plexWell™ 384 Library Preparation Kit
and
plexWell™ 96 Library Preparation Kit
for Illumina® Sequencing Platforms

(Part Nos. PW384, PW096)

User Guide

v20220429

Introduction

plexWell™ 384 and 96 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 and 96 individual DNA samples, respectively. The 384 well kit comes in versions A-F to allow users to multi-plex up to 2304 samples, if required. Multiple DNA types are suitable input for the kit, ranging in size and complexity from amplicons to bacterial genomic DNA. 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 96 and 384 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 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, the i7-barcoded DNA samples are all pooled into a single tube. In the second barcoding step, a single i5-barcoded adapter is inserted into each pool of 96 i7-barcoded DNA samples. Finally, each 96-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 Kits Master Index List found in the resources section on the plexwell product page or refer to Appendix D.

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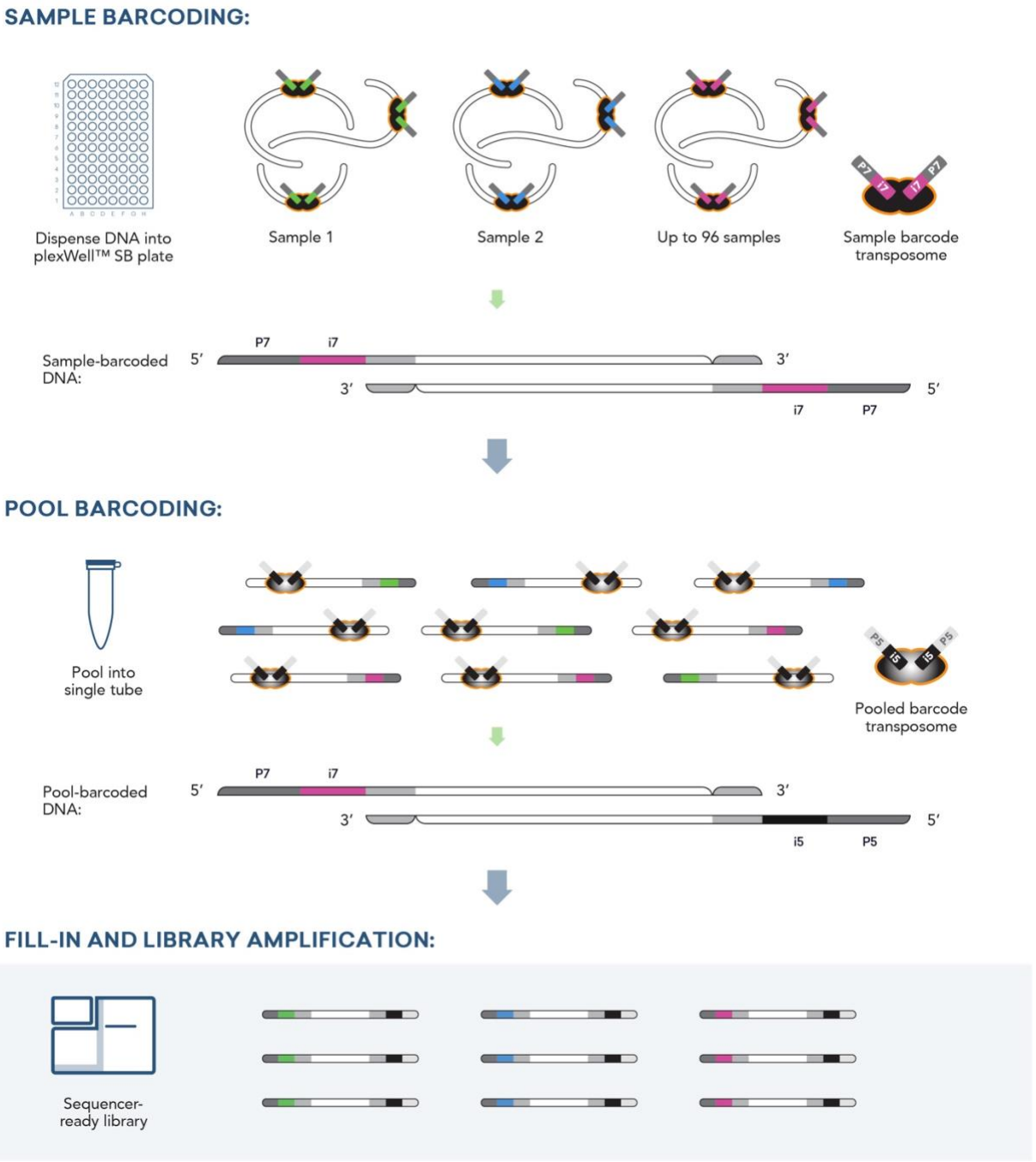


