Application Note

High-Throughput NGS Library Prep for Plasmids and Amplicons Using the ExpressPlex™ Library Preparation Kit on the Tecan Fluent®

Introduction

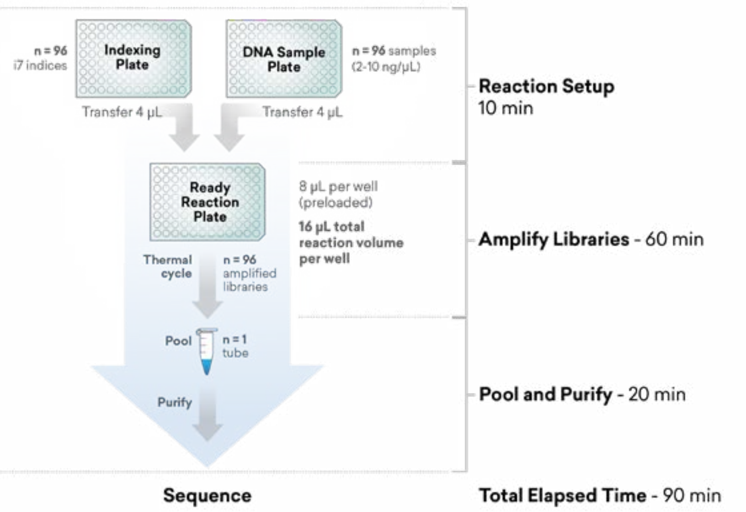
In the field of synthetic biology, the ability to rapidly manufacture large numbers of new constructs and confirm their sequences is of paramount importance. Additionally, to leverage the ever-increasing capacity of sequencing instruments while minimizing the sequencing cost-per-sample, users need to be able to multiplex larger numbers of samples during every run. These dual requirements of reduced turnaround time and higher multiplexing demand highly efficient NGS library preparation methods and reliable, easy-to-implement, automated liquid handling systems. Herein we describe the automation of seqWell's ExpressPlex™ Library Preparation Kit on the Tecan Fluent® liquid handler to create compact and cost-efficient methods for fast screening of synthetic constructs.

ExpressPlex Library Preparation Kit

Introduced in 2023, the ExpressPlex Library Preparation Kit is designed to be the fastest and easiest library prep kit available for creating Illumina-compatible libraries from plasmid or amplicon DNA. Key features of ExpressPlex include:

- 90-minute total time to final library, with only 30 minutes hands-on (for 96 samples)
- Multiplexing up to 1,536 samples in a single run (6,144 barcodes expected early 2024)
- Only 2 reagent additions, followed by a single-tube cleanup step
- Auto-normalization of read counts and insert sizes
- Up to 80% reduction in pipette tips required compared to other methods

ExpressPlex Workflow:



* *Patents Pending*

The ExpressPlex protocol requires only 2 pipetting steps per sample prior to placing the full plate onto the thermocycler. This enables the user to immediately proceed to setting up the next plate. Post-cycling, the user pools all samples from the plate into a single tube and performs one magnetic bead cleanup prior to quantifying the library and loading onto the sequencer. Technicians can easily prepare multiple sets of libraries and begin sequencing in less than half a day, and a high-throughput laboratory can easily go from plasmids or amplicons to sequenced data in 24 hours. However, to take full advantage of the multiplexing available to ExpressPlex users, most will want to incorporate a robotic liquid handling platform to maximize efficiency.

Tecan Fluent Liquid Handler

The Fluent Automation Workstation from Tecan is ideal for laboratories looking for state-of-the-art automation to increase productivity, streamline workflows, optimize assay precision and consistency, and relieve operators from repetitive tasks. This instrument can be found routinely in laboratories throughout the world and is known for its flexibility, ease of use, and high throughput.

ExpressPlex + Fluent

To assess the compatibility of seqWell's library prep solution and the Fluent robotic platform, we created a Fluent method that performs library setup, which is followed by off-deck thermocycling. Post cycling, the plate is moved back to the Fluent instrument for pooling, after which a manual paramagnetic bead cleanup is performed. Final libraries were sequenced on an Illumina MiSeq instrument and data were evaluated for equivalence of read counts across all samples. For comparison purposes, the same samples were prepared and sequenced manually.

Materials and Methods for Automated and Manual Preparation of ExpressPlex Libraries

DNA Template

pUC19 plasmid DNA (New England Biolabs, Ipswich, MA) was diluted to 4 ng/µL and distributed across all wells of a 96-well plate.

