enrichment workflow features standard hybridization v2 reagents. This whole protocol allows you to perform gDNA library preparation (Steps 1–3) in less than 5 hours, followed by QC and pooling for an overnight hybridization (Steps 4–8).

	LIBRARY PREPARATION WORKFLOW	TIME
	MECHANICAL FRAGMENTATION (FRAGMENTED DNA, 200–1000 NG STARTING DNA MATERIAL)	
STEP 1	**Perform End Repair and dA-Tailing** dA-tailed DNA fragments	**1 hour**
STEP 2	**Ligate Twist Universal Adapters and Purify** Ligated gDNA libraries	**2 hours**
STEP 3	**Pre-Capture PCR Amplify, Purify, and Perform QC** Amplified indexed libraries	**2 hours**
	HYBRIDIZATION TARGET ENRICHMENT WORKFLOW (AMPLIFIED INDEXED LIBRARIES)	
STEP 4	**Prepare Libraries for Hybridization** Indexed library pool STOPPING POINT	**1 hour**
STEP 5	**Hybridize Capture Probes With Pools** Hybridized targets in solution	**16 hours**
STEP 6	**Bind Hybridized Targets to Streptavidin Beads** Captured targets on beads	**1.5 hours**
STEP 7	**Post-Capture PCR Amplify, Purify, and Perform QC** Enriched libraries STOPPING POINT	**3 hours**
STEP 8	**Convert Libraries for Long-Read Sequencing Platform** Libraries ready for sequencing on a long-read sequencer platform	**—**

STEP 1 PERFORM END REPAIR AND dA-TAILING

Use mechanically fragmented input gDNA to perform end repair and subsequent dA-tailing to generate dA-tailed DNA fragments. Fragmentation can be done using either Megaruptor or Covaris g-Tube. For optimum performance, analyze the size distribution of the fragmented input gDNA with an Agilent Femto Pulse System before proceeding with end repair and dA-tailing reactions. Ensure the mode of the fragment size distribution is >3000 bp. If more than 10% of fragment size is <3000 bp, please perform beads-based size selection. Perform bead-based size selection using a 3.7X bead ratio of diluted DNA Purification Beads (35% v/v) described in Step 2.19.



DNA fragment size distribution of a gDNA fragment library immediately after mechanical shearing and size selection, as analyzed using an Agilent Femto Pulse Assay.

Reagents Required

- Fragmented gDNA: 200–1000 ng per sample (Twist recommends using either a Megaruptor or Covaris g-Tube, but this can be 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.

STEP	TEMPERATURE	TIME
1	4°C	HOLD
2	20°C	30 minutes
3	65°C	30 minutes
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 20–100 ng/µl with water.

1.4 Add 10 µl of each diluted gDNA sample (200–1000 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 TWIST UNIVERSAL ADAPTERS AND PURIFY

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

Reagents Required

- dA-tailed DNA fragments (from Step 1.10)
- Ethanol
- Molecular biology grade water (chilled)
- From the Twist Library Preparation Mechanical Fragmentation Kit 1:
  - DNA Ligation Mix
  - DNA Ligation Buffer
- From the Twist Library Preparation Kit 2:
  - DNA Purification Beads
- From the Twist Universal Adapter System:
  - Twist Universal Adapters

Before You Begin

- Thaw or place on ice:
  - DNA Ligation Mix
  - DNA Ligation Buffer
- Prepare at least 1.2 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 TWIST UNIVERSAL ADAPTERS

2.1 Add 5 µl Universal Adapters into each sample well or tube containing the dA-tailed DNA fragments from Step 1. Mix gently by pipetting and keep on ice.

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)	15 µl
DNA Ligation Buffer	20 µl
DNA Ligation Mix	10 µl
Total	**45 µl**

**Prepare a master mix for multiple reactions.*

2.3 Add 45 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 1 hour 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.

PURIFY

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

2.7 Add 80 µl of homogenized DNA Purification Beads to each ligation sample from Step 2.5. Mix well by gentle pipetting.

2.8 Incubate the samples for 10 minutes at room temperature.

2.9 Place the samples on a magnetic plate for 5–10 minutes 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 at room temperature, 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 3-5 minutes or until the bead pellet is dry.

⚠ IMPORTANT: Do not overdry the beads. This may result in lower recovery of DNA. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

2.15 Remove the plate or tubes from the magnetic plate and add 37 µl 10mM Tris-HCl pH 8 to each sample. Mix by pipetting until homogenized.

2.16 Incubate at room temperature for 5 minutes.

2.17 Place the plate or tubes on a magnetic plate and let stand for 3-5 minutes or until the beads form a pellet.

2.18 Transfer 35 µl of the clear supernatant containing the ligated 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.

