



plexWell^f LP384 Library Preparation Kit
for Illumina[®] Sequencing Platforms
(Part No. LP384)

User Guide

v20221104

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, C, D, E, and F allowing users to multi-plex up to 2304 samples, if required. Multiple DNA types are suitable input for the kit, ranging in size and complexity for up to 20 million read pairs per sample. plexWell libraries are compatible with the Illumina iSeq, MiniSeq 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 adapters² 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.

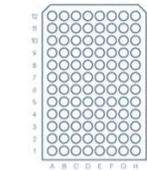
² For a complete list of all i7 and i5 indices in plexwell kits download the plexWell Kit Index List found in the resources section on the plexwell product page.

Table of Contents

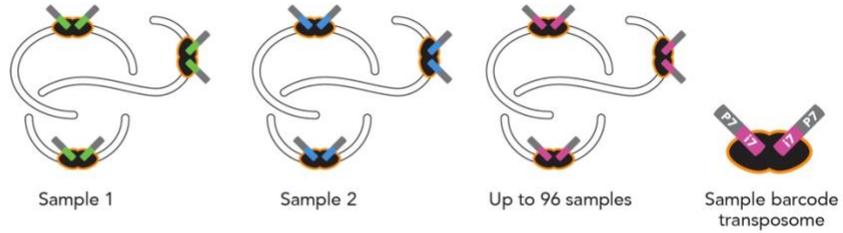
Molecular Diagram	3
Workflow Diagram	4
plexWell Kit Components	5
Considerations before you begin	
Required Equipment, consumables, and reagents	7
Reagent handling	8
plexWell LP384 Library Prep detailed protocol	9
Sample-Barcoding (SB) Reaction Set-up	9
SB Reaction Stop	9
SB Reaction Pooling	10
SB Pool Purification	10
Pool-Barcoding (PB) Reaction Setup	12
PB Reaction Stop	12
PB Reaction Purification	13
Library Amplification	14
Library Purification	15
Library QC	17
Appendices	
A: Adjusting starting sample concentration	19
B: Guidelines for library prep with small pools (<48 samples)	20
C: Alternative purification conditions	21
D: Complete List of indices in PW384 kits	22
E: Sample Sheet and Sequencer Loading Guidelines	23
F: Demonstrated protocol for pooling of 96 samples/library	24
Experienced User Checklist	25

plexWell 384 and 96 Library Prep Molecular Diagram

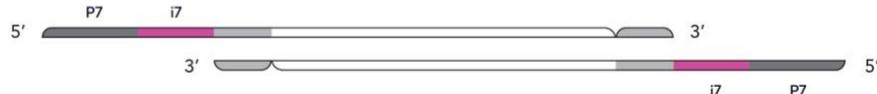
SAMPLE BARCODING:



Dispense DNA into plexWell™ SB plate



Sample-barcoded DNA:



POOL BARCODING:



Pool into single tube



Pool-barcoded DNA:



FILL-IN AND LIBRARY AMPLIFICATION:



Sequencer-ready library



plexWell 384 Low Pass Library Preparation

Workflow Diagram

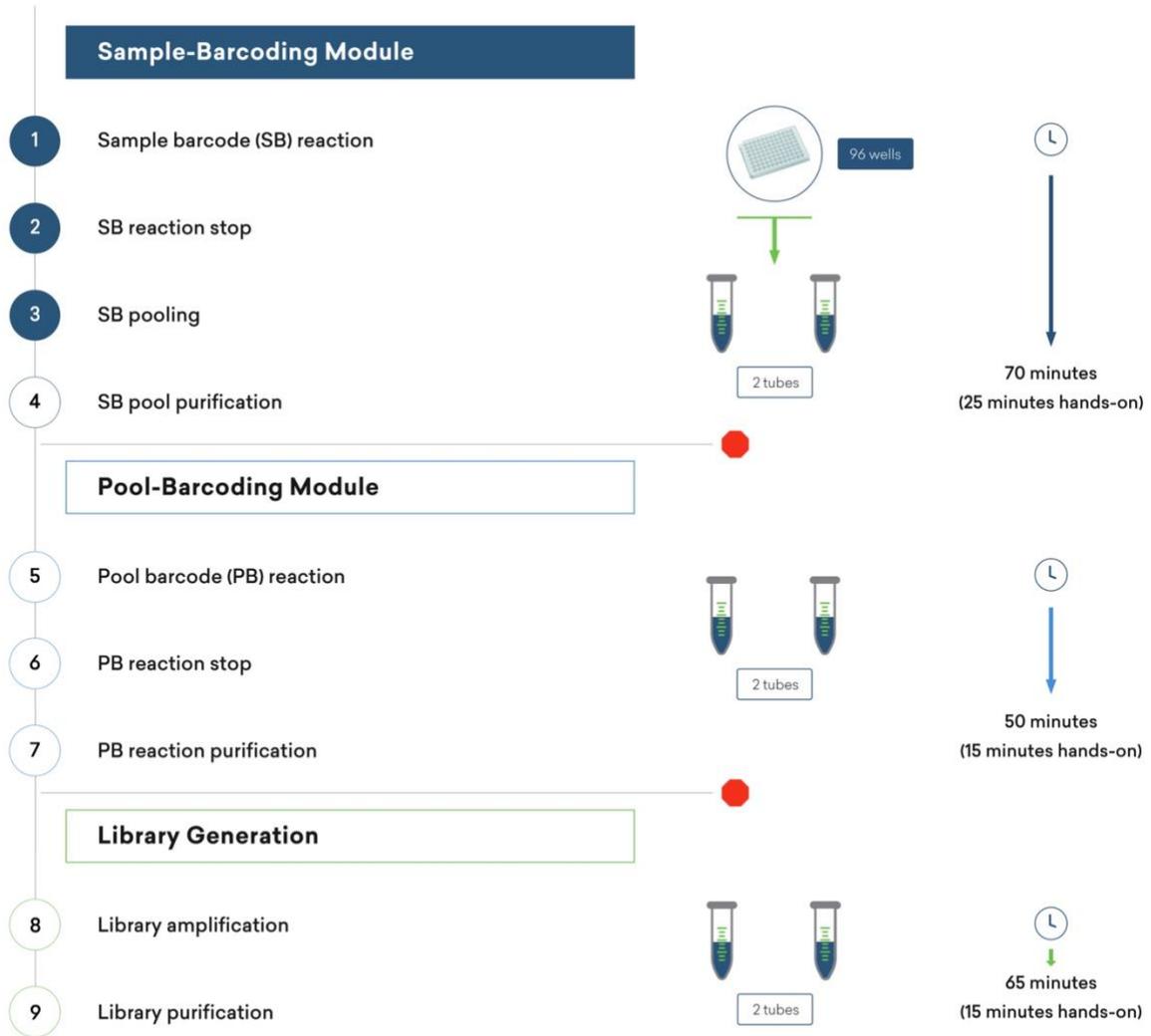


Table 1. plexWell™ Low Pass 384 Library Preparation Kit Configuration

Box Label	Ref	Qty	Shipping	Storage
plexWell™ PW384/LP384 Library Preparation Kit Box 1	PW384-1	1	-20°C	-20°C
plexWell™ PW384/LP384 Library Preparation Kit Box 2	PW384-2	1	ambient	Mixed see box label
plexWell™ PW384/LP384 Library Preparation Kit Index Set X	PW384-3X (X= A, B, C, D, E, or F)	2	-20°C	-20°C

