Modified LongPlex™ Protocol (LongPlex XL): Generating HiFi Read Lengths >10 kb Using PacBio Short Read Eliminator (SRE)

Modified LongPlex >10 kb Protocol Requirements

- Only **high-quality DNA** can be used in this modified protocol. Starting genomic DNA quality **must** be measured by Femto Pulse with a GQN30kb ≥7.
- DNA input must be **500 - 750 ng** of high-quality gDNA per sample.
- The **minimum batch is 8 samples** in each pool to ensure sufficient yield following SRE.
- The SRE kit is a PacBio product. For technical support specific to SRE, please contact your PacBio FAS.

PLEASE REACH OUT
to support@seqwell.com about your project before running supplemental protocol

In this technical note, we describe an alternative method for generating HiFi read lengths of 10-15 kb from high-quality genomic DNA using LongPlex Long Fragment Multiplexing Kit to generate fragmented and barcoded sample pools and leveraging PacBio's Short Read Eliminator (SRE) technology to size-select the pools for fragment >10 kb prior to SMRTbell® library preparation.

LongPlex is a streamlined strategy for multiplexed library construction which uses transposase tagmentation for plate-based fragmentation and sample barcoding. LongPlex eliminates the need for mechanical shearing and enables pooling of barcoded samples prior to SMRTbell library prep, increasing throughput and reducing library prep costs.

The standard protocol for LongPlex is designed to quickly fragment high to medium quality genomic DNA to generate HiFi read lengths of 6-9 kb, ideal for microbial or other smaller genome sequencing applications. However, those users who have higher quality DNA may wish to generate longer HiFi read lengths using LongPlex to achieve higher gigabase yields on their PacBio sequencer.

This modified protocol can only be used with high-quality DNA (Femto Pulse GQN30kb ≥7).

Using degraded DNA will lead to significant sample loss during SRE size selection.

