

Modified LongPlex™ Protocol: Whole-Plasmid PacBio® HiFi Sequencing

Introduction

Plasmids are core tools across biopharma used as expression vectors for therapeutic proteins, gene/cell therapy components, mRNA templates, and CRISPR delivery systems. Their sequence integrity is critical as a single mutation or rearrangement can have impacts ranging from protein yield and stability to therapeutic efficacy and safety. Additionally, errors are propagated at scale supporting the need for sequence confirmation throughout therapeutic development.

Sequencing is often used only to confirm the construct insert, leaving the backbone unverified — a risk given the prevalence of plasmid errors¹. A challenge to using short-read NGS approaches is their struggle with repetitive and structurally complex regions. While long-read sequencing captures the full-plasmid sequence more reliably, scale remains a key limitation: verifying hundreds to thousands of constructs demands multiplexed, cost-effective library prep which many commonly used workflows can't deliver.

seqWell's LongPlex Multiplexing Kit was built to meet this need². LongPlex uses tagmentation-based linearization and barcoding upstream of PacBio® SMRTbell® prep to increase multiplexing and lower per-sample HiFi sequencing costs, making large-scale, full-plasmid long-read sequencing practical.

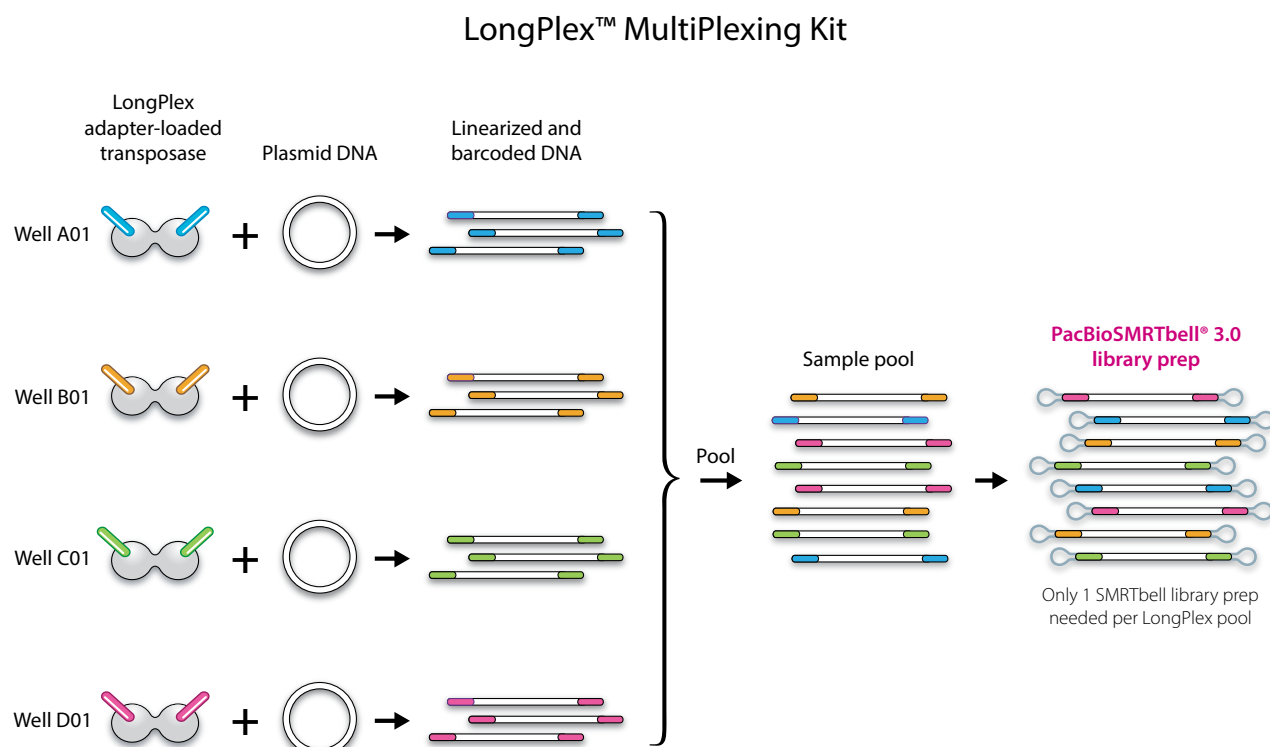
In this technical note, we describe a modified LongPlex protocol to support whole-plasmid PacBio HiFi sequencing.