Table 2. plexWell Low Pass 384 Library Preparation Kit Components

Ref	Component	P/N	Description	Storage	Box Qty	Kit Qty
PW384-1 (Box 1)	Sample Barcode Plate	SBP96	SBP96 Plate: assay ready SB reagent in low profile, fully-skirted 96-well red PCR plate	-20°C	4	4
PW384-2 (Box 2)	3X Coding Buffer	CB0384	2 ml tube, violet cap, 1.5 ml	ambient	2	2
	X Solution	QB0096	2 ml tube, black cap, 1.5 ml	ambient	4	4
	MAGwise™ Paramagnetic Beads	MG10000	15 ml bottle, 10 ml	4°C	1	1
PW384-3X (Index Set)	Pool Barcode Reagent*	PBX###	0.5 ml tube, red cap, 10 µl	-20°C	4	8
	Library Primer Mix	PRM384	0.5 ml tube, natural cap, 24 µl	-20°C	1	2

*Complete list of LP384 Pool Barcode Reagents by index set in Table 3.

Table 3. Pool Barcode Reagents and Indexes by kit

Index Set	Component	P/N	Index – A type sequencer	Index – 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
D	Pool Barcode Reagent X003	PB-X003	TAATTACT	AGTAATTA
	Pool Barcode Reagent X149	PB-X149	TCTAAGAG	CTCTTAGA
	Pool Barcode Reagent X184	PB-X184	CCACCCTA	TAGGGTGG
	Pool Barcode Reagent X193	PB-X193	TGCTGTAG	CTACAGCA
E	Pool Barcode Reagent X116	PB-X116	GACCTAAG	CTTAGGTC
	Pool Barcode Reagent X140	PB-X140	TCCCTAAA	TTTAGGGA
	Pool Barcode Reagent X153	PB-X153	AAATAGAT	ATCTATTT
	Pool Barcode Reagent X178	PB-X178	CATAGGAA	TTCCTATG
F	Pool Barcode Reagent X169	PB-X169	TTGACCGA	TCGGTCAA
	Pool Barcode Reagent X173	PB-X173	AAGGGAAA	TTTCCCTT
	Pool Barcode Reagent X181	PB-X181	CACATGGA	TCCATGTG
	Pool Barcode Reagent X207	PB-X207	CGACGTTG	CAACGTCG

Refer to Appendix E for guidance regarding using Workflow A or Workflow B i5 sequences

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 μ l, 20-200 μ l, 100-1,000 μ l)
- Multi-channel pipettors (1-10 μ l, 10-200 μ l)
- 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 AMP8 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 **SBX96 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 3X Coding Buffer is viscous. Store **3X Coding Buffer** at room temperature. Pipette slowly and do not pre-wet pipette tips to transfer volumes accurately. While adding **3X 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 **3X 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

Before beginning the plexWell LP384 library prep, check that your average sample concentration is 1.7 ng/ μ l (10 ng input). This library prep kit can tolerate up to a 5-fold difference (5-25 ng) in input concentration but the average of all samples should be close to 10 ng. Put in terms of concentration, this means any individual sample can be between 0.83 and 4.2 ng/ μ l but the average should be 1.7 ng/ μ l as measured by a validated dsDNA quantification assay such as PicoGreen.

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 **SBX96 Plate** (through starting the thermal cycler) before proceeding to the next one.

- a. Pulse-spin the **SBX96 Plate**; then remove the seal carefully.
- b. Transfer 6 μ l of input DNA (approximately 1.7 ng/ μ l) to each well (one sample per well) of the **SBX96 Plate**. Mix thoroughly and slowly by pipetting (5 times at 6 μ l), being careful not to introduce excessive bubbles. Use clean tips for addition of each sample.
- c. Carefully pipette 5 μ l of 3X **Coding Buffer** to each well of the **SBX96 Plate**, using new pipette tips for each transfer. Mix thoroughly and slowly by pipetting up and down ten times at 5 μ l, being careful not to introduce excessive bubbles.

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

- d. Seal the **SBX96 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 **SBX96 Plate** and then remove seal.
- c. Add 7.5 μ l of **X Solution** to each well of the **SBX96 Plate**. Pipette up and down slowly 10 times to mix. Change pipette tips for each addition.

