User Guide

ExpressPlex™ Library Preparation Kit

Catalog numbers:
301097; ExpressPlex Library Prep Kit, 96 Reactions
301098; ExpressPlex Library Prep Kit, 384 Reactions, Set 1000
301099; ExpressPlex Library Prep Kit, 384 Reactions, Set 2000
301100; ExpressPlex Library Prep Kit, 384 Reactions, Set 3000
301101; ExpressPlex Library Prep Kit, 384 Reactions, Set 4000

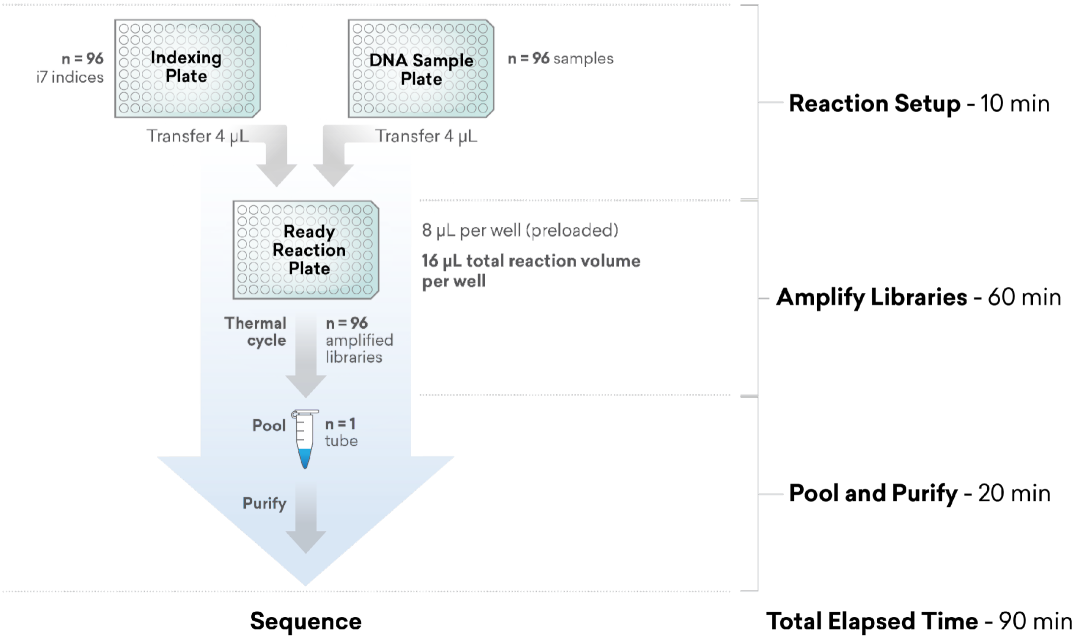


Figure 1. ExpressPlex Library Preparation Workflow Diagram for 96 plasmid or amplicon samples.

Table 1. ExpressPlex Library Preparation Kit Components (96 Reactions)

Catalog No. 301097

Item	Component	P/N	Description	Storage	Qty
1	1001-Indexing Reaction Plate	1001-IRP96	Indexing Reagent Plate (96 wells) in a fully-skirted, white PCR plate	-20° C	1
2	Ready Reaction Mix Plate	RRP96	Ready Reaction Plate (96 wells) in a fully-skirted, red PCR plate	-20° C	1
3	MAGwise paramagnetic beads*	101003	5 ml of MAGwise beads in a 10 ml screwcap tube	4° C	1

Table 2. ExpressPlex Library Preparation Kit Components (384 Reactions)

Set 1000, Catalog No. 301098

Item	Component	P/N	Description	Storage	Qty
1	Indexing Reagent Plates	1001-IRP96 1002-IRP96 1003-IRP96 1004-IRP96	Indexing Reagent Plates (96 wells) in fully-skirted, white PCR plates	-20° C	4
2	Ready Reaction Mix Plates	RRP96	Ready Reaction Plates (96 wells) in fully-skirted, red PCR plates	-20° C	4
3	MAGwise paramagnetic beads*	101003	5 ml of MAGwise beads in a 10 ml screwcap tube	4° C	1

