



plexWell™ WGS 24 Library Preparation Kit  
for Illumina® Sequencing Platforms  
(Part No. WGS24)

User Guide

v20220421

## Introduction

This user guide provides detailed instructions for preparing 24 genomic DNA samples for Illumina Sequencing using the plexWell WGS 24 Library Preparation Kit.<sup>1</sup> The intended use of this plexWell kit is for generating high quality, high complexity libraries from purified human, plant and animal DNA (For Research Use Only).

The primary advantages and benefits of using the plexWell WGS 24 Library Preparation Kit are a truly multiplexed library preparation workflow, superior sequencing performance, and significant labor/consumable cost savings.

This plexWell WGS 24 User Guide is optimized for 200 ng of high quality genomic DNA per sample and typically generates 750 – 1,500 fmoles of purified, size-selected multiplexed library output, ranging from 400 – 1,500 bp in library fragment length.

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<sup>1</sup> Although optimized for the NovaSeq 6000, plexWell WGS multiplexed libraries are compatible with a range of Illumina sequencing platforms. Refer to Illumina technical documentation for specific instructions on how to set up a sequencing run for a Nextera-style single-indexed or dual-indexed library prior to loading a plexWell library on your particular model of sequencing system.

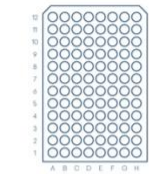
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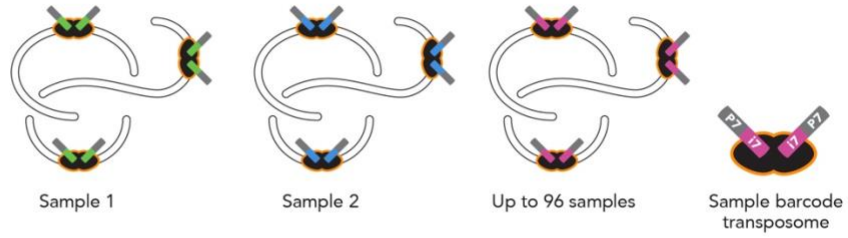
# plexWell WGS-24 Library Preparation

