plexWell™ Plus 24 Library Preparation Kit

for Illumina® Sequencing Platforms

(Part No. PWP24)

User Guide

v20210122

Introduction

The plexWell Plus 24 Library Preparation Kit features a flexible multiplexed workflow for generating dual-indexed Illumina-compatible libraries in pools of 8 - 24 samples. Enough reagents are provided with the kit to prepare libraries from 96 individual DNA samples. Multiple purified DNA types are suitable input for this kit, ranging in size and complexity from plasmid to bacterial genomic DNA. PCR products (≥500 bp) also serve as excellent DNA input for plexWell Plus 24 library prep. plexWell libraries are compatible with the Illumina MiSeq, NextSeq, HiSeq and NovaSeq systems.[1]

The plexWell Plus 24 kit accommodates smaller batch sizes providing extra flexibility for those labs that prepare multiplex libraries in batches of 8 to 24 input samples per day. The supplied Pool Barcode Reagent (n=4) adds a unique i5 barcode to each batch, allowing up to 96 different samples per run. Appendix A (inside back cover) illustrates how plexWell Plus libraries can be conveniently prepared in multiple batches and/or batch sizes, and then loaded on to the same sequencing run. Examples are provided in the procedure for preparing multiplexed libraries in batch sizes of 8, 16, or 24 samples.

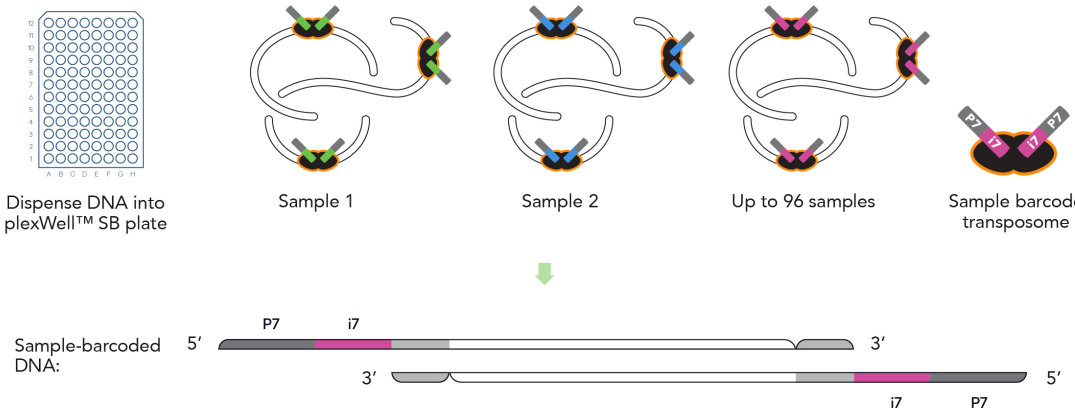
This multiplexed library preparation procedure is optimized for an average of 10 ng of purified input DNA per sample, and typically generates library fragment lengths ranging from 500 – 1,000 bp. The primary advantages and benefits of using the plexWell Plus 24 Library Preparation Kit are rapid processing time (<3 hours), flexible batch size (8 – 24 samples), superior sequencing performance over a wide range of DNA inputs (3 – 30 ng per sample), and significant labor/consumable cost savings.

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 are inserted into each DNA sample in segregated reactions. Next, these i7-barcoded DNA samples are all pooled into a single tube. Then, in the second barcoding step, a single i5-barcoded adapter is inserted into the pool of i7-barcoded DNA samples. Then a moderately to highly multiplexed library can be 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).

