SARS-CoV-2 Surveillance sequencing using plexWell™ 384 or plexWell™ 96 Library Preparation Kits for Illumina® Sequencing Platforms

(Part Nos. PW384, PW096)

Demonstrated Protocol

v20220110

Introduction

SARS-CoV-2 sequencing improves our understanding and ability to respond to the global COVID-19 pandemic by tracking pattern mutations, identifying modes of spread and employing genomic epidemiology. One obstacle to scaling sequence efforts is the ability to prepare and pool 100s to 1000s of samples per day to match the throughput demand and be cost-effective. The plexWell™ 96/384 workflow was designed to specifically address this challenge through scalable and highly multiplexed library preparation, enabling sequencing of up to 2,304 samples in a single sequencing run.

plexWell 384 and 96 Library Preparation Kits come in an assay-ready 96-well configuration to streamline high-throughput multiplexed library preparation. Each kit contains sufficient reagents to prepare dual-indexed Illumina-compatible libraries from 384 and 96 individual DNA samples, respectively. The 384 well kit comes in versions A-F to allow users to multi-plex up to 2304 samples, if required. plexWell libraries are compatible with the Illumina iSeq, MiniSeq MiSeq, NextSeq, HiSeq and NovaSeq systems.[1]

The primary advantages and benefits of using the plexWell 96 and 384 Library Preparation Kits are a streamlined 96 sample multiplexed library preparation workflow that tolerates variation in DNA input concentration and greatly saves on labor and consumable costs. plexWell library preparation kits from seqWell utilize proprietary transposase-based reagents to insert barcoded adapters directly into input DNA in two separate steps. In the first barcoding step, different i7-barcoded adapters[2] are inserted into each of the 96 DNA samples in segregated reactions. Next, the i7-barcoded DNA samples are all pooled into a single tube. In the second barcoding step, a single i5-barcoded adapter is inserted into each pool of 96 i7-barcoded DNA samples. Finally, each 96-plex library is amplified in a single PCR reaction using universal library primers (*i.e.*, P5 and P7 primers), making for a highly efficient multiplexed library prep workflow (see Workflow Diagram).

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

[2] For a complete list of all i7 and i5 indices in plexwell kits download the plexWell Kit Index List found in the resources section on the plexwell product page or refer to Appendix E

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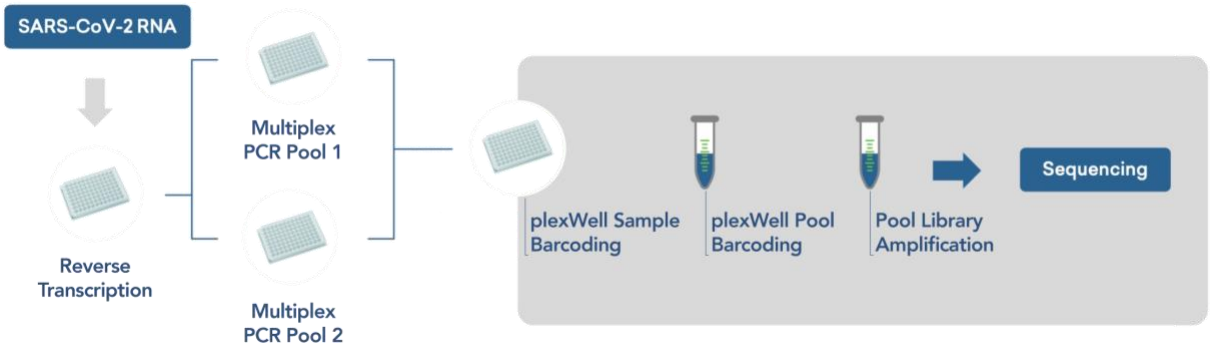
ARTIC + plexWell Workflow