Set 2000, Catalog No. 301099

Item	Component	P/N	Description	Storage	Qty
1	Indexing Reagent Plates	2001-IRP96 2002-IRP96 2003-IRP96 2004-IRP96	Indexing Reagent Plates (96 wells) in fully-skirted, white PCR plates	-20° C	4
2	Ready Reaction Mix Plates	RRP96	Ready Reaction Plates (96 wells) in fully-skirted, red PCR plates	-20° C	4
3	MAGwise paramagnetic beads*	101003	5 ml of MAGwise beads in a 10 ml screwcap tube	4° C	1

Set 3000, Catalog No. 301100

Item	Component	P/N	Description	Storage	Qty
1	Indexing Reagent Plates	3001-IRP96 3002-IRP96 3003-IRP96 3004-IRP96	Indexing Reagent Plates (96 wells) in fully-skirted, white PCR plates	-20° C	4
2	Ready Reaction Mix Plates	RRP96	Ready Reaction Plates (96 wells) in fully-skirted, red PCR plates	-20° C	4
3	MAGwise paramagnetic beads*	101003	5 ml of MAGwise beads in a 10 ml screwcap tube	4° C	1

Set 4000, Catalog No. 301101

Item	Component	P/N	Description	Storage	Qty
1	Indexing Reagent Plates	4001-IRP96 4002-IRP96 4003-IRP96 4004-IRP96	Indexing Reagent Plates (96 wells) in fully-skirted, white PCR plates	-20° C	4
2	Ready Reaction Mix Plates	RRP96	Ready Reaction Plates (96 wells) in fully-skirted, red PCR plates	-20° C	4
3	MAGwise paramagnetic beads*	101003	5 ml of MAGwise beads in a 10 ml screwcap tube	4° C	1

* Larger volumes (15 ml) of MAGwise paramagnetic beads are also available to purchase separately (Catalog No. 101002).

Introduction

The patent-pending ExpressPlex Library Prep Kit comes in a convenient 96-well PCR plate configuration for high-throughput multiplexed library preparation. Amplicons and plasmid DNA are suitable inputs for the kits. ExpressPlex libraries are compatible with the Illumina MiSeq, NextSeq, iSeq, and NovaSeq sequencing platforms.

Each ExpressPlex kit contains sufficient reagents to prepare Illumina-compatible libraries from 384 individual DNA samples. Libraries can be prepared from 8 – 384 samples per batch. There are four different kits available for preparing libraries from 384 samples (Catalog Nos. **301098 - 301101**), providing a total of 1,536 total barcode combinations that can be loaded on a single sequencing run. There is also an ExpressPlex kit for preparing libraries from 96 samples (Catalog No. **301097**).

This multiplexed library preparation procedure is optimized for inputs of 8 - 40 ng of plasmid or amplicon DNA per 16 µl reaction, and typically generates library fragment lengths ranging from 400 – 1,200 bp. Library fragment length will depend on the magnetic bead cleanup ratio used. The primary advantages and benefits of using the ExpressPlex library preparation kits are a streamlined one-step multiplexed library preparation workflow that tolerates variation in DNA input concentration and greatly saves on labor and consumable costs. Using the ExpressPlex kit, a 96-plex library can easily be prepared for library QC and sequencing in under 90 minutes.

ExpressPlex library preparation kits utilize a proprietary mixture of enzymes to add indexed adapters to input DNA and amplify libraries in a single step. Different i7-indexed adapters are attached to each of the 96 DNA samples and libraries are amplified in segregated amplification reactions, making for a highly efficient, one-step multiplexed library prep workflow. Each **Indexing Reaction Plate** contains a different i5 index that is applied to all samples in that plate (see Workflow Diagram in Fig. 1). Multiple plates of samples may be multiplexed in a single sequencing run provided that the user utilizes a different i5 plate for each set of 96 samples.

User-Supplied Reagents, Equipment and Consumables

Reagents

- 100% Ethanol (molecular biology grade)
- Tris-HCl, pH 8.0
- Ultrapure Water (PCR grade)
- Quant-iT™ PicoGreen™ dsDNA Assay Kits (ThermoFisher P/N: P7589), or other validated dsDNA quantification assay

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 and 2 ml, DNA LoBind Tubes)
- PCR plate seals (must be evaporation-resistant)
- 96-well thermal cycler (compatible with fully-skirted PCR plates and 8-tube PCR strips)
- Magnetic stand for 1.5 ml tubes
- 0.2 ml 8-tube PCR strips and caps/seals
- Benchtop centrifuge to pulse-spin tubes and 8-tube PCR strips
- Plate centrifuge
- Vortex mixer

