Implementation and Validation Guidelines for Automating the ExpressPlex™ 2.0 (96- & 384-well) Library Preparation Kits

This document describes liquid-handling parameters, an experimental plan, and key performance criteria which may be used in the validation of seqWell's ExpressPlex 2.0 Library Preparation kits on any liquid handler with 96-multichannel pipetting capability. These same criteria have been used to validate seqWell's own internal systems.

Sequencing-metric data derived from the following automation platforms is included herein: SPT Labtech ***apricot® S3*** and ***firefly®***, Tecan ***Fluent®***, Beckman Coulter ***Biomek® FX^P***, and Complete Genomics ***MGISP-960***. For comparison, a set of manual control data is also included.

For validated methods available through seqWell and implementations on specific platforms not named here, please contact seqWell at support@seqWell.com.

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Introduction

ExpressPlex 2.0 is the fastest library preparation chemistry available. It performs auto-normalization of sample input over a wide range of concentrations, fragments input DNA into sizes suitable for Illumina sequencers, produces highly consistent library insert sizes, and yields uniform read-count statistics. A plate of samples can be taken from library setup through final library purification in less than 120 minutes, with a reduction by ~80% in pipetting steps required and pipette tips consumed in comparison to other traditional chemistries. The ExpressPlex 2.0 formulation, powered with our next generation transposase TnX™, delivers reduced insertion bias and correspondingly better coverage uniformity than its predecessor.

ExpressPlex 2.0 is available in two standard kit configurations, differing in plate formats and throughputs. The ***ExpressPlex 2.0 (96-well) Library Preparation Kit*** provided in a 96-well format supports up to 1536 unique barcodes as combinatorial dual indices (CDIs). It is suitable for automated or manual use, enabling the preparation of as few as 16 samples per batch and up to 96 samples per batch. The ***ExpressPlex 2.0 (384-well) Library Preparation Kit*** provided in a 384-well format supports up to 6144 unique barcodes as CDIs. Manual use and flexible batching are possible, but not recommended for this kit.

Initial Manual-Control Assessment of ExpressPlex 2.0

Prior to developing an automated protocol, seqWell recommends gaining experience with a manual prep, following the protocol described in the ***ExpressPlex 2.0 (96-well) Library Preparation Kit User Guide*** *https://bit.ly/EP2-96-well-UserGuide*. Ideally, identical samples should be used in the manual and automated preparations, to enable direct comparison of outcomes.

When performing a manual preparation, the initial reaction setup is performed slightly differently than described in this document, due to the limited capabilities of manual pipettors as compared with automated liquid-handling instrumentation. The ExpressPlex 2.0 (96-well) User Guide should be followed for manual preparation. Nevertheless, outcomes should be comparable for manual or automated preparations, and any discrepancies are from irregularities in liquid handling.

For ***ExpressPlex 2.0 (384-well)*** customers, we recommend experimenting with manual and automated controls comparison in 96-well format, before embarking on 384-well format work.

ExpressPlex 2.0 (96-well)

Workflow Diagram for Liquid Handlers - *EP Green*

When performed on an automated liquid handler, the ExpressPlex 2.0 (96-well) protocol requires three steps per sample prior to placing the plate onto the thermocycler: (1) an aspirate of 4 µL of DNA template, (2) an aspirate of 4 µL of Indexing Reagent into the same tips, and (3) a dispense of 8 µL with tip mixing into the Ready Reaction Plate. Library construction is completed by sealing the plate and running the tagmentation and amplification reactions in a thermocycler.

Post-thermocycling, all samples are pooled column-wise and then combined into one tube. A single SPRI cleanup is performed, followed by library quantification, sizing, and loading onto the sequencer. (Figure 1.)

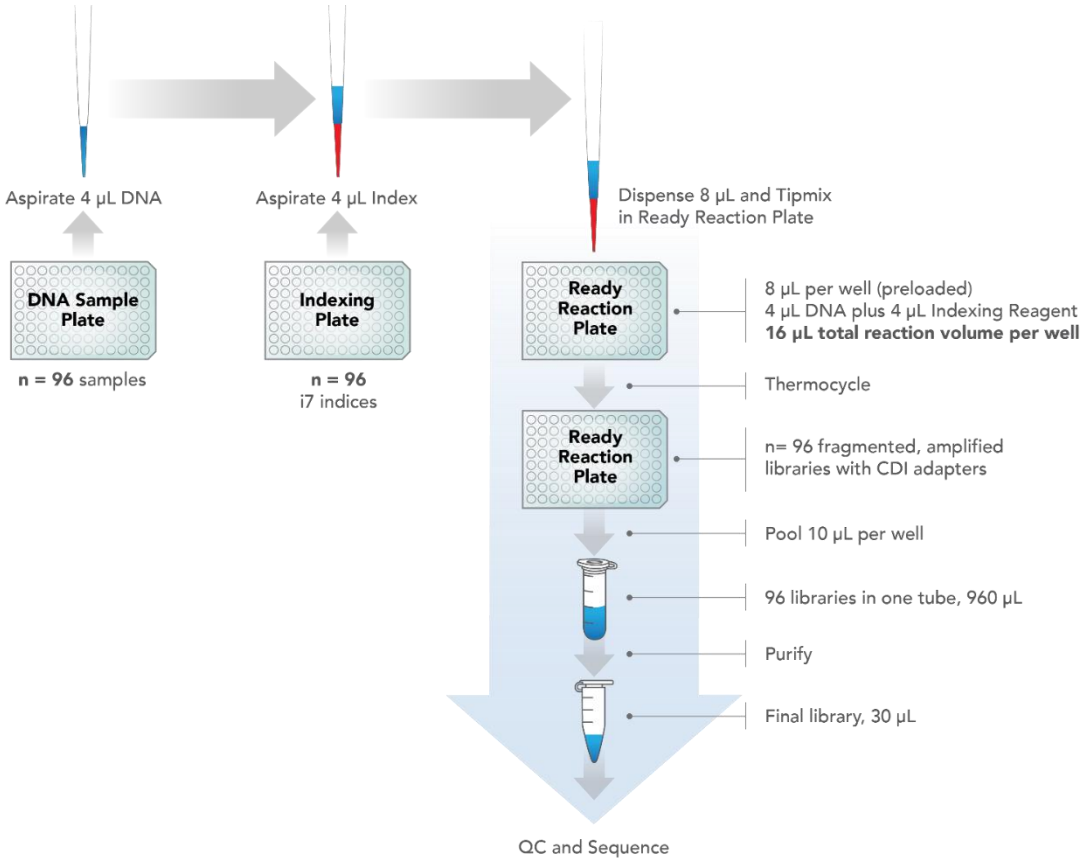


Figure 1 - ExpressPlex 2.0 (96-well) - *EP Green Workflow*. An automated liquid handler combines template DNA, Indexing Reagent, and Ready Reaction Mix. Tagmentation and library amplification occur in an off-deck thermocycler, freeing the liquid handler for other tasks. After thermocycling, the liquid handler pools 96 libraries column-wise. If possible, on the respective liquid handler, the column is pooled by probe into a single tube, otherwise pooled manually. Then, SPRI purification is most efficiently performed manually.

