plexWell Rapid Single Cell RNA Library Prep Kit

for Illumina® Sequencing Platforms

(Part Nos. PWSCR384, PWSCR96)

User Guide

v20211111

Introduction

plexWell Rapid Single Cell RNA Libray Prep Kits enable the construction of 96 sequencing-ready transcriptome libraries in a single day, without the need for specialized equipment. Improvements to the Smart-seq2 method[1] allow for combined and shortened cDNA synthesis and amplification to generate full-length cDNAs from single or multiple eukaryotic cells, or from 2 pg to 10 ng of purified, total RNA. The unique features of the plexWell technology further simplify the process by reducing the effort required to QC and dilute cDNA samples prior to library prep, and collapses 96 cDNAs into a single, multiplexed library. This eliminates major bottlenecks in single-cell transcriptome projects, and significantly reduces overall project costs.

plexWell utilizes proprietary transposase-based reagents to insert barcoded adapters directly into input cDNA in two separate steps. In the first barcoding step, a unique i7-barcoded adapter[2] is inserted into each of the 96 cDNA samples in segregated reactions. Next, the i7-barcoded cDNA samples are pooled into a single tube. In the second barcoding step, a single i5-barcoded adapter is inserted into each pool of cDNA samples. Finally, each 96-plex library is amplified in a single PCR reaction using universal library primers, making for a highly efficient multiplexed library prep workflow (see Workflow Diagram).

Each kit contains sufficient reagents to prepare dual-indexed Illumina-compatible libraries from 384 or 96 samples. The 384 sample kit comes in versions A, B, & C to allow users to multi-plex up to 1152 samples, if required. plexWell libraries are compatible with the Illumina iSeq, MiniSeq, MiSeq, NextSeq, HiSeq and NovaSeq systems.[3]

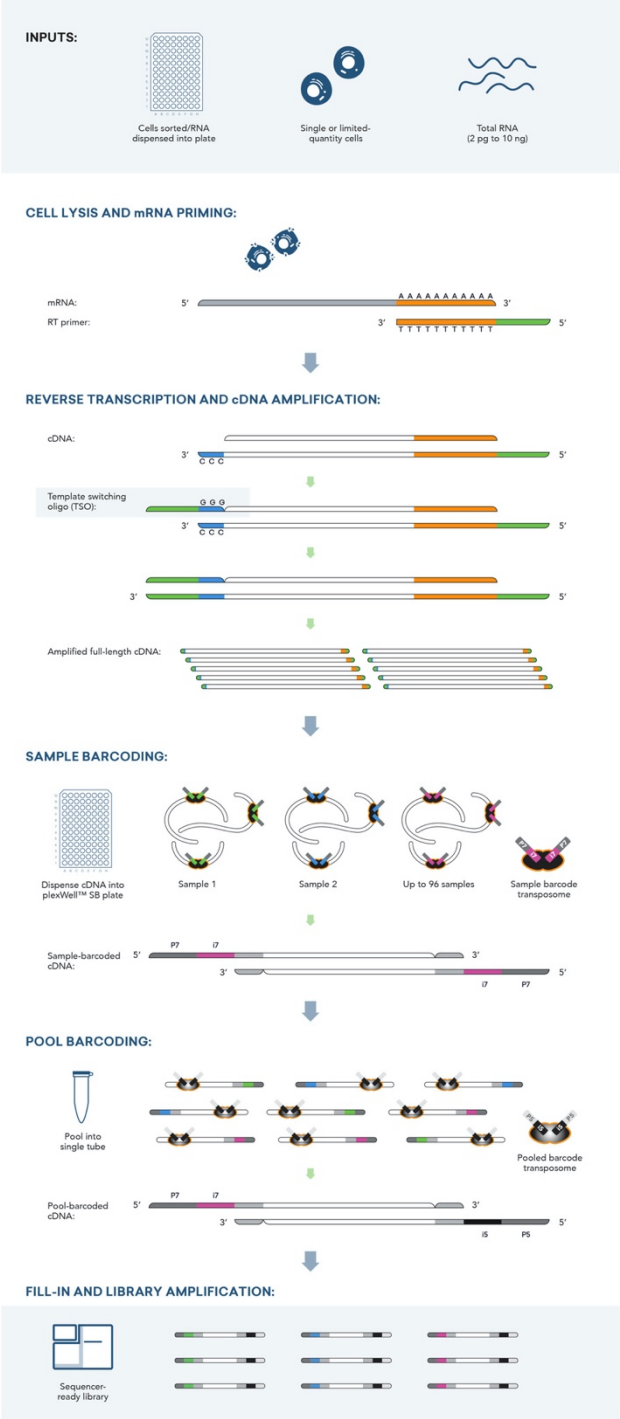
[1] Picelli S, et al. Nat. Protoc. (2014). 9: 171–181.

[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.