## Molecular Diagram

### SAMPLE BARCODING:



Dispense DNA into plexWell™ SB plate



### POOL BARCODING:



Pool into single tube



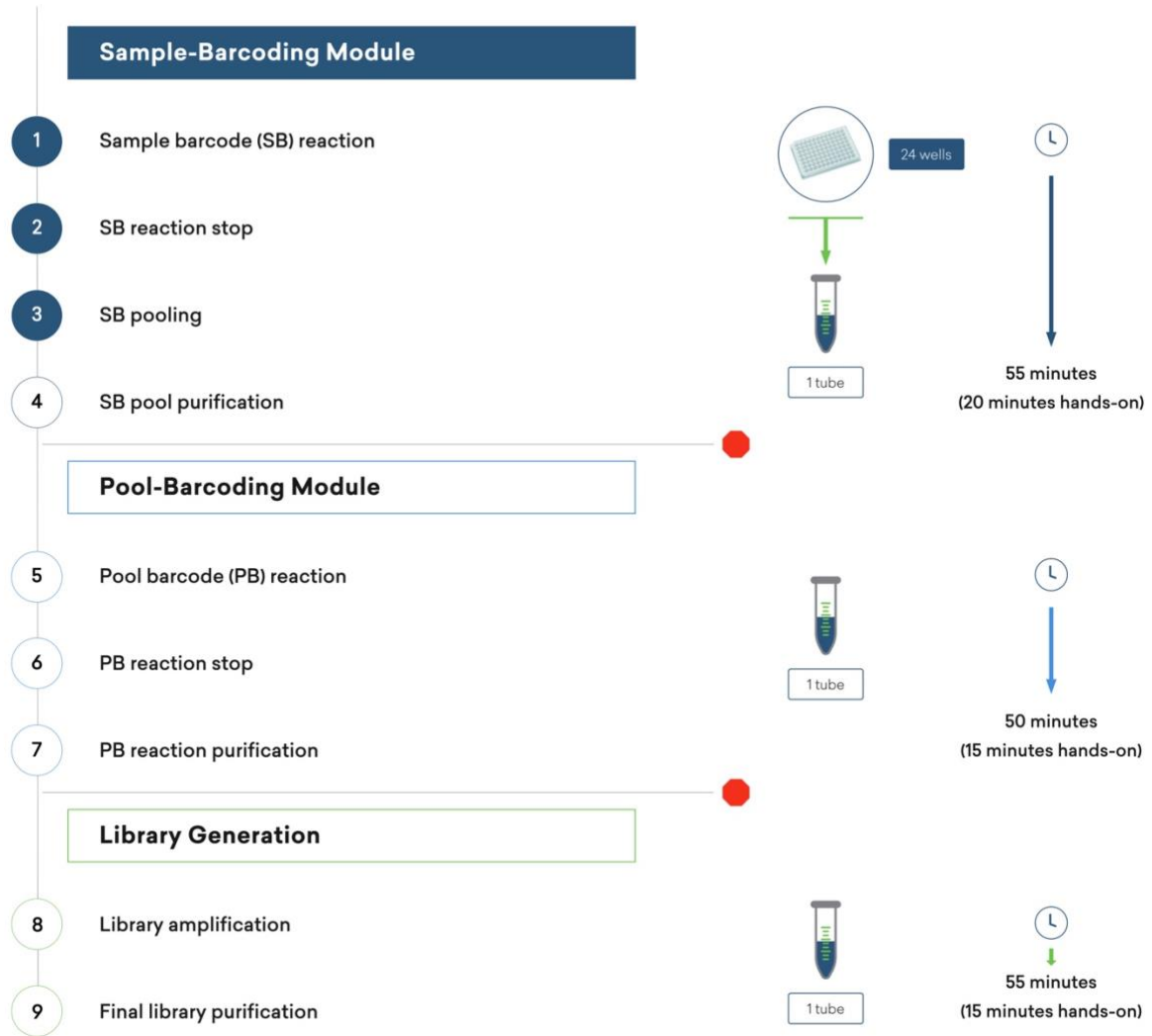
### FILL-IN AND LIBRARY AMPLIFICATION:



Sequencer-ready library



# plexWell WGS-24 Library Preparation Workflow Diagram



**Table 1.** Components of plexWell WGS 24 Library Preparation Kit

Box Ref.	Component	P/N	Description	Storage	Qty
WGS24-1	Sample Barcode Plate	SBW24	<b>SBW24 Plate:</b> assay ready <b>SB</b> reagent in low profile, fully-skirted 96-well green PCR plate (columns 1 – 3)	-20°C	1
WGS24-2	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
WGS24-3	Pool Barcode Reagent W014	PBW014	0.5 ml tube, green cap, 10 µl	-20°C	1
	Library Primer Mix	PRM384	0.5 ml tube, natural cap, 24 µl	-20°C	1

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

### Reagents

- 80% Ethanol (freshly prepared)
- Tris-HCl, pH 8.0
- PicoGreen<sup>®</sup> DNA assay (recommended) or other validated dsDNA quantification assay
- KAPA HiFi HotStart ReadyMix (2X) (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<sup>®</sup> (1.5 and 2.0 ml, DNA LoBind Tubes)
- PCR plate seals (must be evaporation-resistant)
- 96-well thermal cycler (compatible with low profile fully-skirted 96-well PCR plates, Biorad HSP 9641)
- 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 tubes and PCR 8-tube strips
- Plate centrifuge
- Vortex mixer

### Thermal Cycler Programs (all with lid-heating on)

- **TAG:** 55°C for 15 minutes; 25°C hold.
- **STOP:** 68°C for 10 min; 25°C hold.
- **FILL AMP4:**
  - 72°C for 10 minutes (fill-in)
  - 95°C for 3 minutes (initial denaturation)
  - 
  - 98°C for 30 seconds
  - 64°C for 15 seconds
  - 72°C for 30 seconds
  - 
  - 72°C for 3 minutes (final extension)
  - 4°C hold

## Before starting the procedure:

**Adjust input DNA concentration.** Assay the DNA concentration of each sample (n=24) using a PicoGreen assay. Before adding DNA to the PicoGreen assay, we recommend serially-diluting a small aliquot of genomic DNA in 10 mM Tris-HCl, because highly concentrated DNA is notoriously difficult to quantify accurately. This protocol requires approximately 200 ng of purified genomic DNA input per sample. Before starting library preparation, adjust each input DNA concentration to 25 ng/μl in 10 mM Tris-HCl, pH 8.0 (do not dilute input DNA with TE, or other EDTA-containing buffers).

