purePlex™ DNA Library Preparation Kit

for Illumina® Sequencing Platforms

(Part No. 301067)

User Guide

V20220128

Introduction

The purePlex™ DNA Library Preparation Kit enables the streamlined and parallel construction of up to 96 uniquely dual-indexed, sequencing-ready libraries per plate. The use of unique dual indexes (UDIs), where unique i5 and i7 indexes are used to specifically identify each library within a set, allows for the detection and removal of "hopped" reads on patterned flow cells.[1] Additionally, the workflow tolerates a wide DNA input concentration range without the need for protocol modifications and utilizes a novel DNA normalization reagent wherein individual libraries can be pooled and amplified together to reduce final QC burden. Overall, this translates to a reduction in labor and consumable costs. The purePlex kit allows for flexible batch and pool sizes (see Appendix D) and contains sufficient reagents to process and pool 96 samples.

This multiplexed library prep procedure is optimized for inputs of 5 - 50 ng of purified dsDNA per sample, and typically generates library fragment lengths ranging from 300 – 1,500 bp. Multiple DNA types are suitable input for the kit, ranging in size and complexity from amplicons to genomic DNA. purePlex libraries are compatible with all Illumina sequencers including iSeq, MiniSeq, MiSeq, NextSeq, HiSeq and NovaSeq systems.[2]

Library preparation kits from seqWell utilize proprietary transposase-based reagents to insert barcoded adapters directly into input DNA in two separate steps. In the purePlex workflow, a first barcoding step adds unique i7-barcoded adapters into up to 96 DNA samples in segregated reactions. During the second step, unique i5-barcoded transposases and normalization reagent are added to each reaction. At the end of the second step each well contains a normalized number of unique dual indexed library fragments. After a brief stop reaction, samples are pooled in batches of 24,[3] purified, and 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] https://www.illumina.com/techniques/sequencing/ngs-library-prep/multiplexing/index-hopping.html
[2] Refer to Illumina technical documentation for specific instructions on how to set up a sequencing run for a Nextera style 10 nt dual-indexed library prior to loading a plexWell library on your model of sequencing system.
[3] Refer to Appendix D for pooling in batches not equal to 24

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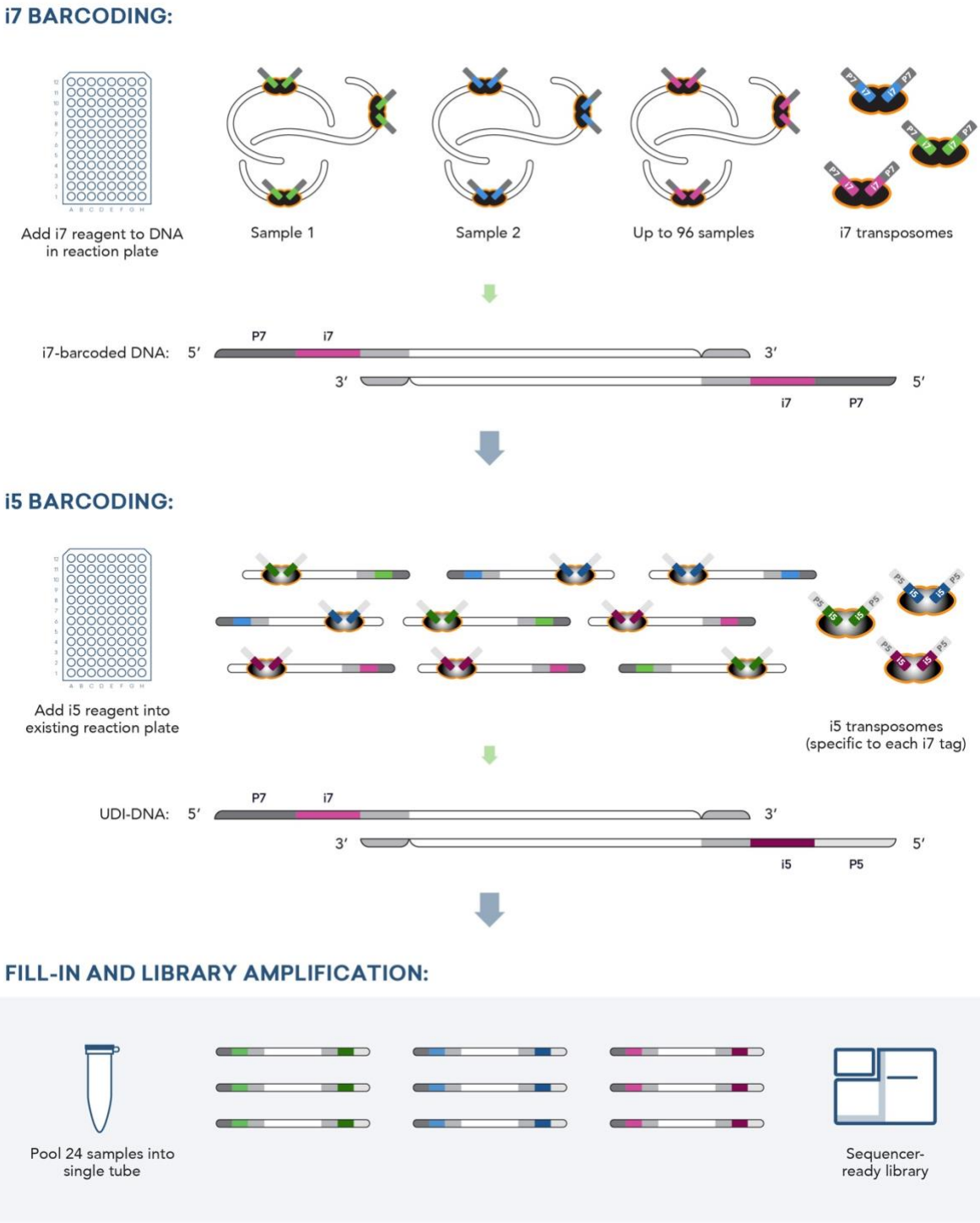
purePlex™ Library Prep - Molecular Diagram



