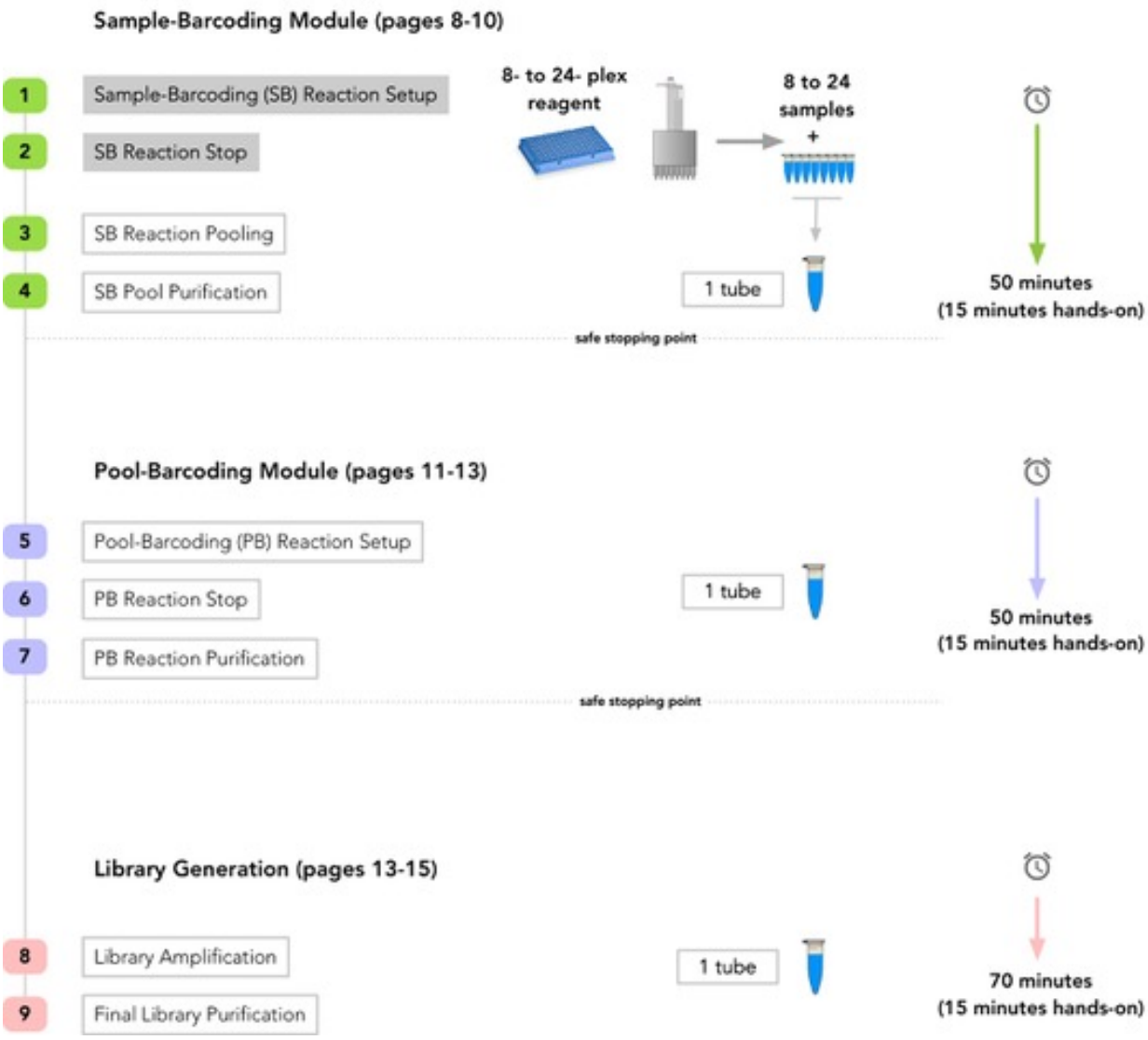

# plexWell™ Plus 24 Library Preparation Kit

## for Illumina® Sequencing Platforms

(Part No. PWP24)

# User Guide

# 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 | Blue fully-skirted 96-well PCR plate (columns 1 – 3) for storing SB reagents. | -20°C | 1 (24 wells) |
| 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, white 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*

- 100% Ethanol (molecular biology grade)
- Tris-HCl, pH 8.0
- Ultrapure Water (PCR grade)
- Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen P/N: P11496), or another validated dsDNA quantification assay
- KAPA HiFi HotStart ReadyMix (2X) (Roche P/N: 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 (compatible with PCR 8-tube strips)
- 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
- 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_AMP 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

# Introduction

The new plexWell Plus 24 Library Preparation Kit features a flexible multiplexed workflow for generating dual-indexed Illumina-compatible libraries in batches of 8 - 24 samples at a time. 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. Supplementary Figure 1 (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 (2.5 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.