**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 and shift location inside containers during shipment or storage. Before opening the **SBW24 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 kit components freeze, 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 the magnetic beads prior to 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 at 37°C for 5 minutes (or longer if necessary). Mix gently by inversion until the precipitate dissolves (do not vortex). Note: **X Solution** contains SDS and will precipitate if stored below room temperature. Overly vigorous mixing will cause foaming.

**Please note that Coding Buffer is viscous.** Store **Coding Buffer (3X)** at room temperature. To transfer volumes accurately, pipette slowly and do not pre-wet pipette. While adding **Coding Buffer** to reactions, mix in completely by pipetting up and down several times with the same pipette tip(s) used for addition. Always change pipette tips before adding **Coding Buffer** to different reactions.

**Prepare 80% ethanol fresh daily.**

**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/PCR grade). Do not use EDTA-containing solutions (e.g., TE) 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 procedure, review the adjusting sample concentration guidelines on the previous page to ensure that each sample has been adjusted to 25 ng/ $\mu$ l using 10 mM Tris-HCl, pH 8.0.

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

- a. After labeling the assay-ready **Sample-Barcode Plate (SBW24 Plate)**, pulse-spin the **SBW24 Plate** in a centrifuge. After centrifugation, visually inspect columns 1 - 3 to confirm that the volume of sample-barcode reagent in all 24 wells appears uniform, and then carefully remove the plate seal from the **SBW24 Plate**. Set-up SB reactions at room temperature.
- b. Add 8  $\mu$ l of input genomic DNA (25 ng/ $\mu$ l) to all wells (one sample per well). Mix the DNA thoroughly with the sample-barcode reagent in each well by pipetting up and down (10 times at 8  $\mu$ l), being careful not to introduce excessive bubbles. Use clean tips for each sample.
- c. Next, carefully pipette 8  $\mu$ l of **Coding Buffer (3X)** to each well (n=24) of the **SBW24 Plate**, using new tips for each transfer. Mix thoroughly and slowly by pipetting up and down (20 times at 8  $\mu$ l), being careful not to introduce excessive bubbles.  
*Useful Tip: Aliquot 30  $\mu$ l of Coding Buffer (3X) into a PCR 8-tube strip, and then use a multichannel pipettor to dispense 8  $\mu$ l into the **SBW24 Plate** and to mix.*
- d. Seal the **SBW24 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 **SBW24 Plate** and then remove seal.

- c. Add 12  $\mu\text{l}$  of **X Solution** to each well of the **SBW24 Plate**. Pipette up and down slowly 10 times to mix, being careful not to introduce excessive bubbles. Use clean tips for each addition of **X Solution**.

**Useful Tip:** Aliquot 45  $\mu\text{l}$  of **X Solution** into a PCR 8-tube strip, and then use a multichannel pipettor to transfer 12  $\mu\text{l}$  from the strip into columns 1 - 3 of the **SBW24 Plate** and to mix.

- d. Securely reseal and pulse-spin the **SBW24 Plate**, 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

### 3. SB Reaction Pooling

- a. Pulse-spin the **SBW24 Plate** and then remove seal. Using a P200 pipettor, transfer 26  $\mu\text{l}$  of stopped SB reaction from every well (n=24) into a 2 ml LoBind tube. The total volume of the SB reaction pool will be approximately 624  $\mu\text{l}$ . Note: It is important to transfer an equal volume from every SB reaction. After pooling, check the volume remaining in the wells of the **SBW24 Plate** to verify that none of the wells were missed during pooling. Do not pool together SB reactions from different **SBW24 Plates**.