SIZE SELECTION

2.19 Prepare diluted DNA Purification Beads (35% v/v) by mixing 3.5 ml DNA Purification Beads and 6.5 ml 10 mM Tris-HCl pH 8. Add 129.5 µl of diluted DNA Purification Beads (3.7x, 35% v/v) to each ligation sample from Step 2.18. Mix well by gentle pipetting.
NOTE: Diluted beads can be stored at 4°C for up to three months.

2.20 Incubate the samples for 10 minutes at room temperature.

2.21 Place the samples on a magnetic plate for 5–10 minutes or until the supernatant is clear.

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 at room temperature, then remove and discard the ethanol.

2.24 Repeat the 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 3–5 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 24 µl 10 mM Tris-HCl pH 8 to each sample. Mix by pipetting until homogenized.

2.28 Incubate at room temperature for 5 minutes.

2.29 Place the plate or tubes on a magnetic plate and let stand for 3–5 minutes or until the beads form a pellet.

2.30 Transfer 22 µl of the clear supernatant containing the ligated 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 ligated gDNA libraries with Twist UDI Primers, purify them, and perform quality control (QC) analysis to complete library preparation.

Reagents Required

- Ligated libraries (from Step 2.30)
- 80% Ethanol (from Step 2)
- Equilibrated DNA Purification Beads (from Step 2)
- Molecular biology grade water
- KOD Xtreme Hot Start DNA Polymerase, 2X XtremeBuffer, dNTPs*
- From the Twist Universal Adapter System:
  - Twist UDI Primers (96-well plate)
- Femto Pulse gDNA 165 kb Analysis Kit
- Qubit dsDNA High Sensitivity or Broad Range Assay

Before You Begin

Thaw on ice:

- Twist UDI Primers (96-well plate with single use primers)
- KOD Xtreme Hot Start DNA Polymerase

** For alternative polymerase guidance, refer to Appendix C (see step C3.1)*

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	94°C	2 minutes	1
2	Denaturation	98°C	10 seconds	6–8*
	Annealing	60°C	30 seconds	
	Extension	68°C	10 minutes	
3	Final Extension	68°C	10 minutes	1
4	Final Hold	4°C	HOLD	–

**6–8 cycles is recommended when starting with 200–1000 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*
2X XtremeBuffer	50 µl
dNTPs (2 mM each)	20 µl
KOD Xtreme Hot Start DNA Polymerase	2 µl
Total	**72 µl**

**Prepare a master mix for multiple reactions.*

PERFORM PCR

3.3 Add 6 µl Twist UDI Primers from the provided 96-well plate to each of the ligated libraries from Step 2.30 and mix well by gentle pipetting. Add 72 µl PCR master mix to each sample. Pulse-spin the sample plate or tube.

3.4 Immediately transfer samples 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 (0.5X) homogenized DNA Purification Beads to the reaction from Step 3.5. Mix well by gentle pipetting.
NOTE: This purification uses standard, undiluted DNA Purification Beads

3.8 Incubate the samples for 10 minutes at room temperature.

3.9 Place the samples on a magnetic plate for 5–10 minutes.

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 at room temperature, 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 3–5 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.

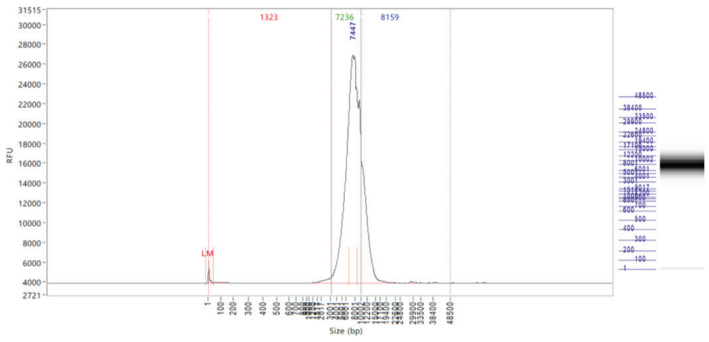
3.16 Incubate at room temperature for 5 minutes.

3.17 Place the plate or tubes on a magnetic plate and let stand for 3–5 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 Femto Pulse gDNA 165 kb Analysis Kit. Final concentration values should be ≥50 ng/µl, and average fragment length should be between 3,000–20,000 bp using a range setting of 300–48,500 bp.