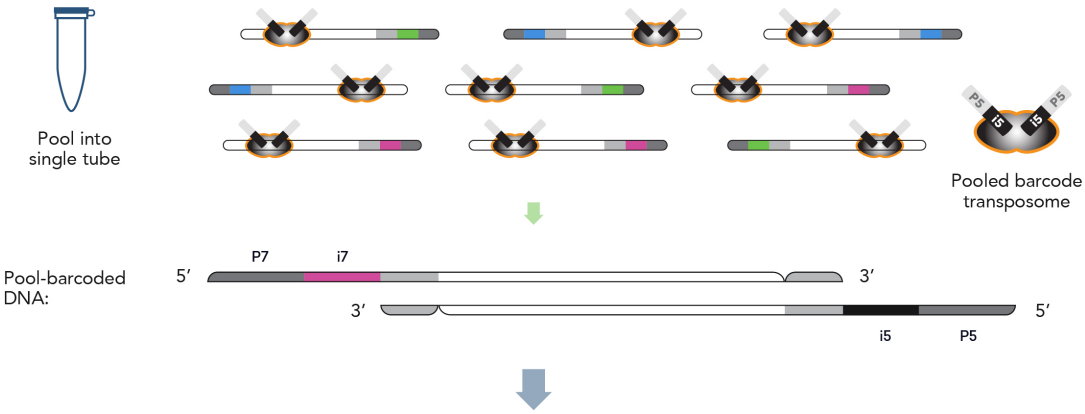
[1] Refer to Illumina technical documentation for specific instructions on how to set up a sequencing run for a Nextera-style dual-indexed library prior to loading a plexWell library on your model of sequencing system.

plexWell Plus 24 Library Prep
Molecular Diagram

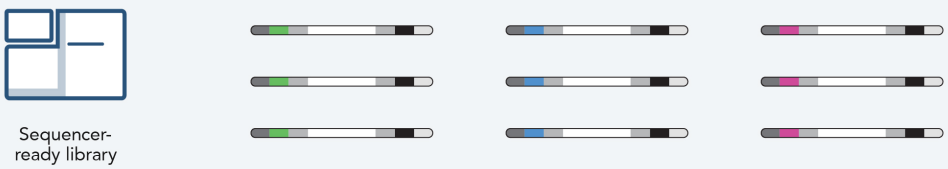
SAMPLE BARCODING:



POOL BARCODING:



FILL-IN AND LIBRARY AMPLIFICATION:



plexWell Plus 24 Library Preparation
Workflow Diagram

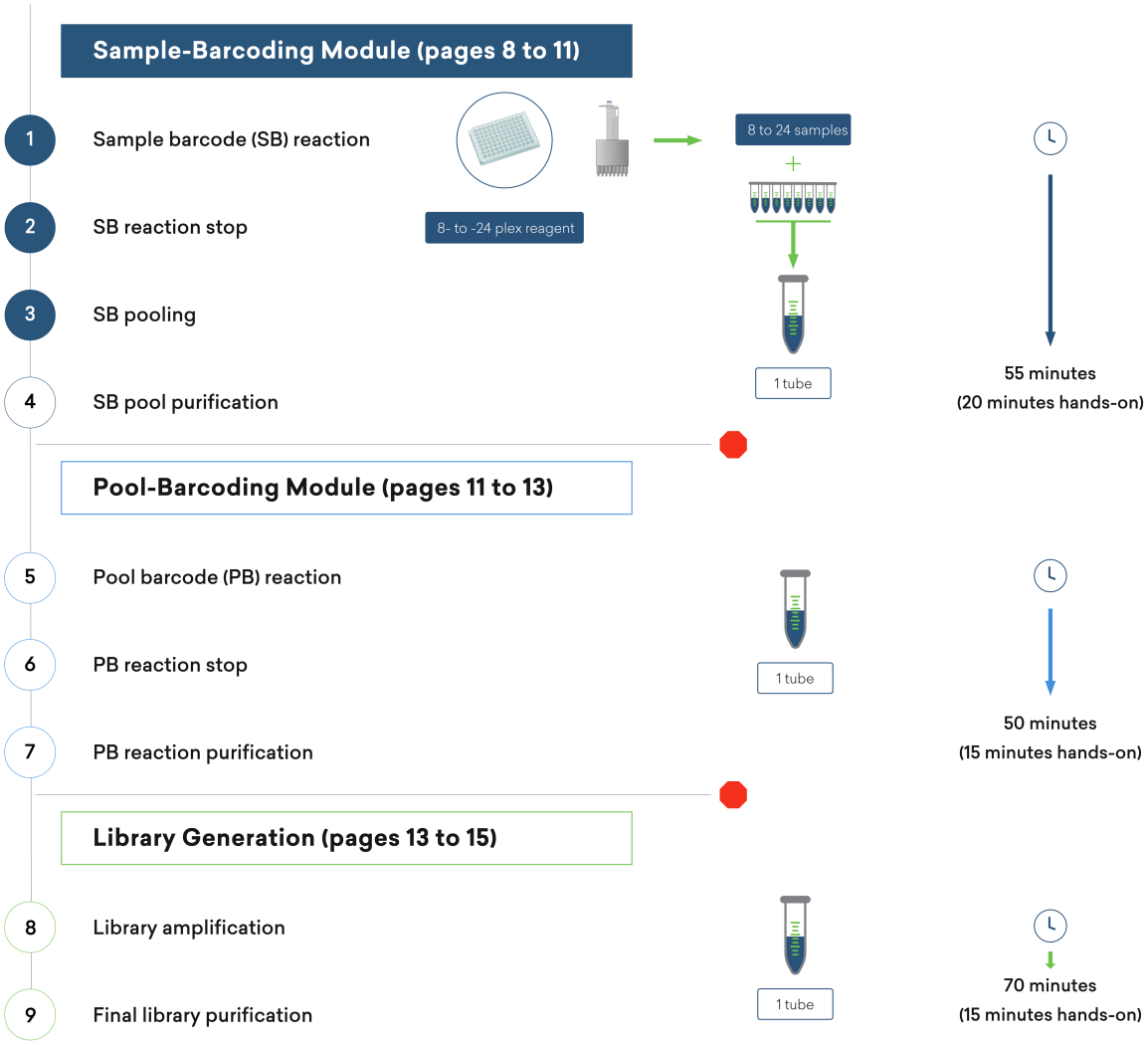


Table 1. Components of plexWell Plus 24 Library Preparation Kit

Box	Component	P/N	Description	Storage	Qty
1	Sample Barcode Plate	SBP24	**SBP24 Plate:** SB reagent in columns 1-3 of a low profile, fully-skirted, blue, 96-well PCR	-20°C	1
2	Coding Buffer (3X)	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, natural cap, 5 ml	4°C	1
3	Pool Barcode Reagent	PBZ012	0.5 ml tube, blue cap, 10 µl	-20°C	1
		PBZ014	0.5 ml tube, blue cap, 10 µl	-20°C	1
		PBZ032	0.5 ml tube, blue cap, 10 µl	-20°C	1
		PBZ047	0.5 ml tube, blue cap, 10 µl	-20°C	1
	Library Primer Mix	PRM052	0.5 ml tube, natural cap, 52 µl	-20°C	1

*Upon arrival, store kit components as indicated in this chart.

User-Supplied Reagents, Equipment & 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, DNA LoBind Tubes)
- PCR plate seals (must be evaporation-resistant at -20°C)
- 96-well thermal cycler
- Magnetic stand for 1.5 ml and 2 ml tubes (ThermoFisher P/N: 12321D, or equivalent)
- 0.2 ml PCR 8-tube strips and caps/seals and/or PCR plate
- Benchtop centrifuge to pulse-spin tubes and PCR 8-tube strips
- Plate centrifuge
- Vortex mixer

Thermal Cycler Programs (all with lid-heating on)

1. TAG Program: 55°C for 15 minutes; 25°C hold.
2. STOP Program: 68°C for 10 minutes; 25°C hold.
3. 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 the procedure:

Measure and adjust input DNA concentration. Spot-check the DNA concentration of several samples using a PicoGreen assay. The plexWell Plus 24 protocol is generally tolerant of a wide range of DNA concentrations, so typically, after determining the concentration of several representative samples, the input DNAs from multiple samples are diluted globally to an average concentration of 2.5 ng/µl using a single dilution factor. However, if the DNA concentration range of samples is extremely variable (*e.g.*, if the concentration difference between any two samples is greater than 10-fold), then outlier samples might need to be individually diluted into a suitable concentration range in order to achieve more uniform read counts across samples. Before starting the procedure, adjust the average DNA concentration to 2.5 ng/µl 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.

