User Guide

ExpressPlex™ HT Library Preparation Kit
Early Access

Catalog numbers:
301140; ExpressPlex HT Library Prep Kit, 384 Reactions – Any Index Set

Introduction

The patent-pending ExpressPlex Library Prep Kit comes in a convenient 384-well PCR plate configuration for ultra-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.

Libraries should be prepared in batches of 384, with multiple batches easily prepared in parallel to allow processing of up to 1,536 samples in less than 3 hours. There are currently four different kits available for preparing libraries from 384 samples providing a total of 1,536 total barcode combinations that can be loaded on a single sequencing run. Additional barcode sets, totaling 6,144 combinations, are in development and will be available in Q4, 2023. Please inquire if more than 1,536 barcodes are needed.

This multiplexed library preparation procedure is optimized for inputs of 4 – 20 ng of plasmid or amplicon DNA per 8 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 384-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. Each 384-well plate contains 96 different i7-indexed adapters arrayed in a 4x96 grid across the plate. Each grid of 96 i7-indexed adapters contains one i5-indexed adapter, for a total of 384 combinatorial indexed samples per plate. During the single thermal cycling incubation step for 384 samples, the i7-indexed adapters are first attached to each of DNA samples; next, the i5-indexed adapters are attached, and subsequently libraries are amplified in segregated amplification reactions. This makes for a highly efficient, one-step multiplexed library prep workflow. Multiple plates of samples may be multiplexed in a single sequencing run, for a total of up to 6,144 samples, provided that the user utilizes a different plate for each set of 384 samples.

