APPLICATION NOTE

Automating the Illumina stranded mRNA prep with the Sciclone NGSx workstation.

Introduction

Illumina® Stranded mRNA Prep, Ligation Kit automation on the Sciclone® G3 NGSx Automation workstation has reduced hands-on time while working with low volumes. This allows a user to experience sequencing library from 25-1000 ng of high-quality total human RNA.

The Sciclone NGSx liquid handling workstation is designed for high throughput, rapid, and reliable NGS library construction that reduces overall operational cost, error rate and sample variability thereby reducing the standard deviation and variance. This automation system when combined with the Illumina® Stranded mRNA prep workflow helps in enhancing capture of both coding RNA and multiple forms of noncoding RNA that are polyadenylated, a PolyA capture to selectively sequence mRNA and unique dual (UD) indexing with the IDT for Illumina® RNA UD Indexes.

The high throughput system enables user to load up to 96 RNA samples on the Sciclone NGSx workstation where libraries are prepared (after purifying and fragmenting mRNA, synthesizing cDNA), amplified, and purified using automation with highly reproducible and reliable sequencing data.

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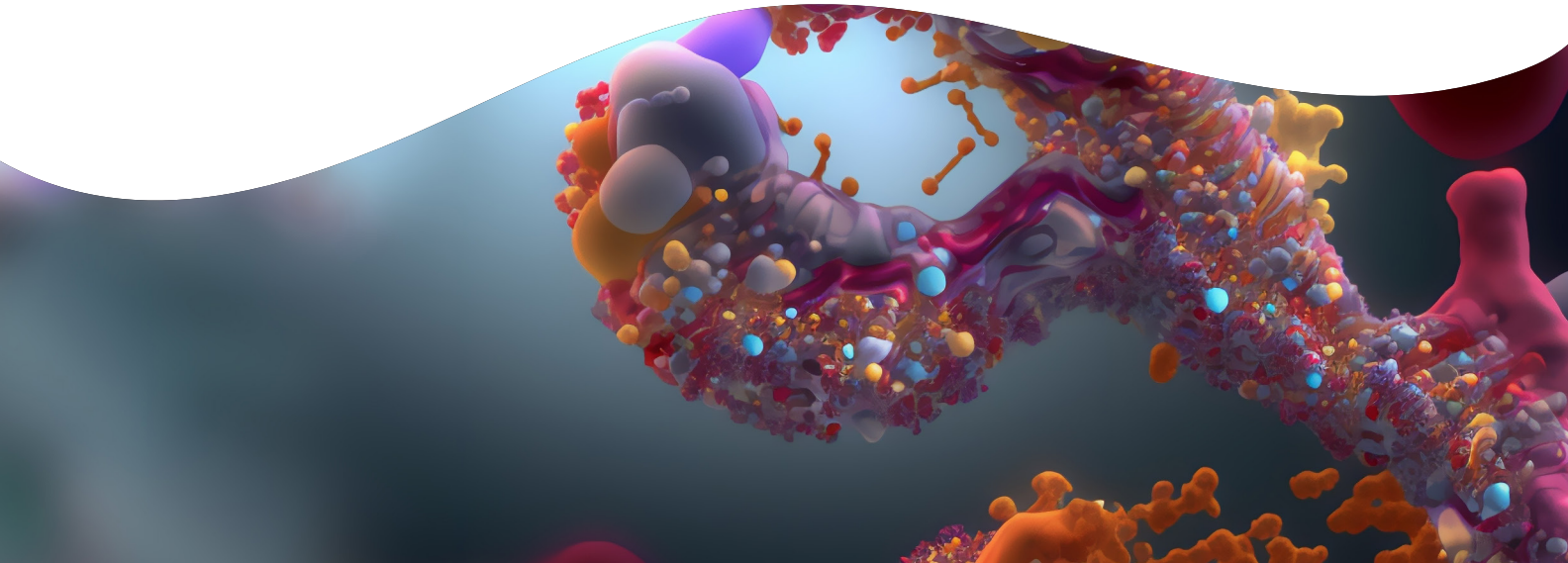

Experimental setup

A set of 48 (low throughput) to 96 (high throughput) libraries were prepared from Human reference RNA samples using the workflow as described in Illumina® Stranded mRNA Prep Reference guide. A total of 100 ng and 50 ng RNA was used as input RNA into the Sciclone NGSx workstation for low throughput and high throughput respectively. For the high throughput run, 48 negative controls were included to study the cross-contamination during the library preparation. The input RNA and purified libraries were quantified using Qubit® RNA HS and dsDNA HS Assay Kit respectively on Qubit® 2 fluorometer (Thermo Fisher Scientific). The fragment distribution was analyzed using the LabChip® GX Touch™ HT instrument.

