Improving NGS library performance with lower input amounts using the NEBNext® Ultra™ II RNA Library Prep Kit for Illumina® (non-directional)

Advances in non-strand-specific RNA library construction in a **poly(A) mRNA enrichment** workflow

This is one of four technical notes available that address directional and non-directional RNA library preparation for both poly(A) mRNA enrichment and rRNA depletion workflows. Additional tech notes:

- NEBNext Ultra II Directional RNA Library Prep Kit in a rRNA depletion workflow
- NEBNext Ultra II Directional RNA Library Prep Kit in a poly(A) mRNA enrichment workflow
- Non-directional NEBNext Ultra II RNA Library Prep Kit in a rRNA depletion workflow

Introduction

RNA-seq has become the most popular method for transcriptome analysis and is widely used to study gene expression, and to detect mutations, fusion transcripts, alternative splicing, and post-transcriptional modifications. It is becoming the method of choice to detect genetic alterations causing diseases, to provide insights on the various molecular pathways perturbed by changes in the transcriptome and study their implications. As RNA-seq is adopted for this growing range of applications, the need for good quality, reproducible library preparation methods using very low amounts of RNA input, or precious clinical samples, is increasing.

To meet these challenges, we have reformulated each step of the RNA library prep workflow to create the **NEBNext Ultra II RNA Library Prep Kit for Illumina (NEB #E7770/E7775)**. This new kit utilizes a fast, streamlined, automatable workflow for high-yield production of superior quality libraries, from as little as 10 ng total RNA input in a poly(A) mRNA enrichment workflow.

Note that libraries constructed using this kit are not strand-specific.

For construction of strand-specific libraries please see the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB #E7760/E7665).

For removal of ribosomal RNA, the kit is compatible with both poly(A) mRNA enrichment and rRNA depletion. **Here we demonstrate the utility of NEBNext Ultra II RNA Library Prep Kit for library construction in a poly(A) mRNA enrichment workflow, with a broad range of input amounts.**

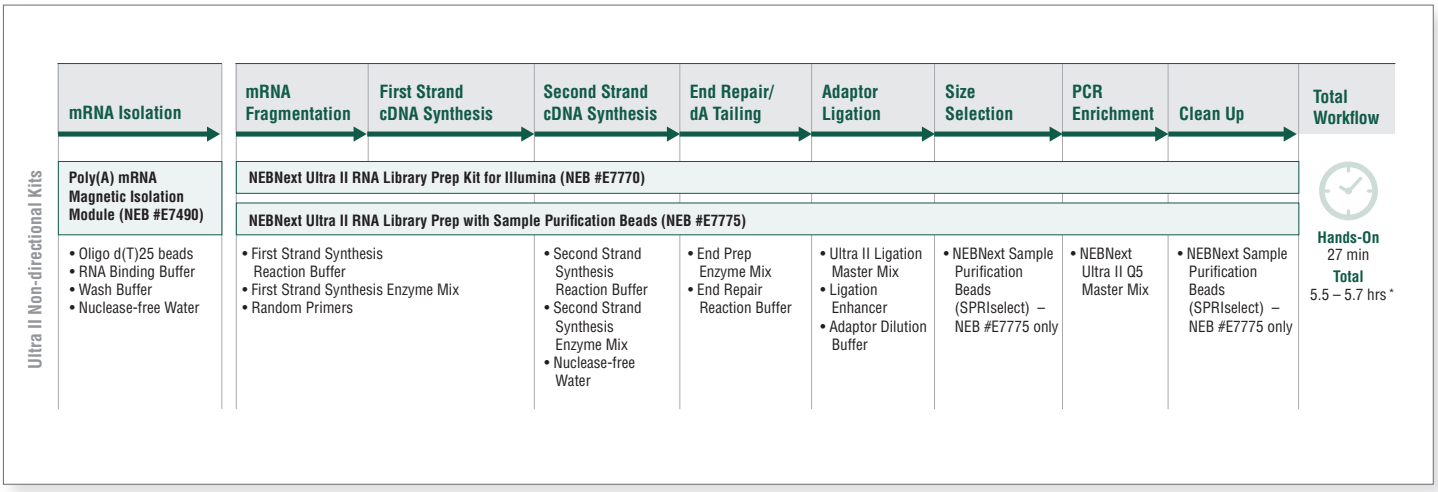
For information on performance in a ribosomal RNA depletion workflow, please refer to the separate technical note on that topic.

