APPLICATION NOTE

Illumina Stranded Total RNA Prep, Ligation with Ribo-Zero Plus automated on the Sciclone G3 NGSx liquid handler.

Introduction

Illumina® Stranded Total RNA Prep, Ligation with Ribo-Zero™ Kit automation on the Sciclone® NGSx workstation has significantly reduced hands-on time while working with reduced volumes. This allows a user to experience sequencing library from 10-100 ng RNA input.

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 Illumina® Stranded Total RNA Prep, Ligation with Ribo-Zero™ Plus workflow converts total RNA into dual-indexed libraries. Depletion reagents bind and deplete abundant transcripts from total RNA, reverse transcription then convert the remaining RNA into cDNA, while subsequent ligation and amplification steps add adapters for clustering on Revvity's Sciclone NGSx workstation and sequencing on an Illumina® NextSeq® 500 system.

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

Sciclone G3 NGSx

For research use only. Not for use in diagnostic procedures.

Experimental setup

A set of 21 (low throughput) to 96 (high throughput) libraries were prepared from Human reference RNA samples using the workflow as described in the Illumina® Stranded Total RNA Prep, Ligation with Ribo-Zero™ Plus Reference guide. A total of 100 ng and 50 ng RNA was used as input RNA into the Sciclone NGSx workstation for low 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 the Thermo Fisher Scientific 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 Total RNA Prep, Ligation with Ribo-Zero™ Plus Workflow on the Sciclone NGSx workstation consists of nine steps:

1. Deplete ribosomal RNA
2. Fragment and denature RNA
3. Synthesize first strand cDNA
4. Synthesize second strand cDNA
5. Adenylate 3′ Ends
6. Ligate anchors
7. Clean up fragments
8. Amplify library
9. Clean up LibrarySubheadline goes here

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

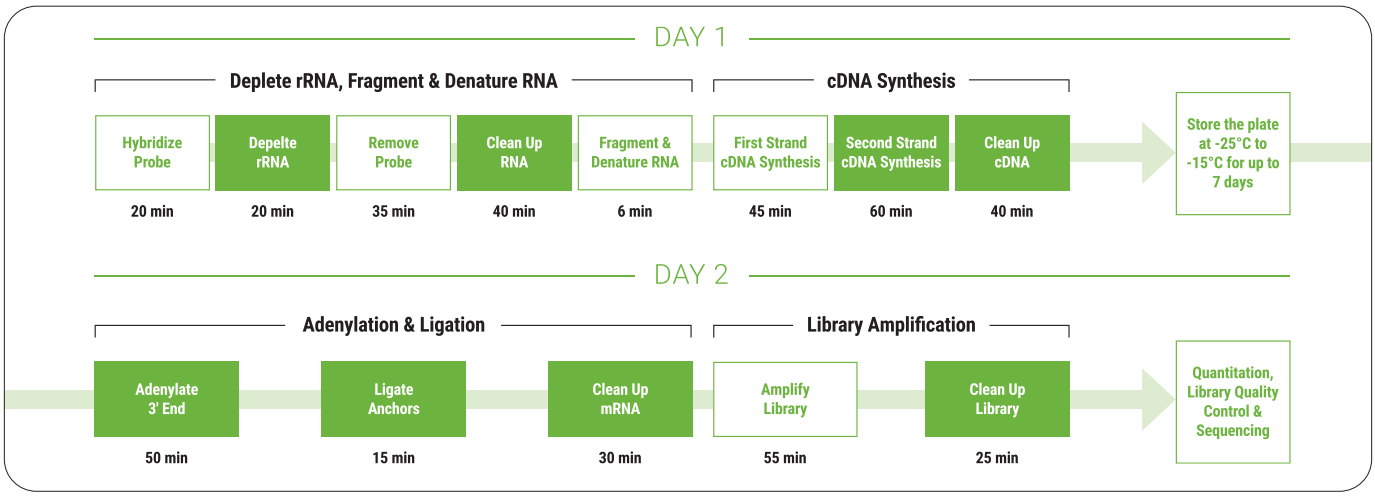

Figure 1: Illumina® Stranded total RNA prep, Ligation with Ribo-Zero™ Plus Workflow along with the time required to complete each step. Solid Blue block represents on-deck incubations and White blocks represent the steps that require off-deck thermocycler incubations.