### 4. SB Pool Purification

- a. Vortex (or vigorously pipette) room temperature **MAGwise Paramagnetic Beads** to ensure that the beads are fully resuspended.
- b. Add 624  $\mu\text{l}$  (approximately 1 volume equivalent) of MAGwise to the pooled SB reactions and mix thoroughly by pipetting. Incubate on bench for  $\geq 5$  minutes to allow DNA to bind.
- c. Incubate in a 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 the bead pellet with 80% ethanol.

- i. With the tube in the magnetic stand, add 1.5 ml of 80% ethanol without disturbing beads.
  - ii. After 30 seconds, slowly remove and discard supernatant, being careful not to disturb the pellet.
- g. Wash beads with 80% ethanol a 2<sup>nd</sup> time.
- i. With tube in the magnetic stand, add 1.5 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  $\geq 30$  seconds, remove and discard supernatant, being careful not to disturb pellet.
    2. Cap tube, pulse-spin and return to magnet, letting beads settle ( $< 30$  seconds). Use a small pipet tip ( $\leq 20 \mu\text{l}$ ) to remove any residual ethanol at the bottom of the tube.
    3. Add  $65 \mu\text{l}$  of 10 mM Tris to bead pellet, remove from magnetic stand and pipet the solution along the inner wall of the tube multiple times to thoroughly resuspend the bead pellet. Place tube in rack on bench. **DO NOT air dry bead pellet** prior to Tris addition or the DNA recovery will be compromised.
- h. Incubate the tube 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  $64 \mu\text{l}$  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  $1 \mu\text{l}$  of the purified SB reaction pool for PicoGreen. The expected concentration is 18-36 ng/ $\mu\text{l}$ . 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  $-20^{\circ}\text{C}$ .**

## 5. Pool-Barcoding (PB) Reaction Setup

- a. Add 5  $\mu\text{l}$  of **Pool-Barcode Reagent** to the PCR tube containing the purified SB reaction pool from the last step. Mix thoroughly by pipetting.
- b. Add 34.5  $\mu\text{l}$  of **Coding Buffer (3X)**, and mix the PB reaction thoroughly by vortexing for 5 seconds or pipetting  $\geq 10$  times at  $\geq 75 \mu\text{l}$ .
- c. Pulse-fuge the PCR tube containing the PB reaction then transfer to a thermal cycler, and run the TAG program, below, with lid-heating on:
  - 55°C for 15 minutes
  - 25°C Hold

## 6. PB Reaction Stop

- a. Pulse-fuge PB reaction, then add 52  $\mu\text{l}$  of **X Solution** to the PB reaction. Mix thoroughly by pipetting 10 times.
- b. Recap the PB reaction tube, 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 the entire volume of each stopped PB reaction to a 1.5 ml LoBind tube.
- b. Vortex (or vigorously pipette) room temperature **MAGwise Paramagnetic Beads** to ensure they are completely resuspended. Add 156  $\mu\text{l}$  (1 volume) of MAGwise beads to the stopped PB reaction and mix thoroughly by pipetting.
- c. Incubate at room temperature on the bench for 5 minutes in a tube rack (non-magnetic) to allow the DNA to bind.
- d. Incubate on bench for at least 5 minutes to allow DNA to bind.
- e. 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.
- f. Remove and discard supernatant with pipette. Be careful not to disturb the pellet.
- g. Wash beads with 80% ethanol.

