plexWell™ LP384 Library Preparation Kit

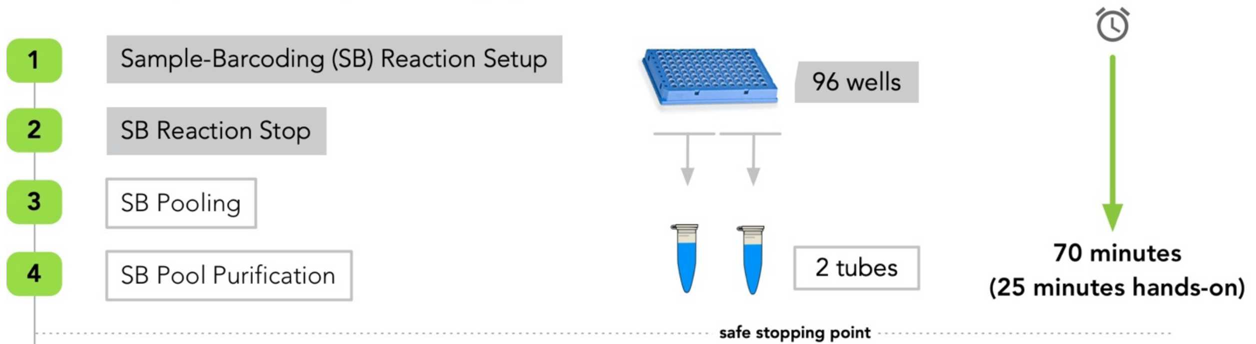
for Illumina® Sequencing Platforms

(Part No. LP384)

User Guide

plexWell 384 Low Pass Library Preparation

Workflow Diagram



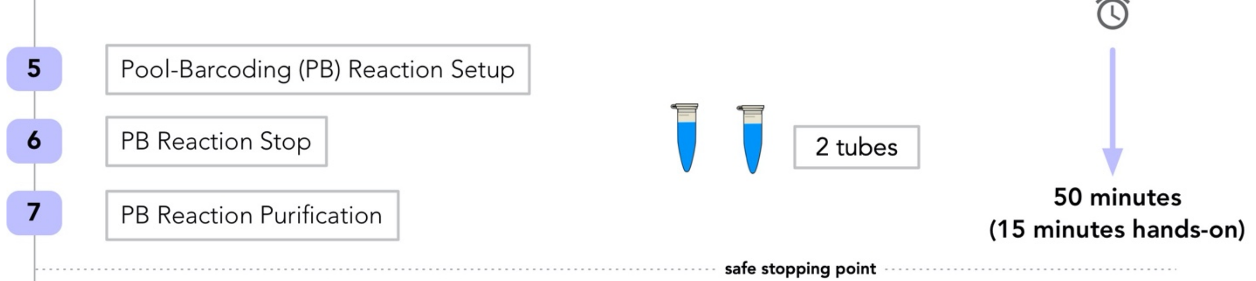


Table 1. Components of plexWell 384 Library Preparation Kit

Box	Component	P/N	Description	Storage	Qty
1	**Sample Barcode Plate***	SBP96 or SBX96	**SBP/X96 Plate:** Assay ready SB reagent in low profile, fully-skirted 96-well red PCR plate	-20°C	4
2	**Coding Buffer (3X)**	CB0384	2 ml tube, violet cap, 1.5 ml	ambient	2
	X Solution	QB0096	2 ml tube, black cap, 1.5 ml	ambient	4
	MAGwise™ Paramagnetic Beads	MG5000	10 ml tube, white cap, 5 ml	4°C	2
3	**Pool Barcode Reagent****	PBX###	0.5 ml tube, red cap, 10 µl	-20°C	8
	Library Primer Mix	PRM052	0.5 ml tube, natural cap, 52 µl	-20°C	1

*Please note we are transitioning from i7 index set SBP96 to SBX96. Confirm your kits index set.

