APPLICATION NOTE

New England Biolabs' NEBNext Ultra II Directional RNA Library Preparation Workflows on Hamilton NGS STAR

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Introduction

New England Biolabs Inc. (NEB), the European Molecular Biology Laboratory (EMBL GeneCore), and Hamilton have developed automated versions of the poly(A) mRNA enrichment and rRNA depletion workflows with a seamless transition into the NEBNext Ultra II Directional RNA Library Prep Kit. The automation solutions of these protocols cater to the needs of users seeking scalable throughput with minimal hands-on time. The streamlined procedures allow for increased sensitivity and specificity in RNA-Seq experiments by choosing between the removal of interfering rRNAs or the enrichment of mRNAs by oligo d(T) beads.

Automation of RNA-Seq library preparation ensures higher reproducibility, reduction in hands-on time and adaptation of sample throughput according to user needs. Developed with automation in mind, NEBNext protocols include fewer components simplifying run setup. The Ultra II Directional RNA Library Preparation workflow also reduces bias for increased accuracy and confidence in your gene expression experiments. The automation solution provides highly efficient and time-saving sample processing as compared to manual preparation and its optimized working times allow users to focus on other urgent matters.

- **Generate high-quality libraries with a broad range of input amounts**
- **Save time with streamlined workflows and maximized walk-away automation**
- **Increase sample throughput with high reproducibility**

Figure 1: The Hamilton NGS STAR with ODTC.

Method Description

Extracted RNA samples are subject to quality control, quantification, and normalization before being provided to the instrument, prior to running the method. In the NEBNext Poly(A) mRNA enrichment workflow, mRNAs are isolated and cleaned-up using magnetic oligo d(T) Beads.
In the rRNA depletion workflow, single-stranded DNA (ssDNA) probes hybridize specifically to rRNA molecules. The hybridized rRNA is subsequently degraded using RNase H. The ssDNA probes are then digested by DNase I, followed by a bead clean-up.
Upon seamless transition from either workflow, samples then undergo fragmentation and priming steps before 1st and 2nd strand cDNA synthesis and a bead clean-up. DNA libraries are then generated through a series of enzymatic reactions including end repair/dA tailing, adaptor ligation and a clean-up step, followed by On-Deck PCR enrichment using the On Deck Thermal Cycler (ODTC) and a final bead clean-up. An option to perform size selection is also included within the method.
The method supports single or dual indexing options for sample multiplexing.
Running the complete end-to-end method is best split into two days and requires (approximately) a total of 12-13 hours for 48 samples.

Automation of New England Biolabs RNA library preparation

Increased sample throughput with high reproducibility

High yield and high-quality libraries with broad range of input amounts

System Description

In addition to the standard NGS STAR deck components (Figure 2), the Ambion Magnet Adapter (PN 10107866, Figure 3) needs to be purchased separately. This part allows for the pipetting of low volumes (≤ 15µl) from a Hard-Shell Plate (HSP) on the magnet position while being compatible with a Deep-Well Storage (MIDI) Plate. The adapter can be easily removed to allow for the adaptation of the deck for other NGS protocols.

Figure 2: Layout of the NGS STAR Deck with ODTC (details can be accessed on the NGS STAR Flyer).

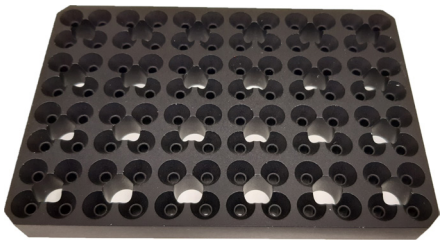

Figure 3: Ambion Magnet Adapter.

Kit Description

NEB's Ultra II Directional RNA Library Prep Kit is suitable for many sample types and makes RNA-Seq achievable for low-input samples. Library preparation can be performed with as little as 10 ng to as much as 1 µg total RNA. The streamlined workflow results in high quality libraries while saving both time and resources

Because ribosomal RNAs (rRNAs) are extremely abundant and constitute approximately 80 - 90% of total RNA, the efficient removal of these contaminants is critical to ensure cost-effective sequencing results.

Oligo d(T)-based mRNA enrichment is ideal for that purpose, if samples are of eukaryotic origin and of high-quality (RIN >7) with intact poly (A) tails.

The depletion of rRNAs is a highly valuable alternative that can ensure the removal of cytoplasmic and mitochondrial rRNA, even for low-quality RNA samples in a time-efficient manner. A high versatility in DNA probe pools allows its use with a broad diversity of sample types, including samples from human, mouse, rat, or bacteria.

