User Guide

Early Access version

ExpressPlex™ 2.0 Library Preparation Kit

for Illumina® Sequencing Platforms

Part numbers:
301176: ExpressPlex Library Prep Kit, 384 Reactions, Set 1000
301177: ExpressPlex Library Prep Kit, 384 Reactions, Set 2000
301178: ExpressPlex Library Prep Kit, 384 Reactions, Set 3000
301179: ExpressPlex Library Prep Kit, 384 Reactions, Set 4000
301170: ExpressPlex Library Prep Kit, 96 Reactions

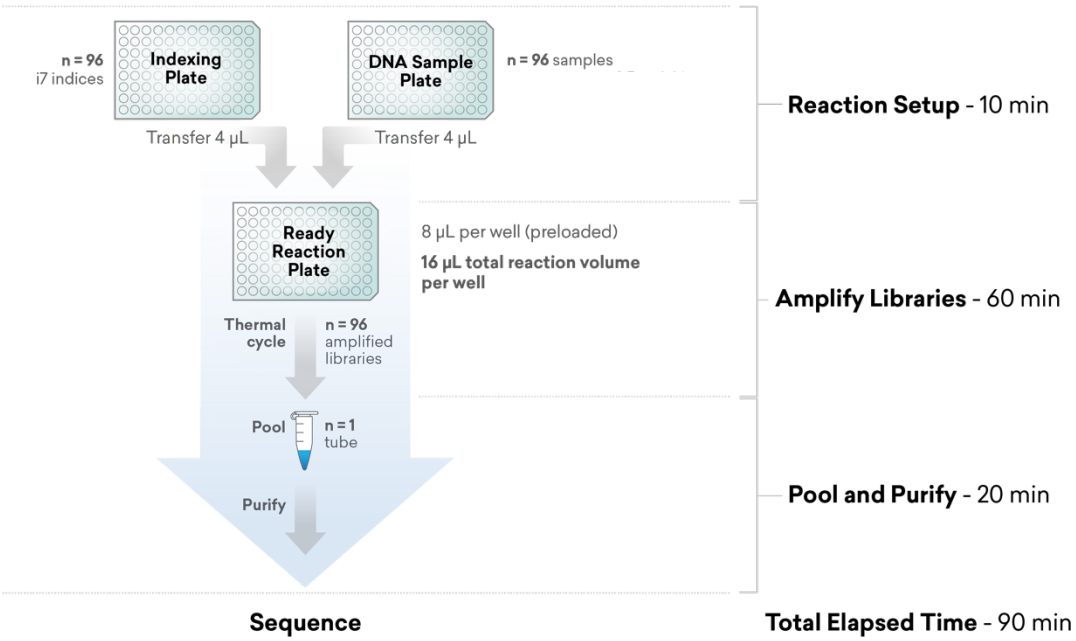


Figure 1. ExpressPlex 2.0 Library Preparation Workflow Diagram for 96 plasmid or amplicon samples.

Table 1. ExpressPlex 2.0 Library Preparation Kit Components (384 Reactions)

ExpressPlex 2.0 Library Preparation Kit, Set 1000, Reorder No.: 301176

Item	Component	P/N	Description	Storage	Qty
1	Box 1, Indexing Reagent Plates 1001, 1002, 1003, 1004	301171	Indexing Reagent Plates(96 wells) in fully-skirted,white PCR plates	-25° to -15°C	4
2	Box 2, Ready Reaction Plates	301175	Ready Reaction Plates (96 wells) in fully-skirted,red PCR plates	-25° to -15°C	4
3	MAGwise paramagnetic beads*	101003	5 ml of MAGwise beads in a 10 ml screwcap tube	2 to 8°C	1

ExpressPlex 2.0 Library Preparation Kit, Set 2000, Reorder No.: 301177

Item	Component	P/N	Description	Storage	Qty
1	Box 1, Indexing Reagent Plates 2001, 2002, 2003, 2004	301172	Indexing Reagent Plates(96 wells) in fully-skirted,white PCR plates	-25° to -15°C	4
2	Box 2, Ready Reaction Plates	301175	Ready Reaction Plates (96 wells) in fully-skirted,red PCR plates	-25° to -15°C	4
3	MAGwise paramagnetic beads*	101003	5 ml of MAGwise beads in a 10 ml screwcap tube	2 to 8°C	1