Developing Automated Protocols the ExpressPlex 2.0 (96-well) Library Preparation Kit

General recommendations for developing automated protocols for library setup and pooling for the ExpressPlex 2.0 (96-well) Library Preparation Kit include:

- Use filter tips throughout the setup and pooling portions of the protocol.
- All volumes are “volumes to deliver”, i.e. volumes actually transferred to their destinations. Since some automated pipettors systematically under-dispense, it is usually necessary to apply an empirically-determined scale factor to the intended transfer volumes, perhaps as large as 1.4 for small volumes.
- Parameters such as pipetting heights, pipetting rates, etc, which are shown below are not intended to be prescriptive and may not even be optimal, but they are parameters which have been successfully used in one or more implementations of the protocol.
- Once the protocol is written, evaluate it stepwise: initial runs can be dry, followed by runs with liquid.
- Refine liquid handling and volume transfers with mock reagents, e.g. 50% glycerol in water as a proxy for Indexing Reagent and Ready Reaction Mix, and tinted water (e.g. 0.5% food coloring in water) as a surrogate for DNA template.
- Confirm that the final reactions are uniformly mixed (i.e. uniformly tinted) and contain minimal bubbles so the plate can be sealed and placed in the thermocycler without centrifuging.
- Products of the library setup step are nearly ideal mock reagents for the pooling step.
- Confirm that all wells of the Ready Reaction Plate are equally depleted after the pooling step.
- Confirm that the volume of the final pooled libraries is close to 960 µL (within 10%).
- If performing the SPRI cleanup directly in the primary tube of pooled libraries, there is no need to vortex prior to adding the SPRI reagent, because you will mix thoroughly after adding the SPRI reagent at the start of the DNA binding step.
- If an aliquot of the pooled libraries will be removed for SPRI cleanup, cap, vortex, and centrifuge the tube of pooled libraries prior to removal, to ensure that all individual libraries are equally represented in the final pool.

Detailed procedures for automated 96-well library setup and pooling may be found in Appendix A.

ExpressPlex 2.0 (384-well)
Workflow Diagram for Liquid Handlers

ExpressPlex 2.0 (384-well) differs from ExpressPlex 2.0 (96-well) in that it is a half-scale version, and thus the liquid handling has been modified to avoid transferring very small volumes.

When performed on an automated liquid handler, the ExpressPlex 2.0 (384-well) protocol requires four steps per sample prior to placing the full plate onto the thermocycler: (1) an aspirate of 4 µL of DNA template, (2) a transfer of that sample to the Indexing Plate with tip mixing, (3) an aspirate of 4 µL of the combined DNA template and Indexing Reagent into the same tips, and (4) a dispense of 4 µL with tip mixing into the Ready Reaction Plate. Library construction is completed by sealing the plate and running the tagmentation and amplification reactions in a thermocycler.

After thermocycling, samples are pooled first by quadrant into a fresh 96-well plate, then pooled column-wise, and finally combined into one tube. A single SPRI cleanup per 384-well plate is performed, followed by library quantification, sizing, and loading onto the sequencer. (Figure 2.)

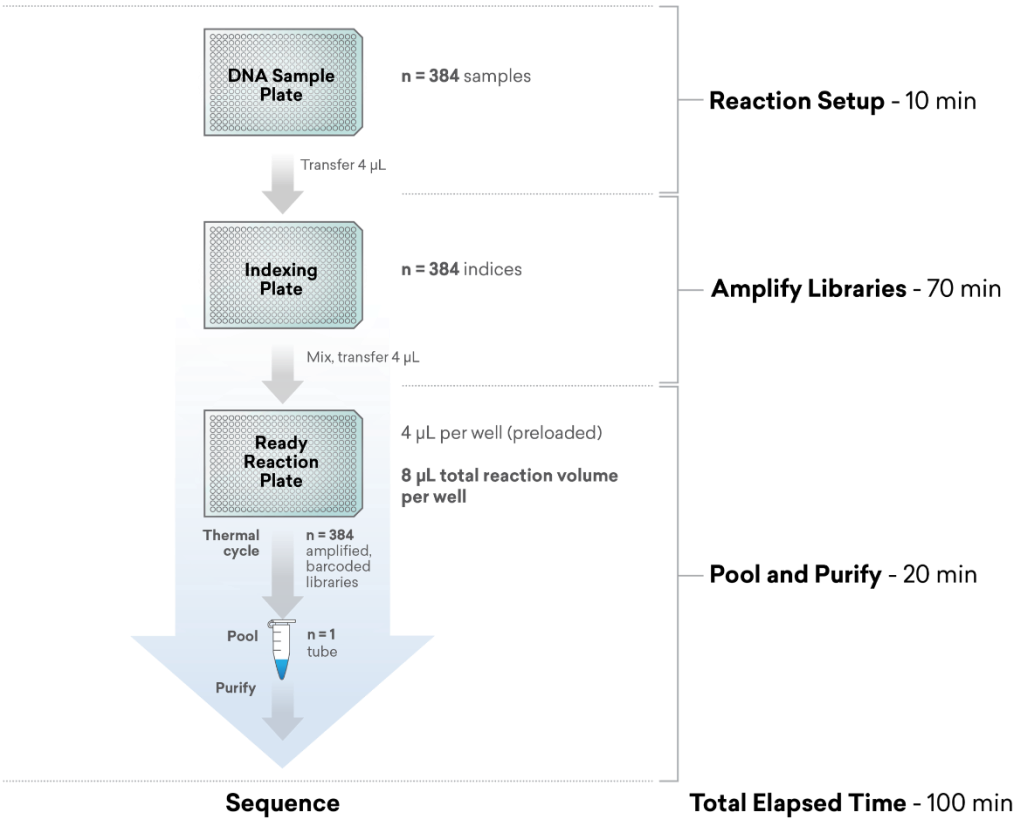


Figure 2 - ExpressPlex 2.0 (384-well) Workflow. An automated liquid handler combines template DNA, Indexing Reagent, and Ready Reaction Mix, either by quadrant or with the use of a native 384-mandrel pipetting head. Tagmentation and library amplification occur in an off-deck thermocycler, freeing the liquid handler for other tasks. After thermocycling, the liquid handler pools by quadrant into a fresh 96-well plate and then column-wise. If possible, on the respective liquid handler, the column is pooled by probe into a single tube, otherwise pooled manually. Then, the SPRI purification is most efficiently performed manually.

Developing Automated Protocols for the ExpressPlex 2.0 (384-well) Library Preparation Kit

General recommendations for developing automated protocols for library setup and pooling for the ExpressPlex 2.0 (384-well) Library Preparation Kit are similar to those for the ExpressPlex 2.0 (96-well) Library Preparation Kit, with the following additional guidance:

- Library setup may be performed using a 384-well multichannel pipetting head in one pass, or with a 96-well multichannel pipetting head by quadrant, requiring four passes per 384-well plate.
- Prior to thermocycling it is better to seal the Ready Reaction Mix plate with adhesive rather than thermal seals, as heat sealing can reduce the diameter of the well openings and make it more difficult to access the bottoms of the wells with automation tips.
- A rapid and effective pooling strategy is to use the 96-multichannel pod to pool all four quadrants into a fresh 96-well plate, and then to use the column-based pooling method described for the ExpressPlex 2.0 (96-well) Library Preparation Kit to complete the process.