Library Construction

Following the ExpressPlex Library Preparation Kit User Guide (seqWell, Beverly, MA), 4 µL of indexing reagent was transferred from each well of an Indexing Reagent Plate to a Ready Reaction Mix Plate, 4 µL of DNA was transferred to all wells of the Ready Reaction Mix Plate, the Ready Reaction Mix Plate was placed on a thermocycler for fragmentation/indexing and amplification, and 10 µL from each well was ultimately pooled into a 2 mL Eppendorf tube.

Post-Pooling Cleanup

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, typically 720 µL MAGwise to 960 µL pooled libraries.

Quantification for Concentration

Final purified libraries were quantified using the Qubit 4 Fluorometer and the Qubit 1X dsDNA HS Assay kit (ThermoFisher Scientific, Waltham, MA). Concentrations are tabulated below.

Library Type	Concentration (ng/µL)	Molarity (nM)
Tecan Fluent	87	179
Manual Control Library	95.8	198

Electrophoresis

Fragment size distributions were characterized using the Agilent TapeStation 4200 and the High Sensitivity D5000 ScreenTape System (Agilent Technologies, Santa Clara, CA). Key metrics provided by the TapeStation Analysis Software include the average fragment size within the range of clusterable (sequenceable) fragment sizes (400 – 1200 bp) and the fraction of the total which lies within the sequenceable range.

Quantification for Molarity

Libraries were diluted to 4 nM in Tris-HCl pH 8.

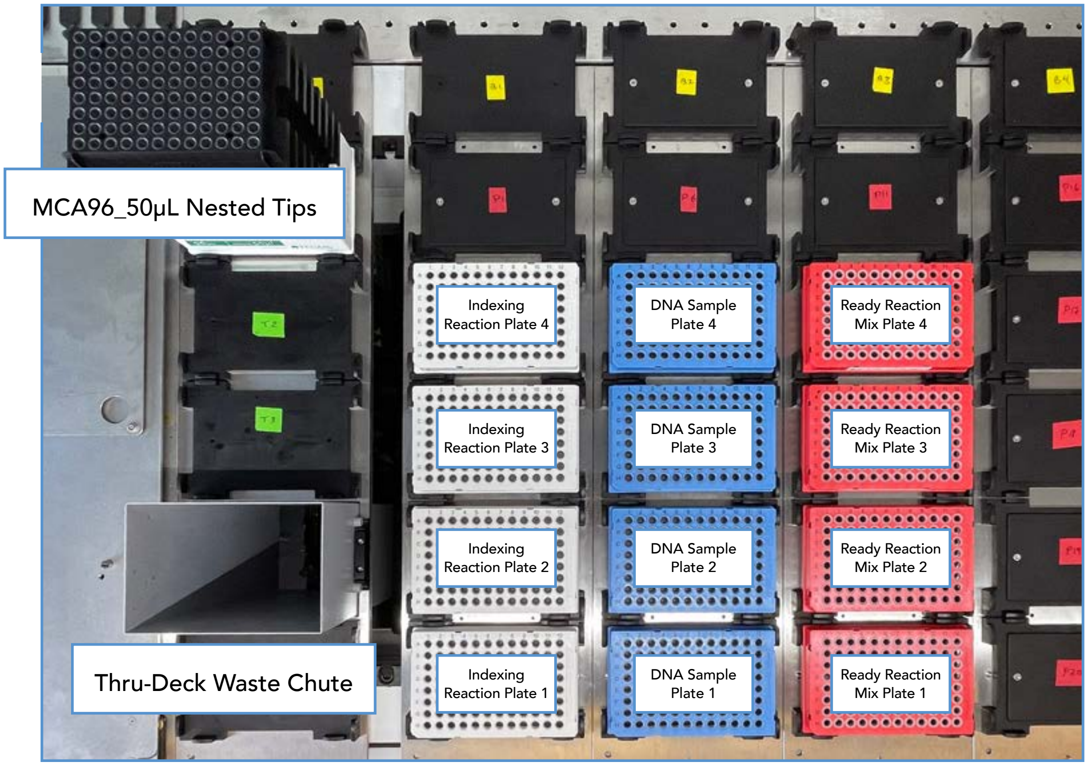
Final Preparation for Sequencing

Aliquots of each 4 nM library were pooled equi-volume and prepared for sequencing following the MiSeq System Denature and Dilute Libraries Guide (Illumina, San Diego, CA). Denatured libraries were diluted to a final loading concentration of 15 pM and sequenced on a MiSeq at 2 X 150 bp using the MiSeq Reagent Kit v2 Micro 300 cycle kit (Illumina).