purePlex™ Library Prep - Workflow Diagram

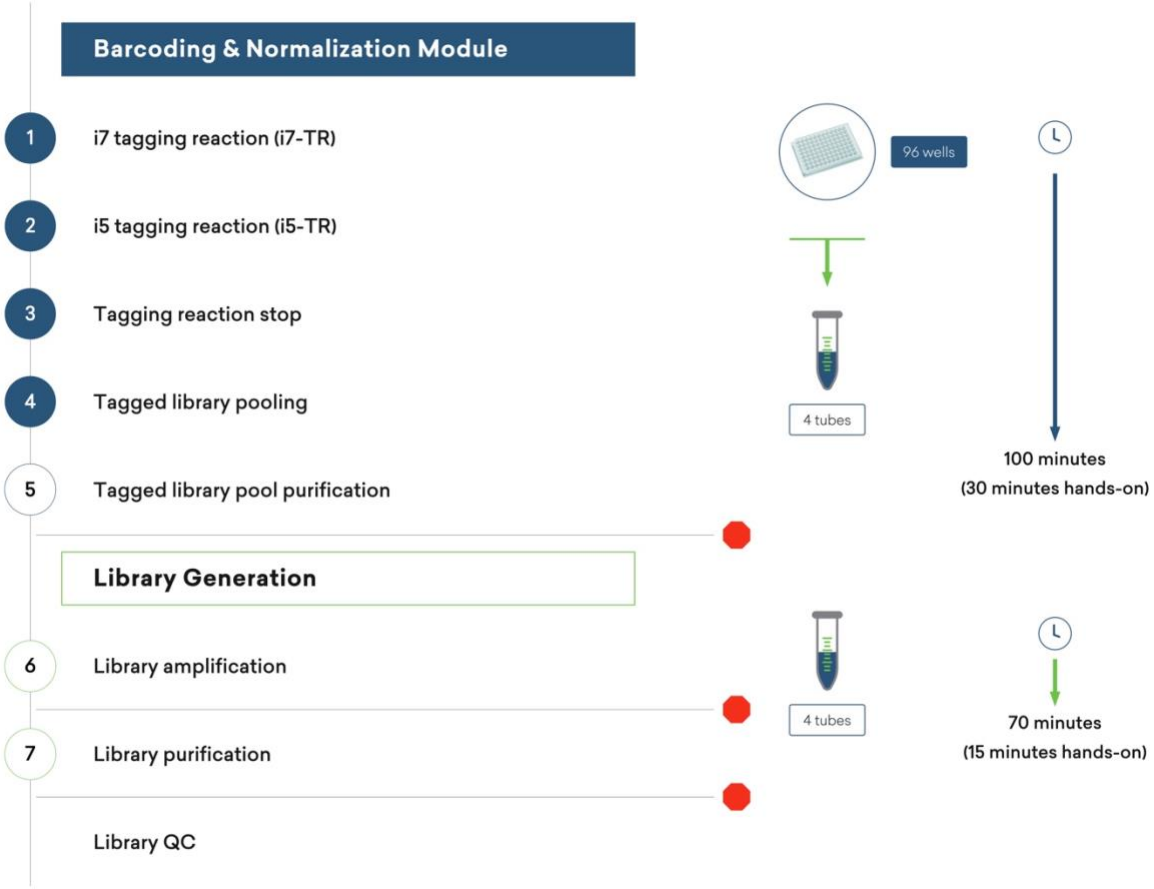


Table 1. Components of purePlex™ Library Preparation Kit

Box	Component	P/N	Description	Storage	Qty
1	**i7 Tagging Reagent Plate**	i7-TR96A	Fully skirted, low profile, 96-well PCR plate with **blue** sticker.	-20°C	1
1	**i5 Tagging Reagent Plate**	i5-TR96A	Fully skirted, low profile, 96-well PCR plate with **yellow** sticker.	-20°C	1
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	8 ml bottle, 5 ml	4°C	1
3	**Normalization Reagent**	NRM60	2 ml tube, yellow cap, 600 µl	-20°C	1
	Library Primer Mix	PRM384	0.5 ml tube, natural cap, 24 µl	-20°C	1