Detailed procedures for automated 384-well library setup and pooling may be found in Appendix B.

Performance Assessment of the ExpressPlex 2.0 Chemistry with Plasmid DNA

A key performance metric of the ExpressPlex 2.0 kits is the uniformity of read counts across a sample set, because reliable representation of all samples allows sequencing runs to be optimized for capacity. Read-count uniformity is best assessed by sequencing multiple replicates of the same DNA of constant concentration. Using constant-concentration inputs also allows for the identification of any plate effects that might arise due to irregularities in liquid handling.

Another performance metric intrinsic to the ExpressPlex 2.0 kits is the robustness of read-count uniformity when challenged with samples of varying concentration, also referred to as the kits' auto-normalization property. Auto-normalization is best assessed by sequencing replicates of the same DNA at variable concentration within the range allowed (0.25-10ng/µl).

Sample Input

There are any number of sample configurations that could be employed to assess the uniformity and robustness of read counts. For convenience we employ a single 96-well plate of pUC19 plasmid samples with:

- fixed DNA inputs across the plate, to evaluate uniformity in read outputs and plate-wise effects
- variable DNA inputs ranging fivefold in concentration, to evaluate auto-normalization.

To construct our DNA test plate, we dilute and distribute pUC19 plasmid DNA (New England Biolabs, Ipswich, MA) as shown in Figure 3 (concentrations in ng/µl). NEB indicates that quantification of their pUC19 product should be done with absorbance for accuracy, due to their internal QC measures of absorbance and the supercoiled nature of the plasmid. pUC19 should be accurately quantified by absorbance by running a serial dilution down to 50ng/µl to generate a stock sample for further dilution. The 50ng/µl stock should then be confirmed by absorbance in triplicate and a true actual concentration should be assigned. Subsequently, the stock should be used to further dilute to the concentrations used in this experimental plan below. Absorbance quantification is inaccurate below 10ng/µl, and should not be used below this threshold.

	1	2	3	4	5	6	7	8	9	10	11	12
A	4	4	4	4	4	4	2	2	2	2	2	2
B	4	4	4	4	4	4	4	4	4	4	4	4
C	4	4	4	4	4	4	4	4	4	4	4	4
D	4	4	4	4	4	4	6	6	6	6	6	6
E	4	4	4	4	4	4	6	6	6	6	6	6
F	4	4	4	4	4	4	8	8	8	8	8	8
G	4	4	4	4	4	4	8	8	8	8	8	8
H	4	4	4	4	4	4	10	10	10	10	10	10

Figure 3 - Configuration of the DNA test plate (ng/µL). Fixed-input pUC19 plasmid DNA (double stranded, 2686 bp) comprises columns 1 through 6 and also all of rows B and C of the test plate, allowing assessment of fixed-input read count across the plate, for any possible plate effects. Varying concentrations of DNA comprise columns 7 through 12 of the test plate, enabling the evaluation of auto-normalization of read counts.

In a full verification or validation run, a sample plate such as the one proposed above could serve as input for an initial manual control run, an automated ExpressPlex 2.0 (96-well) run, and even a by-quad run of ExpressPlex 2.0 (384-well). Alternatively, a simple fixed input plate at 4ng can be used for Express 2.0 (384-well) in either 96-well or 384-well, for a bit easier handling.

Since 4 ul of DNA template is used in each preparation, the total mass of DNA (ng) in each well is:

	1	2	3	4	5	6	7	8	9	10	11	12
A	16	16	16	16	16	16	8	8	8	8	8	8
B	16	16	16	16	16	16	16	16	16	16	16	16
C	16	16	16	16	16	16	16	16	16	16	16	16
D	16	16	16	16	16	16	24	24	24	24	24	24
E	16	16	16	16	16	16	24	24	24	24	24	24
F	16	16	16	16	16	16	32	32	32	32	32	32
G	16	16	16	16	16	16	32	32	32	32	32	32
H	16	16	16	16	16	16	40	40	40	40	40	40

Figure 4 - Configuration of the DNA test plate (total mass of DNA, ng). The per-well mass ranges from 8 to 40 ng, with the majority of wells containing 16 ng.

Library Construction

For this effort, we used the sample plate described above for:

1. ExpressPlex 2.0 (96-well) manual control
2. ExpressPlex 2.0 (96-well) run automated on an SPT Labtech apricot S3
3. ExpressPlex 2.0 (96-well) run automated on an SPT Labtech firefly
4. ExpressPlex 2.0 (96-well) run automated on a Tecan Fluent
5. ExpressPlex 2.0 (96-well) run automated on a Biomek FX^P
6. ExpressPlex 2.0 (96-well) run automated on a Complete Genomics MGISP-960.

Additionally, we used a simple fixed-input (1.25 ng/µL) pUC19 plate (not shown) for an ExpressPlex 2.0 (384-well) run on the same Tecan Fluent utilized in (4).

The manual control run was performed as described in the ***ExpressPlex 2.0 (96-well) Library Preparation Kit User Guide***, and the automation methods were configured as described in this document, in the **Developing Automated Protocols** sections pertinent to each of the ExpressPlex 2.0 96-well and 384-well sections (Appendix A and Appendix B respectively).

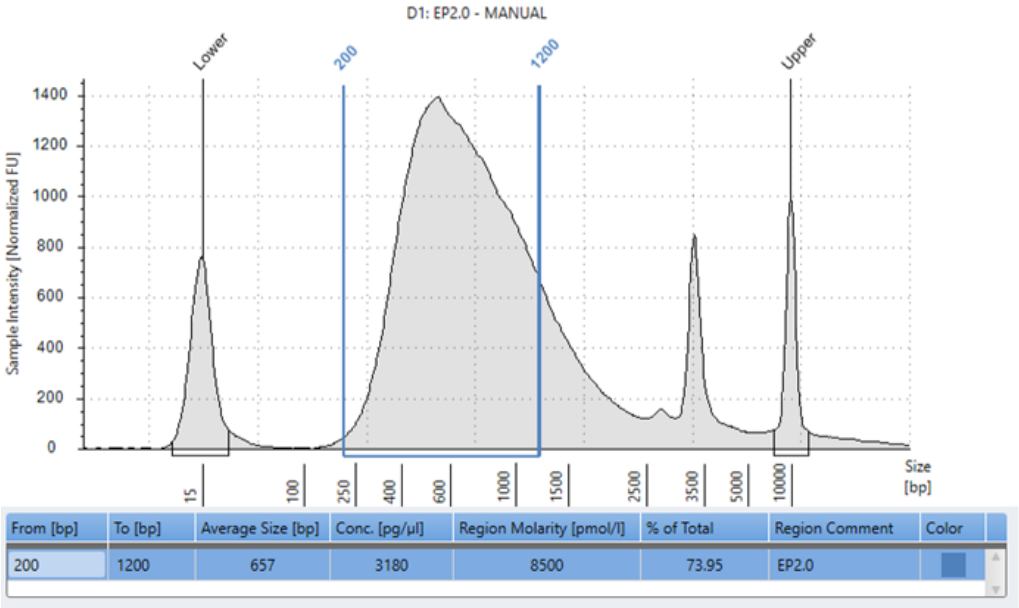
Purification

After pooling, libraries were purified in a conventional SPRI cleanup protocol using a 0.75 X ratio of seqWell's MAGwise paramagnetic beads to pool volume, 720 µL MAGwise to the 960 µL pool, as described in the pertinent user guides.

