ExpressPlex™ 2.0 (96-well) Library Preparation Kit

for Illumina® Sequencing Platforms

Catalog numbers:

301176: ExpressPlex 2.0 (96-well) Library Preparation Kit, 4X96 Reactions - Set 1000
301177: ExpressPlex 2.0 (96-well) Library Preparation Kit, 4X96 Reactions - Set 2000
301178: ExpressPlex 2.0 (96-well) Library Preparation Kit, 4X96 Reactions - Set 3000
301179: ExpressPlex 2.0 (96-well) Library Preparation Kit, 4X96 Reactions - Set 4000
301170: ExpressPlex 2.0 (96-well) Library Preparation Kit, 1X96 Reactions - Any Index

User Guide

v20250103

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Introduction

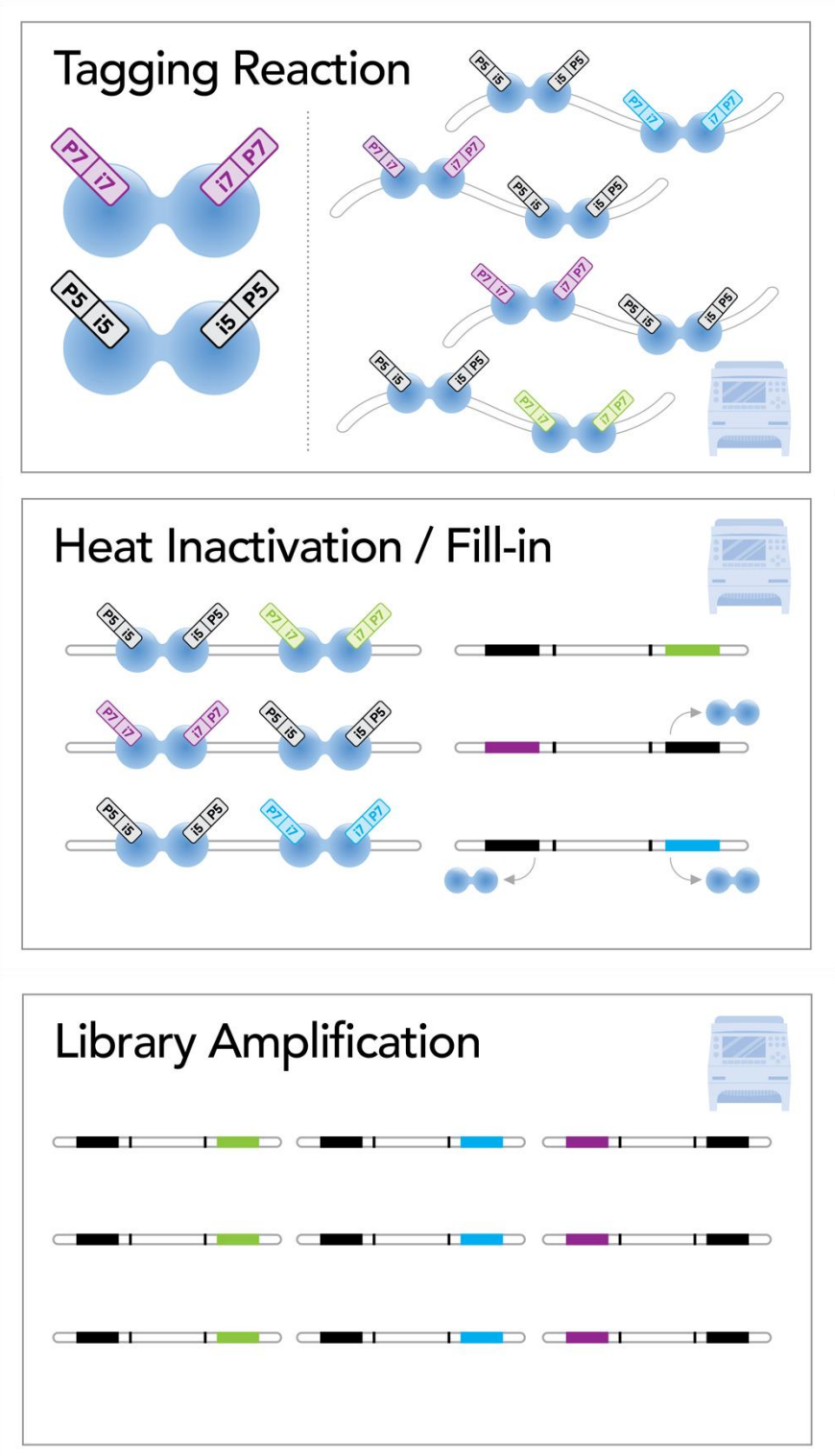
The patent-pending ExpressPlex 2.0 Library Prep Kit comes in both convenient 96-well and 384-well PCR plate configurations for high-throughput multiplexed library preparation. This upgraded version of ExpressPlex uses seqWell's high performance TnX™ transposase that was specifically engineered for NGS library preparation. Amplicons (>350 bp) and plasmid DNA are suitable standard inputs for the kits. Appendix E highlights protocol modifications for small microbial whole genome sequencing. ExpressPlex libraries are compatible with the Illumina MiSeq™, NextSeq™, iSeq™, and NovaSeq™ sequencing platforms.