At the start of the run, the user was prompted to enter the number of columns to process (Figure 2). All the reagents from the kit were either placed on ice or room temperature as per Illumina® Stranded Total RNA Prep, Ligation with Ribo-Zero™ Plus workflow instructions. 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 Total RNA Prep, Ligation with Ribo-Zero™ Plus protocol. An off-deck thermocycler was used to Probe hybridization, fragment and denature RNA, 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 nucleic acid analyzer. Libraries were sequenced on the Illumina® NextSeq™ 500 sequencer using 2x74 bp reads.

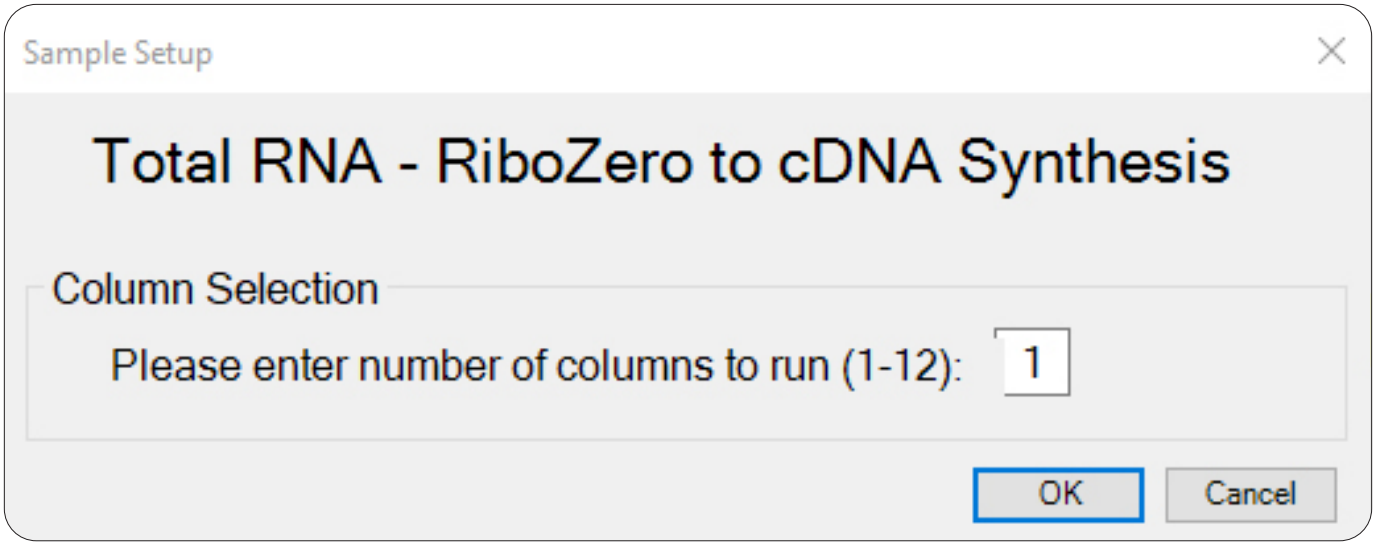

Figure 2: User Interface to select the number of columns to run.

