ExpressPlex™ 2.0 Library Preparation Kit – 384-well

for Illumina® Sequencing Platforms

Catalog numbers:
301141: ExpressPlex 2.0 - 384-well, 4x384 Reactions – Set A
301142: ExpressPlex 2.0 - 384-well, 4x384 Reactions – Set B
301143: ExpressPlex 2.0 - 384-well, 4x384 Reactions – Set C
301144: ExpressPlex 2.0 - 384-well, 4x384 Reactions – Set D
301152: ExpressPlex 2.0 - 384-well, 1x384 Reactions – Any Index

User Guide

v20241216

Table of Contents

Introduction

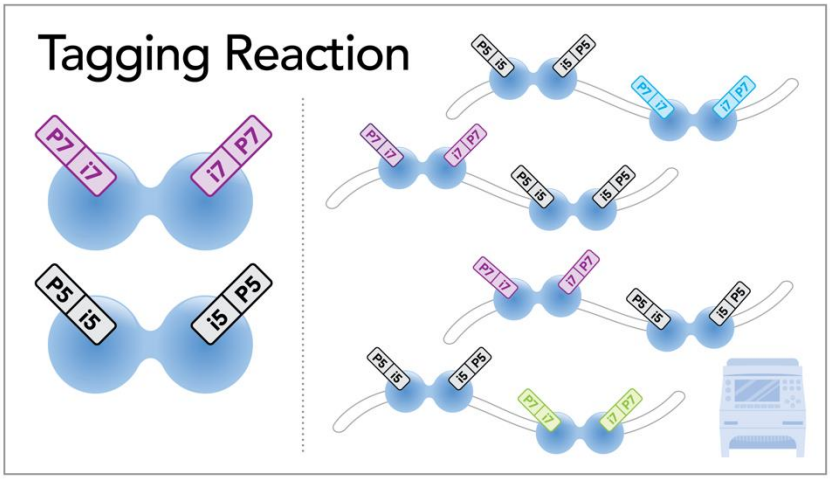
The patent-pending ExpressPlex 2.0 Library Prep Kit comes in a convenient 384-well PCR plate configuration for high-throughput multiplexed library preparation. This upgraded version of ExpressPlex uses seqWell's high performance TnX™ transposase that was specially engineered for NGS library preparation. Amplicons (>350 bp) and plasmid DNA are suitable standard inputs for the kits. Appendix E highlights modifications that can be made for small microbial whole genome sequencing. ExpressPlex libraries are compatible with the Illumina MiSeq™, NextSeq™, iSeq™, and NovaSeq™ sequencing platforms.

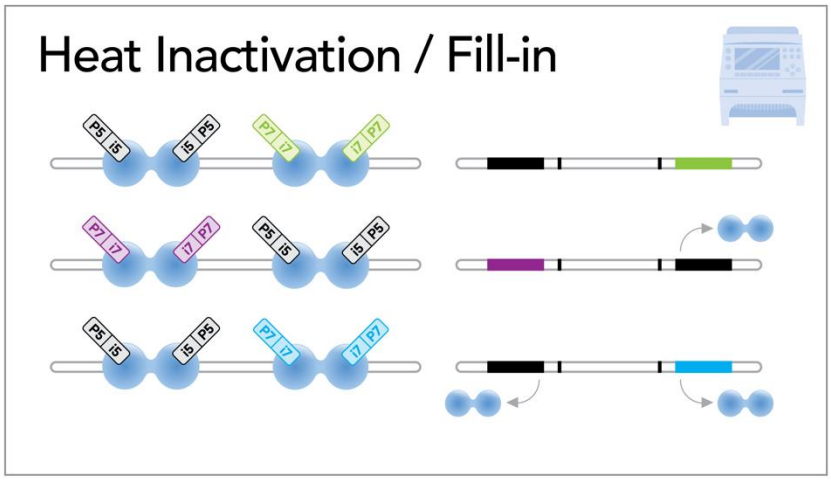
Each ExpressPlex 2.0 – 384-well kit contains sufficient reagents to prepare Illumina-compatible libraries from 384 or 1,536 individual DNA samples. Libraries are standardly prepared with 384 samples per pool, up to 1,536 samples per kit. There are four different kits available for preparing libraries from 1,536 samples, providing a total of 6,144 total barcode combinations that can be loaded on a single sequencing run.