Useful Tip: Aliquot 100 μ l of **X Solution** to an 8-strip tube, then use a multichannel pipette to transfer 7.5 μ l from the strip to each column of the **SBX96 Plate**.

- d. Seal **SBX96 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 **SBX96 Plate** and then remove seal.
- b. Transfer 18 μ l 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 SBX96 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 μ l) 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 μ l (approximately 1 volume equivalent) of **MAGwise** to the pooled SB reactions and mix thoroughly by pipetting. Incubate on bench for \approx 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 ($\leq 20 \mu\text{l}$) to remove any residual ethanol at the bottom of the tube.
 - 3. Add $40 \mu\text{l}$ 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 $39 \mu\text{l}$ 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 μ l of SB eluate. It should be 3.5 to 6.0 ng/ μ l. 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 μ l 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 **SBX96** plate. Use a different **PB reagent** for each **SBX96** plate.

- b. Add 22.5 μ l of **3X Coding Buffer** to each PCR tube containing purified SB pool. Mix thoroughly by vortexing for 5 seconds or pipetting ≥ 10 times at ≥ 50 μ l.
- c. Pulse-fuge the PCR tube containing the PB reaction then transfer to a thermal cycler, and run the TAG program, below, with lid heating on:
55 $^{\circ}\text{C}$ for 15 minutes
25 $^{\circ}\text{C}$ Hold

6. PB Reaction Stop

- a. Pulse-fuge PB reactions, then add 34 μ l of **X solution** to each PB reaction. Mix thoroughly by pipetting slowly 10 times at 50 μ l.
- b. Re-cap PB reactions, pulse-fuge, and transfer to a thermal cycler and run the STOP program, below, with lid heating on:
68 $^{\circ}\text{C}$ for 10 minutes
25 $^{\circ}\text{C}$ Hold

7. PB Reaction Purification

- a. Pulse-spin stopped PB reactions, then transfer entire contents (~101 μ l) 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 μ l (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 μ l 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 μ l 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 μ l) to remove any residual ethanol at the bottom of the tube.
 3. Add 24 μ l 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 μ l 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 μ l of **Library Primer Mix** to each purified PB product.
- b. Add 27 μ l 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_AMP8 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 μ l with 10 mM Tris-HCl, pH 8.0 and mix thoroughly. Transfer 200 μ l to new 1.5 mL LoBind tubes for purification. Set aside 5 μ l 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 μ l (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 μ l of 80% ethanol without disturbing beads.
 - ii. After \geq 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 μ l of 80% ethanol without disturbing beads.
 - ii. Perform the next steps quickly, working 1-2 tubes at a time
 1. After \geq 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 ($\leq 20 \mu\text{l}$) to remove any residual ethanol at the bottom of the tube.
 3. Add $32 \mu\text{l}$ 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 $30 \mu\text{l}$ of DNA eluate, containing the purified, multiplexed library, to a new 1.5 ml LoBind tube. The remaining $2 \mu\text{l}$ of eluate may be used for electrophoretic analysis.

SAFE STOPPING POINT

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

Library QC

Electrophoretic analysis: Run an aliquot of purified library along with an aliquot of unpurified amplified library from step 9b through an electrophoretic analysis such as the 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). If instruments mentioned above are unavailable, run 3 μ l of purified library and 5 μ l of unpurified, diluted, library on a 2% Agarose E-gel EX alongside the 1 kb plus ladder (NEB) to determine the median fragment length.

Typical results on the TapeStation High Sensitivity D5000 assay shown on next page (Figure 1). Follow the manufacturer's instructions for these instruments and dilute the library, if appropriate, prior to running. Typically, LP384 libraries should be diluted 1:4 (unpurified library) and 1:6 (final purified library) prior to loading them on a TapeStation High Sensitivity D5000 assay. For optimal sequencing results, use a region analysis for fragments of 200-1500 bp to determine the average cluster-able fragment length for size adjustment with SYBR based qPCR.