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

Reagents

- 80% Ethanol (freshly prepared)
- Tris-HCl, pH 8.0
- PicoGreen® DNA assay (recommended) or other validated dsDNA quantification assay
- KAPA HiFi HotStart ReadyMix (2X) (P/N: KK2602 or KK2601) for library amplification

Equipment & Consumables

- Single-channel pipettors (1-20 µl, 20-200 µl, 100-1,000 µl)
- Multi-channel pipettors (1-10 µl, 10-200 µl)
- Pipette tips (low-retention barrier tips)
- Eppendorf Tubes® 1.5 ml, DNA LoBind Tubes
- PCR plate seals (must be evaporation-resistant)
- Thermal cycler
- Magnetic stand for 1.5 ml and 2 ml tubes
- 0.2 ml PCR 8-tube strips and caps/seals
- Benchtop centrifuge to pulse-spin tubes and PCR 8-tube strips
- Plate centrifuge
- Vortex mixer

Thermal Cycler Programs (all with lid-heating on)

- **TAG**: 55°C for 15 minutes; 25°C hold.
- **STOP**: 68°C for 10 min; 25°C hold.
- **FILL_AMP8**:

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
8 cycles
72°C for 3 minutes (final extension)
4°C hold

Before starting the procedure:

Adjust input DNA concentration. Assay the DNA concentration of each sample using a PicoGreen assay. This protocol tolerates a range of 5 - 50 ng of purified genomic DNA input per sample. Before starting library preparation, adjust each input DNA concentration to be 1.0 – 10.0 ng/µl in 10 mM Tris-HCl, pH 8.0 (do not dilute input DNA with TE, or other EDTA-containing buffers).

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

Pulse-spin kit components. Liquids can condense and shift location inside containers during shipment or storage. Before opening the **i7 and i5 TR plates** 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. Equilibrate to 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). Mix gently by inversion until the precipitate dissolves **(do not vortex).** Note: **X Solution** contains SDS and will precipitate if stored below room temperature. Overly vigorous mixing will cause foaming.

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

Prepare 80% ethanol fresh daily.

Prepare 10 mM Tris-HCl, pH 8.0. Prepare 10 mM Tris-HCl, pH 8.0 from a concentrated stock solution diluted with ultrapure water (both molecular-biology/PCR grade). Do not use EDTA-containing solutions (e.g., TE) to dilute input DNA or elute DNA from beads as EDTA can inhibit enzymatic activity.

Safe-stopping points are indicated in the procedure. For optimal results, proceed directly to the next step unless an optional safe-stopping point is indicated.

Procedure

Before beginning the procedure, review the adjusting sample concentration guidelines on the previous page to ensure that each sample has been adjusted to 1.0 - 10.0 ng/µl using 10 mM Tris-HCl, pH 8.0.

The protocol herein has been validated for pools of 24 samples, such that columns 1-3, 4-6, 7-9, and 10-12 each have their own pool. If processing <96 samples at one time, take careful note of storage conditions especially for i7 and i5-TR plates. Appendix D includes tips for alternate pooling in plex sizes other than 24.

1. i7 Tagging (i7-TR) Reaction

a. Transfer 5 µl of DNA sample (1.0-10 ng/µl) to a 96-well PCR plate (the REACTION PLATE).

b. Pulse-spin the **i7-TR plate** in a centrifuge. Take care when removing seal to avoid splashing/contamination of reagents. Only remove the seal from the wells that will be used.

Useful Tip: *If processing <96 samples, use a razor blade to cut the plate seal on the i7-TR plate around the wells that will be used. Then remove only that portion of the plate seal.*

c. Next, add 5 µl from the i7 tagging reagent to the DNA samples. Carefully pipette 5 µl from the i7-TR plate and transfer to the REACTION PLATE already containing the DNA. Mix thoroughly and slowly by pipetting up and down (5 times at 5 µl), being careful not to introduce excessive bubbles. Take note of which i7-TR reagent is used for each sample.

Note: If <96 samples are being processed, reseal and store the **i7-TR plate** at -20ºC, marking on the seal what columns have been used.

d. Next, carefully pipette 5 µl of **Coding Buffer (3X)** to each well of the REACTION PLATE, using new tips for each transfer. Mix thoroughly and slowly by pipetting up and down (10 times at 5 µl), being careful not to introduce excessive bubbles.