Methods

Illumina® Stranded mRNA Prep workflow on the Sciclone NGSx workstation consists of eight steps:

1. Purify and Fragment Messenger RNA
2. Synthesize First Strand cDNA
3. Synthesize Second Strand cDNA
4. Adenylate 3′ Ends
5. Ligate Anchors
6. Clean Up Fragments
7. Amplify Library
8. Clean Up Library

Figure 1 demonstrates the workflow along with the time required to complete each step.

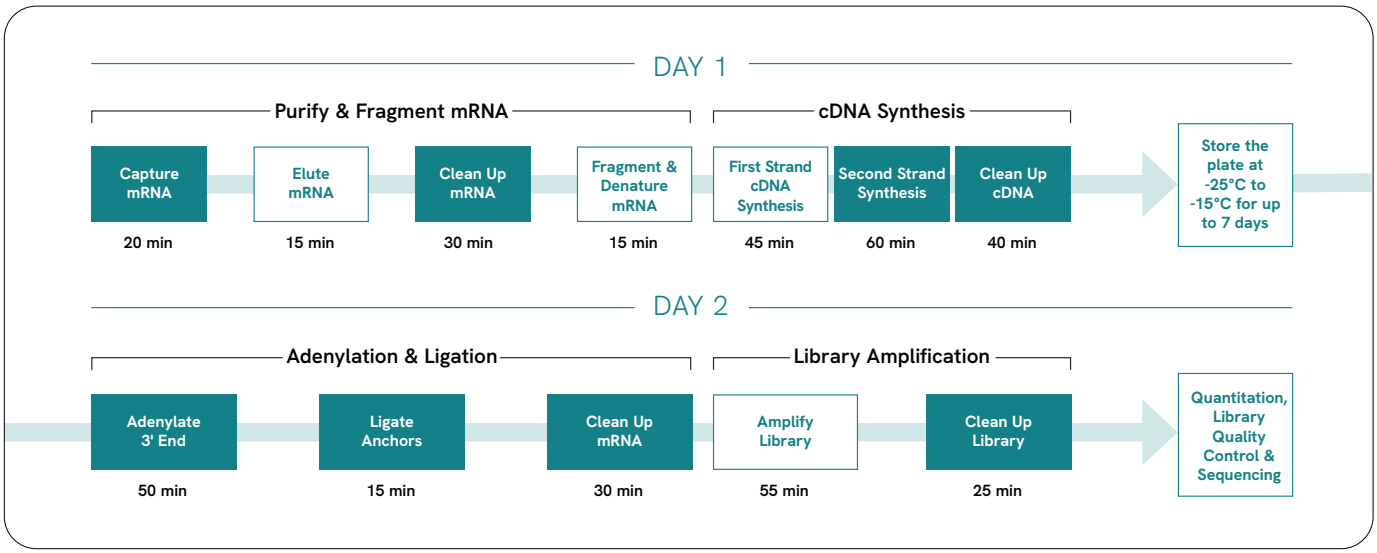

Figure 1: **Illumina® Stranded mRNA Prep, Ligation Kit Workflow along with the time required to complete each step.** Solid Blue block represents on-deck incubations and White blocks represent the steps that require off-deck thermocycler incubations

At the start of the run, the user was prompted to enter the number of columns to process and RNA input amount (Figure 2A). The library prep requires additional information to start the run, therefore, user is prompted to enter the RNA Anchor and the Index adapter plate starting column for the single run (Figure 2B). All the reagents from the kit were either placed on ice or room temperature as per Illumina® Stranded mRNA Prep workflow instructions.