Note: plexWell library preparation kits sometimes produce fragments >1000 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.

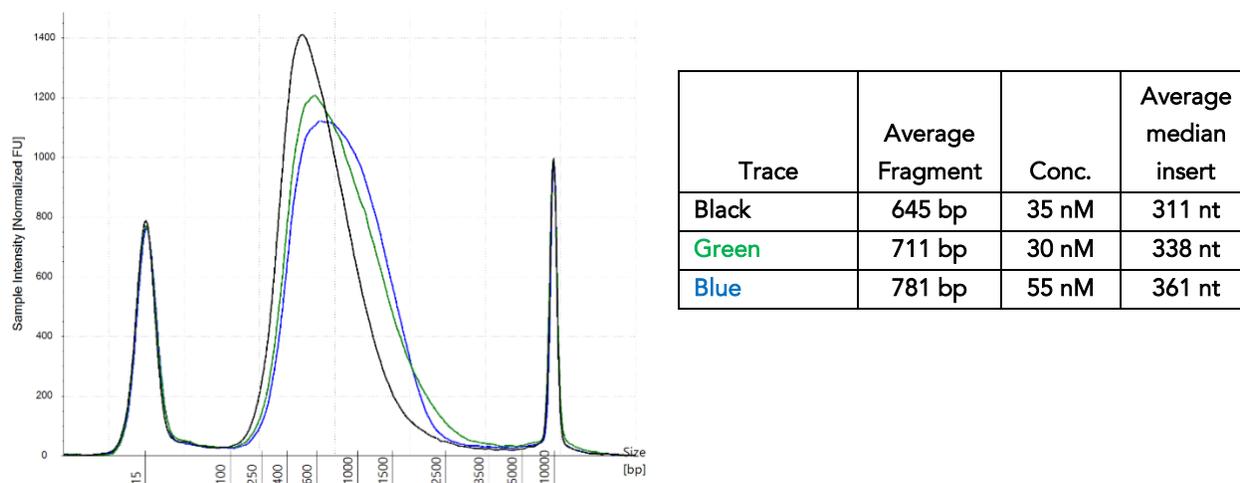
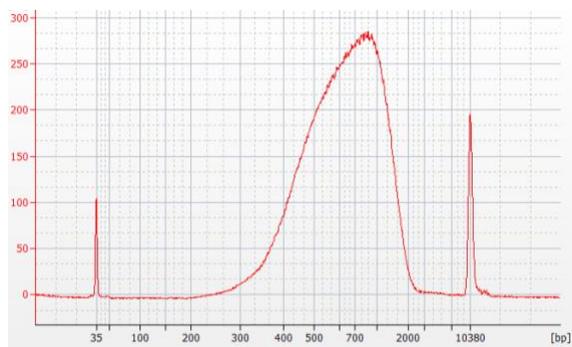
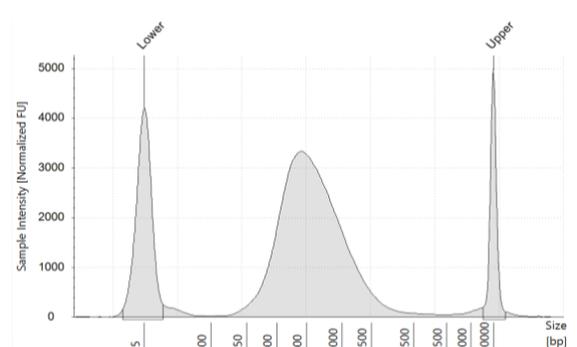


Figure 1. (Left) Representative plexWell LP384 library traces generated using a TapeStation 2200 with High Sensitivity DNA 5000 reagents and tapescreens. Libraries were prepared using human genomic DNA (ATCC NA12878) and purified using 0.8 volume equivalents of MAGwise. Traces selected show the range of “typical” profiles. **(Right)** Table of typical plexWell LP 384 results. Data includes the concentration determined by qPCR using the Kapa Library Quantification kit and the average fragment length (200-1500) for the size adjustment. Insert size was generated by first sequencing the libraries using iSeq v1 chemistry and aligning to the hg38 reference genome, determining the median insert for each of the 48 samples, then taking the average of the 48 values.