Detailed plexWell Workflow

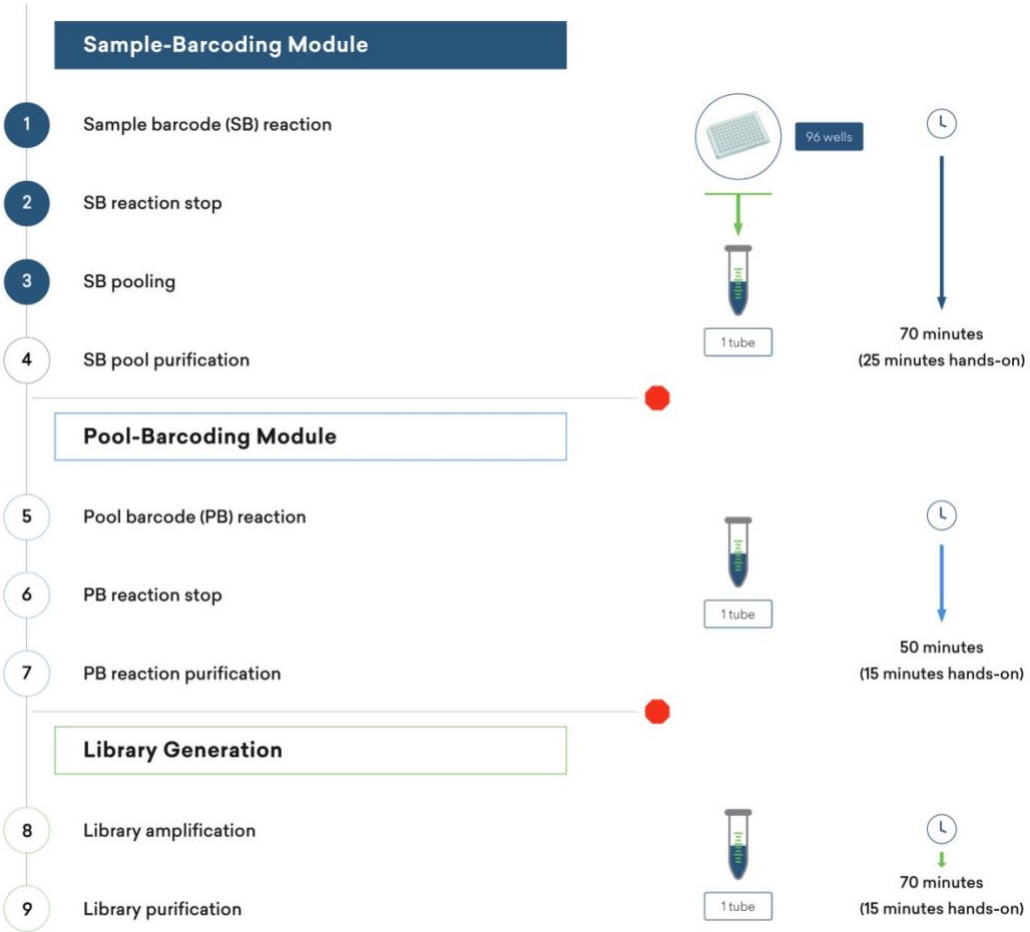


Table 1. plexWell 384 Library Preparation Kit Components

Ref	Component	P/N	Description	Storage	Qty
PW384-1 (Box 1)	**Sample Barcode Plate**	SBX96	**SBX96 Plate:** assay ready SB reagent in low profile, fully-skirted 96-well red PCR plate	-20°C	4
PW384-2 (Box 2)	**3X Coding Buffer**	CB0384	2 ml tube, violet cap, 1.5 ml	ambient	2
	X Solution	QB0096	2 ml tube, black cap, 1.5 ml	ambient	4
	MAGwise™ Paramagnetic Beads	MG10000	15 ml bottle, 10 ml	4°C	1
PW384-3X (Index Set)	**Pool Barcode Reagent***	PBX###	0.5 ml tube, red cap, 10 µl	-20°C	4
	Library Primer Mix	PRM384	0.5 ml tube, natural cap, 24 µl	-20°C	1

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

Table 2. plexWell 96 Library Preparation Kit Components

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

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

Index Set	Component	P/N	Index – A type sequencer	Index – B type sequencer
A	Pool Barcode Reagent X007	PB-X007	CTCTCTAT	ATAGAGAG
	Pool Barcode Reagent X060	PB-X060	TCTCATAT	ATATGAGA
	Pool Barcode Reagent X079	PB-X079	GATCATAG	CTATGATC
	Pool Barcode Reagent X089	PB-X089	CCCTATGG	CCATAGGG
B	Pool Barcode Reagent X021	PB-X021	ATATATGA	TCATATAT
	Pool Barcode Reagent X024	PB-X024	AGGAAACT	AGTTTCCT
	Pool Barcode Reagent X038	PB-X038	TATGGAGG	CCTCCATA
	Pool Barcode Reagent X044	PB-X044	TGATACAT	ATGTATCA
C	Pool Barcode Reagent X048	PB-X048	TCCGACTA	TAGTCGGA
	Pool Barcode Reagent X055	PB-X055	ATGGACAT	ATGTCCAT
	Pool Barcode Reagent X056	PB-X056	TTGCATTG	CAATGCAA
	Pool Barcode Reagent X083	PB-X083	CTCAAATA	TATTTGAG
D	Pool Barcode Reagent X003	PB-X003	TAATTACT	AGTAATTA
	Pool Barcode Reagent X149	PB-X149	TCTAAGAG	CTCTTAGA
	Pool Barcode Reagent X184	PB-X184	CCACCCTA	TAGGGTGG
	Pool Barcode Reagent X193	PB-X193	TGCTGTAG	CTACAGCA
E	Pool Barcode Reagent X116	PB-X116	GACCTAAG	CTTAGGTC
	Pool Barcode Reagent X140	PB-X140	TCCCTAAA	TTTAGGGA
	Pool Barcode Reagent X153	PB-X153	AAATAGAT	ATCTATTT
	Pool Barcode Reagent X178	PB-X178	CATAGGAA	TTCCTATG
F	Pool Barcode Reagent X169	PB-X169	TTGACCGA	TCGGTCAA
	Pool Barcode Reagent X173	PB-X173	AAGGGAAA	TTTCCCTT
	Pool Barcode Reagent X181	PB-X181	CACATGGA	TCCATGTG
	Pool Barcode Reagent X207	PB-X207	CGACGTTG	CAACGTCG

Refer to Appendix D for guidance regarding using Workflow A or Workflow B i5 sequences

