Whole Genome and Transcriptome Analysis for Leukemia Diagnostics

Automated on the Hamilton NGS STAR Platform

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Introduction

The rationale behind the automation of the assay is mainly in terms of a quality improvement and enhancement of productivity, provided by minimizing the bias that might be otherwise introduced by manual library preparation. Moreover, the automated system is sufficiently flexible to adapt and therefore easily meet evolving needs.

- High throughput workflow for 5000 patients
- Standardization of whole genome and transcriptome workflows for sequencing
- Lower bias compared with manual sample preparation

Library preparation is a key requirement for many Next Generation Sequencing (NGS) applications such as the sequencing and analysis of clinical samples; an essential part of medical diagnostics.

Figure 1: Fully-optional configuration of the Hamilton NGS STAR system

The automation of the NGS workflow, including on-deck incubations and plate shaking, plate stacking, and large tip inventory, significantly reduces hands-on time and allows for the processing of up to 96 samples in a single run. Proper placement of reagents, plates and tips is guaranteed by automated barcode verification. The user can also define in-line controls, upload a worklist with the combination of index and samples, select the desired fragmentation time, and define the appropriate index adapters. The automated error handling and the easy-to-use graphical interface ensure a smooth setup of the experimental run, which can also be started and stopped at specific steps within the process.

Protocol

The automation of the TruSeq® DNA PCR-Free library preparation starts with an input of 1 µg genomic DNA, which is fragmented to a 350 bp insert size by shearing with Covaris. The Covaris generates dsDNA fragments with 3' and 5' overhangs, which are then loaded to the Hamilton NGS STAR in 96-well PCR plates.

The on-deck sample processing starts with the end repair process to convert the overhangs from the fragmentation into blunt ends. Subsequently the appropriate library size is automatically selected using different ratios of the Sample Purification Beads (SPB). The blunt 3' ends of the library fragments are then adenylated and ligated with the appropriated adapters.

The automation of the TruSeq® Stranded Total RNA library preparation starts with an input of 0.1-1 ug of total RNA. Following the quantification and dilution of the total RNA, the samples are loaded on the deck in 96-well PCR plates. The automated workflow depletes rRNA (Gold kit) from the total RNA and subsequently fragments and purifies the remaining RNA in order to remove the

mineral oil. The remaining RNA is then primed for cDNA synthesis (sequential 1st and 2nd strand cDNA synthesis). After a further purification step, the 3' ends are adenylated to prevent unspecific binding of the fragments. The newly generated A-overhangs provide the template for the subsequent adapter ligation.

Finally, the library is amplified on an external thermal cycler to increase the amount of DNA. Following the automated workflow, the resulting uniquely indexed paired-end libraries are assessed for both quality and quantity.