- i. With tube in the magnetic stand, add 500  $\mu\text{l}$  of 80% ethanol without disturbing beads.
  - ii. After  $\geq 30$  seconds, remove and discard supernatant, being careful not to disturb pellet.
- h. Wash beads with 80% ethanol a 2<sup>nd</sup> time.
- i. With tube in the magnetic stand, add 500  $\mu\text{l}$  of 80% ethanol without disturbing beads.
  - ii. Perform the next steps quickly, working 1-2 tubes at a time
    1. After  $\geq 30$  seconds, remove and discard supernatant, being careful not to disturb pellet.
    2. Cap tube, pulse-spin and return to magnet, letting beads settle ( $< 30$  seconds). Use a small pipet tip ( $\leq 20$   $\mu\text{l}$ ) to remove any residual ethanol at the bottom of the tube.
    3. Add 101  $\mu\text{l}$  of 10 mM Tris to bead pellet, remove from magnetic stand and pipet the solution along the inner wall of the tube multiple times to thoroughly resuspend the bead pellet. Place tube in rack on bench. **DO NOT air dry bead pellet** prior to Tris addition or the DNA recovery will be compromised.
- i. Incubate the resuspended beads on the bench for at least 5 minutes to elute the purified DNA from the beads.
- j. Return tube to magnetic stand and allow bead pellet to form on the inner wall of the tube (~2 minutes).
- k. When the supernatant has cleared completely, carefully transfer 100  $\mu\text{l}$  of DNA eluate to a clean 1.5 ml 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  $-20^{\circ}\text{C}$ .**

## **8. Library Amplification**

- a. Add 16  $\mu\text{l}$  of **Library Primer Mix** to the eluate in the 1.5 ml tube.
- b. Add 100  $\mu\text{l}$  of Kapa HiFi Hot Start ReadyMix (2X), and mix well by pipetting.

- c. Divide the PCR mix evenly into 4 tubes of a PCR 8-tube strip (54  $\mu$ l x 4 tubes).
- d. Close the strip, pulse-spin and run the FILL\_AMP4 program, below, with lid heating on:
  - Fill-in:** 72°C for 10 min
  - Initial denaturation:** 95°C for 3 min
  - 4 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 by MAGwise purification.

- a. After PCR, pulse-spin and pool the four library amplification reactions together in a 1.5 ml LoBind tube. Measure the total pooled volume.

***Note:** volumes normally change due to film-loss and evaporation during thermal cycling, so it is important to measure the pooled volume prior to purification below.*

- b. Retain 5  $\mu$ L of the unpurified pooled library amplification reactions and set-aside on ice for electrophoretic analysis later.
- c. Vortex (or vigorously pipette) room temperature **MAGwise Paramagnetic Beads** to ensure they are completely resuspended.
- d. Add 0.70-0.75 volume equivalents of MAGwise beads to the pooled, unpurified multiplexed library. Mix thoroughly by pipetting up and down.
- e. Incubate in a tube rack 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. 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. Use a large pipettor to remove most of the supernatant and then use a smaller pipettor (e.g., P20) to remove the residual supernatant.
- h. Wash beads with 80% ethanol.
  - i. With tube in the magnetic stand, add 300  $\mu$ l of 80% ethanol without disturbing beads.
  - ii. After  $\geq$ 30 seconds, remove and discard supernatant, being careful not to disturb pellet.
- i. Wash beads with 80% ethanol a 2<sup>nd</sup> time.
  - i. With tube in the magnetic stand, add 300  $\mu$ l of 80% ethanol without disturbing beads.
  - ii. Perform the next steps quickly, working 1-2 tubes at a time
    - 1. After  $\geq$ 30 seconds, remove and discard supernatant, being careful not to disturb pellet.
    - 2. Cap tube, pulse-spin and return to magnet, letting beads settle (<30 seconds). Use a small pipet tip ( $\leq$ 20  $\mu$ l) to remove any residual ethanol at the bottom of the tube.
    - 3. Add 32  $\mu$ l of 10 mM Tris to bead pellet, remove from magnetic stand and pipet the solution along the inner wall of the tube multiple times to thoroughly resuspend the bead pellet. Place tube in rack on bench. **DO NOT air dry bead pellet** prior to Tris addition or the DNA recovery will be compromised.
- j. Incubate for 5 minutes on the bench to elute the purified library from the magnetic beads.
- k. Return tubes to magnetic stand and allow bead pellet to form on the inner wall of the tube (~ 2 minutes).
- l. When the supernatant has cleared completely, carefully transfer 30  $\mu$ l of DNA eluate to a new 1.5 ml LoBind tube. The transferred eluate contains the purified, size-selected multiplexed library. The remaining 2  $\mu$ l of eluate may be used for electrophoretic analysis.