**Complete list of LP384 Pool Barcode Reagents by index set in Table 2.

Table 2. Pool Barcode Reagents and Indexes by kit

Index Set	Component	P/N	A type sequencer	B type sequencer
A	Pool Barcode Reagent X007	PB-X007	CTCTCTAT	ATAGAGAG
	Pool Barcode Reagent X060	PB-X060	TCTCATAT	ATATGAGA
	Pool Barcode Reagent X079	PB-X079	GATCATAG	CTATGATC
	Pool Barcode Reagent X089	PB-X089	CCCTATGG	CCATAGGG
B	Pool Barcode Reagent X021	PB-X021	ATATATGA	TCATATAT
	Pool Barcode Reagent X024	PB-X024	AGGAAACT	AGTTTCCT
	Pool Barcode Reagent X038	PB-X038	TATGGAGG	CCTCCATA
	Pool Barcode Reagent X044	PB-X044	TGATACAT	ATGTATCA
C	Pool Barcode Reagent X048	PB-X048	TCCGACTA	TAGTCGGA
	Pool Barcode Reagent X055	PB-X055	ATGGACAT	ATGTCCAT
	Pool Barcode Reagent X056	PB-X056	TTGCATTG	CAATGCAA
	Pool Barcode Reagent X083	PB-X083	CTCAAATA	TATTTGAG

Illumina Type A sequencing workflow Instruments: MiSeq, HiSeq 2000/2500, NovaSeq

Illumina Type B sequencing workflow Instruments: iSeq, MiniSeq, NextSeq, HiSeq 3000/4000, HiSeqX

Introduction

plexWell Low Pass 384 Library Preparation Kits come in an assay-ready 96-well configuration to streamline high-throughput multiplexed library preparation. Each kit contains sufficient reagents to prepare dual-indexed Illumina-compatible libraries from 384 individual DNA samples. The kit comes in versions A, B, and C allowing users to multi-plex up to 1152 samples, if required. Multiple DNA types are suitable input for the kit, ranging in size and complexity for up to 10 million read pairs per sample. plexWell libraries are compatible with the Illumina MiSeq, NextSeq, HiSeq and NovaSeq systems.[1]

This multiplexed library preparation procedure is optimized for inputs of 10 ng of purified dsDNA per sample, and typically generates library fragment lengths ranging from 500 – 1,000 bp. The primary advantages and benefits of using the plexWell Library Preparation Kits are a streamlined 96 sample multiplexed library preparation workflow that tolerates variation in DNA input concentration and greatly saves on labor and consumable costs. Using a plexWell low pass 384 kit, multiple libraries can easily be prepared in 96-sample batches and loaded on the same sequencing run---all in a single day.

plexWell library preparation kits from seqWell utilize proprietary transposase-based reagents to insert barcoded adapters directly into input DNA in two separate steps. In the first barcoding step, different i7-barcoded adapters[2] are inserted into each of the 96 DNA samples in segregated reactions. Next, DNA samples are pooled into two tubes, each containing 48 i7-barcoded DNA samples. In the second barcoding step, a single i5-barcoded adapter is inserted into each pool of i7-barcoded DNA samples. Finally, each 48-plex library is amplified in a single PCR reaction using universal library primers (*i.e.*, P5 and P7 primers), making for a highly efficient multiplexed library prep workflow (see Workflow Diagram).

[1] Refer to Illumina technical documentation for specific instructions on how to set up a sequencing run for a Nextera-style dual-indexed library prior to loading a plexWell library on your model of sequencing system.

[2] For a complete list of all i7 and i5 indices in plexwell kits download the plexWell Kit Index List found in the resources section on the plexwell product page.

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

Reagents

- 80% Ethanol (freshly prepared)
- Tris-HCl, pH 8.0
- PicoGreen® DNA assay (recommended) or other validated dsDNA quantification assay
- KAPA Biosystems HiFi HotStart ReadyMix (KK2602 or KK2601) for library amplification

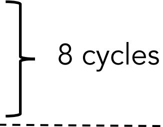
Equipment & Consumables

- 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)
- Eppendorf Tubes® (1.5 ml & 2.0 ml, DNA LoBind Tubes)
- PCR plate seals (must be evaporation-resistant)
- 96-well thermal cycler (compatible with 96 well low profile fully skirted PCR plates, BioRad HSP 9611)
- Magnetic stand for 1.5 ml and 2 ml tubes
- 0.2 ml PCR 8-tube strips and caps/seals
- Benchtop centrifuge to pulse-spin 1.5/2 mL tubes and PCR 8-tube strips
- Plate centrifuge
- Vortex mixer