ExpressPlex Library Preparation on the Fluent

The deck layout for four 96-well plates of ExpressPlex library preparation is shown below.

The deck image below depicts the elements utilized in the automated setup of four plates of ExpressPlex sequencing reactions. The Indexing Reaction Plates and the Ready Reaction Mix Plates are components of the ExpressPlex kit; the DNA Sample Plates are user-supplied plates of template DNA for sequencing; a stack of MCA96_50µL Nested Tips is consumed for each four-plate run; used tips and tip wafers are discarded into the Thru-Deck Waste Chute, shown as a gray rectangle on the lower left of the deck.

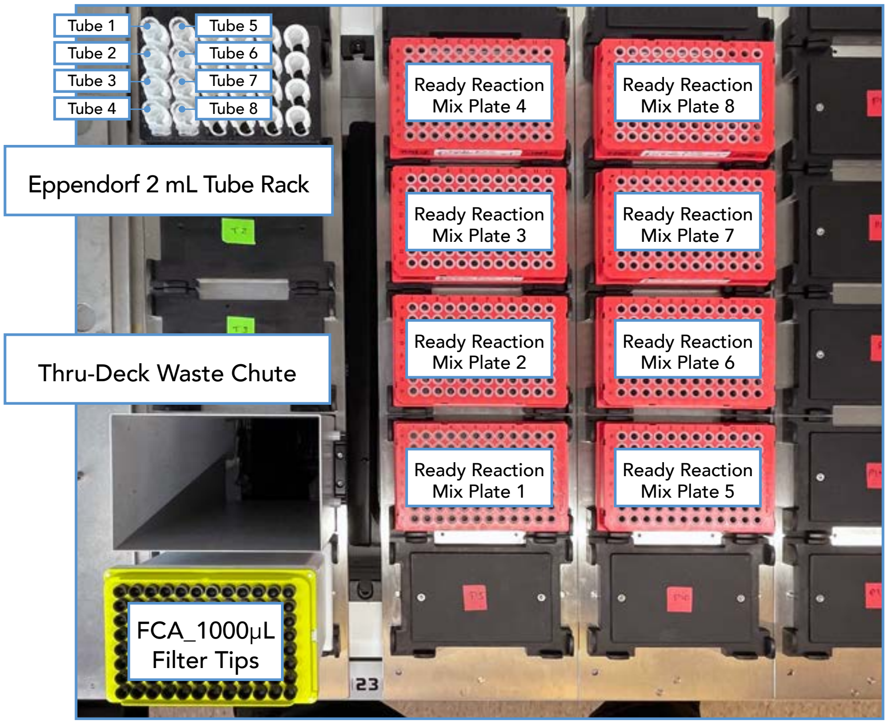


Reagents and labware used for the library setup are as follows:

Name	Supplier	Contents	Labware Type	Catalog Number
Indexing Reaction Plate	seqWell	ExpressPlex Indexing Reagent	Bio-Rad HSP9601	XXXX-IRP96
Ready Reaction Mix Plate	seqWell	ExpressPlex Ready Reaction Mix	Bio-Rad HSP9611	RRP96
DNA Sample Plate	user	~4 ng/u µL DNA	e.g., Bio-Rad HSP9601	-
MCA96_ 50 µL Nested	Tecan	Automation Tips	Nested MCA96 50 µL Tips without Filter	30038609

The deck layout for the sample pooling of eight 96-well plates is shown below.

The deck image below depicts the elements utilized in the automated pooling of eight plates of ExpressPlex sequencing reactions. At the time of pooling, the ***Ready Reaction Mix Plates*** contain the fragmented, tagged, and amplified libraries; the 24-position ***Eppendorf 2 mL Tube Rack*** contains the destination tubes which host the final pooled libraries; ***FCA_1000 µL Filter Tips*** are used for pooling; used tips are discarded into the ***Thru-Deck Waste Chute***. The pooling method has been configured to pool either a single plate per destination tube (10 µL per well pooled – data shown in this presentation); two plates per destination tube (5 µL per well); three plates per destination tube (3.33 µL per well); or four plates per destination tube (2.5 µL per well). The latter has been evaluated (data not shown) – there were no dropouts over the 384 samples.