The NEBNext Ultra II RNA Workflow with poly(A) mRNA Enrichment

The workflow combines enrichment of mRNA using the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #7490) and library construction using the NEBNext Ultra II RNA Library Prep Kit (NEB #E7770/E7775).

The library prep kit's new reverse transcriptase master mix improves first strand synthesis. As in the Ultra II DNA kit (NEB #E7645), combining the End Repair and dA-Tailing steps and minimizing clean up steps makes the workflow both fast (~ 6 hours) and easy to use (Figure 1). The protocol can accommodate 10 ng to 1 µg of Total RNA for the poly(A) mRNA enrichment workflow. As little as 1 ng of previously isolated mRNA can be used directly with the NEBNext Ultra II RNA Library Prep Kit. The protocol is compatible with adaptors and primers from the NEBNext product line ("NEBNext Oligos") or from other sources.

FIGURE 1:
NEBNext Ultra II Directional RNA workflow with poly(A) mRNA enrichment



Library Yields

One measure of the success of library preparation is the yield of the final library. The NEBNext Ultra II RNA Kit produces substantially higher yields compared to another commercially available kit (Figure 2), and compared to NEB's original Ultra RNA kit (Figure 3). The increased reaction efficiencies with the Ultra II kit mean that sufficient library yields can be obtained even with low input amounts, and with fewer PCR cycles.

FIGURE 2: **NEBNext Ultra II RNA produces the highest yields, from a range of input amounts**

Poly(A)-containing mRNA was isolated from Universal Human Reference RNA (Agilent® #740000) and libraries were prepared using the NEBNext Ultra II RNA Kit (plus the NEBNext Poly(A) mRNA Magnetic Isolation Module), and the Illumina TruSeq RNA Sample Preparation Kit v2. Input RNA and number of PCR cycles are indicated. Library yields from an average of three replicates are shown.

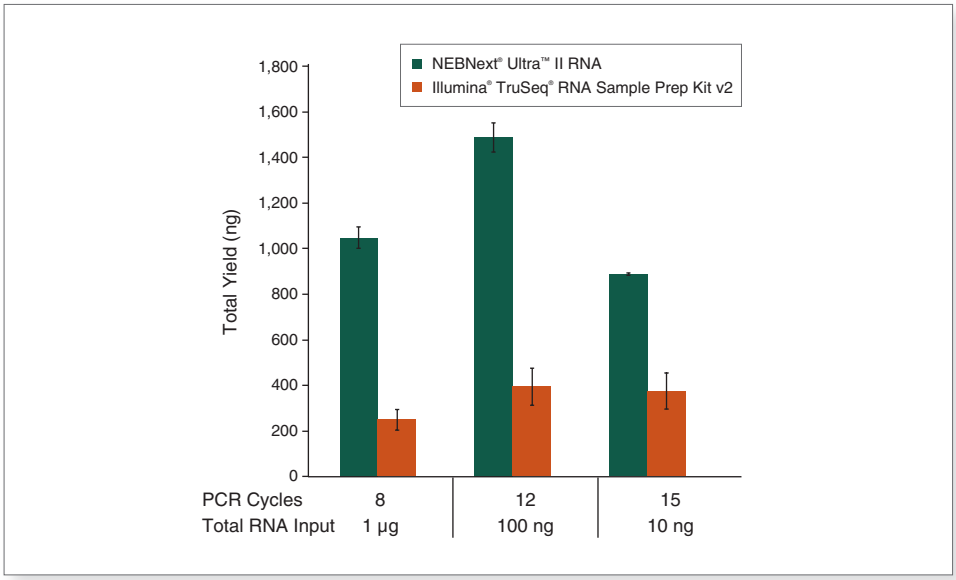
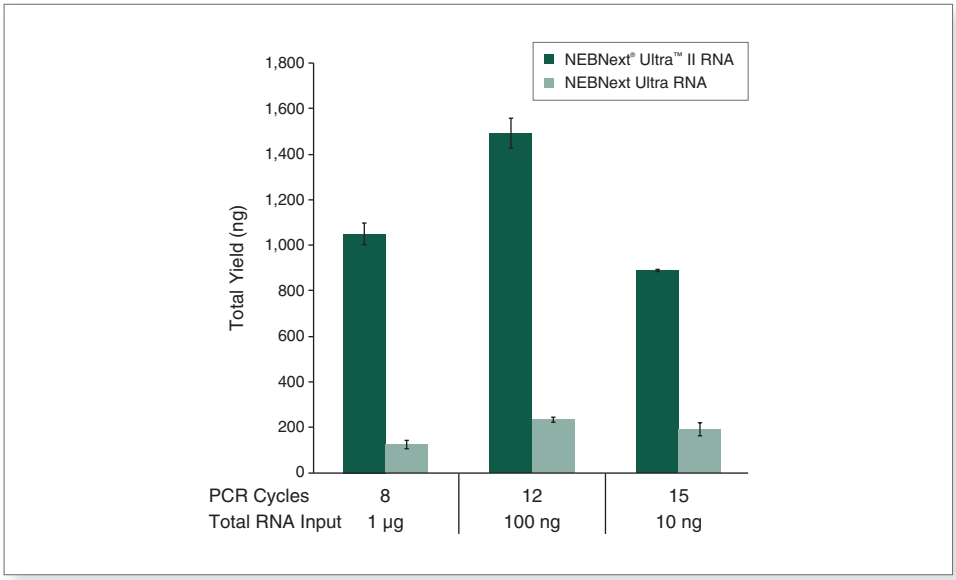


