LongPlex™ Multiplexing Kit

Catalog number:
301315; LongPlex Multiplexing Kit, 1x96, Any Index Set

User Guide

V20251001

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Introduction

The LongPlex Multiplexing kit fragments HMW genomic DNA to the range of 7-10 kb while simultaneously adding barcodes in a rapid transposase-based enzymatic method. Shorter fragments of genomic DNA or large plasmids may be used as well, but the final library size will be up to half the size of the starting material. For example, starting with 10 kb genomic DNA will yield library fragments ≤5 kb. **Please contact support@seqwell.com for protocol modifications if you are planning to use plasmids, amplicons, or DNA ≤10 kb**. 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 Multiplexing Kit is ideal for fragmenting and multiplexing microbial or other small genomes for whole genome sequencing, and large genomes for low-pass sequencing. The kit offers flexibility with both **PCR-plus and PCR-free WGS** library preparation options. Additionally, a protocol is provided for human samples destined for **Targeted Hybrid Capture** libraries using Twist Bioscience's long read targeted panel workflow.

WGS PCR-Plus

The LongPlex Multiplexing kit can be used to prepare 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, 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. Pooling up to 96 samples together to be processed in a single SMRTbell library prep may be possible for some applications, but optimization by the user may be required.

WGS PCR-Free

The LongPlex Multiplexing kit can be used to prepare 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, and 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. Pooling up to 96 samples together to be processed in a single SMRTbell library prep may be possible for some applications, but optimization by the user may be required.

Targeted Hybrid Capture

The LongPlex 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

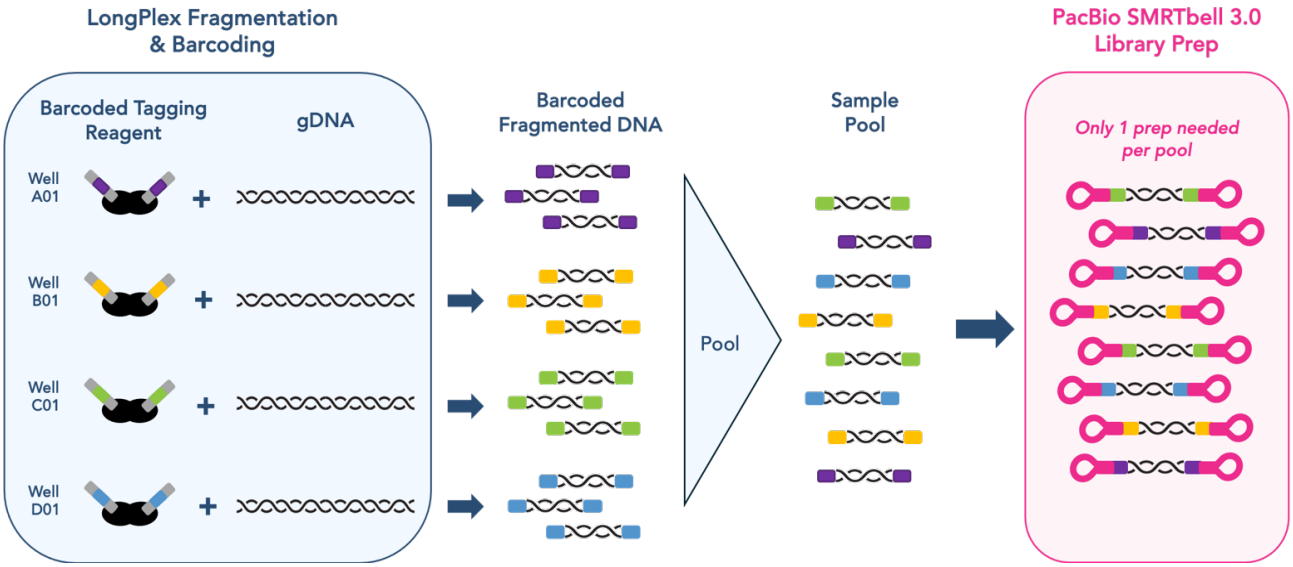
The LongPlex Multiplexing kit comes with reagents needed for fragmentation and barcoding. All AMPure® beads, PCR polymerases, Twist Bioscience's hybrid capture reagents, and PacBio SMRTbell library prep 3.0 reagents must be purchased separately.