The Sciclone NGSx deck was set up as shown in Figure 3. Each consumable required on deck for a setup was filled with volumes of reagent or master mix provided by the application workbook (Figure 4). The workbook calculates the master mix and reagent volume required based on number of columns to run in user-friendly manner. At the end of each run, a prompt reminds the user to place the index plate at 4 °C for future use.

For 96 samples, the number of columns to process was entered twelve. All the master mixes were maintained at 4 °C on CPAC throughout the experiment. The master mixes were either pre-broadcasted to a new plate or broadcasted directly to the sample plate to save time and eliminate manual pipetting errors. Samples were mixed on the on-deck shaker. Incubations were completed on the on-deck CPAC location. The temperature on CPAC was maintained as per the Illumina Stranded mRNA Prep protocol.

An off-deck thermocycler was used to elute mRNA, fragment and denature mRNA, synthesize First strand and amplifying libraries. The user was guided by the prompts at every step that minimizes the time to look back and forth for the protocol. Qubit was used to quantify the libraries after cleanup. Final library size was assessed using the LabChip GX Touch HT instrument. Libraries were sequenced on an Illumina® NextSeq® 500 instrument using 2x74 bp reads.

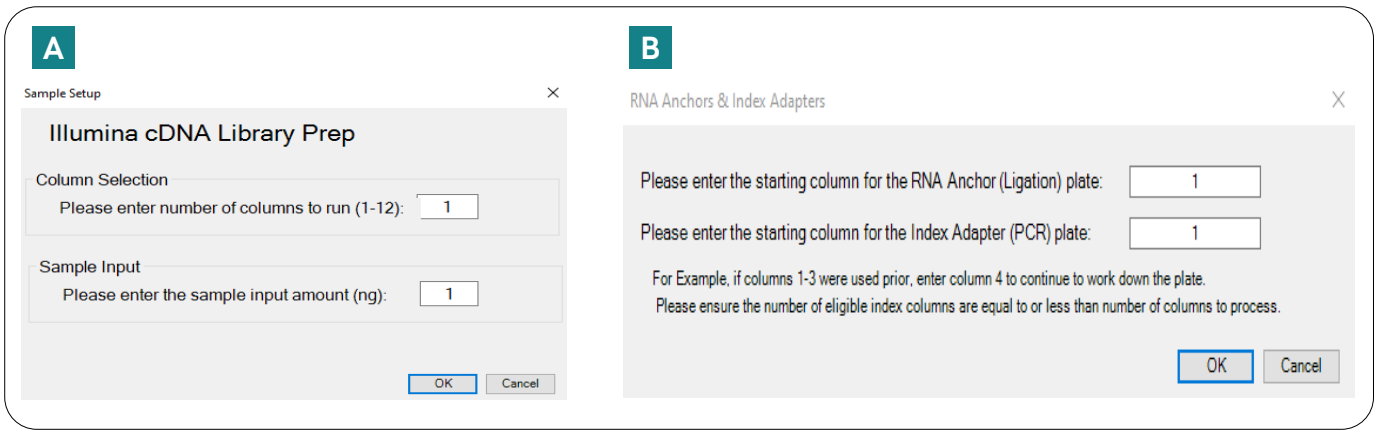

Figure 2: User Interface to select the **(A)** number of columns to run, input RNA and **(B)** starting column for RNA anchor and Index adapter.