Before starting the procedure:

Measure and adjust input DNA concentration. Assay the DNA concentration of each 96 well plate of samples to be processed by PicoGreen or other validated dsDNA assay. Globally adjust the average concentration of input DNA across each plate to 4 ng/µl in 10 mM Tris-HCl, pH 8.0. Do not use EDTA-containing solutions (*e.g.*, TE buffer) to dissolve or dilute input DNA because EDTA can suppress enzymatic activity. *See the Appendix for more detailed information on globally adjusting the average input DNA concentration.*

Program thermal cycler. For convenience, set-up the thermal cycler program described in the Procedure section before starting.

Pulse-spin kit components. Liquids can condense or shift locations inside containers during shipment or storage. Before using the reagent plates and 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.

Prepare 80% ethanol fresh daily.

Prepare 10 mM Tris-HCl, pH 8.0 from a concentrated stock solution diluted with ultrapure water (both molecular-biology grade). 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

1. ExpressPlex Reaction Setup

ExpressPlex reactions can be set up at room temperature. If preparing libraries from 96 samples at a time, complete the setup and thermal cycle directly in the **Ready Reaction Plate**. If preparing libraries from fewer than 96 samples, please refer to subsection 1(f) below:

a. Pre-label each **Ready Reaction Plate** with the number from the **Indexing Reagent Plate** and a name to allow easy identification of your samples.

b. Pulse-spin one **Indexing Reagent Plate** and one **Ready Reaction Plate** in a tabletop centrifuge; then remove the heat seals carefully.

c. Carefully transfer 4 µl of **Indexing Reagent** to each corresponding well of the **Ready Reaction Plate** with a multichannel pipettor, using new tips for each transfer.

d. Next, transfer 4 µl of input DNA (at approximately 4 ng/µl) to each well (one sample per well) of the **Ready Reaction Plate**. Mix thoroughly by pipetting up and down ten times at 4 µl, being careful not to introduce excessive bubbles. Use clean tips for addition of each DNA sample.

Optional: If excessive bubbles are present after reaction set-up, use a tabletop centrifuge to collapse the bubbles prior to proceeding.

e. Seal the **Ready Reaction Plate**, transfer to a thermal cycler, and run the program below, with lid-heating on:

Temperature	Duration	
55°C	5 minutes	(Tagging with indexed adapters)
75°C	5 minutes	(Heat-inactivation & fill-in)
79°C	5 minutes	
83°C	5 minutes	
98°C	3 minutes	(Initial denaturation)
98°C	15 seconds	(Library amplification):
64°C	30 seconds	**12X** PCR cycles for plasmids
72°C	1 minute	**15X** PCR cycles for amplicons
72°C	5 minutes	(Final extension)
4°C	Hold	

SAFE STOPPING POINT

Proceed immediately to the next step or store the amplified libraries at -20°C.

Special instructions for preparing libraries in batches of fewer than 96 samples (for batches of n = 8 – 88 samples):

f. Only peel the heat seal from the wells of the **Indexing Reagent Plate** and **Ready Reaction Plate** corresponding to the total number of samples that will be processed.

   Note: The **Indexing Reagent Plate** and **Ready Reaction Plate** can be thawed and refrozen up to 12 times without adversely impacting performance.

g. Follow the instructions for reaction setup in the **Reaction Ready Plate** above (steps 1a – 1d). After mixing all the reaction components and DNA together in the **Reaction Ready Plate**, transfer all the contents to a clean 8-tube PCR strip(s) or a clean PCR plate.

h. After verifying that the seals on the unused portion of the **Indexing Reagent Plate** and **Ready Reaction Plate** are still intact, cover the used wells to prevent contamination of the unused reagents when the plates are handled again. Then return the sealed/resealed plates to the freezer for later use.

i. Transfer the assembled reactions in the 8-tube PCR strip(s) or plate to the thermal cycler and use the thermal cycling program above for ExpressPlex library amplification.

SAFE STOPPING POINT

Proceed immediately to the next step or store the amplified libraries at -20°C.