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

Reagents (Library Prep)

- 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

Reagents (RT+multiplex PCR)

- Reverse Transcription reagents such as LunaScript® RT SuperMix (New England Biolabs)
- Q5® Hot Start High-Fidelity 2X Master Mix (New England Biolabs)
- SARS-CoV-2 multiplex primer sets such as ARTIC v3 panel (IDT), ARTIC v4 panel (IDT), or Midnight panel (IDT)

OR

- NEBNext® ARTIC SARS-CoV-2 RT-PCR Module (New England Biolabs) which includes all reagents needed to complete the RT and multiplex PCR according to the NEB protocols

Equipment & Consumables (library prep)

- Single-channel pipettors (1-20 µl, 20-200 µl, 100-1,000 µl)
- Multi-channel pipettors (1-10 µl, 10-200 µl)
- Pipette tips (low-retention barrier tips)
- Eppendorf Tubes® (1.5 ml & 2.0 ml, DNA LoBind Tubes)
- PCR plate seals (must be evaporation-resistant)
- 96-well thermal cycler (compatible with 96 well low profile fully skirted PCR plates, BioRad HSP 9611)
- Magnetic stand for 1.5 ml and 2 ml tubes
- 0.2 ml PCR 8-tube strips and caps/seals
- Benchtop centrifuge to pulse-spin 1.5/2 mL tubes and PCR 8-tube strips
- Plate centrifuge
- Vortex mixer

Additional Equipment & Consumables for RT + PCR

- PCR plates
- Mastermix and extraction/sample addition hoods or cabinets (*recommended)*

Thermal Cycler Programs

- **RT program**:

  25 °C for 2 min
  55 °C for 20 min
  95 °C for 1 min
  4°C hold

- **Multiplex PCR program**:

  98°C for 30 seconds (initial denaturation)
  98°C for 15 seconds
  63°C for 5 minutes } 35 cycles
  4°C hold

- **TAG Program**: 55°C for 15 minutes; 25°C hold

- **STOP Program**: 68°C for 10 min; 25°C hold

- **FILL_AMP12 Program**:

  72°C for 10 minutes (fill-in)
  95°C for 3 minutes (initial denaturation)
  98°C for 30 seconds
  64°C for 15 seconds } 12 cycles
  72°C for 30 seconds
  72°C for 3 minutes (final extension)
  4°C hold

Before starting procedure:

Mitigate Contamination Risk. To prevent contamination during the ARTIC (RT+PCR) portion of the workflow, it is highly recommended to do your sample extraction and sample addition in one hood/cabinet and prepare your mastermixes in another. Decontaminate hoods/cabinets between uses.

Program thermal cycler(s). For convenience, set-up the thermal cycler programs listed on the previous page before starting.

Pulse-spin kit components. Liquids can condense or shift locations inside containers during shipment or storage. Before using the **SBX96 Plate**, and before dispensing from reagent tubes, pulse-spin in a suitable centrifuge to gather the reagents at the bottom of the well or tube. If the kit components freeze during shipment or storage, thaw, mix and pulse-spin before use.

Equilibrate MAGwise Paramagnetic Beads to room temperature. MAGwise beads can be stored for up to 2 weeks at room temperature or for longer periods at 2 - 8°C. If stored cold, warm at room temperature for 30 minutes before use. Vortex to thoroughly resuspend beads prior to use. To transfer volumes accurately, pipette slowly and do not pre-wet pipette tips.

Check the X Solution for precipitate before use. If a precipitate is visible, incubate at 37°C for 5 minutes (or longer if necessary). Mix gently by inversion until the precipitate dissolves (do not vortex). Note: **X Solution** contains SDS and will precipitate if stored below room temperature. Overly vigorous mixing will cause foaming.

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

Prepare 80% ethanol fresh daily. You will need ~4 ml per 96-well sample plate.

Prepare 10 mM Tris-HCl, pH 8.0. Prepare 10 mM Tris-HCl, pH 8.0 from a concentrated stock solution diluted with ultrapure water (both molecular-biology grade). You will need ~250 µl per 96-well sample plate. Do not use EDTA-containing solutions (e.g., TE).

Safe stopping points are indicated in the protocol. For optimal results, proceed directly to the next step unless a safe stopping point is indicated.

Reverse Transcription (RT) and enrichment (PCR) for SARS-CoV-2

seqWell has used a variety of ARTIC procedures to generate SARS-CoV-2 multiplex amplicons for utilization with the plexWell 96, 384 and plexWell Plus 24 library preparation kits. Outlined below is the procedure used at seqWell for RT-PCR using LunaScript® (NEB) and ARTIC v3 and v4 (IDT) primer panels. Additional protocols for the following primer panels are linked below.

- NEBNext® ARTIC SARS-CoV-2 RT-PCR module protocol for RT and multiplex PCR with NEB developed primer panels
- MIDNIGHT (IDT) primer set multiplex PCR here, used in conjunction with RT steps outlined below.