Useful Tip*: Aliquot 70 µl of* ***Coding Buffer (3X)*** *into each well of a PCR 8-tube strip, and then use a multichannel pipettor to dispense 5 µl into the REACTION PLATE. The coding buffer can be stored in the PCR strip at room temperature for up to 4 weeks for future use.*

e. Seal the REACTION 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. i5 Tagging (i5-TR) Reaction

a. Pulse-spin the REACTION PLATE in a centrifuge and carefully remove the seal.

b. Add 5 ul of **Normalization Reagent** to each well being used in the REACTION PLATE using new tips for each transfer. Mix thoroughly and slowly by pipetting up and down (10 times at 5 µl), being careful not to introduce excessive bubbles.

***Useful Tip**: Aliquot 70 µl of **Normalization Reagent** into each well of a PCR 8-tube strip, and then use a multichannel pipettor to dispense 5 µl into the REACTION PLATE. If processing < 96 samples, store the remaining **Normalization Reagent** in the strip tube at -20ºC for up to 4 weeks.*

c. Pulse-spin the i5-TR plate in a centrifuge. Take care when removing seal to avoid splashing/contamination of reagents. Only remove the seal from the wells that will be used.

***Useful Tip:** If processing <96 samples, use a razor blade to cut the plate seal on the i5-TR plate around the wells that will be used. Then remove only that portion of the plate seal.*

d. Transfer 5 ul from the i5-TR plate to the REACTION PLATE well that used the i7-TR from the same well. Use new tips for each transfer. Mix thoroughly and slowly by pipetting up and down (10 times at 5 µl), being careful not to introduce excessive bubbles.

Note: The unique dual index i7+i5 combinations have been specifically paired, thus the i7-TR in A01 should ONLY be used with the i5-TR in A01.

Note: If less than 96 samples are being processed, reseal and store the i5-TR plate at -20°C, marking on the seal which columns have been used.

e. Seal the REACTION 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

3. Tagging Reaction Stop

a. Add 12.5 µl of **X Solution** to each well of the REACTION PLATE. Pipette up and down slowly 10 times to mix, being careful not to introduce excessive bubbles. Use clean tips for each addition of **X Solution**.

***Useful Tip*:** *Aliquot 160 µl of* **X Solution** *into a PCR 8-tube strip, and then use a multichannel pipettor to transfer 12.5 µl from the strip to the REACTION PLATE and mix. The **X Solution** can be stored in the PCR strip at room temperature for up to 4 weeks for future use.*

b. Securely reseal and pulse-spin the REACTION PLATE, then transfer to a thermal cycler and run the STOP program, below, with lid heating on:

68°C for 10 minutes
25°C hold

4. Tagged Library Pooling

a. Pulse-spin the REACTION PLATE and then remove seal.

b. For every 24 samples being processed, set out and label a 1.5 ml LoBind tube. Label tubes as A-D if processing 96 samples. for each pool of 24 samples as A-D.

Note: *See Appendix D for recommendations for plex sizes other than 24-plexes.*

c. Transfer 32 µl of stopped tagging reaction from every well of Cols 1-3 into tube A. Repeat the process for the samples in columns 4-6 to tube B, columns 7-9 to tube C, and columns 10-12 to tube D, such that you have 24 samples per LoBind tube.

Useful Tip: *Pool down each column into a row of a deep well or midi plate using a multi-channel pipet then transfer to a LoBind tube.*

d. The total volume of the pool in each tube will be approximately 768 µl.

5. Tagged Library Pool Purification

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

b. Add 576 µl (0.75 volume equivalent) of **MAGwise** to each Library Pool and mix thoroughly by pipetting.

c. Incubate in a tube rack for ≥5 minutes to allow the DNA to bind.

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

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

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

f. Wash the bead pellet with 80% ethanol.

i. With the tube in the magnetic stand and without disturbing the beads, add 1.5 ml of 80% ethanol. The beads should be completely covered by 80% ethanol.

ii. After 30 seconds, slowly remove and discard supernatant, being careful not to disturb the pellet.

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

   i. With the tube in the magnetic stand and without disturbing the beads, add 1.5 ml of 80% ethanol. The beads should be completely covered by 80% ethanol.

   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 48 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 tube(s) on the bench for at least 5 minutes to elute the purified tagged reaction pool from the beads.

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

j. When the supernatant has cleared completely, carefully transfer 46 µl of DNA eluate from each purification tube to a PCR tube (1 for each 24 plex). The transferred eluate contains the purified library pool.