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

plexWell Rapid Single Cell RNA Library Prep

Molecular Diagram



plexWell Rapid Single Cell RNA Library Prep

Workflow Diagram

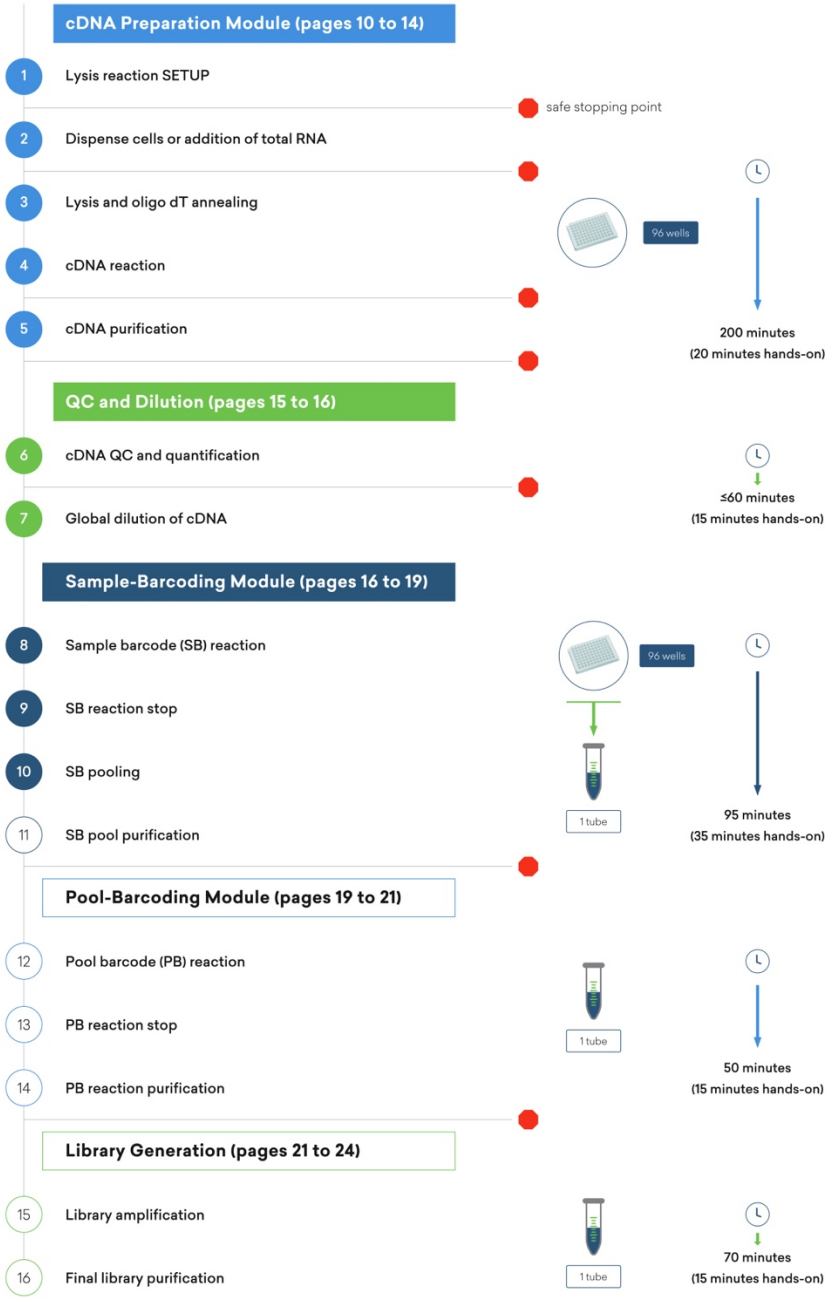


Table 1. Components of plexWell 384 Rapid Single Cell RNA Library Prep Kit

Box Ref.	Component	P/N	Description	Storage	Qty
PWSCR-1	Lysis Buffer	LYB384	2ml tube, blue cap, 895 µl	-20°C	2
	Oligo Mix	OM384	0.5 ml tube, blue cap, 465 µl	-20°C	1
	Rapid cDNA Synthesis Buffer	RSB384	2 ml tube, red cap, 1350 µl	-20°C	2
	Rapid RT	RRT 384	0.5 ml tube, red cap, 120 µl	-20°C	1
	RNase Inhibitor	RNI384	0.5 ml tube, green cap, 295 µl	-20°C	1
PWSCR-2	Sample Barcode Plate*	SBL96	Labeled 96 well PCR Plate	-20°C	4
PWSCR-3	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™	MG7500	10 ml conical transport vial, 7.5 ml	4°C	2
PWRS-4x (X-A, B, C)	Pool Barcode Reagent*	PB-Lxxx	0.5 ml tube, white cap, 10 µl	-20°C	4
	Library Primer Mix	PRM384	0.5 ml tube, natural cap, 24 µl	-20°C	1

*See Table 3 for a list of Pool Barcode Reagent indices.

Table 2. Components of plexWell 96 Rapid Single Cell RNA Library Prep Kit

Box Ref.	Component	P/N	Description	Storage	Qty
PWSCR 96-1	Lysis Buffer	LYB096	2ml tube, blue cap, 450 µl	-20°C	1
	Oligo Mix	OM096	0.5 ml tube, blue cap, 120 µl	-20°C	1
	Rapid cDNA Synthesis Buffer	RSB096	2 ml tube, red cap, 675 µl	-20°C	1
	Rapid RT	RRT096	0.5 ml tube, red cap, 30 µl	-20°C	1
	RNase Inhibitor	RNI096	0.5 ml tube, green cap, 75 µl	-20°C	1
PWSCR 96-2	Sample Barcode Plate*	SBL96	Labeled 96 well PCR Plate	-20°C	1
PWSCR 96-3	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™	MG7500	10 ml conical transport vial, 7.5 ml	4°C	1
PWSCR 96-4	Pool Barcode Reagent L007	PB-L007	0.5 ml tube, white 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 Indices

