plexWell™ WGS 24 Library Preparation Kit

for Illumina® Sequencing Platforms

(Part No. WGS24)

User Guide

Introduction

This user guide provides detailed instructions for preparing 24 genomic DNA samples for loading onto the Illumina NovaSeq™ 6000 Sequencing System using the plexWell WGS 24 Beta Library Preparation Kit.[1] 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.

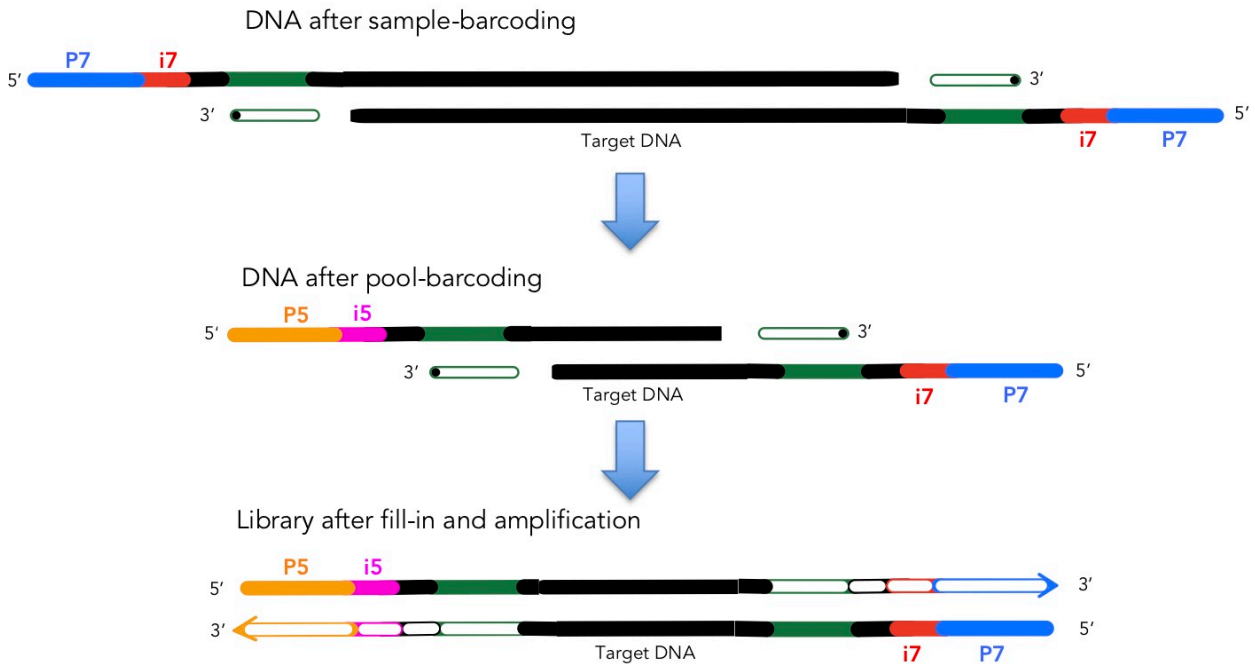
This plexWell WGS 24 User Guide is optimized for 200 ng of high quality genomic DNA per sample and typically generates 750 – 1,500 fmoles of purified, size-selected multiplexed library output, ranging from 500 – 1,000 bp in library fragment length.

[1] 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.