plexWell 96/384 Library Preparation
Workflow Diagram

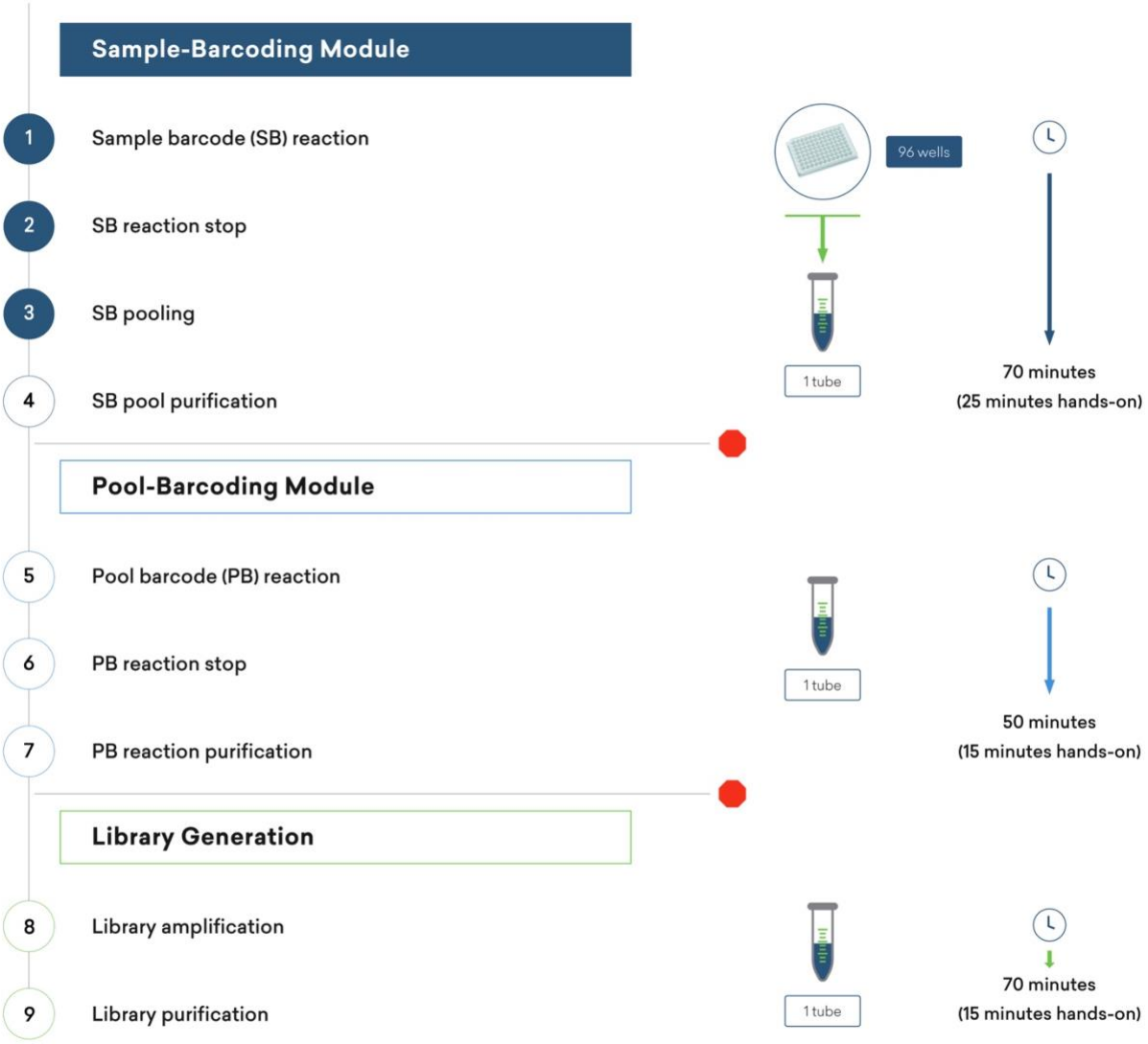


Table 1. plexWell 384 Library Preparation Kit Components

Ref	Component	P/N	Description	Storage	Qty
PW384-1 (Box 1)	**Sample Barcode Plate**	SBX96	**SBX96 Plate:** assay ready SB reagent in low profile, fully-skirted 96-well red PCR plate	-20°C	4
PW384-2 (Box 2)	**3X Coding Buffer**	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	MG10000	15 ml bottle, 10 ml	4°C	1
PW384-3X (Index Set)	**Pool Barcode Reagent***	PBX###	0.5 ml tube, red cap, 10 µl	-20°C	4
	Library Primer Mix	PRM384	0.5 ml tube, natural cap, 24 µl	-20°C	1

*Complete list of PW384 Pool Barcode Reagents by index set in Table 3.

Table 2. plexWell 96 Library Preparation Kit Components

Ref	Component	P/N	Description	Storage	Qty
PW096-1 (Box 1 of 3)	**Sample Barcode Plate**	SBX96	**SBX96 Plate:** Assay ready SB reagent in low profile, fully-skirted 96-well red PCR plate	-20°C	1
PW096-2 (Box 2 of 3)	**3X Coding Buffer**	CB0384	2 ml tube, violet cap, 1.5 ml	ambient	1
	X Solution	QB0096	2 ml tube, black cap, 1.5 ml	ambient	1
	MAGwise™ Paramagnetic Beads	MG5000	10 ml tube, white cap, 5 ml	4°C	1
PW096-3 (Box 3 of 3)	**Pool Barcode Reagent X007**	PBX007	0.5 ml tube, red cap, 10 µl	-20°C	1
	Library Primer Mix	PRM384	0.5 ml tube, natural cap, 24 µl	-20°C	1

Table 3. Pool Barcode Reagents and Indexes by kit (PW384 only)

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 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_AMP12 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
} 12 cycles
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 2.5 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. To transfer volumes accurately, pipette slowly and do not pre-wet pipette. 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 ~4 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 (both 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 384/96 library prep, check that your average sample concentration is 2.5 ng/µl (10 ng input). This library prep kit can tolerate up to a 10-fold difference (3-30 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 0.75 and 7.5 ng/µl but the average should be 2.5 ng/µl 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 4 µl of input DNA (approximately 2.5 ng/µl) to each well (one sample per well) of the **SBX96 Plate**. Mix thoroughly and slowly by pipetting (5 times at 4 µl), being careful not to introduce excessive bubbles. Use clean tips for addition of each sample.