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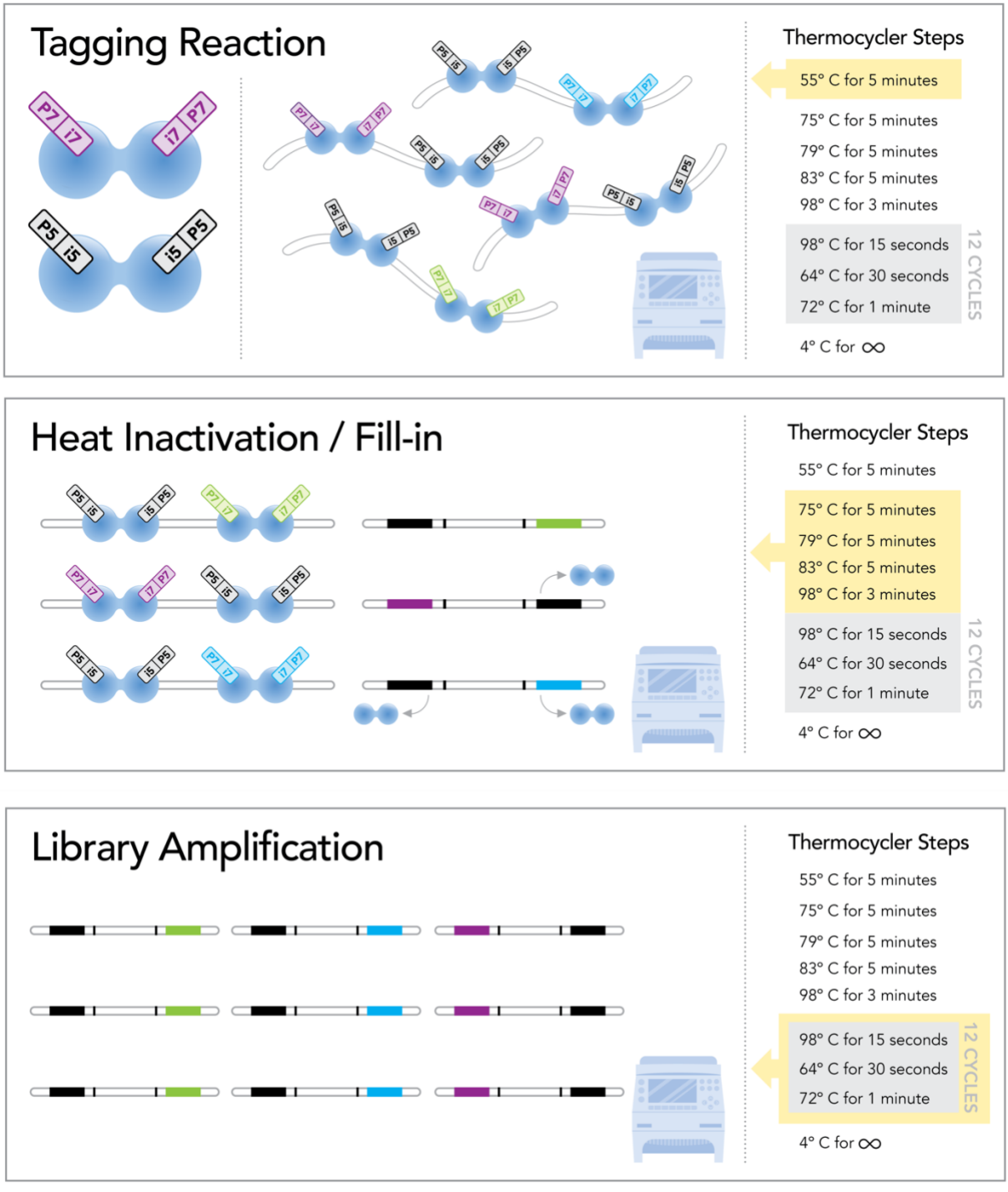
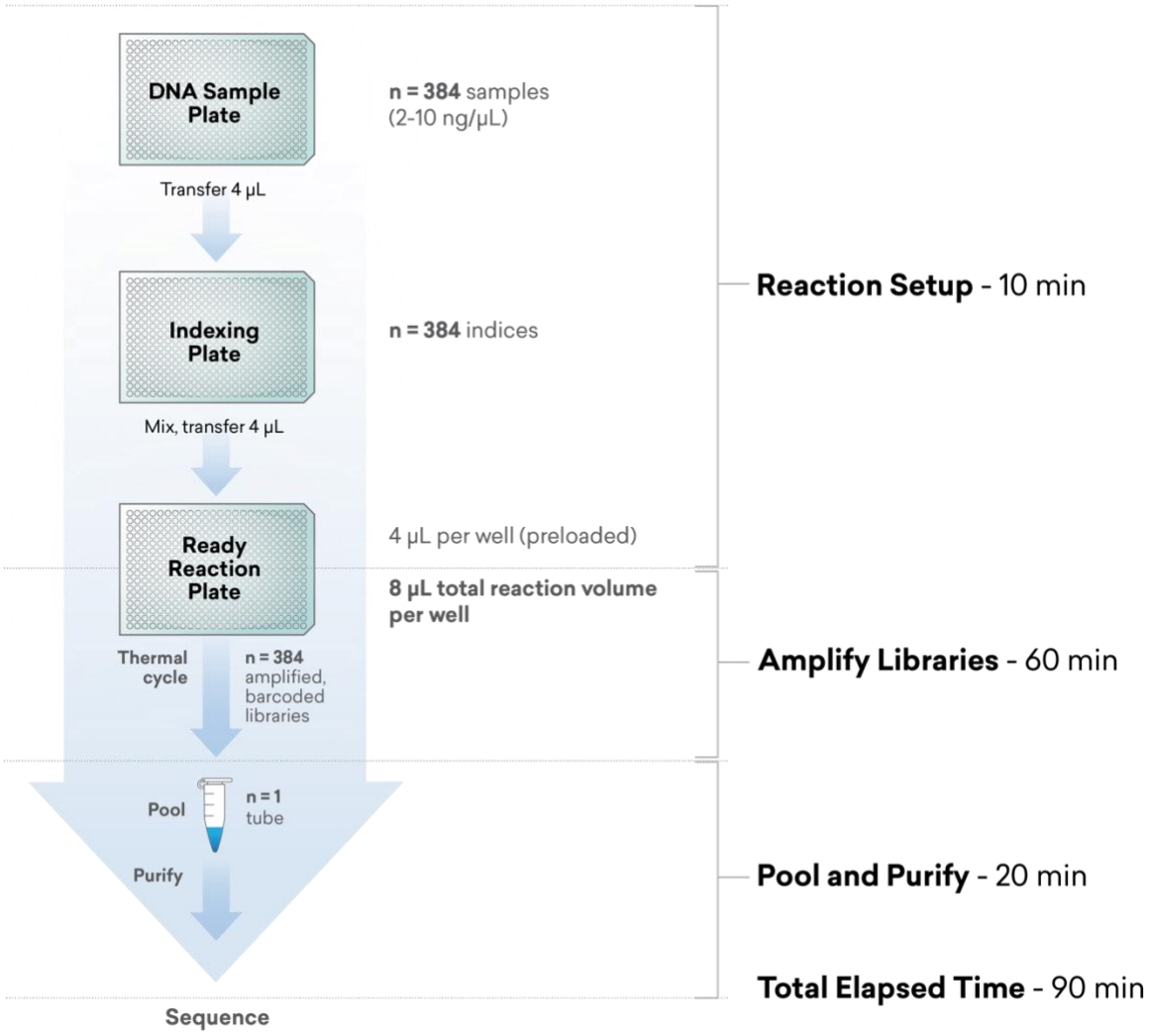