Note: The recommended input for RT reactions is purified nucleic acid from positive COVID-19 clinical samples with Ct values between 18 - 33. This Ct working range assumes that a concentration factor of 3X or greater was applied by eluting in a smaller volume than the initial sample volume used for purification. If the Ct is between 12 - 15, then dilute the sample 100-fold in water, if between 15 - 18 then dilute 10-fold in water. This will reduce the likelihood of PCR-inhibition

1. Reverse Transcriptase with LunaScript® SuperMix

a. Array 8 µl of each purified sample into a PCR plate in the extraction and sample addition cabinet.

b. Add 2 µl **LunaScript® Supermix** to each well.

c. Gently mix by pipetting up and down five times at 8 µl. Seal the PCR plate, then pulse spin the plate to collect liquid at the bottom of the wells.

d. Incubate the RT reaction in a thermal cycler, as follows:

25 °C for 2 min
55 °C for 20 min
95 °C for 1 min
Hold at 4 °C

2. Multiplex PCR to enrich for SARs-CoV-2 Genome

Note: *Pool 1 and Pool 2 reactions **must be amplified separately***

a. In the mastermix hood, set up the Multiplex PCR pool 1 mastermix. You will have two mastermixes, one for pool 1, one for pool 2. The volume of primer pool used differs for ARTIC (v3 and v4 use the same volume) and MIDNIGHT primers. Refer to NEB's protocol for use with NEB primer mixes.

Table 4. Multiplex PCR Pool 1 Reagent Volumes

Reagent	Per Sample (ARTIC)	96 reactions (ARTIC)	Per Sample (MIDNIGHT)	96 reactions (MIDNIGHT)
Q5® Hot Start High-Fidelity 2X Master Mix	12.5 µl	1320 µl	12.5 µl	1320 µl
10 µM Primer Pool 1	3.6 µl	380 µl	1.1 µl	116.2 µl
Nuclease free water	6.4 µl	676 µl	8.9 µl	940

b. Repeat Step 1 with 10 µM Primer Pool 2 (refer to table 4 for volumes), substituting 10 µM Primer pool 2 in place of Primer pool 1.

c. Label one PCR plate for multiplex PCR pool 1. Distribute 22.5 µl of Pool1 multiplex PCR mastermix into the labelled PCR plate

d. Label a 2nd PCR plate for multiplex PCR pool 2. Distribute 22.5 µl of Pool 2 multiplex PCR mastermix into the labelled PCR plate.

e. Transfer multiplex PCR plates to the extraction and sample addition cabinet. Wipe down outside of plates before entering extraction and sample addition cabinet.

f. In the extraction and sample addition cabinet add 2.5 µl of cDNA (from part A or B) to the corresponding wells of the **Pool 1 and Pool 2 Multiplex PCR mastermix** plates. Pipette 5x at 20 µl to mix.

g. Seal the plates and pulse centrifuge the PCR plates to collect the contents at the bottom of the wells.

h. Set-up and run the following PCR program on the thermal cycler, with lead heating on.

Initial denaturation: 98°C for 30 seconds
35 Cycles of: 98°C for 15 seconds
63°C for 5 minutes
Hold: 4°C

Note: Thermal cycling conditions are the same for ARTIC, midnight, and NEB primer sets.

3. Prepare Multiplex PCR products for Library Preparation

The plexWell library preparation kit does not require purification or quantification of the multiplex PCR products greatly speeding up the steps between multiplex PCR and library preparation. The next steps outline methods for combining pools 1 and 2 and suggested dilution points. It is not necessary to apply different dilutions based on Ct values.

a. Combine Pool 1 and Pool 2 PCR products. Label a new PCR plate as "Combined Amplicons". Transfer 3 µl of "pool 1" and 3 µl of "pool 2" to the corresponding well of the "Combined Amplicon" PCR plate. Following 2nd addition, pipet 10x to mix.

b. Dilute combined multiplex PCRs by adding 10 mM Tris to the "Combined amplicons" PCR plate and tip mixing 10x. plexWell kits work optimally with 3-30 ng of input DNA. To reach the optimal input, it is recommended to apply a global dilution to the entire place of PCR products. We have validated a variety of dilutions and recommend for first time users to start at the midpoint on the table below for each primer panel.

Table 5. Recommended Dilution of combined multiplex PCRs prior to library preparation.

Amplicon Set	Range	Volume Tris	Comments
ARTIC v3, v4 & NEB	1:5 to 1:10	24 µl (1:5)	Final library fragment analysis may show residual amplicon peaks.
		42 µl (1:8)	
		54 µl (1:10)	
MIDNIGHT	1:8 to 1:15	42 µl (1:8)	Final library fragment analysis may show residual amplicon peaks.
		54 µl (1:10)	
		66 µl (1:12)	
		84 µl (1:15)	

*Typically residual amplicon peaks do not contribute to sequencing and are not a problem if library concentration is determined by qPCR. Optimization may be required if these peaks are significant and library concentration is derived by picogreen.

Library Preparation

Before beginning the plexWell 384/96 library prep, confirm that you have prepared your SARS-CoV-2 amplicons correctly. This entails combining pools 1 and 2, then diluting them. Refer to table 6, above for suggested dilution points. It is NOT necessary to purify or quantify the SARS-CoV-2 amplicons prior to starting plexWell. A key benefit to the plexWell library preparation kit is auto-normalization which ensures an approximately equal number of reads per index over a 10-fold input range. When combined with the ARTIC workflow, it is not necessary to normalize samples based on Ct prior to starting the library preparation.