c. Carefully pipette 4 µl 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 4 µl, being careful not to introduce excessive bubbles.

Useful Tip**:** *Aliquot 55 µl of 3X* **Coding Buffer** *into each well of an 8-tube strip, and then use a multichannel pipettor to transfer 4 µl (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 6 µl of **X Solution** to each well of the **SBX96 Plate**. Pipette up and down slowly (10 times at 6 µl). Change pipette tips for each addition.

Useful Tip: *Aliquot 85 µl of* **X Solution** *to an 8-strip tube, then use a multichannel pipette to transfer 6 µl 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. Transfer 9 µl of stopped SB reactions from columns 1-12 into an 8-well strip tube, pipetting twice after each dispense to mix after each addition. ***<u>Do NOT</u> 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

b. Transfer entire contents (95-108 µl) from each well of column 1 (or strip tube) to a 2 ml DNA LoBind tube, pipette twice after each dispense to mix. You will have a total of ~800-860 µl.

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 ε5 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 DNA 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: Use 1 µl of the purified SB reaction pool for PicoGreen. The expected concentration is 4-8 ng/µl. If you are outside this range, contact support@seqwell.com

SAFE STOPPING POINT

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

5. Pool Barcoding (PB) Reaction Setup

a. Add 5 µl of **Pool Barcode (PB) Reagent** to the PCR tube containing the purified SB reaction pool. Pipette up and down five times after dispensing to ensure entire volume of **PB Reagent** is dispensed.

Note: **PB Reagent** *contains the i5 index. To enable multiplexing on the same sequencing runs, use a different* **PB Reagent** *for each SB reaction pool.*

b. Add 22 µl of **3X Coding Buffer** to the PCR tube containing the purified SB pool. Mix thoroughly by vortexing for 5 seconds or pipetting ≥ 10 times at ≥50 µl.