Index Set	Component	P/N	Index-A type sequencer	Index-B type sequencer
A	Pool Barcode Reagent L007	PB-L007	CTCTCTAT	ATAGAGAG
	Pool Barcode Reagent L060	PB-L060	TCTCATAT	ATATGAGA
	Pool Barcode Reagent L079	PB-L079	GATCATAG	CTATGATC
	Pool Barcode Reagent L089	PB-L089	CCCTATGG	CCATAGGG
B	Pool Barcode Reagent L021	PB-L021	ATATATGA	TCATATAT
	Pool Barcode Reagent L024	PB-L024	AGGAAACT	AGTTTCCT
	Pool Barcode Reagent L038	PB-L038	TATGGAGG	CCTCCATA
	Pool Barcode Reagent L044	PB-L044	TGATACAT	ATGTATCA
C	Pool Barcode Reagent L048	PB-L048	TCCGACTA	TAGTCGGA
	Pool Barcode Reagent L055	PB-L055	ATGGACAT	ATGTCCAT
	Pool Barcode Reagent L056	PB-L056	TTGCATTG	CAATGCAA
	Pool Barcode Reagent L083	PB-L083	CTCAAATA	TATTTGAG

Illumina Type A sequencing workflow Instruments: MiniSeq Rapid, MiSeq, HiSeq 2000/2500, and NovaSeq v1 reagents

Illumina Type B sequencing workflow Instruments: iSeq, MiniSeq, NextSeq, HiSeq 3000/4000, HiSeqX, and NovaSeq v1.5 reagents

User-Supplied Reagents, Equipment, & Consumables

Reagents

- Nuclease-free water (RNase- and DNase-free)
- 80% Ethanol (freshly prepared)
- Tris-HCl, pH 8.0
- PicoGreen® DNA assay (recommended) or other validated dsDNA quantification assay
- Agilent® High Sensitivity D5000 ScreenTape and Reagents or similar assay
- RNaseZap™ or similar cleaning agent for RNase decontamination
- Dry ice for flash freezing sorted cells in lysis buffer
- KAPA Biosystems HiFi HotStart ReadyMix PCR Kit (KK2602), now distributed by Roche (7958935001)

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 & 2.0 ml, DNA LoBind Tubes)
- 96-well thermal cycler (compatible with 96-well fully skirted PCR plates, BioRad HSP 9601)
- Magnetic stand for 1.5 ml and 2 ml tubes
- Magnetic stand for 96-well plate
- 0.2 ml PCR 8-tube strips and caps
- 96 well PCR plate and evaporation resistant seals
- 96-well, segmented, semi-skirted plates (Thermo Fisher P/N AB0900) for cDNA synthesis module
- Benchtop centrifuge to pulse-spin 1.5/2 ml tubes and PCR 8-tube strips
- Plate centrifuge
- Vortex mixer
- Agilent TapeStation® or similar instrument for analysis of cDNA and NGS libraries
- Cell sorter

Thermal Cycler Programs *(all with lid-heating set to 105°C)*

- **LYSIS**: 72°C for 10 minutes; 4°C hold
- **TAG30**: 55°C for 30 minutes; 25°C hold
- **TAG15**: 55°C for 15 minutes; 25°C hold
- **STOP:** 68°C for 10 min; 25°C hold
- **FILL_AMP12**: 72°C for 10 minutes
  95°C for 3 minutes
  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
- **cDNA**: 50°C for 30 minutes (reverse transcription)
  98°C for 3 minutes
  98°C for 20 seconds
  67°C for 20 seconds
  72°C for 3 minutes[4]
  } n cycles (See Table 4)
  4°C hold

Table 4. cDNA amplification cycles by RNA amount or anticipated RNA mass per cell

RNA Amount	Cycle number	Cell type	Cycle number
2 pg	21	PBMCs single cell	21
10 pg	19	HEK 293 single cell	19
50 pg	17	K562 single cell	19
1 ng	12	Gm12878 single cell	19
10 ng	9		

[4] Detection of very long transcripts (>15kb) is improved by increasing the elongation time from 3 minutes to 6 minutes.

Before starting procedure:

Preventing sample degradation and contamination. Always wear gloves when handling samples. To avoid RNase contamination, decontaminate the work area by wiping down work surfaces and pipettes with 0.5% sodium hypochlorite, RNaseZap™, or similar, followed by DEPC-treated water. Use only RNase- and DNase-free plasticware and water to prepare solutions. Keep reactions at recommended temperatures. To prevent contamination from the lab environment, work in a clean room with positive airflow or in a PCR hood. Use dedicated pipettes and consumables for protocol steps 1- 4.

Use of positive and negative controls: The use of a positive and negative control is encouraged during sample processing. A negative control should be generated using the RNA dilution or cell sorting fluid of choice. An ideal positive control is an intact previously characterized control RNA sample with an input range of 10 pg or greater.

Working with total RNA. This workflow accommodates up to 1 µl of 2 pg/µl to 10 ng/µl of purified, total eukaryotic RNA. Starting RNA quality impacts overall workflow performance. An RNA Integrity Number (RIN) > 8 is recommended to improve 5' coverage. To reduce the potential for degradation, RNA should be in nuclease-free water,stored at -80°C, and undergo minimal freeze-thaws. Work quickly in an RNase-free environment and keep all buffers and enzymes on ice unless otherwise indicated. If starting quantities are not limited, use the maximum amount of RNA (up to 10 ng).

