LongPlex™ Long Fragment Multiplexing Kit

Catalog number:
301310; LongPlex Long Fragment Multiplexing Kit, 96 Reactions

User Guide

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Introduction

The LongPlex™ Long Fragment Multiplexing kit fragments genomic DNA to the range of 7-10 kb while simultaneously adding barcodes in a rapid transposase-based enzymatic method. The initial fragmentation and barcoding steps take only ~35 minutes in a plate-based workflow. The resulting fragmented and barcoded samples contain 10 bp unique dual indexes (UDIs), which enables pooling of samples prior to downstream PacBio™ SMRTbell™ prep kit 3.0 saving both time and library preparation reagent costs.

The LongPlex Long Fragment Multiplexing Kit is ideal for fragmenting and multiplexing microbes or other small genomes for **PCR-free and PCR-plus WGS** libraries, or for human samples destined for **Targeted Hybrid Capture** libraries using Twist Bioscience's long read targeted panel workflow.

WGS PCR-Free

The LongPlex Long Fragment Multiplexing kit can be used to prepare small genomes for downstream long read workflows and sequencing. ***The WGS PCR-Free application is best suited for high quality DNA (DIN ≥8), if methylation data is required, or if downstream inhibitors are unlikely to be present.*** After DNA fragmentation and barcoding, up to 24 samples are pooled together prior to bead-based clean up and size selection. This pool can then be processed in a single SMRTbell library prep for downstream sequencing on the PacBio's Revio or Sequel II/IIe. For a full plate of 96, only 4 pooled SMRTbell libraries need to be made.

WGS PCR-Plus

The LongPlex Long Fragment Multiplexing kit can be used to prepare small genomes for downstream long read workflows and sequencing. ***The WGS PCR-Plus application is best suited for low quality or degraded DNA with a DIN <8, if less than 250 ng is available, if methylation data is not required, or if downstream inhibitors such polysaccharides are likely present.*** After DNA fragmentation and barcode tagging, individual samples go through a bead-based clean up prior to PCR. While PCR is not required for most WGS applications, it may be desired for lower input samples or samples that are highly degraded. PCR amplification using KOD Xtreme Hot Start DNA Polymerase (Millipore-Sigma, user supplied) is included in the workflow. After PCR, up to 24 samples are pooled together for bead-based clean-up and size selection. This pool can then be processed in a single SMRTbell library prep for downstream sequencing on the PacBio's Revio or Sequel II/IIe. For a full plate of 96, only 4 pooled SMRTbell libraries need to be made.

Targeted Hybrid Capture

The LongPlex Long Fragment Multiplexing kit can be used to prepare human genomes compatible with long read hybrid capture workflows and sequencing. After DNA fragmentation and barcode tagging, individual samples go through a bead-based size selection prior to PCR. PCR amplification using KOD Xtreme™ Hot Start DNA Polymerase (Millipore-Sigma, user supplied) is included in the workflow. After PCR, up to 8 samples are pooled together for long read hybrid capture using Twist Bioscience's long read off the shelf or custom panels following the manufacturer's protocol with minor adaptations (Appendix

D). Post hybrid capture, samples can be converted using PacBio's SMRTbell prep kit 3.0 (Appendix C) for downstream sequencing.

Additional Notes

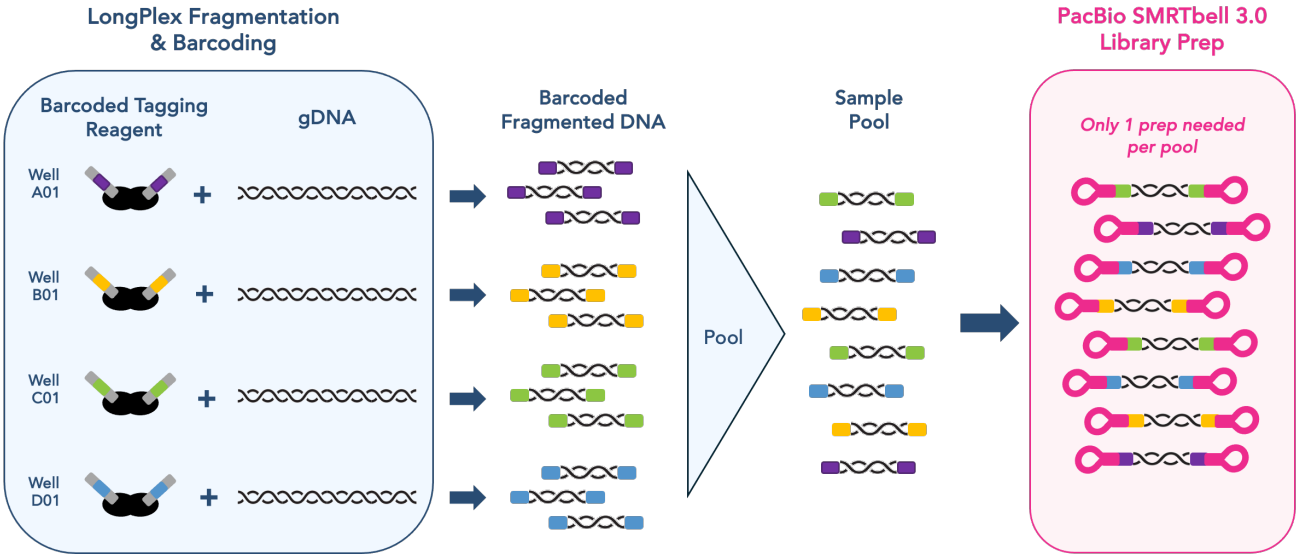
The LongPlex Long Fragment Multiplexing kit comes with reagents needed for fragmentation and barcoding. All AMPure beads, PCR polymerases, Twist Bioscience's hybrid capture reagents, and PacBio SMRTbell library prep 3.0 reagents must be purchased separately.

If gDNA samples are stored in a high EDTA buffer such as TE or contain other suspected inhibitors, refer to Appendix A for a bead-based buffer exchange protocol.

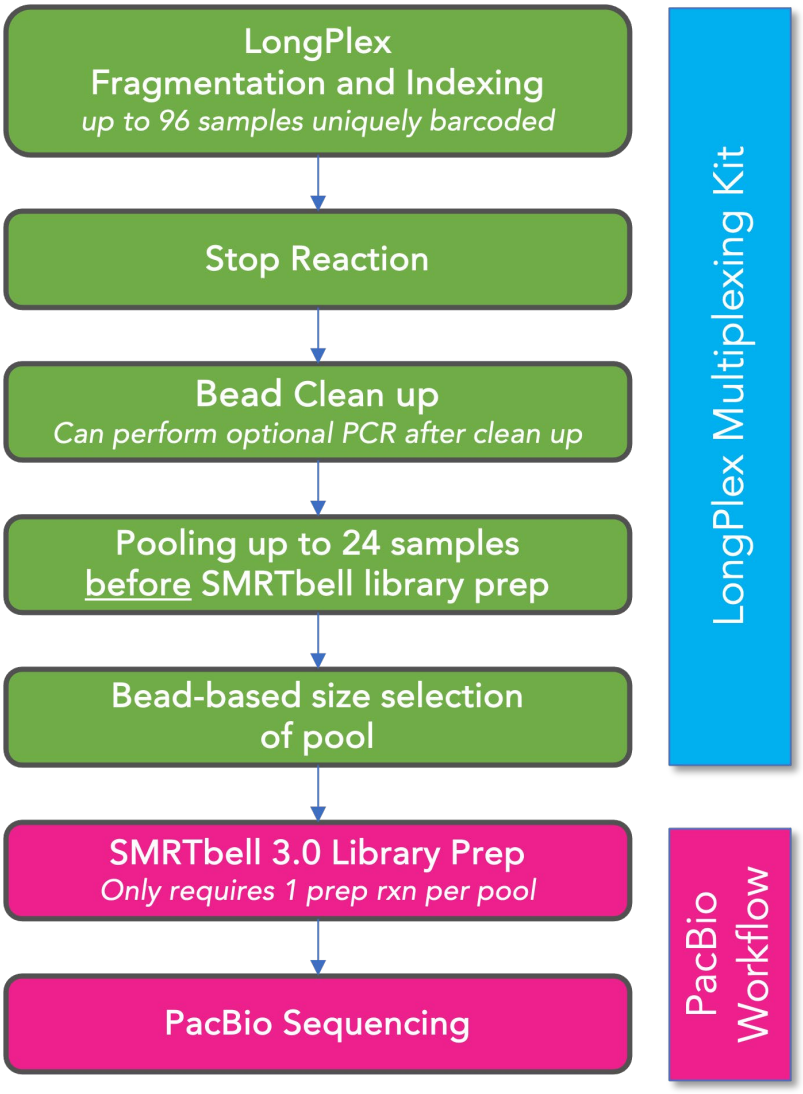
Refer to the following table to select the appropriate method for your application:

Application	**Notes**	**Protocol details**	**Protocol start page**
PCR-free library preparation for microbial and small genome WGS	Best for WGS applications where: ● Methylation data is required. ● DNA is high quality DIN ≥8. ● Samples do not contain known inhibitors of PacBio SMRTbell Prep 3.0.	Input: 250 – 500 ng PCR Required: No	Page 11 and Appendix C
PCR amplified library preparation for microbial and small genome WGS	Best for WGS applications where: ● Methylation data is not required. ● DNA is degraded DIN <8 or less than 250 ng is available. ● Samples contain polysaccharides or other inhibitors of PacBio SMRTbell Prep 3.0.	Input: 150 – 250 ng PCR Required: Yes – 3 to 4 cycles recommended	Page 15 and Appendix C
PCR amplified library prep for human targeted hybrid capture	Best for targeted hybrid capture applications.	Input: 250 – 500 ng PCR Required: Yes – 8 to 10 cycles recommended	Page 20 and Appendix C,D

LongPlex Long Fragment Multiplexing Kit - Molecular Diagram


LongPlex Fragmentation & Barcoding
Barcoded Tagging Reagent
gDNA
Well A01
Well B01
Well C01
Well D01
Barcoded Fragmented DNA
Pool
Sample Pool
PacBio SMRTbell 3.0 Library Prep
Only 1 prep needed per pool

LongPlex Long Fragment Multiplexing Kit - Workflow Diagram: Whole Genome Sequencing



LongPlex Long Fragment Multiplexing Kit - Workflow Diagram: Targeted Hybrid Capture

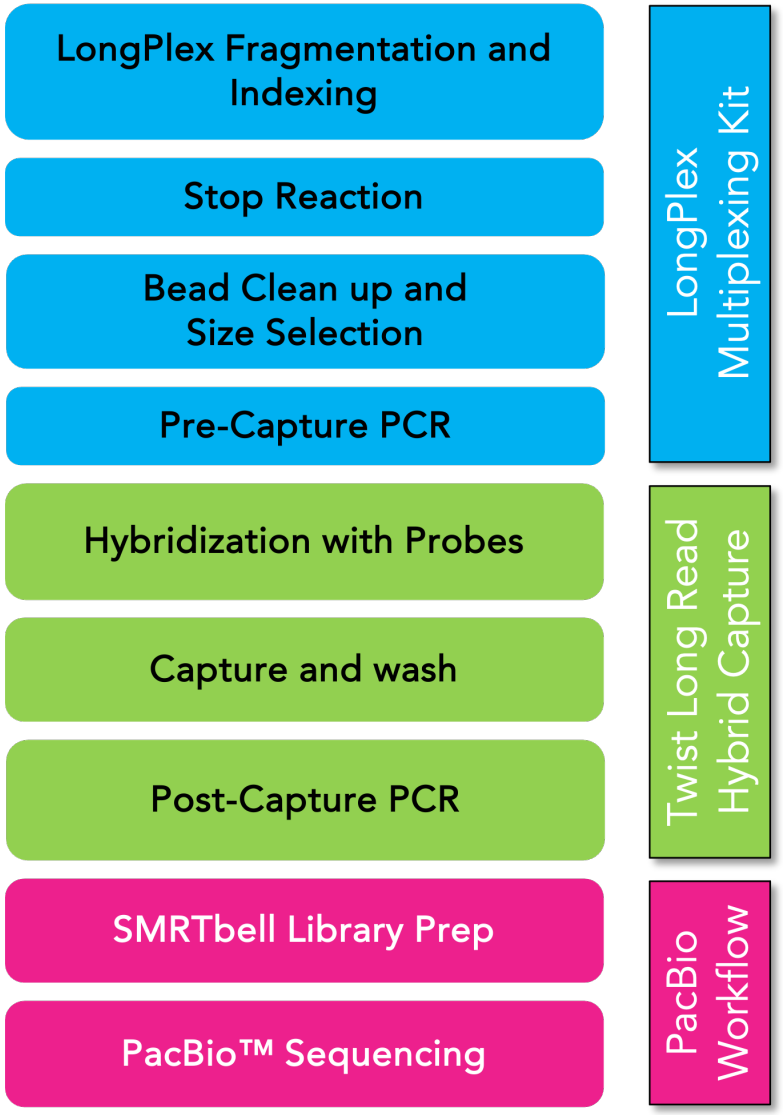


Table 1. LongPlex Long Fragment Multiplexing Kit Components (96 Reactions)

Box	Component	P/N	Description	Storage	Qty
1	UDI Tagging Reagent Plate	301308	Fully-skirted 96-well plate	-20° C	1
	Library Primer Mix (LPM)	300134	0.5 ml tube, clear cap, 700 µl	-20° C	1
2	3X Coding Buffer	101000	2 ml tube, violet cap, 1.5 ml	Ambient	1
	X Solution	101001	2 mL tube, black cap, 750 µl	Ambient	3

User-Supplied Reagents, Equipment & Consumables, and Thermal Cycler Programs

Required Reagents

- AMPure PB beads (PacBio P/N: 100-265-900)* - recommended by PacBio
- 100% Ethanol (molecular biology grade)
- 10 mM Tris-HCl, pH 8.0
- Low TE (10 mM Tris-HCl + 0.1mM EDTA, pH 8.0) - available from PacBio (P/N: 102-178-400) or other vendors
- Ultrapure Water (PCR grade)
- Qubit™ 1X dsDNA High Sensitivity (HS) Assay Kit (ThermoFisher P/N: Q33230), Quant-iT™ PicoGreen™ dsDNA Assay Kits (ThermoFisher P/N: P7589), or other validated dsDNA quantification assay
- Genomic DNA 165 kb Analysis Kit for the Femto Pulse (Agilent P/N: FP-1002-0275)
- **For PacBio library prep reagents:**
    - SMRTbell prep kit 3.0 (PacBio P/N: 102-182-700)
    - Barcoded overhang adapter kit 8A (PacBio P/N: 101-628-400) or 8B (PacBio P/N: 101-628-500)

- **Additional reagents for PCR-plus workflows only**:
    - KOD Xtreme Hot Start DNA Polymerase (Millipore Sigma P/N: 71975-3)

- **Additional reagents required for downstream targeted capture applications only**:
    - Twist Long Read capture panels: Alliance Long Read PGx Panel, Alliance Dark Genes Panel or Twist custom long read panels.
    - Twist buffers and beads for hybrid capture - *see Twist's website for ordering information.*

Equipment & Consumables

- Single-channel pipettors (1-20 µl, 20-200 µl, 100-1,000 µl)
- Multi-channel pipettors (1-10 µl, 10-200 µl)
- Pipette tips (low-retention barrier tips; optional wide bore tips for handling high molecular weight genomic DNA)
- Eppendorf Tubes® (1.5 ml and 2 ml, DNA LoBind® Tubes)
- PCR plate seals (must be evaporation-resistant)
- 96-well thermal cycler (compatible with fully-skirted PCR plates and 8-tube PCR strips)
- Magnetic stand for 1.5 ml tubes and optional for 8-tube PCR strip and/or 96-well plate
- 0.2 ml 8-tube PCR strips and caps/seals
- Benchtop centrifuge to pulse-spin tubes and 8-tube PCR strips
- Plate centrifuge
- Vortex mixer

Thermal Cycler Programs (all with lid-heating on to 105°C)

- **TAG:** 55°C for 15 minutes; 25°C hold
- **STOP:** 68°C for 10 minutes; 25°C hold
- **WGS PCR-plus Amplification:**

  68˚C for 10 minutes (fill-in step)
  94˚C for 2 minutes

  3-4 cycles of:
  98˚C for 10 seconds
  68˚C for 8 minutes

  72˚C for 8 minutes
  4˚C HOLD

- **Targeted Hybrid Capture PCR Amplification:**

  68˚C for 10 minutes (fill-in step)
  94˚C for 2 minutes
  ---
  8 - 11 cycles of:
  98˚C for 10 seconds
  68˚C for 10 minutes

  68˚C for 10 minutes
  4˚C HOLD

Note:
* AMPure XP beads (Beckman P/N: A63880) has been used internally and can be used interchangeably.