SAFE STOPPING POINT

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

6. Library Amplification

a. Add 4 µl of **Library Primer Mix** to each PCR tube containing 46 ul of the purified 24-plex pools.

b. Add 50 µl of Kapa HiFi Hot Start ReadyMix (2X) to each and mix well by pipetting. Total PCR reaction volume is 100 ul.

c. Close the PCR tubes, pulse-spin and run the FILL_AMP8 program, below, with lid heating on:

Fill-in:	72°C for 10 min
Initial denaturation:	95°C for 3 min
8 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.

7. Library Purification

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

a. After PCR, pulse-spin the strip tubes containing the library amplification reactions.

b. Add 25 µl of 10 mM Tris to each reaction to bring the total volume up to 125 µl.

c. Transfer 120 µl of each diluted reaction to its own labelled 1.5 ml LoBind Tube.

Retain the residual ~5 µL of the unpurified diluted library amplification reactions for TapeStation QC.

d. Vortex (or vigorously pipette) room temperature **MAGwise Paramagnetic Beads** to ensure they are completely resuspended.

e. Add 90 µl (0.75x volume equivalent) of MAGwise beads to each 1.5 ml tube containing the amplified library pools. Mix thoroughly by pipetting up and down.

Useful Tip: *Aspirate beads slowly then wipe outside of pipet tip with a kimwipe to ensure accurate transfer of bead volume.*

f. Incubate in a tube rack on the bench for 5 minutes to allow the DNA to bind.

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

h. 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.

i. 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.

j. 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.

k. Incubate for 5 minutes on the bench to elute the purified 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.

SAFE STOPPING POINT

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

Library QC

Electrophoretic analysis: Run an aliquot of purified library through electrophoretic analysis such as the Agilent Bioanalyzer (High Sensitivity DNA or DNA7500 kits), Tapestation (High Sensitivity D5000 or D5000 kits), or Fragment Analyzer (High Sensitivity NGS Fragment Analysis Kit, DNF-474). The majority of library fragments should be between 300 and 1,500 bp in length. Typical results on the Tapestation High Sensitivity D5000 assay shown in Figure 1, below. Follow the manufacturer's instructions for these instruments and dilute the library, if appropriate, prior to running. Typically purePlex™ libraries are 80-100 nM. To determine the average fragment size, use a region analysis for 200-1500 bp and use this value for size adjustment with picogreen based assays or SYBR based qPCR assays for determining library concentration.

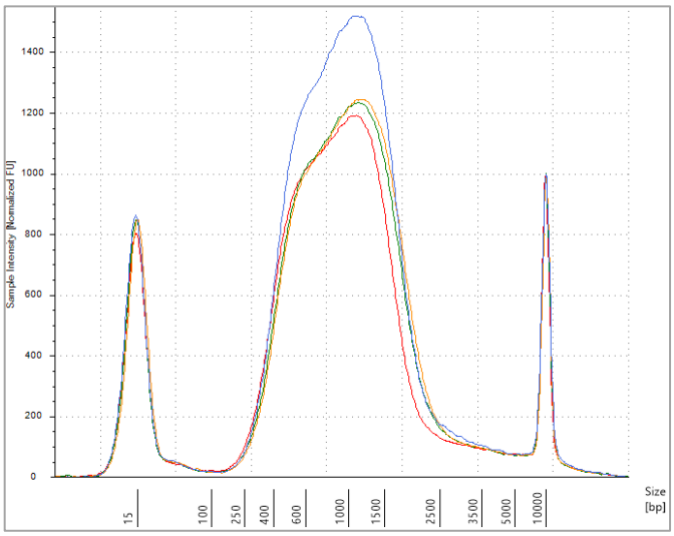


Figure 1. Representative purePlex™ library traces generated using a Tapestation 2200 with HSD5000 DNA reagents and tapescreens. The library was prepared from human genomic DNA (input range of 5-50ng) and used a final purification of 0.75 volume equivalents of MAGwise. Prior to loading on a high sensitivity D5000 tapescreen, the library was diluted 1:10. Average fragment size with 0.75X cleanups is ~800 bp using a region analysis of 200-1500).

***Note*:** purePlex library preparation kits sometimes produce fragments >1000 bp that appear to constitute a large portion of the library on instruments such as the Agilent Bioanalyzer, Tapestation, or Fragment Analyzer. However, these fragments do not appreciably contribute to the library concentration and do not cluster on standard Illumina flow cells. As such it is not necessary to remove them from the library.

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 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 during fragment analysis, above, for the size adjustment. Library concentrations are typically 80 – 100 nM.

Sequencing Loading and Read Configuration

The purePlex™ Library Prep kit libraries are dual indexed using 10 nt indices. Index lists can be found in appendix A or in an excel document that can be requested at support@seqwell.com. The protocol is optimized to generate median inserts of >300 nt to fully utilize 300 cycle Illumina kits. plexWell libraries are identical in structure, as well as adapter and sequencing primer site sequences, to Nextera® libraries. Standard Illumina sequencing primers are utilized. Refer to Illumina technical documentation for specific instructions on how to denature and dilute a purified library on your model of sequencing system[4] and for additional information on setting up the read configuration.