Thermal Cycler Programs (all with lid-heating on)

- **TAG Program**: 55°C for 15 minutes; 25°C hold.
- **STOP Program**: 68°C for 10 min; 25°C hold.
- **FILL_AMP_8 Program**:
    - 72°C for 10 minutes (fill-in)
    - 95°C for 3 minutes (initial denaturation)

    - 98°C for 30 seconds
    - 64°C for 15 seconds
    - 72°C for 30 seconds

    - 72°C for 3 minutes (final extension)
    - 4°C hold

Before starting procedure:

Measure and adjust input DNA concentration. Assay the DNA concentration of each 96 well plate of samples to be processed by PicoGreen or other validated dsDNA assay. Adjust the average concentration of input DNA across each plate to 1.7 ng/µl (10 ng input) in 10 mM Tris-HCl, pH 8.0. Do not use EDTA-containing solutions (*e.g.*, TE buffer) to dissolve or dilute input DNA because EDTA can inhibit enzymatic activity. *See Appendix A for more detailed information on adjusting input DNA concentration.*

Program thermal cycler(s). For convenience, set-up the thermal cycler programs listed on the previous page before starting.

Pulse-spin kit components. Liquids can condense or shift locations inside containers during shipment or storage. Before using the **SBP/X96 Plate**, and before dispensing from reagent tubes, pulse-spin in a suitable centrifuge to gather the reagents at the bottom of the well or tube. If the kit components freeze during shipment or storage, thaw, mix and pulse-spin before use.

Equilibrate MAGwise Paramagnetic Beads to room temperature. MAGwise beads can be stored for up to 2 weeks at room temperature or for longer periods at 2 - 8°C. If stored cold, warm at 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 if necessary). 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 Coding Buffer is viscous. Store **Coding Buffer (3X)** at room temperature. Pipette slowly and do not pre-wet pipette tips to transfer volumes accurately. While adding **Coding Buffer** to reactions, mix in completely by pipetting up and down several times with the same pipette tip(s) used for addition. Always change pipette tips before adding **Coding Buffer** to different reactions.

Prepare 80% ethanol fresh daily. You will need ~11 ml per 96-well sample plate.

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). You will need ~250 µl per 96-well sample plate. Do not use EDTA-containing solutions (e.g., TE).

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

Procedure

1. Sample-Barcoding (SB) Reaction Set-up

SB reactions should be set-up at room temperature. If processing more than one plate, complete the set-up of one **SBP/X96 Plate** (through starting the thermal cycler) before proceeding to the next one.

a. Pulse-spin the **SBP/X96 Plate**; then remove the seal carefully.

b. Transfer 6 µl of input DNA (approximately 1.7 ng/µl) to each well (one sample per well) of the **SBP/X96 Plate**. Mix thoroughly and slowly by pipetting (5 times at 6 µl), <u>being careful not to introduce excessive bubbles</u>. Use clean tips for addition of each sample.

c. Carefully pipette 5 µl of **Coding Buffer (3X)** to each well of the **SBP/X96 Plate**, using new pipette tips for each transfer. Mix thoroughly and slowly by pipetting up and down ten times at 5 µl, <u>being careful not to introduce excessive bubbles</u>.

   Useful Tip: *Aliquot 70 µl of* ***Coding Buffer (3X)*** *into each well of an 8-tube strip, then use a multichannel pipettor to transfer 5 µl (and mix) into each SB reaction.*

d. Seal the **SBP/X96 Plate**, pulse-spin, then transfer to a thermal cycler, and run the TAG program, below, with lid-heating on:

   55°C for 15 minutes;
   25°C hold

2. SB Reaction Stop

a. Confirm there is no precipitate in the **X Solution.** Slowly pipette contents of tube 5 times to ensure everything is mixed.

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

b. Pulse-spin **SBP/X96 Plate** and then remove seal.

c. Add 7.5 µl of **X Solution** to each well of the **SBP/X96 Plate**. Pipette up and down slowly 10 times to mix. Change pipette tips for each addition.