Before starting procedure:

Measure and adjust input DNA concentration. Assay the DNA concentration of each sample using Qubit, PicoGreen or other validated dsDNA assay. Adjust input DNA concentration for each application using 10 mM Tris-HCl, pH 8.0, if necessary. DO NOT use EDTA-containing solutions (*e.g.*, TE buffer) to dissolve or dilute input DNA because EDTA can suppress enzymatic activity. See Appendix A for more detailed information on purifying samples stored in TE buffer. If the sample quantity is below the recommended range for the application, refer to the FAQs or **contact support@seqwell.com**.

Program thermal cycler. For convenience, set-up all applicable thermal cycler programs described in the protocol before starting.

Pulse-spin kit components. Liquids can condense or shift locations inside containers during shipment or storage. Before dispensing from reagent tubes, pulse-spin in a suitable centrifuge to gather the reagents at the bottom of the tube. If the kit components freeze during shipment or storage, thaw, mix and pulse-spin before use.

Equilibrate AMPure PB Beads to room temperature. Bring beads to room temperature for 30 minutes before use. Vortex to thoroughly resuspend beads prior to use. To transfer volumes accurately, pipette slowly and DO NOT pre-wet pipette tips.

Check the X Solution for precipitate before use. If a precipitate is visible, incubate at 37°C for 5 minutes (or longer). Mix gently by inversion until the precipitate dissolves (**DO NOT** vortex).

NOTE: **X Solution** contains SDS and will precipitate if stored below room temperature. Overly vigorous mixing will cause foaming.

Please note that the 3X Coding Buffer is viscous. Store 3X Coding Buffer at room temperature. To transfer volumes accurately, pipette slowly and do not pre-wet pipette. While adding 3X Coding Buffer to reactions, mix completely by slowly pipetting up and down several times with the same pipette tip(s) used for addition. Always change pipette tips before adding 3X Coding Buffer to different reactions.

Prepare 80% ethanol fresh daily. Volume required will depend on the number of samples processed.

Prepare 10 mM Tris-HCl, pH 8.0. Prepare 10 mM Tris-HCl, pH 8.0 from a concentrated stock solution diluted with ultrapure water (molecular-biology grade).

Safe stopping points are indicated in the protocol. For optimal results, proceed directly to the next step unless a safe stopping point is indicated.

Section 1: WGS PCR-Free

This protocol describes the use of LongPlex Long Fragment Multiplexing Kit to prepare small genomes for downstream long read workflows and sequencing. The WGS PCR-Free application is best suited for **high quality DNA (DIN ≥8 recommended)**, if methylation data is required, or if downstream inhibitors are unlikely present.

Before beginning the procedure, ensure that each sample concentration has been adjusted to **16.7 - 33.3 ng/µl (total DNA input of 250 - 500 ng)** using 10 mM Tris-HCl, pH 8.0.

NOTE: To ensure that enough yield is generated for SMRTbell library preparation, the minimum number of samples to be processed is 8.

1. Tagging Reaction - Fragmentation and UDI barcoding

If preparing libraries from fewer than 96 samples, please refer to subsection 1e below prior to starting:

a. Pulse-spin the UDI Tagging Reagent plate in a centrifuge. Remove heat seal carefully to avoid splashing/contamination of reagents.
NOTE: Each well in the UDI Tagging Reagent plate is a single-use well containing transposomes loaded with 96 unique dual indexes (UDIs).

b. To a new 96-well plate or 8-tube PCR strip(s) labeled REACTION PLATE or TUBE, set up the tagging reaction for each sample by adding the following, **in order**, mixing after each addition by pipette up and down (≥10x at the transfer volume) slowly:

Genomic DNA (250-500 ng)	15.0 µl
UDI Tagging Reagent	10.0 µl
3X Coding Buffer	12.5 µl
Total Volume	37.5 µl

***NOTE*:** 3X Coding Buffer is viscous; pipet slowly to avoid introducing excessive bubbles.

c. Heat seal REACTION PLATE or close the REACTION TUBE, pulse-spin, and transfer the REACTION PLATE or TUBE to a thermal cycler and run the TAG program below, with lid-heating on:

55°C for 15 minutes

25°C hold

d. Once the program is complete, proceed directly to the next step.

Special instructions for preparing libraries in batches of fewer than 96 samples:

e. Using a scalpel or razor blade, only open and peel the heat seal from the wells of the UDI Tagging Reagent plate corresponding to the total number of samples that will be processed.

f. Follow the instructions for reaction setup in the UDI Tagging Reagent plate above (steps 1a – 1b).

g. After verifying that the seals on the unused portion of the UDI Tagging Reagent plate are still intact, cover the used wells to prevent contamination of the unused reagents when the plates are handled again. Then return the sealed/resealed plates to the freezer for later use.

h. Continue following the instructions for steps 1c – 1d to the REACTION PLATE or TUBE.

2. Stop Reaction

a. Pulse-spin the REACTION PLATE or TUBE in a centrifuge and carefully open the tube or remove the seal.

b. Add 18.75 µl X Solution to each well of the REACTION PLATE or TUBE. Mix thoroughly and slowly by pipetting up and down (10 times at 18.75 µl), being careful not to introduce excessive bubbles.

***NOTE**: This solution contains SDS and vigorous mixing will cause it to foam. Pipetting slowly and under the surface of the solution will give the best results.*

c. Securely seal and pulse-spin the REACTION PLATE or TUBE, then transfer to a thermal cycler and run the STOP program, below, with lid heating on 105°C:

68°C for 10 minutes

25°C hold

d. Once the program is complete, proceed directly to the next step.

3. Tagged DNA Pooling and Purification

a. Pulse-spin the REACTION PLATE or TUBE in a centrifuge and carefully open the tube or remove the seal.

b. Vortex (or vigorously pipet) room temperature AMPure beads to ensure that the beads are fully resuspended.

c. For every 24 samples being processed, transfer **50 µl** of each sample into a 2 ml tube**.** ***NOTE:*** If less than 24 samples are pooled, record volume to determine the volume of AMPure bead needed.

d. Add 0.6 volumetric equivalent of AMPure beads to each tube (e.g., add 720 µl to 1200 µl of pooled stopped tagging reaction for 24 samples), and mix thoroughly by pipetting up and down at least 10 times. Incubate on the bench for 5 minutes to allow the DNA to bind.

e. Place the tube(s) on a magnetic stand and let the bead pellet form on one side of the tube and wait until the supernatant appears completely clear (~5 minutes).

f. Remove and discard the supernatant with pipette. Be careful not to disturb the bead pellet.

g. Wash beads with 80% ethanol.

   i. With the tube still in the magnetic stand, add sufficient 80% ethanol to cover the bead pellet.

   ii. After ≥30 seconds, remove and discard the supernatant being careful not to dislodge the bead pellet.

   iii. Repeat the previous step for a total of 2 washes with 80% ethanol. Remove and discard the supernatant.

h. Air-dry the bead pellet on the magnetic stand for 2 minutes or until the bead pellet is dry. **IMPORTANT:** Do not over dry the beads.

i. Remove the tube(s) from the magnetic stand.

j. Add 34 **µl** 10mM Tris to each tube and mix by pipetting the solution along the inner wall of the tube multiple times to thoroughly resuspend the bead pellet.

k. Incubate the resuspended beads on the bench for at least 5 minutes to elute the purified tagged DNA from the beads.

l. Return tube to magnetic stand and allow a bead pellet to reform on the inner wall of the tube (~2 minutes).

m. When the supernatant has completely cleared, carefully transfer 30 µl of DNA eluate from each tube to a fresh tube. The transferred supernatant contains the purified, pool of tagged DNA products.