[4] The sequencing primers provided in TruSeq v3 Cluster kits are incompatible with Nextera-style libraries, including purePlex 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: purePlex™ index information (Set A)

The most up-to-date list of indices for all plexWell kits is available in the Resources section for each product. Click on the "purePlex™ Master index list" to download an excel workbook containing all i7 indices (in list and plate layout) and i5 indices. The purePlex master index list available on our website under *Resources > Product Information* is the easiest way to copy and paste index sequences into sample sheets or for demultiplexing.

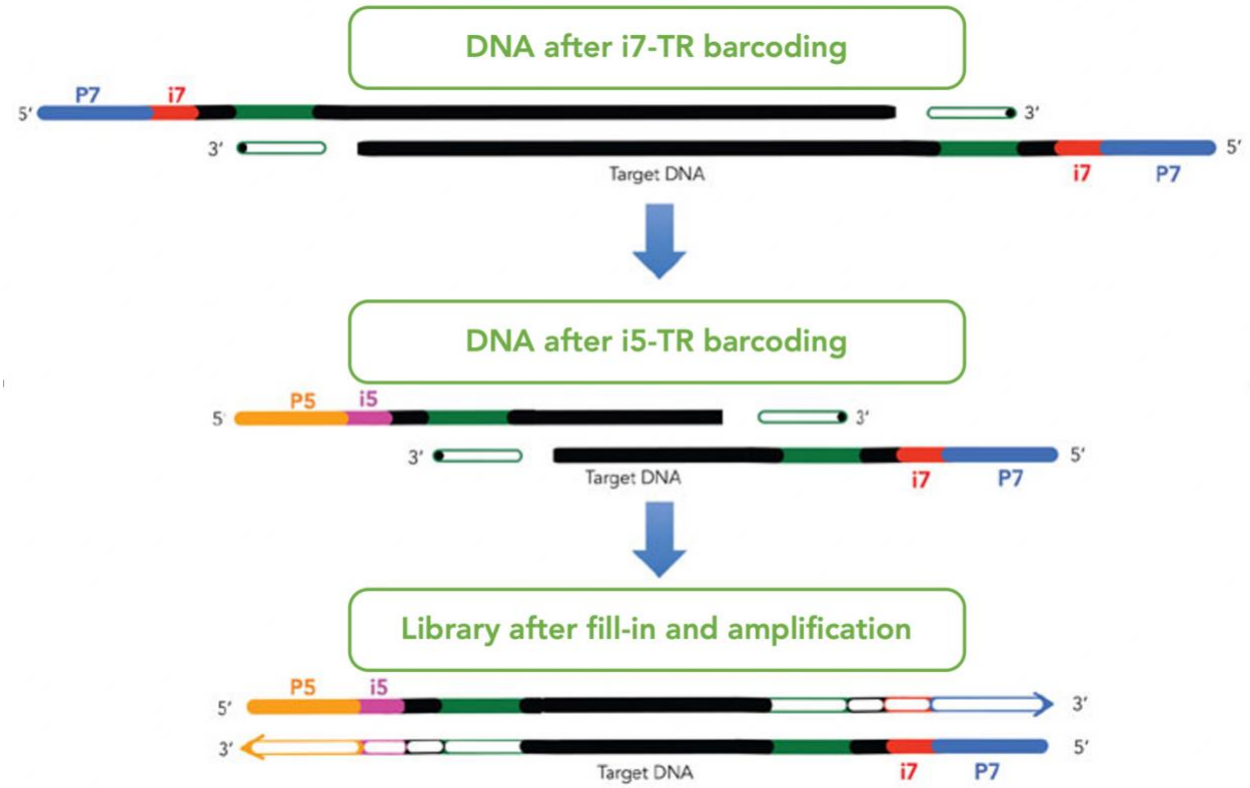
i7-TR plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	GTCAAGTCCA	CAACTAACTC	ATAACCTGAC	CAGGTACTTC	AACCGAGCCA	CAACGTCATT	ATTGGTCAGA	ATGTTGCGGA	CACCAATAAC	TGTCCGTCTT	CGAAGGACTG	TAGTTATCGC
B	TATCTCTTCC	GTACTGGATT	TGCGGTTCCA	CAGGAATATG	TGGATTCAAG	AAGAACGATG	AAGACCTGTT	AATGCTAACC	GCGTCCACAA	CATGAGTAAC	TCTACCGTCA	TGCAGGTGAT
C	CCGCGAAGAA	CATCGGAGGA	AATACTTGCC	AACGCACAAT	CAACAGATAC	CTGCAATTAC	CGCTAATGAA	GTAACACGTA	ATGTGCGCTT	CTGCGCGAAT	GGTAATATCG	TGTGAAGCTA
D	CCTACTCGGA	ATTCTGATGG	AATCGCGGAA	ATTGAGAAGG	AAGGTAACTC	CGCCGATGAT	CAGAGTGCAT	GATATACGGA	TATCTAGTGC	CTGTAGTATG	TGTGCGAGTT	CAACTCCTGA
E	TTCGTATCAC	TATCGTTACC	CAGAACGCGA	ATTGCACCTT	TGACAATACG	CGGACAAGAC	GGTAAGCTGA	ATCCGAGAGG	GCCTACAATG	GAGCCGTACA	TTATCGCTGA	TGTAGCAACG
F	TCCTCCATCC	TGGTCTGTTA	TGAACCAAGG	AAGTGGATAC	GATTGTGCAT	CGTACTCCTC	GTATTCAGTG	CAATTCACAC	AAGCTCAGTT	CATTCTTAGG	GGTTGAGTTC	CGGTAACGCA
G	ATAACATCGC	TGCCAACATG	TGTTAGTCAG	GATAAGATGC	GCACACCATT	TGCATGAGTT	GCGCTACGTT	TTGTCAGTTC	TACGCTTAGA	TGACCGACAA	ATTGGACGCC	GTAGCAGCAG
H	GATATGCGTT	TCATTACACG	CAGTAGGTAA	TACCTCGACA	TCGTTATTCC	AATCTGGAGC	TTGTCATAGC	ATACGCCATT	ATCCACTAGG	TGTGTAACCG	TATGTGTGTG	CTACAGCCGA