Peak	Size (bp)	Conc. (ng/uL)	From (bp)	To (bp)	Avg. Size (bp)	CV%	RFU	Corr. Peak Area
1	1 (LM)	0.0006	0	41	4	228.98	2232	16.421
2	7447	0.2132	6679	8839	7651	7.68	23012	575.406

TIC:	0.2132	ng/uL
TIM:	0.0459	nmole/L
Total Conc.:	0.5295	ng/uL
GQN:	2.0	
Threshold:	10000	

Smear Analysis						
	1 bp to 3000 bp	0.0107 ng/ul	2.0 %Total	0.0133 nmole/L	1323 Avg. Size (bp)	91.75 %CV
	3000 bp to 48500 bp	0.5218 ng/ul	98.5 %Total	0.1053 nmole/L	8159 Avg. Size (bp)	32.51 %CV
	3000 bp to 10000 bp	0.4174 ng/ul	78.8 %Total	0.0950 nmole/L	7236 Avg. Size (bp)	20.85 %CV

Electropherogram generated by an Agilent Femto Pulse gDNA 165 kb Analysis of gDNA library samples that were prepared as described. Note the single prominent peak at ~7000 bp.

3.19 (cont.)

You can proceed with concentrations lower than 50 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.

STEP 4 PREPARE LIBRARIES FOR HYBRIDIZATION

This step involves aliquoting the appropriate amount of amplified, indexed libraries (generated previously in library preparation) and preparing the hybridization reaction solution.

- When multiplexing, follow the pooling guidelines included in the Appendix A.
- If vacuum concentrator is unavailable, see Appendix B.

Reagents Required

- Amplified, indexed library

ALIQUOT AND DRY DOWN THE LIBRARY

This protocol supports a single or multiplex (up to 8-plex) hybridization capture. The amount of indexed library to use depends on the number of indexed samples per pool.

4.1

Use the concentration of each amplified, indexed library to calculate the volume (in µl) of each library needed for hybridization:

- Determine the amount of each indexed library per pool from the table below.
- Divide the amount of each indexed library per pool by the concentrations measured in ng/µl from the library preparation QC.

For example: If multiplexing eight libraries per hybridization reaction, the amount of each library will be 500 ng and the total mass of the pool will be 4,000 ng.

NUMBER OF INDEXED SAMPLES PER POOL	AMOUNT OF EACH INDEXED LIBRARY PER POOL	TOTAL MASS PER POOL
1	1,500 ng	1,500 ng
2	1,500 ng	3,000 ng
3	1,000 ng	3,000 ng
4	1,000 ng	4,000 ng
8	500 ng	4,000 ng

NOTES:

- If the amount of library you have is insufficient, you can use a smaller amount; using less, however, may result in decreased library complexity and a low capture yield.
- More than 4 µg total DNA may lead to reduced performance of the enrichment.

4.2 Transfer the calculated volumes from each amplified indexed library to an indexed library pool reaction tube for each hybridization being performed. Clean thin-walled PCR 0.2-ml strip-tube or well of a 96- well thermal cycling plate are recommended to avoid unnceccesary transfers in downstream steps.

NOTE: Check for a proper seal on the tube(s) as evaporation may occur, leading to decreased performance.

4.3 Pulse-spin the indexed library pool tube(s) to minimize the amount of bubbles present.

4.4 Dry the indexed library pool(s) using a vacuum concentrator using low or no heat.

NOTE: If an alternate method to drydown is desired, proceed to Appendix B: Alternate Pre-Hybridization DNA Concentration Protocol.

STOPPING POINT: If not proceeding immediately to Step 5, store the dried indexed library pool at –20°C for up to 24 hours.

STEP 5 HYBRIDIZE CAPTURE PROBES WITH POOLS

Use the dried indexed library pool(s) from Step 4 for performing the hybridization reaction.
⚠ IMPORTANT: Before proceeding with this step, test the compatibility of your thermal cycler and PCR tubes or plates by incubating them at 95°C for up to 5 minutes to ensure they 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.

Reagents Required

- Indexed library pool(s) from Step 4
- Twist fixed or custom panel
- Twist custom secondary (spike-in) panel(s) (optional)
- From Twist Hybridization Reagents:
  - Hybridization Mix
  - Hybridization Enhancer
- From Twist Universal Blockers:
  - Universal Blockers
  - Blocker Solution (If using a non-human capture panel, replace with species-specific blocking solution, not provided)

Before You Begin

- Thaw all required reagents on ice, then pulse-vortex for 2 seconds to mix and then pulse-spin.
- Set a heat block to 65°C.