ExpressPlex™ HT Library Prep - Workflow Diagram



ExpressPlex™ HT Library Prep Components

Table 1. ExpressPlex™ HT Library Preparation Kit Components, 384 Reactions

Catalog No. **301140**

Item	Component	P/N	Description	Storage	Qty
1	Indexing Reaction Plate	X-XXXX[1]	Indexing Reagent Plate (384 wells) in a fully-skirted, yellow PCR plate	-20° C	1
2	Ready Reaction Mix Plate	RRP384	Ready Reaction Plate (384 wells) in a fully-skirted, red PCR plate	-20° C	1
3	MAGwise paramagnetic beads[2]	101003	5 ml of MAGwise beads in a 10 ml screwcap tube	4° C	1

[1]Plate P/N provided during the early access period may vary. Please confirm P/N provided matches the appropriate index list for demultiplexing.
[2]A larger 15ml bottle of MAGWise paramagnetic beads is available (P/N 101002) for purchase if desired for easier automation.

User-Supplied Reagents, Equipment and Consumables

Reagents

- 80% 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)
- Convential liquid handler with a 96- or 384-tip pipetting head
- Pipette tips (low-retention barrier tips)
- Eppendorf Tubes® (1.5 ml and 2 ml, DNA LoBind Tubes)
- PCR plate seals (must be evaporation-resistant)
- 384-well thermal cycler (compatible with fully-skirted BioRad HSP 3901 or equivalent PCR plates)
- Magnetic stand for 1.5 ml tubes
- 0.2 ml 8-tube PCR strips and caps/seals
- 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)
- Benchtop centrifuge to pulse-spin tubes and 8-tube PCR strips
- Plate centrifuge
- Vortex mixer

Thermal Cycler Program (all with lid-heating on at 105°C)

- EP_TAG_AMP:

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): **12X** PCR cycles for plasmids **15X** PCR cycles for amplicons
64°C	30 seconds	
72°C	1 minute	
72°C	5 minutes	(Final extension)
4°C	Hold	

Before starting the procedure:

Measure and adjust input DNA concentration. It is recommended that the user assay the DNA concentration of each plate of samples to be processed by PicoGreen or other validated dsDNA assay. This can be accomplished by measuring the concentration of a representative selection of samples. Globally adjust the average concentration of input DNA across each plate to 4 ng/µl (8ng calculated input into final reaction) 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 liquid handler. For assistance, contact support@seqwell.com

Program thermal cycler. For convenience, set-up the thermal cycler program described in the previous page 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. You will need ~5 ml per 384-well sample plate.