LongPlex™ XL

Long Fragment Multiplexing

HiFi read lengths >10 kb

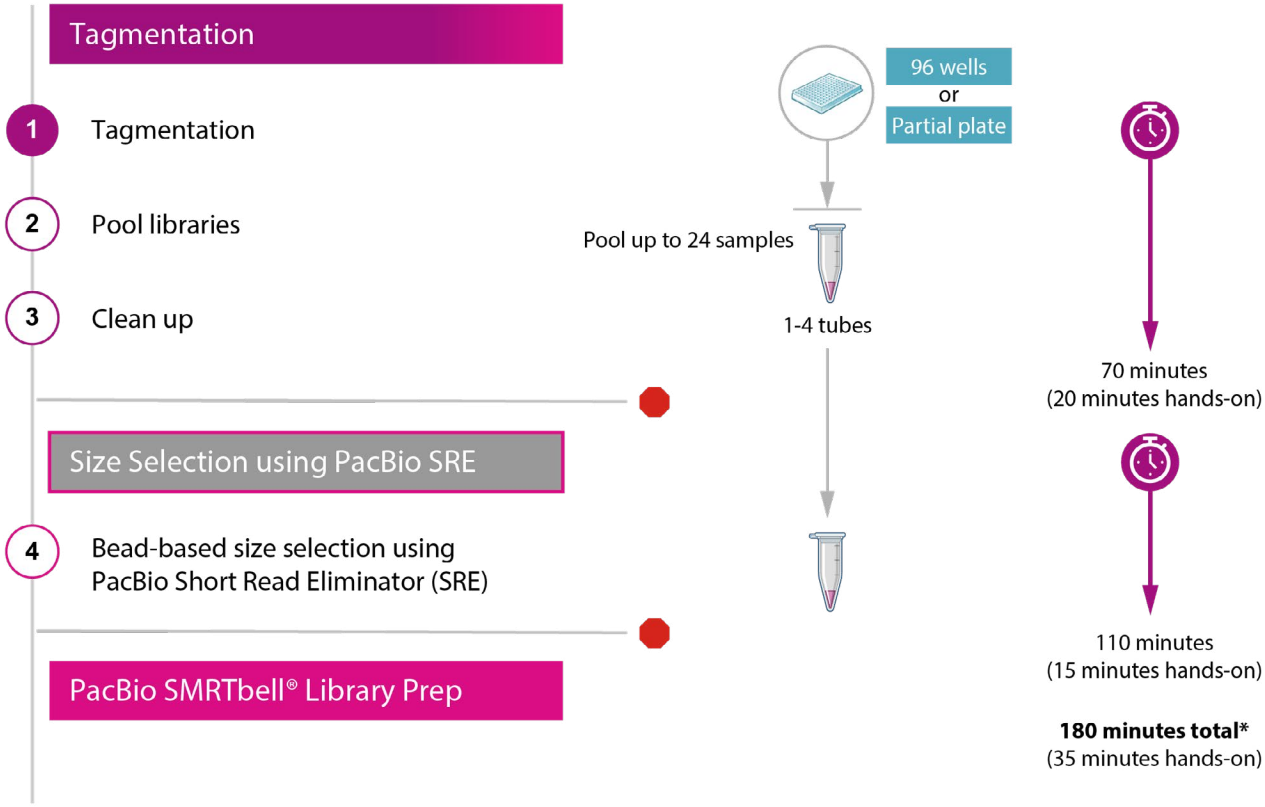


Figure 1. LongPlex >10 kb modified workflow, incorporating SRE size selection of the fragmented sample pool prior to SMRTbell library preparation.

Materials and Methods

Required Reagents

- LongPlex Long Fragment Multiplexing kit - seqWell, Cat. No. 301315
  - Includes UDI Tagging Reagent plate, 3X Coding Buffer, X-Solution, and Library Primer Mix
- AMPure® PB Beads – PacBio, Cat. No. 100-265-900 (Ampure XP beads may be substituted)
- SRE Kit – PacBio, Cat. No. 102-208-300
- SMRTbell prep kit 3.0 – PacBio, Cat. No. 102-182-700
- Qubit™ 1X dsDNA High Sensitivity (HS) Assay Kit – ThermoFisher, Cat. No. Q33230
- Genomic DNA 165 kb Analysis Kit for the Femto Pulse – Agilent, Cat No. .FP-1002-0275
- 80% Ethanol, 10 mM Tris-HCl, Low TE (10 mM Tris-HCl + 0.1mM EDTA, pH 8.0), and Ultra Pure water

Equipment & Consumables

- Single-channel pipettors (1-20 µl, 20-200 µl, 100-1,000 µl) and multi-channel pipettors (1-10 µl, 10-200 µl)
- Pipette tips (low-retention barrier tips; wide bore tips for handling high molecular weight gDNA)
- Eppendorf Tubes® - 1.5 ml DNA LoBind® Tubes
- 96-well PCR plates and seals, or 0.2 ml 8-tube PCR strips with caps
- Thermal cycler with heated lid compatible with above PCR plates or 8-tube PCR strips
- Magnetic stand for 1.5 ml tubes
- Benchtop centrifuge for 1.5 ml tubes capable of 10,000 x g speed
- Vortex mixer
- Heat block set to 50°C for SRE incubation

LongPlex 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

**Refer to PacBio's SMRTbell Prep Kit 3.0 manual for required thermal cycler programs for that kit*

Protocol in brief

Important – Please read before you begin

- **Using high quality genomic is crucial to ensure efficient recovery during SRE size selection. DNA quality must be measured by Femto Pulse with a Genomic Quality Number of GQN30kb ≥7.**
- The minimum batch size is 8 samples to ensure sufficient yield following size selection.
- DNA input is 500-750 ng per sample at a concentration of 33.3-50 ng/µl in 15 µl total volume.
- Please also refer to seqWell's LongPlex user guide for more details on the LongPlex protocol.

1. Modified LongPlex Tagging reaction – Fragmentation and barcoding

a. Pulse-spin UDI Tagging Reagent plate and remove seal carefully to avoid splashing/contamination.

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, pipette mixing slowly 10x after each addition:

Genomic DNA (750 ng)	15.0 µl
UDI Tagging Reagent	8.0 µl
3X Coding Buffer	12.5 µl
Water	2.0 µl
Total Volume	**37.5 µl**

c. Seal REACTION PLATE or TUBE, pulse-spin, transfer to a thermal cycler, and run the **TAG** program with lid-heating on: ***55°C for 15 minutes, 25°C hold***.

d. If <96 reactions are being prepared, carefully reseal the LongPlex tagging reagent plate containing unused wells and return to the freezer for future use. The tagging reagent is stable for up to 12 freeze thaw cycles.

e. 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 of 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.