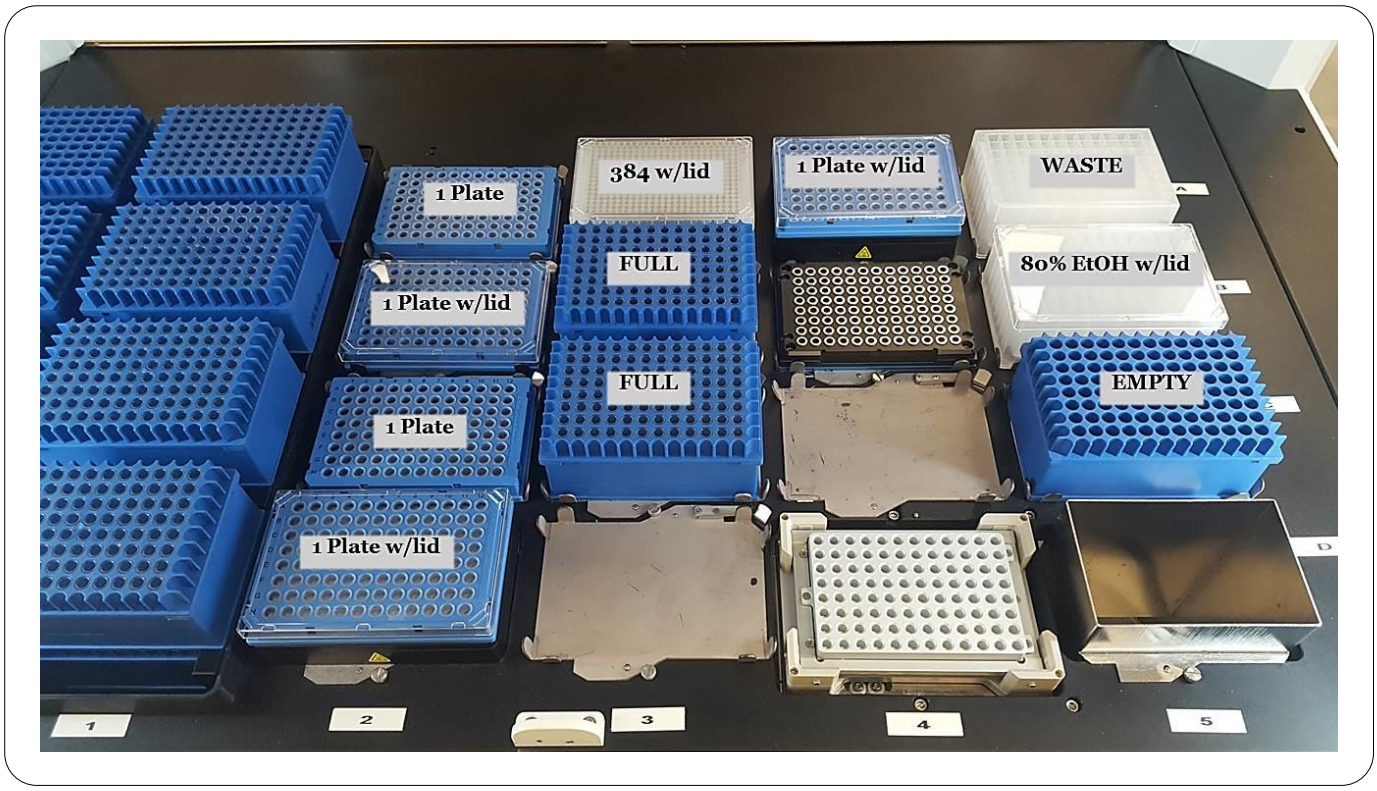

Figure 3: Deck layout to Start the Illumina® Stranded mRNA Prep application on Sciclone NGSx workstation.