Working with cells. This workflow accommodates eukaryotic cell inputs (single cells through hundreds, depending on cell type) in a volume of 1 µl of nuclease free water or PBS. When working with cultured cells, it is important to ensure the selected culture media does not impact workflow performance and/or that cells are washed in PBS prior to isolation. This workflow has been evaluated using nuclease-free water and PBS as sheath fluid options. Plant cells have not yet been evaluated with this workflow. For alternate volumes or buffers please contact support@seqwell.com.

Sample Preparation for first time users. Each kit contains sufficient reagents to prepare dual-indexed Illumina-compatible libraries from 384 or 96 cells or RNA samples. First time users should begin by processing ≤16 samples through cDNA amplification. Once the 16 samples through cDNA amplification and purification. Proceed with the remaining 80 samples once the first 16 samples have been QC'd and verified.

Batching considerations. The following protocol assumes a singlular sample type or RNA input are loaded across a 96 well plate. If different sample types or a range of RNA input

amounts are being co-processed on a single plate, please visit Appendix A for further guidance.

Program thermal cycler(s). For convenience, setup 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 **SBL96 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. Warm MAGwise beads to 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 Coding Buffer is viscous. Store **Coding Buffer (3X)** at room temperature. To transfer volumes accurately, pipette slowly and do not pre-wet pipette tips. While adding **Coding Buffer** to reactions, mix reactions 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. You will need ~30 ml per 96-well sample plate for both cDNA and Library Preparation.

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 (molecular-biology grade). You will need ~2.5 ml 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.

Procedure

cDNA Preparation

For first time users, it is recommended to process only 16 samples through steps 1-6. After cDNA QC, prepare the remaining 80 samples before starting the library prepration (step 7). To facilitate this process all master mix formulations are provided per sample, as well as for 16, 80 and 96 samples.

1. Lysis Reaction Setup

This workflow was validated using 96-well, segmented, semi-skirted plates (Thermo Fisher P/N AB0900). Use of other labware may impact cDNA yield and fragment distribution.

a. Thaw the **Lysis Buffer** and **Oligo Mix** at room temperature and move to ice once the reagents have thawed (**RNase Inhibitor** does not freeze at -20 and thus is thawed). Pulse spin to collect contents to the bottom of the tubes.

b. Prepare a Lysis Master Mix on ice with the following components:

Table 5. Lysis Master Mix. Volumes for >1 sample include a 12.5% overage.

Reagent	Per Sample	16 samples	80 samples	96 samples
Lysis Buffer	3.85 µl	69.3 µl	346.5 µl	416 µl
RNase Inhibitor	0.15 µl	2.7 µl	13.5 µl	16.2 µl
Oligo Mix	1 µl	18 µl	90 µl	108 µl

c. Mix thoroughly by vortexing or by pipetting up and down 10 times.

d. Pulse spin to collect the contents to the bottom of the tube. Distribute 5 µl of Lysis Master Mix into each well of a 96-well, segmented, semi-skirted plates (Thermo Fisher P/N AB0900).
Useful Tip for 96 samples: *Aliquot 65 µl of Lysis Master Mix into each well of an 8-well strip then distribute across the plate using a multichannel pipette.*

e. Pulse spin the plate (10 - 20 seconds) to collect the Lysis Master Mix to the bottom of each well. Store the Lysis Plate or proceed immediately to the next step.

SAFE STOPPING POINT

Proceed immediately with cell sorting or addition of total RNA, or seal the 96-well plate and store at -20°C.

2. Dispense cells or addition of total RNA

If the lysis plate was frozen, allow lysis mater mix to thaw completely at room temperature, then pulse spin the lysis plate.

The following protocol assumes a singlular sample type or RNA input are loaded across a 96 well plate. If different cell types or a range of RNA input amounts are being co-processed, please visit Appendix A for further guidance.

For cells:

Note: To improve yield, sort cells in the presence of both a positive viability indicator and cell death marker. Work quickly in an RNase-free environment and keep all buffers and enzymes on ice unless otherwise is indicated.

a. Sort cells in ≤1 µl of nuclease-free water or PBS into each well of the lysis plate.
b. Pulse spin the lysis plate to collect cells to the bottom of the wells.
c. Seal the plate and flash freeze on dry ice. Keep cells on dry ice or at -80°C until ready to proceed with lysis.

For purified, total RNA (2 pg/µl – 10 ng/µl in nuclease-free water):

Note: High quality (RIN ≥8) of total RNA is critical. Work quickly in an RNase-free environment and keep all buffers and enzymes on ice unless otherwise is indicated.

a. Distribute 1 µl of total RNA into each well of the lysis plate using a multichannel pipette. Ensure even liquid levels across tips, and flush tips to ensure a complete dispense.
b. Mix thoroughly by pipetting up and down 10 times with a multi-channel pipette set to 5 µl.
c. Seal the plate, pulse spin to collect contents to the bottom of each of the wells, and keep on ice until ready to proceed with lysis.

Note: Lysis Buffer contains a detergent, avoid bubbles when mixing

SAFE STOPPING POINT

Proceed immediately with lysis or store 96-well plates with cells or RNA in Lysis Buffer at -80°C for up to 3 months.

3. Lysis and Oligo dT Annealing

Note: Perform Lysis and Oligo dT annealing steps even if working with purified, total RNA.

a. Transfer the plate to a thermocycler and run the **LYSIS** program with lid-heating on: 72°C for 10 minutes;
4°C Hold

Useful tip: *Prepare the cDNA Master Mix in step 4 during this incubation.*

b. Remove plates from the thermocycler, immediately place on ice, pulse spin, then return to ice. Proceed immediately to **cDNA Reaction.**

4. cDNA Reaction

a. Thaw the **Rapid cDNA Synthesis Buffer** and **2X KAPA HiFi HotStart Ready Mix** at room temperature and move to ice once the reagents have thawed. Pulse spin to collect contents to the bottom of the tubes.

b. Prepare a cDNA Master Mix on ice with the following components, ensuring **Rapid RT** is added last:

Table 6. cDNA Master Mix component volumes. Volumes for 16, 80 and 96 samples include a 12.5% overage.