**SAFE STOPPING POINT**

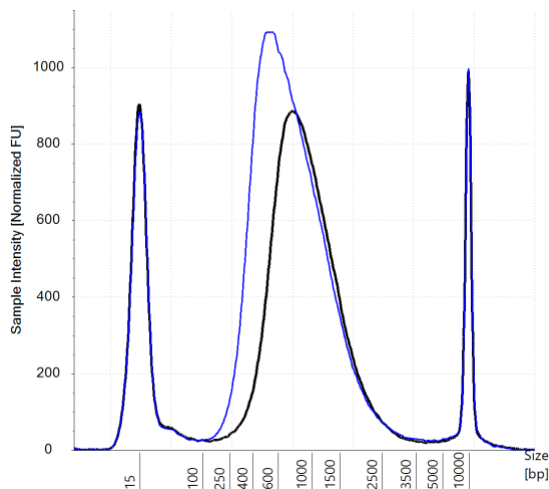
**Store the purified, multiplexed library at -20°C, or proceed directly to library QC.**

## Library QC

**Electrophoretic analysis:** Run an aliquot of purified library along with an aliquot of unpurified amplified library from step 9b through an electrophoretic analysis such as the Agilent Bioanalyzer (High Sensitivity DNA or DNA7500 kits), TapeStation (High Sensitivity D5000 or D5000 kits), or Fragment Analyzer (High Sensitivity NGS Fragment Analysis Kit, DNF-474). Alternatively, run 3  $\mu$ l of purified library and 5  $\mu$ l of unpurified, diluted, library on a 2% Agarose E-gel EX alongside the 1 kb plus ladder (NEB) to determine the median fragment length.

The majority of library fragments should be between 500 and 1,000 bp in length. However, 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.

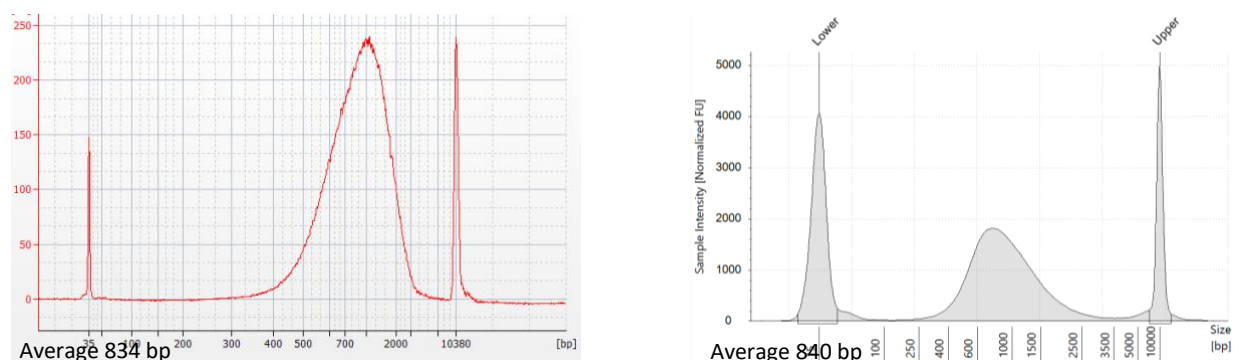
Typical results on the TapeStation High Sensitivity D5000 assay shown in Figure 1, below. Follow the manufacturer's instructions for these instruments and dilute the library, if appropriate, prior to running. Typically WGS24 libraries should be diluted  $\sim$ 1:7 (unpurified library) and  $\sim$ 1:10 (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	Removes fragments	Average Fragment	Conc.	Average median insert
0.75 volumes	<250	716 bp	38 nM	322 nt
0.70 volumes	<400	849 bp	41 nM	420 nt

**Figure 1. (Left)** Representative plexWell library traces generated using a TapeStation 2200 with High Sensitivity DNA 5000 reagents and tapescreens. Libraries were prepared from 24 replicates of human genomic DNA (ATCC NA12878) using a final library purification of 0.7 (black) and 0.75 (blue) volume equivalents of MAGWise. **(Right)** Table of typical plexWell WGS24 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 the hg38 reference genome, determining the median insert for each of the 24 samples, then taking the average of the 24 values.