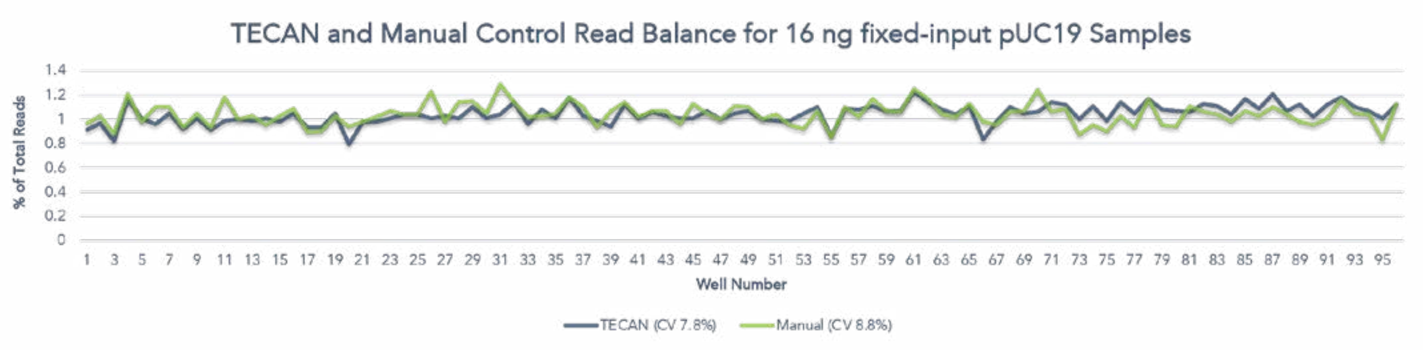


Reagents and labware used for library pooling are as follows:

Name	Supplier	Contents	Labware Type	Catalog Number
Ready Reaction Mix Plate	seqWell	Individual ExpressPlex Libraries	Bio-Rad HSP9611	-
Eppendorf 2 mL Tube Rack	Beckman Coulter	Inserts and Tubes	24 Position Tube Rack Holder	BCI 373661
Tube Rack Inserts	Beckman Coulter	Tubes	11-mm Diameter Insert, White	BCI 373696
Eppendorf 2 mL Tubes	Millipore Sigma	Final Pooled Libraries	Eppendorf LoBind 2 mL Tubes	22431048
FCA_ 1000µL Filter Tips	Tecan	-	1000µL, with Filter, Conductive	30057817

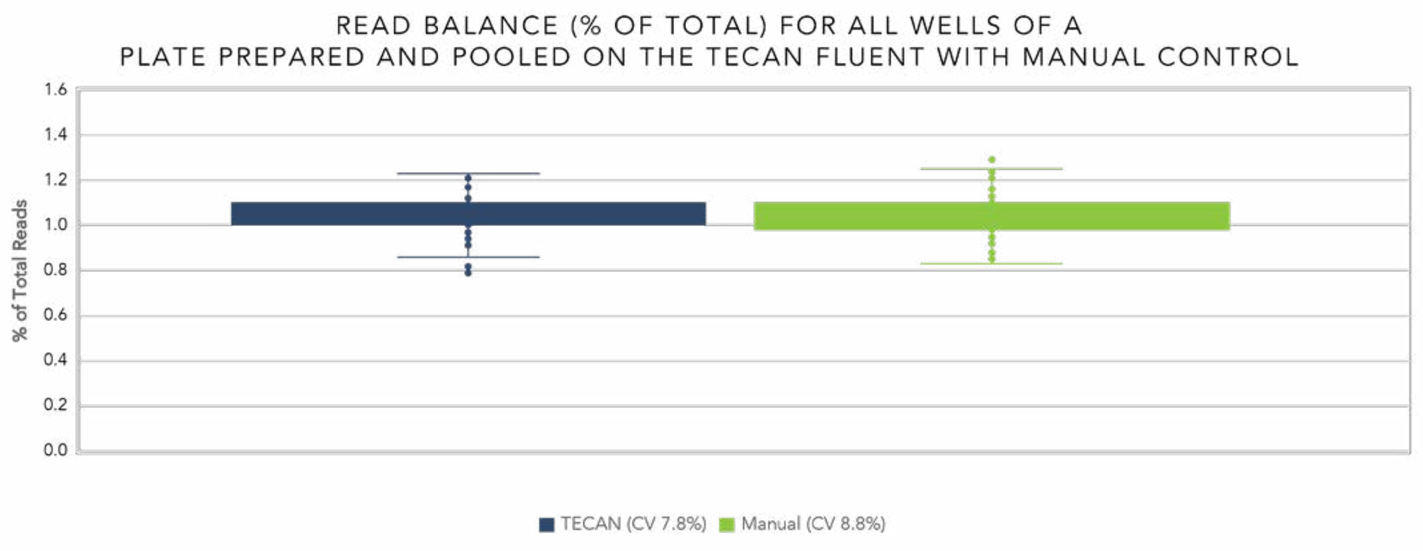
Results

96 libraries were prepared on the Tecan Fluent, as described above, and 96 libraries were prepared manually. The number of sequencing reads per well, as a percent of the total reads recovered, reflects the consistency of the library preparation across all samples. As seen in Data Figure 1, the balance of sample reads is highly consistent for the automated and manual preparations, with coefficients of variation (CVs) of 7.8% and 8.8% respectively.