Visual Workflow

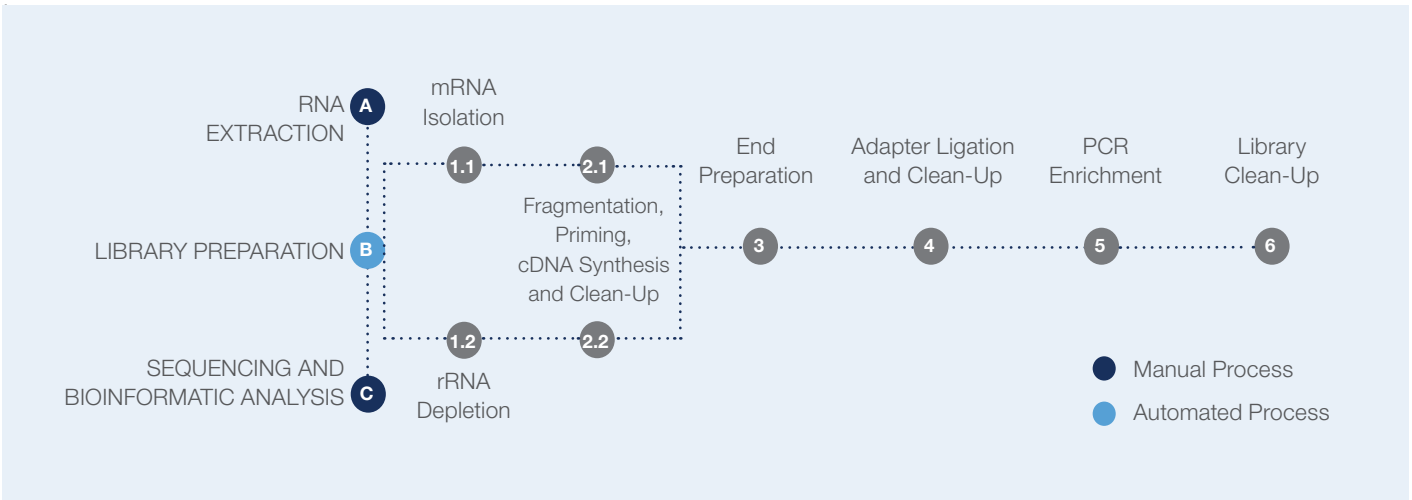

Figure 3: Graphical Overview of the NEBNext Ultra II Directional RNA Library Prep workflow including the two separate branches for Poly(A) mRNA enrichment or rRNA Depletion.

Qualification Setup

Both RNA workflows were tested with a total RNA input amount of 500 ng (Universal Human Reference RNA from Agilent Technologies, cat 740000, lot 0006305182) and qualified for the processing of 48 samples. No size selection was performed, and PCR cycles were chosen as indicated in Table 1.

The sequencing of final libraries for the Poly(A) mRNA Enrichment workflow was performed on an Illumina MiSeq in paired-end run mode. For the rRNA depletion workflow, sequencing was performed in single read mode on an Illumina NextSeq 500.

Eight selected samples of a 48 sample run were used for sequencing and data generated were downsampled to one million reads per sample for analysis.

Technology

The method uses the ODTC to enable maximal walk-away times by performing the necessary PCR steps on the instrument. Additionally, the method utilizes the capacitive Liquid Level Detection (cLLD) and pressure-based Liquid Level Detection (pLLD) technology of Hamilton CO-RE tips.

Results

After completion of the library preparation workflows on the NGS STAR, final library concentrations were measured using the Qubit 1x dsDNA HS Assay Kit (Cat. No. Q33230). Individual library size distributions were estimated using an Agilent 4150 TapeStation System (PN G2992AA), using the High Sensitivity D1000 ScreenTape Assay.
The results obtained indicated sufficient library yields for downstream processing and an expected narrow size distribution around 300 bp (Table 1).

After sequencing of the libraries, the quality of the data was assessed with regard to strand-specificity, mapping rate, and remaining rRNA (Figure 4). The data demonstrates a very high ability to determine the originating strand from which the RNA was transcribed. Further, only very few reads did not map to the reference genome, and contaminating rRNA species were very low, demonstrating superior depletion performance.

Run Metrics (tested with 48 samples)	Poly(A) Enrichment	rRNA Depletion
Input RNA Amount [ng]	500	500
Size selection	Not implemented	Not implemented
ODTC	Yes	Yes
PCR Cycles	12	10
Average Library Yields [ng/µl]	7.3 (±4.1)	16.7 (±5.6)
Average Library Yields [nM]	30.8 (±17.5)	86.2 (±25.6)
Average Library Size Distribution [bp]	360 (±8.0)	303 (±8.0)