Reagent	Per Sample	16 samples	80 samples	96 samples
Rapid cDNA Synthesis Buffer	5.77 µl	103.9 µl	519 µl	623 µl
2X KAPA HiFi HotStart Ready Mix	12.5 µl	225 µl	1125 µl	1350 µl
RNase Inhibitor	0.48 µl	8.64 µl	43.2 µl	51.8 µl
Rapid RT	0.25 µl	4.5 µl	22.5 µl	27 µl

c. Mix thoroughly by vortexing or by pipetting up and down 10 times.

d. Pulse spin to collect the contents to the bottom of the tube.

e. Distribute 19 µl of cDNA Master Mix into each well.

Useful Tip: *Aliquot cDNA Master Mix into an 8-well strip or column of a deepwell plate by adding 245 µl to each well, then distribute across the plate using a multichannel pipette.*

f. Mix thoroughly by pipetting up and down 10 times with a multi-channel pipette set to 19 µl.

g. Seal the plate and pulse spin to collect reactions at the bottom of each well

h. Place plate in thermocycler and run the **cDNA** program, below, with lid-heating on:

50°C for 30 minutes (reverse transcription)
98°C for 3 minutes

98°C for 20 seconds
67°C for 20 seconds
72°C for 3 minutes[5]
} n cycles (See Table 7)

4°C hold

Table 7. cDNA amplification cycles by RNA input amount or anticipated RNA mass (dependent on cell type)

RNA Amount	Cycle number	cell type	Cycle number
2 pg	21	PBMCs single cell	21
10 pg	19	HEK 293 single cell	19
50 pg	17	K562 single cell	19
1 ng	12	Gm12878 single cell	19
10 ng	9		

SAFE STOPPING POINT

Proceed immediately with cDNA Purification or store amplified cDNA at -20°C for up to 7 days .

5. cDNA Purification

Note: DO NOT allow bead pellets to air dry.

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

[5] Detection of very long transcripts (>10kb) is improved by increasing the elongation time from 3 minutes to 6 minutes.

b. Remove the plate of cDNA reactions from the thermocycler or storage, pulse spin the plate, and remove the plate seal.

c. Add 20 µl (0.8 equivalents) of **MAGwise Paramagnetic Beads** to each cDNA reaction using a multichannel pipette. Ensure even liquid levels across tips, and flush tips to ensure a complete dispense. Mix thoroughly.

   Useful Tip: *Dispense 2.2 ml to a trough then distribute across the plate using a multichannel pipette. Ensure that beads don't settle in the trough by pipette mixing intermittently.*

d. Incubate on bench for ≥5 minutes to allow cDNA to bind.

e. Place the plate on a magnet and let the beads settle completely, about 2 minutes. A bead pellet should form on the inner walls of each well and the supernatant should be visibly clear.

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

g. Wash beads once with 80% ethanol.
   i. With the plate on the magnetic stand, add 200 µl of freshly prepared 80% ethanol to each well without disturbing the beads.
   ii. After ≥30 seconds, remove and discard supernatant, without disturbing the bead pellet. **DO NOT** air dry bead pellet or cDNA recovery may be compromised. Proceed immediately to the next steps through Tris addition.
   iii. Remove any residual ethanol at the bottom of the well

h. Add 20 µl of 10 mM Tris to each well. Remove the plate from the magnetic stand and pipette the solution along the inner wall of the wells multiple times to thoroughly resuspend the bead pellet.

i. Incubate at room temperature for at least 2 minutes to elute the purified cDNA off the beads.

j. Return the plate to the magnetic stand and allow beads to pellet on the inner walls of the wells (~2 minutes).

k. When the supernatant has completely cleared, carefully transfer 18 µl of cDNA eluate from each well to a fresh plate. The transferred supernatant contains the purified cDNA product

SAFE STOPPING POINT

Proceed immediately with cDNA QC or store purified cDNA at -20°C .

6. cDNA QC and Quantification

For each 96 well plate processed, spot check cDNA quality and quantity of a subset of samples on a High Sensitivity D5000 Agilent DNA ScreenTape or similar assay and PicoGreen DNA Assay, respectively.

a. Quantify cDNA from 24 wells using a PicoGreen DNA Assay (recommended) or similar assay. Expected concentrations are approximately 600 - 1400 pg/µl, though this can vary as PCR cycle numbers are modified based in input amount and cell type.

b. Evaluate cDNA from 12 wells on a High Sensitivity D5000 Agilent DNA ScreenTape or similar assay following the manufacturer's instructions. Example traces are provided below:

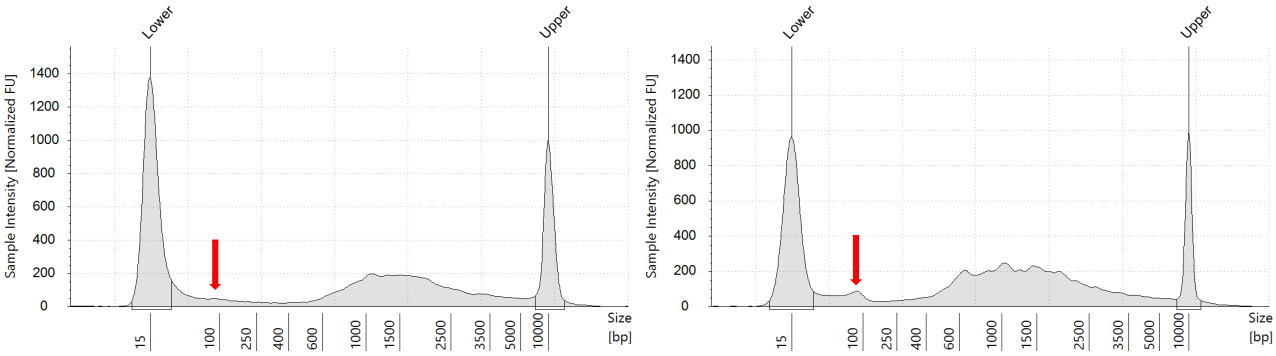


Figure 1. Example cDNA traces. cDNA traces for a single HEK293 cell (left) and 10 pg RNA input (right).

Note: After cDNA synthesis a a peak ~88 bp (primer dimer) may be visible. These short molecules are removed during downstream steps and do not negatively impact the library preparation or sequencing. Please visit Appendix B or contact support@seqwell.com for additional information.

SAFE STOPPING POINT

Proceed immediately with Library Preparation or store purified cDNA at -20°C .

Library Preparation

7. Global Dilution of cDNA

The reagents in this kit are formulated to perform optimally with an average input of 1 ng cDNA per well. Individually adjusting each sample to input 1 ng is not necessary as this kit will typically tolerate up to a 10-fold difference in sample input (300 to 3000 pg) across 96 samples when the average input is 1 ng.

If different cell types or a range of RNA input amounts are to be co-processed on a single plate, please visit Apendix A or contact support@seqwell.com for further guidance.

a. Determine the average concentration of cDNA across 24 samples using the collected PicoGreen data.

b. Determine the Global Dilution Factor using the following formula:

$$Global\ Dilution\ Factor = Average\ Concentration/0.25$$

Note: *Alternatively, seqWell's Global Dilution Calculator can be used to calculate the appropriate Global Dilution Factor.*

c. Dilute the cDNA equally across the plate using 10 mM Tri-HCl. Do not use EDTA-containing solutions (*e.g.*, TE buffer) because EDTA can inhibit enzymatic activity.

   i. Determine the volume of 10 mM Tris to dilute 4 µl of cDNA:

$$Tris\ Volume = (4 * Global\ Dilution\ Factor) - 4$$

   ii. Transfer the Tris Volume calculated above into a fresh plate.

   iii. Transfer 4 µl of each cDNA into the dilution plate, and tip mix 10 times with appropoximately half the total dilution volume

d. Store leftover diluted and undiluted cDNA at -20°C.

8. Sample-Barcode (SB) Reaction

SB reactions should be setup at room temperature. If processing more than one plate, complete the setup of one **SBL96 Plate** (through starting the thermal cycler) before proceeding to the next one.

a. Pulse-spin the **SBL96 Plate**; then remove the seal carefully.

b. Transfer 4 µl of diluted cDNA (from step 7c-iii) to each well (one sample per well) of the **SBL96 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 **Coding Buffer (3X)** to each well of the **SBL96 Plate**, using new pipette tips for each transfer. Mix thoroughly and slowly by pipetting up and down 10 times at 4 µl, being careful not to introduce excessive bubbles.

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

d. Seal the **SBL96 Plate**, pulse-spin, then transfer to a thermal cycler, and run the **TAG30** program, below, with lid-heating on:

   55°C for 30 minutes;
   25°C Hold

9. 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. Remove the **SBL96 Plate** from thermal cycler, pulse-spin and then remove the seal.

c. Add 6 µl of **X Solution** to each well of the **SBL96 Plate**. Pipette up and down slowly 10 times to mix. Change pipette tips for each addition.

   Useful Tip: *Aliquot 85 µl of* **X Solution** *into each well of an 8-strip tube, then use a multichannel pipette to transfer 6 µl from the strip to each column of the* **SBLI96 Plate**.

d. Seal the **SBL96 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

10. SB Pooling (within plate)

a. Pulse-spin the **SBL96 Plate** and then remove the seal.

b. Transfer 9 µl of stopped SB reactions from columns 1-12 into an 8-well strip tube. ***Do NOT pool samples from different SBL96 plates together***!

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

c. Transfer entire contents (95-108 µl) from each well of the strip tube into a 2 ml 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.

11. SB Pool Purification

a. Vortex (or vigorously pipette) *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 ≥5 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 before continuing.

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.
   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.
ii. Perform the next steps quickly, working 1-2 tubes at a time to prevent the beads from drying out. . **DO NOT air dry bead pellet** prior to Tris addition or the DNA recovery will be compromised.
    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 pipette 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 pipette the solution along the inner wall of the tube multiple times to thoroughly resuspend the bead pellet. Place tube in rack on bench.

g. Incubate the resuspended beads at room temperature for 15 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 eluate to a PCR tube. The transferred eluate contains the purified SB reaction pool.