SAFE STOPPING POINT

Proceed immediately to the next step, or store the purified pool at -20ºC.

4. Tagged DNA Pool Size Selection

***NOTE*:** Below is a PacBio developed size selection method using diluted AMPure beads that removes small fragments <3 kb.

a. Calculate the total volume of 35% AMPure beads needed for all sample pools. Each tagged DNA pool requires 3.7X volume equivalent of 35% diluted AMPure beads (e.g., for 1 pool of tagged DNA = 30 µl of purified pool x 3.7 volume equivalent = 111 µl of 35% diluted AMPure beads needed).

b. Vortex (or vigorously pipet) room temperature AMPure beads to ensure that the beads are fully resuspended.

c. Prepare 35% diluted AMPure beads by diluting AMPure beads with 10 mM Tris-HCl pH 8.0 using a ratio of 35:65, as follows:

   i. To prepare 500 µl of 35% diluted AMPure beads needed, add 175 µl of AMPure beads to 325 µl of 10 mM Tris.

   ii. Adjust the total volume needed as necessary using a ratio of 35:65.

   iii. Vortex well to mix.

d. Add 3.7 volumetric equivalent of 35% diluted AMPure beads to each tube containing purified pool tagged DNA (e.g., add 111 µl to 30 µl of purified tagged DNA pool), and mix thoroughly by pipetting up and down at least 10 times. Incubate on the bench for 5 minutes to allow the DNA to bind.

e. Place the tube(s) on a magnetic stand and let the bead pellet form on one side of the tube and wait until the supernatant appears completely clear (~5 minutes).

a. Remove and discard the supernatant with pipette. Be careful not to disturb the bead pellet.

b. Wash beads with 80% ethanol.

   i. With the tube still in the magnetic stand, add sufficient 80% ethanol to cover the bead pellet.

   ii. After ≥30 seconds, remove and discard the supernatant being careful not to dislodge the bead pellet.

   iii. Repeat the previous step for a total of 2 washes with 80% ethanol. Remove and discard the supernatant.

c. Air-dry the bead pellet on the magnetic stand for 2 minutes or until the bead pellet is dry. **IMPORTANT:** Do not over dry the beads.

d. Remove the tube(s) from the magnetic stand.

e. Add 32 µl Low TE buffer to each tube and mix by pipetting the solution along the inner wall of the tube multiple times to thoroughly resuspend the bead pellet.

f. Incubate the resuspended beads on the bench for at least 5 minutes to elute the purified tagged DNA from the beads.

g. Return tube to magnetic stand and allow a bead pellet to reform on the inner wall of the tube (~ 2 minutes).

h. When the supernatant has completely cleared, carefully transfer 30 µl of DNA eluate from each tube to a fresh tube. The transferred supernatant contains the purified, size-selected library.

i. Proceed to downstream sizing QC (Appendix B) and subsequent conversion to PacBio SMRTbell libraries (Appendix C).

Section 2: WGS PCR-Plus

This protocol describes the use of LongPlex Long Fragment Multiplexing Kit to prepare small genomes for downstream long read workflows and sequencing. The WGS PCR-Plus application is best suited for lower quality or degraded DNA with a DIN ≥ 6.5 recommended, if less than 250 ng is available (minimum of 150 ng), if methylation data is not required, or if downstream inhibitors such polysaccharides are likely present.

Before beginning the procedure, ensure that each sample concentration has been adjusted to **10.0 - 33.3 ng/µl (total DNA input of 150 - 500 ng)** using 10 mM Tris-HCl, pH 8.0.

1. Tagging Reaction - Fragmentation and UDI barcoding

If preparing libraries from fewer than 96 samples, please refer to subsection 1e below prior to starting:

a. Pulse-spin the UDI Tagging Reagent plate in a centrifuge. Remove heat seal carefully to avoid splashing/contamination of reagents.
NOTE: Each well in the UDI Tagging Reagent plate is a single-use well containing transposomes loaded with 96 unique dual indexes (UDIs).

b. To a new 96-well plate or 8-tube PCR strip(s) labeled REACTION PLATE or TUBE, set up the tagging reaction for each sample by adding the following, **in order**, mixing after each addition by pipet up and down (≥10x at the transfer volume) slowly:

Genomic DNA (150-500 ng)	15.0 µl
UDI Tagging Reagent	10.0 µl
3X Coding Buffer	12.5 µl
Total Volume	37.5 µl

***NOTE*:** The 3X Coding Buffer is viscous; pipette carefully to avoid introducing excessive bubbles.

c. Heat seal REACTION PLATE or close the REACTION TUBE, pulse-spin, and transfer the REACTION PLATE or TUBE to a thermal cycler and run the TAG program below, with lid-heating on:

55°C for 15 minutes

25°C hold

d. Once the program is complete, proceed directly to the next step.

Special instructions for preparing libraries in batches of fewer than 96 samples:

e. Using a scalpel or razor blade, only open and peel the heat seal from the wells of the UDI Tagging Reagent plate corresponding to the total number of samples that will be processed.

f. Follow the instructions for reaction setup in the UDI Tagging Reagent plate above (steps 1a – 1b).

g. After verifying that the seals on the unused portion of the UDI Tagging Reagent plate are still intact, cover the used wells to prevent contamination of the unused reagents when the plates are handled again. Then return the sealed/resealed plates to the freezer for later use.

h. Continue following the instructions for steps 1c – 1d to the REACTION PLATE or TUBE.

2. Stop Reaction

a. Pulse-spin the REACTION PLATE or TUBE in a centrifuge and carefully open the tube or remove the seal.

b. Add 18.75 µl X Solution to each well of the REACTION PLATE or TUBE. Mix thoroughly and slowly by pipetting up and down (10 times at 18.75 µl), being careful not to introduce excessive bubbles.

***NOTE**: This solution contains SDS and vigorous mixing will cause it to foam. Pipetting slowly and under the surface of the solution will give the best results.*

c. Securely seal and pulse-spin the REACTION PLATE or TUBE, then transfer to a thermal cycler and run the STOP program, below, with lid heating on:

68°C for 10 minutes

25°C hold

d. Once the program is complete, proceed directly to the next step.

3. Tagged DNA Purification

a. Pulse-spin the REACTION PLATE or TUBE in a centrifuge and carefully open the tube or remove the seal.

b. Vortex (or vigorously pipet) room temperature AMPure beads to ensure that the beads are fully resuspended.

c. Add 33.75 µl of AMPure beads (0.6 volumetric equivalent) of AMPure beads to each well, and mix thoroughly by pipetting up and down at least 10 times. Incubate on the bench for 5 minutes to allow the DNA to bind.

d. Place the tube(s) on a magnetic stand and let the bead pellet form on one side of the tube and wait until the supernatant appears completely clear (~5 minutes).

e. Remove and discard the supernatant with pipette. Be careful not to disturb the bead pellet.

f. Wash beads with 80% ethanol.

- With the tube still in the magnetic stand, add sufficient 80% ethanol to cover the bead pellet.
- After ≥30 seconds, remove and discard the supernatant being careful not to dislodge the bead pellet.