   Useful Tip: *Aliquot 100 µl of* ***X Solution*** *to an 8-strip tube, then use a multichannel pipette to transfer 7.5 µl from the strip to each column of the* **SBP/X96** ***Plate****.*

d. Seal **SBP/X96 Plate**, pulse-spin, then transfer to a thermal cycler and run the STOP program, below, with lid-heating on:

68°C for 10 minutes
25°C hold

3. SB Pooling (within plate)

a. Pulse-spin **SBP/X96 Plate** and then remove seal.

b. Transfer 18 µl of stopped SB reactions from columns 1-6 into an 8-well strip tube, pipetting twice after each dispense to mix after each addition. Repeat Transfer step from columns 7-12 into a 2nd strip tube. ***Do NOT pool samples from different* SBP/X96 *plates together***!

Optional: If bubbles are present after pooling stopped SB reactions in strip tube, use a tabletop centrifuge to remove bubbles prior to proceeding.

c. Transfer entire contents (95-108 µl) from each well of a strip tube to a 2 ml DNA LoBind tube, pipette twice after each dispense to mix. Repeat process for the 2nd strip tube, pooling into a separate 2 ml DNA LoBind tube.

Optional: If bubbles are present after pooling use a tabletop centrifuge to remove bubbles prior to proceeding.

4. SB Pool Purification

a. Vortex (or vigorously pipet) *room temperature* **MAGwise Paramagnetic Beads** to ensure that the beads are fully resuspended.

b. Add 850 µl (approximately 1 volume equivalent) of MAGwise to the pooled SB reactions and mix thoroughly by pipetting. Incubate on bench for ≥5 minutes to allow DNA to bind.

c. Place tube on magnetic stand and let beads settle, 5 minutes. A pellet should form on one side of the tube and the supernatant should be visibly clear after 5 minutes.

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

Useful tip: *Use a large pipette tip to remove most of the supernatant and then if necessary, use a smaller one to remove the remaining supernatant.*

e. Wash beads with 80% ethanol.

   i. With tube in the magnetic stand, add 1.7 ml of 80% ethanol without disturbing beads. If this volume is insufficient to cover the bead pellet, add a larger volume.
   ii. After ≥30 seconds, remove and discard supernatant, being careful not to disturb pellet, to complete the wash step.

f. Wash beads with 80% ethanol a 2nd time.
   i. With tube in the magnetic stand, add 1.7 ml of 80% ethanol without disturbing beads. If this volume is insufficient to cover the bead pellet, add a larger volume.
   ii. Perform the next steps quickly, working 1-2 tubes at a time
      1. After ≥30 seconds, remove and discard supernatant, being careful not to disturb pellet**.**
      2. Cap tube, pulse-spin and return to magnet, letting beads settle (<30 seconds). Use a small pipet tip (≤20 µl) to remove any residual ethanol at the bottom of the tube.
      3. Add 40 µl of 10 mM Tris to bead pellet, remove from magnetic stand and pipet the solution along the inner wall of the tube multiple times to thoroughly resuspend the bead pellet. Place tube in rack on bench. **DO NOT air dry bead pellet** prior to Tris addition or the DNA recovery will be compromised.

g. Incubate the resuspended beads on the bench for at least 5 minutes to elute the purified SB reaction pool from the beads.

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

i. When the supernatant has cleared completely, carefully transfer 39 µl of eluate to a PCR tube. The transferred eluate contains the purified SB reaction pool.

   Note: *Transfer of some beads with the purified SB reaction pool will not inhibit the Pool Barcode reaction.*

   Optional QC point to check SB recovery: *Picogreen 1 µl of SB eluate. It should be 3.5 to 6.0 ng/µl. If not, contact support@seqWell.com before proceeding.*

SAFE STOPPING POINT

Proceed immediately to the next step or store the purified SB reaction pool at -20°C.

5. Pool Barcoding (PB) Reaction Setup

a. Add 6 µl of **Pool Barcode (PB) Reagent** to each purified SB reaction pool. Pipette five times to ensure entire volume of **PB Reagent** is dispensed.