Average fragment 697 bp



Average fragment 682 bp

Figure 2. These data demonstrate that the same library may yield similar fragment size distribution on two different electrophoretic analysis. Library were prepared using 96 replicates of *E. coli* genomic DNA with final library purification using 0.8 volume equivalents of MAGwise. **(Left)** Representative plexWell 096 library traces generated using Agilent Bioanalyzer 2100 with a High Sensitivity DNA. **(Right)** Representative of the same plexWell 096 library traces generated using a TapeStation 2200 with High Sensitivity DNA 5000 reagents and tapescreens.

qPCR assay: Use 2 μ l 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.

Sequencer Loading and Read Configuration

Refer to Illumina technical documentation for specific instructions on denaturation and dilution of purified library for your Illumina sequencing system. plexWell 384/96 libraries are dual indexed using 8 nt indices. Each pool contains 96 sample-specific i7 indices and a single pool-specific i5 index. Refer to Appendix E for index sequences. These libraries should be sequenced as dual index (R1, i7, i5, R2) if sequencing more than 96 samples on a single run. However, if only a single pool is being sequenced, they can be run as a single index (R1, i7, R2). The libraries are sequenced using the same primers as Nextera[®] libraries.³ For information regarding setting up a run configuration, refer to Appendix E and Illumina technical documentation.

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, GAIIx, 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/ μ l 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/ μ l (*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/ μ l 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 with small 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/ μ l
- 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 ($6 \times$ number of samples used/48). Scale library primer mix volume to $N/12 \mu$ l, where N= number of sample wells used.
- <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 $\pm 0.05X$ 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: Complete List of indices in PW384 kits

The most up-to-date list of indices for all plexWell kits is available in the Resources section for this product. Click on the “All-plexWell index list” to download an excel workbook containing all i7 indices (in list by row and column formats as well as in plate layout) and i5 indices. The All-plexWell index list is the easiest way to copy and paste index sequences into sample sheets or for demultiplexing.

i7 indices for SBX plates

	1	2	3	4	5	6	7	8	9	10	11	12
A	ACTCACCG	GGCTCCTA	GTTGACAG	CCATTGCG	TACAGAGT	GTTTCGTCT	ACGAAGCG	CAGAGTGG	ATGGAACA	CATCTCT	TCCTCAGA	TTCATTTC
B	CCTTATGT	CAGAAGAA	AATGTGCC	TTCACACT	CTTGTGG	CCAGGTAA	CTCTCAGG	TTGGCTGC	CTAACAC	ACATCTCT	ACGCTGCA	CTAAGGCG
C	ATAGATCC	CAGGAAGG	AAGTACCT	ATGGTCCG	TGTAAGAC	CACAGTCT	CACCGCAA	GATGAGAA	CCATACTC	ACACAACA	CGATGGCA	GTATCGA
D	GGAGCTAT	CGTCTGAA	CGACTAGC	TCCTATCT	CTGGTCGT	TGGTACAG	TGCTCGT	ATGACACC	TCCTTGGC	CAGGCCAT	CAACCGTG	TGGACAAC
E	TGGTGACT	ACTCGAAT	GTTAAGCA	CACATGGT	CTCGTACA	AACGGTTG	CGAGCATT	TGTTGCAC	TCACTCAC	CAACTCCG	TCAACTGA	CTATTCCA
F	CCGAGTTA	GTACCAGC	AACCAATC	GGTGTGAC	CGTAATTC	ATTCCGTA	ACCGTTCC	ATTCTCCA	CAGGCTTC	ACCGACCA	CAGTAGT	CTGCGAAC
G	TGGTGGAA	ACTTCAAC	TCTATTGG	CCACAATG	ATTGCGAG	CGCTCTTG	TCAAGGAT	CGCAACAG	CCTACACA	GTGCGAST	GTGTCCAT	GCCAGTGT
H	CTGTACGC	CCTGTAC	TGAATGTG	TCAGATAC	ACCTGAGC	TGAACTCT	CAAGTGAC	CTTCTGGC	CGCGTGAT	ATGCCGCT	CTAGCCGA	GTGCGTTC