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°C for 15 minutes;
25°C hold

6. PB Reaction Stop

a. Pulse-fuge PB reaction, then add 33 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 reaction, then transfer entire contents (~99 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 99 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. A pellet should form on one side of the tube and the supernatant should be visibly cleared after 3 minutes.

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

f. Wash beads with 80% ethanol.

   i. With tube in the magnetic stand, add 300 µl of 80% ethanol 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 tube in the magnetic stand, add 300 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 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 to new labelled PCR tube. 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 reaction at -20ºC.

8. Library Amplification

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

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

c. Close the PCR tube, pulse-spin and run the FILL_AMP12 program, below, with lid heating on:

Fill-in:	72°C for 10 minutes
Initial denaturation:	95°C for 3 minutes
12 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 B for suggestions on altering MAGwise ratios.

a. After PCR, pulse-spin and transfer the library 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 thus it is necessary to measure the reaction volume post-cycling.*

b. Dilute the library amplification reaction to a final volume of 205 µl with 10 mM Tris-HCl, pH 8.0 and mix thoroughly. Transfer 100 µl to a new 1.5 mL LoBind tube for purification. Set aside 5 µl of unpurified material for a control. Store the remaining 100 µl at -20 C as unpurified material until library QC is complete.

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

d. Add 75 µl (0.75 volume equivalent) MAGwise to the 1.5 ml LoBind tube containing 100 µl of diluted amplified library. Mix thoroughly by pipetting up and down.

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

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

h. Wash beads with 80% ethanol.

   i. With tube in the magnetic stand, add 300 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 tube in the magnetic stand, add 300 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 30 µl of DNA eluate, containing the purified, multiplexed library, to a new 1.5 ml LoBind tube. The remaining 2 µl of eluate may be used for electrophoretic analysis.

SAFE STOPPING POINT

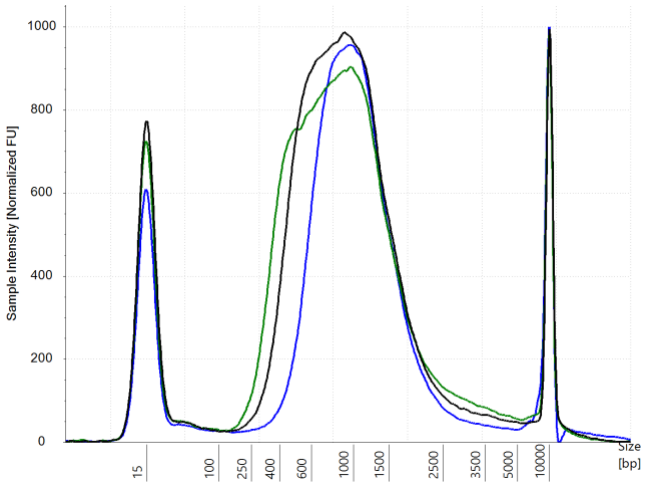
Store the purified, multiplexed library at -20ºC, or proceed directly to library QC.

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 µl of purified library and 5 µl of unpurified, diluted, library on an 2% Agarose E-gel EX alongside the 1 kb plus ladder (NEB) to determine the median fragment length.

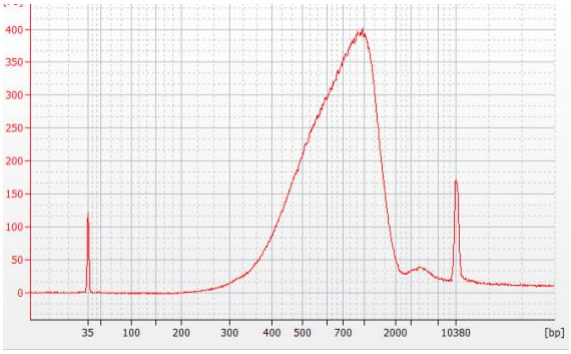
Typical results on the TapeStation High Sensitivity D5000 assay shown on next page (Figure 1). Follow the manufacturer's instructions for these instruments and dilute the library, if appropriate, prior to running. Typically, PW384/96 libraries should be diluted 1:8 (unpurified library) and 1:12 (final 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.