PREPARE THE HYBRIDIZATION SOLUTION

5.1

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

STEP	TEMPERATURE	TIME	# OF CYCLES
1	95°C	HOLD	–
2	95°C	5 minutes	1
3	90°C	5 minutes	1
4	85°C	5 minutes	1
5	80°C	5 minutes	1
6	75°C	5 minutes	1
7	70°C	HOLD	–

5.2 Heat the Hybridization Mix at 65°C in the heat block for 10 minutes or until all precipitate is dissolved. Then cool it to room temperature on the benchtop for 5 minutes.

5.3 Resuspend the Dried Indexed Library Pool by adding the reagents described below. Mix this entire Hybridization Reaction thoroughly by pipetting, making sure not to generate bubbles. Pulse-spin to ensure all solution is at the bottom of the tube.

NOTE: Hybridization Mix is very viscous. Pipette slowly to ensure accurate pipetting volume.

REAGENT	VOLUME PER REACTION
Dried Indexed Library Sample	—
Blocker Solution*	5 µl
Universal Blockers	7 µl
Hybridization Mix	20 µl
Twist Fixed or Custom Panel	4 µl
Optional: Secondary Panel (in place of water)	4 µl
Water (up to Total Volume)	(0-4) µl
Total	**40 µl**

* ⚠ IMPORTANT: If using a non-human capture panel, replace with species-specific blocking solution, not provided.

PERFORM THE HYBRIDIZATION REACTION

5.4 Pulse-spin the tube(s) to ensure all solution is at the bottom of the tube(s).

5.5 Add 30 µl Hybridization Enhancer to the top of the entire capture reaction.

5.6 Pulse-spin the tube(s) to ensure no bubbles are present.

5.7 Transfer the Hybridization Reaction into the thermal cycler and start the thermal cycler program. Incubate for 16 hours at the 70°C HOLD step.

⚠ IMPORTANT: Seal the tube(s) tightly to prevent excess evaporation over the 16-hour incubation.

NOTE: Halting hybridization between 15–17 hours will not affect downstream capture quality.

PROCEED TO STEP 6: BIND HYBRIDIZED TARGETS TO STREPTAVIDIN BEADS

STEP 6 BIND HYBRIDIZED TARGETS TO STREPTAVIDIN BEADS

Reagents Required

- Hybridization reactions (from Step 5.7)
- From the Twist Wash Buffers:
  - Binding Buffer
  - Standard Wash Buffer 1
  - Wash Buffer 2
- From the Twist Library Preparation Kit 2:
  - DNA Purification Beads
- From third party vendor:
  - Invitrogen Dynabeads M-270 Streptavidin Beads
  - 2 N NaOH
  - KOD Xtreme Hot Start DNA Polymerase, 2X XtremeBuffer, dNTPs*

Before You Begin

- Preheat the following tubes at 48°C until any precipitate is dissolved:
  - Binding Buffer
  - Standard Wash Buffer 1
  - Wash Buffer 2
- For each hybridization reaction:
  - Equilibrate 800 µl Binding Buffer to room temperature
  - Equilibrate 225 µl Std Wash Buffer 1 to 68°C
  - Leave 700 µl Wash Buffer 2 at 48°C
- Equilibrate the Dynabeads M-270 Streptavidin Beads to room temperature for at least 30 minutes
- Make fresh 0.2 N NaOH using 100 µl of 2 N NaOH into 900 µl of molecular biology grade water
- In preparation for Step 7.4 (Post-Capture PCR Amplify, Purify, and Perform QC):
  - Thaw on ice:
    - KOD Xtreme Hot Start DNA Polymerase, 2X XtremeBuffer, dNTPs*
    - Amplification Primers
- Equilibrate DNA Purification Beads to room temperature for at least 30 minutes

PREPARE THE BEADS

6.1 Vortex the pre-equilibrated Streptavidin Binding Beads until mixed.

6.2 Add 100 µl Streptavidin Binding Beads to a 1.5-ml microcentrifuge tube. Prepare one tube for each hybridization reaction.

6.3 Add 200 µl Binding Buffer to the tube(s) and mix by pipetting.

6.4 Place the tube(s) on a magnetic stand for 1 minute, then remove and discard the clear supernatant. Make sure to not disturb the bead pellet. Remove the tube from the magnetic stand.

** For alternative polymerase guidance, refer to Appendix C (see Step C 7.1)*

6.5 Repeat the wash (Steps 6.3 and 6.4) two more times for a total of three washes.

6.6 After removing the clear supernatant from the third wash, add a final 200 µl Binding Buffer and resuspend the beads by vortexing until homogenized.

6.7 Heat the resuspended beads at 68°C for at least 10 min before continuing to step 6.8.

BIND THE TARGET

6.8 After the hybridization (Step 5.10) is complete, open the thermal cycler lid and directly transfer the volume of each hybridization reaction into a corresponding tube of preheated Streptavidin Binding Beads from Step 6.7. Mix by pipetting and flicking.