Data Figure 1. **Read Balances for pUC19 samples prepared and pooled on the Fluent, with manual control data.** An ideal read balance would be 100%/96 wells, or 1.04% of total reads per well for all wells of each plate. The Fluent produces read balances which are close to the best attainable in actual practice, and highly comparable to read balances produced manually.

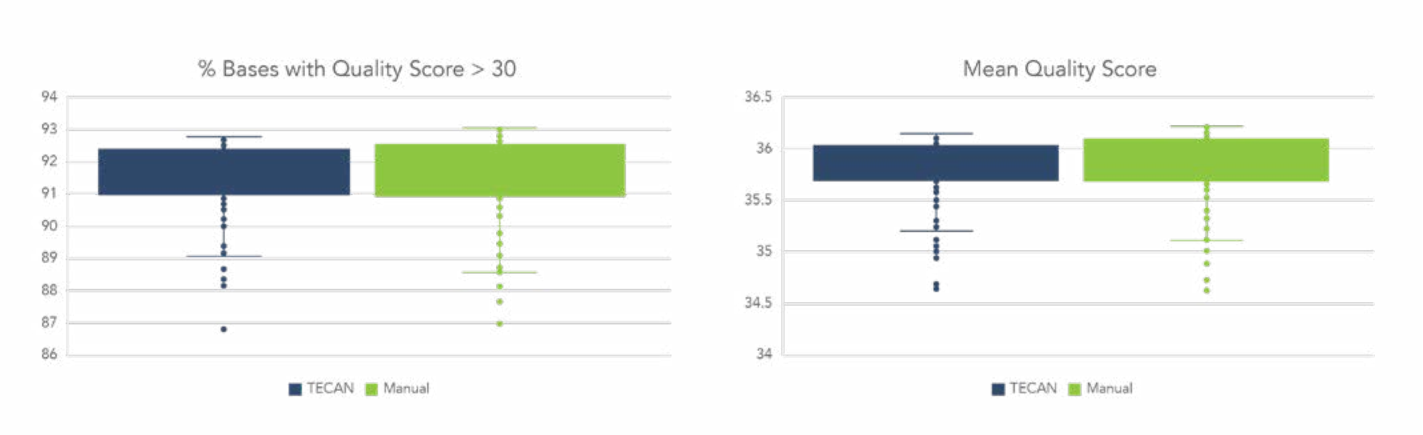
Visualized differently, in Data Figure 2, the medians and distributions of the read-balance data for libraries prepared using the Fluent and libraries prepared manually are virtually indistinguishable.



Data Figure 2. **Read balances for libraries prepared using the Fluent and libraries prepared manually** are practically indistinguishable.

Highly similar read counts for pooled sample libraries is a result of the auto-normalization engineered into the ExpressPlex Library Preparation Kit, and validates the ability to proceed directly from cleanup into final sequencing preparation without the need to normalize sample libraries on a well-by-well basis.