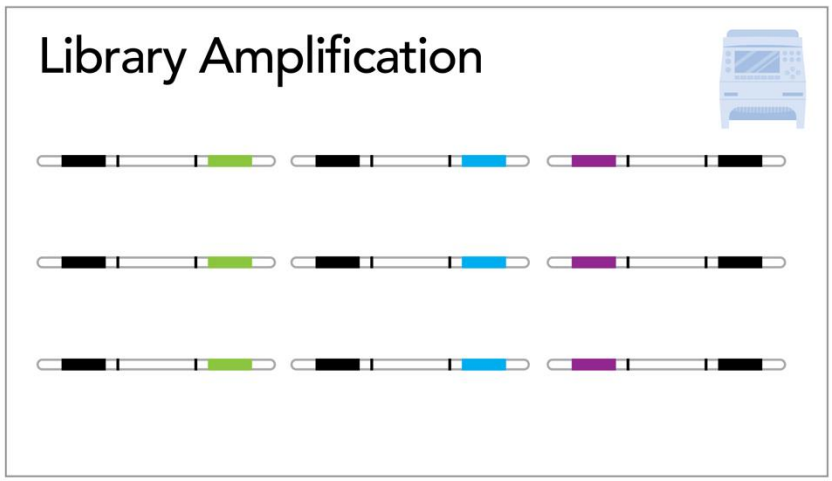
This multiplexed library preparation procedure is optimized for inputs of 0.5 - 20 ng of plasmid or amplicon DNA per 8 µl reaction, and typically generates library fragment lengths ranging from 400 – 1,200 bp. Library fragment length will depend on the quality of DNA and the magnetic bead cleanup ratio used. The primary advantages and benefits of using the ExpressPlex library preparation kits are a streamlined, one-step multiplexed library preparation workflow that automatically normalizes read output per sample across a 40-fold range of DNA input concentration, while minimizing labor and consumable costs. Using the ExpressPlex 2.0 – 384-well kit, a 384-plex library can be prepared for library QC and sequencing in under 120 minutes, with less than 30 minutes of hands-on time.

ExpressPlex 2.0 library preparation kits utilize a proprietary mixture of enzymes to tag input DNA with indexed adapters and amplify libraries all in a single reaction. 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 and i5-indexed adapters are simultaneously attached to each of the DNA samples; 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.