Each ExpressPlex 2.0 (96-well) kit contains sufficient reagents to prepare Illumina-compatible libraries from 384 or 96 individual DNA samples. Libraries are typically prepared with 48 – 96 samples per pool, up to 384 samples per kit. For running fewer than 48 samples, please refer to Appendix E. There are four different kits available for preparing libraries from 384 samples, providing a total of 1,536 barcode combinations that can be loaded on a single sequencing run. Set 1000 is recommended if running fewer than four 96-well plates (384 samples) per run with Illumina XLEAP-SBS chemistry. Guidelines for optimal batching can be found on our website. Please refer to Illumina's guidelines for optimal color balancing for different sequencing chemistries.

This multiplexed library preparation procedure is optimized for inputs of 1 - 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 quality and size of input DNA and 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 (96-well) kit, a 96-plex library can be prepared for library QC and sequencing in under 120 minutes, with less than 30 minutes of hands-on time.

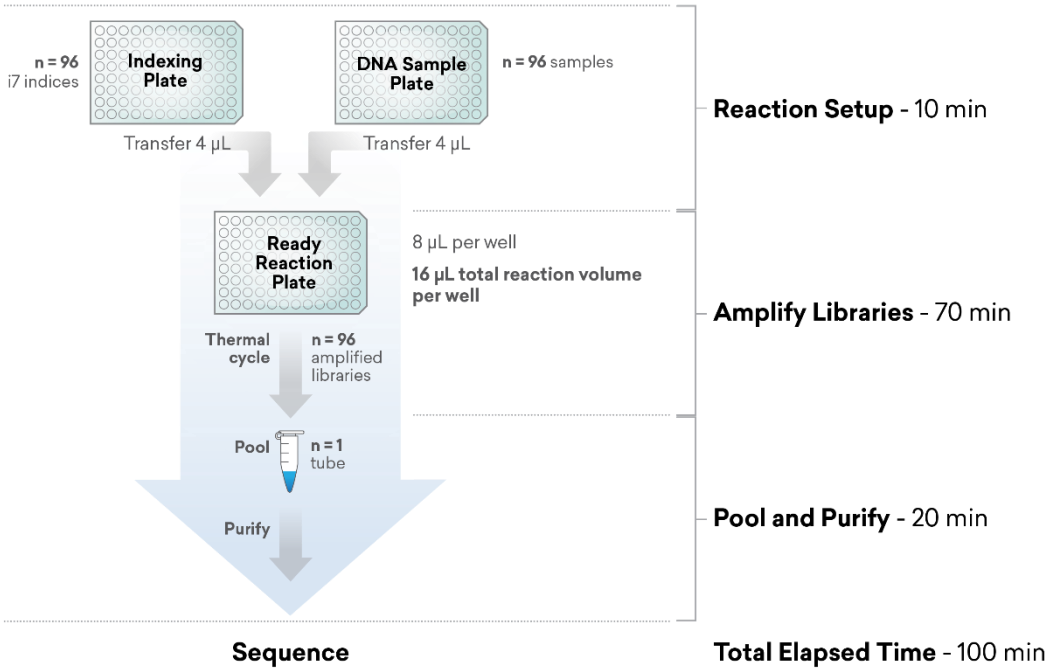
ExpressPlex 2.0 library preparation kits utilize a proprietary mixture of enzymes to tag input DNA with indexed adapters and amplify libraries all in a single reaction. Different full-length i7-indexed adapters tag the 96 DNA samples and barcoded libraries are amplified in separate wells, making for a highly efficient, one-step multiplexed library prep workflow. Each Indexing Reagent Plate contains a different i5 index that is applied to all samples within that plate (Workflow Diagram and Appendix C).