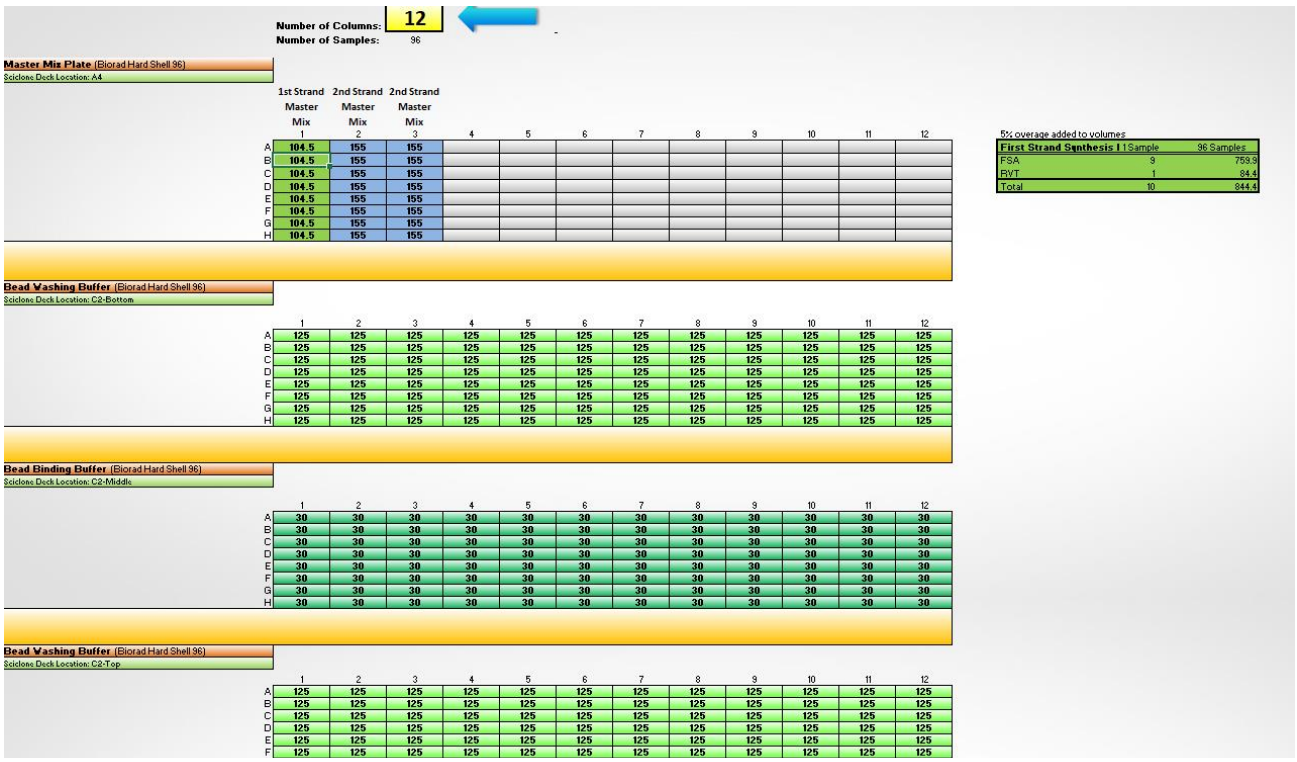

Figure 4: The excel workbook for setting up the Illumina® Stranded mRNA Prep application on Sciclone NGSx workstation.

Results

The Sciclone NGSx workstation was used to prepare 48 libraries using 100 ng input DNA. It produced libraries averaging 7.6 µg with 32.7 nM concentration (Figure 5A and 5B). On the other hand, the 96 libraries prepared using 50 ng input DNA produced libraries averaging 11.5 µg with 50 nM concentration (Figure 5C and 5D). No cross-contamination was observed in the negative control wells during the high throughput run. The gel image and LabChip trace of low throughput and high throughput along with negative control has been demonstrated in Figure 6.

The 32 and 29 enriched multiplex libraries from low throughput and high throughput run were sequenced on an Illumina® NextSeq® 500 using 2x74 bp reads respectively. The data resulted in average 23.39x fold coverage in the coding region, 91.7% total alignment, 8.3% Percent abundance, 0.57 Median CV Coverage, 98.7% Percent stranded and 8% Duplication rate for low throughput run. The high throughput run resulted in 29.26x average fold coverage in the coding region, 92.96% total alignment, 8.3% Percent abundance, 0.52 Median CV Coverage, 98.7% Percent stranded and 8.9% Duplication rate (Figure 7).