   Note: ***PB Reagent*** *contains the i5 index. Pools from columns 1-6 have i7 indices that are distinct from those in columns 7-12, thus the same PB reagent should be used for both pools from the same* **SBP/X96** *plate. Use a different PB reagent for each* **SBP/X96** *plate.*

b. Add 22.5 µl of **Coding Buffer** to each PCR tube containing the purified SB pool. Mix thoroughly by pipetting.

c. Cap each PCR tube containing the PB reactions, transfer to a thermal cycler, and run the TAG program, below, with lid heating on:

   55°C for 15 minutes
   25°C Hold

6. PB Reaction Stop

a. Pulse-fuge PB reactions, then add 34 µl of **X solution** to each PB reaction. Mix thoroughly by pipetting slowly 10 times at 50 µl.

b. Re-cap PB reactions, pulse-fuge, and transfer to a thermal cycler and run the STOP program, below, with lid heating on:

   68°C for 10 minutes
   25°C Hold

7. PB Reaction Purification

a. Pulse-spin stopped PB reactions, then transfer entire contents (~101 µl) of each stopped PB reaction to its own 1.5 ml LoBind tube.

b. Briefly vortex or pipette MAGwise to ensure beads are suspended. Then add 101 µl (1 volume equivalent) of MAGwise to each stopped PB reaction and mix thoroughly by pipetting.

c. Incubate on bench for at least 5 minutes to allow DNA to bind.

d. Place tube in magnetic stand and let beads settle, 3 minutes. A pellet should form on one side of the tube and the supernatant should be visibly clear.

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

   Useful tip: *Use a large pipette tip to remove most of the supernatant and then use a smaller one to remove the remaining supernatant.*

f. Wash beads with 80% ethanol.
   i. With tubes in the magnetic stand, add 400 µl of 80% ethanol to each without disturbing beads.
   ii. After ≥30 seconds, remove and discard supernatant, being careful not to disturb pellet.

g. Wash beads with 80% ethanol a 2nd time.
   i. With tubes in the magnetic stand, add 400 µl of 80% ethanol to each without disturbing beads.
   ii. Perform the next steps quickly, working 1-2 tubes at a time
      1. After ≥30 seconds, remove and discard supernatant, being careful not to disturb pellet.
      2. Cap tube, pulse-spin and return to magnet, letting beads settle (<30 seconds). Use a small pipet tip (≤20 µl) to remove any residual ethanol at the bottom of the tube.
      3. Add 24 µl of 10 mM Tris to bead pellet, remove from magnetic stand and pipet the solution along the inner wall of the tube multiple times to thoroughly resuspend the bead pellet. Place tube in rack on bench. **DO NOT air dry bead pellet** prior to Tris addition or the DNA recovery will be compromised.

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

i. Return tubes to magnetic stand and allow bead pellet to form on the inner wall of the tube (~2 minutes).

j. When supernatant has cleared completely, carefully transfer 23 µl of DNA eluate from each tube to new labelled PCR tubes. The transferred supernatant contains the purified PB product.
   Note: *Transfer of some beads with the purified PB product will not inhibit library amplification.*

SAFE STOPPING POINT

Proceed immediately to the next step or store the purified PB reactions at -20°C.

8. Library Amplification

a. Add 4 µl of **Library Primer Mix** to each purified PB product.

b. Add 27 µl of Kapa HiFi Hot Start ReadyMix (2X) to each and mix well by pipetting.

c. Close the PCR tubes, pulse-spin and run the FILL_AMP_8 program, below, with lid heating on:

Fill-in:	72°C for 10 minutes
Initial denaturation:	95°C for 3 minutes
8 Cycles of:	98°C for 30 seconds
	64°C for 15 seconds
	72°C for 30 seconds
1 Cycle of:	72°C for 3 minutes
	4°C hold

SAFE STOPPING POINT

Proceed immediately to the next step, or freeze the amplified library at -20°C.