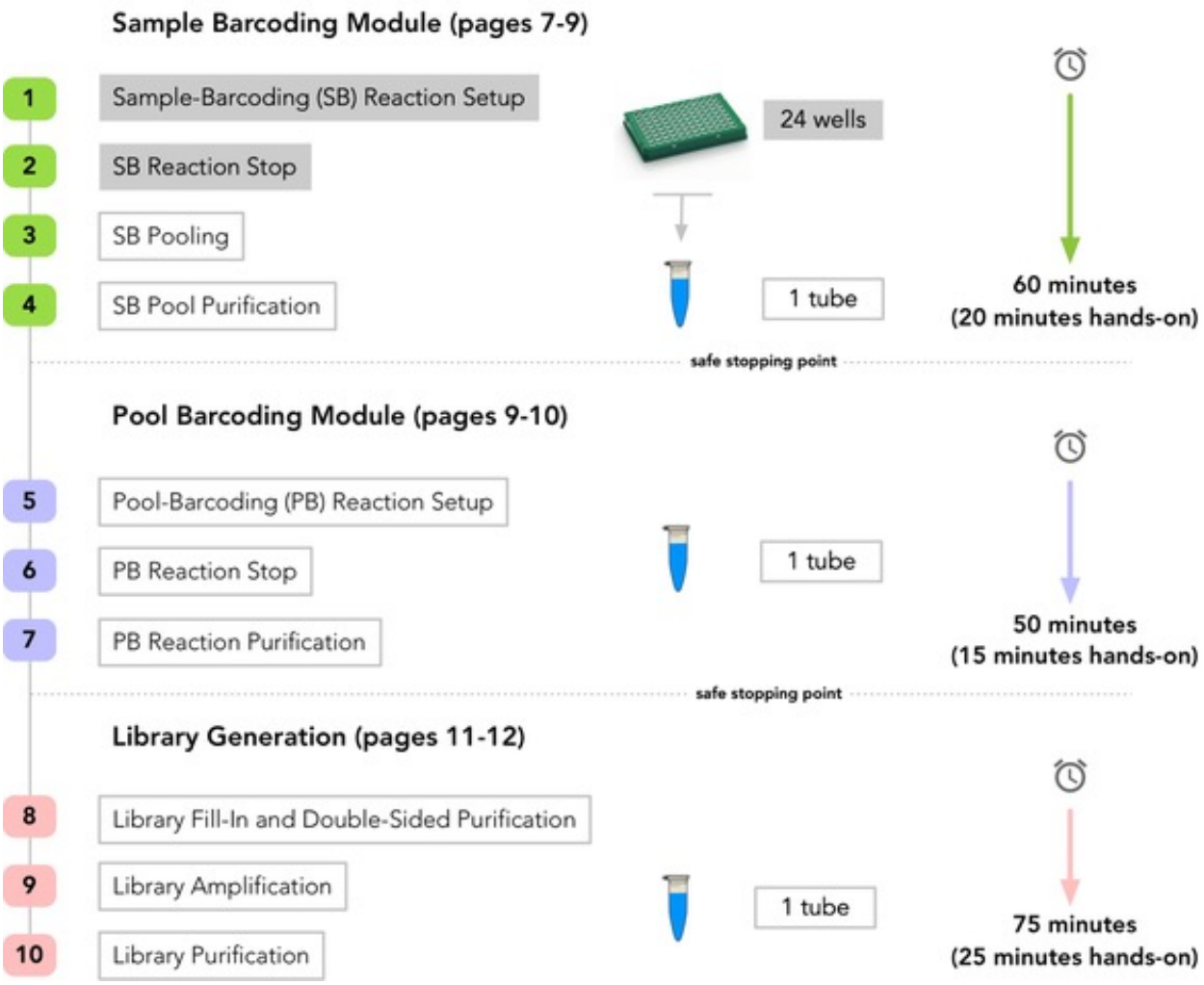
Table 1. Components of plexWell WGS 24 Library Preparation Kit

Box	Component	P/N	Description	Storage	Qty
1	**Sample Barcode Plate**	SBW24	**SBW24 Plate:** Input DNA-ready **SB** reagent in a green fully-skirted 96-well PCR plate (columns 1 – 3)	-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**	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

plexWell Library Preparation Intermediates



plexWell™ WGS24 Library Prep Workflow



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

Reagents

- 100% Ethanol (molecular biology grade)
- Tris-HCl, pH 8.0
- Ultrapure Water (PCR grade)
- PicoGreen® DNA assay (recommended), or another 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® (1.5 and 2.0 ml, DNA LoBind Tubes)
- PCR plate seals (must be evaporation-resistant)
- 96-well thermal cycler (compatible with fully-skirted 96-well PCR plates)
- 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.
- FILLAMP:
    - 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

  ] 4 cycles

    - 72°C for 2 minutes (final extension)
    - 4°C hold

Before starting the procedure:

Adjust input DNA concentration. Assay the DNA concentration of each sample (n=96) 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 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 concentrated Coding Buffer is viscous. Store **Coding Buffer (3X)** at room temperature. Pipette slowly and do not pre-wet pipette tips to transfer volumes accurately. 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) 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

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 µl of input genomic DNA (25 ng/µl) to all wells (one sample per well). Mix the DNA thoroughly with the sample-barcode reagent in each well by pipetting up and down ten times at 8 µl, being careful not to introduce excessive bubbles. Use clean tips for each sample.