Instead a global dilution (same dilution for all wells) is applied to the entire plate of combined amplicons prior to starting library prep.

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

SB reactions should be set-up at room temperature. If processing more than one plate, complete the set-up of one **SBX96 Plate** (through starting the thermal cycler) before proceeding to the next one.

a. Pulse-spin the **SBX96 Plate**; then remove the seal carefully.

b. Transfer 4 µl of input DNA (approximately 2.5 ng/µl) to each well (one sample per well) of the **SBX96 Plate**. Mix thoroughly and slowly by pipetting (5 times at 4 µl), being careful not to introduce excessive bubbles. Use clean tips for addition of each sample.

c. Carefully pipette 4 µl of 3X **Coding Buffer** to each well of the **SBX96 Plate**, using new pipette tips for each transfer. Mix thoroughly and slowly by pipetting up and down ten times at 4 µl, being careful not to introduce excessive bubbles.

Useful Tip**:** *Aliquot 55 µl of 3X* ***Coding Buffer*** *into each well of an 8-tube strip, and then use a multichannel pipettor to transfer 4 µl (and mix) into each SB reaction.*

d. Seal the **SBX96 Plate**, pulse-spin, then transfer to a thermal cycler, and run the TAG program, below, with lid-heating on:

55°C for 15 minutes;
25°C hold

2. SB Reaction Stop

a. Confirm there is no precipitate in the **X Solution.** Slowly pipette contents of tube 5 times to ensure everything is mixed.

Note: *This solution contains SDS and vigorous mixing will cause it to foam. Pipetting slowly and under the surface of the solution will give the best results.*

b. Pulse-spin **SBX96 Plate** and then remove seal.

c. Add 6 µl of **X Solution** to each well of the **SBX96 Plate**. Pipette up and down slowly (10 times at 6 µl). Change pipette tips for each addition.

Useful Tip: *Aliquot 85 µl of* **X Solution** *to an 8-strip tube, then use a multichannel pipette to transfer 6 µl from the strip to each column of the* **SBX96 Plate**.

d. Seal **SBX96 Plate**, pulse-spin, then transfer to a thermal cycler and run the STOP program, below, with lid-heating on:

68°C for 10 minutes;
25°C hold

3. SB Pooling (within plate)

a. Pulse-spin **SBX96 Plate** and then remove seal. Transfer 9 µl of stopped SB reactions from columns 1-12 into an 8-well strip tube, pipetting twice after each dispense to mix after each addition. ***Do NOT pool samples from different* SBX96 *plates together***!

Optional: If bubbles are present after pooling stopped SB reactions in strip tube, use a tabletop centrifuge to remove bubbles prior to proceeding

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

Optional: If bubbles are present after pooling use a tabletop centrifuge to remove bubbles prior to proceeding

4. SB Pool Purification

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

b. Add 850 µl (approximately 1 volume equivalent) of MAGwise to the pooled SB reactions and mix thoroughly by pipetting. Incubate on bench for ε5 minutes to allow DNA to bind.

c. Place tube on magnetic stand and let beads settle, 5 minutes. A pellet should form on one side of the tube and the supernatant should be visibly clear after 5 minutes.

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

Useful tip: *Use a large pipette tip to remove most of the supernatant and then if necessary, use a smaller one to remove the remaining supernatant.*

e. Wash beads with 80% ethanol.

   i. With tube in the magnetic stand, add 1.7 ml of 80% ethanol without disturbing beads. If this volume is insufficient to cover the bead pellet, add a larger volume.
   ii. After ≥30 seconds, remove and discard supernatant, being careful not to disturb pellet, to complete the wash step.

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

   i. With tube in the magnetic stand, add 1.7 ml of 80% ethanol without disturbing beads. If this volume is insufficient to cover the bead pellet, add a larger volume.
   ii. Perform the next steps quickly, working 1-2 tubes at a time
      1. After ≥30 seconds, remove and discard supernatant, being careful not to disturb pellet**.**
      2. Cap tube, pulse-spin and return to magnet, letting beads settle (<30 seconds). Use a small pipet tip (≤20 µl) to remove any residual ethanol at the bottom of the tube.
      3. Add 40 µl of 10 mM Tris to bead pellet, remove from magnetic stand and pipet the solution along the inner wall of the tube multiple times to thoroughly resuspend the bead pellet. Place tube in rack on bench. **DO NOT air dry bead pellet** prior to Tris addition or the DNA recovery will be compromised.

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

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

i. When the supernatant has cleared completely, carefully transfer 39 µl of DNA eluate to a PCR tube. The transferred eluate contains the purified SB reaction pool.