FIGURE 3: **NEBNext Ultra II RNA produces yields several fold higher than the original Ultra RNA Kit**

Poly(A)-containing mRNA was isolated from Universal Human Reference RNA (Agilent #740000) using the NEBNext Poly(A) mRNA Magnetic Isolation Module, and libraries were made using either the Ultra II RNA Kit or the original Ultra RNA Kit. Significantly higher yields were achieved with the Ultra II RNA kit than with the original Ultra RNA Kit.



Library Quality

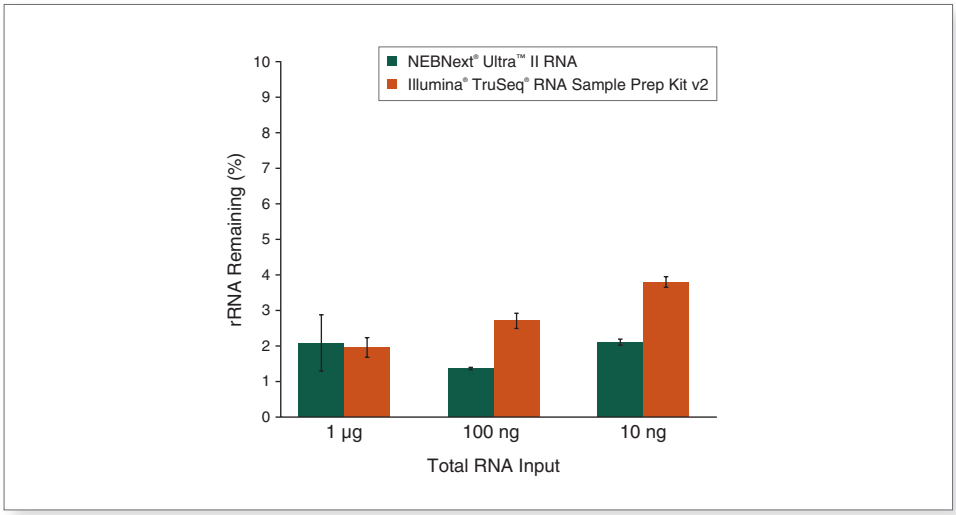
While sufficient yield of a library is required for successful sequencing, quantity alone is not enough. The quality of a library is also critical, regardless of the input amount or GC content of the sample RNA. A high-quality library will have uniform representation of the RNA of interest in the original sample, as well as even coverage across the GC spectrum.

Levels of Ribosomal RNA Remaining After poly(A) mRNA Enrichment

Ribosomal RNAs (rRNAs) are extremely abundant, constituting 80 - 90% of total RNA, and so one measure of the efficiency of enrichment of poly(A) mRNAs is the level of rRNA present in the sample after enrichment.