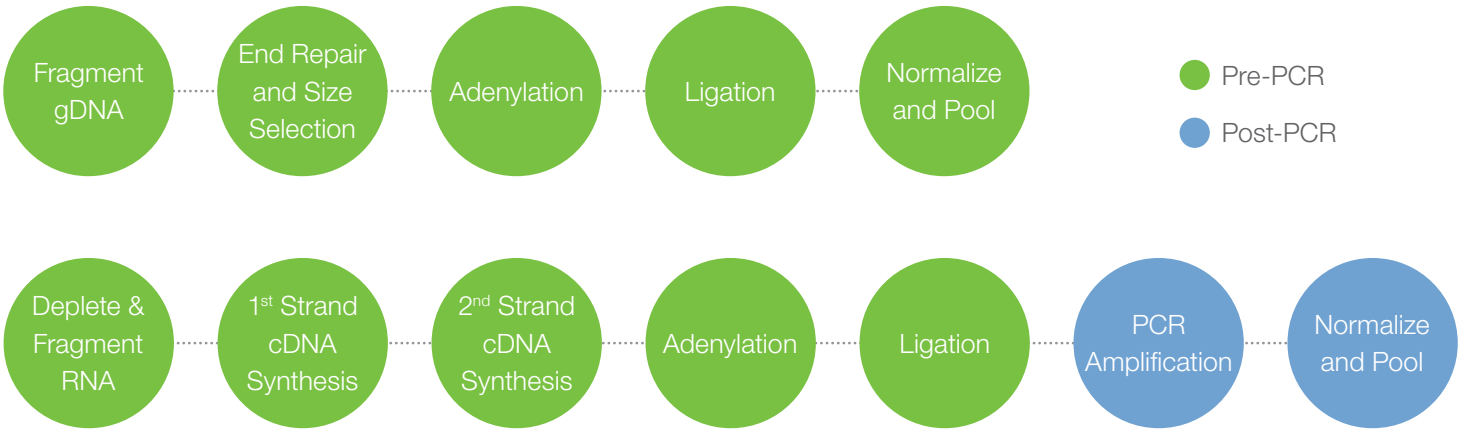


Figure 2: On top, the TruSeq® Nano DNA workflow; below, TruSeq® stranded total RNA workflow

Method Decription

The Whole Genome Sequencing (WGS) application is based on the automation of the TruSeq® DNA PCR-Free High Throughput Library Prep Kit (Illumina); while the Whole Transcriptome analysis is based on the automation of the TruSeq® Stranded Total RNA Library Prep Gold kit (Illumina).

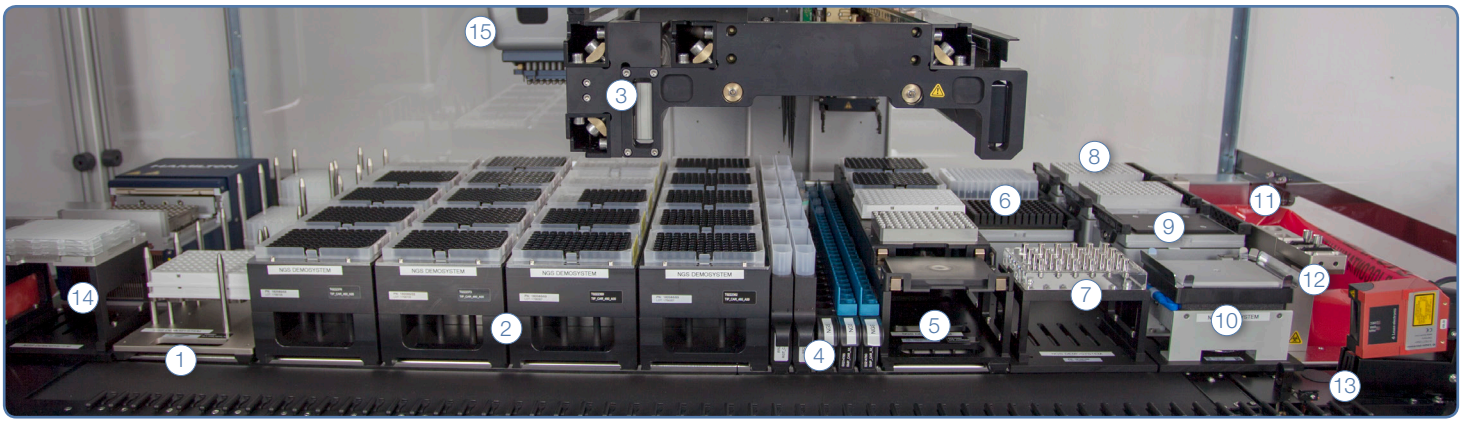
The methods start with DNA or RNA samples provided in a 96-well PCR plate. In order to reduce the loss of volumes during the pipetting, reagents are loaded on the system in their original containers. This applies, with the exception of large volume reagents, such as Ethanol, Beads and RSB buffers, which are stored in Hamilton reagent troughs. Both automated workflows can be used from 4 to 96 samples per run.

Moreover, to reduce hands-on time, the kit provider has validated the use of mineral oil, which enables the on-deck high tem-pertaure incubation of RNA fragments within the TruSeq® Stranded total RNA protocol.