c. Next, carefully pipette 8 µl 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 twenty times at 8 µl, being careful not to introduce excessive bubbles.
Useful Tip: *Aliquot 40 µl of* ***Coding Buffer (3X)*** *into a PCR 8-tube strip, and then use a multichannel pipettor to dispense 8 µl into the* ***SBW24 Plate*** *and to mix.*

d. Seal the **SBW24 Plate** securely with a plate seal, 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 12 µl of **X Solution** to each well of the **SBW24 Plate**. Pipette up and down slowly 5 times to mix, being careful not to introduce excessive bubbles. Use clean tips for each addition of **X Solution**.
Useful Tip: *Aliquot 60 µl of* **X Solution** *into a PCR 8-tube strip, and then use a multichannel pipettor to transfer 12 µl from the strip into columns 1 - 3 of the* ***SBW24 Plate*** *and to mix.*

b. Securely reseal and pulse-spin the **SBW24 Plate**; 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 **SBW24 Plate**.

3. SB Reaction Pooling

a. Using a P200 pipettor, transfer 26 µl 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 µl. 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 µl (approximately 1 volume equivalent) of MAGwise beads to the SB reaction pool in the 2 ml LoBind tube, and mix thoroughly by pipetting.

c. Incubate in a 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 for 5 minutes. 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 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. Repeat previous step for a total of 2 washes with 80% ethanol. Use a small pipettor (e.g., P20) to remove the residual ethanol after the second wash. **Do not air-dry** the pellet.

h. Remove the tube from magnetic stand and immediately pipette 65 µl of 10 mM Tris-HCl, pH 8.0 on top of the bead pellet. Pipette the solution along the inner wall of the tube multiple times to thoroughly resuspend the bead pellet.

i. Incubate the tube on the bench for at least 5 minutes to elute the purified SB reaction pool from the beads.

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

k. When the supernatant has cleared completely, carefully transfer 64 µl of DNA eluate to a PCR tube. The transferred eluate contains the purified SB reaction pool.

SAFE STOPPING POINT

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

5. Pool-Barcoding (PB) Reaction Setup

a. Add 5 µl 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 µl of **Coding Buffer** (3X), and mix the PB reaction again 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 52 µl of **X Solution** to the PB reaction. Mix thoroughly by pipetting 10 times while being careful not to produce excessive bubbles.

b. Recap 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 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 µl (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. Transfer the 1.5 ml tube to a magnetic stand and let the beads settle completely for 3 minutes. A bead pellet will form along one side of the tube and the supernatant should appear completely clear after 3 minutes.

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

f. Wash with 80% ethanol.
   i. With the tube in the magnetic stand, add 500 µl of 80% ethanol without disturbing the beads.
   ii. After 30 seconds, slowly remove and discard supernatant, being careful not to disturb the pellet.

g. Repeat the previous step for a total of 2 washes with 80% ethanol. Use a large pipettor to remove most of the ethanol waste, and then use a smaller pipettor (e.g., P20) to remove the residual ethanol that collects at the bottom of the tube. **Do not air-dry**.

h. Remove tube from magnetic stand and immediately add 101 µl of 10 mM Tris-HCl, pH 8.0. Pipette the liquid along the inside of the tube several times to thoroughly resuspend the bead pellet.

i. Incubate 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 µl 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 fill-in and amplification.

SAFE STOPPING POINT

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

8. Library Amplification

a. Add 16 µl of **Library Primer Mix** to the eluate in the 1.5 ml tube.

b. Add 100 µl 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 µl x 4 tubes).

d. Close the strip, and run the following program (FILLAMP) 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 2 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 µL 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 0.7 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 for approximately 3 minutes. A bead pellet will form along one side of the tube and the supernatant should appear completely clear after 3 minutes.

g. Slowly remove the supernatant with a pipettor and discard. Be careful not to disturb the bead pellet. 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 with 80% ethanol.
   i. With the tube in the magnetic stand, add 500 µl of 80% ethanol without disturbing the beads.
   ii. After 30 seconds, slowly remove and discard supernatant, being careful not to disturb the pellet.

i. Repeat previous step for a total of 2 washes with 80% ethanol. Use a small pipettor (e.g., P20) to remove the residual ethanol after the second wash. Do not air-dry the pellet or the DNA recovery may be compromised.

j. Remove tube from magnetic stand and add 35 µl of 10 mM Tris-HCl, pH 8.0. Pipette the liquid along the inside of the tube several times to thoroughly disperse the beads.

k. Incubate for 5 minutes in a tube rack 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.