2. Library Pooling

a. After library amplification, pulse-spin the **Ready Reaction Plate** (or the 8-tube PCR strips if processing fewer than 96 samples), and then open the seal/cap.

b. Using a multichannel pipette, pool 10 µl of each amplified library from each column into a single prelabeled 8-tube PCR strip.

c. After mixing by pipetting, transfer the entire volume from each well of the 8-tube PCR strip(s) into a prelabeled 2 ml LoBind tube.

   Note: After pooling, measure the total volume of pooled library by pipetting because there will be some loss due to dead volume and surface wetting.

Optional: If excessive bubbles are present after pooling, use a tabletop centrifuge to collapse the bubbles prior to proceeding. Also, you may freeze any unpurified amplified libraries remaining in the **Ready Reaction Plate**, providing an option to purify more library later if any sample(s) should require additional sequencing depth.

3. Library Pool Purification

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

b. Add 0.75 volumetric equivalent of **MAGwise** to the pooled ExpressPlex library (*e.g.*, add 720 µl of MAGwise to 960 µl of pooled library), and mix thoroughly by pipetting. Incubate on the bench for 5 minutes to allow the DNA to bind.

c. Place the tube on a magnetic stand and let the bead pellet form on one side of the tube and wait until the supernatant appears completely clear (approximately 5 minutes).

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

e. Wash beads with 80% ethanol.
   i. With the tube still in the magnetic stand, add sufficient 80% ethanol to cover the bead pellet.
   ii. After ≥30 seconds, remove and discard the supernatant being careful not to dislodge the bead pellet.

f. Repeat the previous step for a total of 2 washes with 80% ethanol. [Do not air dry the bead pellets---proceed immediately to the next step]

   Useful tip: *After using a large pipette tip to remove the waste ethanol from the second wash, briefly pulse-spin, and then use a smaller pipette tip to remove any residual volume, if visible.*

g. Immediately remove the tube from the magnetic stand and pipette 32 µl of 10 mM Tris-HCl, pH 8 on top of the bead pellet. Pipette the solution along the inner wall of the tube multiple times to thoroughly resuspend the bead pellet.

h. Incubate the tube on the bench for at least 5 minutes to elute the libraries from the beads.

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

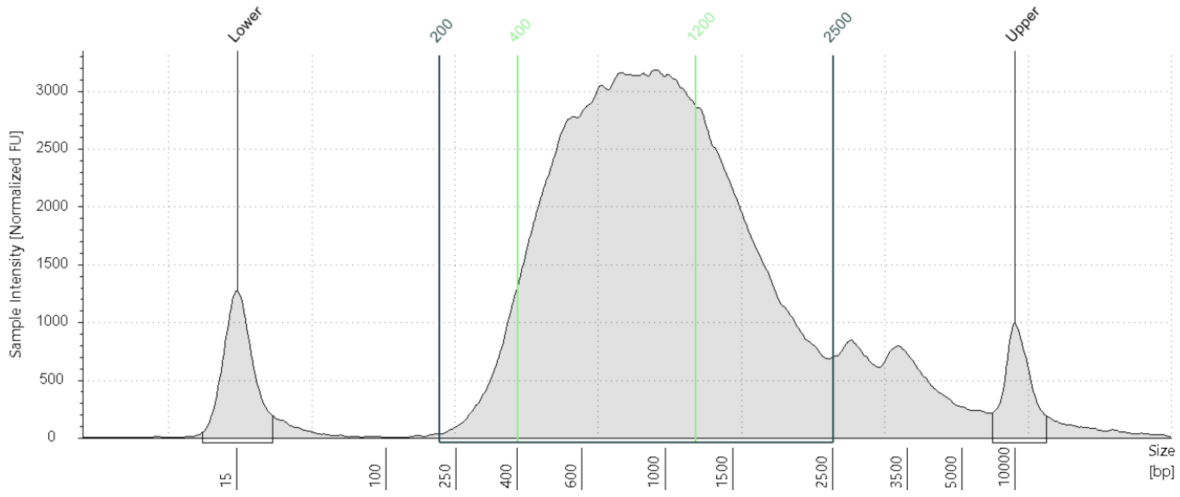
j. When the supernatant has cleared completely, carefully transfer 30 µl of DNA eluate to a clean 1.5 ml LoBind tube. The transferred eluate contains the purified pooled library.

SAFE STOPPING POINT

Proceed immediately to the next step or store the pooled purified library at -20°C.