System Description

NGS STAR basic configuration, developed with Microlab® Vector software (VENUS III), which includes the method itself, labware definitions and liquid classes (see deck layout description on the next page). Below is the labware requirement table.

Labware Requirements	Provider	Part Number
NGS STAR	Hamilton	806600
50 µL filtered tips	Hamilton	235948
300 µL filtered tips	Hamilton	235903
1000 µL filtered tips	Hamilton	235905
Biohazard waste bags	Hamilton	199202
50 ml reagent troughs	Hamilton	56694-01
MIDI (0.8 ml) 96-well plates	Thermo Fisher Scientific	AB-0859
HSP - Hard Shell Plate (PCR) 96-well plates	Bio-Rad	HSP-9601
2 ml screw cap micro-tubes	Sarstedt	72.694.xxx



1. Plate Stacker (HSP and MIDI plates)
2. Tip Carriers (50, 300 & 1000 µl filter tips)
3. Modular Arm 4/8/12/96MPH
   (equipped with 8x channels 1000 µl)
4. Reagent Carriers (Troughs, Tubes, and Vials)
5. Plates and Tips Carrier (HSP and MIDI Plates)
6. Hamilton Heater Shakers (HHS) with Adapters for MIDI Plates
7. Magnetic Stand (Thermo Fisher - Ambion)
8. Hamilton Heater Shakers (HHS) with Adapters for PCR Plates
9. Hamilton Heater Shakers (HHS) with flat bottom
10. Inheco CPAC
11. Liquid Waste
12. CO-RE Gripper
13. Autoloader with Barcode Reader
14. Inheco ODTC (On-Deck Thermal Cycler) with lid parking position *(optional and not included in this method)*
15. 96 Multi-Probe Head
    (optional configuration not used to generate the current data)

Kit Description

TruSeq® DNA PCR-Free High Throughput Library Prep Kit with IDT for Illumina Unique Dual Indexes: the kit provides simple, all-inclusive library preparation for whole-genome sequencing applications.

TruSeq® Stranded Total RNA Gold with IDT for Illumina - TruSeq® RNA UD Indexes (which allows multiplexing of up to 96 samples): the kit prepares the input samples for total RNA analysis with sequencing and removes both cytoplasmic and mitochondrial rRNA.

Technology

The NGS STAR is an ideal platform for the implementation of the TruSeq® DNA PCR-Free High Throughput Library Prep Kit and the TruSeq® Stranded Total RNA Library Prep Gold protocol, as it is equipped with all the positions and devices required for this NGS workflow. In combination with proven Hamilton technologies like CO-RE, MAD, and cLLD the process can be run with the high robustness and process-safety required for oncology applications.

Results

In total, 2688 WGS (TruSeq® DNA PCR-free) and 3414 WTS (TruSeq® Stranded Total RNA – Gold) libraries were generated, to date. In the TruSeq® DNA PCR-free workflow, 1 µg input genomic DNA was fragmented to a 350 bp insert size before loading on the deck in 96-well plates for the library preparation process.

The completion of the entire workflow required 9 hours for 48 and 9.5 hours for 96 samples. After the library preparation was completed, the uniquely indexed paired-end libraries were quantified with qPCR, by using the KAPA Library Quantification Kit.

In the TruSeq® Stranded Total RNA workflow, the total RNA is quantified and diluted, according to the protocol, before loading on the deck in 96-well plates. The workflow was completed in 13 hours for 96 samples. TruSeq® Stranded Total RNA libraries were subsequently assayed for quality and quantity using the Agilent TapeStation System.

Yield and Quality

The best performance of the TruSeq® DNA PCR-Free libraries was achieved by processing 48 samples in a single run. The average concentration of the final libraries was 9.26 nM, addressing the library yield requirements within the range of 2-20 nM. The average concentration of undiluted libraries was also compared between the manual approach and the automated workflow (Figure 3).