Outcomes of the Manual and Automated Library Preparations

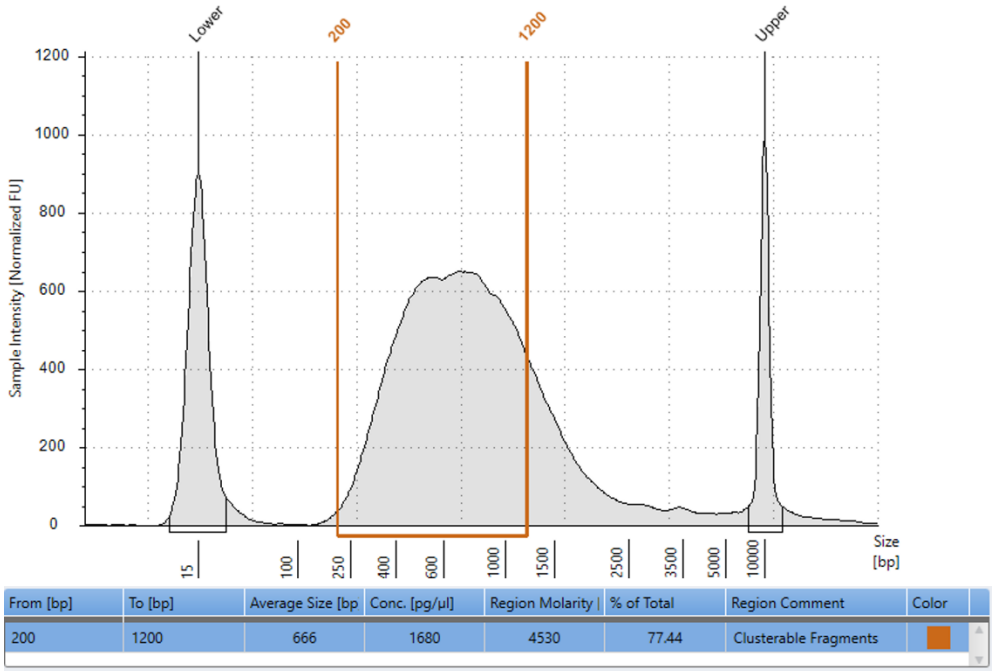
Electrophoresis

After SPRI purifications, fragment size distributions were characterized using the Agilent TapeStation 4200 and the High Sensitivity D5000 ScreenTape System. A region of sequenceable (i.e. clusterable) library (200-1200bp) should be added to the region table in the analysis software. Key metrics provided by the TapeStation Analysis Software include the average fragment size within the sequenceable region (200 – 1200 bp), concentration (pg/µl) within the sequenceable region, and the percentage of the total mass contained within the sequenceable region. See Figure 5 for an electropherogram of the manual control libraries prepared using the DNA test plate described above, and Figure 6 for an electropherogram of libraries prepared with a fixed-input plate.



From [bp]	To [bp]	Average Size [bp]	Conc. [pg/µl]	Region Molarity [pmol/l]	% of Total	Region Comment	Color
200	1200	657	3180	8500	73.95	EP2.0	

Figure 5 - Electropherogram of the Manual Control library created for this analysis. Sequenceable library (200-1200 bp) comprises over 73% of the total DNA in this sample, with an average library size of 657 bp. The peak at ~3500 bp derives from input pUC19 plasmid, untouched by transposons, which migrates more slowly than a linear fragment of the same molecular weight. The small peak at ~2700 bp represents pUC19 which has been cleaved by a single transposon, and which migrates as a linear fragment. Neither of these will anneal to the sequencing flow cell. The average input is above the recommended 5ng, but all within range, and sequencing quality is not impacted. See Figure 6 and Appendix C for more information.



From [bp]	To [bp]	Average Size [bp	Conc. [pg/µl]	Region Molarity	% of Total	Region Comment	Color
200	1200	666	1680	4530	77.44	Clusterable Fragments	

Figure 6 - Electropherogram of an ExpressPlex 2.0 library created from a fixed-input sample plate. In this case, sample inputs (4ng) are near recommended average input (5ng) for all wells, and only small traces of uncleaved and singly-cleaved pUC19 remain.

Quantification for Molarity

Concentration of the sequenceable region on the TapeStation should be used to calculate molarity of the library, taking into account the library dilution made for the TapeStation. For example, in Figure 6, the concentration of the library in the sequenceable range is 4530 pMol/L, or 4.53 nMol/L, and the pre-electrophoresis dilution is fortyfold, so the concentration of the undiluted library is ~181 nM. On this basis, libraries can be diluted to an appropriate loading concentration, often 4 nM in 10 mM Tris-HCl pH 8.

Final Preparation for Sequencing

Aliquots of each library are pooled equimolar and sequenced on either a NextSeq™ 2000 or a MiSeq™ (Illumina, San Diego, CA).

Sequencing Outcomes: Read Count Data

Samples were demultiplexed and sorted by their associated indexes. Raw read counts were organized into their original plate format, converted to per-well percentages of sequencing reads, and rendered as color-gradient images. Data for manual-control and SPT Labtech apricot S3 samples in 96-well format are shown in Figure 7, below. Despite the challenge of variable inputs in columns seven through twelve, sample counts are uniform across the plate.

EP 2.0 - Manual Control - 96-well

	1	2	3	4	5	6	7	8	9	10	11	12	% of Total
A	1.00	0.96	0.93	1.03	1.08	0.98	1.09	1.06	1.02	1.06	1.07	1.07	1.4
B	1.16	1.00	1.10	1.06	1.03	0.96	0.90	1.13	0.97	1.10	1.20	1.09	1.3
C	0.89	1.05	1.10	1.10	1.00	1.07	1.04	1.08	1.15	1.09	1.04	1.18	1.2
D	1.06	1.10	0.94	1.13	1.04	1.07	0.94	1.08	1.13	1.07	1.08	1.01	1.1
E	1.01	1.07	0.98	1.18	1.01	1.04	1.13	1.11	0.93	1.10	1.14	1.00	1.0
F	1.10	1.08	0.94	0.98	0.89	1.11	0.98	1.00	0.93	1.07	1.08	1.02	0.9
G	0.97	1.05	1.08	1.06	0.97	1.11	0.98	1.08	0.98	1.06	1.10	0.96	0.8
H	0.86	1.08	1.02	1.17	1.00	1.10	1.03	1.00	1.05	1.01	0.95	0.98	0.7