i5-TR plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	GTAACACAGA	CAAGAGCGTG	ATCTCCACGG	TTACTGCTGC	CCGAGGTTAG	CTGATTAGGA	TGGAGCGATG	TCATACGTTC	TTGTCCTTCC	TCTCAACAAG	CGTGATACTT	CACACAGTCG
B	TAAGTTGTGG	AATACTCGGC	CTCGCTGCTT	TTGTTCTGCG	CCGTAATCGA	CAATGCGGAG	TTCGGTGCAT	TTGTGTCCGA	GGAGTTCTAT	TCGCATGACC	TACAATCCGG	TACCATCTAG
C	CACCTACCTC	GTATCTTAGG	CTCAGTAGAC	TCGCGGACAT	CCATTCACTT	ATTCCGCTTA	CCTCTGAACA	CTTATCCAGG	ATCTTAACCG	TACGAAGCAA	TAACCATTCG	TTGTAGCATC
D	ATATCAGTCC	TGGTCTGAGG	CTTGGTCCTT	ATCGAATTGC	TCCGACTACC	CTGACAACGG	TCCACCATAG	CGGTAACTCC	GGAGCTTGTT	CCAACGCTTA	TGCTACTGTT	TTCTTCCGTA
E	GGTTAAGTAG	CAAGGACATT	CGTCCAAGAG	TGTAGGATAG	TAGAACCTGA	GCATGTTAAG	CAAGCATGAA	TAGGACCATG	GTTACAGGTT	CAGTTAGCTC	CGTCAATTCG	TCTAGCGTTG
F	TGGTGGATAT	CGGTTGAGAA	GTATGGAACA	ATGTTGCTGA	ATATGGTGCA	TCGAGAAGCG	TCAGTTGGTT	CACAATTACG	CAACCAACAT	GTATGCTAAC	TTCACGTTGC	TCTAAGTCTG
G	TAGCTGGCAC	TACCTCGCTA	AATCGGTACG	TGAGGATGAA	TTGAGTATGG	TGCAGAGTGA	CCATTATACC	AACGCGAATA	ATGTTCCGTT	GTTGAGGCAC	GGCTTGAATT	CTCATATCCA
H	TTACCGTTGG	TAAGCTCGTT	CTAATGTCGA	TGTCTTCCAA	ATTCTGGACA	TACACAACCG	AAGTGCAAGG	TCACCAGTAT	CGAGGAACAG	AACAGAACGG	GTTCCTTCTT	TGGAGGTATA

Appendix B: purePlex™ Library Intermediates



Appendix C: Sample Sheet and Sequencer Loading Guidelines

Illumina sequencing systems differ in their use of sample sheets, availability of on-instrument demultiplexing, and run setup methods. If you have questions for your specific sequencer, contact Illumina tech support for the best guidance on setting up your run using your sequencing platform.

purePlex™ libraries use the Nextera Adapter sequences and are unique dual-index libraries using 10 nt indices for both the i7 and i5 index sequences. purePlex libraries do NOT require custom sequencing primers.

All Illumina sequencers read the i7 index in the forward direction (as listed in Appendix A or excel index list). 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 and using a Workflow B sequencer, enter the reverse complement of the i5 index (provided in the excel under Workflow B).