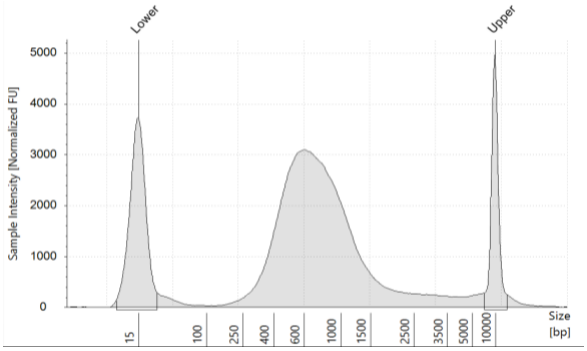
***Note*:** plexWell library preparation kits sometimes produce fragments >1000 bp that appear to constitute a large portion of the library on instruments such as the Agilent Bioanalyzer, TapeStation, or Fragment Analyzer. However, these fragments do not appreciably contribute to the library concentration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



Purification	Average Fragment	Conc.	Average median insert
0.80 volumes	**790 bp**	**56 nM**	**315 nt**
0.75 volumes	**825 bp**	**38 nM**	**387 nt**
0.70 volumes	**905 bp**	**41 nM**	**509 nt**

Figure 1. ***(Left)*** Representative plexWell 384/96 library traces generated using a TapeStation 2200 with High Sensitivity DNA 5000 reagents and tapescreens. Libraries were prepared using E. coli genomic DNA and put through a final library purification using 0.7 (blue), 0.75 (black) and 0.8 (green) volume equivalents of MAGwise. (***Right)*** Table of typical plexWell 384/96 library 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 MiSeq v2 chemistry and aligning to an E. Coli reference genome, determining the median insert for each of the 96 samples, then taking the average of the 96 values.





Average fragment 734bp **Average fragment 719 bp**

Figure 2. This data demonstrates 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 2200 with a High Sensitivity DNA. ***(Rigth)*** 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 µl of purified 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, GA*II*x, and HiScanSQ.

Appendix A: Adjusting starting sample concentration

plexWell 96 and plexWell 384 kits perform optimally with 10 ng of dsDNA per well, however, individually adjusting each sample to 2.5 ng/µl is not necessary as plexWell library preparation kits are formulated to tolerate up to a 10-fold difference in sample input (3 to 30 ng) within a 96 well plate. To achieve the best library performance, apply a global dilution factor to the input samples in a 96-well plate such that the average DNA concentration across all samples will be 2.5 ng/µl (*i.e.,* average of 10 ng per input 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 10-fold), then outlier samples may need to be individually diluted into the acceptable 10-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 10-fold range, or, if an average sample concentration of 2.5 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 (<96 samples):

- When making plexWell libraries from fewer than 96 samples, only pool SB reactions from wells that receive input DNA.
- >80 samples; follow the plexWell 96 procedure as written, pooling only SB reactions from wells that receive input DNA
- 64-80 samples; change SB pooling volume to 11 µl per well (instead of 9 µl per well), adjust volume of MAGwise beads for SB pool purification to 1 volume equivalent of the total pool volume.
- 48-60 samples; change SB pooling volume to 14 µl per well (instead of 9 µl per well), adjust volume of MAGwise beads for SB pool purification to 1 volume equivalent of the total pool volume.
- <48 samples; consider running replicates of samples to fill all wells of the **SBX96 Plate**.
- For processing any number of samples <96, also consider using the plexWell Plus 24 Library Preparation Kit from seqWell (P/N: PWP24), featuring a flexible multiplexed workflow for generating libraries in batches of 8 - 24 samples at a time. Enough reagents are provided with the plexWell Plus 24 kit to prepare libraries from 96 individual DNA samples.

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. This user guide calls for purifying only half of your total amplified multiplexed library. However, if the fragment size distribution of the library purified under standard conditions (0.75 volume equivalent of MAGwise) does not meet your specific application needs, the remaining 100 µl of unpurified library amplification can be used to optimize size selection. See below for general purification guidelines.

MAGwise for final purification

Adjusting the MAGwise volume equivalent by ±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)	Average clusterable Fragment *	Median insert *
0.80	>325	**790 bp**	**315 nt**
0.75 (recommended)	**>400**	**825 bp**	**387 nt**
0.70	>475	**905 bp**	**509 nt**

*Average clusterable fragment was obtained using a 200-1500 bp smear analysis on a TapeStation 2200. Libraries were diluted 1:12 prior to running them on a HSD5000 tapescreen. Insert size was determined from sequencing on the MiSeq using MiSeq v2 reagents.