ExpressPlex 2.0 Molecular Diagram

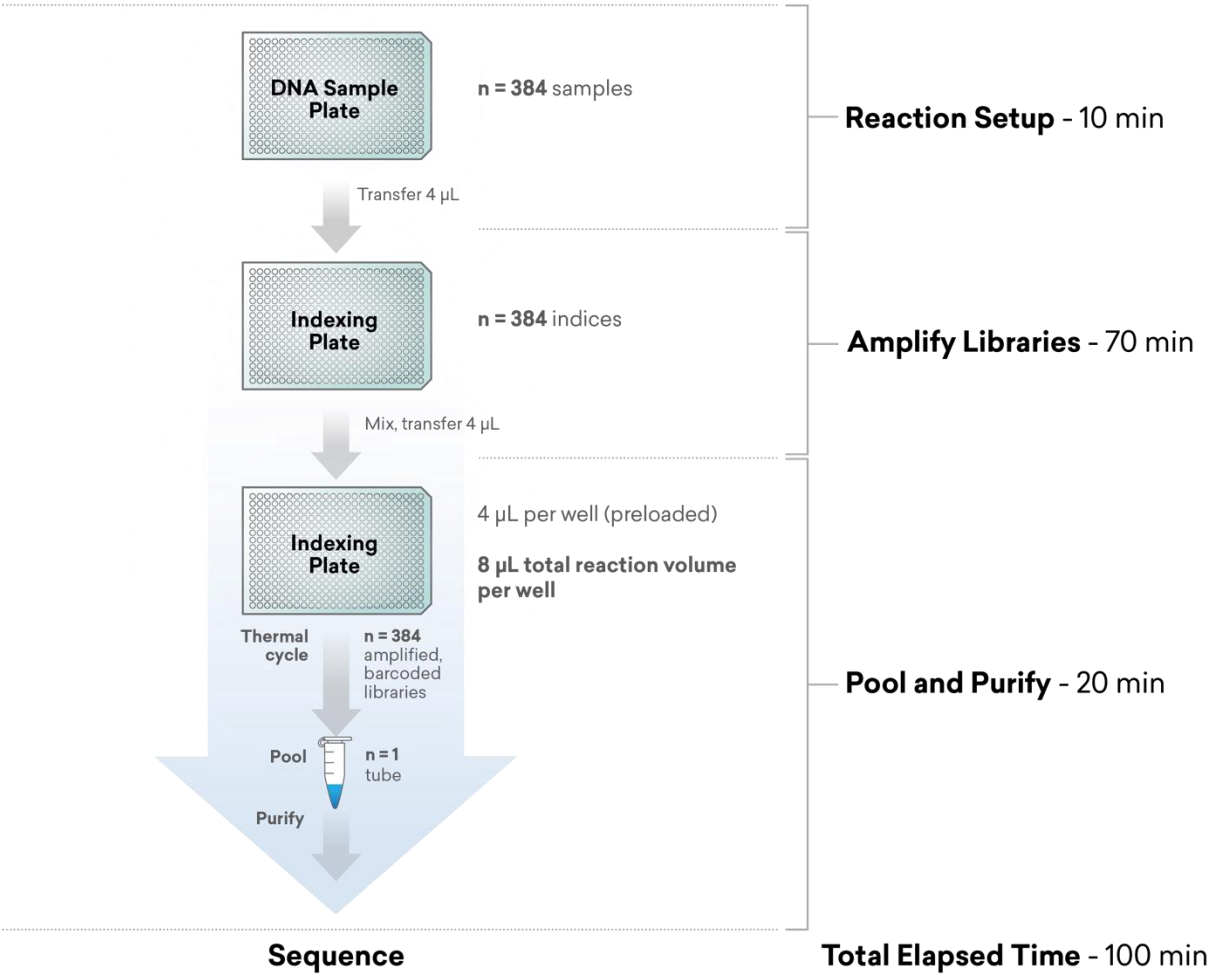






ExpressPlex 2.0– 384-well

Workflow Diagram



Kit Components

Table 1. ExpressPlex 2.0 Library Preparation Kit Components, 384-well (4X384 Reactions)

Off the Shelf – 4 x 384 - Set A:

ExpressPlex 2.0 Library Preparation Kit - 384-well, 4x384 - Set A

Catalog No. 301141

Item	Component	REF	Description	Storage	Qty
1	Box 1 - 4X384 Indexing Reaction Plates – A-1000, A-2000, A-3000, A-1000	301147	Indexing Reagent Plates (384-well) in a full-skirt, colored PCR plates	-20° C	4
2	Box 2 - 4X384 Ready Reaction Mix Plates	301151	Ready Reaction Plates (384-well) in a full-skirt, clear PCR plates	-20° C	4
3	MAGwise paramagnetic beads*	101003	5 ml of MAGwise beads in a 10 ml screwcap tube	4° C	1

Made to Order – 4x384 - Set B, C, D:

ExpressPlex 2.0 Library Preparation Kit - 384-well, 4X384 - Set B

Catalog No. 301142

Item	Component	REF	Description	Storage	Qty
1	Box 1 - 4X384 Indexing Reaction Plates – B-1000, B-2000, B-3000, B-4000	301148	Indexing Reagent Plates (384-well) in a full-skirt, colored PCR plates	-20° C	4
2	Box 2 - 4X384 Ready Reaction Mix Plates	301151	Ready Reaction Plates (384-well) in a full-skirt, clear PCR plates	-20° C	4
3	MAGwise paramagnetic beads*	101003	5 ml of MAGwise beads in a 10 ml screwcap tube	4° C	1

ExpressPlex 2.0 Library Preparation Kit - 384-well, 4x384 - Set C

Catalog No. 301143

Item	Component	REF	Description	Storage	Qty
1	Box 1 - 4X384 Indexing Reaction Plates – C-1000, C-2000, C-3000, C-4000	301149	Indexing Reagent Plates (384-well) in a full-skirt, colored PCR plates	-20° C	4
2	Box 2 - 4X384 Ready Reaction Mix Plates	301151	Ready Reaction Plates (384-well) in a full-skirt, clear PCR plates	-20° C	4
3	MAGwise paramagnetic beads*	101003	5 ml of MAGwise beads in a 10 ml screwcap tube	4° C	1

ExpressPlex 2.0 Library Preparation Kit - 384-well, 4x384 - Set D

Catalog No. 301144

Item	Component	REF	Description	Storage	Qty
1	Box 1 - 4X384 Indexing Reaction Plates – D-1000, D-2000, D-3000, D-4000	301150	Indexing Reagent Plates (384-well) in a full-skirt, colored PCR plates	-20° C	4
2	Box 2 - 4X384 Ready Reaction Mix Plates	301151	Ready Reaction Plates (384-well) in a full-skirt, clear PCR plates	-20° C	4
3	MAGwise paramagnetic beads*	101003	5 ml of MAGwise beads in a 10 ml screwcap tube	4° C	1

*A larger 15ml bottle of MAGWise paramagnetic beads is available (REF 101002) for purchase if desired for easier automation.