ExpressPlex 2.0 Library Preparation Kit, Set 3000, Reorder No.: 301178

Item	Component	P/N	Description	Storage	Qty
1	Box 1, Indexing Reagent Plates 3001, 3002, 3003, 3004	301173	Indexing Reagent Plates(96 wells) in fully-skirted,white PCR plates	-25° to -15°C	4
2	Box 2, Ready Reaction Plates	301175	Ready Reaction Plates (96 wells) in fully-skirted,red PCR plates	-25° to -15°C	4
3	MAGwise paramagnetic beads*	101003	5 ml of MAGwise beads in a 10 ml screwcap tube	2 to 8°C	1

ExpressPlex 2.0 Library Preparation Kit, Set 4000, Reorder No.: 301179

Item	Component	P/N	Description	Storage	Qty
1	Box 1, Indexing Reagent Plates 4001, 4002, 4003, 4004	301174	Indexing Reagent Plates(96 wells) in fully-skirted,white PCR plates	-25° to -15°C	4
2	Box 2, Ready Reaction Plates	301175	Ready Reaction Plates (96 wells) in fully-skirted,red PCR plates	-25° to -15°C	4
3	MAGwise paramagnetic beads*	101003	5 ml of MAGwise beads in a 10 ml screwcap tube	2 to 8°C	1

* Larger volumes (15 ml) of MAGwise paramagnetic beads are also available to purchase separately (P/N: 101002).

Table 2. ExpressPlex 2.0 Library Preparation Kit Components (96 Reactions)

ExpressPlex 2.0 Library Preparation Kit, Reorder No.: 301170

Item	Component	P/N	Description	Storage	Qty
1	Indexing Reagent Plate (any index)	301163	Indexing Reagent Plate(96 wells) in a fully-skirted, white PCR plates	-25° to -15°C	1
2	Ready Reaction Plate	301167	Ready Reaction Plate (96 wells) in a fully-skirted,red PCR plates	-25° to -15°C	1
3	MAGwise paramagnetic beads*	101003	5 ml of MAGwise beads in a 10 ml screwcap tube	2 to 8°C	1

Introduction

The patent-pending ExpressPlex 2.0 Library Prep Kit comes in a convenient 96-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 inputs for the kits. ExpressPlex libraries are compatible with the Illumina MiSeq™, NextSeq™, iSeq™, and NovaSeq™ sequencing platforms.

Each ExpressPlex 2.0 kit contains sufficient reagents to prepare Illumina-compatible libraries from 384 or 96 individual DNA samples. Libraries can be prepared from 48 – 384 samples per batch. There are 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. Set 1000 is recommended if running fewer than four 96-well plates (384 samples) per run on the NextSeq 1000/2000 sequencing platforms. 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 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 kit,a 96-plex library can be prepared for library QC and sequencing in under 120 minutes.

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 (see the Workflow Diagram in Fig. 1 and Appendix C).

User-Supplied Reagents, Equipment and Consumables

Reagents

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

Equipment & Consumables

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

Before starting the procedure:

Measure and adjust input DNA concentration. Assay the DNA concentration of each 96 well plate of samples to be processed by PicoGreen or other validated dsDNA assay. Globally adjust the average concentration of input DNA across each plate to 1.25 ng/µl in 10 mM Tris-HCl, pH 8.0. Higher concentrations of DNA could be more suitable if some of the samples are unusually dilute. Do not use EDTA-containing solutions (*e.g.*, TE buffer) to dissolve or dilute input DNA because EDTA can suppress enzymatic activity. *See the Appendix for more detailed information on globally adjusting the average input DNA concentration.*

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

Pulse-spin kit components. Liquids can condense or shift locations inside containers during shipment or storage. Before using the reagent plates and tubes, pulse-spin in a suitable centrifuge to gather the reagents at the bottom of the well or tube. If the kit components freeze during shipment or storage, thaw, mix and pulse-spin before use.