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.

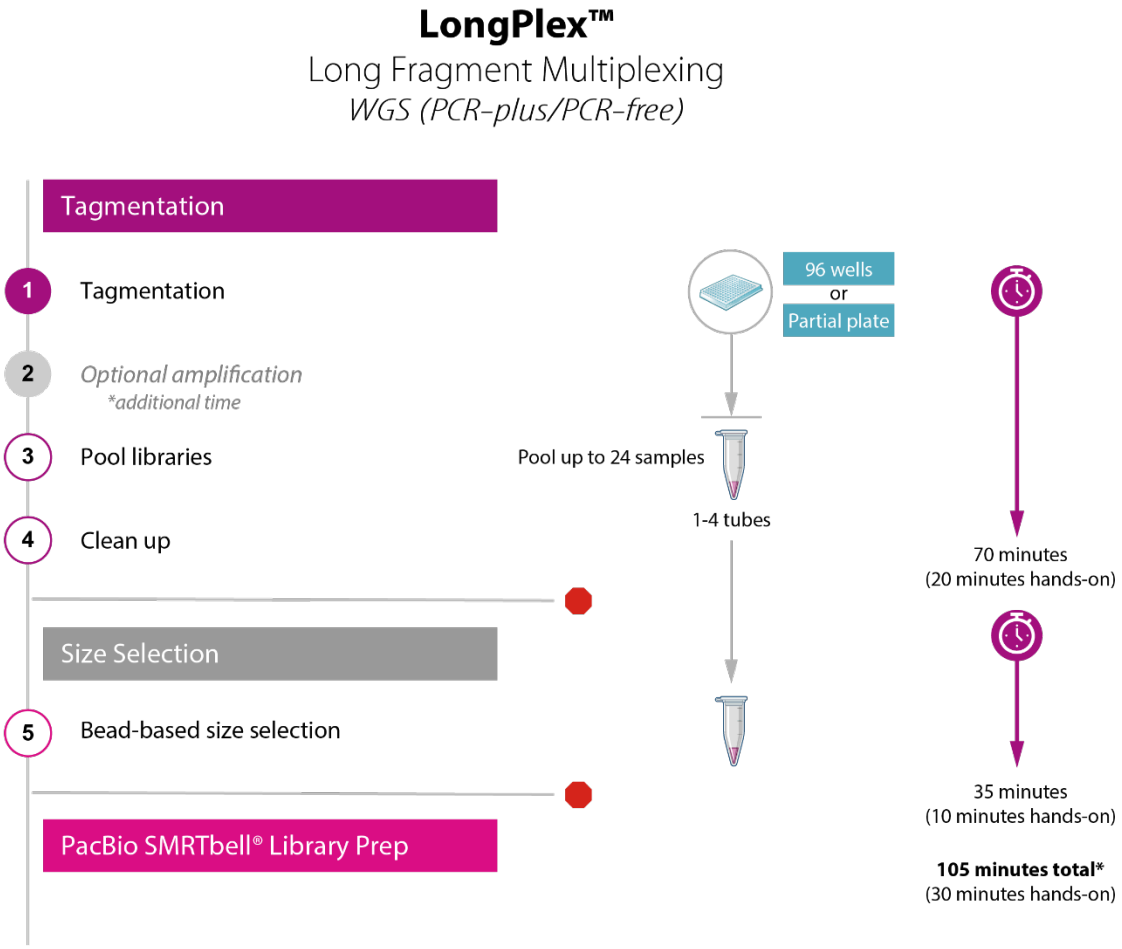
Refer to the following table to select the appropriate method for your application:

Application	Notes	Protocol details	Protocol start page
PCR amplified library preparation for microbial, small genome WGS, and large genome low-pass 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.	Batch range: 8 – 96 samples Input: 150 – 500 ng PCR Required: Yes – 3 to 4 cycles recommended	Page 13 and Appendix C
PCR-free library preparation for microbial, small genome WGS, and large genome low-pass 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.	Batch range: 8 – 96 samples Input: 250 – 500 ng PCR Required: No	Page 20 and Appendix C
PCR amplified library prep for human targeted hybrid capture	Best for targeted hybrid capture applications.	Batch range: 1- 96 samples Input: 250 – 500 ng PCR Required: Yes – 8 to 12 cycles recommended	Page 26 and Appendix C, Appendix D

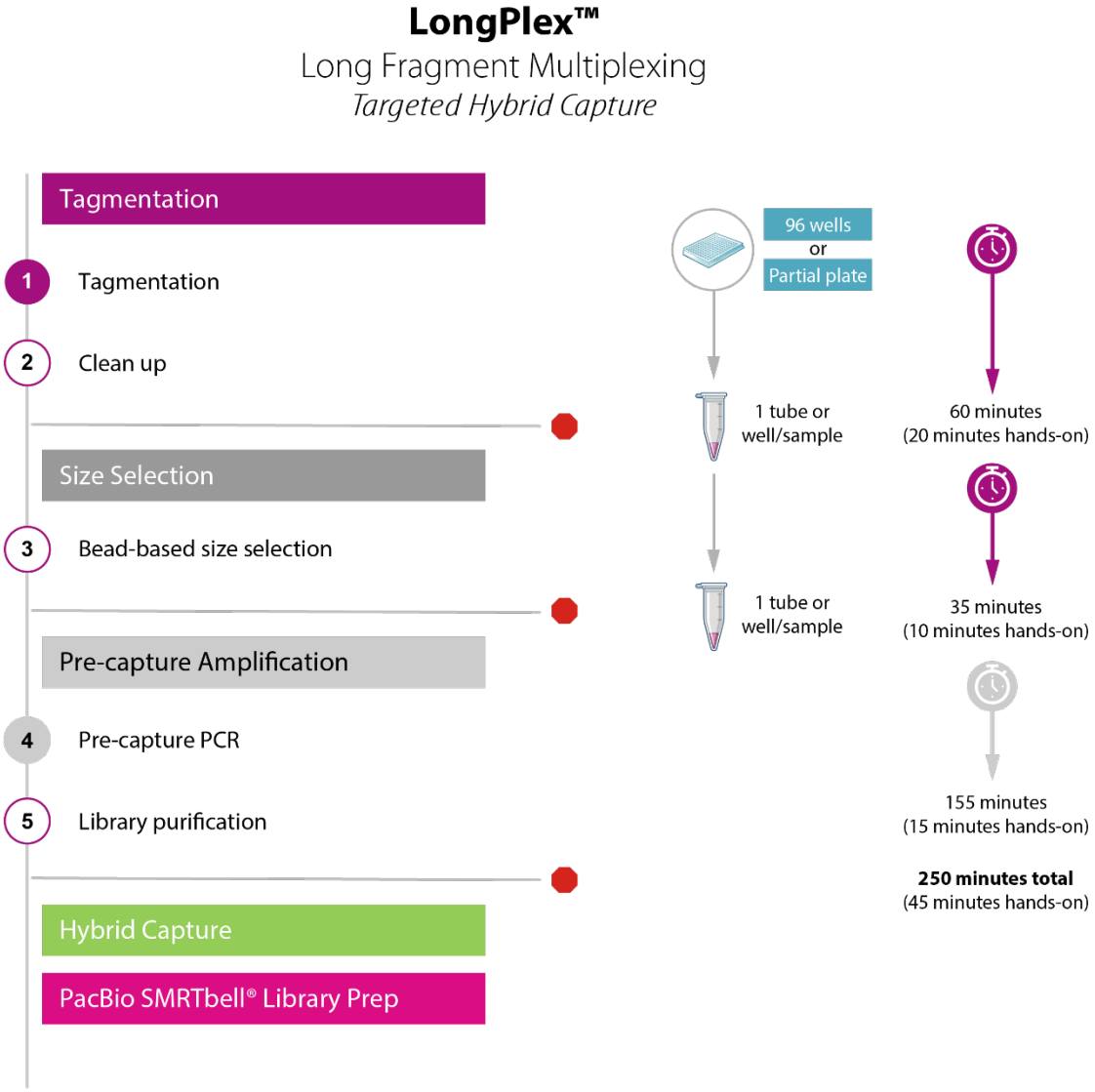
LongPlex Multiplexing Kit - Molecular Diagram