ExpressPlex 2.0 Molecular Diagram



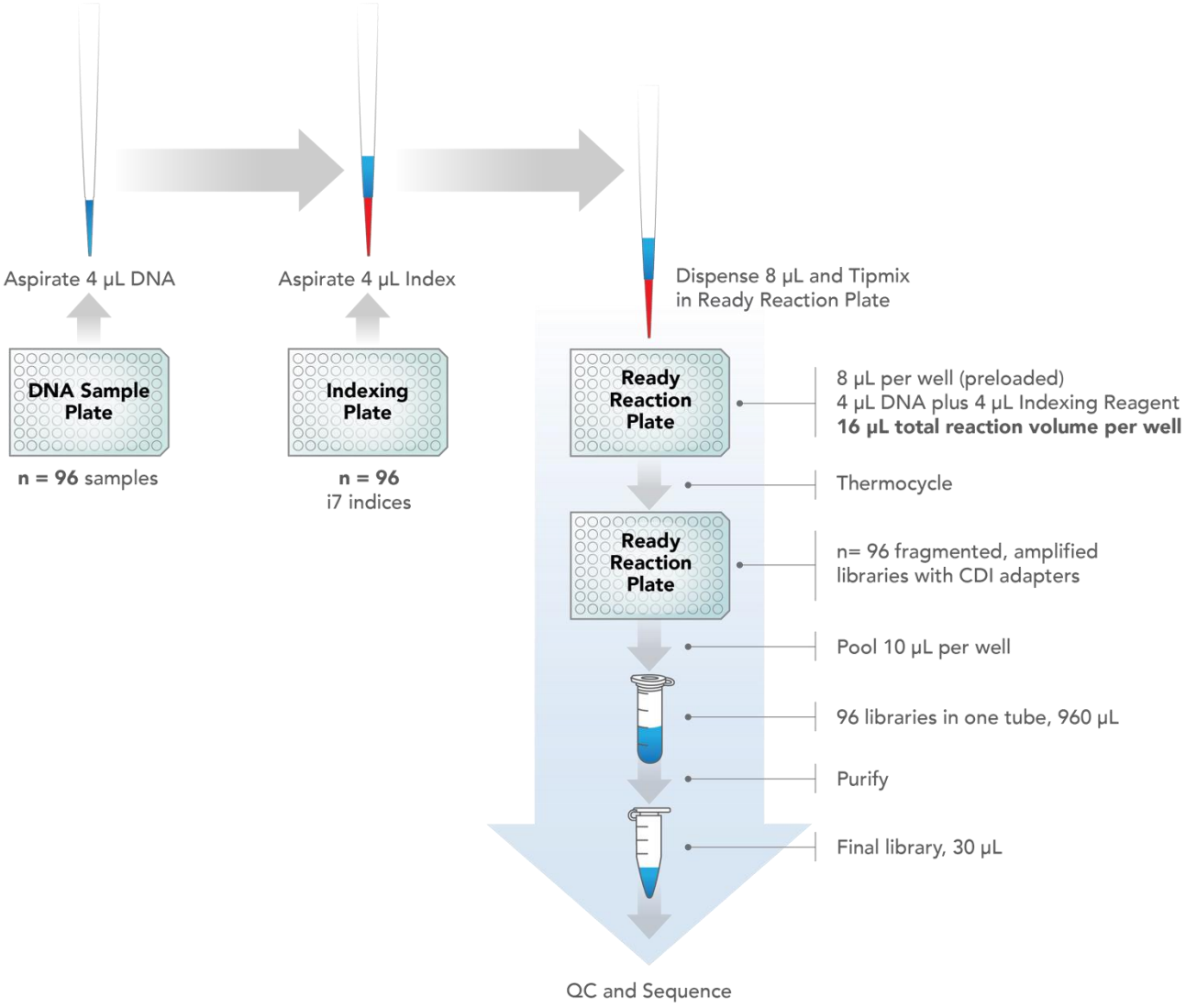
ExpressPlex 2.0 (96-well)