Equilibrate MAGwise Paramagnetic Beads to room temperature. MAGwise beads can be stored for up to 2 weeks at room temperature or for longer periods at 2 - 8°C. Ifstored cold, warm at room temperature for 30 minutes before use. Vortex to thoroughly resuspend beads prior to use. To transfer volumes accurately, pipette slowly and do not pre-wet pipette tips.

Prepare 80% ethanol fresh daily.

Prepare 10 mM Tris-HCl, pH 8.0. 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)

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

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

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	

Special instructions for preparing libraries in batches of <u>fewer</u> than 96 samples (for batches of n = 48 – 88 samples):

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

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

g. Follow the instructions for reaction setup in the **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 thermal cycling program above for ExpressPlex library amplification.

2. ExpressPlex Reaction Setup using automated liquid handlers

Skip to Section 3 if the reactions were already setup manually and thermal-cycled. ExpressPlex reactions can be setup 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, the plastic waste and time required for setup can be dramatically reduced. Optimal throughput is achieved using 96-channel instruments.

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 ExpressPlex thermal cycling program.

SAFE STOPPING POINT

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

3. Library pooling

f. After library amplification, pulse-spin the **Ready Reaction Plate** and then open the seal/cap.

g. 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 a 2 ml LoBind tube.

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

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

Optional: If excessive bubbles are present after pooling, use a tabletop centrifuge to collapse the bubbles prior to proceeding. Also, you may freeze any unpurified amplified libraries remaining in the **Ready Reaction Plate**, providing an option to purify more 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. Incubate on the bench for 5 minutes to allow the DNA to bind.

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

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

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

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

f. 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 thesecond wash, briefly pulse-spin, and then use a smaller pipette tip to remove anyresidual volume, if visible.*

g. 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 wallof 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.

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

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

j. 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 -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. For a more accurate measurement of the concentration of clusterable library fragments, use qPCR (see below).

See figures below for representative trace for pooled, purified libraries run on the TapeStation or an agarose gel. Also see *Appendix D* for alternative quantification methods for ExpressPlex 2.0 libraries.