Record the sample destinations and the barcodes for library prep. Plan out the sample destinations within a PCR 8-tube strip(s) and the corresponding source wells from the **SBP24 Plate**. Make a map, and clearly label the PCR 8-tube strip(s) before starting the procedure. Also, select the Pool Barcode Reagent that will be used to barcode the batch of samples. Note: For planning, most labs find it helpful to keep track of how much of each SB and PB reagent has been consumed from the plexWell Plus 24 Library Prep kit. The supplied nominal volume of SB reagent (per well) is 40 µl, and the supplied nominal volume of PB reagent (per tube) is 10 µl.

Reagents in the kit are intended for multiple uses: do not discard until empty. Contamination of all kit reagents/components should be carefully avoided, and the unused portions should be returned to storage at the temperature indicated on the label. The **SBP24 Plate** supplied in the plexWell Plus 24 kit contains sufficient SB reagent to prepare libraries from up to 96 samples, so after setting-up the SB reactions in PCR 8-tube strip(s), the **SBP24 Plate** should be carefully resealed, and stored at -20°C until the next use.

Program thermal cycler(s). For convenience, set up the thermal cycler programs before starting.

Pulse-spin kit components. Liquids can condense or shift locations inside containers during shipment or storage. Before every use of the **SBP24 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 and vortex MAGwise thoroughly to resuspend the magnetic beads before use. Pipette slowly and do not pre-wet pipette tips to transfer volumes accurately.

Check the X Solution for precipitate before use. If a precipitate is visible, incubate the tube 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 (3X) is viscous. Store **Coding Buffer (3X)** at room temperature. To transfer Coding Buffer accurately, pipette slowly and do not pre-wet pipette tips. While adding **Coding Buffer** to reactions, mix in the coding buffer completely by pipetting up and down several times with the same pipette tip(s) that was used for addition.

Prepare 80% ethanol fresh daily.

Prepare 10 mM Tris-HCl, pH 8.0 from a concentrated stock solution diluted with ultrapure water (both molecular-biology/PCR grade). Do not use EDTA-containing solutions (*e.g.*, TE buffer) to dissolve or dilute input DNA because EDTA can inhibit enzymatic activity.

Safe-stopping points are indicated in the Procedure. For optimal results, proceed directly to the next step unless an optional safe-stopping point is indicated.

Procedure

Before beginning the plexWell Plus 24 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

a. Pulse-spin the **SBP24 Plate** in a centrifuge. After centrifugation, carefully remove the plate seal from the **SBP24 Plate**, and discard the plate seal (do not reuse plate seals!).

b. Transfer 8 µl of sample barcode (SB) reagent from the **SBP24 Plate** to a pre-labeled PCR 8-tube strip(s) or PCR plate using an accurate multichannel pipettor. Use clean tips for each transfer and visually confirm that the volume of SB reagent appears equal. After dispensing the sample barcode reagent, reseal the **SBP24 Plate** securely with a new plate seal, and return the plate to the freezer (-20°C storage).

c. Transfer 4 µl of input DNA (2.5 ng/µl) to each tube (one sample per tube) using an accurate multichannel pipettor. Mix the DNA thoroughly with the sample barcode reagent in each tube by pipetting up and down (5 times at 4 µl), being careful not to introduce excessive bubbles. Use clean tips for addition of each sample.

d. Next, carefully pipette 6 µl of **Coding Buffer (3X)** to each tube using new pipette tips for each transfer. Mix thoroughly but slowly by pipetting up and down (10 times at 6 µl), being careful not to introduce excessive bubbles.

   Useful Tip: *Aliquot **Coding Buffer (3X)** into a separate PCR 8-tube strip, and then use a multichannel pipettor to transfer 6 µl into each SB reaction (and to mix).*

e. Cap or seal the SB reactions, 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. Pulse-spin the SB reactions and then uncap/seal the reactions.

b. Add 9 µl of **X Solution** to each SB reaction. Pipette slowly up and down (10 times at 9 µl) to mix, being careful not to introduce excessive bubbles. Use clean tips for each addition of **X Solution**.