EP 2.0 - Automated Apricot S3 - 96-well

	1	2	3	4	5	6	7	8	9	10	11	12	% of Total
A	1.34	0.85	0.95	1.22	0.96	0.86	1.35	1.15	1.15	1.18	1.26	1.20	1.4
B	1.40	0.92	1.33	1.20	1.02	1.06	0.97	1.21	1.05	0.94	1.20	1.02	1.3
C	0.82	0.90	1.21	0.94	0.91	1.03	1.04	1.09	0.99	1.02	0.80	1.30	1.2
D	1.06	1.26	0.88	1.09	1.23	0.93	0.90	0.83	1.09	1.15	1.04	1.16	1.1
E	1.01	1.06	1.10	1.19	0.89	1.08	1.14	1.30	0.96	0.96	1.09	1.17	1.0
F	1.27	1.19	0.91	0.83	0.70	1.07	0.85	0.98	0.97	1.01	1.08	1.24	0.9
G	1.07	0.90	1.17	1.29	0.87	0.81	0.77	0.96	0.93	1.03	1.04	0.86	0.8
H	0.90	1.26	0.90	1.19	1.12	1.18	0.83	0.67	0.88	0.90	0.93	1.05	0.7

Figure 7 - Color-gradient maps of per-well percentage of reads for the Manual Control and SPT apricot S3 libraries created for this analysis. In a perfectly balanced run, each well would represent 100% / 96, or 1.04%, of the total number of reads. Reads are uniform for each of these libraries, with coefficients of variation (CV) of 6.8% and 15.3% respectively, despite the challenge of variable inputs (from 8 to 40 ng) in columns seven through twelve.

Visualized in a box plot, the medians and distributions about the medians of the read-balance data for 96-well libraries prepared manually and using the SPT Labtech ***apricot S3*** and ***firefly***, Tecan ***Fluent***, Beckman Coulter ***Biomek FX^P***, and Complete Genomics ***MGISP-960*** are shown in Figure 8.

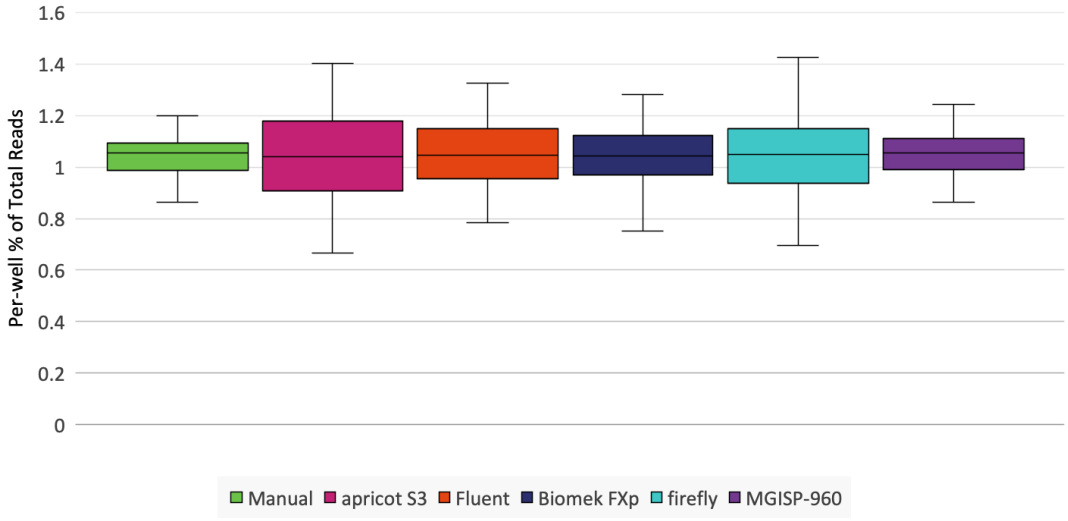


Figure 8 - Box plot of read balance data acquired for all 96-well libraries prepared for this analysis. Medians cluster near the expected mean of 1.04%, and the heights of the interquartile range, in which 50% of the data points occur, scale with the calculated CV.

Read Count Data for ExpressPlex 2.0 (384-well)

Samples were demultiplexed with associated indexes. Raw read counts for automated library preparations were organized into their original plate format and rendered as color-gradient maps. An example is shown in Figure 9, below.

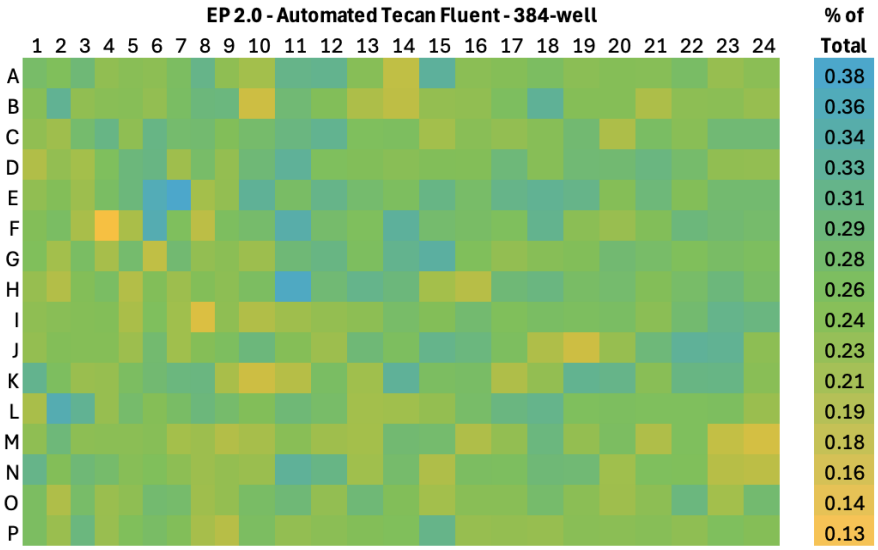


Figure 9 - A color-gradient map of per-well reads for an ExpressPlex 2.0 (384-well) run created on the Tecan Fluent using fixed inputs (5 ng pUC19 per well). In a perfectly balanced run, each well would represent 100% / 384, or .26%, of the total number of reads. Read counts are uniform and consistent: the CV for the entire 384-well plate is 14.2%, and the by-quadrant CVs are 13.2%, 14.9%, 14.1%, and 15.0%.

Acceptance Criteria for ExpressPlex 2.0 (96-well) Libraries

seqWell's quality-control metrics for ExpressPlex 2.0 (96-well) libraries are intended to set standards for library yield and quality prior to sequencing, and to set standards and expectations for read-count balance in the final sequencing outcomes.

Pre-sequencing criteria are assessed by electrophoresis, and include:

- adequate yield (library concentration ≥ 30 nM)
- appropriate fragment-size distribution such that ≥ 50% of the library molecules are between 200bp and 1200 bp in size so they cluster efficiently on the flow cell.

Post-sequencing criteria include well-to-well read count uniformity, specifically that:

- coefficient of variation of read counts is ≤ 20% across all samples
- constraints on extreme values, specifically that the ***range*** (the average of the three highest wells divided by the lowest of the three wells) is < 2.5
- constraint on minimum and maximum read counts, such that the minimum read count must be > 30% of the average, and that the maximum read count must be < 160% of the average.