Library QC

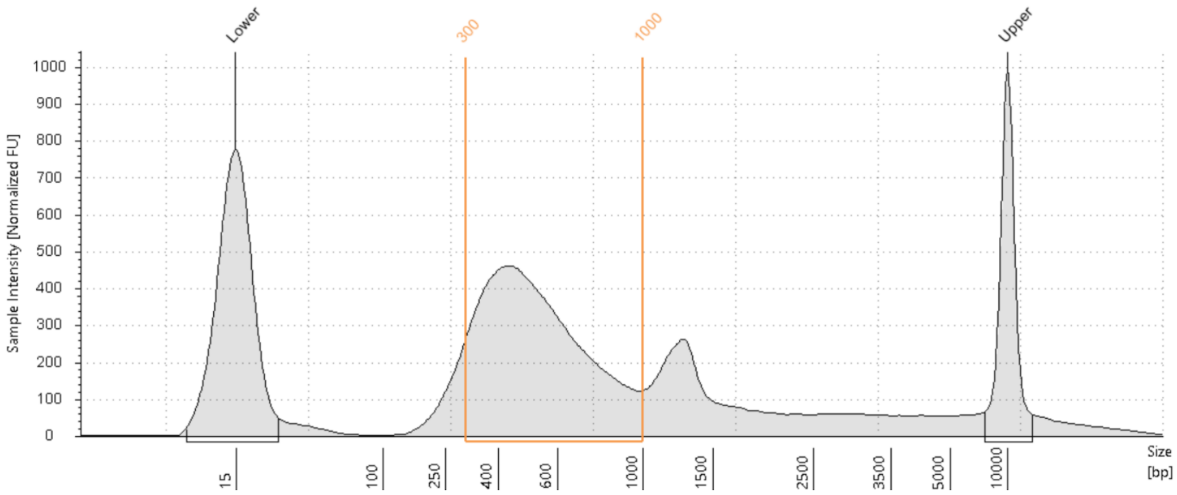
Electrophoretic analysis: Dilute the pooled, purified ExpressPlex library at least 10-fold with 10 mM Tris-HCl, pH 8 before running on the Agilent TapeStation (High Sensitivity D5000 or D5000 kits), Bioanalyzer (High Sensitivity DNA or DNA7500 kits), or Fragment Analyzer (High Sensitivity NGS Fragment Analysis Kit, DNF-474) following the manufacturer's instructions for these instruments. For optimal sequencing results with ExpressPlex plasmid libraries, use a region analysis for fragments of 400 - 1,200 bp to determine the average clusterable fragment length for size adjustment. See Figure 2 (below) for a representative trace for a pooled, purified library run on the TapeStation.



Region Table

From [bp]	To [bp]	Average Size [bp]	Conc. [pg/µl]	Region Molarity [pmol/l]	% of Total	Region Comment	Color
200	2500	1002	7700	15000	88.08	EP	
400	1200	766	5100	11200	58.42	Sequenceable Lib	

Figure 2. Representative TapeStation electropherogram of a pooled, purified 96-plex plasmid ExpressPlex library.



Region Table

From [bp]	To [bp]	Average Size [bp]	Conc. [pg/µl]	Region Molarity [pmol/l]	% of Total	Region Comment	Color
300	1000	544	692	2150	57.49		

Figure 3. Representative TapeStation electropherogram of a pooled, purified 96-plex amplicon ExpressPlex library. The library was analyzed over a region inclusive of fragment lengths between 300 and 1000 bp, to remove the contribution from untagged amplicons (i.e., the peak visible between 1,000 and 1,500 bp).

Library quantification: Use 2 µl to quantify the pooled, purified library using the PicoGreen assay. Follow the manufacturers' instructions for the appropriate dilutions. Use the average fragment size as determined by electrophoresis to calculate the library concentration and the dilution factor required for loading your sequencing system.

General library loading guidelines: If only relying on Quant-iT PicoGreen dsDNA assays to determine the library concentration, ExpressPlex libraries are typically loaded onto the sequencer at 1.5X higher concentrations than other libraries to generate optimal cluster densities on the flow cell.

> *Note:* The optimal library loading concentration can vary depending on the library fragment size distribution and whether there is a significant amount of untagged target DNA present in your final library.