Table 1: Run parameters and library yields

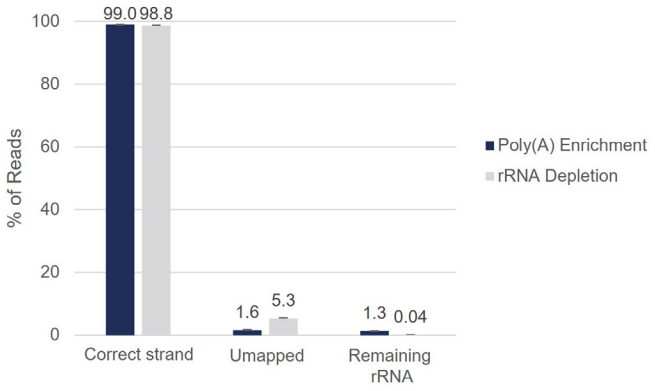

Figure 4: Sequencing results demonstrating excellent directionality and mapping rate, as well as the efficient depletion of rRNA.

High quality libraries are consistent across replicates, as shown in both poly(A) mRNA enrichment and rRNA depletion workflows. The poly(A) enriched libraries reflect the distribution of GC content of an exonic RNA population. In rRNA depletion, there is a broader diversity of RNA species represented (including non-coding), with a significant fraction at 60% GC (Figure 5A).

The libraries also display even coverage across the entire transcript length. A view of the 5' to 3' coverage of highly abundant transcripts measured using Picards RnaSeqMetrics shows end-to-end coverage for both workflows. Higher coverage at the 3' end for rRNA depletion libraries is a result of single-read sequencing (Figure 5B) used for these libraries vs. the paired-end sequencing used for the poly(A) mRNA enrichment libraries.

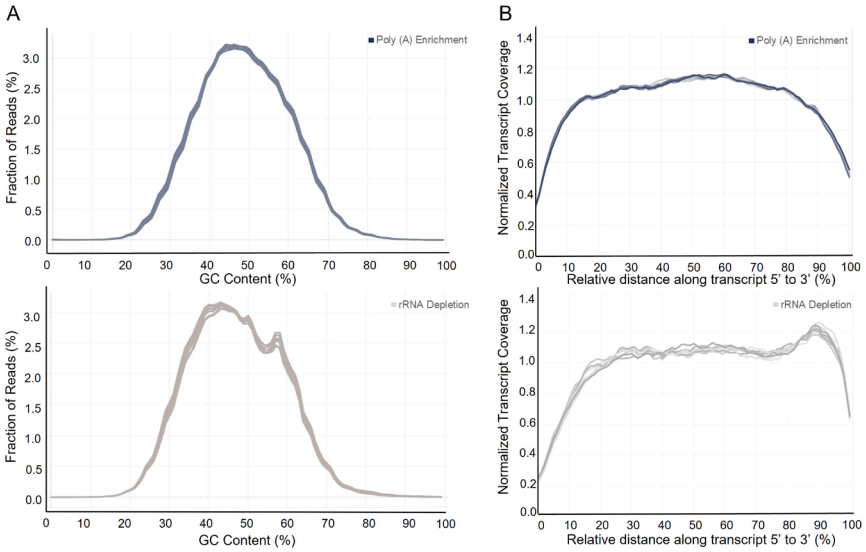

Figure 5: Unbiased sequencing independent of GC content and uniform coverage across gene body of transcripts.

Summary

Samples processed on the Hamilton NGS STAR using the NEBNext Ultra II Directional RNA Library Prep Kit were found to yield high-quality libraries with great performance on Illumina sequencing platforms.

Using automated library preparation enables the user to increase the number of samples that can be processed and thereby raise the number of RNA-Seq experiments to be performed.

Additionally, automating the directional methods for RNA sequencing substantially enhances the value gained from gene expression experiments. While in the poly(A) mRNA enrichment workflow only coding transcripts are considered, the combination with ribosomal RNA depletion further allows identification of antisense transcripts, determination of the transcribed strand of non-coding RNA, and measurement of expression levels of coding or non-coding overlapping transcripts

Others

System Requirements	Part Number
NGS STAR	806603
NGS STAR ODTC 96 Kit	806604
iSWAP Plate Handler	190220

Labware Requirements	Part Number	Provider
50 µL CO-RE Tips Filter	235984	Hamilton Bonaduz AG
300 µL CO-RE Tips Filter	235903	Hamilton Bonaduz AG
1000 µL CO-RE Tips Filter	235905	Hamilton Bonaduz AG
60 ml PP Reagent Trough with Lid	56694-01	Hamilton Bonaduz AG
PCR ComfortLid	814300	Hamilton Bonaduz AG
Hard-Shell 96-Well PCRPlate (HSP)	HSP-9601	Bio-Rad
Abgene 96-Well 0.8 mlPolypropylene Deep-Well Storage Plate	AB-0859	Thermo Fisher Scientific
2 ml Screw Cap Micro Tubes	72.694.006	Sarstedt

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