Note: *Transfer of some beads with the purified SB reaction pool will not inhibit the Pool Barcode reaction.*

Optional QC: Use 1 µl of the purified SB reaction pool for picogreen. The expected concentration with the ARTIC workflow is 4-20 ng/µl. If outside this range, contact support@seqwell.com

SAFE STOPPING POINT

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

5. Pool Barcoding (PB) Reaction Setup

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

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

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

c. Pulse-fuge the PCR tube containing the PB reaction then transfer to a thermal cycler, and run the TAG program, below, with lid heating on:

55°C for 15 minutes;
25°C hold

6. PB Reaction Stop

a. Pulse-fuge PB reaction, then add 33 µl of **X solution** to each PB reaction. Mix thoroughly by pipetting slowly 10 times at 50 µl.

b. Re-cap PB reactions, pulse-fuge, and transfer to a thermal cycler and run the STOP program, below, with lid heating on:

68°C for 10 minutes;
25°C hold

7. PB Reaction Purification

a. Pulse-spin stopped PB reaction, then transfer entire contents (~99 µl) of each stopped PB reaction to its own 1.5 ml LoBind tube.

b. Briefly vortex or pipette MAGwise to ensure beads are suspended. Then add 99 µl (1 volume equivalent) of MAGwise to each stopped PB reaction and mix thoroughly by pipetting.

c. Incubate on bench for at least 5 minutes to allow DNA to bind.

d. Place tube in magnetic stand and let beads settle. A pellet should form on one side of the tube and the supernatant should be visibly cleared after 3 minutes.

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

f. Wash beads with 80% ethanol.

   i. With tube in the magnetic stand, add 300 µl of 80% ethanol without disturbing beads.
   ii. After ≥30 seconds, remove and discard supernatant, being careful not to disturb pellet.

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

   i. With tube in the magnetic stand, add 300 µl of 80% ethanol without disturbing beads.
   ii. Perform the next steps quickly, working 1-2 tubes at a time
      1. After ≥30 seconds, remove and discard supernatant, being careful not to disturb pellet**.**
      2. Cap tube, pulse-spin and return to magnet, letting beads settle (<30 seconds). Use a small pipet tip (≤20 µl) to remove any residual ethanol at the bottom of the tube.
      3. Add 24 µl of 10 mM Tris to bead pellet, remove from magnetic stand and pipet the solution along the inner wall of the tube multiple times to thoroughly resuspend the bead pellet. Place tube in rack on bench. **DO NOT air dry bead pellet** prior to Tris addition or the DNA recovery will be compromised.

h. Incubate the resuspended beads on the bench for at least 5 minutes to elute the purified DNA from the beads.

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

j. When supernatant has cleared completely, carefully transfer 23 µl of DNA eluate to new labelled PCR tube. The transferred supernatant contains the purified PB product.

Note: *Transfer of some beads with the purified PB product will not inhibit library amplification.*

SAFE STOPPING POINT

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

8. Library Amplification

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

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

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

Fill-in:	72°C for 10 minutes
Initial denaturation:	95°C for 3 minutes
12 Cycles of:	98°C for 30 seconds
	64°C for 15 seconds
	72°C for 30 seconds
1 Cycle of:	72°C for 3 minutes
	4°C hold

SAFE STOPPING POINT

Proceed immediately to the next step, or freeze the amplified library at -20°C.

9. Library Purification

Following library amplification, it is necessary to remove residual primers and, in some cases, shorter 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 thus it is necessary to measure the reaction volume post-cycling.

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

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

d. Add MAGwise (see below) to the 1.5 ml LoBind tube containing 100 µl of diluted amplified library and mix thoroughly by pipetting up and down.

Table 6. Recommended purification conditions based on starting primer panel.

Primer Set	Amplicon Size	MAGWise vol. equivalent	MAGWise Volume
V3 (IDT, NEB)	380-400 bp	0.9 – 1.0	90-100 µl
V4 (IDT), VarSkip Short (NEB)	540-560 bp	0.85-0.9	85-90 µl
Midnight (IDT)	1200 bp	0.75-0.8	75-80 µl

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

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

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

h. Wash beads with 80% ethanol.

   i. With tube in the magnetic stand, add 300 µl of 80% ethanol without disturbing beads.
   ii. After ≥30 seconds, remove and discard supernatant, being careful not to disturb pellet.

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

i. With tube in the magnetic stand, add 300 µl of 80% ethanol without disturbing beads.
ii. Perform the next steps quickly, working 1-2 tubes at a time
    1. After ≥30 seconds, remove and discard supernatant, being careful not to disturb pellet**.**
    2. Cap tube, pulse-spin and return to magnet, letting beads settle (<30 seconds). Use a small pipet tip (≤20 µl) to remove any residual ethanol at the bottom of the tube.
    3. Add 32 µl of 10 mM Tris to bead pellet, remove from magnetic stand and pipet the solution along the inner wall of the tube multiple times to thoroughly resuspend the bead pellet. Place tube in rack on bench. **DO NOT air dry bead pellet** prior to Tris addition or the DNA recovery will be compromised.

j. Incubate for 5 minutes on the bench to elute the purified library from the magnetic beads.

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

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

SAFE STOPPING POINT

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

Expected Performance, recommended QC and sequencing

Best practice for QC is to run a fragment analysis of the final library (Bioanalyzer, Tapestation, Fragment Analyzer etc.) followed by qPCR for concentration determination. For more information on library QC methods, refer to **Appendix A** Standard plexWell 384 Library QC.