Read configuration: The ExpressPlex Library Prep kit libraries are dual indexed with 10 base indices. Index lists can be found in appendix C. ExpressPlex libraries are sequenced using the same primers as standard Illumina libraries, and consequently, custom sequencing primers are not needed. Longer reads deliver greater read depth, and read lengths of 2 x 150 or greater are recommended for *de novo* assembly. The i7 and i5 index read must be 10 bases long for ExpressPlex libraries, although the non-index read lengths can be adjusted for different sequencing kits, speed or read depth requirements.

Appendix A: Adjusting the starting sample concentration

ExpressPlex library kits perform optimally with 8 - 40 ng of total dsDNA input per 16 µl reaction so individual adjustment of each sample concentration is not necessary.

If the method used to purify DNA upstream of library prep is well-characterized and generates relatively consistent concentrations of DNA per sample, it may be sufficient to only assay several samples from a 96-well plate (*i.e.,* spot-check the dsDNA concentration using a PicoGreen Assay to estimate the average DNA concentration across all samples). If all your samples already fall within the 2 – 10 ng/µl range, no adjustment is required. If, however, the average concentration of your samples exceeds 10 ng/µl, calculate the global dilution factor using the formula below:

$$\text{Global dilution factor (X)} = \frac{\text{Average assayed dsDNA concentration (ng/µl)}}{\text{4 ng/µl}}$$

The global dilution factor is applied to the input samples in a 96-well plate so that the average DNA concentration across all samples will be approximately 4 ng/µl (*i.e.,* resulting in an average of 16 ng of input being added per full ExpressPlex reaction volume).

Note: If the DNA concentration of your input samples is not easily confined to a ~5-fold range (max conc./min conc.), or if an average sample concentration of ≥2 ng/µl cannot be routinely achieved, consider optimizing the method used to generate input DNA.

Important Reminder: Do not use EDTA-containing solutions (*e.g.*, TE buffer) to dissolve or dilute input DNA because EDTA can suppress enzymatic activity.

Appendix B: Sample Sheet and Sequencer 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.

ExpressPlex adapters are similar to Nextera adapters and carry 10 base indices for both the i7 and i5 index sequences. ExpressPlex libraries do NOT require custom sequencing primers.

All Illumina sequencers read the i7 index in the forward direction (as listed in Appendix C). 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 shown in Appendix C.

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)
- NovaSeq X

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 C: ExpressPlex index information

ExpressPlex utilizes a combinatorial dual indexing (CDI) strategy: all 16 Indexing Reagent Plates have the same 96 i7 indices but each plate has a different i5 index, providing 1,536 barcode combinations in total. Please refer to the ExpressPlex index list and convenient sample sheet template on our website for a complete list of all i7 indices.

i7 Index Plate Map (Workflows A & B) for ExpressPlex

	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 Index Read

Set	Index name	i5 workflow A*	i5 workflow B*
Set A	1001	GTAACACAGA	TCTGTGTTAC
	1002	CAAGAGCGTG	CACGCTCTTG
	1003	CCGAGGTTAG	CTAACCTCGG
	1004	TGGAGCGATG	CATCGCTCCA
Set B	2001	ATCTCCACGG	CCGTGGAGAT
	2002	ATTCCGCTTA	TAAGCGGAAT
	2003	TTGTTCTGCG	CGCAGAACAA
	2004	CCTCTGAACA	TGTTCAGAGG
Set C	3001	CTGATTAGGA	TCCTAATCAG
	3002	CAATGCGGAG	CTCCGCATTG
	3003	GTATCTTAGG	CCTAAGATAC
	3004	TCGCGGACAT	ATGTCCGCGA
Set D	4001	TAAGTTGTGG	CCACAACTTA
	4002	CCGTAATCGA	TCGATTACGG
	4003	CTCAGTAGAC	GTCTACTGAG
	4004	CTTATCCAGG	CCTGGATAAG

* **Workflow A:** MiSeq, HiSeq 2000/2500, and NovaSeq 6000 (v1 reagents)
* **Workflow B:** iSeq 100, MiniSeq, NextSeq, HiSeq X/3000/4000, and NovaSeq 6000 (v1.5 reagents)

Important Note: Illumina sample sheets for on-board demultiplexing use the Workflow A sequence. The software will automatically generate the reverse complement for Workflow B sequencers.

Version	Release Date	Prior Version	Description of changes
v20230419	April 19, 2023	N/A	First version

Technical Assistance

For technical assistance with ExpressPlex Library Preparation, please contact seqWell Technical Support.
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