⚠ IMPORTANT: Rapid transfer directly from the thermal cycler at 70°C is a critical step for minimizing off-target binding. Do not remove the tube(s) of hybridization reaction from the thermal cycler or otherwise allow it to cool to less than 70°C before transferring the solution to the washed Streptavidin Binding Beads. Allowing to cool to room temperature for less than 5 minutes will result in as much as 10–20% increase in off-target binding.

6.9 Incubate the tube(s) of the hybridization reaction with the Streptavidin Binding Beads for 10 minutes at 68°C, agitation is not required.

NOTE: **Do not vortex.** Aggressive mixing is not required.

6.10 Remove the tube(s) containing the hybridization reaction with Streptavidin Binding Beads from the mixer and pulse-spin to ensure all solution is at the bottom of the tube(s).

6.11 Place the tube(s) on a magnetic stand for 1 minute.

6.12 Remove and discard the clear supernatant including the Hybridization Enhancer. Do not disturb the bead pellet.

NOTE: Some Hybridization Enhancer may be visible after supernatant removal and throughout each wash step. It will not affect the final capture product.

6.13 Remove the tube(s) from the magnetic stand and add 200 µl 68°C Standard Wash Buffer 1. Mix by pipetting.

⚠ IMPORTANT: Ensure that beads are mixed well by pipetting or flicking.

6.14 Incubate the tube(s) for 5 minutes at 68°C.

6.15 Pulse-spin to ensure all solution is at the bottom of the tube(s).

6.16 Transfer the entire volume from Step 6.15 (~200 µl) into a new 1.5-ml microcentrifuge tube, one per hybridization reaction. Place the tube(s) on a magnetic stand for 1 minute.

⚠ IMPORTANT: This step reduces background from non-specific binding to the surface of the tube.

6.17 Remove and discard the clear supernatant. Make sure to not disturb the bead pellet.

6.18 Remove the tube(s) from the magnetic stand and add 200 µl of 48°C Wash Buffer 2. Mix by pipetting, then pulse-spin to ensure all solution is at the bottom of the tube(s).

6.19 Incubate the tube(s) for 5 minutes at 48°C.

6.20 Place the tube(s) on a magnetic stand for 1 minute.

6.21 Remove and discard the clear supernatant. Make sure to not disturb the bead pellet.

6.22 Repeat the wash (Steps 6.18–6.21) two more times, for a total of three washes.

6.23 After the final wash, use a 10 µl pipette to remove all traces of supernatant. Proceed immediately to the next step. Do not allow the beads to dry.
NOTE: Before removing supernant, the bead pellet may be briefly spun to collect supernatant at the bottom of the tube or plate and returned to the magnetic plate.

6.24 Remove the tube(s) from the magnetic stand and add 12 µl water. Mix by pipetting until homogenized, then place this solution, hereafter referred to as the Streptavidin Binding Bead slurry, on ice.

6.25 Add 12 µl 0.2 N NaOH (freshly diluted from 2 N NaOH) to the Streptavidin Binding Bead slurry. Vortex and spin down. Incubate at room temperature for 5 minutes.

6.26 Add 24 µl 200 mM Tris-HCl pH 8 to the mixture from 6.25. Vortex and spin down.

6.27 Place the mixture from 6.26 on a magnetic rack and transfer the supernatant into a clean thin-walled PCR 0.2-ml strip-tube or well of a 96-well thermal cycling plate. Discard the beads.

PROCEED TO STEP 7: POST-CAPTURE PCR AMPLIFY, PURIFY, AND PERFORM QC

STEP 7 POST-CAPTURE PCR AMPLIFY, PURIFY, AND PERFORM QC

Reagents Required

- Supernatant from Step 6.27
- Ethanol
- Molecular biology grade water
- Reagents thawed and equilibrated in Step 6:
  - DNA Purification Beads
  - KOD Xtreme Hot Start DNA Polymerase, 2X XtremeBuffer, dNTPs*
  - Amplification Primers
- Femto Pulse gDNA 165 kb Analysis Kit
- Qubit dsDNA High Sensitivity Quantitation Assay

Before You Begin

- Prepare 500 µl 80% ethanol for each supernatant (from Step 6.27) to be processed.