Prepare 10 mM Tris-HCl, pH 8.0. Prepare 10 mM Tris-HCl, pH 8.0 from a concentrated stock solution diluted with ultrapure water (both molecular-biology grade). Do not use EDTA-containing solutions (e.g., TE). You will need ~50 µl per 384-well sample plate.

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

Before beginning the ExpressPlex library prep, check that your average sample concentration is approximately 4 ng/µl (8ng calculated input into final reaction). This library prep kit can tolerate up to a 5-fold difference (4-20 ng) in input mass. Put in terms of concentration, this means any individual sample can be between 2 and 10 ng/µl as measured by a validated dsDNA quantification assay such as PicoGreen.

Note: the user will pipette 4 µl of DNA into the tagging reagent, resulting in a 1:1 dilution of the DNA sample prior to beginning the next step.

1. ExpressPlex HT Reaction Setup

ExpressPlex reactions can be set up at room temperature. Performing setup via automated liquid handler is highly recommended. Processing of multiple plates at one time is possible with properly programmed liquid handler platforms. However, the user should only process as many plates at one time as there are thermal cyclers available for immediate continuation of the protocol.

a. Pulse-spin one **Indexing Reagent Plate** and one **Ready Reaction Plate** in a tabletop centrifuge for 1 minute at 1000 x g.

b. Tagging Reaction Setup:
   i. Complete the setup and thermal cycling directly in the **Ready Reaction Plate**. Pre-label each **Ready Reaction Plate** with the number from the **Indexing Reagent Plate** and a name to allow easy identification of your samples.
   ii. Transfer 4 µl of input DNA to each well (one sample per well) of the **Indexing Reagent 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.
   iii. Using the same tips, immediately transfer 4 µl of the resulting **DNA/Indexing Reagent mixture** to each corresponding well of the **Ready Reaction Plate** with a multichannel pipettor. Mix thoroughly by pipetting up and down ten times at 4 µl, being careful not to introduce excessive bubbles. Note: 4 µL of unused volume will remain behind in the Indexing Plate after transfer, and should be discarded.

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

c. Seal the reaction plate, pulse-spin, then transfer to a thermal cycler, and run the EP_TAG_AMP program below, with lid-heating on at 105°C:

Temperature	Duration	
55°C	5 minutes	(Tagging with indexed adapters)
75°C	5 minutes	
79°C	5 minutes	(Heat-inactivation & fill-in)
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.

2. Library pooling

a. After library amplification, pulse-spin the reaction plate, and then remove the plate seal.

b. Using a liquid handler, pool 4 µl of each amplified library from each 96-well quadrant into a single prelabeled 96-well PCR plate. The four quadrants will be combined, 4 samples per well, in this plate.

Note: The same pipette tips may be reused for pooling because the samples are fully barcoded and amplified.

c. Using 10 µl from each combined well, proceed to pooling all samples, by column, into a separate plate or 8-tube PCR strip.

d. After mixing by pipetting, transfer the 100 µl from each well of the plate or 8-tube PCR strip(s) into a prelabeled 2 ml LoBind tube. The total volume should be approximately 800 µl.

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Optional: If excessive bubbles are present after pooling, use a tabletop centrifuge to collapse the bubbles at 1,000 x g for 1 minute 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 600 µl of MAGwise to 800 µl of pooled library), and mix thoroughly by pipetting. For the most accurate cleanup, measure the amount of pooled library via pipette prior to adding the 0.75x equivalent of MAGwise.

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

d. 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).

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

f. Wash beads with 80% ethanol.
   i. With the tube still in the magnetic stand, add 1.5 ml 80% ethanol to cover the bead pellet.
   ii. After ≥30 seconds, remove and discard the supernatant being careful not to dislodge the bead pellet.

g. 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.*

h. 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.

