FAQs - LongPlex™ Long Fragment Multiplexing Kit

1. What applications are recommended for the LongPlex Long Fragment Multiplexing kit?

    - The LongPlex kit is recommended for long read microbial and small genome WGS, low pass sequencing, metagenomics, targeted hybridization capture, or any application that requires DNA constructs <10 kB.

2. Can LongPlex libraries be run on sequencers other than PacBio™ instruments?

    - All current protocols are geared and optimized to run on PacBio sequencers, but there is a possibility to load these libraries on other systems. Please reach out to support@seqwell.com for more information.

3. How many samples are processed with a single kit?

    - A single LongPlex kit provides enough reagents to process up to 96 samples. We recommend pooling up to 24 samples (WGS) and 8 samples (targeted capture) for each PacBio SMRTbell™ library preparation.

4. Are all required adapters, indices, and amplification primers included in the LongPlex Long Fragment Multiplexing Kit?

    - The LongPlex kit includes all the indexed adapters and amplification primers necessary to make fragmented indexed libraries. PacBio SMRTbell reagents or other long read instrument specific reagents are not provided. Additionally, if running the target hybrid capture assay, hybridization reagents are also not provided.

5. Are any additional reagents, consumables, or equipment needed?

    - Reagents: AMPure XP beads (Beckman cat no. A63880), 10 mM Tris-HCl, pH 8.0, ultra-pure water, ethanol, reagents for DNA quantification (PicoGreen™), and reagents for gel electrophoresis (Femto Pulse). Optional for PCR plus workflow – KOD™ Xtreme™ Hot Start DNA Polymerase (Millipore Sigma cat no. 71975-3). Additionally, if running on a PacBio instrument - SMRTbell 3.0 library prep kit and barcoded SMRTbell adapters.

    - Consumables: 2 mL LoBind tubes; PCR plate, PCR strip tubes or individual tubes; pipette tips; plate seals.

    - Equipment: Table-top vortex; plate centrifuge; minifuge; appropriate pipettors, magnets (suitable for 2 mL LoBind tubes + plates/strip tubes) for bead-based purification steps; a thermal cycler, equipment for assessing library size by gel electrophoresis (Femto Pulse (recommended)) and library concentration (fluorometer or qPCR instrument).

- Additionally, we have found that samples with SMRTbell adapters can be pooled and/or cleaned up prior to sequencing with Qiagen's DNeasy® PowerClean® CleanUp Kit to maximize sequencing efficiency.

6. If I am processing <96 samples, can I reuse the remaining reagents?

   - Yes, if not using the entire plate, you may use a razor blade to cut the seal for the columns you wish to use. To re-store, place a foil adhesive seal over the plate and store at -20˚C.

DNA Input:

1. What is the recommended DNA input range for the LongPlex Kit?
   - Microbial/Small Genome WGS PCR-free workflow: 250-500 ng
   - Microbial/Small Genome WGS PCR-plus workflow: 150-250 ng
   - Hybrid Capture: 250-500 ng

2. What DNA quality is recommended for the LongPlex kit?

   - Best results for all protocols will be obtained with DIN ≥8. For DIN 6.5 - 8, it is recommended to work with the PCR plus protocol. For DIN < 6.5, a large portion of the DNA is likely already fragmented and targeting ~7-10 kB may be difficult. Please reach out to support@seqwell.com for more assistance.

3. The concentration of DNA sample input is variable. Can the samples still be prepped together?

   - As long as the DNA inputs fall within the recommended range, proceed with no issue. In general, higher DNA inputs will lead to higher yield and longer libraries.

   - If DNA inputs fall outside of the range, please contact support@seqwell.com for further assistance.

4. What quantification methods are recommended?

   - Fluorometric methods for dsDNA (e.g., PicoGreen, Qubit™) are advised over absorbance methods (e.g. Nanodrop™).

5. Is it okay if there is EDTA in my sample buffer?

    - EDTA concentrations ≤0.1mM are compatible with the LongPlex workflow.

    - EDTA concentrations higher than this may lead to little or no fragmentation. In order to remove the EDTA, perform a buffer exchange on the genomic DNA (see Appendix A in the User Guide). In brief, the suggested method is as follows: re-purify by adding 3X volumetric equivalent of AMPure XP beads and follow Beckman's standard SOP for AMPure XP, eluting in 10 mM Tris-HCl. To improve yield, incubate the final elution at 37˚C for 15 minutes. There will be some loss in total DNA so it is recommended to purify twice the amount of DNA than you need.

Library QC:

1. Can I QC sizing using an electrophoretic instrument other than the FemtoPulse?

    - All QC in the protocol is done with the Femto Pulse instrument. Other electrophoretic instruments could size fragments ~ 8kb. However, the fragment size might be reported differently than what is in the protocol.

2. What is the expected fragment size of the LongPlex library?

    - For the LongPlex WGS PCR free workflow, fragment size should be between 8-12 kB on the Femto Pulse. For the LongPlex PCR plus workflow, fragment size should be between 6-8 kB.

3. Why is my fragment size outside of the expected range?

    - If very little to no fragmentation is occurring, it's possible that too much EDTA is in your sample buffer, proceed with the suggested buffer exchange explained above.

    - If fragmentation occurs but the size is >12kb, it is likely that there is too much DNA in your reaction. Re-quantify genomic DNA (using fluorometric methods like Qubit or PicoGreen are recommended) and ensure you are using the target total input.

    - If fragmentation occurs but the size is <7kb, there might be too little DNA in the reaction, re-quantify and ensure it is at the target input. Smaller sizes can also be a result of poor-quality DNA (DIN <6.5). For degraded samples, increase the input up to 500 ng to increase the fragment size. If still having issues, please reach out to support@seqwell.com for further assistance.

Bioinformatics:

1. Do *lima* demultiplexing parameters need to be modified?

    - Yes. Standard *lima* settings look for fragments that have an i7/P7 adapter on one side and an i5/P5 adapter on the other. However, due to the nature of transposase-based fragmentation and tagging, there is some proportion of fragments that will have P5-P5 and P7-P7 adapter instead.

    - While PCR amplification will enrich for P7-P5 ends, there is still a proportion of P5-P5 and P7-P7 fragments that varies based on PCR efficiency, etc. Adjusting the settings in *lima* to look for fragments from all 3 populations (P7-P5, P5-P5, and P7-P7) will increase the total yield of properly demultiplexed reads.

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