- Repeat the previous step for a total of 2 washes with 80% ethanol. Remove and discard the supernatant.

g. Air-dry the bead pellet on the magnetic stand for 2 minutes or until the bead pellet is dry. **IMPORTANT:** Do not over dry the beads.

h. Remove the tube(s) from the magnetic stand.

i. Add 14 **µl** 10mM Tris to each tube and mix by pipetting the solution along the inner wall of the tube multiple times to thoroughly resuspend the bead pellet.

j. Incubate the resuspended beads on the bench for at least 5 minutes to elute the purified tagged DNA from the beads.

k. Return tube to magnetic stand and allow a bead pellet to reform on the inner wall of the tube (~2 minutes).

l. When the supernatant has completely cleared, carefully transfer 11 µl of DNA eluate from each tube to a fresh tube. The transferred supernatant contains the purified, tagged DNA product.

Optional: Use 2 µl of the purified tagged DNA for Qubit DNA. The expected concentration is 7-18 ng/µl. If you are outside this range, contact support@seqwell.com.

SAFE STOPPING POINT

Proceed immediately to the next step, or store the purified tagged DNA at -20ºC.

4. Library Amplification

a. To a new 96-well plate or 8-tube PCR strip(s), set up the PCR reaction for each sample by adding the following, **in order**, mixing after each addition by pipet up and down (≥10x at transfer volume) slowly:

Unamplified library	11 µl
2X KOD Xtreme Buffer	25 µl
dNTPs (2 mM each)	10 µl
Library Primer Mix	3 µl
KOD Xtreme Hot Start DNA Polymerase	1 µl
Total Volume	50 µl

b. Seal or cap, and transfer to the plate or tube to a thermal cycler and run the KOD Xtreme cycling program below, with lid-heating on to 105˚C:

68˚C for 10 minutes (fill-in step)
94˚C for 2 minutes
3-4 cycles of:
    98˚C for 10 seconds
    68˚C for 8 minutes
72˚C for 8 minutes
4˚C HOLD

***NOTE*:** This program takes ~2 hours as it is optimized to maximize the fragment size with long per cycle extension times.

SAFE STOPPING POINT

Proceed immediately to the next step, or store the unpurified PCR reactions at -20ºC for up to 24 hours.

5. Library Pooling and Purification with Size Selection

***NOTE*:** Below is a PacBio developed size selection method using diluted AMPure beads that removes small fragments <3 kb. However, if you do not wish to perform a size selection, you may instead perform a standard AMPure bead cleanup using 0.6 volumetric equivalent ratio of undiluted AMPure to each pool.

a. Pulse-spin the 96-well plate or 8-tube PCR strip(s) in a centrifuge and carefully open the tube or remove the seal.

b. Vortex (or vigorously pipet) room temperature AMPure beads to ensure that the beads are fully resuspended.

c. For every 24 libraries being processed, transfer **16 µl** of each library into a 2 ml tube**.** ***NOTE:*** If less than 24 libraries are pooled, record volume to determine the volume of 35% diluted AMPure beads needed.

c. Calculate the total volume of 35% AMPure beads needed for all library pools. Each library pool requires 3.7X volume equivalent of 35% diluted AMPure beads (e.g., for 1 pool of 24 libraries = 24 x 16 µl of purified library x 3.7 volume equivalent = 1420.8 µl of 35% diluted AMPure beads needed).

d. Prepare 35% diluted AMPure beads by diluting AMPure beads with 10 mM Tris-HCl pH 8.0 using a ratio of 35:65, as follows:

   i. To prepare 1000 µl of 35% diluted AMPure beads needed, add 350 µl of AMPure beads to 650 µl of 10 mM Tris.

   ii. Adjust the total volume needed as necessary using a ratio of 35:65.

   iii. Vortex well to mix.

e. Add 3.7 volumetric equivalent of 35% diluted AMPure beads to each tube containing purified library pool (e.g., add 1420.8 µl **of 35% diluted AMPure beads** to 384 µl of purified library pool), and mix thoroughly by pipetting up and down at least 10 times. Incubate on the bench for 5 minutes to allow the DNA to bind.

f. Place the tube(s) on a magnetic stand and let the bead pellet form on one side of the tube and wait until the supernatant appears completely clear (~5 minutes).

a. Remove and discard the supernatant with pipette. Be careful not to disturb the bead pellet.

b. Wash beads with 80% ethanol.

- With the tube still in the magnetic stand, add sufficient 80% ethanol to cover the bead pellet.
- After ≥30 seconds, remove and discard the supernatant being careful not to dislodge the bead pellet.
- Repeat the previous step for a total of 2 washes with 80% ethanol. Remove and discard the supernatant.

c. Air-dry the bead pellet on the magnetic stand for 2 minutes or until the bead pellet is dry. **IMPORTANT:** Do not over dry the beads.

d. Remove the tube(s) from the magnetic stand.

e. Add 32 **µl** Low TE buffer to each tube and mix by pipetting the solution along the inner wall of the tube multiple times to thoroughly resuspend the bead pellet.

f. Incubate the resuspended beads on the bench for at least 5 minutes to elute the purified tagged DNA from the beads.

g. Return tube to magnetic stand and allow a bead pellet to reform on the inner wall of the tube (~ 2 minutes).

h. When the supernatant has completely cleared, carefully transfer 30 µl of DNA eluate from each tube to a fresh tube. The transferred supernatant contains the purified, size-selected library.

i. Proceed to downstream sizing QC (Appendix B) and subsequent conversion to PacBio SMRTbell libraries (Appendix C).

Section 3: Targeted Hybrid Capture

This protocol describes the use of LongPlex Long Fragment Multiplexing Kit to prepare human genomes compatible with long read hybrid capture workflows and sequencing.

Before beginning the procedure, ensure that samples are **high quality DNA (DIN ≥8 recommended)** and that each sample concentration has been adjusted to **16.7 - 33.3 ng/µl (total DNA input of 250 - 500 ng)** using 10 mM Tris-HCl, pH 8.0.

1. Tagging Reaction - Fragmentation and UDI barcoding

If preparing libraries from fewer than 96 samples, please refer to subsection 1e below prior to starting:

a. Pulse-spin the UDI Tagging Reagent plate in a centrifuge. Remove heat seal carefully to avoid splashing/contamination of reagents.
NOTE: Each well in the UDI Tagging Reagent plate is a single-use well containing transposomes loaded with 96 unique dual indexes (UDIs).

b. To a new 96-well plate or 8-tube PCR strip(s) labeled REACTION PLATE or TUBE, set up the tagging reaction for each sample by adding the following, **in order**, mixing after each addition by pipet up and down (≥10x at transfer volume) slowly:

Genomic DNA (250-500 ng)	15.0 µl
UDI Tagging Reagent	10.0 µl
3X Coding Buffer	12.5 µl
Total Volume	37.5 µl

***NOTE*:** The 3X Coding Buffer is viscous; pipet carefully to avoid introducing excessive bubbles.

c. Heat seal REACTION PLATE or close the REACTION TUBE, pulse-spin, and transfer the REACTION PLATE or TUBE to a thermal cycler and run the TAG program below, with lid-heating on:

55°C for 15 minutes

25°C hold

d. Once the program is complete, proceed directly to the next step.

Special instructions for preparing libraries in batches of fewer than 96 samples:

e. Using a scalpel or razor blade, only open and peel the heat seal from the wells of the UDI Tagging Reagent plate corresponding to the total number of samples that will be processed.

f. Follow the instructions for reaction setup in the UDI Tagging Reagent plate above (steps 1a – 1b).

g. After verifying that the seals on the unused portion of the UDI Tagging Reagent plate are still intact, cover the used wells to prevent contamination of the unused reagents when the plates are handled again. Then return the sealed/resealed plates to the freezer for later use.

h. Continue following the instructions for steps 1c – 1d to the REACTION PLATE or TUBE.