FIGURE 4: **NEBNext Ultra II RNA with NEBNext poly(A) mRNA isolation results in lower ribosomal RNA remaining**

Poly(A)-containing mRNA was isolated from Universal Human Reference RNA (Agilent #740000) and libraries were prepared using the NEBNext Ultra II RNA Kit (plus the NEBNext Poly(A) mRNA Magnetic Isolation Module), and the Illumina TruSeq RNA Sample Preparation Kit v2. Input RNA amount is indicated. Libraries were sequenced on an Illumina® NextSeq® 500 using paired-end mode (2x76 bp). Read pairs were assessed to be ribosomal if they contain 6 or more 32 base matches to 18S, 28S, 5S, 5.8S, 16S or 12S human rRNA sequences (mirabait 4.9). Percent rRNA remaining was calculated by dividing rRNA reads by the total number of reads passing instrument quality filtering. Percent rRNA remaining is shown from an average of three replicates.

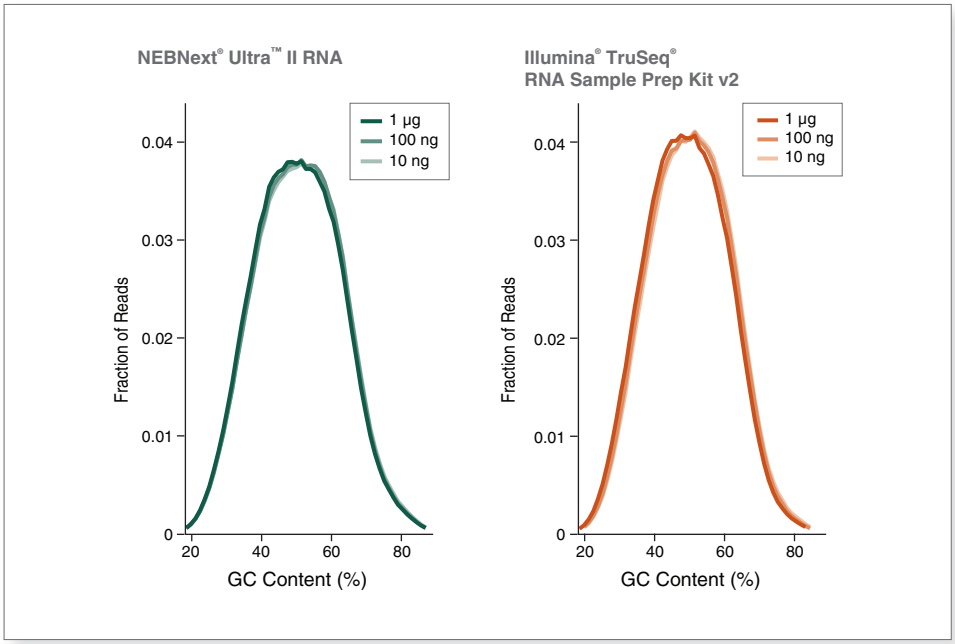


Uniformity of GC Content Distribution

During the entire library construction workflow, and especially when amplification is required to obtain sufficient library yields, it is important to ensure that no bias is introduced, and that representation of GC-rich and AT-rich regions is not skewed in the final library. Uniformity of GC representation can be more challenging to maintain with lower input amounts.

FIGURE 5: **NEBNext Ultra II RNA libraries provide uniform GC content distribution, at a broad range of input amounts**

Poly(A)-containing mRNA was isolated from Universal Human Reference RNA (Agilent #740000), and libraries were prepared using the NEBNext Ultra II RNA Kit (plus the NEBNext Poly(A) mRNA Magnetic Isolation Module), and the Illumina RNA Sample Preparation Kit v2. Input RNA amount is indicated. Libraries were sequenced on an Illumina NextSeq 500 using paired-end mode (2x76 bp). Reads were mapped to the hg19 reference genome using Hisat 2.0.3. GC content distribution for each library was calculated using mapped reads.