Useful Tip: *Aliquot* **X Solution** *into a separate PCR 8-tube strip, and then use a multichannel pipettor to transfer 9 µl into each SB reaction (and mix).*

c. Securely re-cap or seal the SB reactions, 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 Reaction Pooling

a. Pulse-spin the PCR 8-tube strip or plate containing the stopped SB reactions. Using a P20 pipettor, transfer 18 µl of stopped SB reaction from each PCR tube into a single 1.5 ml LoBind tube. The final volume of the stopped SB reaction pool is determined by the batch size (see examples of approximate pool volumes in step 4b. below).

Note: *Do not pool samples with the same SB together. Maximum pool size is 24 samples.*

Useful Tip: *After pooling, visually compare the volume remaining in the PCR 8-tube strip(s) to confirm that none of the samples were inadvertently missed during pooling.*

4. SB Pool Purification

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

b. Add 1 volume equivalent of MAGwise beads to the stopped SB reaction pool in the 1.5 ml LoBind tube, and mix thoroughly by pipetting (see below).

MAGwise Purification of Stopped SB Reaction Pool

Batch size (samples per pool)	8-plex	16-plex	24-plex
Volume of stopped SB reaction pool	144 µl	288 µl	432 µl
Add MAGwise (1 vol. equivalent)	144 µl	288 µl	432 µl

Note: If working with a sample number (N) not listed in the table, the amount of pooled material is equal to N*18. Use 1 volume equivalent of MAGwise.

c. Incubate in a non-magnetic tube rack on the bench for ≥5 minutes to allow the DNA to bind.

d. 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.

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 if necessary, use a smaller one to remove the remaining supernatant.*

f. Wash beads with 80% ethanol.
   i. With tube in the magnetic stand, add 900 µl 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.

g. Wash beads with 80% ethanol a 2nd time.
   i. With tube in the magnetic stand, add 900 µl 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 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 SB reaction pool from the beads.

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

j. When the supernatant has cleared completely, carefully transfer 24 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.*

SAFE-STOPPING POINT

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

5. Pool-Barcoding (PB) Reaction Set-up

a. Set up a PB reaction in the PCR tube containing the purified SB reaction pool from the last step. Add the following amounts of **PB Reagent**, **10 mM Tris-HCl, pH 8,** and **Coding Buffer (3X)**, depending on the batch size (see below).

Note: If you are working with a sample number (N) not listed in the table, the amount of PB required is (N*0.2), the amount of Tris required is (4.8-PB reagent volume).

PB Reaction Set-up:

Batch size (samples per pool)	8-plex	16-plex	24-plex
Purified SB reaction pool	24 µl	24 µl	24 µl
10 mM Tris-HCl, pH 8	3.2 µl	1.6 µl	N/A
PB Reagent	1.6 µl	3.2 µl	4.8 µl
Coding Buffer (3X)	14.4 µl	14.4 µl	14.4 µl

Note: The pool barcode (PB) reagent carries the i5 index. To enable multiplexing on the same sequencing runs, use different PB Reagent. for each SB reaction pool.

b. Mix the PB reaction thoroughly by pipetting.

c. Cap the PCR tube containing the PB reaction, pulse-fuge, 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. Add 21.6 µl of **X Solution** to the PB reaction. Mix thoroughly by pipetting 10 times while being careful not to produce excessive bubbles.

b. Re-cap the PCR tube containing the PB reaction, 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. Transfer the entire volume of the stopped PB reaction to a single 1.5 ml LoBind tube.

b. Vortex (or vigorously pipette) room temperature **MAGwise Paramagnetic Beads** to ensure they are completely resuspended. Add 65 µl (1 volume equivalent) of MAGwise beads to the stopped PB reaction in the 1.5 ml LoBind tube from the previous step, and mix thoroughly by pipetting.

c. Incubate in a tube rack (non-magnetic) on the bench for ≥5 minutes to allow the DNA to bind.

d. Transfer the tube to a magnetic stand and let the beads settle completely before proceeding to the next step. (A bead pellet will form along one side of the tube and the supernatant should appear completely clear after ≤ 5 minutes).

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

f. Wash beads with 80% ethanol.

   i. With tube in the magnetic stand, add 0.5 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.

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

   i. With tube in the magnetic stand, add 0.5 ml 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 in a tube rack (non-magnetic) on the bench for at least 5 minutes to elute the purified DNA from the beads.