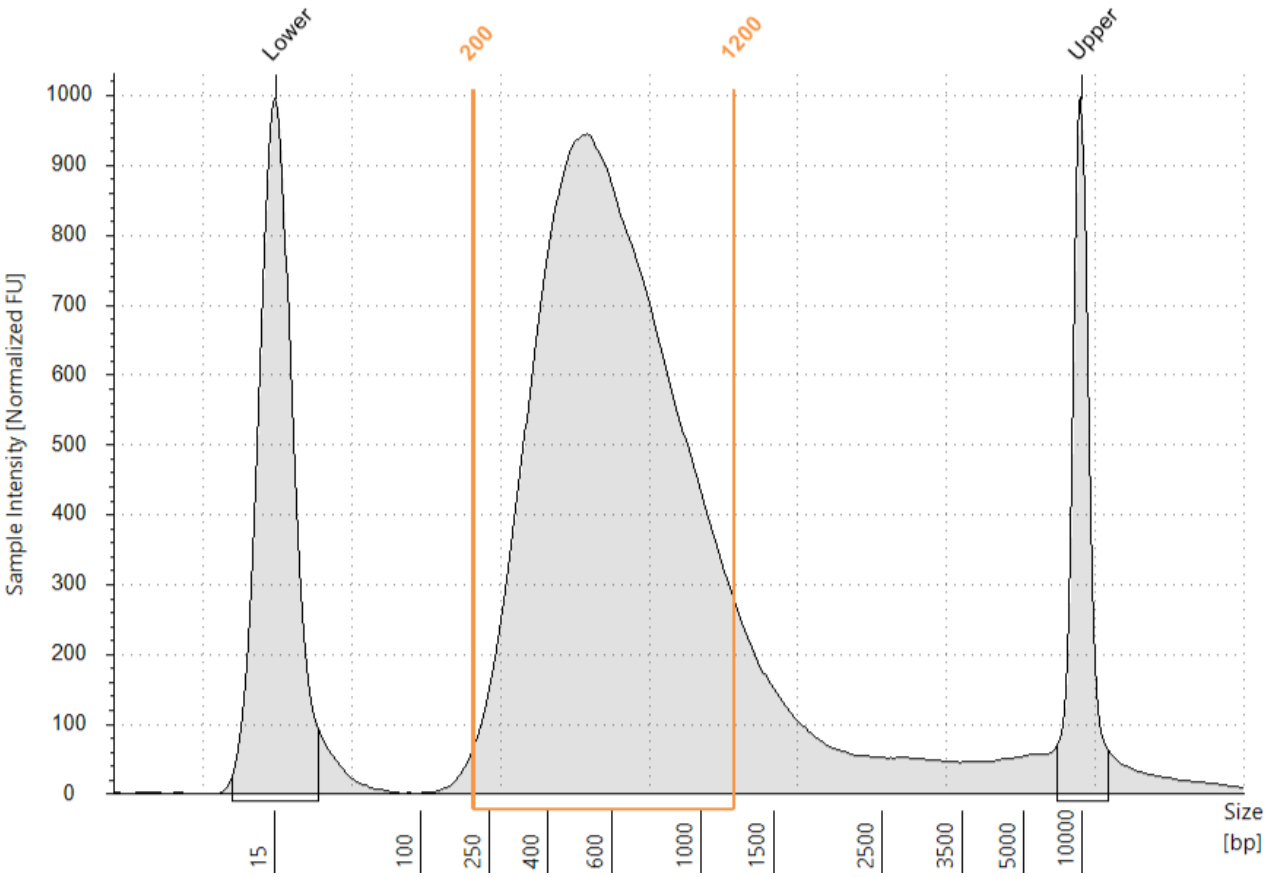


Figure 2. Representative TapeStation electropherogram of a pooled, purified 96-plex ExpressPlex 2.0 plasmid library (diluted 20-fold prior to electrophoresis). Region (orange) shows the range of clusterable fragments.