9. Library Purification

Following library amplification, it is necessary to remove residual primers and short library fragments. To adjust the size of the final library, please see Appendix C for suggestions.

a. After PCR, pulse-spin and transfer each amplification reaction to a 1.5 ml LoBind tube. Measure the total volume. **Note:** volumes normally change due to film-loss and evaporation during thermal cycling so it is important to measure the volume.

b. Dilute each library amplification reaction to a final volume of 205 µl with 10 mM Tris-HCl, pH 8.0 and mix thoroughly. Transfer 200 µl to new 1.5 mL LoBind tubes for purification. Set aside 5 µl of unpurified material from each for a control.

c. Vortex (or vigorously pipette) room temperature MAGwise to ensure beads are completely resuspended.

d. Add 150-160 µl (0.75-0.8 equivalents will typically remove fragments <400 and <300 bp, respectively) MAGwise to the diluted amplified library. Mix thoroughly.

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

f. Transfer the 1.5 ml tubes to a magnetic stand and let the beads settle completely, approximately 3 minutes. A bead pellet will form along one side of the tubes and the supernatant should appear completely clear after 3 minutes.

g. Slowly remove the supernatant with a pipettor and discard. Be careful not to disturb the bead pellets.

h. Wash beads with 80% ethanol.

   i. With tubes in the magnetic stand, add 400 µl of 80% ethanol without disturbing beads.
   ii. After ≥30 seconds, remove and discard supernatant, being careful not to disturb pellet.

i. Wash beads with 80% ethanol a 2nd time.

   i. With tubes in the magnetic stand, add 400 µl of 80% ethanol without disturbing beads.
   ii. Perform the next steps quickly, working 1-2 tubes at a time
       1. After ≥30 seconds, remove and discard supernatant, being careful not to disturb pellet.
       2. Cap tube, pulse-spin and return to magnet, letting beads settle (<30 seconds). Use a small pipet tip (≤20 µl) to remove any residual ethanol at the bottom of the tube.
       3. Add 32 µl of 10 mM Tris to bead pellet, remove from magnetic stand and pipet the solution along the inner wall of the tube multiple times to thoroughly resuspend the bead pellet. Place tube in rack on bench. DO NOT air dry bead pellet prior to Tris addition or the DNA recovery will be compromised.

j. Incubate for 5 minutes on the bench to elute the purified library from the magnetic beads.

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

l. When the supernatant has cleared completely, carefully transfer 28 µl of DNA eluate, containing the purified, multiplexed library, to a new 1.5 ml LoBind tube. The remaining 4 µl of eluate may be used for electrophoretic analysis.

   Optional: Repeat Elution Step to maximize total recovered library. Add 30 µl of 10 mM Tris to bead pellet and pipet to resuspend. Follow steps *j-l*, above, to complete a 2nd elution. A 2nd elution recovers 10-25% of the first elution.

SAFE STOPPING POINT

Store the purified, multiplexed library at -20°C, or proceed directly to library QC.

Library QC

Electrophoretic analysis: Run 4 µl of purified library on a gel along with 5 µl of unpurified amplified library from step 9b. Alternatively, an Agilent Bioanalyzer (High Sensitivity DNA or DNA7500 kits), Tapestation (High Sensitivity D5000 or D5000 kits), or Fragment Analyzer (High Sensitivity NGS Fragment Analysis Kit, DNF-474) can be used following the manufacturer's instructions for these instruments. For optimal sequencing results, use a region analysis for fragments of 300-1300 bp to determine the average cluster-able fragment length for size adjustment with SYBR based qPCR. See Figure 1 (below) for representative traces for purified libraries run on the Fragment analyzer, as well as a representative image of size distribution by gel electrophoresis. Typical recovered fragments range from 350 bp to 1200 bp.

***Note*:** plexWell library preparation kits sometimes produce fragments around 1000-1500 bp that appear to constitute a large portion of the library on instruments such as the Agilent Bioanalyzer, TapeStation, or Fragment Analyzer. However, these fragments do not appreciably contribute to the library concentration and do not cluster on standard Illumina flow cells. As such it is not necessary to remove them from the library. For sequencing on Illumina patterned flow cells, it may be necessary to remove fragments >1200 bp.

qPCR assay: Use 2 µl of each library for qPCR analysis. Follow kit and instrument documentation for appropriate conditions and dilutions. For KAPA Library Quantification kits, prepare a 1/100,000 dilution of the libraries. Use the average fragment size as determined by electrophoresis to calculate the library concentration.