Workflow Diagram



ExpressPlex 2.0 (96-well)

EP Green Automation Workflow Diagram



Kit Components

Table 1. ExpressPlex 2.0 (96-well) Library Preparation Kit Components (4X96 Reactions)

ExpressPlex 2.0 (96-well) Library Preparation Kit, 4X96 - Set 1000
Catalog No.: 301176

Item	Component	REF	Description	Storage	Qty
1	Box 1 – 4X96 Indexing Reagent Plates - 1001, 1002, 1003, 1004	301171	Indexing Reagent Plates(96-well) in full-skirt,white PCR plates	-20°C	4
2	Box 2 – 4X96 Ready Reaction Plates	301175	Ready Reaction Plates (96-well) in full-skirt,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

ExpressPlex 2.0 (96-well) Library Preparation Kit, 4X96 - Set 2000
Catalog No.: 301177

Item	Component	REF	Description	Storage	Qty
1	Box 1 – 4X96 Indexing Reagent Plates - 2001, 2002, 2003, 2004	301172	Indexing Reagent Plates(96-well) in full-skirt,white PCR plates	-20°C	4
2	Box 2 – 4X96 Ready Reaction Plates	301175	Ready Reaction Plates (96-well) in full-skirt,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

ExpressPlex 2.0 (96-well) Library Preparation Kit, 4X96 - Set 3000

Catalog No.: 301178

Item	Component	REF	Description	Storage	Qty
1	Box 1 – 4X96 Indexing Reagent Plates - 3001, 3002, 3003, 3004	301173	Indexing Reagent Plates (96-well) in full-skirt,white PCR plates	-20°C	4
2	Box 2 – 4X96 Ready Reaction Plates	301175	Ready Reaction Plates (96-well) in full-skirt,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

ExpressPlex 2.0 (96-well) Library Preparation Kit, 4X96 - Set 4000

Catalog No.: 301179

Item	Component	REF	Description	Storage	Qty
1	Box 1 - 4X96 Indexing Reagent Plates - 4001, 4002, 4003, 4004	301174	Indexing Reagent Plates (96-well) in full-skirt,white PCR plates	-20°C	4
2	Box 2 – 4X96 Ready Reaction Plates	301175	Ready Reaction Plates (96-well) in full-skirt,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 (REF: 101002).

Table 2. ExpressPlex 2.0 (96-well) Library Preparation Kit Components (1X96 Reactions)

ExpressPlex 2.0 (96-well) Library Preparation Kit, 1X96 – Any Index
Catalog No.: 301170

Item	Component	REF	Description	Storage	Qty
1	Indexing Reagent Plate (any index); Ready Reaction Plate	301169	Indexing Reagent Plate (96-well) in full-skirt,white PCR plate; Ready Reaction Plate (96-well) in full-skirt,red PCR plate	-20°C	1
2	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 (REF: 101002).

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

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
- Electrophoretic analysis reagents - Agilent TapeStation (High Sensitivity D5000 kit), 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)
- Multichannel 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 Bio-Rad Hard-Shell® 96-Well 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
- Electrophoretic analysis equipment - Agilent TapeStation, Bioanalyzer, or Fragment Analyzer
- Fluorometer for dsDNA quantification assay

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

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

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. *Refer to Appendix A for more detailed information on globally adjusting the average input DNA concentration. Refer to the Appendix E if working with <48 samples and/or other applications such as microbial WGS.*

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 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).

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 (Manual)

Note: **Key Items Before Starting:**

- Refer to **Appendix A** for instructions to dilute input samples.
- Refer to **Appendix E** for instructions to prepare samples for batches fewer than 48 samples and/or 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).