2. Stop Reaction

a. Pulse-spin the REACTION PLATE or TUBE in a centrifuge and carefully open the tube or remove the seal.

b. Add 18.75 µl X Solution to each well of the REACTION PLATE or TUBE. Mix thoroughly and slowly by pipetting up and down (10 times at 18.75 µl), being careful not to introduce excessive bubbles.

***NOTE**: This solution contains SDS and vigorous mixing will cause it to foam. Pipetting slowly and under the surface of the solution will give the best results.*

c. Securely seal and pulse-spin the REACTION PLATE or TUBE, then transfer to a thermal cycler and run the STOP program, below, with lid heating on:

68°C for 10 minutes

25°C hold

d. Once the program is complete, proceed directly to the next step.

3. Tagged DNA Purification

a. Pulse-spin the REACTION PLATE or TUBE in a centrifuge and carefully open the tube or remove the seal.

b. Vortex (or vigorously pipet) room temperature AMPure beads to ensure that the beads are fully resuspended.

c. Add 56.25 µl of AMPure beads (1 volumetric equivalent) of AMPure beads to each well, and mix thoroughly by pipetting up and down at least 10 times. Incubate on the bench for 5 minutes to allow the DNA to bind.

d. Place the tube(s) on a magnetic stand and let the bead pellet form on one side of the tube and wait until the supernatant appears completely clear (~5 minutes).

e. Remove and discard the supernatant with pipette. Be careful not to disturb the bead pellet.

f. Wash beads with 80% ethanol.

i. With the tube still in the magnetic stand, add sufficient 80% ethanol to cover the bead pellet.

ii. After ≥30 seconds, remove and discard the supernatant being careful not to dislodge the bead pellet.

iii. Repeat the previous step for a total of 2 washes with 80% ethanol. Remove and discard the supernatant.

g. Air-dry the bead pellet on the magnetic stand for 2 minutes or until the bead pellet is dry. **IMPORTANT:** Do not over dry the beads.

h. Remove the tube(s) from the magnetic stand.

i. Add 30 **µl** 10mM Tris to each tube and mix by pipetting the solution along the inner wall of the tube multiple times to thoroughly resuspend the bead pellet.

j. Incubate the resuspended beads on the bench for at least 5 minutes to elute the purified tagged DNA from the beads.

k. Return tube to magnetic stand and allow a bead pellet to reform on the inner wall of the tube (~2 minutes).

l. When the supernatant has completely cleared, carefully transfer 30 µl of DNA eluate from each tube to a fresh tube. The transferred supernatant contains the purified, tagged DNA product.

4. Tagged DNA Size Selection

***NOTE*:** Below is a PacBio developed size selection method using diluted AMPure beads that removes small fragments <3 kb.

a. Calculate the total volume of 35% AMPure beads needed for all sample pools. Each tagged DNA requires 3.3X volume equivalent of 35% diluted AMPure beads (e.g., for 1 sample of tagged DNA = 30 µl of purified pool x 3.3 volume equivalent = 99 µl of 35% diluted AMPure beads needed).

b. Vortex (or vigorously pipet) room temperature AMPure beads to ensure that the beads are fully resuspended.

c. Prepare 35% diluted AMPure beads by diluting AMPure beads with 10 mM Tris-HCl pH 8.0 using a ratio of 35:65, as follows:

i. To prepare 2000 µl of 35% diluted AMPure beads needed, add 700 µl of AMPure beads to 1300 µl of 10 mM Tris.

ii. Adjust the total volume needed as necessary using a ratio of 35:65.

iii. Vortex well to mix.

d. Add 3.3 volumetric equivalent of 35% diluted AMPure beads to each tube containing purified tagged DNA (e.g., add 99 µl of 35% diluted AMPure beads to 30 µl of purified tagged DNA pool), and mix thoroughly by pipetting up and down at least 10 times. Incubate on the bench for 5 minutes to allow the DNA to bind.

e. Place the tube(s) on a magnetic stand and let the bead pellet form on one side of the tube and wait until the supernatant appears completely clear (~5 minutes).

a. Remove and discard the supernatant with pipette. Be careful not to disturb the bead pellet.

b. Wash beads with 80% ethanol.

   i. With the tube still in the magnetic stand, add sufficient 80% ethanol to cover the bead pellet.

   ii. After ≥30 seconds, remove and discard the supernatant being careful not to dislodge the bead pellet.

   iii. Repeat the previous step for a total of 2 washes with 80% ethanol. Remove and discard the supernatant.

c. Air-dry the bead pellet on the magnetic stand for 2 minutes or until the bead pellet is dry. **IMPORTANT:** Do not over dry the beads.

d. Remove the tube(s) from the magnetic stand.

e. Add 25 **µl** 10 mM Tris to each tube and mix by pipetting the solution along the inner wall of the tube multiple times to thoroughly resuspend the bead pellet.

f. Incubate the resuspended beads on the bench for at least 5 minutes to elute the purified tagged DNA from the beads.

g. Return tube to magnetic stand and allow a bead pellet to reform on the inner wall of the tube (~2 minutes).

h. When the supernatant has completely cleared, carefully transfer 22 µl of DNA eluate from each tube to a fresh tube. The transferred supernatant contains the purified, size-selected tagged DNA product.

   Optional: Use 2 µl of the purified tagged DNA for Qubit DNA. The expected concentration is 3-15 ng/µl. If you are outside this range, contact support@seqwell.com.

SAFE STOPPING POINT

Proceed immediately to the next step, or store the purified product at -20ºC.

5. PCR Amplification

a. To a new 96-well plate or 8-tube PCR strip(s), set up the PCR reaction for each sample by adding the following, **in order**, mixing after each addition by pipet up and down (≥10x at transfer volume) slowly:

Unamplified library	22 µl
2X KOD Xtreme Buffer	50 µl
dNTPs (2 mM each)	20 µl
Library Primer Mix	6 µl
KOD Xtreme Hot Start DNA Polymerase	2 µl
Total Volume	100 µl

b. Seal or cap, and transfer to the plate or tube to a thermal cycler and run the KOD Xtreme cycling program below, with lid-heating on to 105˚C:

68˚C for 10 minutes (fill-in step)
94˚C for 2 minutes
8 - 11 cycles of:
98˚C for 10 seconds
68˚C for 10 minutes
68˚C for 10 minutes
4˚C HOLD

***NOTE*:**

- This program takes ~2 hours as it is optimized to maximize the fragment size with long per cycle extension times.
- LongPlex libraries amplified with 9 cycles should yield sufficient libraries to pool 500 ng per sample (into an 8-Plex) for Twist targeted capture. If two Twist hybrid capture attempts are desired, 10 - 11 cycles should be used.

SAFE STOPPING POINT

Proceed immediately to the next step, or store the unpurified PCR reactions at -20ºC for up to 24 hours.

6. Library Purification

a. Pulse-spin the 96-well plate or 8-tube PCR strip(s) in a centrifuge and carefully open the tube or remove the seal.

b. Vortex (or vigorously pipet) room temperature AMPure beads to ensure that the beads are fully resuspended.

c. Add 60 µl of AMPure beads to each well (0.6 volumetric equivalent), and mix thoroughly by pipetting up and down at least 10 times. Incubate on the bench for 5 minutes to allow the DNA to bind.

d. Place the plate or tube(s) on a magnetic stand and let the bead pellet form on one side of the tube and wait until the supernatant appears completely clear (~5 minutes).

e. Remove and discard the supernatant with pipette. Be careful not to disturb the bead pellet.

f. Wash beads with 80% ethanol.

i. With the tube still in the magnetic stand, add sufficient 80% ethanol to cover the bead pellet.

ii. After ≥30 seconds, remove and discard the supernatant being careful not to dislodge the bead pellet.

iii. Repeat the previous step for a total of 2 washes with 80% ethanol. Remove and discard the supernatant.

g. Air-dry the bead pellet on the magnetic stand for 2 minutes or until the bead pellet is dry. **IMPORTANT:** Do not over dry the beads.

h. Remove the tube(s) from the magnetic stand.

i. Add 32 **µl** 10mM Tris to each well and mix by pipetting the solution along the inner wall of the tube multiple times to thoroughly resuspend the bead pellet.

j. Incubate the resuspended beads on the bench for at least 5 minutes to elute the purified tagged DNA from the beads.

k. Return the plate or tube(s) to a magnetic stand and allow a bead pellet to reform on the inner wall of the tube (~2 minutes).

l. When the supernatant has completely cleared, carefully transfer 30 µl of DNA eluate from each tube to a fresh tube. The transferred supernatant contains the purified LongPlex library.

m. Proceed to downstream sizing QC (Appendix B) and subsequent Twist Bioscience's Long Read Library Preparation and Standard Hyb v2 Enrichment (Appendix D) and then conversion to PacBio SMRTbell libraries (Appendix C).