Table 2. ExpressPlex 2.0 Library Preparation Kit Components, 384-well (1x384 Reactions)

Off the Shelf – 1x384 - Any Index

ExpressPlex 2.0 Library Preparation Kit - 384-well, 1x384 – Any Index

Catalog No. 301152

Item	Component	REF	Description	Storage	Qty
1	Indexing Reaction Plate (any index); Ready Reaction Mix Plate	301153	Indexing Reagent Plate (384-well) in a full-skirt, colored PCR plate; Ready Reaction Plate (384-well) in a full-skirt, clear PCR plate	-20° C	1
3	MAGwise paramagnetic beads*	101003	5 ml of MAGwise beads in a 10 ml screwcap tube	4° C	1

User-Supplied Reagents, Equipment, Consumables, and Thermal Cycler Program

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
- Electrophoretic analysis reagents - 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)

Equipment & Consumables

- Single-channel pipettors (1-20 µl, 20-200 µl, 100-1,000 µl)
- Multi-channel pipettors (1-10 µl, 10-200 µl)
- Conventional 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 full-skirt BioRad HSP 3901 or equivalent PCR plates)
- 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
- Electrophoretic analysis equipment - Agilent Tapestation, Bioanalyzer, or Fragment Analyzer
- Fluorometer for dsDNA quantification assay

Thermal Cycler Program EP2_TAG_AMP (all with lid-heating on at 105°C):

15	min	55ºC	Tagging
5	min	75ºC	Fill-in/Heat-kill
3	min	68ºC	
5	min	79ºC	
3	min	68ºC	
5	min	83ºC	
1	min	98ºC	Initial denaturation
15	sec	98ºC	Amplification (PCR) **Use 12 cycles for plasmids** **Use 15 cycles for amplicons**
30	sec	64ºC	
1	min	72ºC	
5	min	72ºC	Final extension
Hold		4ºC	

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 1.25 ng/µl (2.5 ng 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). User 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

1. ExpressPlex Reaction Setup

Note: **Key Items Before Starting:**

- Performing setup via automated liquid handler is highly recommended.
- Refer to **Appendix A** for instructions to dilute input samples.
- Refer to **Appendix E** for instructions to prepare samples for other applications other than plasmids and amplicons sequencing (e.g. small bacterial genome whole-genome sequencing (WGS)).
- If processing more than one plate, multiple reaction plates may be set up and thermal cycled at the same time before proceeding to subsequent steps. (Immediate continuation after cycling is recommended)
- 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.

ExpressPlex reactions can be set up at room temperature. Performing setup via automated liquid handler is highly recommended.

If preparing libraries from fewer than 96 samples, refer to subsection 1(f) below.

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

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

c. 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.

d. Using the same tips, immediately transfer 4 µl of the resulting **DNA/Indexing Reagent mixture** to each corresponding 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. Note: 4 µL of unused volume will remain behind in the Indexing Plate after transfer. This volume should be discarded.

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

e. Seal the reaction plate, pulse-spin, then transfer to a thermal cycler, and run the **EP2_TAG_AMP** program below, with lid-heating on at 105°C:

15	min	55ºC	Tagging
5	min	75ºC	Fill-in/Heat-kill
3	min	68ºC	
5	min	79ºC	
3	min	68ºC	
5	min	83ºC	
1	min	98ºC	Initial denaturation
15	sec	98ºC	Amplification (PCR) **Use 12 cycles for plasmids** **Use 15 cycles for amplicons**
30	sec	64ºC	
1	min	72ºC	
5	min	72ºC	Final extension
Hold		4ºC	

SAFE STOPPING POINT

Proceed immediately to the next step or

store the amplified libraries at -20ºC.

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 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. Approximately 20 µl will be left behind in each well or strip tube and may be discarded.

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 -20ºC.

Library Quantification and QC

Library quantification and QC with electrophoretic analysis: Run the pooled, purified ExpressPlex 2.0 library 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 2.0 plasmid libraries, use a region analysis for fragments of **200 - 1,200 bp** to determine the average clusterable fragment length for size adjustment. For amplicon libraries, adjust the region size based on the input amplicon length to exclude unfragmented template DNA.