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

j. When the supernatant has cleared completely, carefully transfer 23 µl of DNA eluate to a clean PCR tube. The transferred eluate contains the DNA purified from the PB reaction, and it is now ready for library amplification.

SAFE-STOPPING POINT

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

8. Library Amplification

a. Add 4 µl of **Library Primer Mix** to the eluate in the PCR tube from the last step.

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

c. Cap 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.

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 so it is important to measure the volume prior to the size selection steps below.

b. Dilute the library amplification reaction to a final volume of 205 µl with 10 mM Tris-HCl, pH 8.

c. Remove and retain 5 µl of unpurified library for use during Library QC step.

d. Vortex (or vigorously pipette) room temperature **MAGwise Paramagnetic Beads** to ensure they are completely resuspended.

e. Add 160 µl of MAGwise beads (0.8 volume equivalents) to the diluted multiplexed library. Mix thoroughly by pipetting up and down.

   Note: Use 0.85 volume equivalents (or more) of MAGwise if libraries were made from short PCR products (≤500 bp).

   Note: Use 0.75 volume equivalents (or less) of MAGwise to remove fragments >300

f. Incubate in a non-magenetic tube rack on the bench for ≥5 minutes to allow the DNA to bind.

g. Transfer the tube to a magnetic stand and let the beads settle completely before proceeding to the next step. (A bead pellet will form along one side of the tube and the supernatant should appear completely clear after ≤ 5 minutes).

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

i. Wash beads with 80% ethanol.

   i. With tube in the magnetic stand, add 0.5 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.

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

   i. With tube in the magnetic stand, add 0.5 ml 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 32µl 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

k. Incubate for 5 minutes in a tube rack (non-magnetic) on the bench to elute the size-selected multiplexed library from the magnetic beads.

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

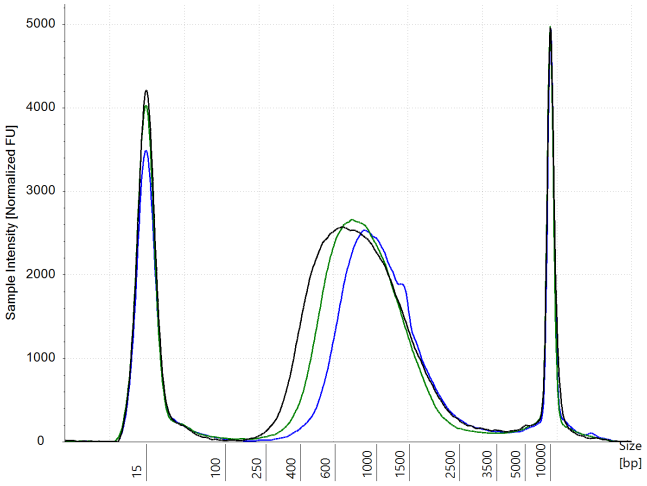
m. When the supernatant has cleared completely, carefully transfer 30 µl of DNA eluate to a new 1.5 ml LoBind tube. The transferred eluate contains the purified, size-selected multiplexed library. The remaining 2 µl of eluate may be used for electrophoretic analysis.

SAFE-STOPPING POINT

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

Library QC and Quantification

Electrophoretic analysis: Run an aliquot of purified library along with an aliquot of unpurified amplified library from step 9b through an electropheretic 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). Typical results on the Tapestation D5000 assay shown below (Figure 1). Follow the manufacturer's instructions for these instruments and dilute the library, if appropriate, prior to running. Typically PWP24 libraries should be diluted 1:4-1:6 (unpurified library) and 1:8-1:12 (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.



Purification	Average Fragment	Conc.	Average median insert
0.80 volumes	766 bp	48 nM	341 nt
0.75 volumes	828 bp	33 nM	422 nt
0.70 volumes	926 bp	23 nM	501 nt

Figure 1. ***(Left)*** Representative plexWell Plus 24 library traces generated using a Tapestation 2200 with DNA 5000 reagents and tapescreens. Library were prepared using 8 replicates of E. coli genomic DNA with final library purification using 0.7 (blue), 0.75 (green) and 0.8 (black) volume equivalents of MAGWise. (***Right)*** Table of typical plexWell Plus 24 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 chemsitry and aligning to an E. Coli reference genome, determining the median insert for each of the 8 samples, then taking the average of the 8 values.