For specific applications and starting DNA (such as cDNA or amplicons ≤500 bp), it is advisable to use a higher ratio of MAGwise beads to preserve more of the shorter fragments. In these cases, it is reasonable to use up to 0.9 volume equivalents during the final library purification.

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 "plexWell Kits Master 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 plexWell Kits Master 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	GTTCGTCT	ACGAAGCG	CAGAGTGG	ATGGAACA	CATCTTCT	TCCTCAGA	TTCCATTC
B	CCTTATGT	CAGAAGAA	AATGTGCC	TTCACACT	CTTGTTGG	CCAGGTAA	CTCTCAGG	TTGGCTGC	CTAACAAC	ACATCCTT	ACGCTGCA	CTAAGGCG
C	ATAGATCC	CAGGAAGG	AAGTACCT	ATGGTCCG	TGTAAGAC	CACAGTCT	CACCGCAA	GATGAGAA	CCATACTC	ACACAACA	CGATGGCA	GTTATCGA
D	GGAGCTAT	CGTCTGAA	CGACTAGC	TCCTATCT	CTGGTCGT	TGGTACAG	TGCTCCGT	ATGACACC	TCCTTGGC	CAGGCCAT	CAACCGTG	TGGACAAC
E	TGGTGACT	ACTCGAAT	GTTAAGCA	CACATGGT	CTCGTACA	AACGCTTG	CGAGCATT	TGTTGCAC	TCACTCAC	CAACTCCG	TCAACTGA	CTATTCCA
F	CCGAGTTA	GTACCAGC	AACCAATC	GGTGTGAC	CGTAATTC	ATTCCGTA	ACCGTTCC	ATTCTCCA	CAGGCTTC	ACCGACCA	CAAGTAGT	CTGCGAAC
G	TGGTGGAA	ACTTCAAC	TCTATTGG	CCACAATG	ATTCGCAG	CGCTCTTG	TCAAGGAT	CGCAACAG	CCTACACA	GTGCGAGT	GTGTCCAT	GCCAGTGT
H	CTGTACGC	CCTGTTAC	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	AGTTTCCT	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	CCACCCTA	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

Experienced User Checklist

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

Sample Barcoding

- ❑ Centrifuge **SBX96** plate
- ❑ Add 4 µl sample to **SBX96** and pipette 5x
- ❑ Add 4 µl 3X coding buffer to **SBX96** and pipette 10x
- ❑ Run TAG incubation
- ❑ Add 6 µl X-solution to SB reactions (**SBX96** plate) and pipette 10x
- ❑ Run STOP incubation
- ❑ Pool 9 µl from each SB reaction
- ❑ Complete SB purification (1 volume equivalent), eluting 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

- ❑ Add 5 µl PB Reagent to 39 µl of purified SB pool and pipette 5x
- ❑ Add 22 µl 3X coding buffer to PB reaction tube and vortex 5 seconds
- ❑ Run TAG incubation
- ❑ Add 33 µl X-solution to PB reaction and pipette 10x
- ❑ Run STOP incubation
- ❑ Complete PB purification (1 volume equivalent), eluting 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_Amp12 program
- ❑ Dilute amplification to 205 µl with 10 mM Tris. Transfer 100 µl to purification tube.
- ❑ Complete library purification (0.75 volume equivalent), eluting with 32 µl of 10 mM Tris

Revision History

Version	Release Date	Prior Version	Description of changes
20220429	20220506	20210609	● Table of content added ● Added library QC information with Bioanalyzer and TapeStation library profiles ● Appendix E, workflow B updated ● Appendix F removed
20210609	20210609	20210402	● Updated PB indices
20210402	20210402	20210122	● Updated Component table for new packaging configuration ● Add appendix F for reference to old configuration ● Updated PB reaction mixing conditions
20210122	20210125	20200127	● Removed references to part number SBP96 ● Updated library QC metrics, amp program name, kit contents and workflow diagram ● Added Appendices D and E, molecular diagram
20200127	10FEB2020	20190813	● Updated to include part number SBX96 ● Addition of optional QC step following SB purification

Technical Assistance

For technical assistance, contact seqWell Technical Support.
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