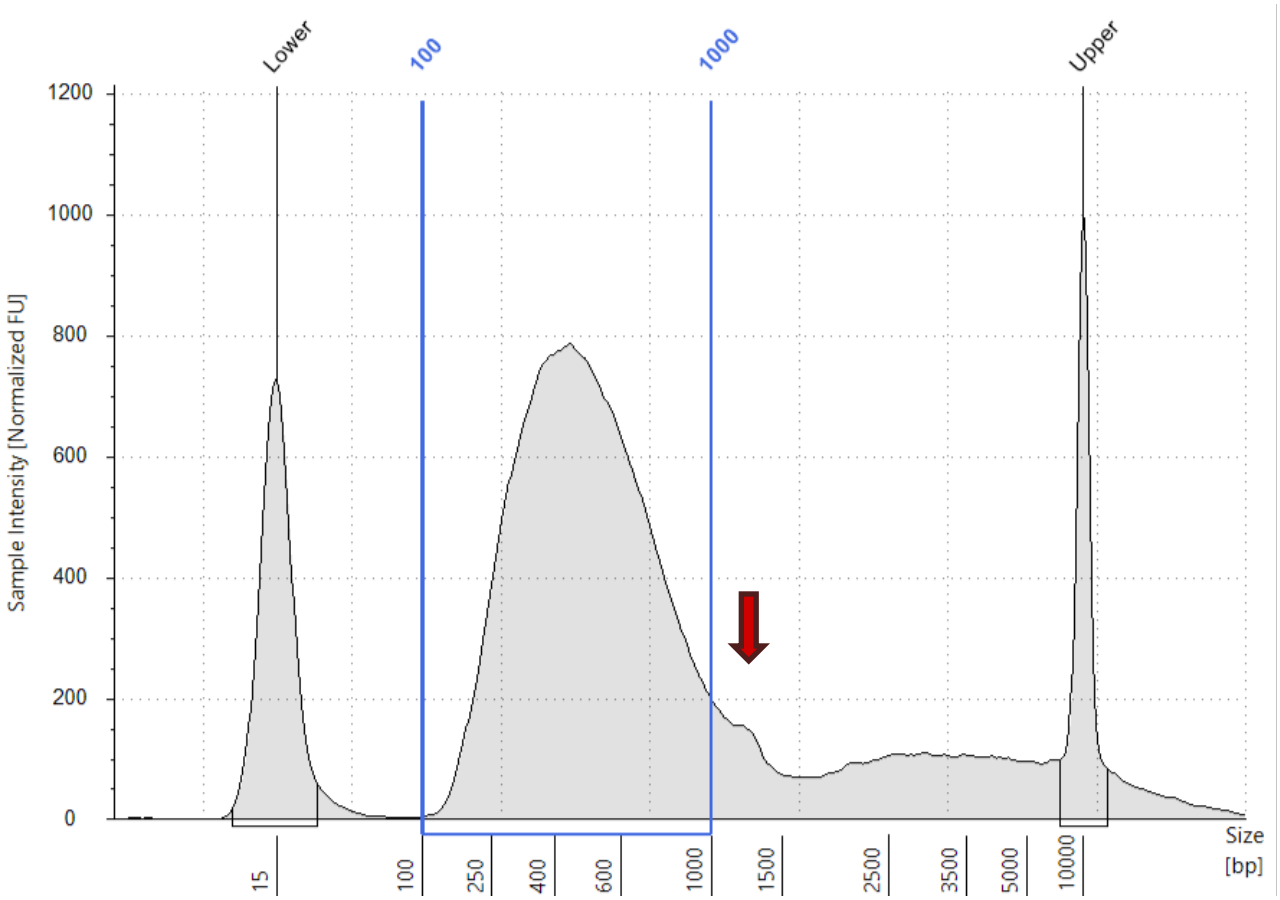


Figure 3. Representative TapeStation electropherogram of a pooled, purified 96-plex ExpressPlex 2.0 amplicon library (diluted 20-fold prior to electrophoresis). Region (blue) shows the range of clusterable fragments. Arrow (red) shows a small amount of unfragmented amplicon DNA.

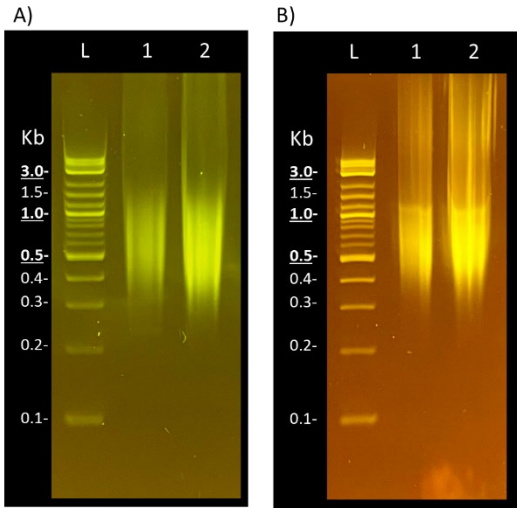


Figure 4. Representative gel image of A) a pooled, purified 96-plex ExpressPlex plasmid library, and B) a pooled, purified 96-plex ExpressPlex amplicon library run on an Invitrogen™ E-Gel EX with SYBR Gold II, 2% Agarose. Lane L – 1 kb Plus DNA Ladder; Lane 1: 2 µL library; Lane 2: 5 µL library.

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.

See table below for starting concentration and recommended loading concentrations.

Table 3.

As of August 2021:

Sequencing System	Starting Concentration (nM)	Recommended Final Loading Concentration (pM)
iSeq 100	2	150~200
MiniSeq	2	1.2
MiSeq (v3)	4	12
MiSeq (v2/v2 Nano/v2 Micro)	4	10~11
NextSeq 550 and NextSeq 500	2	1.1~1.2
NextSeq 2000	2	550~750
NovaSeq 6000	2	See NovaSeq 6000 System Guide

Appendix A: Adjusting the starting sample concentration

ExpressPlex 2.0 library kits perform optimally with 1 - 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 0.25 – 10 ng/µl range, no adjustment is required. If, however, the average concentration of your 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 your input samples is not easily confined to a ~40-fold range (max conc./min conc.), 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 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.