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

SAFE STOPPING POINT

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

12. Pool Barcode (PB) Reaction Setup

a. Add 5 µl of **PB Reagent** to each purified SB reaction pool. Pipette 5 times 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 each PCR tube containing the purified SB pool. Vortex 5 seconds, or pipett ≥10 times at ≥ 50 µl, to thoroughly mix. Pulse-fuge.

c. Pulse-fuge the PCR tube containing the PB reaction then transfer to a thermal cycler, and run the **TAG15** program, below, with lid heating on:

55°C for 15 minutes;
25°C Hold

13. PB Reaction Stop

a. Pulse-spin the PB reactions, then add 33 µl of **X Solution** to each PB reaction. Mix thoroughly by pipetting slowly 10 times.

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

68°C for 10 minutes;
25°C Hold

14. PB Reaction Purification

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

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 ≥5 minutes to allow DNA to bind.

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

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 use a smaller one to remove the remaining supernatant.*

f. Wash beads with 80% ethanol.

i. With tubes in the magnetic stand, add 400 µl of 80% ethanol to each 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 tubes in the magnetic stand, add 400 µl of 80% ethanol to each without disturbing beads.
      1. Perform the next steps quickly, working 1-2 tubes at a time. **DO NOT air dry bead pellet** prior to Tris addition or the DNA recovery will be compromised.
      2. After ≥30 seconds, remove and discard supernatant, being careful not to disturb pellet**.**
      3. Cap tube, pulse-spin and return to magnet, letting beads settle (<30 seconds). Use a small pipette tip (≤20 µl) to remove any residual ethanol at the bottom of the tube.
      4. Add 24 µl of 10 mM Tris to bead pellet, remove from magnetic stand and pipette the solution along the inner wall of the tube multiple times to thoroughly resuspend the bead pellet. Place tube in rack on bench.

h. Incubate the resuspended beads on the bench for ≥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 from each tube into new 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 reactions at -20°C.

15. Library Amplification

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

b. Add 27 µl of 2X KAPA HiFi HotStart Ready mix to each and mix well by pipetting.

c. Close the PCR tubes, pulse-spin and run the **FILL_AMP_12** program, below, with lid heating on:

72°C for 10 minutes	
95°C for 3 minutes	
98°C for 30 seconds	12 cycles
64°C for 15 seconds	
72°C for 30 seconds	
72°C for 3 minutes (final extension)	
4°C hold	

SAFE STOPPING POINT

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

16. Final Library Purification

Following library amplification, it is necessary to remove residual primers and short library fragments.

a. After PCR, pulse-spin and transfer 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.

b. Dilute each library amplification reaction to a final volume of 205 µl with 10 mM Tris-HCl, pH 8.0 and mix thoroughly. Transfer 200 µl to new 1.5 mL LoBind tubes for purification. Set aside 5 µl of unpurified material from each for a control.

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

d. Add 160 µl (0.8 equivalents will typically remove fragments <300 bp) MAGwise to the diluted amplified library. Mix thoroughly.

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

f. Transfer the 1.5 ml tubes to a magnetic stand and let the beads settle completely, approximately 3 minutes. A bead pellet will form along one side

of the tubes 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 pellets.

h. Wash beads with 80% ethanol.
   i. With tubes in the magnetic stand, add 400 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 tubes in the magnetic stand, add 400 µl of 80% ethanol without disturbing beads.
      1. Perform the next steps quickly, working 1-2 tubes at a time. **DO NOT air dry bead pellet** prior to Tris addition or the DNA recovery will be compromised.
      2. After ≥30 seconds, remove and discard supernatant, being careful not to disturb pellet**.**
      3. Cap tube, pulse-spin and return to magnet, letting beads settle (<30 seconds). Use a small pipette tip (≤20 µl) to remove any residual ethanol at the bottom of the tube.
      4. Add 32 µl of 10 mM Tris to bead pellet, remove from magnetic stand and pipette the solution along the inner wall of the tube multiple times to thoroughly resuspend the bead pellet. Place tube in rack on bench.

j. Incubate for 5 minutes on the bench to elute the purified library from the 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 28 µl of DNA eluate, containing the purified, multiplexed library, to a new 1.5 ml LoBind tube. The remaining 4 µl 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 and Sequencing

Electrophoretic analysis: An 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) can be used following the manufacturer's instructions for these instruments. For optimal sequencing results, use a region analysis for fragments of 300-1300 bp to determine the average cluster-able fragment length for size adjustment with SYBR based qPCR. See Figure 2 (below) for a representative trace for purified libraries run on the TapeStation. Typically the recovered fragments range from 250 bp to 2000 bp.

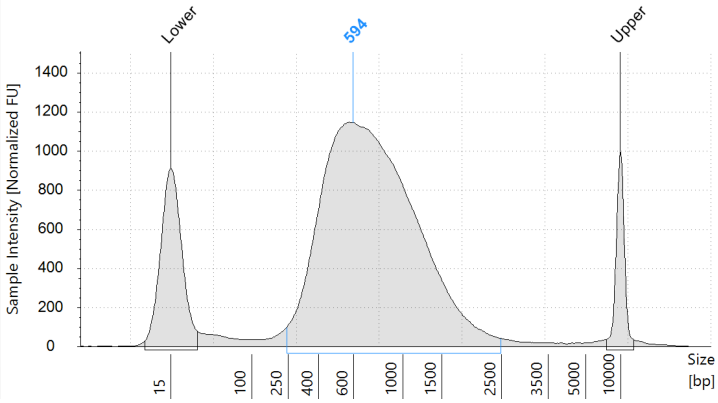


Figure 2. Representative final library electropherogram. Trace was generated using the TapeStation with a High Sensistivity D5000 kit, with average size 802 bp.

qPCR assay: Use 2 µl of each 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. The expected library concentration is >20 nM.