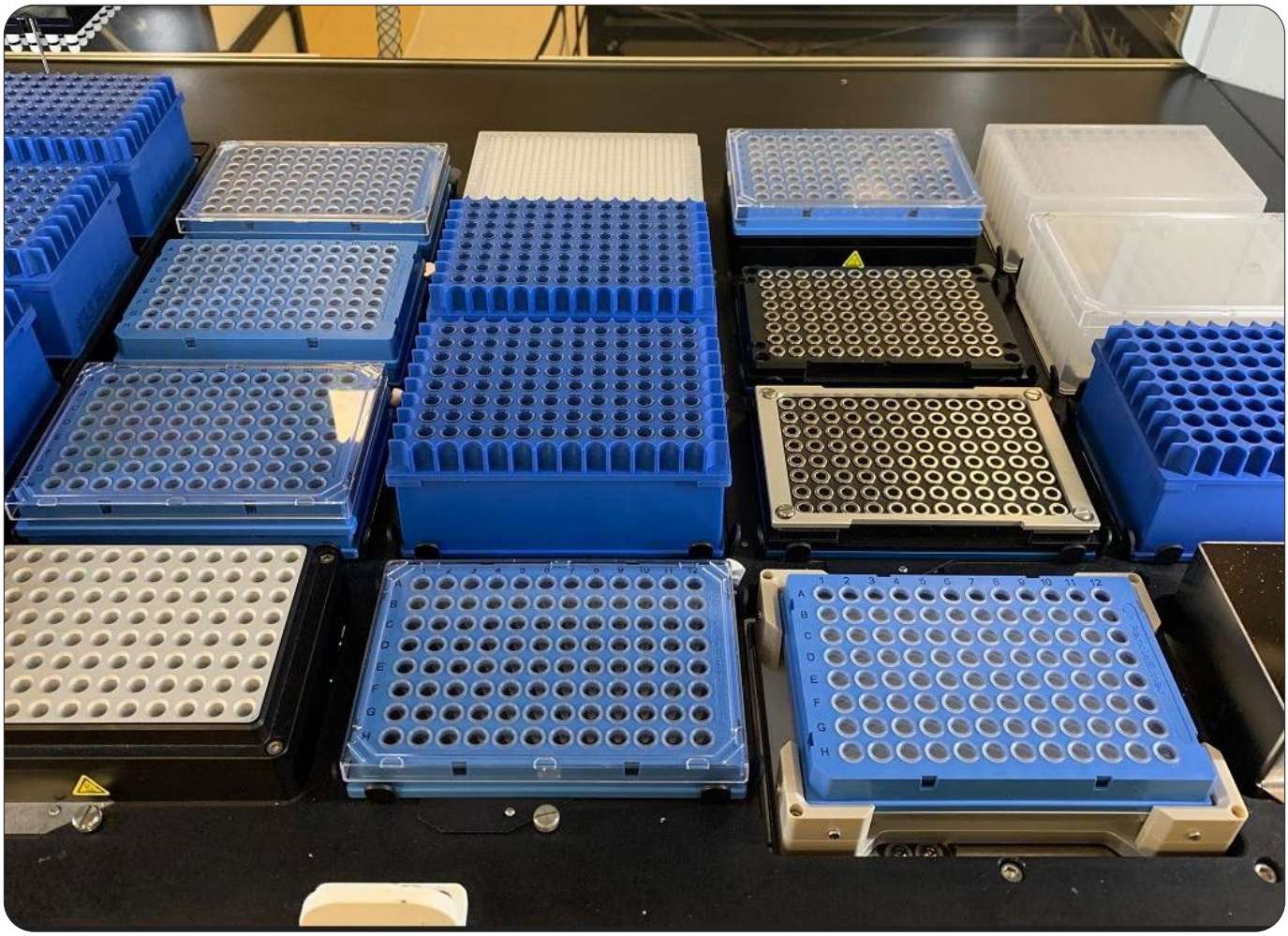

Figure 3: Deck layout to Start the Illumina® Stranded Total RNA Prep, Ligation with Ribo-Zero™ Plus application on Sciclone NGSx workstation.