Workflow Diagram: Whole Genome Sequencing



Workflow Diagram: Targeted Hybrid Capture



Kit Components

Table 1. LongPlex Multiplexing Kit Components (96 Reactions)

LongPlex Multiplexing Kit, 1x96, Any Index Set

Catalog No.: 301315

Box	Component	REF	Description	Storage	Qty
1	LongPlex Indexed Tagging Reagent	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	101284	2 ml tube, violet cap, 1.5 ml	Ambient	1
	X Solution	101323	2 mL tube, black cap, 750 µl	Ambient	3

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

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

****NOTE:** AMPure XP beads (Beckman P/N: A63880) or SMRTbell cleanup beads (PacBio P/N 102.158-300) can be used interchangeably. Any beads other than AMPure PB, AMPure XP, or SMRTbell cleanup cannot be used with this protocol.*

Thermal Cycler Programs (all with lid-heating set 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:**

10	min	68°C	Fill-in Step
2	min	94°C	Initial denaturation
10	sec	98°C	Library Amplification
8	min	68°C	**3-4 Cycles**
8	min	72°C	Final extension
Hold		4°C	

- **Targeted Hybrid Capture PCR Amplification:**

10	min	68°C	Fill-in Step
2	min	94°C	Initial denaturation
10	sec	98°C	Library Amplification
10	min	68°C	**8-12 Cycles**
10	min	68°C	Final extension
Hold		4°C	

Before starting procedure:

Review FAQs. FAQs can be found here: https://seqwell.com/resource-category/faqs/. Review these prior to your first run.

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-Plus

This protocol describes the use of LongPlex Multiplexing Kit to prepare small and large 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 as polysaccharides are likely present.

DNA must not be stored or diluted in EDTA-containing solutions. If needed, see Appendix A for a protocol describing how to perform a buffer exchange.

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.

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

a. Pulse-spin the Indexed Tagging Reagent plate in a centrifuge. Make a note of which index set (1, 2, or 3) you are using.

b. If processing a full plate (96 samples): Peel and remove the entire heat seal carefully to avoid splashing/contamination of reagents.

   If processing fewer than 96 samples: Using a scalpel or razor blade, only open and peel the heat seal from the wells of the Indexed Tagging Reagent plate corresponding to the total number of samples that will be processed.

   Each well in the Indexed Tagging Reagent plate is a single-use well containing transposomes loaded with a unique i7 and i5 barcode.

c. 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:

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

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

d. If processing a full plate (96 samples): The indexing plate may be discarded.

   If processing fewer than 96 samples: After verifying that the seals on the unused portion of the Indexed 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. The tagging reagent is stable for up to 12 freeze thaw cycles.

e. Securely seal the 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 set to 105°C:

   55°C for 15 minutes
   25°C hold

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

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.

   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 set to 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 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 plate or tube(s) on a magnetic stand and let the bead pellet form on one side of the well or 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 plate or 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 plate or tube(s) from the magnetic stand.

i. Add 14 µl 10mM Tris to each well or tube and mix by pipetting the solution along the inner wall of the well or 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 to magnetic stand and allow a bead pellet to reform on the inner wall of the well or tube (~2 minutes).

l. When the supernatant has completely cleared, carefully transfer 11 µl of DNA eluate from each well or tube to a new 96-well plate or 8-tube PCR strip(s). 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 -20°C.