Appendix A: Performing a buffer exchange on genomic DNA samples containing EDTA or other likely inhibitors

If LongPlex libraries are showing little to no fragmentation, there may be inhibitors in the genomic DNA samples present. One very common inhibitor to transposase enzymes is EDTA. **DO NOT use TE or other buffers containing EDTA to dilute your DNA,** instead use 10 mM Tris-HCl pH 8.0.

However, if the genomic DNA is already in TE or other buffer containing suspected inhibitors, a bead-based buffer exchange can be performed (see below). However, this will lead to a loss of DNA, up to 50% in some cases.

To compensate, we recommend performing buffer exchange on twice the amount of DNA than is needed for downstream processing (e.g., for 250 ng DNA input going into LongPlex™ Long Fragment Multiplexing Kit, perform a bead-based buffer exchange on ≥ 500 ng of genomic DNA).

Performing a bead-based buffer exchange with AMPure beads:

1. Vortex (or vigorously pipet) room temperature AMPure beads to ensure that the beads are fully resuspended.
2. Transfer the desired volume of genomic DNA to a fresh plate, strip tube, or 1.5 ml tube.

   NOTE: Use twice the quantity of DNA input needed for downstream library preparation to compensate for any DNA loss during the bead-based buffer exchange process.
3. Add 3X volumetric equivalent of room temperature AMPure beads to each DNA sample needed buffer exchange, and mix thoroughly by pipetting up and down at least 10 times until mixture is homogeneous.
4. Incubate on the bench for 15 minutes to allow the DNA to bind.
5. Place the plate or tube(s) on a magnetic stand and let the bead pellet form on one side of the tube and wait until the supernatant appears completely clear (~5 minutes). For larger volumes, this could take longer than 5 minutes.
6. Remove and discard the supernatant with pipette. Be careful not to disturb the bead pellet.
7. Wash beads with 80% ethanol.
   - With the tube still in the magnetic stand, add sufficient 80% ethanol to cover the bead pellet.
   - After ≥30 seconds, remove and discard the supernatant being careful not to dislodge the bead pellet.
   - Repeat the previous step for a total of 2 washes with 80% ethanol. Remove and discard the supernatant.
8. Air-dry the bead pellet on the magnetic stand for 2 minutes or until the bead pellet is dry. **IMPORTANT:** Do not over dry the beads.
9. Remove the tube(s) from the magnetic stand.
10. Add 22 **µl** 10mM Tris to each well and mix by pipetting the solution along the inner wall of the tube multiple times to thoroughly resuspend the bead pellet.
11. Incubate the resuspended beads on the bench for ≥ 15 minutes at 37˚C to elute the purified DNA from the beads.

12. Return the plate or tube(s) to a magnetic stand and allow a bead pellet to reform on the inner wall of the tube and wait until the supernatant appears completely clear.
13. When the supernatant has completely cleared, carefully transfer 20 µl of DNA eluate from each tube to a fresh tube.
14. Verify DNA concentration using Qubit or other method and proceed with LongPlex™ Long Fragment Multiplexing Kit on remaining buffer exchanged DNA.

Appendix B: Library QC

For each LongPlex library processed, check library quality on a Femto Pulse using Genomic DNA 165 kb Analysis Kit and its quantity on Qubit™ 1X dsDNA High Sensitivity (HS) Assay, Quant-iT™ PicoGreen™ dsDNA Assay Kits or other validated dsDNA quantification assay.

PCR-free and PCR-plus WGS:

- Quantify LongPlex library using a Qubit™ 1X dsDNA High Sensitivity (HS) Assay, Quant-iT™ PicoGreen™ dsDNA Assay Kits or other validated dsDNA quantification assay prior conversion to PacBio SMRTbell libraries (Appendix C).

- Evaluate LongPlex library size on a Femto Pulse using Genomic DNA 165 kb Analysis Kit. Follow the manufacturer's instructions for these instruments and dilute the library, if appropriate, prior to running. Example traces are provided below:

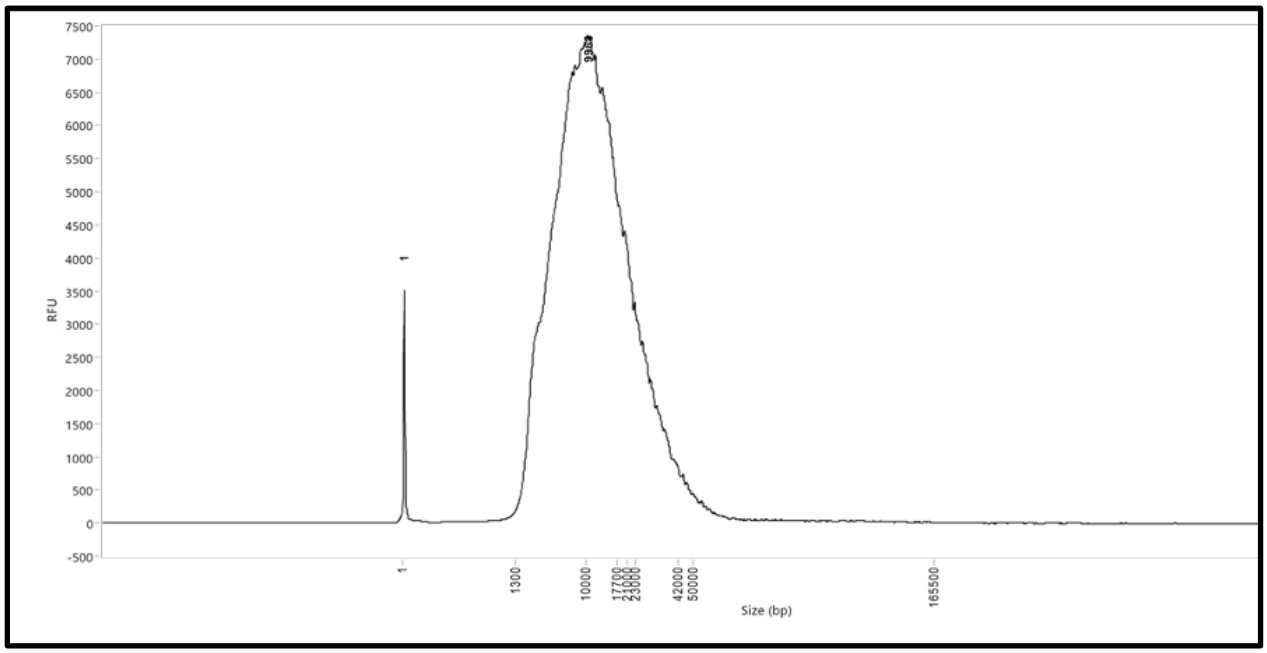


Figure 1. Example of the electropherogram generated by an Agilent Femto Pulse gDNA 165kb Analysis of a LongPlex library.

The average peak size should be >7-10 kb.

Targeted Hybrid Capture

- Quantify LongPlex library using a Qubit™ 1X dsDNA High Sensitivity (HS) Assay, Quant-iT™ PicoGreen™ dsDNA Assay Kits or other validated dsDNA quantification assay prior to Twist Bioscience's Long Read Library Preparation and Standard Hyb v2 Enrichment (Appendix D) and conversion to PacBio SMRTbell libraries (Appendix C).