**Figure 2.** This data demonstrates that the same library may yield similar fragment size distribution on two different electrophoretic analysis. Libraries were prepared from 24 replicates of human genomic DNA (ATCC NA12878) using a final library purification of 0.7 volume equivalents of MAGwise. **(Left)** Representative plexWell WGS 24 library traces generated using an Agilent Bioanalyzer 2100 with High Sensitivity DNA reagents. **(Right)** Representative of the same plexWell WGS 24 library traces generated using a TapeStation 2200 with High Sensitivity DNA 5000 reagents and tapescreens.

**qPCR assay:** Use 2  $\mu$ l 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 determined in 11 a., above, for the size adjustment value used for calculating the purified, multiplexed library concentration. Library concentrations are typically 30 – 60 nM.

## Sequencing Loading and Read Configuration

plexWell WGS24 libraries are dual indexed using 8 nt indices. Each pool contains 24 unique sample-specific i7 indices (Appendix B) and single pool-specific i5 indices. These libraries can be sequenced in dual index (R1, i7, i5, R2) if multiplexed with other libraries or single index (R1, i7, R2) if sequenced alone. plexWell libraries are sequenced using the same primers as Nextera<sup>®</sup> libraries<sup>2</sup> and contain the same adapter configuration. Refer to Illumina technical documentation for specific instructions on how to denature and dilute a purified library on your model of sequencing system<sup>3</sup> and for additional information on setting up the read configuration.

<sup>2</sup> The primers provided in TruSeq v3 Cluster kits are incompatible with Nextera-style libraries, including plexWell libraries. The TruSeq Dual Index Sequencing Primer Box from Illumina is required for sequencing plexWell libraries on older systems, such as the HiSeq 2500, HiSeq 2000, HiSeq 1500, GAIIx, and HiScanSQ.

<sup>3</sup> The sequencing primers provided in TruSeq v3 Cluster kits are incompatible with Nextera-style libraries, including plexWell libraries. The TruSeq Dual Index Sequencing Primer Box from Illumina is required for sequencing plexWell libraries on older systems, such as the HiSeq 2500, HiSeq 2000, HiSeq 1500, GAIIx, and HiScanSQ.

## Appendix A: plexWell WGS-24 index information

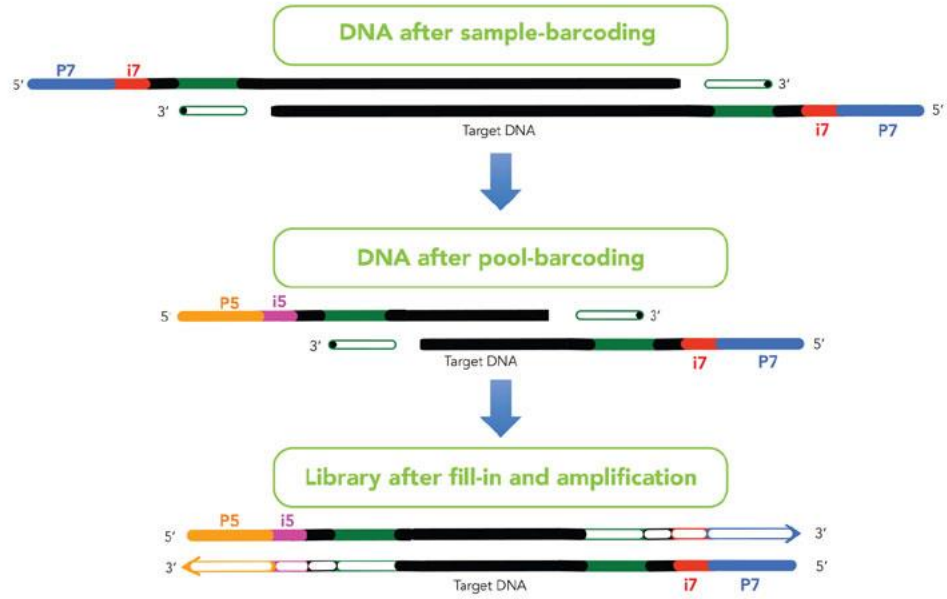
The most up-to-date list of indices for [all plexWell kits](#) is available in the Resources section for this product. Click on the “plexWell Kit Master Index List” to download an excel workbook containing all i7 indices (in list and plate layout) and i5 indices. The master index list is the easiest way to copy and paste index sequences into sample sheets or for demultiplexing.