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

Protocol in brief (continued)

3. Pooling and bead purification

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

b. Create pools of 8 to 12 samples by pooling 50 µl from each well into a 1.5 ml tube.
   - Record the total volume of each pools – i.e., for an 8-plex pool the volume will be 400 µl.
   - **Note: We do not recommend pooling less than 8 samples as yields may be too low for downstream SMRTbell prep.**
   - Pooling more than 12 samples results in the volume after bead addition being larger than 1.5 ml. If more than 12 samples must be pooled, you may split the pool into two for 1X bead purification. See notes at steps h and k below.

c. Vortex room temperature AMPure beads and ensure they are fully resuspended. To each pool, add **1X volume of AMPure** (i.e 400 µl beads tor a 400 µl 8-plex pool), mixing 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 on magnetic stand and allow beads to pellet until the supernatant appears completely clear (~5 minutes). Remove and discard the supernatant with pipette. Be careful not to disturb the bead pellets.

f. Wash beads with 80% ethanol:
   - With tube or plate still on the magnetic stand, add sufficient 80% ethanol to cover the bead pellet
   - After ≥30 seconds, remove and discard supernatant being careful not to dislodge the bead pellet
   - Repeat 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. Once dry, remove the tube(s) from the magnetic stand.

h. Add 65 µl of 10mM Tris-HCl to each tube and mix by pipetting the solution along the inner wall of the tube multiple times to thoroughly resuspend the bead pellet.
   - If you pooled >12 samples and split the pool into 2 for bead clean up, please elute each clean up in 33 µl.

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

j. Return tube to magnetic stand and allow beads to pellet (~2 minutes).

k. Carefully transfer 60 µl of DNA eluate from each tube to a fresh tube. The residual 5 µl may be used for Qubit quantification and Femto sizing.
   - **We highly recommend running a Femto Pulse QC on your LongPlex pool to ensure that step or fragments are >10 kb before proceeding to SRE.**

SAFE STOPPING POINT

Proceed immediately to SRE step, or store the purified pools at -20°C.

Protocol in brief (continued)

4. SRE for removal of small fragments from LongPlex Pools

Please refer to PacBio's SRE user manual for full instructions on performing SRE. No significant changes have been made to the standard SRE protocol except that here we start with a pool of fragmented samples.

seqWell's suggested tips when using SRE:

- The SRE technology is a centrifugation-based size selection method. We highly recommend running a few practice SRE preps using control genomic DNA to gain familiarity with the method.
- In SRE, the DNA pellet generated after centrifugation is typically clear and very hard to visualize. Take extreme caution when removing supernatant to avoid dislodging the pellet.
- PacBio does not recommend the use of pellet paint to dye the DNA pellet in SRE as it can disrupt down stream SMRT sequencing. However, it may be beneficial during your practice runs to include pellet paint to help you visualize and locate the pellet. For that purpose, we suggest adding 1 µl of Pellet Paint® NF Co-Precipitant from Millipore Sigma (Part no. 0748-3) into your practice SRE reactions. However, do not include it in any subsequent SRE selections that are destined for sequencing.
- For additional tips and support on SRE, please contact your PacBio FAS and support team.

seqWell's suggested tips when using SRE:

a. Add 60 µL of Buffer SRE to the sample. Vortex to mix for 5 seconds at max speed.

b. Incubate the tube for 1 hour at 50°C in a heating block.

c. Load tube into centrifuge with the hinge facing toward the outside of the rotor.

d. Centrifuge at 10,000 x g for 30 mins at room temperature.
   - If using a centrifuge with temperature control (i.e., cooling function), turn this function off

e. Carefully remove supernatant from tube without disturbing the pellet. Place the pipette tip on the thumb lip side of the tube (see Figure 1 in SRE user manual).
   - The DNA pellet will have formed on bottom of tube under the hinge region but may not be visible.
   - Leaving up to 10 µL of supernatant is acceptable to be sure the pellet is not disturbed.

f. Add 50 µL of Buffer LTE to the tube and incubate at room temperature for 20 minutes.

g. After incubation, pipette-mix 20 times and vortex the tube for 5s to ensure that the DNA is properly resuspended and mixed.

h. Quantify the size selected DNA using Qubit and check the fragment size via Femto Pulse.

i. Recovery from SRE size selection should be >40% if a sufficient quantity of high-quality DNA was used

SAFE STOPPING POINT

Proceed immediately to SRE step, or store the purified pools at -20°C.

Protocol in brief (continued)

5. SMRTbell library prep kit 3.0

Please refer to PacBio's SMRTbell Prep Kit 3.0 user manual for full instructions for making SMRTbell libraries.

