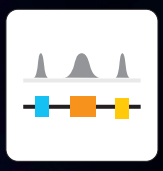
NGS DreamPrep™ – automated mRNA library preparation and quantification on the Fluent® Automation Workstation.

Application Note



A SIMPLIFIED, AUTOMATED WORKFLOW FOR mRNA LIBRARY PREPARATION, QUANTIFICATION, NORMALIZATION AND POOLING IN LESS THAN NINE HOURS

INTRODUCTION

Universal Plus mRNA-Seq library preparation kits with NuQuant® offer a novel, streamlined workflow for the generation of quantified libraries for sequencing on Illumina instruments (Figure 1). The kits feature a rapid workflow with a wide input range – from 10 ng to 1 µg of total RNA – and allow researchers to determine the library molarity without using time-consuming methods, such as qPCR or capillary electrophoresis, that are subject to sample-to-sample variability. Universal Plus mRNA libraries can be prepared, quantified, normalized and pooled in less than nine hours on the Fluent Automation Workstation, compared to more than fourteen hours for manual or automated library preparation and qPCR quantification with other kits.

NGS DreamPrep is a simplified solution designed and optimized for fully automated NGS protocols, including mRNA library preparation and quantification. It combines the Universal Plus mRNA-Seq library preparation kits with a Fluent Automation Workstation and integrated Infinite® F Nano+ plate reader, allowing users to transform up to 96 RNA samples into readily normalized libraries. The instrument is equipped with an eight-channel Flexible Channel Arm™, a Multiple Channel Arm with a 96-channel head and a Robotic Gripper Arm™ – as well as integrated temperature controlled-devices and an ODTC® 96 thermal cycler (INHECO) – reducing manual steps and allowing longer walkaway times. Tecan's TouchTools™ touchscreen interface simplifies run set-up and makes deck preparation more intuitive, reducing the need for operator training.

This application note describes the results of a fully automated protocol for the generation of quantified mRNA libraries, including QC and library normalization, using the NGS DreamPrep workstation and the Universal Plus mRNA-Seq library preparation kit. It enables highly reproducible mRNA library preparation with minimal user intervention, quantifying and normalizing the libraries without sample loss using the integrated plate reader (Figure 2). Unlike other automated solutions, this set-up eliminates the need for additional library quantification steps saving significant time, resources and valuable library material.

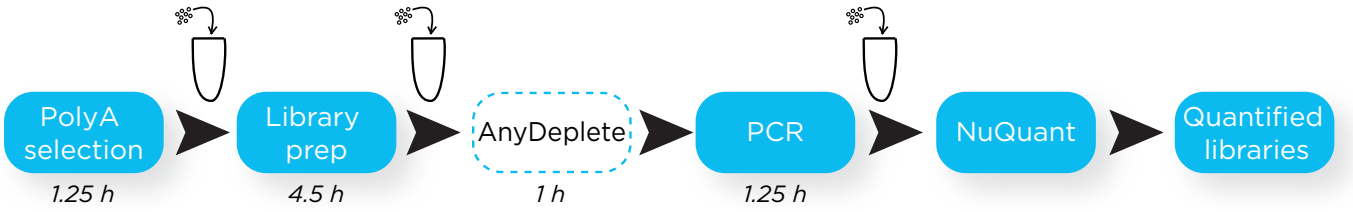


Figure 1: The Universal Plus mRNA-Seq library preparation kit with NuQuant offers a streamlined workflow for the generation of sequencing-ready quantified libraries. The workflow can include an optional and customizable transcript depletion step.

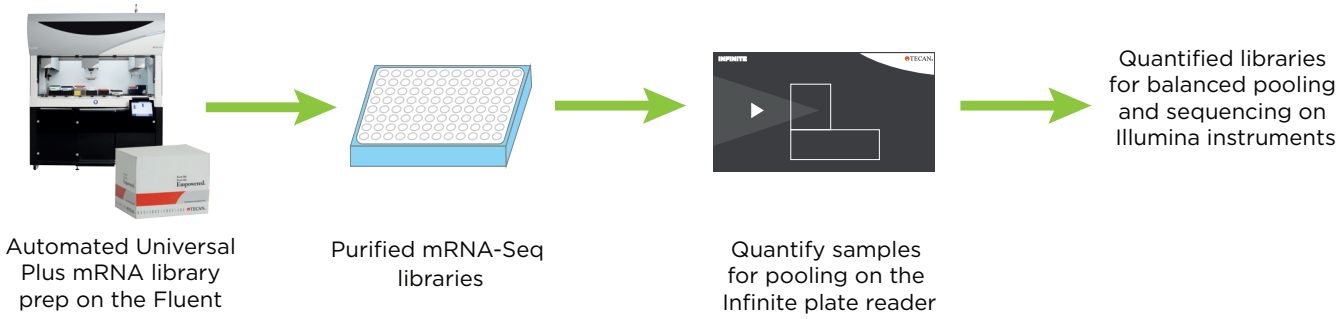


Figure 2: After final purification on the Fluent Automation Workstation, fluorescence measurements of the libraries and standards in 96-well plates are performed on the Infinite plate reader. The molar concentrations determined for the libraries can be used for balanced pooling and subsequent sequencing on Illumina instruments.