LongPlex Multiplexing

- Eliminates mechanical shearing that limits users' ability to economically scale long read library preparation.
- Utilizes a transposase-based workflow to simultaneously fragment and barcode DNA samples in a rapid and streamlined workflow.
- Enables users to efficiently multiplex up to 24 or more samples in a single SMRTbell library prep, saving time, labor, and cost.

Please
reach out to
support@seqwell.com
with questions about
this modified
protocol

LongPlex Molecular Diagram

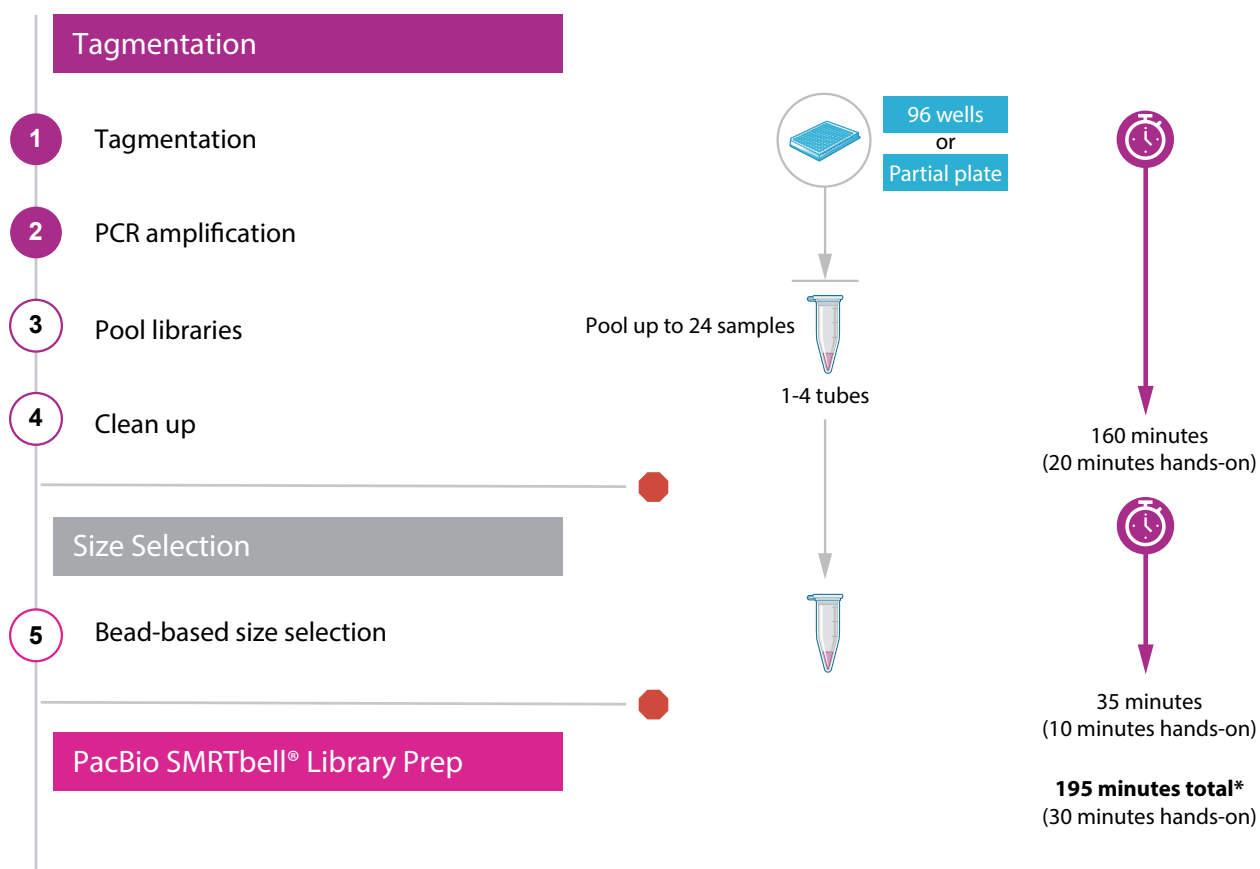


LongPlex simultaneously linearizes and barcodes plasmid DNA upstream of PacBio SMRTbell library prep

LongPlex Workflow

LongPlex™

Long Fragment Multiplexing
for Whole-Plasmid Sequencing



Materials and Methods

Required Reagents

- LongPlex Multiplexing kit, 96 reactions - seqWell, Cat. No. 301310
- Modified Tn5 Storage Buffer, 1.5 ml (sold separately) - seqWell, Cat. No. 300145
- AMPure® PB beads PacBio, Cat. No. 100-265-900
- 100% Ethanol (molecular biology grade)
- 10 mM Tris-HCl, pH 8.0
- Low TE (10 mM Tris-HCl + 0.1mM EDTA, pH 8.0)
- Ultra Pure Water (PCR grade)
- Qubit™ 1X dsDNA High Sensitivity (HS) Assay Kit - ThermoFisher, Cat. No. Q33230, Quant-iT™ PicoGreen™ dsDNA Assay Kits - ThermoFisher, Cat. No. P7589, or other validated dsDNA quantification assay
- Genomic DNA 165 kb Analysis Kit for the Femto Pulse for post fragmentation sizing - Agilent Cat. No. FP-1002-0275
- KOD Xtreme Hot Start DNA Polymerase - Millipore Sigma Cat. No. 71975-3
- For downstream PacBio library prep:
 - SMRTbell prep kit 3.0 - PacBio Cat. No. 102-182-700
 - Optional: To increase multiplexing capabilities, PacBio barcoded SMRTbell adapters may be substituted for the standard non-barcoded adapters provided in the 3.0 kit.

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

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 User Manual for required thermal cycler programs for that kit

Protocol in brief