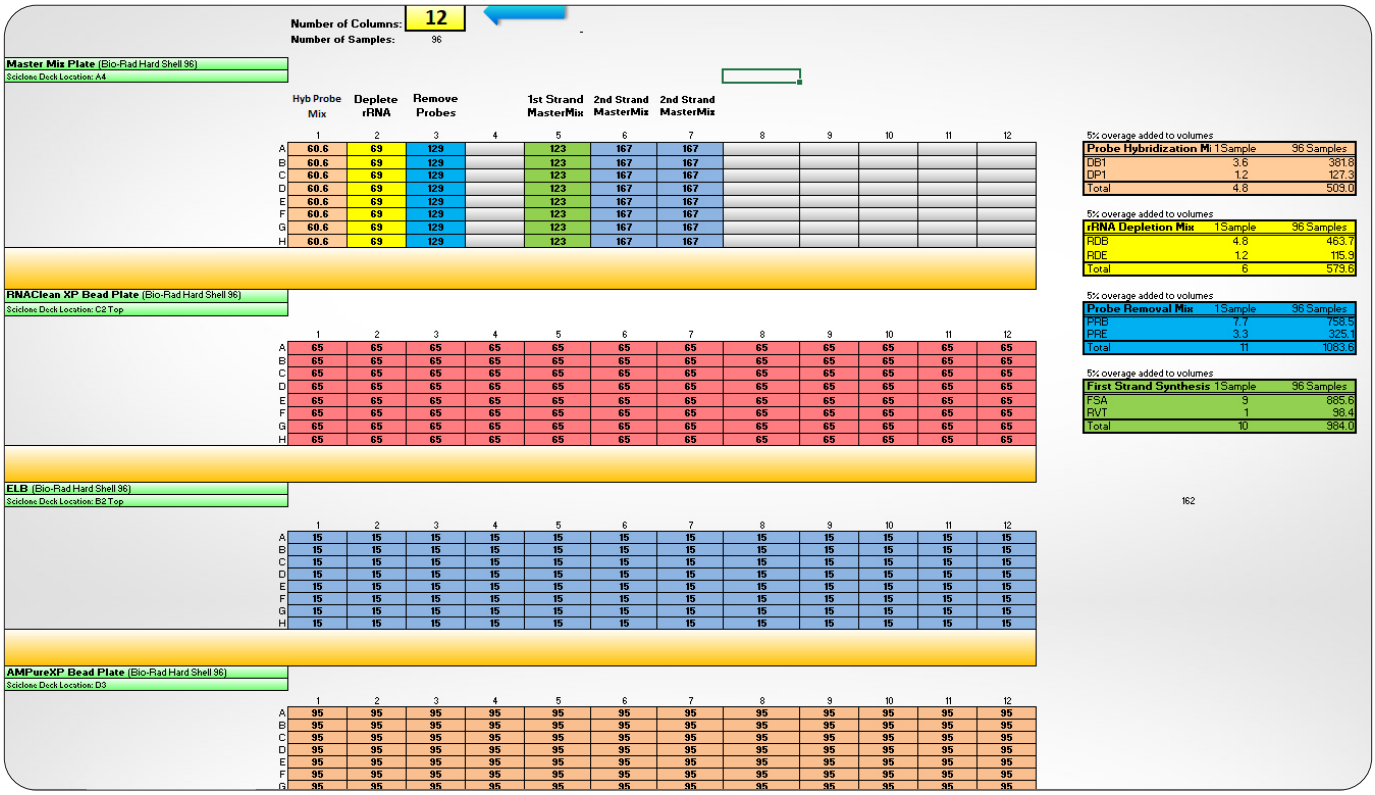

Figure 4: The Excel workbook for setting up the Illumina® Stranded Total RNA Prep, Ligation with Ribo-Zero™ Plus application on Sciclone NGSx workstation.

Results

The Sciclone NGSx workstation was used to prepare 21 libraries using 100 ng input DNA, produced libraries averaging 6.4 µg with 27.7 nM concentration (Figure 5A and 5B). On the other hand, the 96 libraries prepared using 50ng input DNA produced libraries averaging 28.6 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 21 and 18 enriched multiplex libraries from low and high throughput run were sequenced on the Illumina® NextSeq® 500 sequencer using 2x74 bp reads respectively. The data resulted in an average of 4.3x fold coverage in the coding region, 90.6% total alignment, 4.5% Percent abundance, 0.75 Median CV Coverage, 98.7% Percent stranded and 4.5% Duplication rate for low throughput run. The high throughput run resulted in 4.31x average fold coverage in the coding region, 93.6% total alignment, 4.42% Percent abundance, 0.58 Median CV Coverage, 98.5% Percent stranded and 5.4% Duplication rate (Figure 7).