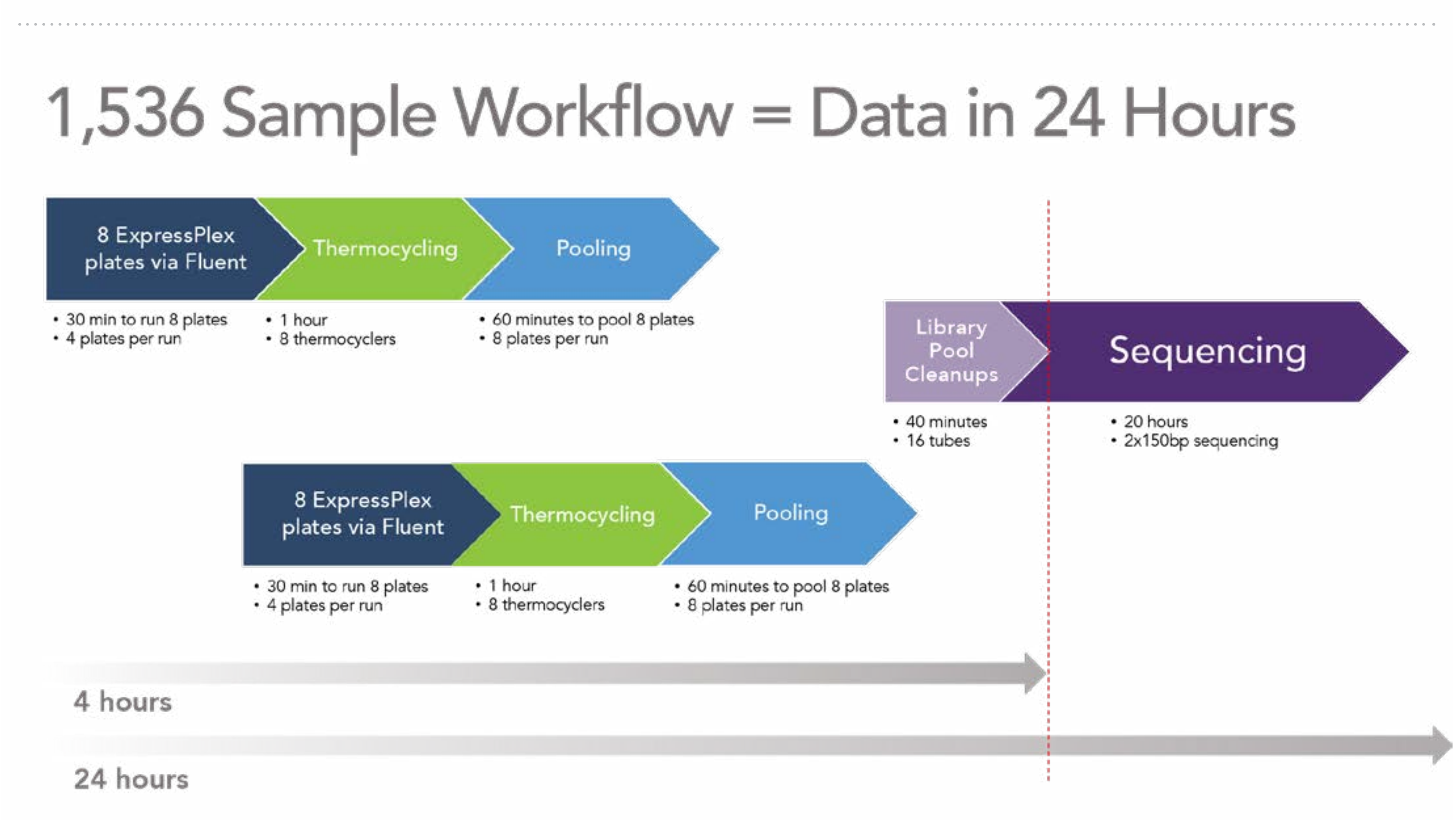
Finally, we evaluated both sets of libraries for quality metrics. Outcomes are of high quality, with no significant differences between manually-prepared libraries and libraries prepared on the Fluent.



Data Figure 3. **% Bases with Quality Score >30 and Mean Quality Score for libraries prepared using the Fluent and libraries prepared manually** show no significant differences between the methods.

Discussion

Using the ExpressPlex Library Preparation Kit, the Tecan Fluent, 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/ pooling steps to maximize instrument and thermocycler usage. To expedite the library preparation process, up to four 96-well plates can be set up for sequencing in a single Fluent run, and up to eight plates may be pooled in a Fluent pooling run.



Quick, reproducible sequencing of synthetic construct libraries can enable synthetic biology researchers to rapidly determine if their sequences of interest are present. With the strategy described in this application note, high-throughput laboratories can efficiently screen large numbers of constructs and focus on the important discovery aspects of their work.

Conclusions

- ExpressPlex libraries created on the Tecan Fluent are highly consistent, maintaining highly balanced read counts without manual normalization.
- Users can prepare and sequence 1,536 or more samples in 24 hours, which enables speedy discovery via rapid data turnaround.
- Through high-throughput screening of synthetic constructs, researchers can increase their rates of discovery, thus allowing them to focus on more important aspects of their work.

The following scientists at seqWell are responsible for the work shown in this application note:
John Palys, Jenna Couture, Stella Huang, and Becca Feeley

Would you like more information about ExpressPlex or associated automation methods?

Contact seqWell: sales@seqwell.com