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
- NextSeq 550
- NextSeq 1000/2000
- NovaSeq 6000 (v1.5 reagents)

Appendix D: Sample pooling for plexities ≠24

The purePlex™ library preparation kit workflow has been optimized and validated using 24 samples per pool, with columns 1-3 in pool A, columns 4-6 in pool B, columns 7-9 in pool C and columns 10-12 in pool D. However, because both the i7 and i5 tagging steps are performed on individual samples, the purePlex kit allow flexibility in pooling and batch sizes. Alternate pooling strategies can be employed and recommendations for best practices are outlined below.

Pooling >24 samples

For pooling of >24 samples, follow the user guide as written through tagging reaction stop. At step 4, tagging reaction pooling, pool all samples into a single tube. Be sure to choose an appropriate tube size to accommodate the total pool volume (32 µl per sample) plus MAGwise beads (see MAX volume in table below). After pooling all samples, add MAGwise (0.75 volume equivalents) and mix thoroughly. Divide total contents into 1.5 or 2.0 mL tubes for purification. Examples are provided below:

Total Samples	MAX volume with bead addition	# of tubes for purification	Tube type for purification	Elution Volume per pool
25-32	1792	1	2.0 mL	(N/24)*48
33-47	2632	2	1.5 mL	48
48	2688	2	1.5 mL	48
72	4032	3	1.5 mL	48
96	5376	4	1.5 mL	48

Make the following adjustments during pool purification.

- Ensure supernatant clears entirely before removal of supernatant or transfer of eluate.
- Ensure 80% ethanol washes completely cover the bead pellet
- Elute each pool as specified and transfer 2 µl less than total elution volume to its own PCR tube.

For amplification, add 4 µl of library primer mix to each PCR tube, then add 1 volume equivalent (transferred eluate volume + 4 µl) of KAPA HiFi Hotstart Readymix. Run amplification reaction.

Following the amplification, combine all reactions into a single tube. Remove 5 µl and set it aside for troubleshooting. Measure the remaining volume of combined amplified material. Add 0.75 volume equivalents of MAGwise for purification. Follow incubation times etc. for the purification and adjust Ethanol volumes to completely cover bead pellet. For ≥48 samples, increase elution volume to 50 µL.

Pooling <24 samples

In some cases, it may be desirable to pool/process <24 samples at a time. Depending on pooling size, it is recommended to user higher inputs of DNA. Specifically, for plexes between 12-23 samples, it is recommended to use 10-50 ng of input DNA per sample and for plexes of <12, it is recommended to use 25-50 ng of input DNA per sample.

Follow steps 1-3 as written in the user guide. At step 4, reaction pooling, combine the samples to be pooled into a single tube and measure volume (32 µl per sample). Add 0.75 volume equivalents of MAGwise. Follow incubation times and reagent volumes as outlined in the user guide. Continue following the user guide for steps 6 and 7 (library amplification and purification). Note that for pools of <24 samples the library yield may be lower than what is typically expected for a 24-plex pool.

For processing of <8 samples, additional PCR amplification cycles may be needed to achieve high enough concentrations for sequencer loading.

Experienced User Checklist

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

i7 Tagging Reaction

- ❑ Use a new 96-well PCR plate (REACTION PLATE)
- ❑ Add 5 µl sample to REACTION PLATE and pipette 5x
- ❑ Pipette 5 µl from i7-TR plate to each well being used of the REACTION PLATE and pipette 5x
- ❑ Add 5 µl 3X coding buffer to the REACTION PLATE and pipette 10x
- ❑ Run TAG incubation

i5 Tagging Reaction

- ❑ Add 5 µl Normalization Reagent and pipette 10x
- ❑ Pipette 5 µl from i5-TR plate (same wells used from i7-TR plate) to each well being used of the REACTION PLATE and pipette 10x
- ❑ Run TAG incubation
- ❑ Add 12.5 µl X-solution to each well of the REACTION PLATE and pipette 10x
- ❑ Run STOP incubation
- ❑ Pool 32 µl from each reaction for every 24 samples (4 tubes for 96 samples)
- ❑ Complete purification (0.75 volume equivalent), eluting with 48 µl of 10 mM Tris and transferring 46 µl to amplification tube.

Library amplification

- ❑ Add 4 µl Library Primer Mix to amplification tube
- ❑ Add 50 µl of KAPA HiFi HotStart ReadyMix and pipette to mix
- ❑ Run Fill_Amp8 program
- ❑ Dilute reaction to 125 µl with 10 mM Tris. Transfer 120 µl to purification tube. Retain remaining 5 µl for electrophoretic analysis.
- ❑ Complete library purification (0.75 volume equivalent), eluting with 32 µl of 10 mM Tris

Version	Release Date	Prior Version	Description of changes
V20220128	January 28, 2022	N/A	First version

Technical Assistance

For technical assistance, contact seqWell Technical Support.
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