1. Modified LongPlex Tagging Reaction – Fragmentation and UDI Barcoding

- a.** Begin with the recommended starting input for plasmid applications (see below). Transposase tagging is DNA concentration dependent, so it is important to normalize plasmid DNA to the target concentration, when possible, to ensure consistency in size and yield:
 - 50 ng of total DNA at 3.33 ng/μl in 15 μl
 - DIN ≥ 6.5 recommended
- b.** Remove the Tagging Reagent plate from the freezer and pulse spin.
 - i) The Tagging Reagent plate contains transposomes loaded with 96 unique dual indexes (UDIs). Each well is single use.
 - ii) If not using the entire plate, you may use a razor blade to cut the seal for just the columns you wish to use. To re-store, place a foil adhesive seal over the plate and store at -20°C.
- c.** Dilute the UDI Tagging Reagent 5-fold using Modified Tn5 Storage Buffer from seqWell as the diluent before setting up tagging reaction for each sample as follows:
 - i) The volume of Diluted Tagging Reagent needed in the tagging reaction is 10 μl. Suggested dilution method: Transfer 3 μl of Undiluted Tagging Reagent from the provided source plate to a new destination plate (or strip tube(s) and adding 12 μl of Modified Tn5 Storage Buffer to achieve a 5-fold dilution (total volume 15 μl).
 - ii) Mix thoroughly via slow pipette mixing ≥10x before moving to next step. Try to avoid generating excess bubbling and foaming.
- d.** To a separate 96-well plate or strip tube(s), set up the Tagging Reaction for each sample as follows. Reagents must be added in the following order, gently pipette mixing ≥10x between each addition:

Reagents	Volume
Plasmid DNA (at 3.33 ng/μl)	15 μL
5-Fold Diluted UDI Tagging Reagent	10 μL
3X Coding Buffer	12.5 μL
Total Volume	37.5μL

- e.** Seal the plate or strip tubes and spin down briefly.
- f.** Run TAG Program: 55°C for 15 minutes with heated lid followed by 25°C hold.
- g.** Once the program is complete, immediately proceed to the next step.