plexWell libraries are sequenced using the same primers as Nextera® libraries. Refer to Illumina technical documentation for specific instructions on how to set up a sequencing run for a Nextera-style dual-indexed library prior to loading a plexWell library on your model of sequencing system.[3]

[3] The sequencing primers provided in TruSeq v3 Cluster kits are incompatible with Nextera-style libraries, including plexWell libraries. The TruSeq Dual Index Sequencing Primer Box from Illumina is required for sequencing plexWell libraries on older systems, such as the HiSeq 2500, HiSeq 2000, HiSeq 1500, GA*II*x, and HiScanSQ.

Appendix A: Adjusting starting sample concentration

plexWell LP384 kits perform optimally with 10 ng of dsDNA per well, however, individually adjusting each sample to 1.7 ng/µl is not necessary as plexWell LP library preparation kits are formulated to tolerate up to a 5-fold difference in sample input (5 to 25 ng) within a SB pool. To achieve the best library performance, apply a global dilution factor to the input samples in a 96-well plate such that the average and median DNA concentration across all samples is 1.4-2 ng/µl (*i.e.,* 8-12 ng input per sample).

If the method used to produce input DNA for library prep is well-characterized and generates consistent amounts of DNA per sample (*i.e.,* low CV), it may be adequate to assay only several or a few dozen samples from a 96-well plate (*i.e.,* spot-check the DNA concentration using a PicoGreen Assay). However, if the DNA concentration is extremely variable across the samples (*e.g.*, if the concentration difference between any two samples is greater than 5-fold), then outlier samples may need to be individually diluted into the acceptable 5-fold concentration range in order to achieve more uniform read counts across samples.

If the DNA concentration of your input samples cannot easily be confined to a 5-fold range, or, if an average sample concentration of 1.7 ng/µl cannot be easily achieved, consider improving the method used to produce input DNA and assaying samples more rigorously before starting the plexWell library prep procedure.

Important Reminder: Do not use EDTA-containing solutions (*e.g.*, TE buffer) to dissolve or dilute input DNA because EDTA can inhibit enzymatic activity.

Appendix B: *Guidelines for library prep in smaller batches (<48 samples):*

- When processing <48 samples per pool, the normalization performance may be impacted, thus it is recommended to normalize each sample to 1.7 ng/µl
- When making plexWell libraries with fewer than 48 samples per pool, only pool SB reactions from wells that receive input DNA.
- >40 samples; follow the plexWell 96 procedure as written, pooling only SB reactions from wells that receive input DNA and adjusting SB purification MAGwise volume to 1 volume equivalent
- 24-40 samples; pool only SB reactions from wells that receive input DNA, adjust SB purification MAGwise volume to 1 volume equivalent. Scale the volume of PB reagent (7.5*number of samples used/48)
- <24 samples; consider running replicates of samples to fill all wells of the 48 plex pool.

Appendix C: Alternate purification conditions

We strongly recommend using the MAGwise purification conditions specified in the user guide for library purification, especially for first-time users. Depending on your application, however, you may wish to bias your library toward larger or smaller insert sizes. This appendix provides some general guidelines for modifying MAGwise purification conditions. **Note:** *In addition to fragment size distribution, other library properties (e.g., library complexity, yield, etc.) are impacted by purification conditions.*

Bead-based size-selection depends on several factors, including the binding conditions and the starting population of fragments. See below for general purification guidelines.

MAGwise for final purification

Adjusting the MAGwise volume equivalent by ±0.04X changes the cut-off by approximately 50-100 basepairs as shown in the table below.*

Volume Equivalents of MAGwise (added to diluted library amp)	**Fragments retained (bp)**
0.85	>300
0.80 (recommended)	**>375**
0.75	>475

* **Guidelines only**: Individual results may vary based on the initial size distribution and concentration of the unpurified library.

Appendix D: Demonstrated protocol for pooling of 96 samples/library

This appendix describes modifications to the user guide that will allow you to pool all 96 samples on a plate in a single library. The protocol has been demonstrated to produce sequence-able libraries with similar performance as the 48 plex libraries.

Follow the user guide for **Sample Barcode reaction** (1) and **stop** (2).