If using electrophoretic analysis for library quantification, use the region analysis function and calculate the library concentration from the clusterable fragment region. Note that this calculation may not be the most accurate. Alternatively, use Quant-iT™ PicoGreen™ dsDNA Assay, Qubit™ dsDNA HS Assay (see below) or other validated dsDNA quantification assay to quantify the library pool, and calculate amount of clusterable fragments by multiplying by the the percentage of sample in the region analysis (200-1200bp) for sequencer loading. For a more accurate measurement of the concentration of clusterable library fragments, use qPCR (see below).

Refer to the figures below for representative trace for pooled, purified libraries run on the Tapestation. Refer to *Appendix D* for quantification methods by Agilent Tapestation for ExpressPlex 2.0 libraries.

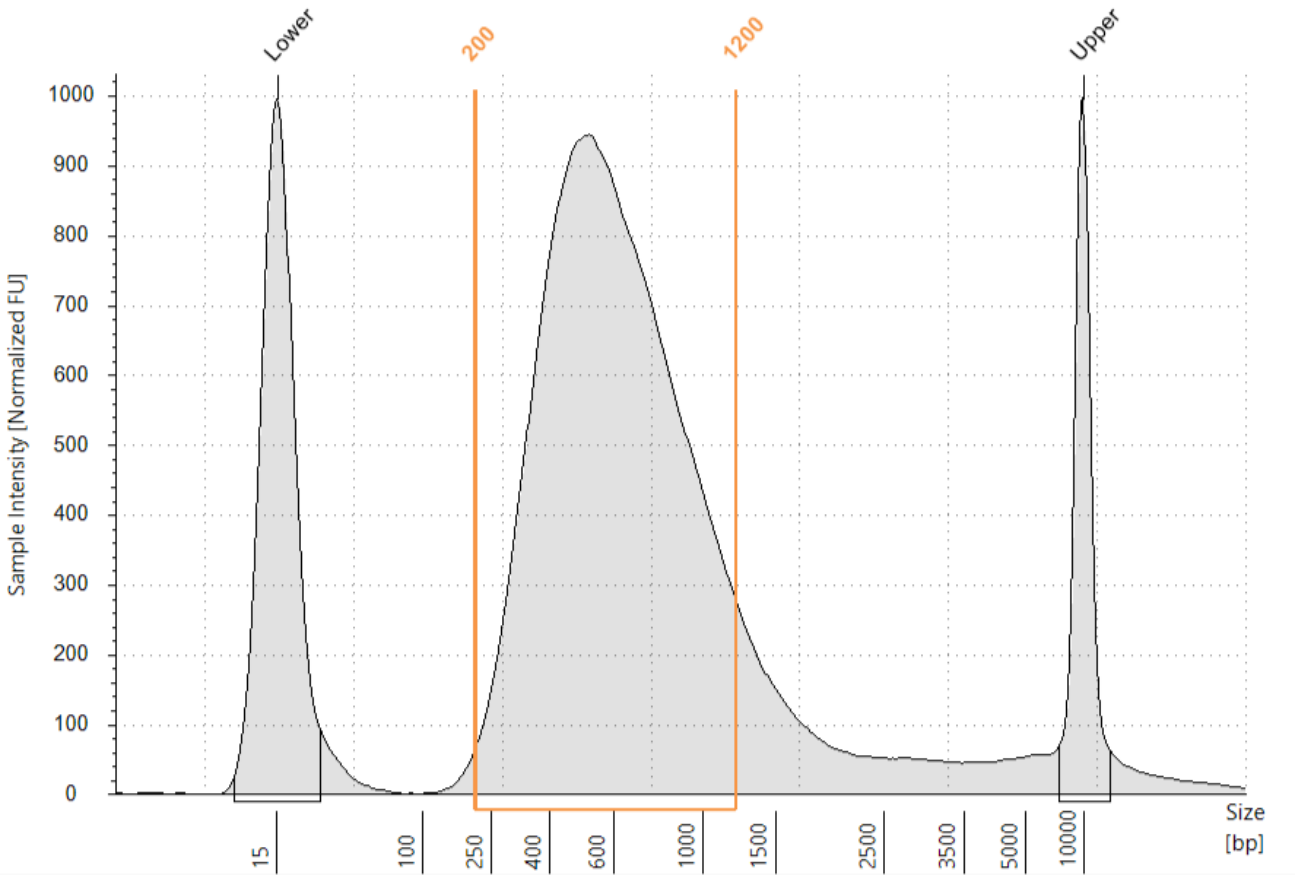


Figure 1. Representative Tapestation electropherogram of a pooled, purified 96-plex ExpressPlex 2.0 plasmid library (diluted 20-fold prior to electrophoresis). Region (orange) shows the range of clusterable fragments.