2. Stop Reaction

- a.** Remove plate or strip tube from the cyclor and spin down briefly.
- b.** Add 18.75 μl of X-Solution buffer to each well, pipette mix 10x.
- c.** Seal and run STOP program (68°C for 10 minutes with heated lid, 25°C hold).
- d.** Once the program is complete, proceed directly to the next bead clean up step.

Protocol in brief (continued)

3. Post Stop AMPure PB Bead Clean Up

- a.** Add 56.25 μ l of AMPure PB to each well following Stop Reaction (1X bead volume ratio).
- b.** After adding beads, mix well by pipetting up and down at least 10 times.
Incubate the samples for 5 minutes at room temperature.
- c.** Place the samples on a magnetic stand for 5–10 minutes or until the supernatant is clear. Remove and discard the supernatant.
- d.** Keeping the plate or strip tube on the magnetic stand, wash the bead pellet by adding 180 μ l 80% ethanol. Incubate for 30 seconds at room temperature, then remove and discard the ethanol.
- e.** Repeat the wash for a total of two washes. After removing the ethanol after the 2nd wash, air-dry the bead pellet on the magnet for 2 minutes or until the bead pellet is dry. IMPORTANT: Do not overdry the beads.
- f.** Remove the plate or tubes from the magnetic stand and add 14 μ l 10mM Tris-HCl pH 8 to each sample and mix by pipetting to fully resuspend beads. Ensure the bead pellet is fully resuspended from the wall of the well/tube. Incubate at room temperature for at least 5 minutes.
- g.** Place the plate or tubes on a magnetic stand for 1-2 minutes or until the beads are fully pelleted.
- h.** Transfer 11 μ l to a new PCR plate or strip tube and proceed with PCR amplification, or store unamplified libraries at -20C.

SAFE STOPPING POINT

Protocol in brief (continued)

4. PCR Amplification

KOD Xtreme Amplification set up:

- a.** In a PCR plate or strip tube, set up the PCR reaction for each sample as follows:

Component	Volume
Unamplified Library	11 µl
KOD Buffer	25 µl
dNTPs (2 mM each)	10 µl
Library Primer Mix	3 µl
KOD Xtreme Pol	1 µl
Total Volume	50 µl

- b.** Pipette mix well, seal or cap, and transfer to the cycler and run the KOD Xtreme cycling program.

- i) Cycling program for KOD Xtreme:

NOTE: Tn5 leaves a 9 nt gap that is filled with an initial incubation at 68°C prior to cycling, see program below:

68°C for 10 minutes (fill-in step)

94°C for 2 minutes

8 cycles of:

98°C for 10 seconds

68°C for 8 minutes

72°C for 8 minutes

4°C HOLD

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

- c.** Proceed directly to the post-PCR clean up step, or you may store unpurified PCR reactions at -20°C for up to 24 hours.

5. Post Amplification Sample Pooling and AMPure PB Bead Clean Up of Pool

- a.** Pool 16 µl of each well into a 2 ml tube - up to 24 wells per tube

- i) For >24 samples, pool into multiple tubes, with a max of 24 samples per tube
ii) Record the total volume of the pooled samples (i.e. for 24 wells: 16 µl x 24 = 384 µl).

- b.** Add 1X volume of AMPure PB beads to each tube.

- i) For a pool of 24 (see above): Add 384 µl of AMPure PB beads to the 384 µl sample volume.
ii) For other pool plexities, add 1X volume based on the recorded total volume (also see above).