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.

If electrophoretic 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.

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 Plus 24 libraries are dual indexed using 8 nt indices. Each pool contains 96 sample-specific i7 indices and a single pool-soecific 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 libararies are sequenced using the same primers as Nextera® libraries.[2] For information regarding setting up a run configuration, refer to Appendix E and Illumina technical documentation.

[2] 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: Reagent Barcodes for Dual-Indexed Sequencing

The plexWell Plus 24 Library Preparation Kit generates dual-indexed, Illumina-compatible multiplexed libraries with up to 24 unique eight-base Index 1 (i7) sequences, and up to 4 unique eight-base Index 2 (i5) sequences. The complete list of index sequences is shown in Tables 2 and 3). 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]

i7 indices on SBP24 plate

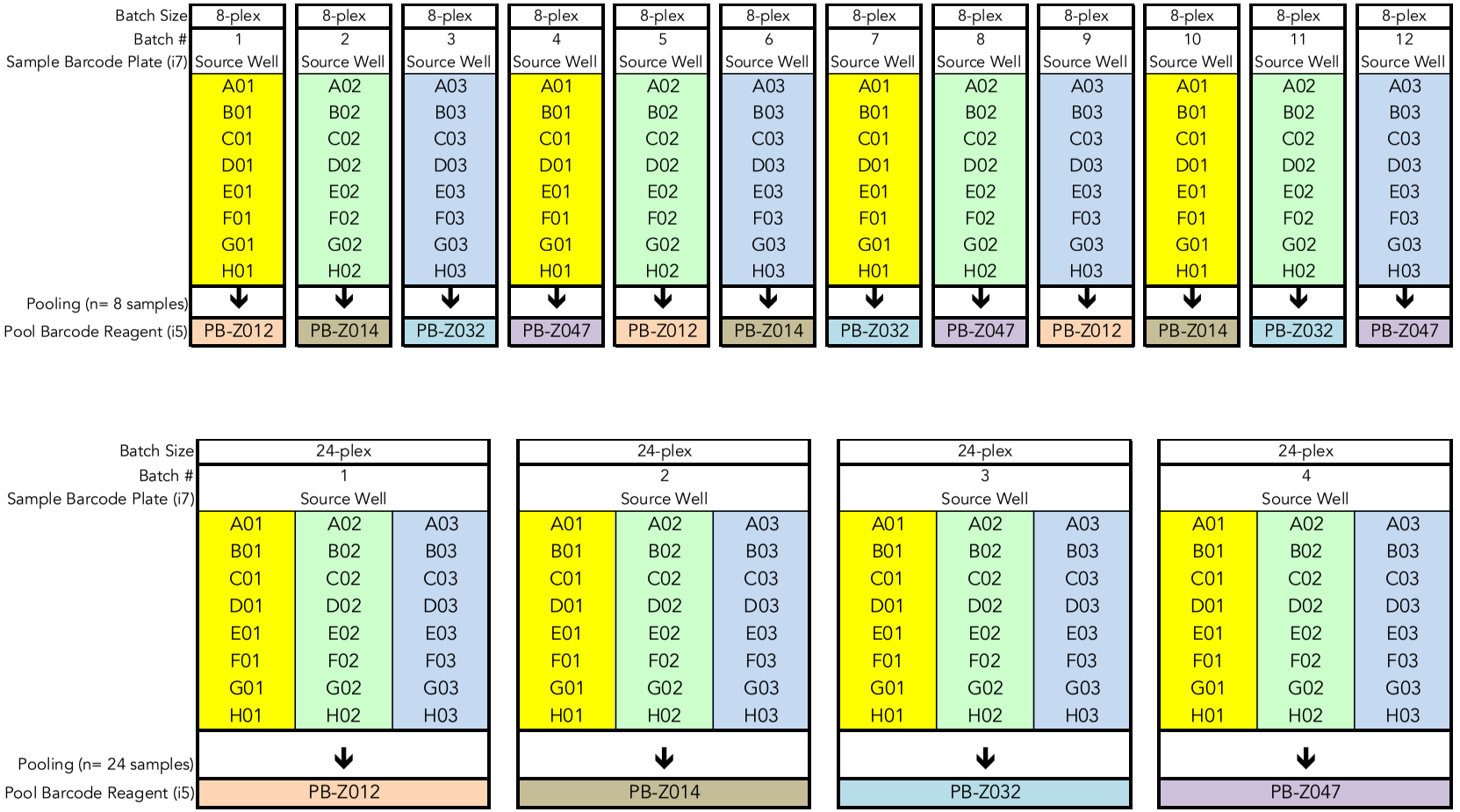
	1	2	3	4	5	6	7	8	9	10	11	12
A	TTGGAATG	CCATATCC	GGTACCGA									
B	TTAATGCG	GTAGTCAC	ATAGCACA									
C	AGCTACGT	CGGAGATA	TCAGACGG									
D	GCCTCCTG	CACTCTCA	GGATGCAT									
E	GGGACAAC	GAGTTCTC	ACACGGTT									
F	TATCCCAC	TCGCCAGC	CTTAGAGT									
G	CAACTGTG	TGTGACTA	TTCCTCAT									
H	ATGACTAG	TTGACGTC	CGTGCTGG									