During **SB pooling** (3), pool 18 µl from each column together into a single 2.0 mL tube. Mix gently by pipetting, then divide the mixed pooled SB reactions into two 2.0 mL DNA LoBind tubes of ~800-850 µl each.

Follow the **SB purification protocol** (4) for each tube through step e. Make the following alterations in the SB purification protocol:

(f) Following the ethanol washes in 4f, resuspend each pellet in 30 µl of 10 mM Tris.

(i) Transfer 29 µl of cleared eluate from each of the tubes into the same 0.2 ml PCR tube. The PCR tube will contain 58 µl of purified SB tagged material

For the **Pool Barcoding Module**, make the following changes in the following steps

(5a) Add 12 µl of PB Reagent, you will need to use 2 tubes of the same PB, to the purified SB reaction pool.

(5b) Add 35 µl of Coding Buffer to the reaction. Mix thoroughly by pipetting.

(6a) Add 52.5 µl of X Solution to the reaction. Mix by pipetting slowly 10 times.

For the **Pool Barcode Purification** (7) implement the following changes:

(7a) Transfer entire contents (~157 µl) to a 1.5 mL LoBind tube

(7b) Add 157 µl of MAGwise and mix thoroughly by pipetting.

(7g) After removal of the ethanol, resuspend the bead pellet in 48 µl of 10 mM Tris

(7j) Transfer 46 µl to a new PCR tube.

During **Library Amplification** (8) make the following changes

(8a-c) Add 8 µl of library primer mix and 54 µl of Kapa HiFi Hot Start ReadyMix. Mix well by pipetting, then transfer 54 µl to a 2nd PCR tube. Cap tubes, pulse-spin and run the FILL_AMP_8 program with lid heating on.

During **Library Purification** (9), first combine both PCRs into 1 tube and add 10 mM Tris to bring the volume up to 205 µl, then follow the steps in the protocol to remove smaller fragments.

Experienced User Checklist

Sample Barcoding

- Centrifuge **SBP/X96** plate
- Add 6 µl sample to **SBP/X96** and pipette 5x
- Add 5 µl coding buffer to **SBP/X96** and pipette 10x
- Seal plate, centrifuge. Run TAG incubation
- Add 7.5 µl X-solution to SB reactions (**SBP/X96** plate) and pipette 10x
- Seal plate, centrifuge. Run STOP incubation
- Pool 18 µl from each SB reaction into two pools
- Complete SB purification (1 volume equivalent). Elute with 40 µl of 10 mM Tris.
- Transfer 39 µl of purified SB pool to 0.2 ml PCR tube (PB reaction tube)

Pool Barcode Reaction

- Vortex and pluse-fuge Pool Barcode Reagent
- Add 7.5 µl Pool Barcode Reagent to 39 µl of purified SB pool and pipette 5x
- Add 23.5 µl coding buffer to PB reaction tube and pipette 10x
- Pulse-fuge, run TAG incubation
- Add 35 µl X-solution to PB reaction and pipette 10x
- Pulse-fuge, run STOP incubation
- Complete PB purification (1 volume equivalent). Elute with 24 µl of 10 mM Tris.
- Transfer 23 µl of purified PB pool to 0.2 ml PCR tube (amplification tube)

Library Amplification

- Add 4 µl Library primer mix to amplification tube
- Add 27 µl of KAPA HiFi HotStart ReadyMix and pipette to mix
- Run Fill_Amp_8 program
- Dilute amplified Library to 205 µl
- Retain 5ul of unpurified library for QC
- Complete Library Purification (0.75 volume equivalent) with 100 µl of diluted PCR

Revision History

Version	Release Date	Prior Version	Description of changes
20200212	10FEB2020	20191203	• Updated to include part number SBX96 • Addition of optional QC step following SB purification • Updated Introduction to include index set A, B, and C

Technical Assistance

For technical assistance, contact seqWell Technical Support.

Email: support@seqwell.com

Website: https://seqwell.com/products/plexwell-lp-384/

seqWell Inc.
376 Hale St.
Beverly, MA 01915
USA
+1-855-737-9355
support@seqwell.com
https://seqwell.com/

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