Protocol in brief (continued)

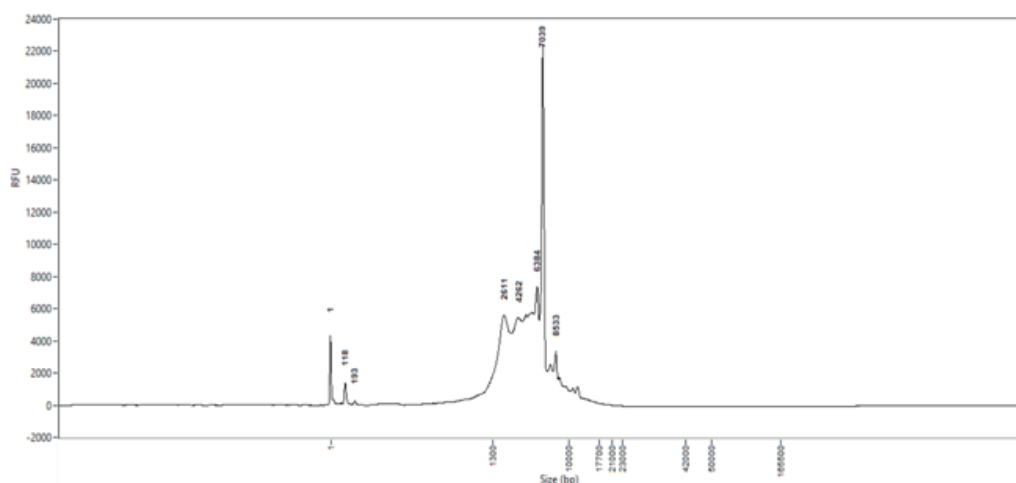
- c. After adding beads, mix well by pipetting up and down at least 10 times.
- d. Incubate the samples for 5 minutes at room temperature.
- e. Place the samples on a magnetic stand for 5–10 minutes or until the supernatant is clear. Remove and discard the supernatant.
- f. Keeping the tubes or plate on the magnetic stand, wash by adding sufficient 80% ethanol to cover the bead pellet. Incubate for 30 seconds at room temperature, then remove and discard the ethanol.
- g. Repeat the wash once, for a total of two washes. After removing the ethanol after the 2nd wash, air-dry the bead pellet on the magnetic stand for 2-5 minutes or until the bead pellet is dry. IMPORTANT: Do not overdry the beads.
- h. Remove the tube(s) from the magnetic stand and add 32 μ l of Lo TE to each sample and mix by pipetting to fully resuspend beads. Incubate at room temperature for at least 5 minutes.
- i. Place the tube(s) on the magnetic stand for 1-2 minutes or until the beads are fully pelleted.
- j. Transfer 30 μ l of the eluted library to a new tube and proceed to downstream library QC and pooling (if desired).
- k. The purified pools can be used directly as the starting material for SMRTbell prep 3.0 (see section 7 for details).

SAFE STOPPING POINT

Protocol in brief (continued)

6. LongPlex Pool QC

- Post LongPlex, we highly recommend using an Agilent Femto Pulse to size long fragments as it is the most accurate method. If using the Agilent Tapestation, fragments will appear much larger and more variable.
- We also recommend quantifying the pool by Qubit or Pico Green to ensure you have sufficient quantity for downstream PacBio SMRTbell 3.0 prep. We suggest using 1-2 µg of LongPlex pooled samples as the starting input for SMRTbell 3.0 prep.



Example of the electropherogram generated by an Agilent Femto Pulse gDNA 165kb Analysis of a 24-plex LongPlex plasmid library, starting from a mixed of 24 plasmid samples with 8-14kb sizes.