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 48-88 samples, refer to subsection 1(f) below. For automated reaction setup, proceed directly to Section 2.

a. Pre-label each **Ready Reaction Plate** 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 1.25 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 setup, 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 EP2_TAG_AMP thermal cycling program below, with lid-heating on to 105°C:

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

Proceed to Section 3 if the reactions are already set up and thermal-cycled.

SAFE STOPPING POINT

Proceed immediately to the next step or

store the amplified libraries at -20°C.

Instructions for preparing libraries in batches of 48 – 88 samples: (refer to Appendix E for <48 samples)

f. With a razor blade, cut the seals up to the sample number being processed. 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 **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.

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 EP2_TAG_AMP thermal cycling program described above for ExpressPlex 2.0 library amplification.

2. ExpressPlex Reaction Setup (Using Automated liquid handlers)

ExpressPlex reactions can be set up at room temperature directly in the **Ready Reaction Plate** using a 96-channel pipetting head, an 8-channel pipetting head, or even an automated single-channel pipetting device. By using the same pipette tips to aspirate indexing reagents and the DNA samples, also known as the *EP Green Method,* the plastic waste and time required for setup can be dramatically reduced (see the *EP Green Automation Workflow Diagram*). Optimal throughput is achieved using 96-channel liquid handlers.

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 and remove the heat seals carefully.

c. Aspirate 4 µl of approximately 1.25 ng/µl input DNA into all channels of the pipettor. Using the same tips, aspirate 4 µl of **Indexing Reagent** into all channels of the pipettor.

   Note: Although sufficient overage is provided to reliably aspirate 4 µl of **Indexing Reagent**, each well of the **Indexing Reagent Plate** is intended for one use only.

d. Dispense 8 µl from the tips into the **Ready Reaction Plate.**

e. Mix thoroughly by rapidly aspirating and dispensing an 8 µl volume ten times, being careful not to introduce bubbles. If excessive bubbles are present after reaction setup, use a tabletop centrifuge to collapse the bubbles prior to proceeding.

f. Seal the **Ready Reaction Plate**, transfer to a thermal cycler, and run the EP2_TAG_AMP thermal cycling program described above.

SAFE STOPPING POINT

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

3. Library Pooling

a. After library amplification, pulse-spin the **Ready Reaction Plate** and then remove the plate seal.

b. Using a multichannel pipette or an automated 8-channel pipetting head, pool 10 µl of each amplified library from each column into a single prelabeled 8-tube PCR strip. The same pipette tips may be used for pooling multiple reactions.

   Optional: If processing multiple plates with a 96-channel pipetting head, stamp the contents of multiple 96-well plates into one 96-well PCR plate and mix by pipetting before pooling into four strip tubes and subsequently four 2 ml LoBind tubes.

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

4. 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 up and down ≥10X.

   Note: Use 1 volumetric equivalent of **MAGwise** for libraries derived from amplicons in order to recover the shorter fragments.

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 the inner wall of the tube and wait until the supernatant appears completely clear (5 minutes or less).

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

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

g. Repeat 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 magnetic stand and pipette 30 µl of 10 mMTris-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.

   Optional: Lower elution volumes (<30 µl) can be used to increase the concentration of libraries prepared from 48 – 88 samples.

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 the 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 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 kit), 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 amplicons.

If using electrophoretic analysis for library quantification, use the region analysis function and calculate the library concentration from the clusterable fragment region. 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 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.