** For alternative polymerase guidance, refer to Appendix C (see Step C7.1)*

PREPARE THE BEADS, THERMAL CYCLER, AND PCR MIX

7.1

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

	STEP	TEMPERATURE	TIME	# OF CYCLES
1	Initialization	94°C	2 minutes	1
2	Denaturation	98°C	10 seconds	Varies
	Annealing	60°C	30 seconds	
	Extension	68°C	10 minutes	
3	Final Extension	68°C	10 minutes	1
4	Final Hold	4°C	HOLD	–

PANEL SIZE	# OF CYCLES MULTIPLEX
10–50 Mb	12
1–10 Mb	13
500–1,000 kb	14
100–500 kb	15
50–100 kb	16
<50 kb	17

NOTE: Number of amplification cycles may vary depending on hybridization reaction size. If low yield is observed, increase number of cycles by 3-5. Use the minimum cycles necessary to produce sufficient yield; increasing amplification can lead to a shorter final library.

7.2

Place the strip-tubes or plate containing the supernatant from Step 6.27 on ice until ready to use in the next step.

⚠ IMPORTANT: If you recover <50 µl, add molecular biology grade water to bring the volume to 50 µl.

7.3

Prepare a PCR mixture by adding the following reagents to the tube(s) containing the supernatant. Mix by pipetting.

7.3 (cont.)

REAGENT	VOLUME PER REACTION
Supernatant from Step 6.27	50 µl
Amplification Primers	6 µl
2X XtremeBuffer	100 µl
dNTPs (2mM each)	40 µl
KOD Xtreme Hot Start DNA Polymerase	4 µl
Total	**200 µl**

PCR AMPLIFY

7.4 Split the 200 µl reaction into two 100 µl aliquots in a strip tube or plate. When processing multiple samples mark the two wells with an appropriate label. Pulse-spin the tubes, transfer them to the thermal cycler and start the cycling program.

7.5 When the thermal cycler program is complete, remove the tube(s) from the block and immediately proceed to the Purify step.

PURIFY

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

7.7 Combine the two separated 100 µl reactions into one 1.5-ml tube. Add 100 µl (0.5X) DNA Purification Beads to the 1.5-ml tube(s). Mix well by gentle pipetting.

7.8 Incubate for 10 minutes at room temperature.

7.9 Place the tube(s) on a magnetic plate for 5–10 minutes or until the supernatant is clear.

7.10 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 clear supernatant.

7.11 Wash the bead pellet by gently adding 500 µl freshly prepared 80% ethanol (do not disturb the pellet). Incubate for 1 minute at room temperature, then remove and discard the ethanol.

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

7.13 Carefully remove all remaining ethanol using a 10 µl pipette, making sure to not 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.

7.14 Air-dry the bead pellet on the magnetic plate for 3–5 minutes or until the bead pellet is dry. Do not overdry the bead pellet.

7.15 Remove the tube(s) from the magnetic plate and add 42 µl water, 10 mM Tris-HCl pH 8, or Buffer EB to each capture reaction. Mix by pipetting until homogenized.

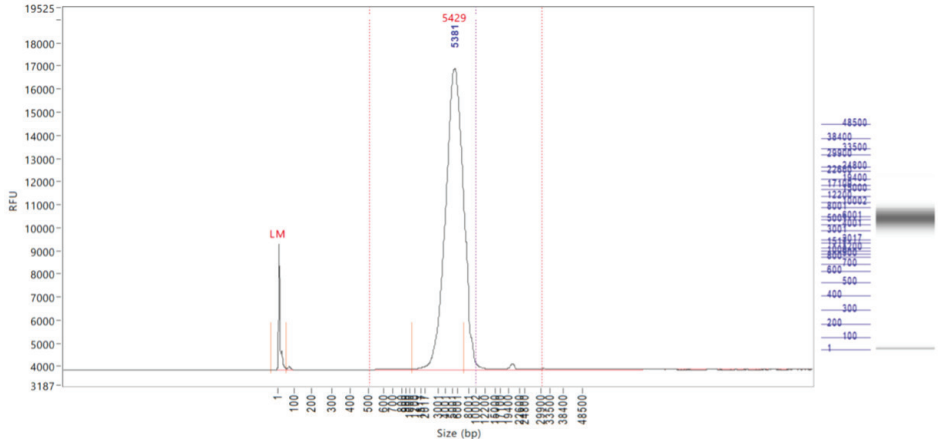
7.16 Incubate at room temperature for 5 minutes.

7.17 Place the plate or tube(s) on a magnetic plate and let stand for 3–5 minutes or until the beads fully pellet.

7.18 Transfer 40 µl of the clear supernatant containing the enriched library to a clean thin-walled PCR 0.2-ml strip-tube or well of a 96-well thermal cycling plate, making sure to not disturb the bead pellet.