4. Library Amplification

a. To the PCR plate or tubes of purified, tagged DNA product, 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 (**WGS PCR-Plus Amplification**) below, with lid-heating set to 105°C:

NOTE: *This program takes ~1 hour as it is optimized to maximize the fragment size with long per cycle extension times.*

10	min	68°C	Fill-in Step
2	min	94°C	Initial denaturation
10	sec	98°C	Library Amplification
8	min	68°C	**3-4 Cycles**
8	min	72°C	Final extension
Hold		4°C	

SAFE STOPPING POINT

Proceed immediately to the next step or store the unpurified PCR reactions at -20°C 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**.**

   If less than 24 libraries are pooled, record volume to determine the volume of 35% diluted AMPure beads needed.

d. 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).

e. 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.

f. Add 3.7X 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.

g. Incubate on the bench for 5 minutes to allow the DNA to bind.

h. 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).

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

j. 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.

k. 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.

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

m. 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.

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

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

p. 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.

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

Section 2: WGS PCR-Free

This protocol describes the use of LongPlex Multiplexing Kit to prepare small and large 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, and if downstream inhibitors are unlikely present.

DNA must not be stored or diluted in EDTA-containing solutions. If needed, see Appendix A for a protocol describing how to perform a buffer exchange.

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

a. Pulse-spin the Indexed Tagging Reagent plate in a centrifuge. Make a note of which index set (1, 2, or 3) you are using.

b. If processing a full plate (96 samples): Peel and remove the entire heat seal carefully to avoid splashing/contamination of reagents.

   If processing fewer than 96 samples: Using a scalpel or razor blade, only open and peel the heat seal from the wells of the Indexed Tagging Reagent plate corresponding to the total number of samples that will be processed.

   Each well in the Indexed Tagging Reagent plate is a single-use well containing transposomes loaded with a unique i7 and i5 barcode.

c. 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:

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

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

d. If processing a full plate (96 samples): The indexing plate may be discarded.

   If processing fewer than 96 samples: After verifying that the seals on the unused portion of the Indexed 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. The tagging reagent is stable for up to 12 freeze thaw cycles.

e. Securely seal the 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 set to 105°C:

   55°C for 15 minutes
   25°C hold

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

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.

   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 set to 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**.**

   If less than 24 samples are pooled, record volume to determine the volume of AMPure beads 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.

e. 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).

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

h. 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.

i. 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.

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

k. 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.

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

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

n. 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 -20°C.

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 3.7X volumetric equivalent of 35% diluted AMPure beads to each tube containing purified pool of 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.

e. 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).

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

h. 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.

i. 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.

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

k. 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.

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

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

n. 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.

o. 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 Multiplexing Kit to prepare human genomes compatible with long read hybrid capture workflows and sequencing.

DNA must not be stored or diluted in EDTA-containing solutions. If needed, see Appendix A for a protocol describing how to perform a buffer exchange.

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

a. Pulse-spin the Indexed Tagging Reagent plate in a centrifuge. Make a note of which index set (1, 2, or 3) you are using.

b. If processing a full plate (96 samples): Peel and remove the entire heat seal carefully to avoid splashing/contamination of reagents.

   If processing fewer than 96 samples: Using a scalpel or razor blade, only open and peel the heat seal from the wells of the Indexed Tagging Reagent plate corresponding to the total number of samples that will be processed.

   Each well in the Indexed Tagging Reagent plate is a single-use well containing transposomes loaded with a unique i7 and i5 barcode.

c. 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:

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

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

d. If processing a full plate (96 samples): The indexing plate may be discarded.

   If processing fewer than 96 samples: After verifying that the seals on the unused portion of the Indexed 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. The tagging reagent is stable for up to 12 freeze thaw cycles.

e. Securely seal the 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 set to 105°C:

   55°C for 15 minutes
   25°C hold

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

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.