## 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

## 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) 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 ten 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 ten 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 the SB reactions, transfer to a thermal cycler, and run the following program (**TAG**) with lid-heating on:

   55°C for 15 minutes;
   25°C hold

## 2. SB Reaction Stop

a. Add 9 µl of **X Solution** to each SB reaction. Pipette slowly up and down 5 times 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).*

b. Securely re-cap and pulse-spin the PCR 8-tube strip. Transfer to a thermal cycler; and run the following program (**STOP**) with lid-heating on:

68°C for 10 minutes;
25°C hold

c. Pulse-spin the PCR 8-tube strip containing the stopped SB reactions again.

## 3. SB Reaction Pooling

a. 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. 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 0.9 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 0.9 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 24µl 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 tube in a tube rack (non-magnetic) 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 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 for the samples. To enable multiplexing on the same sequencing runs, use a 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, transfer to a thermal cycler, and run the following program (TAG) 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, transfer to a thermal cycler, and run the following program (STOP) 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, and run the following program (FILL_AMP) 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).

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 35µl 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 5 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

a. **Electrophoretic analysis:** Use an agarose gel, AATI Fragment Analyzer (High Sensitivity NGS Fragment Analysis kit), Agilent Bioanalyzer (High Sensitivity DNA or DNA 7500 kits), or Agilent TapeStation (High Sensitivity D5000 or D5000 kits) to analyze the fragment size distribution of the purified, multiplexed library. The majority of library fragments are typically between 500 and 1,000 bp in length (see Figure 1, below). The unpurified library (5ul) should also be analyzed using this method to observe what the library fragment distribution was prior to library cleanup.

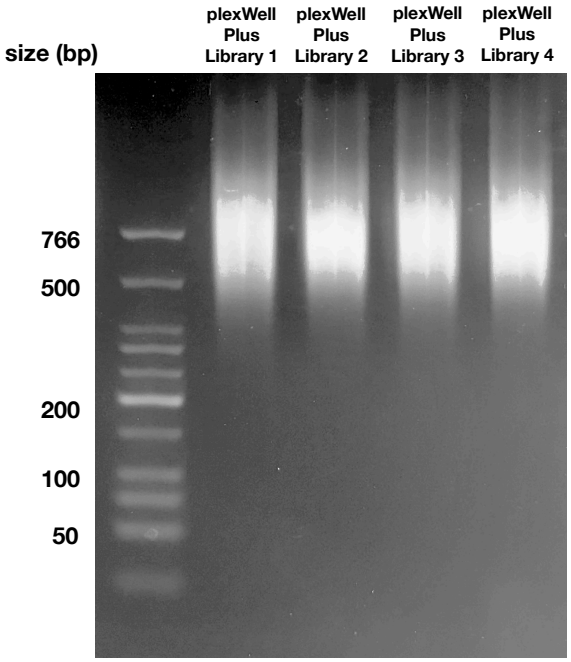



**Figure 1.** Four plexWell Plus 24-plex libraries were prepared from 24 bacterial gDNA samples and electrophoresed on a 2% agarose gel (3 µl purified library per lane).

b. **qPCR assay:** Use 2 µl of the purified, multiplexed library for qPCR analysis. Follow kit and instrument documentation for appropriate conditions and dilutions. For KAPA Library Quantification kits, prepare a 1/100K dilution of the multiplexed library. Use the average fragment size estimated by electrophoresis for the size adjustment value to calculate the purified, multiplexed library concentration. Library concentrations are typically 50 – 150 nM.

-or-

c. **PicoGreen assay:** As an alternative to qPCR, quantify 5 µl of the purified, multiplexed library using the PicoGreen assay and serially diluting the library while picogreen multiple dilutions. Use the average fragment size estimated by electrophoresis for the size adjustment value to calculate the purified, multiplexed library concentration.