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-soecific i5 index. Refer to Appendix E for index sequences. These libraries should be sequenced as dual index (R1, i7, i5, R2) if sequencing more than 96 samples on a single run. However, if only a single pool is being sequenced, they can be run as a single index (R1, i7, R2). The libararies are sequenced using the same primers as Nextera® libraries. [6] For information regarding setting up a run configuration, refer to Appendix E and Illumina technical documentation.

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

Appendix A: Processing of samples from mixed types or sources

cDNA synthesis module:

Multiple sample types or a range of RNA input amounts can be co-processed. Check the tables to see if they will require a different number of PCR cycles during cDNA synthesis. If so, it's recommended to set the samples up on different plates starting with cell lysis. Proceed to cDNA synthesis in different plates by using the appropriate number of PCR cycles for each condition. cDNA concentrations may vary between different cell types. During cDNA QC, quantify a subset of cDNA from each type and apply a per-sample-type dilution factor to each cell type or RNA input amount on each plate. Careful determination of cDNA concentration minimizes library prep's variation and leads to high sequencing library quality.

plexWell module:

The plexWell module (conversion of cDNA to a multiplex Illumina library) requires an average input per well of 1 ng of purified cDNA. As such, when multiple sample types were processed through cDNA generation, it is recommended to apply a global dilution for each sample type (to an average concentraton of 0.25 ng/µl) then transferring the diluted samples to a single SBL96 plate for library preparation.

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

cDNA Synthesis:

Adjust the master mix formulations for the correct number of samples.

plexWell module:

plexWell library preparation has been optimized to work with 96 samples. When processing fewer samples, the read count CV and performance may deviate from the kit specifications. When possible, use replicates to fill in a full 96 samples. In cases where it is not possible to process 96 wells, the following parameters should be used as best practices.

- Only pool SB reactions from wells containing input DNA
- The pooling volume and PB volumes added should be adjusted based on the number of samples being processed
    - >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 SBP/X96 Plate.

Appendix C: Cleanup of Amplified cDNA

In some cases a peak ~88 bp (primer dimer) is visible in the cDNA traces. These molecules are removed during the library preparation process and do not impact library quality or sequencing results. The traces below demonstrate varying amounts of the 88 bp peak. These samples were utilized in the library preparation workflow and sequenced. The sequencing results were equal in quality to samples that did not contain the 88 bp peak. For additional information or guidance, please contact support@seqwell.com

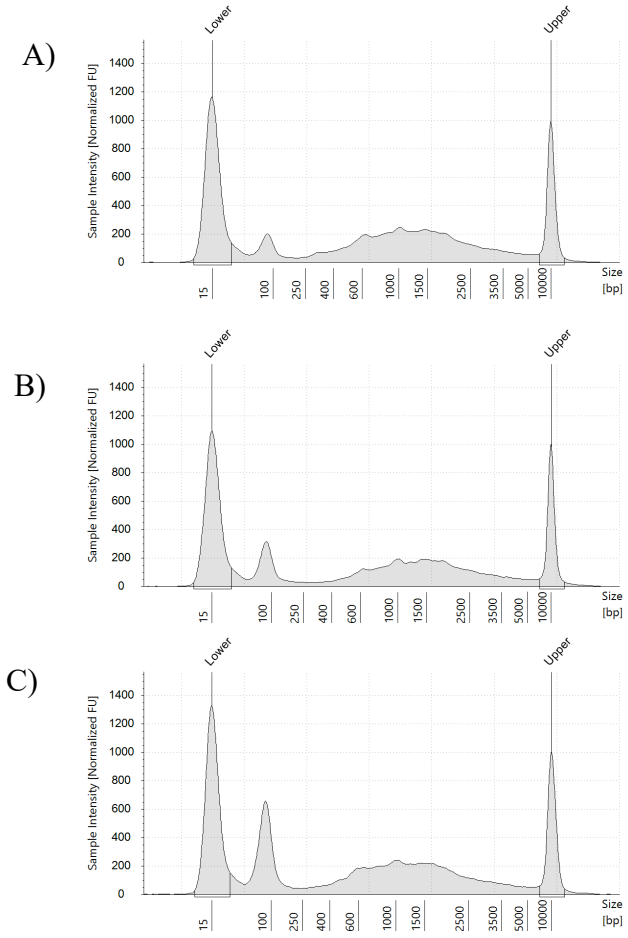


Figure 3. Varying degrees of peak's severity. Traces were generated using the TapeStation with a High Sensistivity D5000 kit for a A) low, B) moderate and C) high 88 bp peak severity.

Appendix D: Complete List of indices in PW384 kits

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 SBL plate

	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 PBL reagents

Set A		Set B		Set C	
PB-	**i5 sequence**	**PB-**	**i5 sequence**	**PB-**	**i5 sequence**
L007	CTCTCTAT	L021	ATATATGA	L048	TCCGACTA
L060	TCTCATAT	L024	AGGAAACT	L055	ATGGACAT
L079	GATCATAG	L038	TATGGAGG	L056	TTGCATTG
L089	CCCTATGG	L044	TGATACAT	L083	CTCAAATA

Appendix E: 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 D 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 Wofklow B column in the All 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
20211111	20211111	20210402	● Corrected part numbers in tables 1 and 2
20210402	20210402	20210205	● Update PB mix conditions ● Added box references
20210205	20210208	NA	● First Version

Technical Assistance

For technical assistance, contact seqWell Technical Support.

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