Figure 5: (A and B) Quantification of 21 libraries **(C and D)** 96 libraries obtained from a Thermo Fisher Scientific Qubit® instrument.

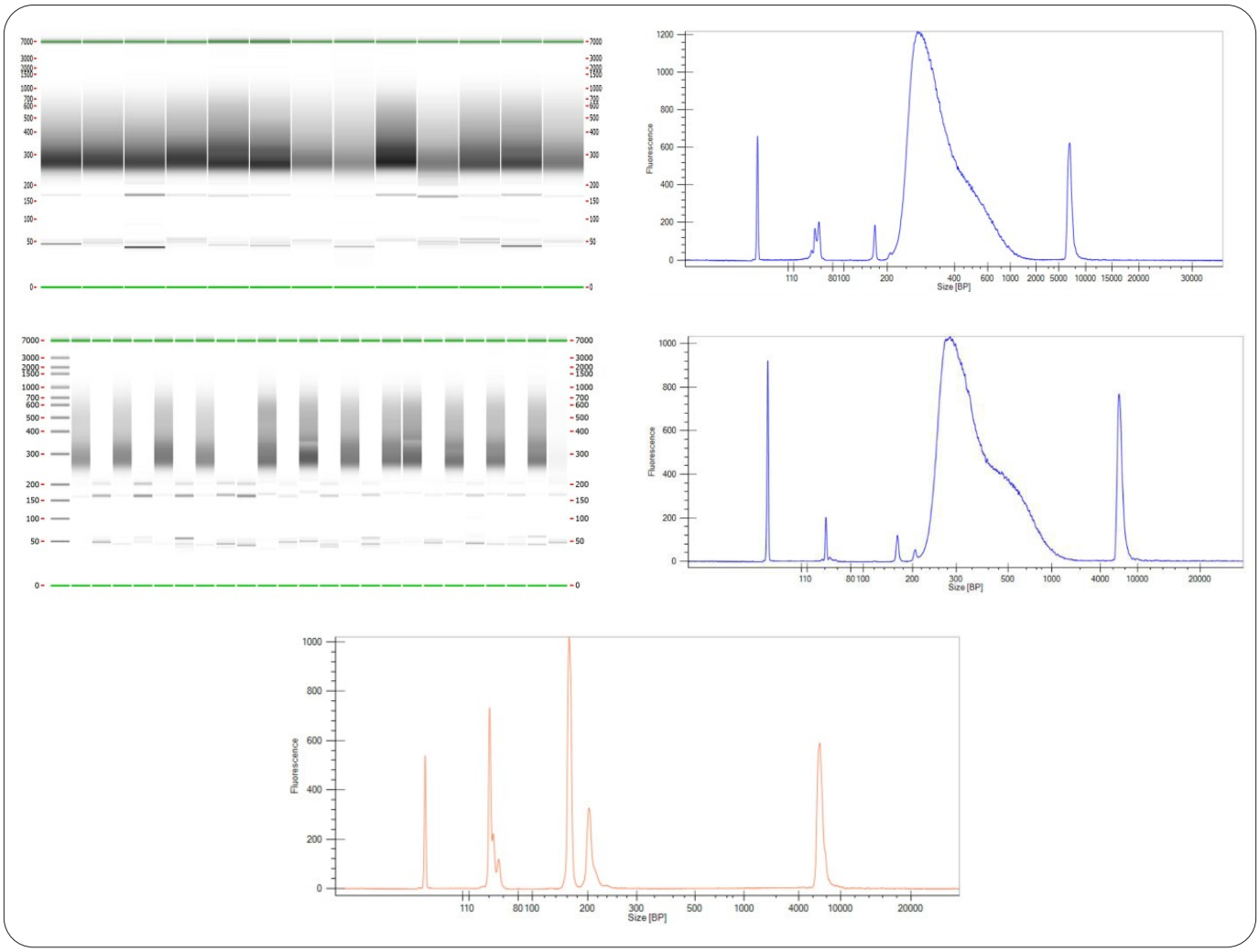

Figure 6: (A) Gel image and **(B)** LabChip trace of low throughput; **(C)** Gel image, **(D)** LabChip trace of high throughput Enriched Multiplex libraries obtained from LabChip GX Touch HT and **(E)** LabChip trace of Negative Control.