   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 set to 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 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.

d. Incubate on the bench for 5 minutes to allow the DNA to bind.

e. Place the plate or tube(s) on a magnetic stand and let the bead pellet form on one side of the well or 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 plate or 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 plate or tube(s) from the magnetic stand.

j. Add 30 µl 10mM Tris to each well or tube and mix by pipetting the solution along the inner wall of the well or 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 the plate or tube to magnetic stand and allow a bead pellet to reform on the inner wall of the well or tube (~2 minutes).

m. When the supernatant has completely cleared, carefully transfer 30 µl of DNA eluate from each well or tube to a new 96-well plate or 8-tube PCR strip(s). 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 3.3X volumetric equivalent of 35% diluted AMPure beads to each well or 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.

e. Incubate on the bench for 5 minutes to allow the DNA to bind.

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

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

h. Wash beads with 80% ethanol:

   i. With the plate or 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.

i. 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.

j. Remove the plate or tube(s) from the magnetic stand.

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

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

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

n. When the supernatant has completely cleared, carefully transfer 22 µl of DNA eluate from each well or tube to a new 96-well plate or 8-tube PCR strip(s). 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 -20°C.

5. PCR Amplification

a. To the PCR plate or tubes of purified, tagged DNA product, 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 (**Targeted Hybrid Capture PCR Amplification**) below, with lid-heating set to 105°C:

NOTES:

- *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 with high quality input DNA should yield sufficient libraries to pool 500 ng per sample (into an 8-Plex) for Twist hybrid capture. With lower quality DNA, a different plex, or for two Twist hybrid capture attempts, 10 - 12 cycles should be used.*

10	min	68°C	Fill-in Step
2	min	94°C	Initial denaturation
10	sec	98°C	Library Amplification
10	min	68°C	**8-12 Cycles**
10	min	68°C	Final extension
Hold		4°C	

SAFE STOPPING POINT

Proceed immediately to the next step, or store the unpurified PCR reactions at -20°C 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 or tube (0.6 volumetric equivalent), and mix thoroughly by pipetting up and down at least 10 times.

d. Incubate on the bench for 5 minutes to allow the DNA to bind.

e. Place the plate or tube(s) on a magnetic stand and let the bead pellet form on one side of the well or 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 plate or 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 plate or tube(s) from the magnetic stand.

j. Add 32 µl 10mM Tris to each well or tube and mix by pipetting the solution along the inner wall of the well or 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 the plate or tube(s) to a magnetic stand and allow a bead pellet to reform on the inner wall of the well or tube (~2 minutes).

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

n. 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 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.

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

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

c. 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 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 library quantity on Qubit 1X dsDNA High Sensitivity (HS) Assay, Quant-iT PicoGreen dsDNA Assay Kits or other validated dsDNA quantification assay.

The Femto Pulse is strongly recommended because other methods of library size evaluation may not be sufficient. The TapeStation® or similar instruments may not display any difference between input DNA and final library. See example TapeStation and Femto Pulse traces below.