7. Notes for Starting with LongPlex Pools in SMRTbell Library Prep Kit 3.0

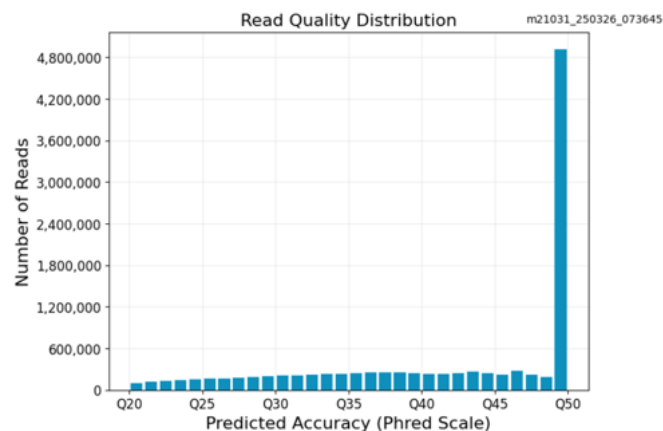
- LongPlex libraries can be pooled and processed directly through PacBio's SMRTbell prep kit 3.0 for whole genome sequencing on PacBio's Sequel IIe, Revio, or Vega. Follow the recommendations below for preparing PacBio libraries.
- Reagents supplied by user from PacBio: PacBio SMRTbell 3.0 prep kit
- LongPlex libraries are already fragmented, barcoded, and pooled, thus can be treated as amplicons in downstream PacBio library preparation.
- Refer to PacBio's Procedure & checklist – Preparing multiplexed amplicon libraries using SMRTbell prep kit 3.0 protocol for all methods and additional required materials.
 - **Follow methods starting at “2. Repair & A-tailing”**
 - We suggest a starting input of 1-2 µg of LongPlex samples pools
 - PacBio's barcoded SMRTbells may be used to increase the number of samples to be pooled on a single SMRTcell. Combining LongPlex's 96 UDIs + PacBio's 96 barcoded SMRTbells provides a theoretical maximum of 9,216 samples on a single SMRTcell.

Appendix A – Example of Plasmid Sequencing Summary using seqWell's LongPlex on a PacBio Vega5.

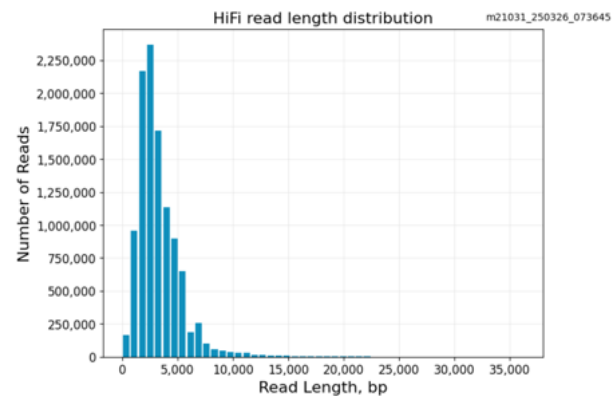
Summary

HiFi reads	10.9 M
HiFi reads yield	36.03 Gb
HiFi reads length (mean)	3,30 kb
HiFi reads length (median, bp)	2,815
HiFi Read Length N50 (bp)	3,829
HiFi Read Quality (median)	Q47
HiFi Read Quality (median)	47
Base Quality Q30 (%)	96.99%
HiFi Number of Passes (mean)	27
Missing adapters (%)	3.22%

Read Quality Distribution



HiFi read length distribution



Example of the HiFi read length distribution generated by a PacBio Vega of a 24-plex LongPlex plasmid library, starting from a mixed of 24 plasmid samples with 8-14kb sizes.