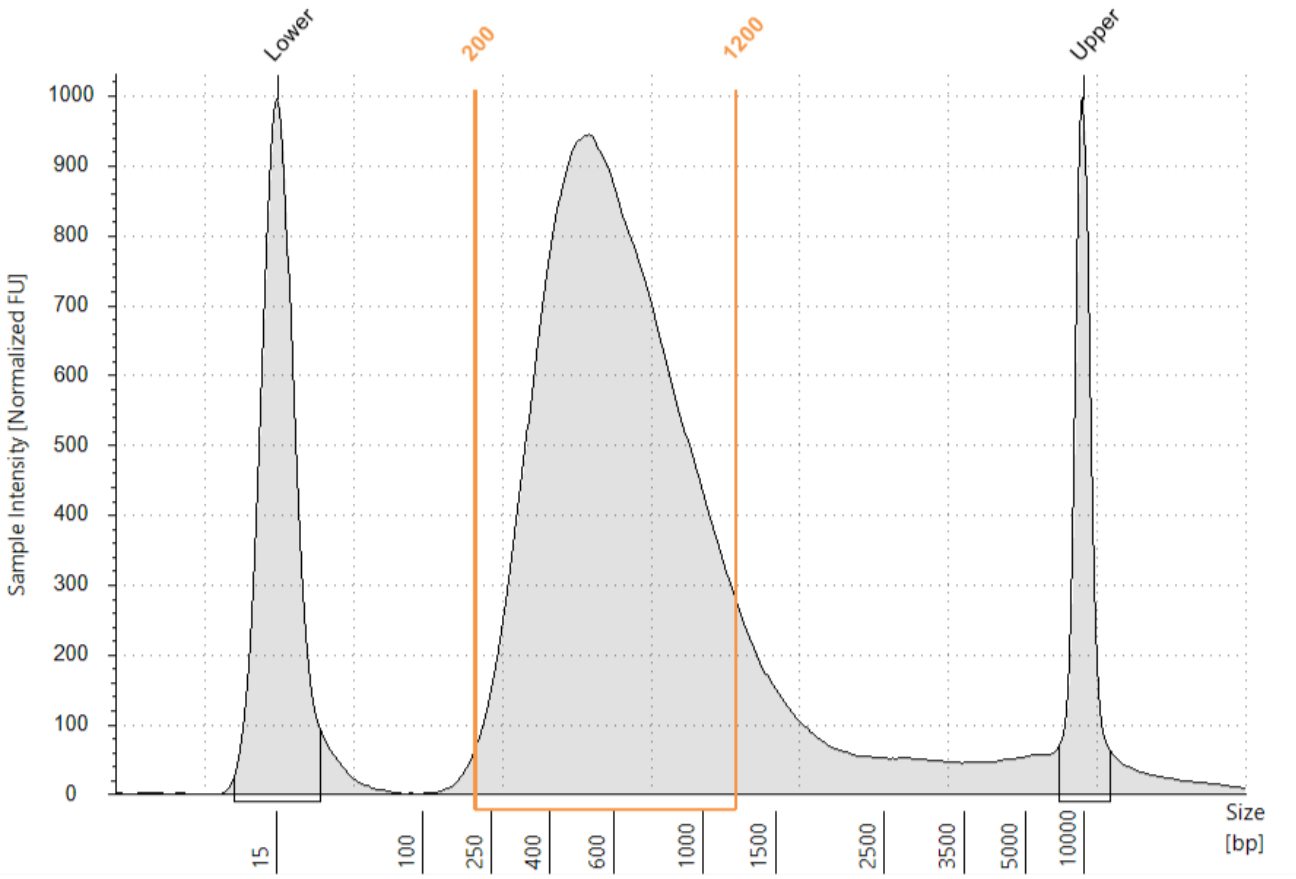


Figure 1. Representative TapeStation electropherogram of a pooled, purified ExpressPlex 2.0 plasmid library (diluted 20-fold prior to electrophoresis). The analysis region (orange) shows the range of clusterable fragments.