The result indicates the increase in uniformity in the automated workflow, supporting the assumption that an automated procedure increases the standardization and minimizes between sample variance. The sample-wide quality of the read depth and coverage distribution was also examined. The coverage of the chromosomes within a representative sample was mapped on an order 5 Hilbert curve. The chromosomes were binned, and each dot represents the median coverage of 175 kbp (Figure 4).

Homogenous results were obtained for TruSeq® Stranded Total RNA libraries, with an average concentration of 31.7 nM.

Throughput and Capacity

The methods can process between 4 and 96 samples per run. The lower-limit is set to ensure adequate pipetting volumes for some critical reagents.

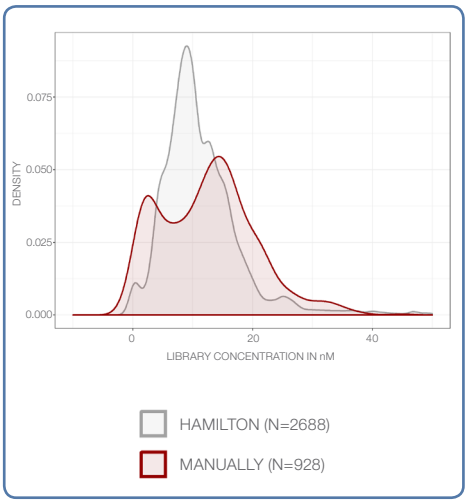


Figure 3: Density plot of library concentrations comparing the manual approach (dark red) with the automated workflow (gray).

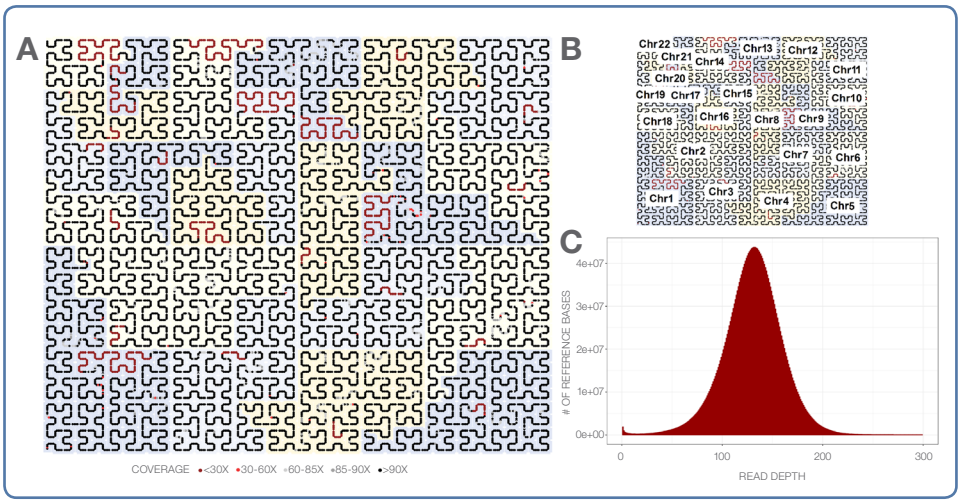


Figure 4: Read depth and coverage distribution of a patient's whole genome sequencing data.
(A) Coverage of the chromosomes is mapped on an order 5 Hilbert curve. The chromosomes are binned and each dot represents the median coverage of 175 kbp. The color of the dots corresponds to the coverage as indicated in the legend at the bottom.
(B) Schematic representation of the location of each chromosome on the Hilbert curve. The background colors define the areas of the individual chromosomes.
(C) Read depth of the sample over all chromosomes.

Discussion

Hamilton and Munich Leukemia Laboratory have developed a validated method for fully automated TruSeq® DNA PCR-Free library preparation and TruSeq® Stranded Total RNA library preparation with reliable yield and quality. This validated method qualifies for inclusion into an accredited diagnostic process, according to ISO 15189.

The flexible system provided by Hamilton can be easily adapted to meet changing needs, and to use a wide variety of different labwares. Additional options such as library dilution, library preparation for qPCR, and library pooling are also available.

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