- Evaluate LongPlex library size on a Femto Pulse using Genomic DNA 165 kb Analysis Kit. Follow the manufacturer's instructions for these instruments and dilute the library, if appropriate, prior to running. Example traces are provided below:

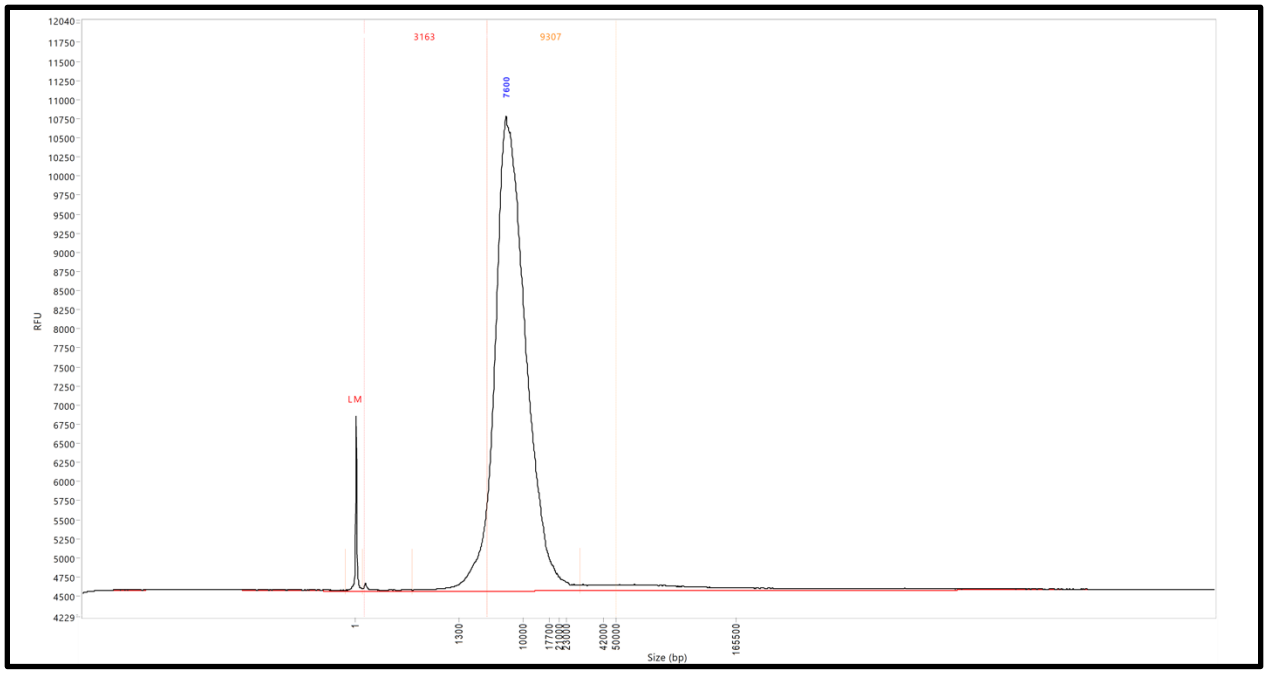


Figure 2. Example of the electropherogram generated by an Agilent Femto Pulse gDNA 165kb Analysis of a LongPlex library suitable for Targeted Hybrid Capture.

The average peak size should be >6-10 kb.

Appendix C: Converting LongPlex multiplexed libraries into PacBio libraries using the SMRTbell prep 3.0 kit

LongPlex libraries can be pooled and processed directly through PacBio's SMRTbell prep kit 3.0 for whole genome sequencing on PacBio's Sequel IIe or Revio. Follow the recommendations below for preparing PacBio libraries.

Reagents supplied by user from PacBio:

- PacBio SMRTbell 3.0 prep kit
- Barcoded Overhang Adapter Kit 8A or Barcoded Overhang Adapter Kit 8B

Using LongPlex barcoded libraries for WGS

1. LongPlex libraries are already fragmented, barcoded, and pooled, thus can be treated as amplicons in downstream PacBio library preparation.

    a. Refer to PacBio's Procedure & checklist - Preparing multiplexed amplicon libraries using SMRTbell prep kit 3.0 protocol for all methods and additional required materials.

    b. Follow methods starting at "2. Repair & A-tailing" on page 9.

    c. We suggest a DNA input of 1 µg (up to 2 µg) in 46 µl.

2. Multiplexing considerations

    a. We recommend pooling up to 24 WGS samples or 1 hybrid capture sample (8-plex) per SMRTbell library prep.

    NOTE: The ideal number of samples to pool will depend on the size of the genome, the coverage required per sample, and the number of total SMRTcells to be loaded. **You may need to experimentally determine the right pooling scheme for your application.**

    b. PacBio's barcoded SMRTbells may be used to increase the number of samples to be pooled on a single SMRTcell.

        i. PacBio provides up to 96 barcoded SMRTbell adapters. Refer to their website for ordering information.

        ii. Utilizing different barcoded SMRTbells enables pooling of pools onto the same SMRTcell.

        iii. Combining LongPlex's 96 UDIs + PacBio's 96 barcoded SMRTbells provides a theoretical max of 9,216 samples on a single SMRTcell.

Appendix D: Using LongPlex Libraries in Twist Bioscience's Long Read Library Preparation and Standard Hyb v2 Enrichment

Refer to Twist Bioscience's Long Read Library Preparation and Standard Hyb v2 Enrichment protocol for all hybrid capture and post processing methods.

LongPlex adapters contain transposase specific adapter sequences that are not fully blocked by using Twist Bioscience's Universal Blockers. Here, we provide information on suggested modifications to the hybridization reaction set up and alternative blockers that can improve % on target.

Reagents supplied by user from Twist:

- Twist Long Read Sequencing Panels - Twist Dark Genes, PGx, or Custom long read panel
- Twist Standard Hybridization V2 Reagent Kit – hybrid capture and wash buffers
    - ***NOTE:*** **DO NOT** order Twist Bioscience's Universal Blocking module if using alternative transposase specific blockers (see below).

Reagents supplied by user from other sources:

- IDT xGen™ Universal Blockers, NXT
- IDT xGen™ Human Cot DNA

Using LongPlex barcoded libraries for Twist Hybrid Capture

1. You may start the Twist long read hybridization workflow at "Step 4 -PREPARE LIBRARIES FOR HYBRIDIZATION" on page 18 of the Long Read Library Preparation and Standard Hyb v2 Enrichment Protocol.
2. Follow all steps as outlined by Twist for pooling and concentrating libraries.
3. For the hybridization reaction set up, use the following substitutions to improve % on target when using LongPlex libraries:
    a. Substitute 5 µl of Twist Blocking Solution with 5 µl of Human Cot DNA.
    b. Substitute 7 µl of Twist Universal Blockers with 1 µl of IDT xGen Universal NXT blockers + 6 µl of water.
4. Once these substitutions have been made, continue with the Twist protocol.
5. Increase the number of amplification cycles by 3-5 (concordance with Twist Bioscience's protocol) to improve yield if pooling less than 8 samples for a single hybrid capture reaction.
6. **Make sure the final elution prior to SMRTbell 3.0 library prep is in low TE.**
7. Proceed as instructed to PacBio SMRTbell 3.0 library prep (Appendix C).

Appendix E: LongPlex Demultiplexing Guidelines for PacBio HiFi Sequencing

Demultiplexing of PacBio HiFi BAM files obtained by sequencing of LongPlex libraries can be performed with *lima* using specific workflow parameters and barcode manifest files that correspond to transposase-based barcode sequences utilized in the LongPlex kit.

Demultiplexing workflow scripts and instructions for use can be downloaded and viewed at https://github.com/seqwell/LongPlex.

Version	Release Date	Prior Version	Description of changes
v081324	**08-13-2024**	**N/A**	**First version**

Technical Assistance

For technical assistance with LongPlex Library Preparation, please contact seqWell Technical Support.

Email: support@seqwell.com

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