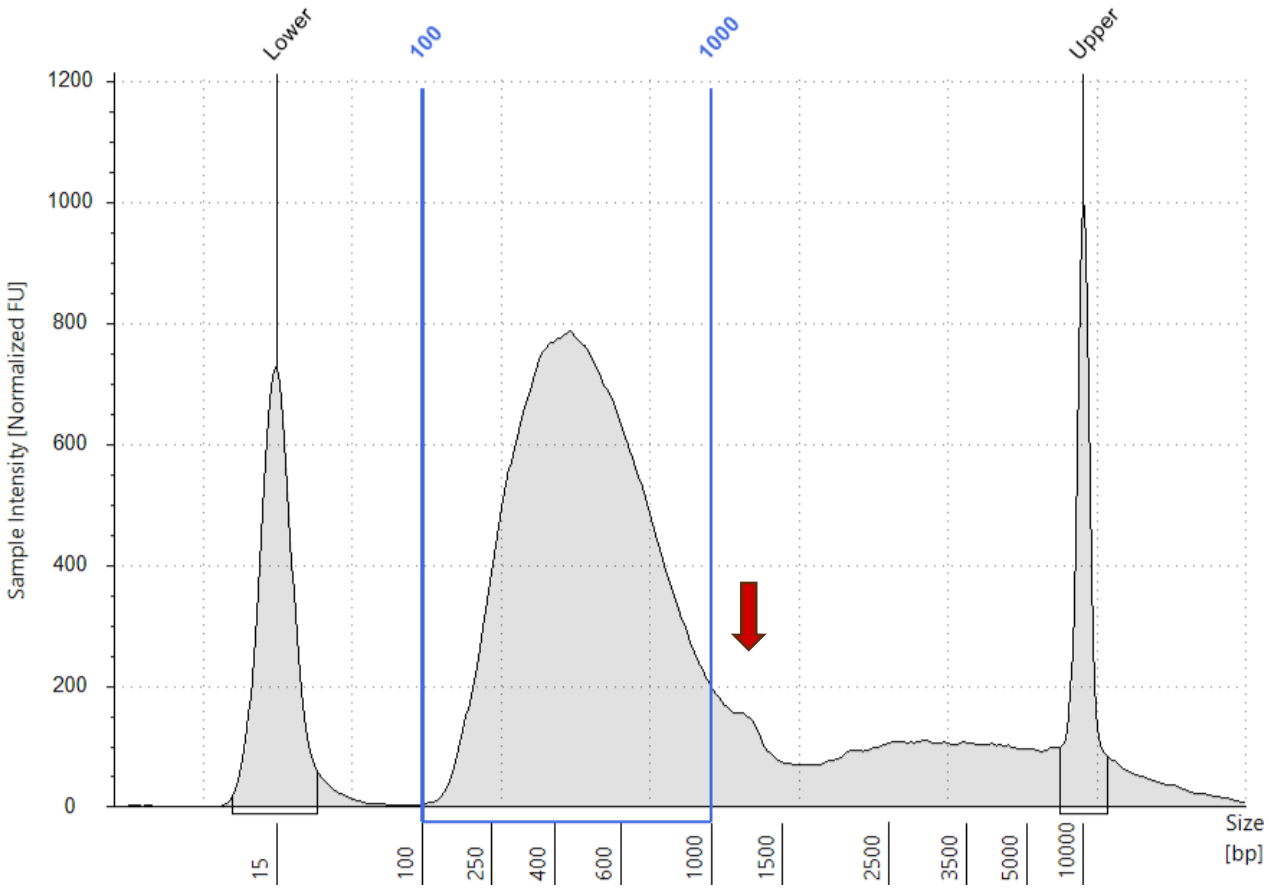


Figure 2. Representative TapeStation electropherogram of a pooled, purified ExpressPlex 2.0 amplicon library (diluted 20-fold prior to electrophoresis). The analysis region (blue) shows the range of clusterable fragments. Arrow (red) shows a small amount of unfragmented amplicon DNA.

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 clusterable 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 quantifying 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 1 described on Illumina's website here (https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/qpcr/sequencing-library-qpcr-quantification-guide-11322363-c.pdf). 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 indexes. Index lists can be found in Appendix C and in the index spreadsheet on the seqWell website. 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 with a single i5 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 a 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 (96-well) Library Preparation Kits perform optimally with 1 - 40 ng of total dsDNA input per 16 µl reaction, and 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 the DNA sample 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 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 technical 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 indexes 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 the ExpressPlex 2.0 index list on seqWell's website: https://seqwell.com/expressplex-2-0-library-prep-kit/.

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: all 16 Indexing Reagent Plates have the same 96 i7 indexes but each plate has a different i5 index, providing 1,536 barcode combinations in total. For a complete list of all i7 indexes (listed by row and column formats as well as in plate layout) and the i5 indexes, please refer to the ExpressPlex 2.0 index list in spreadsheet format on seqWell's website: https://seqwell.com/expressplex-2-0-library-prep-kit/.

i7 Index Plate Map 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 forward*	i5 reverse*
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

Important Note: Illumina sample sheets for on-board demultiplexing use the i5 index forward read sequence. The software will automatically generate the reverse complement for sequencers that rely on the i5 index reverse read sequence. Please refer to Illumina's recommendations for optimal color balancing for different sequencing chemistries.