## 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.[2]

### Table 2. SBP24 plate map, i7 index layout

| | 1 | 2 | 3 |
|---|---|---|---|
| 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 |

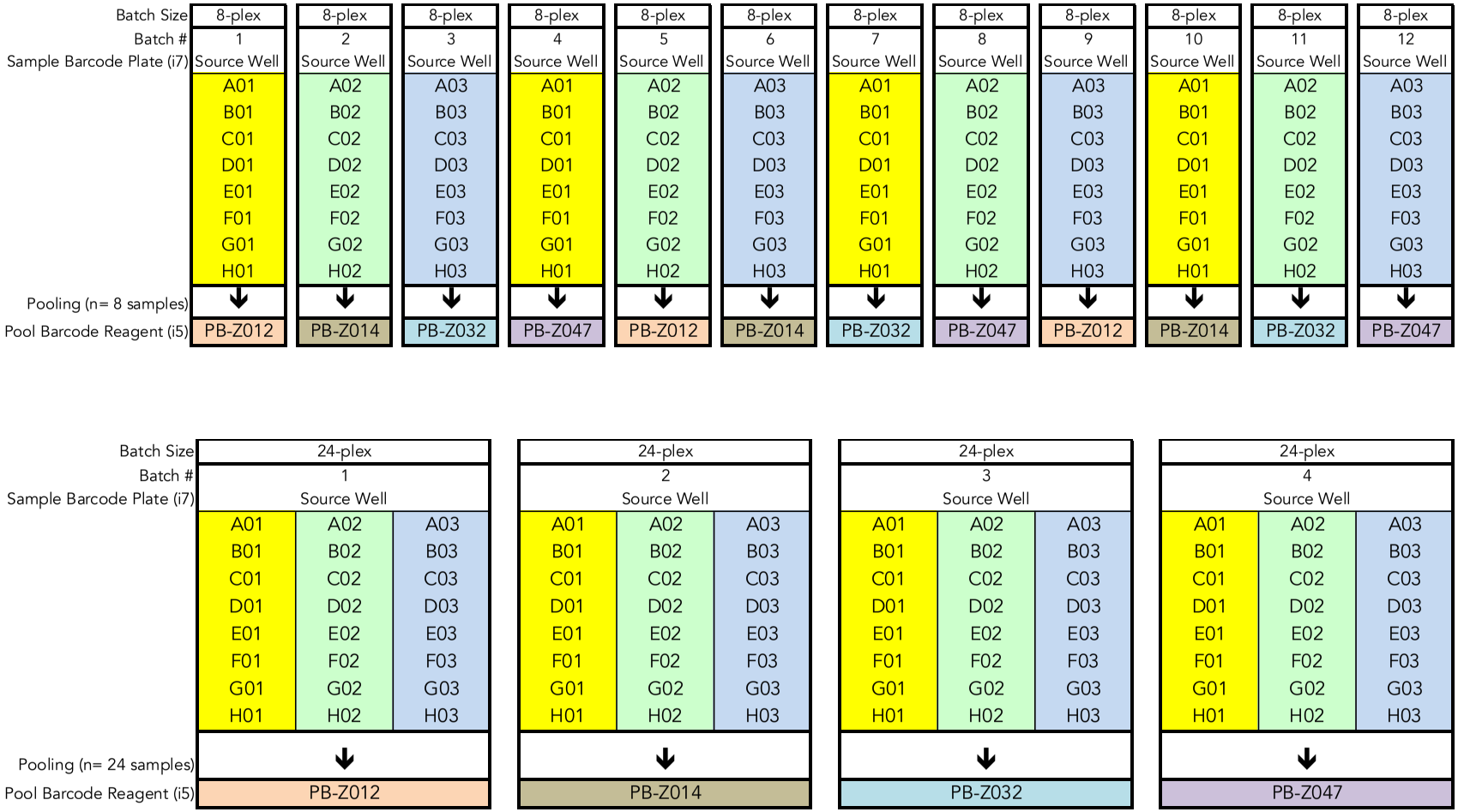
The sample barcode (SB) reagents in columns 1 – 3 of the SBP24 Plate encode twenty-four sample-specific i7 indices.

### Table 3. PB Reagents, i5 indices

| Pool Barcode Reagent | i5 index |
|---|---|
| Z012 | GTCAGTTG |
| Z014 | CCTATTGA |
| Z032 | GCTGATCG |
| Z047 | CAGCGGTG |

The pool barcode (PB) reagents supplied in the kit encode four pool-specific i5 indices.

[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.




**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)

## Revision History

| Version | Release Date | Prior Version | Description of changes |
|---|---|---|---|
| 20200225 | 25FEB2020 | 20190807 | • Updated PB module to keep total PB reaction volume fixed regardless of plexing<br>• Updated final purification to 0.8x from 0.75x<br>• Updated workflow diagram |

## Technical Assistance

For technical assistance, contact seqWell Technical Support.

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