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

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

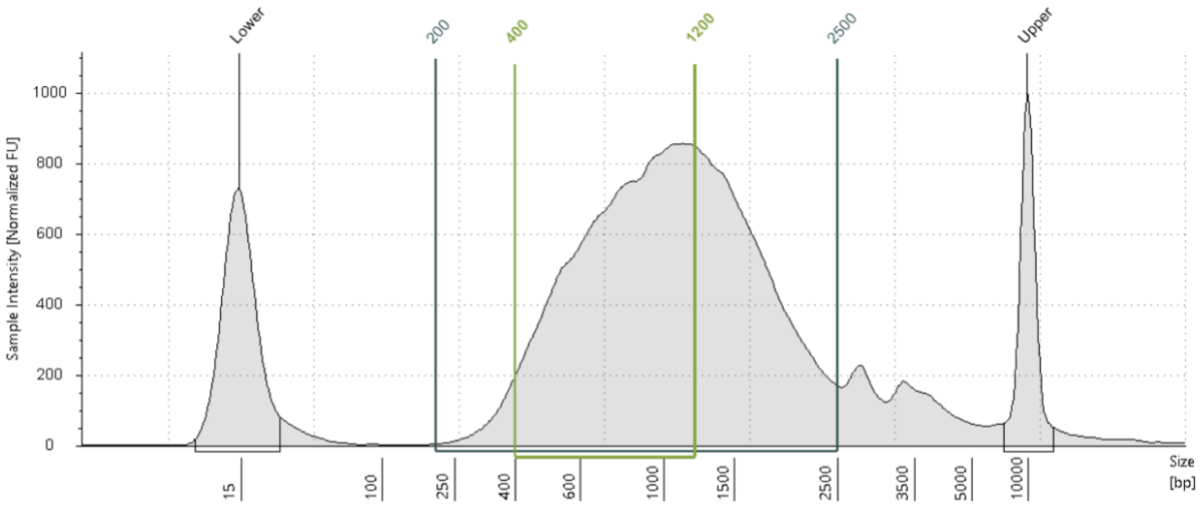
k. When the supernatant has cleared completely, carefully transfer 30 µl of DNA eluate to a clean 1.5 ml LoBind tube. The transferred eluate contains the purified pooled library.

SAFE STOPPING POINT

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

Library QC and Sequencer Loading

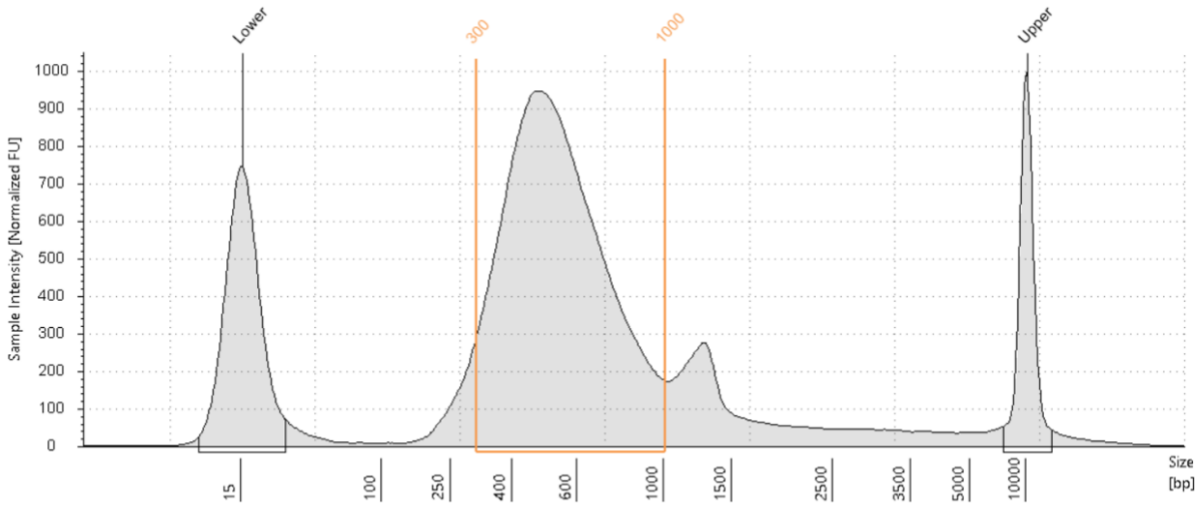
Electrophoretic analysis: Dilute the pooled, purified ExpressPlex library at least 10-fold with 10 mM Tris-HCl, pH 8 before running on the Agilent TapeStation (High Sensitivity D5000 or D5000 kits), Bioanalyzer (High Sensitivity DNA or DNA7500 kits), or Fragment Analyzer (High Sensitivity NGS Fragment 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	1112	2250	3870	88.01	EP	
400	1200	810	1360	2830	53.14		