MATERIALS AND METHODS

Materials

- Leukemia (K-562) Total RNA (Thermo Fisher Scientific)
- Universal human reference (UHR) RNA (Agilent Technologies)
- Rat brain total RNA (Takara USA)
- Universal Plus mRNA-Seq library preparation kit with NuQuant (Tecan)
- HS NGS Fragment Kit 1bp-6000bp (Agilent Technologies)

Instruments:

- Fluent Automation Workstation integrated with an Infinite F Nano+ plate reader and ODTC 96 thermal cycler
- MiSeq® System (Illumina)

Experimental design and protocol

The Universal Plus mRNA-Seq library preparation kit was used to prepare libraries from 100 ng aliquots of K-562 total RNA (n=9), rat brain total RNA (n=9) and human UHR total RNA (n=6). Following mRNA capture, all samples were heat fragmented according to the user guide. Libraries were amplified by PCR for fifteen cycles, with post-amplification bead purification at a 1:1 bead-to-sample ratio. Finished libraries in their original plates were quantified using the NuQuant method and the integrated Infinite plate reader. The molarity of each library was determined by fluorescence measurement (excitation 620 nm, emission 680 nm) and comparison with a standard curve. Libraries were then pooled according to the molarity and sequenced on the MiSeq System.

RESULTS AND ANALYSIS

Library size and QC

Electrophoretic analysis of the individual finished libraries was performed with the HS NGS Fragment Kit. Figure 3 illustrates consistent size distribution from four representative samples of 100 ng of rat brain RNA, UHR RNA and K-562 RNA. The average library size from 24 samples was 257.6 bp, with a standard deviation of 5.9 (Table 1). The Universal Plus mRNA-Seq library preparation kit offers the flexibility to tailor the fragment size by varying the fragmentation time and the final bead purification ratio. Automated processing of the Universal Plus mRNA-Seq kit on the Fluent platform achieved consistent size distribution across sample types with no library failures and minimal adaptor dimers.

Sample	# of replicates	Avg. library size	Avg. % total aligned	Avg. % uniquely aligned
K-562 RNA	9	259 bp (2.0 %CV)	97.0 (0.42 %CV)	92.2 (0.1 %CV)
UHR RNA	6	256 bp (3.0 %CV)	98.7 (0.26 %CV)	92.4 (0.3 %CV)
Rat brain RNA	9	257 bp (2.1 %CV)	98.8 (0.16 %CV)	91.4 (0.4 %CV)

Table 1: Sequencing results from 100 ng of K-562, UHR and rat brain RNA.

The molar concentration of the purified libraries was determined using the NuQuant library quantification method. Figure 4 shows a standard curve derived from fluorescence measurements of diluted NuQuant standards, which was used to determine the library molarity. Figure 5 shows the library yield from 100 ng of K-562, UHR and rat brain RNA, demonstrating reproducibility between different sample types. No library failures were observed, and the molarities determined using NuQuant were used to pool the samples for sequencing.

Automating the library preparation and quantification protocol resulted in high yielding libraries with minimal variability across sample types. In addition, no plate edge effects were observed.