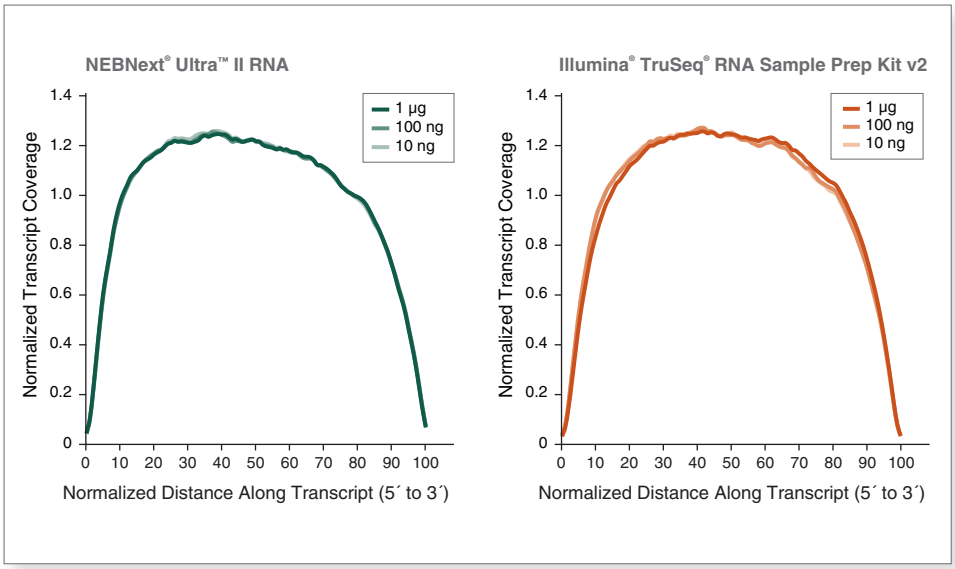


Uniformity of Transcript Coverage

A high-quality library will not only include all transcripts from the original sample, but cover those transcripts completely from 5' to 3'. Examination of global transcript coverage (gene body) can highlight differences between transcript coverage at different input amounts, and between different library kits.

FIGURE 6: **NEBNext Ultra II RNA libraries provide uniform coverage across the gene body of transcripts**

Poly(A)-containing mRNA was isolated from Universal Human Reference RNA (Agilent #740000), and libraries were prepared using the NEBNext Ultra II RNA Kit (plus the NEBNext Poly(A) mRNA Magnetic Isolation Module), and the Illumina TruSeq RNA Sample Preparation Kit v2. Input RNA amount is indicated. Libraries were sequenced on an Illumina NextSeq 500 using paired-end mode (2x76 bp). This view of the 5´ to 3´ coverage of RefSeq (1) transcripts reveals consistent coverage for Ultra II RNA libraries as input RNA is decreased from 1 µg to 10 ng.