Figure 2. Representative TapeStation electropherogram of a pooled, purified 384-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	547	1400	4250	75.20		

Figure 3. Representative TapeStation electropherogram of a pooled, purified 384-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 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)
- MiSeqf
- HiSeq® 2000/2500
- NovaSeqf 6000 (v1 reagents)
- NovaSeq X

The following Illumina sequencers use type B workflow (i5 read as reverse complement):

- iSeqf 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. Each 384-well plate contains 96 different i7-indexed adapters, each placed in 4 adjacent wells. Each placement of an individual i7-indexed adapter is combined with 4 different i5-indexed adapters, resulting in 384 CDI combinations per plate. See figure 4 below. All plates from set A (plates A-1000, A-2000, A-3000, and A-4000) contain the same 96 i7's, but utilize 16 different i5's (4 per plate) across the set to achieve 1,536 different index combinations. Each subsequent set (B, C, or D) contains a different set of 96 i7-indexed adapters than any other set, but repeats the 16 different i5-indexed adapters. If using all four sets (16 plates), 6,144 total index combinations are available to multiplex into a single run. Please refer to the ExpressPlex index list and convenient sample sheet template on our website for a complete list of all i7 and i5 indices.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	89	89
B	1	1	9	9	17	17	25	25	33	33	41	41	49	49	57	57	65	65	73	73	81	81	89	89
C	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	90	90
D	2	2	10	10	18	18	26	26	34	34	42	42	50	50	58	58	66	66	74	74	82	82	90	90
E	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	91	91
F	3	3	11	11	19	19	27	27	35	35	43	43	51	51	59	59	67	67	75	75	83	83	91	91
G	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	92	92
H	4	4	12	12	20	20	28	28	36	36	44	44	52	52	60	60	68	68	76	76	84	84	92	92
I	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	93	93
J	5	5	13	13	21	21	29	29	37	37	45	45	53	53	61	61	69	69	77	77	85	85	93	93
K	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	94	94
L	6	6	14	14	22	22	30	30	38	38	46	46	54	54	62	62	70	70	78	78	86	86	94	94
M	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	95	95
N	7	7	15	15	23	23	31	31	39	39	47	47	55	55	63	63	71	71	79	79	87	87	95	95
O	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	96	96
P	8	8	16	16	24	24	32	32	40	40	48	48	56	56	64	64	72	72	80	80	88	88	96	96

Numbers 1-96 = i7 location

i5-1	i5-2
i5-3	i5-4

Figure 3. Representative layout of the i7- and i5-indexed adapters on an individual plate.

Version	Release Date	Prior Version	Description of changes
v20231214	Dec. 14, 2023	N/A	Revised name of product from ExpressPlex 384-well Library Preparation Kit to ExpressPlex HT Library Prep Kit, revised Molecular Diagram on page 3.
v20231019	Oct. 19, 2023	N/A	First version

Technical Assistance

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