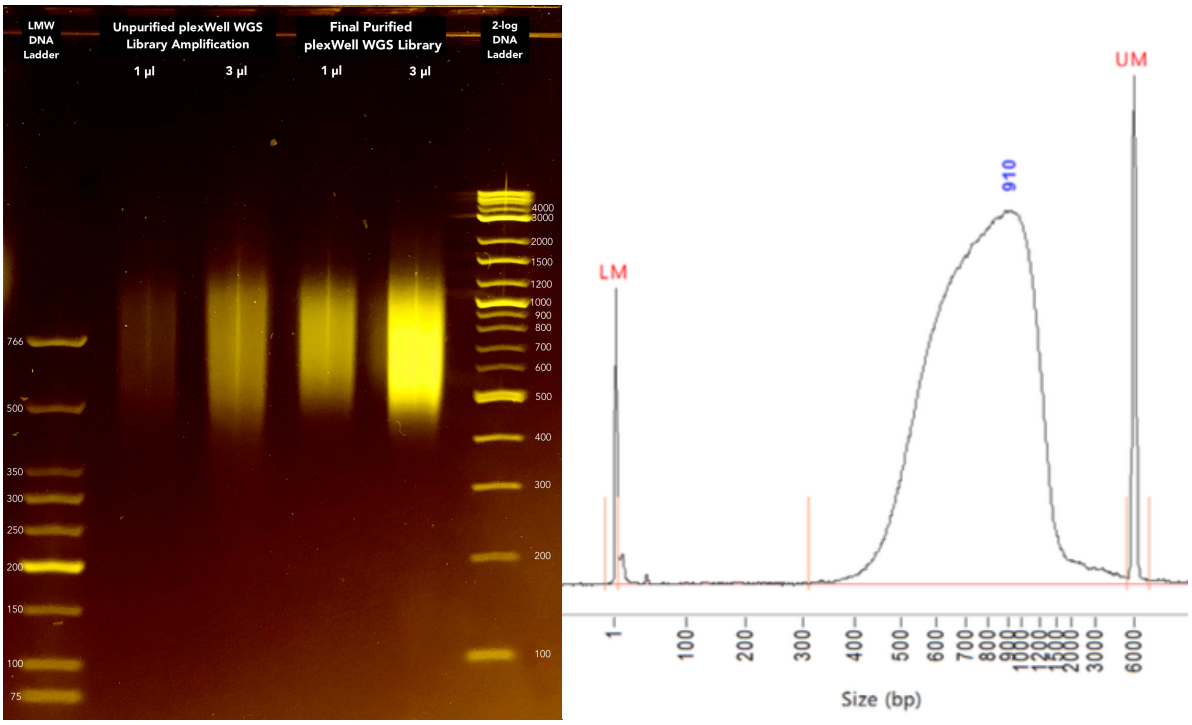
SAFE STOPPING POINT

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 unpurified and purified, multiplexed library. The majority of library fragments should be between 500 and 1,000 bp in length (see typical results on next page).

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 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.



Electrophoretic analysis/QC of a typical plexWell WGS multiplexed library. Unpurified and purified multiplexed library after electrophoresis on a 2% agarose gel (top, gel image); purified multiplexed library after capillary electrophoresis on an AATI fragment analyzer (bottom, electropherogram).

Read Configuration

plexWell WGS24 libraries are dual indexed using 8 nt indices. Each pool contains 24 unique sample-specific i7 indices (Table 2) 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® libraries[2] and contain the same adapter configuration.

Table 2. SBW24 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

Refer to Illumina technical documentation for instructions on how to denature and dilute a purified library prior to loading the NovaSeq 6000 Sequencing System.

[2] 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, GA*II*x, and HiScanSQ.

Revision History

Version	Release Date	Prior Version	Description of changes
20200320	20200325	20190807	• Removed separate fill-in step and reagents • Updated workflow diagram
20190701	02JUL2019	20181196	• Updated input from 300 ng to 200ng • Changed PB volume • Removed double-sided size selection following fill-in

Technical Assistance

For technical assistance, contact seqWell Technical Support.

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