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.

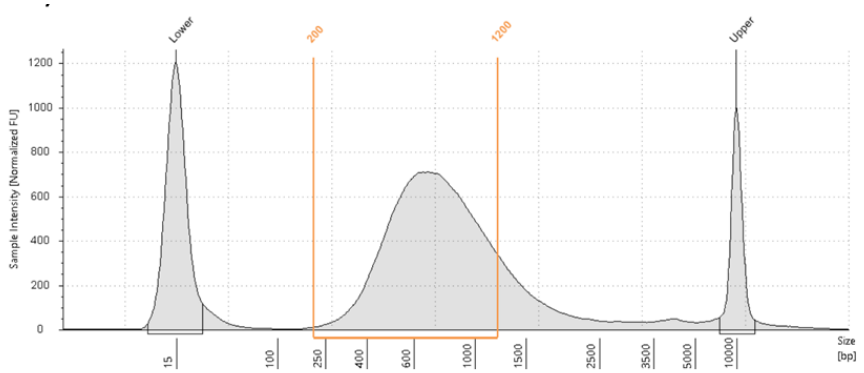
Refer to *Illumina's mismatch guidelines here:*
https://knowledge.illumina.com/software/general/software-general-reference_material-list/000007484

For the XLEAP chemistries:
For NovaSeq X, NextSeq 2000, and MiSeq i100 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 recover all expected data for each barcode.

Appendix D: Library Quantification Options

In this example, Agilent TapeStation was employed to quantify a pooled, purified 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 3. Example of library quantification results for a pooled, purified 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 Number of Sample ≤ 48 and/or 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 prepare less than 48 samples and/or to utilize the ExpressPlex 2.0 Library Preparation Kit for other applications, such as small microbial whole genome sequencing. Alternate pooling strategies for number of samples fewer than 48 samples can be employed and recommendations for best practices are outlined below.

Before beginning the preparation of libraries in batches of fewer than 48 samples and/or other applications than amplicons and plasmids sequencing (*eg*., small microbial genome samples), **ensure that the criteria for the modified protocol below are met:**

- To ensure that enough yield is generated for sequencing, the minimum number of samples to be processed is 16. Fewer than 16 samples may be processed **ONLY IF** an experienced user is comfortable with final elution volume of 15 µl.

- **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 in batches of <u>fewer</u> than 48 samples and/or other applications besides amplicons and plasmids sequencing, follow the modified protocol below:

1. ExpressPlex 2.0 Modified Reaction Setup

a. With a razor blade, cut the seals up to the sample number being processed. 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 **<u>modified</u>** ExpressPlex 2.0 library amplification.

e. Run the **<u>modified</u>** ExpressPlex 2.0 thermal cycling program – ***MOD_EP2_TAG_AMP*** below, with lid-heating on to 105°C:

Thermal Cycler Program MOD_EP2_TAG_AMP (with lid-heating on at 105°C):

30	min	55°C	Tagging Reaction
5	min	75°C	Heat Inactivation / Fill-in
3	min	68°C	
5	min	79°C	
3	min	68°C	
5	min	83°C	
1	min	98°C	Initial denaturation
15	sec	98°C	Library Amplification (PCR) **Use 12 cycles for small microbial genomes samples** **Use 15 cycles for <48 amplicons or plasmid samples**
30	sec	64°C	
1	min	72°C	
5	min	72°C	Final extension
Hold		4°C	

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

SAFE STOPPING POINT

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

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

Note: Lower elution volumes (<30 µl) can be used to increase the concentration of libraries prepared from fewer than 48 samples. Fewer than 16 samples may be processed **ONLY IF** an experienced user is comfortable with final elution volume of 15 µl.

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

Version	Release Date	Prior Version	Description of changes
V20250103	January 3, 2025	N/A	First version

Technical Assistance

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

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