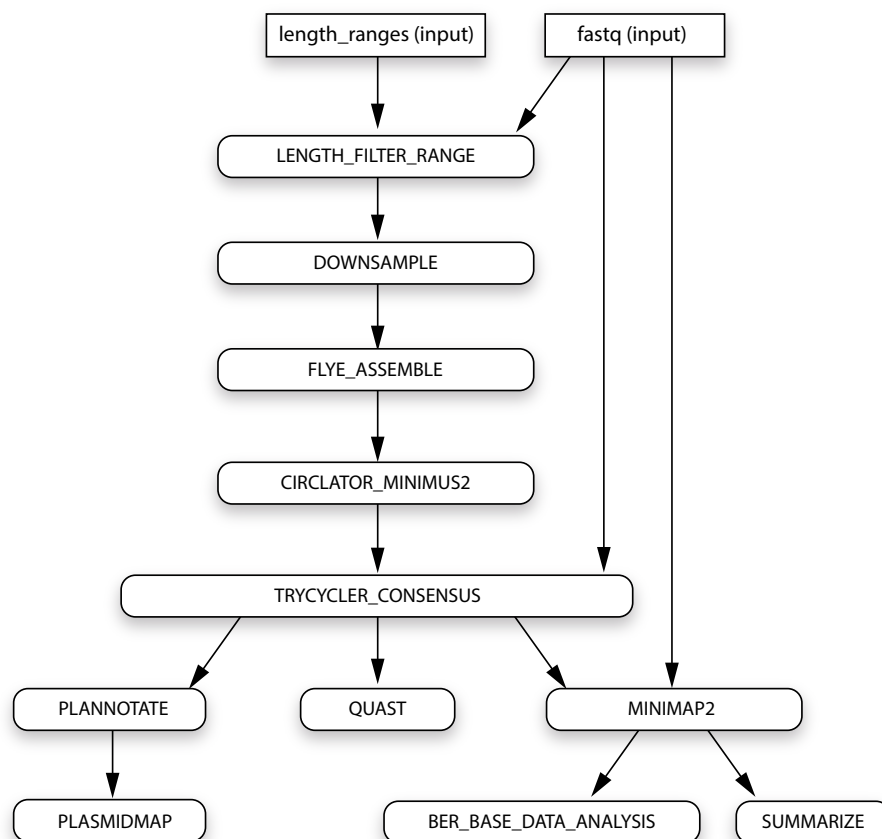
Appendix B – Demultiplexing LongPlex Indexes Using LIMA and Downstream Plasmid Assembly

Demultiplexing LongPlex libraries via seqWell's workflow:

- Using Pacbio's LIMA demultiplexing workflow, we have built tools to demultiplex data using our LongPlex UDIs. The workflow uses the HiFi bam output from SMRTlink, and is packaged as a Nextflow pipeline
- [Link to LongPlex Github page](#)

Downstream Plasmid Assembly Tools:

- Following demultiplexing, plasmid assembly tools are also available on GitHub (see workflow below): <https://github.com/seqwell/nextflow-pacbio-pasmid-flye-assembly>



Summary

Plasmids are key tools across the biopharma industry. Their sequence integrity is a critical attribute and is frequently QC'ed during therapeutic development pipelines. Whole-plasmid sequencing via long-read has emerged as a leading means to confirm the sequence of full plasmids including the backbone and regions that can be problematic to sequence using short-read NGS.

This technical note introduces a modified protocol for fragmenting and multiplexing plasmids using the

LongPlex™ Long Fragment Multiplexing Kit upstream of PacBio SMRTbell library preparation for HiFi sequencing of whole plasmids. The LongPlex method uses transposase-based tagmentation to simultaneously linearize and barcode plasmids followed by sample pooling. A single SMRTbell library prep is needed per multiplexed LongPlex pool. The combination of LongPlex and HiFi barcodes enables a high degree of multiplexing for increased throughput and lower total sequencing cost per sample.

Key LongPlex Benefits:

- **Speed:**

Simple, automation-friendly workflow requiring <1-hour that seamlessly integrates with PacBio SMRTbell library prep

- **Performance:**

Simultaneous linearization and barcoding without compromising library quality

- **Cost -Effective:**

Multiplexed LongPlex pools require only a single SMRTbell prep significantly reducing cost

- **Throughput Gains:**

Large multiplexing capabilities - 96 LongPlex Unique Dual Indexes (UDIs) can be combinatorially expanded HiFi barcodes

References

1. Bai X, Hong JF, Yu S, et al (2024) Prevalence of errors in lab-made plasmids across the globe. bioRxiv 2024.06.17.596931. <https://doi.org/10.1101/2024.06.17.596931>
2. LongPlex Multiplexing Kit [Product Brochure](#)

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