PERFORM QC

7.19 Validate and quantify each enriched library using a Femto Pulse gDNA 165 kb Analysis Kit (or Bioanalyzer High Sensitivity DNA Kit) and a Qubit dsDNA High Sensitivity or Broad Range Quantitation Assay.



Electropherogram generated by a Femto Pulse gDNA 165kb Analysis of the enriched gDNA library samples that were prepared as described. Note the single prominent peak.

7.20 PacBio users should continue on to preparing multiplexed amplicon libraries using the SMRTbell® prep kit 3.0 protocol with workflow “Primer barcoded samples.” See the PacBio protocol for input quantity requirements for SMRTbell library construction.

For PacBio Sequencing refer to: pacb.com/products-and-services/applications/targeted-sequencing

Oxford Nanopore users should continue with Oxford Nanopore Ligation sequencing amplicons v14. See the ONT protocol for input quantity requirements during library construction.

For Oxford Nanopore Sequencing refer to: nanoporetech.com/products/sequence

END OF WORKFLOW

APPENDIX A: UDI SEQUENCES AND POOLING GUIDELINES

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 Universal Adapter System: TruSeq Compatible, 16 Samples (101307)

	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 A: UDI SEQUENCES AND POOLING GUIDELINES

Twist UMI Adapter System: TruSeq Compatible, 96 Samples, Plates A to D (101308, 101309, 101310, 101311).

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

END OF APPENDIX A

APPENDIX B: ALTERNATE PRE-HYBRIDIZATION DNA CONCENTRATION PROTOCOL

Reagents Required

- Amplified, indexed library pool(s) from Step 3.19
- Ethanol
- Molecular biology grade water
- DNA Purification Beads
- From Twist Universal Blockers:
  - Universal Blockers
  - Blocker Solution

Before You Begin

- Equilibrate DNA Purification Beads to room temperature for at least 30 minutes.
- Vortex the pre-equilibrated DNA Purification Beads until well mixed.
- Prepare 500 µl fresh 80% ethanol for each sample to be processed.

CONCENTRATE THE DNA LIBRARIES

B.1 Add 1.8x homogenized DNA Purification Beads to the tube(s) containing the DNA library(ies) from Step 4.3. Mix well by vortexing.
NOTE: For amplified, indexed library pool(s) with a volume of less than 10 µl, bring volume up to 10 µl with water.

B.2 Incubate for 10 minutes at room temperature.

B.3 Pulse spin to ensure all the solution is at the bottom of the tube(s) and place the tube(s) on a magnetic plate or rack for 5–10 minutes or until the solution is clear.

B.4 The DNA Purification Beads form a pellet, leaving a clear supernatant. Without removing the plate or tube(s) from the magnetic plate or rack, remove and discard the clear supernatant.

B.5 Wash the bead pellet by gently adding 200 µl freshly prepared 80% ethanol (do not disturb the pellet). Incubate for 1 minute at room temperature, then remove and discard the ethanol.

B.6 Repeat this wash once, for a total of two washes, while keeping the tube on the magnetic plate.

B.7 Carefully remove all remaining ethanol using a 10 µl pipette, making sure to not disturb the bead pellet.
NOTE: If necessary, pulse spin to ensure complete removal of ethanol.

B.8 Air-dry the bead pellet on a magnetic plate for 1–5 minutes or until the bead pellet is dry. Do not overdry the bead pellet.

B.9 Remove the tube(s) from the magnetic plate or rack and add 7 µl Universal Blockers and 5 µl Blocker Solution*. Mix by pipetting until homogenized.

*⚠ IMPORTANT: If using a non-human capture panel, replace with a species-specific blocking solution, not provided.

B.10 Proceed to Step 5.1 and continue the protocol omitting the addition of the universal blockers and blocker solution to the master mix in Step 5.3.

END OF APPENDIX B

APPENDIX C: ALTERNATIVE POLYMERASE GUIDANCE

This protocol has been validated with an additional 3rd-party polymerase PrimeSTAR GXL. To use this polymerase, modify the PCR master mix and cycling conditions in library preparation and post-capture amplification.

Required Components:

- Adapter-ligated library from Step 2.30 or supernatant from Step 6.27
- Molecular biology grade water
- From Twist Hybridization Reagents:
  - Amplification Primers
- From Twist Universal Adapter System:
  - Twist UDI Primers (96-well plate)
- PrimeSTAR GXL Reagents
  - 5X PrimeSTAR GXL Buffer
  - dNTP Mixture
  - PrimeSTAR GXL DNA Polymerase

Before You Begin

- Thaw all required reagents on ice, then pulse-vortex for 2 seconds to mix and then pulse-spin.