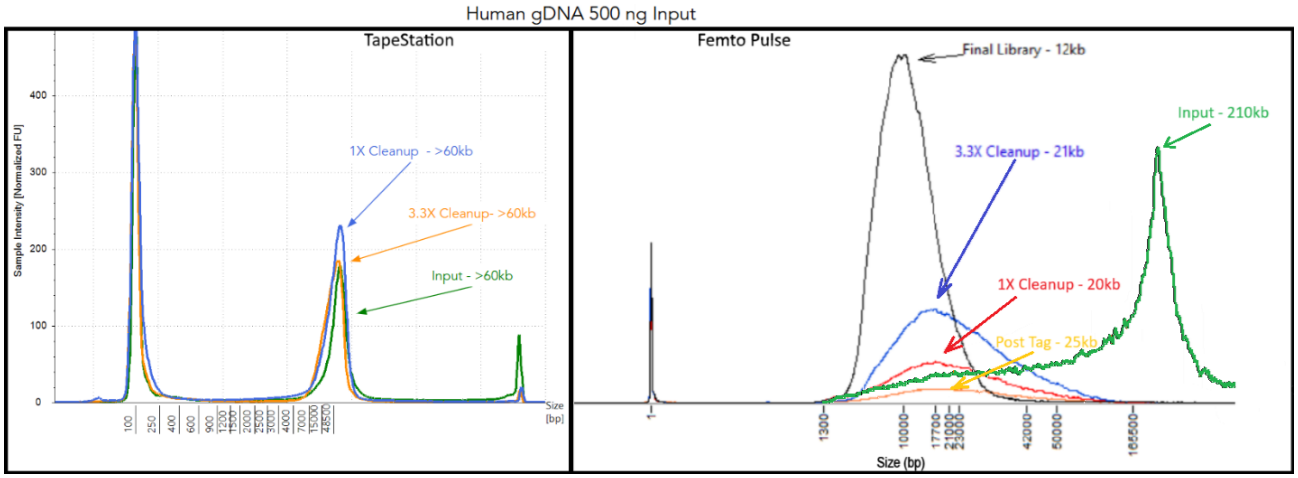


Figure 2. Comparison between TapeStation (left) and Femto Pulse (right) traces at different stages of library preparation. The TapeStation is not able to differentiate input DNA from intermediate or final libraries, while the Femto Pulse clearly resolves input from all intermediate and final libraries.

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:

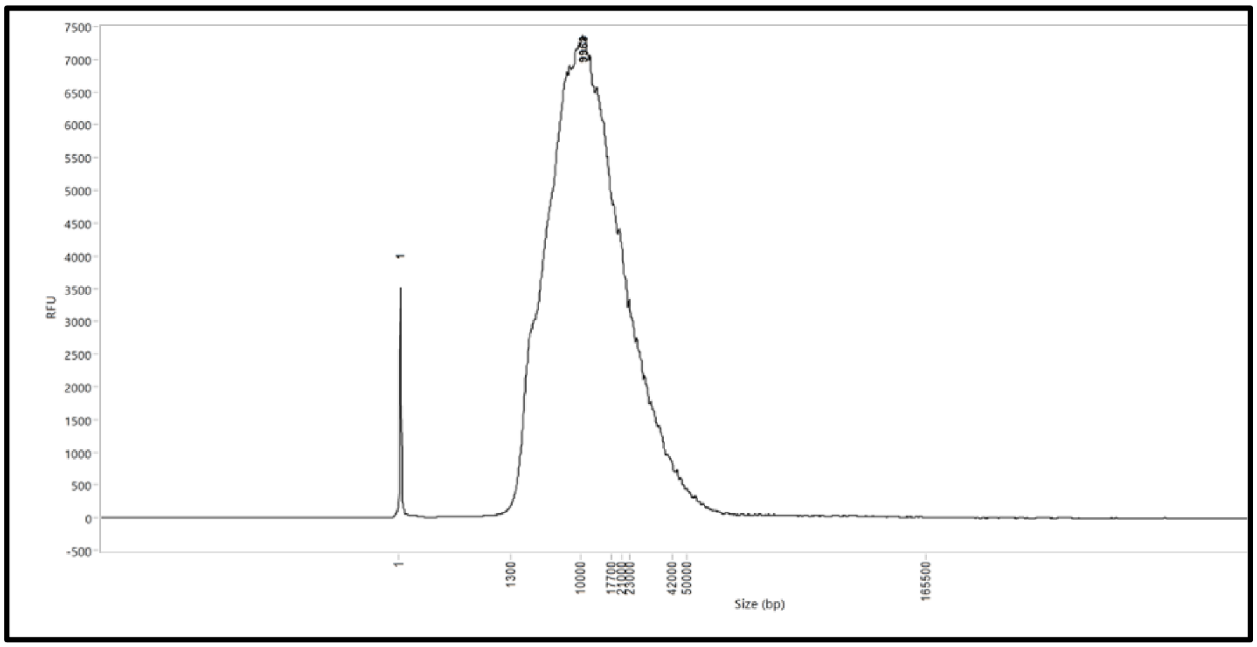


Figure 1. Example of the electropherogram generated by an Agilent Femto Pulse gDNA 165kb Analysis of a LongPlex library.