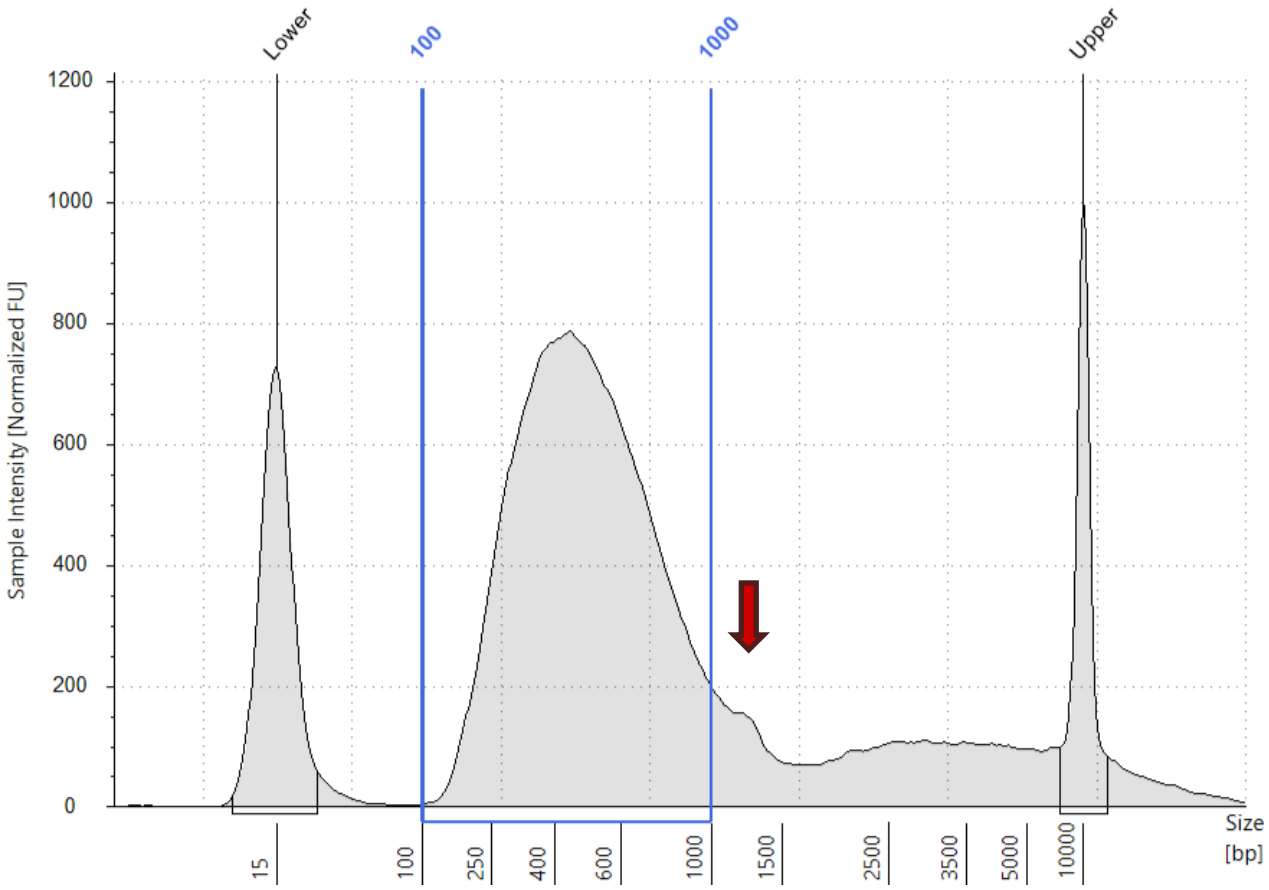


Figure 2. Representative Tapestation electropherogram of a pooled, purified 96-plex ExpressPlex 2.0 amplicon library (diluted 20-fold prior to electrophoresis). Region (blue) shows the range of clusterable fragments. Arrow (red) shows a small amount of unfragmented amplicon DNA.