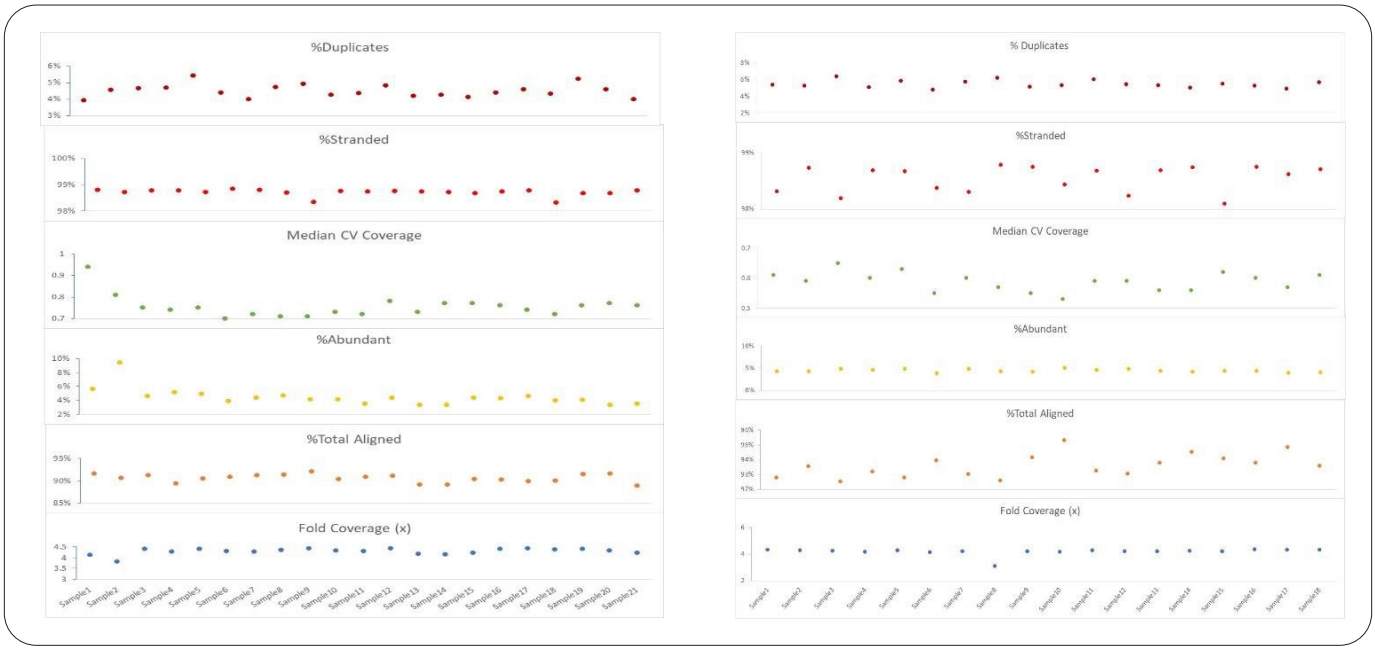

Figure 7: Key sequencing metrics obtained from Illumina® NextSeq® 500 sequencer. **(A)** Low throughput **(B)** High throughput.

Conclusion

The Illumina® Stranded Total RNA Prep, Ligation with Ribo-Zero™ library construction is automatable on the Sciclone NGSx Automation workstation. The library yield and library size were within the expected range as per the Illumina® Stranded Total RNA Prep, Ligation with Ribo-Zero™ Plus 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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