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:

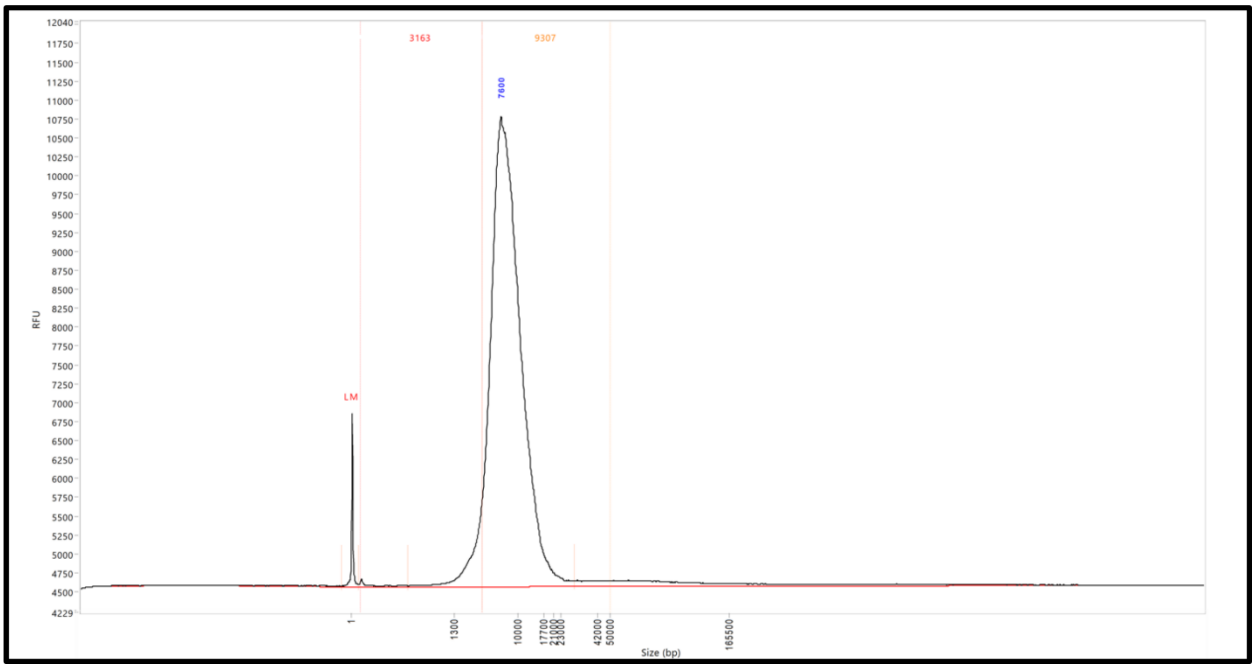


Figure 3. Example of the electropherogram generated by an Agilent Femto Pulse gDNA 165kb Analysis of a LongPlex library suitable for Targeted Hybrid Capture.

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, Revio, or Vega. 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.

    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 maximum 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.

Technical Assistance

Please review FAQs at https://seqwell.com/resource-category/faqs/.

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

Email: support@seqwell.com

Version	Release Date	Prior Version	Description of changes
V20251001	**October 1, 2025**	**V081324**	**1. Changed product name and catalog numbers.** **2. Added language to clarify that low-pass WGS for large genomes is a supported application.** **3. Increased maximum number of PCR cycles for hybrid capture protocol.** **4. Corrected thermal cycler time for WGS PCR-Plus.** **5. Added Femto Pulse vs. TapeStation graphics and links to FAQs.** **6. Rearranged protocol sections.**
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