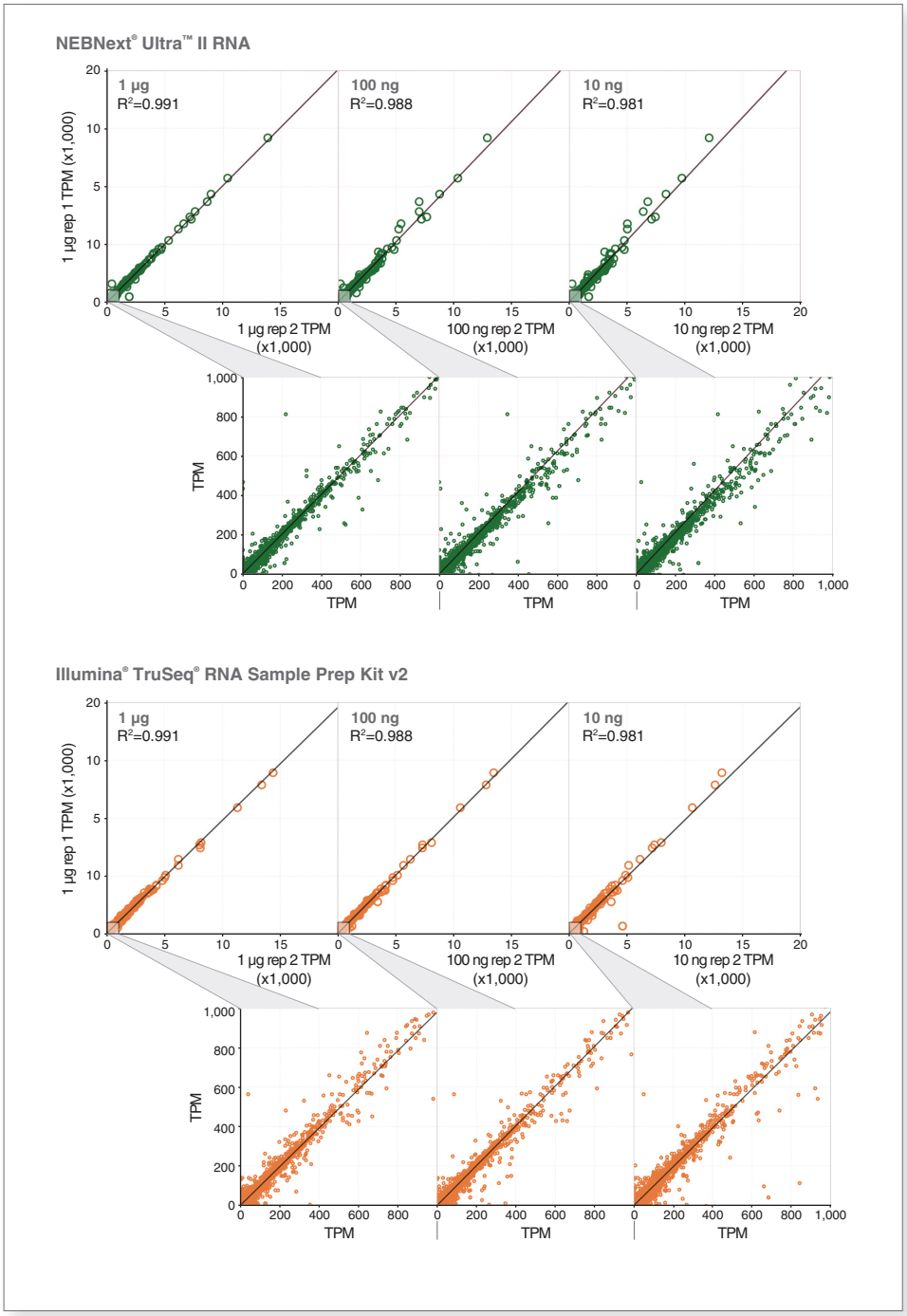
1. O'Leary, N. A., et al. (2016) *Nucleic Acid Res.* 44 (D1), D733–D745.

Superior Library Complexity, Even at Low Input Amounts

As described above, an ideal library will represent completely and proportionally the sequence of the input RNA of interest. When library preparation is inefficient or when input amounts for a library are very low, there is a risk that the resulting library will lack this diversity, and that some sequences will be over- or under-represented. Comparison of transcript abundance achieved with libraries constructed from different input amounts of RNA is a useful measure to determine the effect of input amounts on coverage. The increased efficiency of each step in the NEBNext Ultra II RNA library workflow provides consistent library composition as input amounts are decreased from 1 µg to 100 ng and 10 ng.

FIGURE 7: **Low input NEBNext Ultra II RNA libraries retain superior complexity**

Poly(A)-containing mRNA was isolated from Universal Human Reference RNA (Agilent #740000), and libraries were prepared using the NEBNext Ultra II RNA Kit (plus the NEBNext Poly(A) mRNA Magnetic Isolation Module), and the Illumina TruSeq RNA Sample Preparation Kit v2. Salmon 0.4.0 was used for read mapping and quantification of all GENCODE v25 transcripts. TPM = Transcript Per Kilobase Million. R^2 values for the linear fit are shown. For each set, the lower figures show an expanded view of the 0–1,000 TPM range. Correlation analysis of the transcripts indicates very good transcript expression correlation between the different input amounts.



Conclusion

The NEBNext Ultra II RNA Library Prep Kit for Illumina represents a substantial advance in non-directional library preparation for RNA sequencing in conjunction with poly(A) mRNA enrichment. Improved reagents and workflow steps increase efficiencies of each step, and enable users to overcome many of the challenges previously associated with successful library preparation, such as:

- The use of input amounts of Total RNA from low nanograms to 1 microgram
- Generation of higher yields, with the use of fewer PCR cycles
- Uniformity of transcript coverage, and high library complexity, even at very low input amounts
- Uniform GC coverage of the sample
- Fast, streamlined library preparation that is automation-friendly

For performance data and other information on the NEBNext Ultra II RNA Library Prep Kit in rRNA depletion workflows, see the separate application note.

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