- ARTIC v3 libraries typically have an average fragment size of 400 bp which can be used for size correction for SYBR based qPCR analysis. Residual peaks of the multiplex PCR amplicons may be visible in the fragment analysis and can influence the average size when using a smear or region analysis, thus it is recommended to use a size of 400 bp.
- ARTIC v4 and NEB VarSkip short libraries typically have an average fragment size of 500 bp.
- MIDNIGHT libraries typically have an average fragment of 650-750.
- Typical library concentrations are >50 nM for a pool of 96 samples
- Recommended read configurations for an ARTIC based library are 2x76 while those for a MIDNIGHT library are 2x151
- Load the Illumina sequencer according to Illumina's recommendations.
- For surveillance sequencing purposes, we recommend >50K read pairs per sample. This equates to 96-192 samples per MiSeq (v2 300 or v3 150) flow cell or 384-768 samples per NextSeq 550 mid output flow cell.

Example Final Library traces on D5000 tapescreen analysis

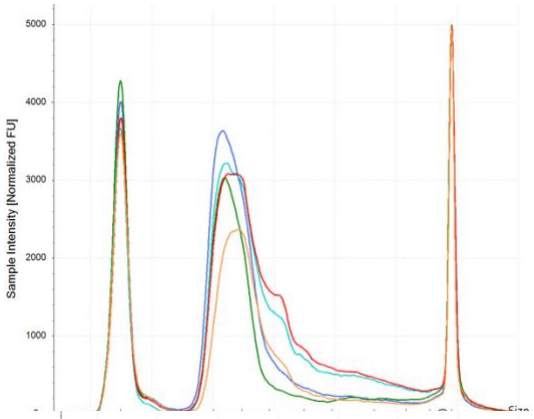


Figure 1. ARTIC v3 library fragment distribution varies depending on how the multiplex ARTIC PCRs are diluted prior to library preparation. All traces generated using D5000 tapescreen analysis (Tapestation). All dilution points generate usable library with slight variations in insert size. 1:12 dilution (green) of ARTIC multiplex amplicons resulted in a median insert of 140-150 nt; 1:10 dilution (turquoise, blue) of amplicons resulted in a median insert of 150-160 nt; 1:8 dilution (red) and 1:5 dilution (orange) results in a median insert of 170-175 nt.

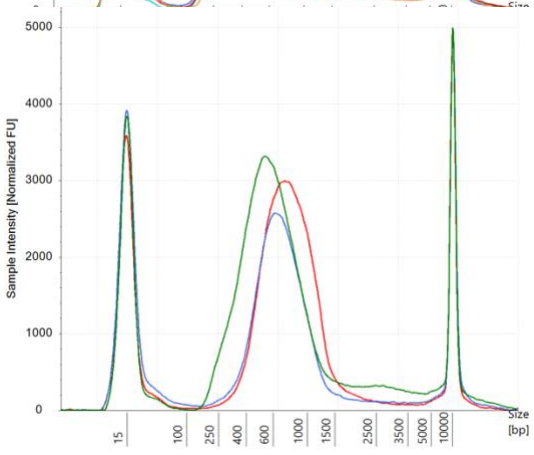


Figure 2. Midnight library fragment distribution varies depending on how the multiplex MIDNIGHT amplicons are diluted prior to library preparation. All traces generated using D5000 tapescreen analysis (Tapestation). All dilution points generate usable library with slight variations in insert size. 1:15 dilution (green) of Midnight multiplex amplicons resulted in a median insert of 285 nt; 1:10 dilution (blue) resulted in a median insert of 340-380; 1:5 dilution (red) resulted in a median insert of 430 nt.

Appendix A: Standard plexWell 96/384 Library QC

Electrophoretic analysis: Run an aliquot of purified library along with an aliquot of unpurified amplified library from Library preparation 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 High Senstitivity D5000 assay shown on next page (Figure 1). Follow the manufacturer's instructions for these instruments and dilute the library, if appropriate, prior to running. Typically PW384/96 libraries should be diluted 1:8 (unpurified library) and 1:12 (final purified library) prior to loading them on a Tapestation High Sensitivity D5000 assay. For optimal sequencing results, use a region analysis for fragments of 200-1500 bp to determine the average cluster-able fragment length for size adjustment with SYBR based qPCR. These conditions may need to be adjusted based on the starting primer panel chosen. If instruments mentioned above are unavailable, run 3 µl of purified library and 5 µl of unpurified, diluted, library on an 2% Agarose E-gel EX alongside the 1 kb plus ladder (NEB) to determine the median fragment length.

qPCR assay: Use 2 µl of purified library for qPCR analysis. Follow kit and instrument documentation for appropriate conditions and dilutions. For KAPA Library Quantification kits, prepare a 1/100,000 dilution of the libraries. Use the average fragment size as determined by electrophoresis to calculate the library concentration.

Sequencer Loading and Read Configuration

Refer to Illumina technical documentation for specific instructions on denaturation and dilution of purified library for your Illumina sequencing system. plexWell 384/96 libraries are dual indexed using 8 nt indices. Each pool contains 96 sample-specific i7 indices and a single pool-specific i5 index. Refer to Appendix E for index sequences. These libraries should be sequenced as dual index (R1, i7, i5, R2) if sequencing more than 96 samples on a single run. However, if only a single pool is being sequenced, they can be run as a single index (R1, i7, R2). The libraries are sequenced using the same primers as Nextera® libraries.[3] For information regarding setting up a run configuration, refer to Appendix D and Illumina technical documentation.