**17 indices by well of SBW 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	GAGTTC TC	ACACGGTT									
F	TATCCCAC	TCGCCAGC	CTTAGAGT									
G	CAACTGTG	TGTGACTA	TTCTCAT									
H	ATGACTAG	TTGACGTC	CGTGCTGG									

**PBW014 (i5) index sequence (5' to 3'): CCTATTGA**

## Appendix B: plexWell Library Preparation Intermediates



## Appendix C: 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)
- NextSeq System

## Experienced User Checklist

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

### Sample Barcoding

- Centrifuge **SBW24** plate
- Add 8  $\mu$ l sample (@25 ng/ $\mu$ l) to **SBW24 plate** and pipette 10x
- Add 8  $\mu$ l coding buffer to **SBW24 plate** and pipette 10x
- Run TAG incubation
- Add 12  $\mu$ l X-solution to SBW24 plate and pipette 10x
- Run STOP incubation
- Pool 26  $\mu$ l from each SB reaction
- Complete SB purification (1 volume equivalent), eluting with 65  $\mu$ l
- Transfer 64  $\mu$ l of purified SB pool to 0.2 ml PCR tube (PB reaction tube)

### Pool Barcode Reaction

- Add 5  $\mu$ l PB Reagent to 64  $\mu$ l of purified SB pool and pipette 5x
- Add 34.5  $\mu$ l coding buffer to PB reaction tube and pipette 10x
- Run TAG incubation
- Add 52  $\mu$ l X-solution to PB reaction and pipette 10x
- Run STOP incubation
- Transfer Stopped PB reaction to a 1.5 ml LoBind tube and complete PB purifications (1 volume equivalent), eluting with 101  $\mu$ l of 10 mM Tris
- Transfer 100  $\mu$ l of purified PB pool to LoBind tube

### Library Amplification

- Add 16  $\mu$ l Library Primer Mix to PB eluate
- Add 100  $\mu$ l of KAPA HiFi HotStart ReadyMix and pipette to mix
- Aliquot 54  $\mu$ l of amplification mix into each of 4 x 0.2 ml PCR tubes. Pulse-fuge
- Run Fill\_Amp4 program
- Combine amplification reactions into a 1.5 ml LoBind tube.
- Remove 3  $\mu$ l of reaction for QC analysis. Measure remaining volume.
- Complete Library Purification (0.7 volume equivalent), eluting with 32  $\mu$ l of 10 mM Tris, pH 8.0

**Revision History**

<b>Version</b>	<b>Release Date</b>	<b>Prior Version</b>	<b>Description of changes</b>
202200421	20220429	20210402	<ul style="list-style-type: none"> <li>● Table of Contents added</li> <li>● Added library QC information with BioAnalyzer and TapeStation library profile</li> </ul>
20210402	20210402	20210122	<ul style="list-style-type: none"> <li>● Updated PB reaction mixing steps</li> </ul>
20210122	20210125	20200320	<ul style="list-style-type: none"> <li>● Updated workflow diagram, purification steps, and amplification program name</li> <li>● Added molecular diagram, appendices and experienced user checklist</li> </ul>
20200320	20200325	20190807	<ul style="list-style-type: none"> <li>● Removed separate fill-in step and reagents</li> <li>● Updated workflow diagram</li> </ul>
20190701	02JUL2019	20181196	<ul style="list-style-type: none"> <li>● Updated input from 300 ng to 200ng</li> <li>● Changed PB volume</li> <li>● Removed double-sided size selection following fill-in</li> </ul>

## Technical Assistance

For technical assistance, contact seqWell Technical Support.

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