See Table 1 for pre-sequencing and post-sequencing outcomes attained by each of the automation platforms evaluated, along with manual data.

EP2.0 Validated Automated Methods Specs						
	Pre-Sequencing		Post-Sequencing			
Liquid Handler	Concentration >30nM	>50% mass in 200-1200bp	<20% CV	<2.5 fold range	Min read count >30% of average	Max read count <160% of average
Manual	170	73.95	6.8	1.34	82	115
SPT Labtech apricot s3	121.4	64.23	15.3	1.91	64	134
Tecan Fluent	98	69.3	11.3	1.59	72	123
Beckman Coulter Biomek FX^P	142.4	75.2	12	1.65	75	127
SPT Labtech firefly	131.2	77.12	13.9	1.83	67	137
Complete Genomics MGISP-960	100	78	8.78	1.55	72	119

Table 1 - Pre- and post-sequencing metrics for libraries created by manual and automated means. Green shading in each cell indicates that the datum obtained for the corresponding instrument (or operator, in the case of the manually-derived libraries) meets or exceeds specifications. Fold range is calculated by dividing the average of the three highest read counts by the average of the three lowest read counts.

Acceptance Criteria for ExpressPlex 2.0 (384-well) Libraries

Pre-sequencing criteria include adequate yield such that:

- each 384-plex library concentration ≥ 25 nM
- appropriate fragment-size distribution such that ≥ 50% of the library molecules are between 200bp and 1200 bp in size so they cluster efficiently on the flow cell.

Post-sequencing criteria include well-to-well read count uniformity, specifically that:

- coefficient of variation of read counts is ≤ 20% across all samples.

ExpressPlex 2.0 (384-well) libraries are often prepared "by quadrant". To facilitate quadrant-to-quadrant comparisons, read counts may be expressed as per-well percentages of the total number of reads in each quadrant rather than as absolute counts. Well-to-well read count uniformity should also be assessed by quandrant, specifically that:

- coefficient of variation of read counts is ≤ 20% across the full quadrant.

ExpressPlex 2.0 (384-well) data on the Tecan Fluent shown in Figure 9 exceed all acceptance criteria. The library molecules measured by Agilent TapeStation between 200bp and 1200bp are 76.2% of the total. The coefficient of variation (CV) for the entire 384-well plate is 14.2%, and the by-quadrant CVs are 13.2%, 14.9%, 14.1%, and 15.0%.

Batching and Pooling Strategies

Using the ExpressPlex 2.0 (96-well) Library Preparation Kit, effective automation, and sufficient thermocyclers, a single user can easily prepare 1,536 total libraries and take them through sequencing in approximately 24 hours. One possible scenario divides the 16 plates comprising 1536 samples into two cohorts of 8 plates apiece, and staggers the library preparation, thermocycling, and pooling steps to maximize instrument and thermocycler usage. (See Figure 10.) To expedite the library preparation process, multiple 96-well plates can be set up for sequencing in a single automation run. Similarly, multiple plates may be pooled in an automated pooling run.

Note that seqWell *does not recommend the use of on-deck thermocyclers for these protocols*, as the reaction setup and pooling times are brief, and incubations on a thermocycler are best performed on stand-alone device to free up the liquid handler.

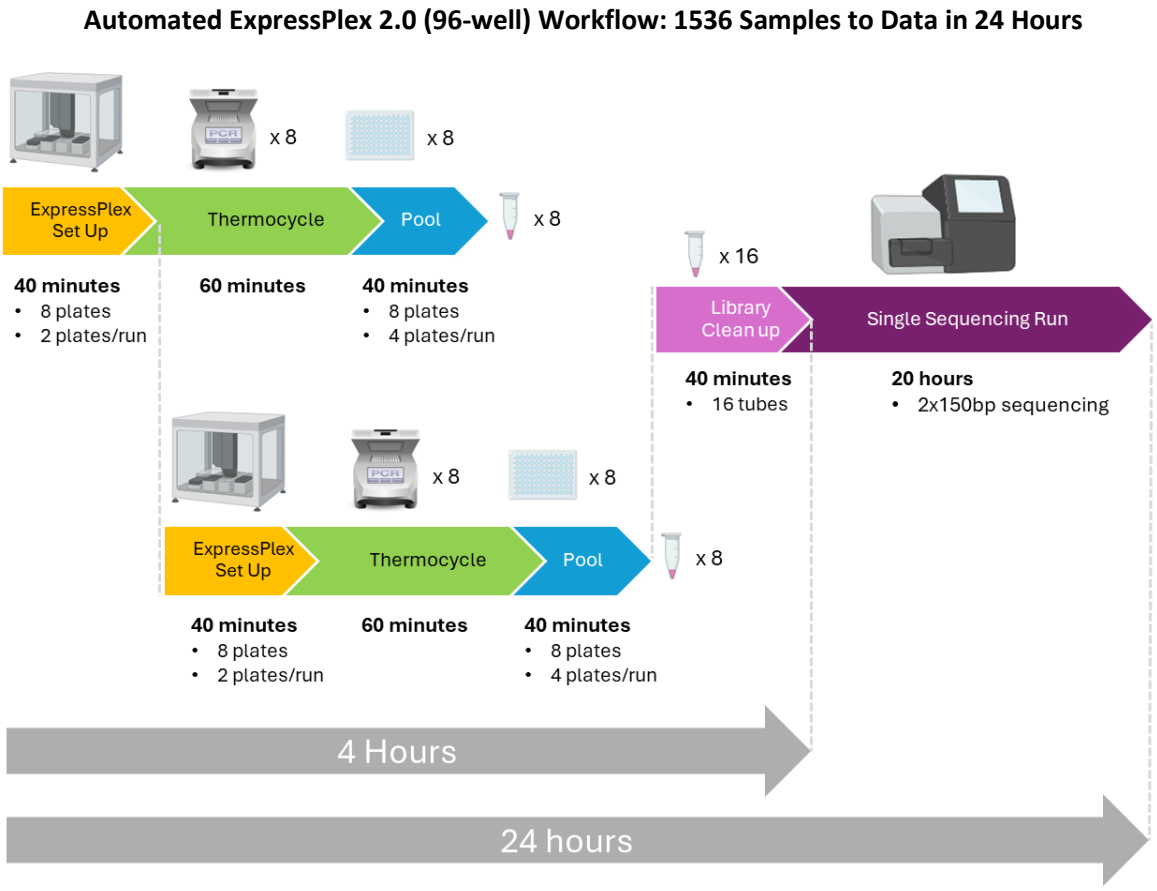


Figure 10 - A workflow diagram corresponding to the 8-thermocycler strategy described in the text. Reaction setup occurs in groups of 8 plates: while the first group cycles the second group is prepared. After pooling, all libraries may be purified, quantified, and then loaded onto the sequencer.

ExpressPlex 2.0 (384-well) in Ultra-High-Throughput Applications

Using contact-free dispensing and miniaturized reactions it is possible to achieve economies of scale and ultra high-throughput workflows, multiplexing as many as all 6144 indices of the ExpressPlex 2.0 (384-well) kit in a single sequencing run. Contact seqWell at support@seqWell.com for more information.