[3] The sequencing primers provided in TruSeq v3 Cluster kits are incompatible with Nextera-style libraries, including plexWell libraries. The TruSeq Dual Index Sequencing Primer Box from Illumina is required for sequencing plexWell libraries on older systems, such as the HiSeq 2500, HiSeq 2000, HiSeq 1500, GA*I*x, and HiScanSQ.

Appendix B: Guidelines for library prep in smaller batches (<96 samples):

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

Appendix C: PW384 Kit Indices

The most up-to-date list of indices for all plexWell kits is available in the Resources section for this product. Click on the "All-plexWell index list" to download an excel workbook containing all i7 indices (in list by row and column formats as well as in plate layout) and i5 indices. The All-plexWell index list is the easiest way to copy and paste index sequences into sample sheets or for demultiplexing.

i7 indices for SBX plates

	1	2	3	4	5	6	7	8	9	10	11	12
A	ACTCACCG	GGCTCCTA	GTTGACAG	CCATTGCG	TACAGAGT	GTTCGTCT	ACGAAGCG	CAGAGTGG	ATGGAACA	CATCTTCT	TCCTCAGA	TTCCATTC
B	CCTTATGT	CAGAAGAA	AATGTGCC	TTCACACT	CTTGTTGG	CCAGGTAA	CTCTCAGG	TTGGCTGC	CTAACAAC	ACATCCTT	ACGCTGCA	CTAAGGCG
C	ATAGATCC	CAGGAAGG	AAGTACCT	ATGGTCCG	TGTAAGAC	CACAGTCT	CACCGCAA	GATGAGAA	CCATACTC	ACACAACA	CGATGGCA	GTTATCGA
D	GGAGCTAT	CGTCTGAA	CGACTAGC	TCCTATCT	CTGGTCGT	TGGTACAG	TGCTCCGT	ATGACACC	TCCTTGGC	CAGGCCAT	CAACCGTG	TGGACAAC
E	TGGTGACT	ACTCGAAT	GTTAAGCA	CACATGGT	CTCGTACA	AACGCTTG	CGAGCATT	TGTTGCAC	TCACTCAC	CAACTCCG	TCAACTGA	CTATTCCA
F	CCGAGTTA	GTACCAGC	AACCAATC	GGTGTGAC	CGTAATTC	ATTCCGTA	ACCGTTCC	ATTCTCCA	CAGGCTTC	ACCGACCA	CAAGTAGT	CTGCGAAC
G	TGGTGGAA	ACTTCAAC	TCTATTGG	CCACAATG	ATTCGCAG	CGCTCTTG	TCAAGGAT	CGCAACAG	CCTACACA	GTGCGAGT	GTGTCCAT	GCCAGTGT
H	CTGTACGC	CCTGTTAC	TGAATGTG	TCAGATAC	ACCTGAGC	TGAACTCT	CAAGTGAC	CTTCTGGC	CGCGTGAT	ATGCCGCT	CTAGCCGA	GTGCGTTC

i5 indices for PBX Reagents

Set A			set B			set C		
PB	i5 sequence - A type sequencer	i5 sequence - B type sequencer	PB	i5 sequence - A type sequencer	i5 sequence - B type sequencer	PB	i5 sequence - A type sequencer	i5 sequence - B type sequencer
X007	CTCTCTAT	ATAGAGAG	X021	ATATATGA	TCATATAT	X048	TCCGACTA	TAGTCGGA
X060	TCTCATAT	ATATGAGA	X024	AGGAAACT	AGTTTCCT	X055	ATGGACAT	ATGTCCAT
X079	GATCATAG	CTATGATC	X038	TATGGAGG	CCTCCATA	X056	TTGCATTG	CAATGCAA
X089	CCCTATGG	CCATAGGG	X044	TGATACAT	ATGTATCA	X083	CTCAAATA	TATTTGAG

Set D			set E			set F		
PB	i5 sequence - A type sequencer	i5 sequence - B type sequencer	PB	i5 sequence - A type sequencer	i5 sequence - B type sequencer	PB	i5 sequence - A type sequencer	i5 sequence - B type sequencer
X003	TAATTACT	AGTAATTA	X116	GACCTAAG	CTTAGGTC	X169	TTGACCGA	TCGGTCAA
X149	TCTAAGAG	CTCTTAGA	X140	TCCCTAAA	TTTAGGGA	X173	AAGGGAAA	TTTCCCTT
X184	CCACCCTA	TAGGGTGG	X153	AAATAGAT	ATCTATTT	X181	CACATGGA	TCCATGTG
X193	TGCTGTAG	CTACAGCA	X178	CATAGGAA	TTCCTATG	X207	CGACGTTG	CAACGTCG

Appendix D: 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 C 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)

Revision History

Version	Release Date	Prior Version	Description of changes
20220110	20220110	20211220	● Corrected volumes on page 10
20211220	20211220	20210625	● Combined ARTIC protocol and existing PW96/384 workflow into one document

Technical Assistance

For additional questions or technical assistance, contact seqWell Technical Support.

Email: support@seqwell.com

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