i5 indices for PBX Reagents

Set A			set B			set C		
PB	i5 sequence - A type sequencer	i5 sequence - B type sequencer	PB	i5 sequence - A type sequencer	i5 sequence - B type sequencer	PB	i5 sequence - A type sequencer	i5 sequence - B type sequencer
X007	CTCTCTAT	ATAGAGAG	X021	ATATATGA	TCATATAT	X048	TCCGACTA	TAGTCGGA
X060	TCTCATAT	ATATGAGA	X024	AGGAAACT	AGTTTCCCT	X055	ATGGACAT	ATGTCCAT
X079	GATCATAG	CTATGATC	X038	TATGGAGG	CCTCCATA	X056	TTGCATTG	CAATGCAA
X089	CCCTATGG	CCATAGGG	X044	TGATACAT	ATGTATCA	X083	CTCAAATA	TATTTGAG

Set D			set E			set F		
PB	i5 sequence - A type sequencer	i5 sequence - B type sequencer	PB	i5 sequence - A type sequencer	i5 sequence - B type sequencer	PB	i5 sequence - A type sequencer	i5 sequence - B type sequencer
X003	TAATTACT	AGTAATTA	X116	GACCTAAG	CTTAGGTC	X169	TTGACCGA	TCGGTCAA
X149	TCTAAGAG	CTCTTAGA	X140	TCCCTAAA	TTTAGGGA	X173	AAGGGAAA	TTTCCCTT
X184	CCACCTTA	TAGGGTGG	X153	AAATAGAT	ATCTATTT	X181	CACATGGA	TCCATGTG
X193	TGCTGTAG	CTACAGCA	X178	CATAGGAA	TTCCTATG	X207	CGACGTTG	CAACGTCG

Appendix E: Sample Sheet and Sequencer Loading Guidelines

Illumina sequencing systems differ in their use of sample sheets, availability of on-instrument demultiplexing, and run setup methods. If you have questions for your specific sequencer, contact Illumina tech support for the best guidance on setting up your run using your sequencing platform.

plexWell libraries use the Nextera Adapter sequences and are combinatorial dual-index libraries using 8 nt indices for both the i7 and i5 index sequences. plexWell libraries do NOT require custom sequencing primers.

All Illumina sequencers read the i7 index in the forward direction (as listed in Appendix D and in the plexWell index list available under the resources section of all plexWell products at seqWell.com. The i5 index, however, is read differently on different sequencers depending on the version/chemistry of the sequencing kits. In this case, if using an Illumina sample sheet template, enter the i5 index in the forward direction as the sample sheet will auto-generate the reverse complement if needed. If demultiplexing using bcl2fastq, enter the reverse complement of the i5 index (provided in the Illumina Workflow B column in the plexWell index list).

As of January 1, 2021

The following Illumina sequencers use type A workflow (i5 read in forward direction):

- MiniSeq (rapid only)
- MiSeq
- HiSeq 2000/2500
- NovaSeq 6000 (v1 reagents)

The following Illumina sequencers use type B workflow (i5 read as reverse complement)

- iSeq 100
- MiniSeq
- HiSeq X
- HiSeq 3000/4000
- NovaSeq 6000 (v1.5 reagents)
- NextSeq Systems

Appendix F: 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 μ l 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 \sim 800-850 μ l 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 μ l of 10 mM Tris.
- (i) Transfer 29 μ l of cleared eluate from each of the tubes into the same 0.2 ml PCR tube. The PCR tube will contain 58 μ l of purified SB tagged material