LongPlex specific SMRTbell protocol notes - these can also be found in the standard LongPlex user guide:

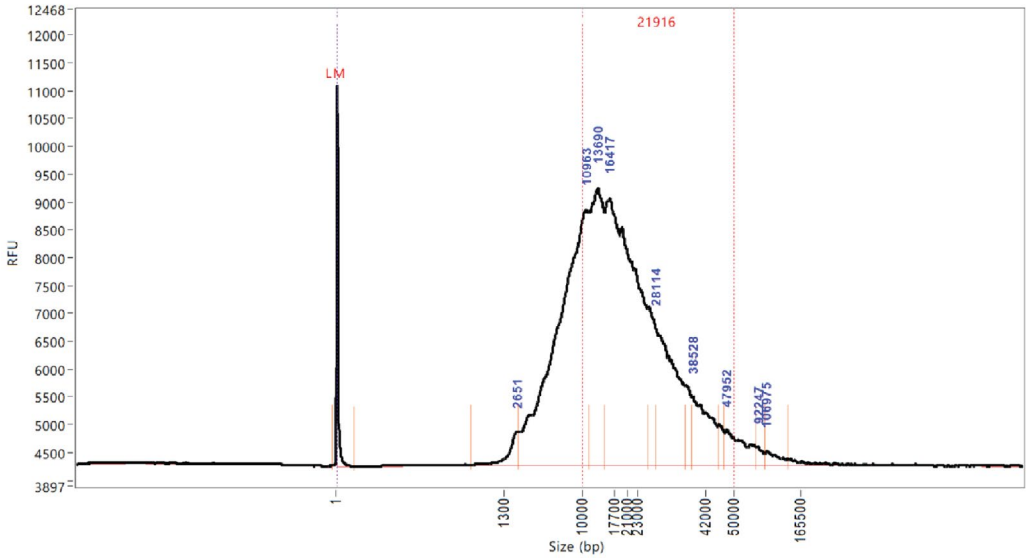
- As LongPlex pools have already been fragmented, please start at the **Repair and A-tailing step** on page 10 of the above linked SMRTbell Prep Kit user manual.
- Use 46 µl of the SRE size selected pool as the starting input to the Repair and A-tailing reaction.
- Because the LongPlex tagged samples contain indexed adapters, you may use the unindexed adapter that comes in the SMRTbell prep kit 3.0. However, Indexed SMRTbell adapters (sold separately) may also be used to increase multiplexing capabilities and flexibility of SMRT cell loading.
- Following PacBio sequencing, the HiFi bam file output from SMRT Link must be further demultiplexed using the LongPlex inline barcodes. Please refer to seqWell's GitHub page for all required demultiplexing information. This page includes files containing the sequences of the 10 bp dual indexes.

Please reach out to support@seqwell.com for any questions relating to this modified protocol.

Appendix – Typical fragment size and yields post SRE

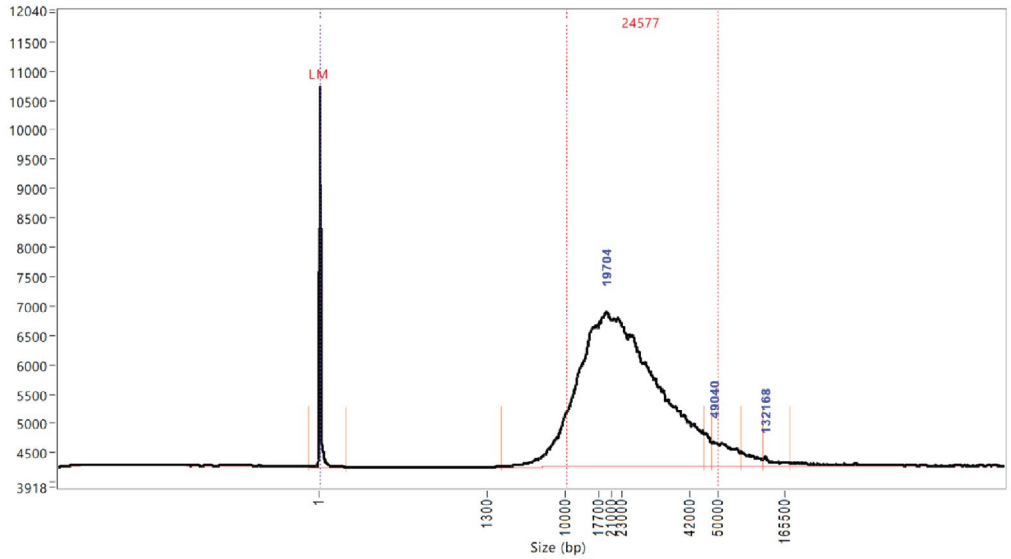
Example Femto Pulse trace of pool before SRE size-selection