Appendix A - Detailed Procedures for Library Setup and Pooling for the ExpressPlex 2.0 (96-well) Library Preparation Kit

Library setup

Initialize

- Load tips;
- Aspirate a small air gap, e.g. 0.5 µL, to minimize the introduction of bubbles which will be amplified during tip mixing.

Aspirate DNA

- Move to ~ 1 mm from the bottom of the wells of the DNA plate;
- Aspirate a nominal 4 µL of DNA at 4 µL/second;
- Pause briefly, e.g. 500 millisecond;
- Retract slowly, e.g. at 10 mm/second.

Aspirate Indexing Reagent (identical to preceding)

- Move to ~ 1 mm from the bottom of the wells of the Indexing Plate;
- Aspirate a nominal 4 µL at 4 µL/second;
- Pause briefly, e.g. 500 milliseconds;
- Retract slowly, e.g. at 10 mm/second.

Dispense to Ready Reaction Plate and mix

- Move to ~ 2 mm from the bottom of the wells of the Ready Reaction Plate;
- Dispense a nominal 8 µL at 100 µL/second;
- Loop for 10 tip mixes
    - Pause briefly, e.g. 250 milliseconds;
    - Aspirate a nominal 8 µL at 100 µL/second;
    - Pause briefly, e.g. 250 milliseconds;
    - For the first 9 tip mixes
        - Dispense a nominal 8 µL at 200 µL/second;
    - For the last tip mix
        - Dispense a nominal 8 µL at 4 µL/second;
- Move above the liquid;
- Pause briefly, e.g. 250 milliseconds;
- Empty tips slowly, e.g. 2uL/second;
- Move just under the liquid level slowly, e.g. at 4 mm/second;
- Pause briefly, e.g. 250 milliseconds;
- Move out of the well slowly, e.g. at 5 mm/second.

Finalize

- Unload tips.

Perform thermocycling

- Seal the plate and run the tagmentation and amplification reactions in a thermocycler as specified in the ExpressPlex 2.0 (96-well) Library Preparation Kit User Guide.

Pool libraries into a single tube

1. *Detailed procedure for library pooling when the automation platform supports pipetting with independent probes*

Initialize
- Load a single column of tips;
- Aspirate a moderate air gap, e.g. 20 µL.

Aspirate column-wise
- For each column
    - Move near the bottom of the well, e.g. 2 mm above;
    - Aspirate a nominal 10 µL slowly, e.g. at 3 µL/second;
    - Pause briefly, e.g. for 1000 milliseconds;
    - Retract slowly, e.g. at 5 mm/second.
- Aspirate a small trailing air gap slowly, e.g. 2 µL at 5 µL/second.

Dispense into a single tube
- For all probes
    - If probe 1
        - Move to 10 mm above the bottom of the tube;
    - Otherwise
        - Move to 10 mm above the liquid;
    - Dispense the trailing air gap plus a nominal 120 µL moderately, e.g. at 50 µL/second;
    - Pause briefly, e.g. for 1000 milliseconds;
    - Empty tips slowly, e.g. at 5 µL/second, to clear the initial air gap;
    - Move just under liquid level;
    - Pause briefly, e.g. for 500 milliseconds;
    - Move out of the liquid moderately, e.g. at 10 mm/second;
    - Aspirate a moderate air gap, e.g. 10 µL.

Finalize
- Unload tips.

2. *Detailed procedure for library pooling when the automation platform does not support independent probes but does allow a single column of tips to be loaded on a multichannel pod*

Initialize

- Load a single column of tips;
- Aspirate a moderate air gap, e.g. 20 µL.

Aspirate Column-wise

- For each column
    - Move near the bottom of the well, e.g. 2 mm above;
    - Aspirate a nominal 10 µL slowly, e.g. at 3 µL/second;
    - Pause briefly, e.g. for 1000 milliseconds;
    - Retract slowly, e.g. at 5 mm/second.

Dispense into a Single Column of a fresh plate

- Move to 1 mm above the bottom of the wells;
- Dispense the trailing air gap plus a nominal 120 µL moderately, e.g. at 50 µL/second, tracking to follow liquid level;
- Pause briefly, e.g. for 1000 milliseconds;
- Move above the liquid level slowly, e.g. 5 mm/second;
- Empty tips slowly, e.g. at 5 µL/second, to clear the initial air gap;
- Move just under liquid level;
- Pause briefly, e.g. for 500 milliseconds;
- Move out of the liquid moderately, e.g. at 10 mm/second.

Finalize

- Unload tips.

At this point the 8 sub-pools will need to be pooled manually into the final 2 mL tube for SPRI cleanup.

Perform SPRI cleanup

- As specified in the ExpressPlex 2.0 (96-well) Library Preparation Kit User Guide.

Appendix B - Detailed Procedures for Library Setup and Pooling for the ExpressPlex 2.0 (384-well) Library Preparation Kit

Library setup

The following operations occur once per plate if pipetting with a 384-channel pipetting head, or four times per plate if pipetting with a 96-channel pipetting head.

Initialize

- Load tips;
- Aspirate a small air gap, e.g. 0.5 µL, to minimize the introduction of bubbles which will be amplified during tip mixing.

Aspirate DNA

- Move to ~ 1 mm from the bottom of the wells of the DNA plate;
- Aspirate a nominal 4 µL of DNA at 4 µL/second;
- Pause briefly, e.g. 500 millisecond;
- Retract slowly, e.g. at 10 mm/second.

Dispense to Indexing Plate and mix

- Move to ~ 1.5 mm from the bottom of the wells of the Indexing Plate;
- Dispense a nominal 4 µL at 150 µL/second;
- Loop for 15 tip mixes
    - Pause briefly, e.g. 1000 milliseconds;
    - Aspirate a nominal 4 µL at 100 µL/second;
    - Pause briefly, e.g. 1000 milliseconds;
    - Move 3 mm higher in the wells;
    - For the first 14 tip mixes
        - Dispense a nominal 4 µL at 20 µL/second;
    - For the last tip mix
        - Dispense a nominal 4 µL at 4 µL/second;
    - Move 3 mm lower in the wells;
- Pause briefly, e.g. 1000 milliseconds;
- Move above the liquid;
- Empty tips slowly, e.g. 2uL/second;
- Pause briefly, e.g. 1000 milliseconds;
- Move just under the liquid level slowly, e.g. at 4 mm/second;
- Pause briefly, e.g. 1000 milliseconds;
- Move out of the well slowly, e.g. at 5 mm/second.

Aspirate an aliquot of DNA and Indexing Reagent

- Move to ~ 2.5 mm from the bottom of the wells of the Indexing plate;
- Aspirate a nominal 4 µL of DNA at 4 µL/second;
- Pause briefly, e.g. 250 millisecond;
- Retract slowly, e.g. at 5 mm/second.