The sample barcode (SB) reagents in columns 1 – 3 of the SBP24 Plate encode twenty-four sample-specific i7 indices.

i5 indices (written as 5'-3') for PWP24 PBZ

PB-	i5 sequence
Z012	GTCAGTTG
Z014	CCTATTGA
Z032	GCTGATCG
Z047	CAGCGGTG

[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.



Supplementary Figure 1. plexWell Plus multiplexed libraries that are prepared in different batches or batch sizes can be pooled and sequenced together on the same run as long as there are no samples that share both the same i7 and i5 barcode. Two examples of compatible batching strategies are shown above. (**Run 1** = 8-plex x 12 batches; **Run 2** = 24-plex x 4 batches)

Appendix B: 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 A 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)

Experienced User Checklist

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

Sample Barcoding

- ❑ Centrifuge SBP24 plate
- ❑ Transfer 8 µl of sample barcode (SB) reagent from the SBP24 Plate to SB reaction strip tubes or plate
- ❑ Add 4 µl sample to each tube and pipette 10x
- ❑ Add 6 µl coding buffer to each tube and pipette 10x
- ❑ Run TAG incubation
- ❑ Add 9 µl X-solution to SB reaction and pipette 10x
- ❑ Run STOP incubation
- ❑ Pool 18 µl from each SB reaction
- ❑ Complete SB purification (1 volume equivalent), eluting with 24 µl of 10 mM Tris
- ❑ Transfer 24 µl of purified SB pool to 0.2 ml PCR tube (PB reaction tube)

Pool Barcode Reaction

- ❑ Add PB Reagent (0.2 µl * number of samples) and Tris (4.8 µl–volume PB reagent)
- ❑ Add 14.4 µl coding buffer to PB reaction tube and pipette 10x
- ❑ Run TAG incubation
- ❑ Add 21.6 µl X-solution to PB reaction and pipette 10x
- ❑ Run STOP incubation
- ❑ Complete PB purifications (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 each amplification tube
- ❑ Add 27 µl of KAPA HiFi HotStart ReadyMix and pipette to mix
- ❑ Run Fill Amp program
- ❑ Complete Library Purification (0.8 volume equivalent), eluting with 32 µl of 10 mM Tris

Revision History

Version	Release Date	Prior Version	Description of changes
20210122	20210125	20200225	• Updated workflow diagram, library QC metrics • Added molecular diagram, appendices, and experienced user checklist
20200225	25FEB2020	20190807	• Updated PB module to keep total PB reaction volume fixed regardless of plexing • Updated final purification to 0.8x from 0.75x • Updated workflow diagram

Technical Assistance

For technical assistance, contact seqWell Technical Support.

Email: support@seqwell.com

Website: https://seqwell.com/products/plexwell-kit/

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