LIBRARY PREPARATION AMPLIFICATION

C3.1 Program a thermal cycler with the following conditions. Set the temperature of the heated lid to 105°C.

	STEP	TEMPERATURE	TIME	# OF CYCLES
1	Initialization	94°C	1 minute	1
2	Denaturation	98°C	10 seconds	6–8*
	Annealing	60°C	15 seconds	
	Extension	68°C	10 minutes	
3	Final Extension	68°C	1 minute	1
4	Final Hold	4°C	HOLD	–

**6–8 cycles is recommended when starting with 200–1000 ng of high-quality gDNA.*

C3.2 Prepare a PCR mix in a 1.6-ml microcentrifuge tube on ice as indicated below. Mix well by gentle pipetting.

REAGENT	VOLUME PER REACTION*
Water	42 µl
5X PrimeSTAR GXL Buffer	20 µl
dNTP Mix	8 µl
PrimeSTAR GXL DNA Polymerase	2 µl
Total	**72 µl**

**Prepare a master mix for multiple reactions.*

C3.3 Proceed to Step 3.3 and continue with the protocol.

POST-CAPTURE AMPLIFICATION

C7.1 Program a thermal cycler with the following conditions. Set the temperature of the heated lid to 105°C.

	STEP	TEMPERATURE	TIME	# OF CYCLES
1	Initialization	94°C	1 minute	1
2	Denaturation	98°C	10 seconds	Varies
	Annealing	60°C	15 seconds	
	Extension	68°C	10 minutes	
3	Final Extension	68°C	1 minute	1
4	Final Hold	4°C	HOLD	–

PANEL SIZE	# OF CYCLES MULTIPLEX
10–50 Mb	12
1–10 Mb	13
500–1,000 kb	14
100–500 kb	15
50–100 kb	16
<50 kb	17

C7.2 Place the strip-tubes or plate containing the supernatant from Step 6.27 on ice until ready to use in the next step.

⚠ IMPORTANT: If you recover <50 µl, add molecular biology grade water to bring the volume to 50 µl.

C7.3 Prepare a PCR mixture by adding the following reagents to the tube(s) containing the supernatant. Mix by pipetting.

C7.3 (cont.)

REAGENT	VOLUME PER REACTION
Supernatant from Step 6.27	50 µl
Water	84 µl
Amplification Primers	6 µl
5X PrimeSTAR GXL Buffer	40 µl
dNTP Mix	16 µl
PrimeSTAR GXL DNA Polymerase	4 µl
Total	**200 µl**

C7.4 Proceed to Step 7.4 and continue with the protocol.

END OF APPENDIX C

LAST REVISED: March 13, 2025

REVISION	DATE	DESCRIPTION
3.0	Mar 13, 2025	• Typo fixes, styling changes, and other edits throughout document (including protocol steps) for clarity • Adjusted language in Step 7.20 to include Oxford Nanopore Sequencing • Added Long-Read and Dark Genes panels as examples in Protocol Components table • Added PrimeSTAR GXL Polymerase as alternative in Materials Supplied by User section • For Step 1 intro paragraph, added guidance on performing bead-based size selection • Various steps in Step 2 Purify and Size Selection (Steps 2.6 to 2.30) had waiting/incubation times, mixing instructions, and volumes modified • Step 3 Pre-Capture PCR Amplify, Purify, and Perform QC had several steps modified. Various changes to thermal cycler conditions, reagent volumes, mixing instructions, transferring instructions, and waiting/incubation times • Step 5 Hybridize Capture Probes With Pools modified. Includes new thermal cycler conditions, hybridization reaction mixture, and other steps • Step 6.9 had a change to incubation time • Step 7 Post-Capture PCR Amplify, Purify, and Perform QC had several steps modified, including adjustments to thermal cycler conditions, transferring instructions, mixing instructions, and waiting/incubation times. • Appendix B steps modified regarding waiting/incubation times and where in protocol to proceed after concentrating libraries • Replaced Appendix C with new appendix titled Alternative Polymerase Guidance
2.0	Feb 2, 2023	• Various typos fixed and styling changes throughout document for clarity • Updated protocol components table to include all Twist reagents required • Added notes regarding mixing instructions and ensuring DNA is RNA free to General Notes and Precautions section • Added note regarding proper drying method to Step 2.14 • Updated Step 4.1 regarding mass of libraries in a pool • Added new steps and modified some existing steps in Step 3 (Pre-Capture PCR Amplify, Purify, and Perform QC), Step 6 (Bind Hybridized Targets to Streptavidin Beads), and Step 7 (Post-Capture PCR Amplify, Purify, and Perform QC) • Added Appendix C Temperature Gradient Hybridization Setup