As of January 1, 2021:

The following Illumina sequencers read the i5 index sequence in the forward direction:

- MiniSeq™ (rapid only)
- MiSeq™
- HiSeq® 2000/2500
- NovaSeq™ 6000 (v1 reagents)

The following Illumina sequencers read the reverse complement of the i5 index sequence:

- iSeq™ 100
- MiniSeq
- HiSeq X
- HiSeq 3000/4000
- NextSeq™ 550
- NextSeq 1000/2000
- NovaSeq 6000 (v1.5 reagents)

Appendix C: ExpressPlex index information

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

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

i5 forward read: MiSeq, HiSeq 2000/2500, and NovaSeq 6000 (v1 reagents)

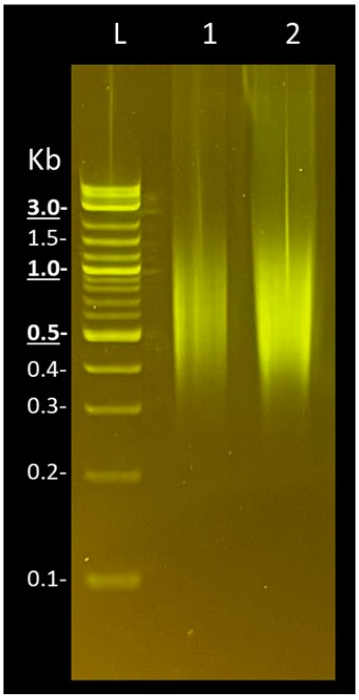
i5 reverse read: iSeq 100, MiniSeq, NextSeq, HiSeq X/3000/4000, and NovaSeq 6000 (v1.5 reagents)

Important Notes: 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.

Appendix D: Library Quantification Options

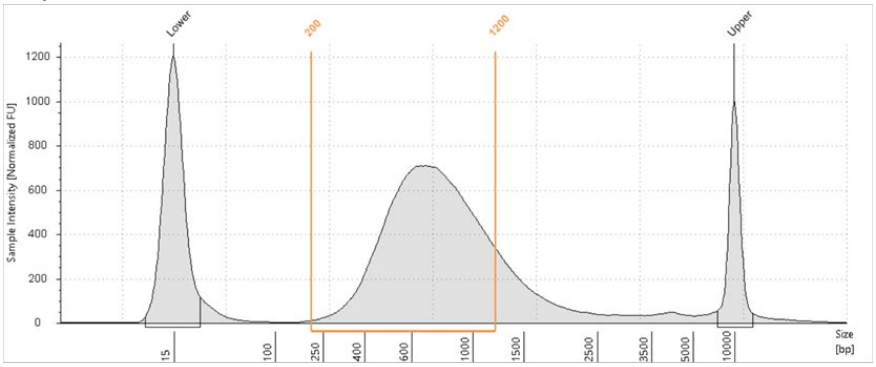
In this example, two library quantification methods (A & B) were employed for a pooled, purified 96-plex plasmid ExpressPlex™ 2.0 library. Refer to the figure and calculations below to compare the library QC results.

A)



Land L – 1 kb Plus DNA Ladder, 20µL
Lane 1 – Purified library, 2uL
Lane 2 – Purified library, 5uL

B)



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. A) Library QC by E-Gel and Qubit, and B) Library QC by TapeStation.

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

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

Method A. Library quantification by 2% Agarose E-Gel and Qubit™ dsDNA HS Assay (library not diluted):

- Library concentration by Qubit: 26.6 [ng/µL]
- Estimated average library size by E-Gel: 700 [bp]
- Estimated library concentration: $\frac{26.6\ [\text{ng/µL}]}{660[\text{g/mol}] \times 700\ [\text{bp}]} \times 10^6 = 57.6\ [\text{nM}]$

Method B. 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: $\frac{24.0\ [\mathrm{ng/\mu L}]}{660[\mathrm{g/mol}]\times 717\ [\mathrm{bp}]} \times 10^6 = 50.7\ [\mathrm{nM}]$

Technical Assistance

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

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