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

- (5a) Add 12 μ l of PB Reagent, you will need to use 2 tubes of the same PB, to the purified SB reaction pool.
- (5b) Add 35 μ l of 3X Coding Buffer to the reaction. Mix thoroughly by pipetting.
- (6a) Add 52.5 μ l 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 (\sim 157 μ l) to a 1.5 mL LoBind tube
- (7b) Add 157 μ l of MAGwise and mix thoroughly by pipetting.
- (7g) After removal of the ethanol, resuspend the bead pellet in 48 μ l of 10 mM Tris
- (7j) Transfer 46 μ l to a new PCR tube.

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

- (8a-c) Add 8 μ l of library primer mix and 54 μ l of Kapa HiFi Hot Start ReadyMix. Mix well by pipetting, then transfer 54 μ l to a 2nd PCR tube. Cap tubes, pulse-spin and run the FILL_AMP8 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 μ l, then follow the steps in the protocol to remove smaller fragments.

Experienced User Checklist

Centrifuge all reagents prior to opening. Pulse-fuge all reactions before and after incubations.

Sample Barcoding

- Centrifuge **SBX96** plate
- Add 6 μ l sample to **SBX96** and pipette 5x
- Add 5 μ l 3X coding buffer to **SBX96** and pipette 10x
- Run TAG incubation
- Add 7.5 μ l X-solution to SB reactions (**SBX96** plate) and pipette 10x
- Run STOP incubation
- Pool 18 μ l from each SB reaction into two pools
- Complete SB purification (1 volume equivalent), eluting with 40 μ l of 10 mM Tris.
- Transfer 39 μ l of purified SB pool to 0.2 ml PCR tube (PB reaction tube)

Pool Barcode Reaction

- Add 6 μ l Pool Barcode Reagent to 39 μ l of purified SB pool and pipette 5x
- Add 22.5 μ l 3X coding buffer to PB reaction tube and vortex 5 seconds
- Pulse-fuge, run TAG incubation
- Add 34 μ l X-solution to PB reaction and pipette 10x
- Pulse-fuge, run STOP incubation
- Complete PB purification (1 volume equivalent), eluting with 24 μ l of 10 mM Tris
- Transfer 23 μ l of purified PB pool to 0.2 ml PCR tube (amplification tube)

Library Amplification

- Add 4 μ l Library primer mix to amplification tube
- Add 27 μ l of KAPA HiFi HotStart ReadyMix and pipette to mix
- Run Fill_Amp8 program
- Dilute amplified Library to 205 μ l
- Retain 5ul of unpurified library for QC
- Complete Library Purification (0.75 volume equivalent) with 200 μ l of diluted PCR, eluting with 32 μ l of 10 mM Tris

Revision History

Version	Release Date	Prior Version	Description of changes
20221104	20221108	20220429	<ul style="list-style-type: none"> • Updated checklist
20220429	20220506	20210609	<ul style="list-style-type: none"> • Updated the introduction • Added the table of content • Added library QC information with Bioanalyzer and TapeStation library profiles • Appendix E, workflow B updated • Appendix G removed
20210609	20210609	20210402	<ul style="list-style-type: none"> • Updated PB indices
20210402	20210402	20210122	<ul style="list-style-type: none"> • Updated Component table for new packaging configuration • Add appendix G for reference to old configuration • Updated PB reaction mixing conditions
20210122	20210125	20200212	<ul style="list-style-type: none"> • Updated workflow diagram and library QC sections • Removed reference to SBP96 • Added information for index sets D and E; molecular diagram, added/updated appendices
20200212	10FEB2020	20191203	<ul style="list-style-type: none"> • 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.

66 Cherry Hill Drive

Beverly, MA 01915

USA

+1-855-737-9355

support@seqwell.com

<https://seqwell.com/>

©2019 seqWell Inc., All rights reserved. Patents pending.
For Research Use Only. Not for use in diagnostic procedures.