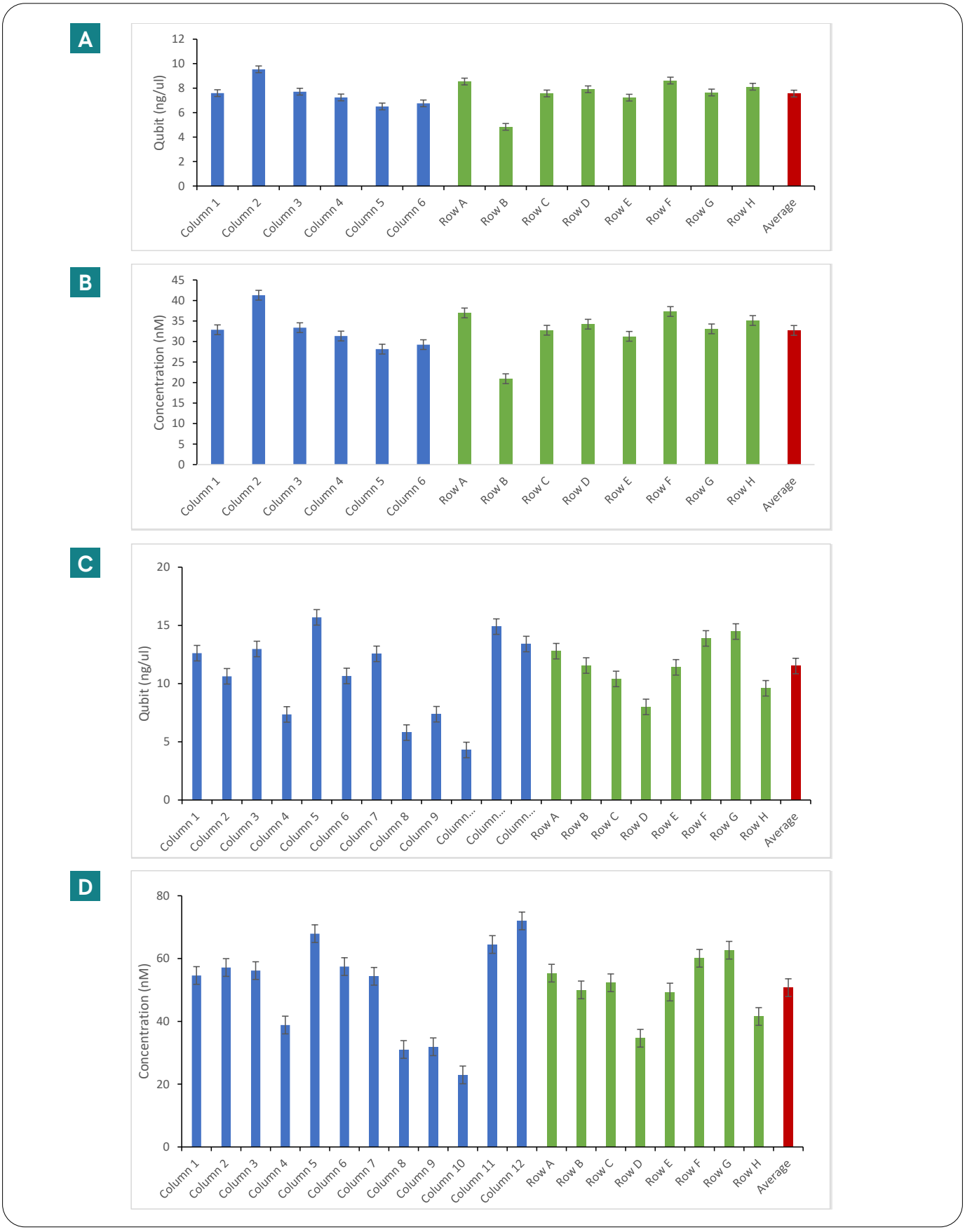

Figure 5: **(A and B)** Quantification of 48 libraries **(C and D)** 96 libraries obtained from Qubit® fluorometer (Thermo Fisher Scientific).