Dispense to Ready Reaction Plate and mix (Identical to *Dispense to Indexing Plate and mix,* above)

- Move to ~ 1.5 mm from the bottom of the wells of the Ready Reaction Plate;
- Dispense a nominal 4 µL at 150 µL/second;
- Loop for 15 tip mixes
    - Pause briefly, e.g. 1000 milliseconds;
    - Aspirate a nominal 4 µL at 100 µL/second;
    - Pause briefly, e.g. 1000 milliseconds;
    - Move 3 mm higher in the wells;
    - For the first 14 tip mixes
        - Dispense a nominal 4 µL at 200 µL/second;
    - For the last tip mix
        - Dispense a nominal 4 µL at 4 µL/second;
    - Move 3 mm lower in the wells;
- Pause briefly, e.g. 1000 milliseconds;
- Move above the liquid;
- Empty tips slowly, e.g. 2uL/second;
- Pause briefly, e.g. 1000 milliseconds;
- Move just under the liquid level slowly, e.g. at 4 mm/second;
- Pause briefly, e.g. 1000 milliseconds;
- Move out of the well slowly, e.g. at 5 mm/second.

Finalize

- Unload tips.

Perform thermocycling

- Seal the plate and run the tagmentation and amplification reactions in a thermocycler as specified in the ExpressPlex 2.0 (384-well) Library Preparation Kit User Guide.

Pool libraries

1. Detailed procedure to pool libraries by quadrant into a 96-well plate

Initialize

- Load a single column of tips;
- Aspirate a small air gap, e.g. 2 µL, to minimize the introduction of bubbles which will be amplified during tip mixing.

The Aspirate Libraries operations occur four times, once per quadrant, using a 96-channel pipetting head.

Aspirate libraries

- Move the tips just above the wells of the thermocycled Ready Reaction plate;
- Move ~2 mm downwards at 0.5 mm/second;
- Move to ~ 3 mm from the bottom of the wells of the thermocycled Ready Reaction plate at ~5 mm/second;
- Aspirate a nominal 4 µL at 4 µL/second;
- Pause briefly, e.g. 1000 millisecond;
- Move just above the wells of the thermocycled Ready Reaction plate at ~2 mm/second.

The Dispense Libraries operation occurs once.

Dispense libraries to 96-well plate and mix

- Move to ~ 1.5 mm from the bottom of the wells of the 96-well pooling plate;
- Dispense a nominal 16 µL at 4 µL/second;
- Pause briefly, e.g. 1000 milliseconds;
- Move above the liquid level at 5 mm/second;
- Pause briefly, e.g. 1000 milliseconds;
- Empty tips at 2 µL/second;
- Move to ~ 1.5 mm from the bottom of the wells of the 96-well pooling plate;
- Loop for 16 tip mixes
    - Pause briefly, e.g. 1000 milliseconds;
    - Aspirate a nominal 11 µL at 100 µL/second;
    - Pause briefly, e.g. 1000 milliseconds;
    - For the first 15 tip mixes
        - Dispense a nominal 11 µL at 200 µL/second;
    - For the last tip mix
        - Dispense a nominal 11 µL at 4 µL/second;
- Pause briefly, e.g. 1000 milliseconds;
- Move out of the well slowly, e.g. at 4 mm/second.

Finalize

- Unload tips.

2. Detailed procedure to pool libraries into a single tube

This is identical to the **Pool libraries into a single tube** procedure described in the workflow for the ExpressPlex 2.0 (96-well) Library Preparation Kit. Please reference the appropriate section (above, in **Developing Automated Protocols for Library Setup and Pooling for the ExpressPlex 2.0 (96-well) Library Preparation Kit**).

Perform SPRI cleanup

- As specified in the ExpressPlex 2.0 (384-well) Library Preparation Kit User Guide

Appendix C - Electropherograms of pUC19 Sample

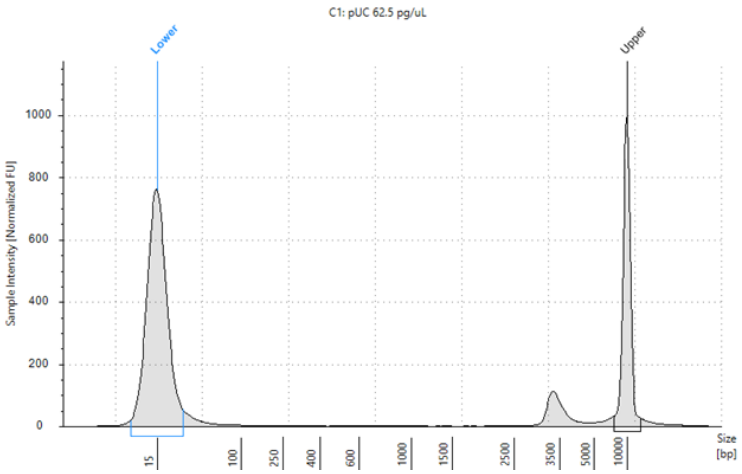


Figure 11 - Electropherogram of pUC19 sample used in this analysis. The peak at ~3500 bp represents pUC19. In this configuration it migrates at a rate slower than a linear 2700-bp molecule (such as a sizing molecule) would migrate under the same conditions.

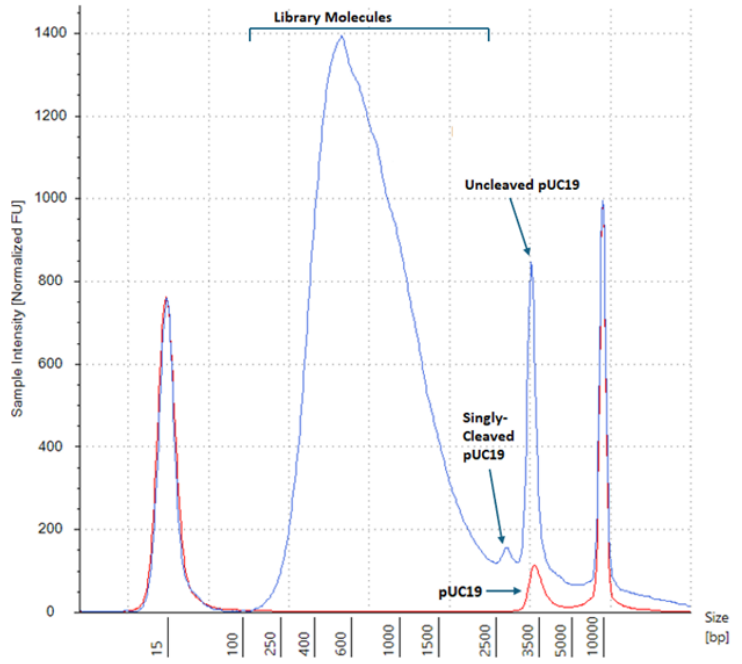


Figure 12 - Electropherogram of manual control library (blue), overlain with pUC19 sample (red). After tagmentation and amplification a peak at ~3500 bp remains, representing unmodified pUC19, and a new peak at ~2700 bp emerges, representing singly-cleaved pUC19 which migrates as a linear molecule. These peaks arise because the transposase is the limiting reagent in the ExpressPlex 2.0 kit, and they derive from the sample wells in the test plate which are at higher concentration than average 1.25ng/µl recommended, although all within range of the kit. The additional peaks have no effect on sequencing metrics since they will not amplify nor cluster.

Version	Release Date	Prior Version	Description of changes
v20250127	**January 27, 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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