Library quantification with dsDNA specific fluorometric method: ExpressPlex™ 2.0 libraries are double stranded. Use 2 µL to quantify the pooled, purified library using the Qubit™ dsDNA assay, Quant-iT™ PicoGreen™ dsDNA assay or other validated dsDNA quantification 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.

Library quantification with qPCR assay: qPCR is a very sensitive method of measuring library fragments that have both adaptor sequences on either end which will subsequently form clusters on a flow cell. Use 2 µl of the purified, multiplexed ExpressPlex™ 2.0 library for qPCR analysis with Illumina qPCR primer 1.1/2.1. Follow kit and instrument instructions for appropriate conditions and dilutions.

Sequencing on Illumina platforms

Read configuration: 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 reads must be 10 bases long for ExpressPlex libraries, although the index reads and non-index reads can be adjusted for different sequencing kits, speed or read depth requirements. For example, the sequencing run can be demultiplexed using only the unique i7 barcodes when running 96 or fewer ExpressPlex libraries on a sequencer.

Library dilution, denature and sequencing: Follow Illumina's guidelines for appropriate dilution to the starting concentration and loading procedures specific to your sequencing system. Not all DNA fragments can efficiently generate clusters. Longer library fragments generally do not cluster as efficiently on flow cell. It is essential to optimize the loading concentration based on your sample type, library QC methods and library fragment distribution. Try adjusting loading concentration over subsequent sequencing runs to optimize cluster density and sequencing run performance.

Appendix A: Adjusting the Input Sample Concentration

ExpressPlex 2.0 – 384-well library prep kits perform optimally with 0.5 - 20 ng of total dsDNA input per 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 of the samples already fall within the 0.25 – 10 ng/µl range, no adjustment is required. If, however, the average concentration of all of the samples exceeds 5 ng/µl, calculate the global dilution factor using the formula below:

$$\text{Global dilution factor (X)} = \frac{\text{Average assayed dsDNA concentration (ng/µl)}}{1.25 \text{ ng/µl}}$$

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 1.25 ng/µl (*i.e.,* resulting in an average of 5 ng of input being added per full ExpressPlex reaction volume).

Note: If the DNA concentration of the input samples is not easily confined to an approximately 40-fold range (maximum concentration divided by minimum concentration), or, if an average sample concentration of ≥0.25 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 and chemistries differ in their use of sample sheets, availability of on-instrument demultiplexing, optimal color balancing 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 2.0 libraries are similar to the Nextera Adapter sequences and are dual-indexed using 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 sequencer that reads the reverse complement of the i5 index sequence, enter the reverse complement of the i5 index shown in Appendix C.

Please refer to Illumina's website for most up to do index sequencing guidelines: https://support.illumina.com/downloads/indexed-sequencing-overview-15057455.html

Appendix C: ExpressPlex Index Information and Demultiplexing Guidance

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 in spreadsheet form 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.

Optimal Demultiplexing Guidance:

In accordance with Illumina's guidelines, it is recommended to allow for 1 mismatch in the barcode reads during demultiplexing. This allows for the capture of many more reads than 0 mismatch at a reduced risk, due to sufficient hamming distances between barcodes.

Refer to *Illumina's mismatch guidelines here:*
https://knowledge.illumina.com/software/general/software-general-reference_material-list/000007484

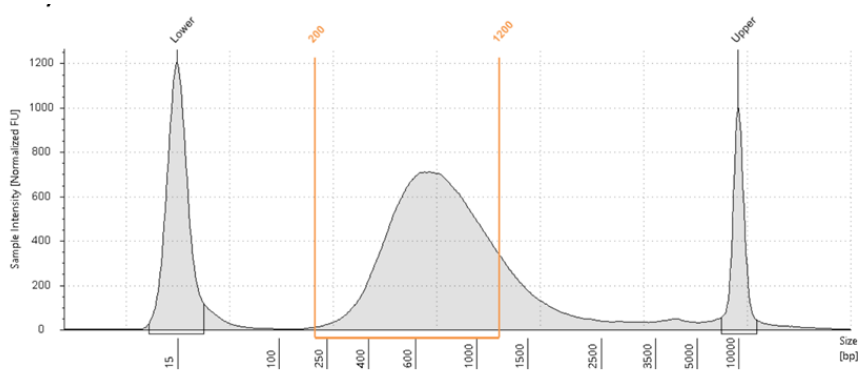
For the XLEAP chemistries:

For NovaSeq X, NextSeq 2000, and MiSeqi100 running XLEAP-SBS chemistry, an increased rate of 1 mismatch in the barcode reads has been observed. Hence, it is especially important to allow for 1 mismatch on these instruments to capture all expected data for each barcode.