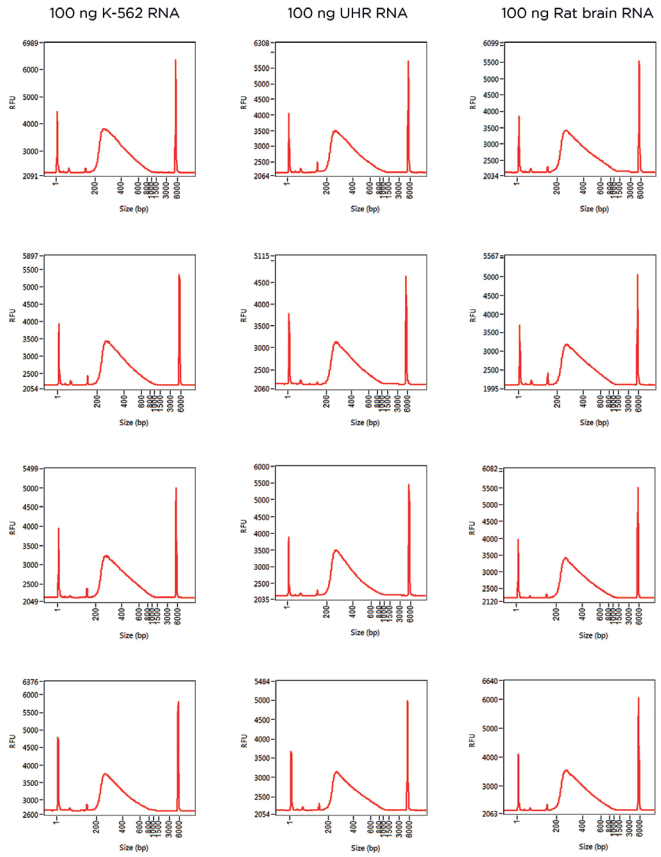


Figure 3: Library size distribution from 100 ng input of K-562, UHR and rat brain RNA.

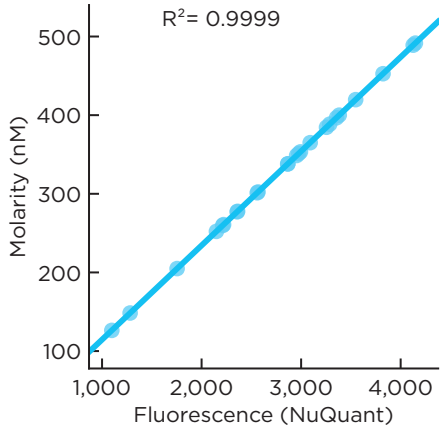


Figure 4: Standard curve generated using NuQuant standards and used to determine library molarity for sample pooling.

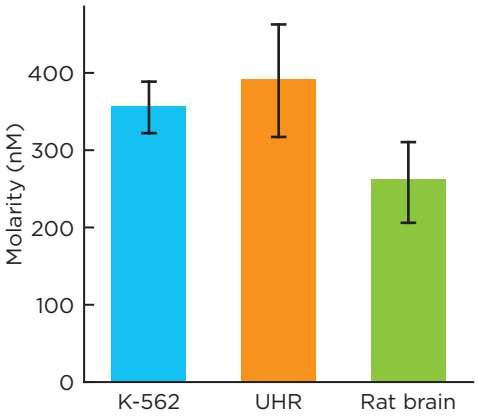


Figure 5: Library yield from 100 ng input of K-562, UHR and rat brain RNA with fifteen PCR cycles. Molarity was determined by NuQuant, and the final library volume for all inputs was 20 µl.

Sequencing results

Sequencing results showing the alignment rates from 100 ng inputs of K-562, UHR and rat brain RNA are given in Table 1. For all three sample types, the average alignment was 98 %, with 92 % uniquely aligned reads. The automated library preparation gave high alignment rates for all samples. The equimolar parsing of total reads from these samples, quantified by NuQuant, had a %CV of 9.4. The % exon of K-562 and UHR RNA was 77.2 % and 75.4 % respectively.

SUMMARY

The results presented in this application note highlight the benefits of automating mRNA library preparation, quantification, normalization and pooling using Tecan's NGS reagents and automation solutions. The protocol can be completed in less than nine hours and generates quantified libraries for balanced pooling and sequencing with minimal manual intervention. The sequencing results demonstrate robust library quantification and pooling using NuQuant, with CVs of less than 10 % (n=24) from 100 ng input for K-562, UHR and rat brain RNA. The results also show a high alignment rate for all sample types.

About the authors

Mike Benway has been automating laboratory workflows for over two decades, and NGS Sample preparation exclusively since 2010. During that time he has served in dozens of sequencing centers and NGS research labs throughout the US and Europe. He has taken his degrees in Molecular Biology and Computer Science from The University of Massachusetts at Boston. He has come to Tecan from TTP Labtech, and is now helping to automate the entire Tecan NGS sample prep reagent portfolio.

Joe Don Heath has been a scientific leader and customer advocate at Tecan since 2003, in which time he has had several responsibilities, including leadership roles in technical service, training, collaborations, and marketing. He presently is responsible for technical support, bioinformatics, and automation. JD earned his PhD in biochemistry and molecular biology from the University of Texas Health Science Center in Houston and completed a postdoctoral fellowship in plant-microbe interactions at the University of Washington in Seattle.

The NGS DreamPrep method needs to be validated in your lab based on your workflow and according to your desired use and laboratory protocols. Tecan makes no claims regarding the performance of the example methods. For research use only. Not for use in diagnostic procedures.

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