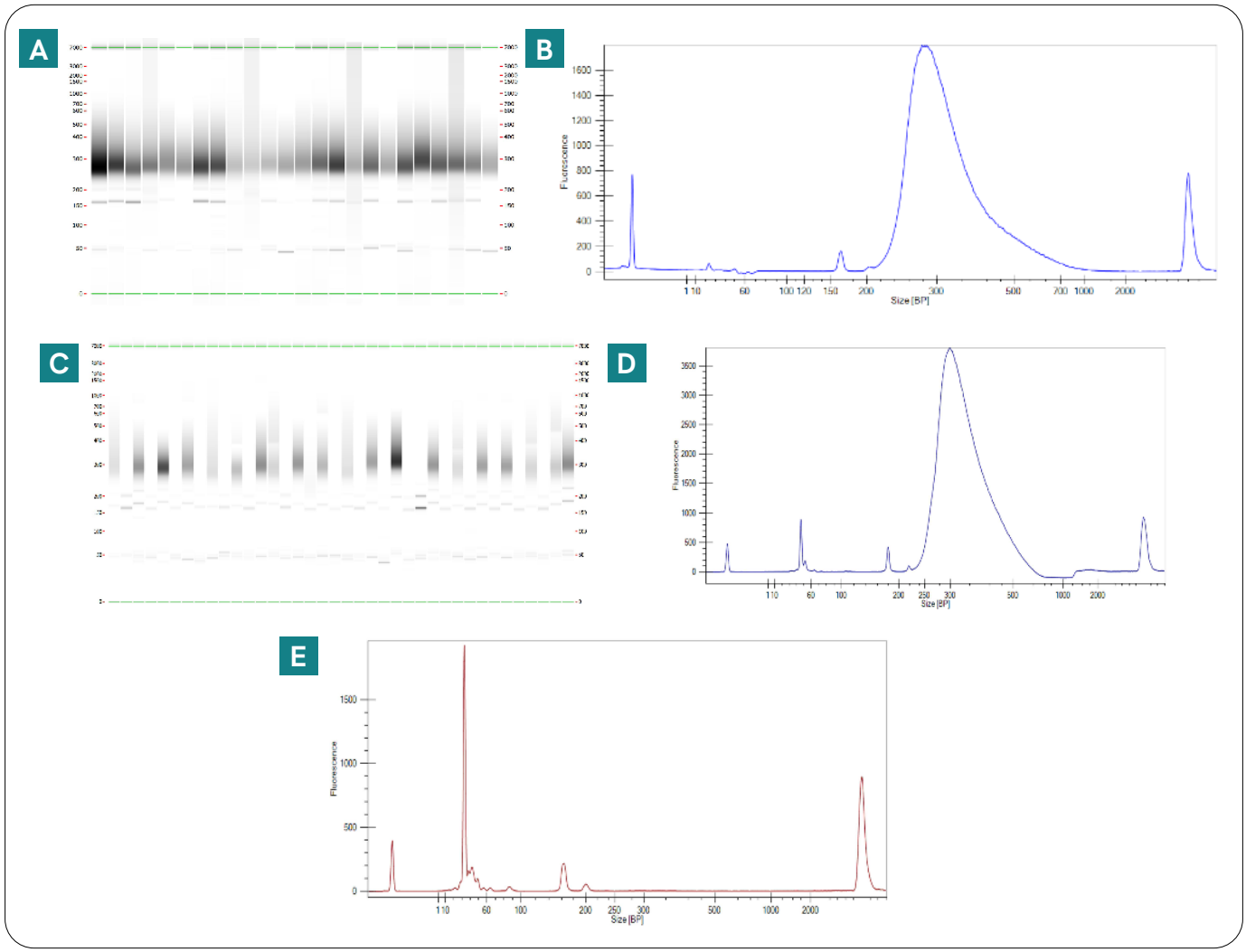

Figure 6: **(A)** Gel image and **(B)** LabChip trace of low throughput; **(C)** Gel image, **(D)** LabChip trace of high throughput Enriched Multiplex libraries obtained from LabChip GX Touch HT instrument and **(E)** LabChip trace of Negative Control.

Figure 7: Key sequencing metrics obtained from Illumina® NextSeq® 500 sequencer. **(A)** Low throughput **(B)** High throughput.

Conclusion

The Illumina® Stranded mRNA library construction is automatable on the Sciclone NGSx workstation. The library yield and library size were within the expected range as per the Stranded mRNA prep workflow protocol. Data obtained from technical replicates as well as different input amount demonstrated reproducibility with minimum variation. Also, no cross-contamination was observed between the wells. The data obtained from low-throughput was comparable to high-throughput assay indicating that the Sciclone NGSx workstation can be used both for low- and high-throughput settings. Automating this workflow significantly reduced hands-on time and risk of errors while working with very reduced volumes. Automation can help avoid pipetting errors and cross contamination during a run. The Sciclone NGSx liquid handling workstation is designed for high throughput, rapid and reliable NGS library construction and can significantly reduce overall operational cost, error rate and sample variability.

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