Appendix D: Library Quantification Options

In this example, Agilent Tapestation was employed to quantify a pooled, purified 96-plex plasmid ExpressPlex™ 2.0 library for sequencer loading.

Refer to the figure below for library QC results.



From [bp]	To [bp]	Average Size [bp]	Conc. [pg/µL]	Region Molarity [pmol/L]	% of Total
200	1200	717	1200	2860	78.39

Figure 4. Example of library quantification results for a pooled, purified 96-complex plasmid library by Library QC on Agilent Tapestation.

Use the following formula to convert library concentration from ng/µl to nM:

$$\text{Library concentration [nM]} = \frac{\text{Library concentration [ng/µL]}}{660[\text{g/mol}] \times \text{Average fragment size [bp]}} \times 10^6$$

Library quantification by Tapestation **ONLY** (Library diluted 20-fold prior to electrophoresis):

- Clusterable region average library size: 717 [bp]
- Clusterable region library concentration: 1200 [pg/µl] × 20 = 24.0 [ng/µl]
- Estimated library concentration: $\frac{24.0\ [\text{ng/µl}]}{660[\text{g/mol}] \times 717\ [\text{bp}]} \times 10^6 = 50.7\ [\text{nM}]$

Appendix E: Protocol Modifications for Small Microbial WGS Applications

The ExpressPlex 2.0 Library Preparation Kit workflow has been optimized and validated for amplicons and plasmids sequencing in high-throughput settings. Though in some cases, it may be desirable to utilize the ExpressPlex 2.0 Library Preparation Kit for other applications, such as small microbial whole genome sequencing. Recommendations for best practices are outlined below.

Before beginning the preparation of libraries for applications or than amplicons and plasmids sequencing (eg. small microbial genome samples), **ensure that the criteria for below modified protocol are met:**

- **Measure and adjust input DNA concentration.** Assay the DNA concentration of each sample to be processed by PicoGreen or other validated dsDNA assay. Adjust the concentration of input DNA to a minimum concentration of 2.5 ng/µl and a maximum of 10 ng/µl in 10 mM Tris-HCl, pH 8.0. Depending on pooling size, it is recommended to use higher inputs of DNA. Do not use EDTA-containing solutions (*e.g.*, TE buffer) to dissolve or dilute input DNA because EDTA can suppress enzymatic activity. **For WGS we recommend using high quality DNA input (DIN >7) for optimal results.** Reach out to support@seqwell.com if processing samples with lower quality DNA.

To prepare libraries for applications besides amplicons and plasmids sequencing, follow the modified protocol below:

1. ExpressPlex 2.0 Modified Reaction Setup

a. 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.

b. Follow the instructions for reaction setup in the **Ready Reaction 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.

c. 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.

d. Transfer the assembled reactions in the 8-tube PCR strip(s) or plate to the thermal cycler and use the thermal cycling program below for **modified** ExpressPlex 2.0 library amplification.

e. Run the **modified** ExpressPlex 2.0 thermal cycling program below, with lid-heating on to 105ºC:

30	min	55ºC	Tagging
5	min	75ºC	Fill-in/Heat-kill
3	min	68ºC	
5	min	79ºC	
3	min	68ºC	
5	min	83ºC	
1	min	98ºC	Initial denaturation
15	sec	98ºC	Amplification (PCR) **Use 12 cycles for small microbial genomes samples**
30	sec	64ºC	
1	min	72ºC	
5	min	72ºC	Final extension
Hold		4ºC	

Note: Highlighted sections reflect the changes in the modified protocol. Important to note that increasing the tagging reaction time will shorten the insert sizes and resulting in smaller library fragment size.

SAFE STOPPING POINT

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

f. Follow the instructions for library pooling and library pool purification as in **Section 3 and 4** of the Standard Protocol above.

Proceed immediately to the Library Quantification and QC or store the amplified libraries at -20ºC.

Version	Release Date	Prior Version	Description of changes
V20241216	**Dec 16, 2024**	**N/A**	**First version**

Technical Assistance

For technical assistance with ExpressPlex 2.0 Library Preparation Kit, contact seqWell Technical Support.

E-mail: support@seqwell.com

seqWell, Inc.
66 Cherry Hill Drive
Beverly, MA 01915
USA
+1-855-737-9355
https://seqwell.com/