8-plex LongPlex pool starting with 500 ng of high-quality DNA. Total DNA quantity pre-SRE = 2226 ng



Example Femto Pulse trace of pool following SRE size-selection

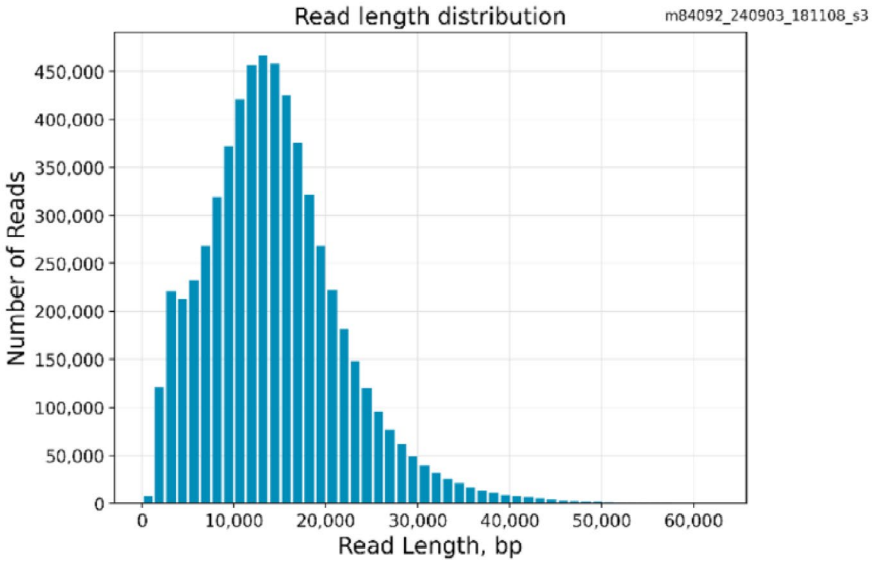
Same 8-plex pool following SRE size selection. Total DNA quantity post-SRE = 1030 ng (~46% recovery)



Appendix – Example Revio SMRT Sequencing Output

SMRT cell sequencing performance

Sample ID	HiFi Reads	HiFi Yield (Gb)	HiFi Read Length (mean, bp)	HiFi Read Quality (median)
SEQW102-S102-0001	6,110,413	87.98	14,400	Q36



Post demultiplexing and alignment mean insert size length per pool (8 samples per pool):

Pool ID	Mean Insert Size (bp)	StdDev across 8 samples (bp)
Pool A	13,786	807
Pool B	15,149	533
Pool C	15,039	777

Summary

This technical note introduces a modified protocol for generating HiFi read lengths greater than 10 kb using the LongPlex™ Long Fragment Multiplexing Kit in combination with PacBio's Short Read Eliminator (SRE) technology. The LongPlex method utilizes transposase-based tagmentation to enable efficient fragmentation and sample barcoding while eliminating the need for mechanical shearing. This modified protocol extends the capabilities of the standard LongPlex workflow by enabling the processing of high-quality DNA to generate longer read lengths, ultimately enhancing sequencing throughput and data quality.

By leveraging the modified LongPlex protocol, researchers can maximize read lengths and sequencing data yield while maintaining high efficiency, reducing preparation time, and lowering overall sequencing costs. This approach ensures improved throughput and optimized performance for applications requiring long-read sequencing.

Key protocol highlights include:

- **High-Quality DNA Requirement:**
  Only high-quality genomic DNA (Femto Pulse GQN30kb ≥7) can be used to ensure sufficient recovery post-SRE selection.
- **Optimized Fragmentation and Pooling:**
  The method supports 8–24 sample pools, ensuring efficiency in size selection and sequencing.
- **Integration with PacBio SRE Technology:**
  The use of SRE removes short DNA fragments (<10 kb), resulting in improved read length and sequencing efficiency.
- **Seamless SMRTbell Library Prep:**
  The modified protocol aligns with PacBio's SMRTbell Prep Kit 3.0 workflow, allowing for straightforward high-throughput sequencing.