

# Obtain superior NGS library performance with lower input amounts using the NEBNext<sup>®</sup> Ultra<sup>™</sup> II Directional RNA Library Prep Kit for Illumina<sup>®</sup>

Substantial advances in strand-specific RNA library construction in a **ribosomal RNA 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 Directional RNA Library Prep Kit for Illumina (NEB #E7760/E7765)**. This new kit utilizes a fast, streamlined, automatable workflow for high-yield production of superior quality libraries, from as little as 5 ng total RNA input.

Strand-specificity is important for correct annotation of genes, identification of antisense transcripts with potential regulatory roles, and accurate determination of gene expression levels in the presence of antisense transcripts. Enhanced sensitivity to detect transcripts with uniform coverage across their length offers a non-biased approach for accurate quantitation of transcript levels.

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

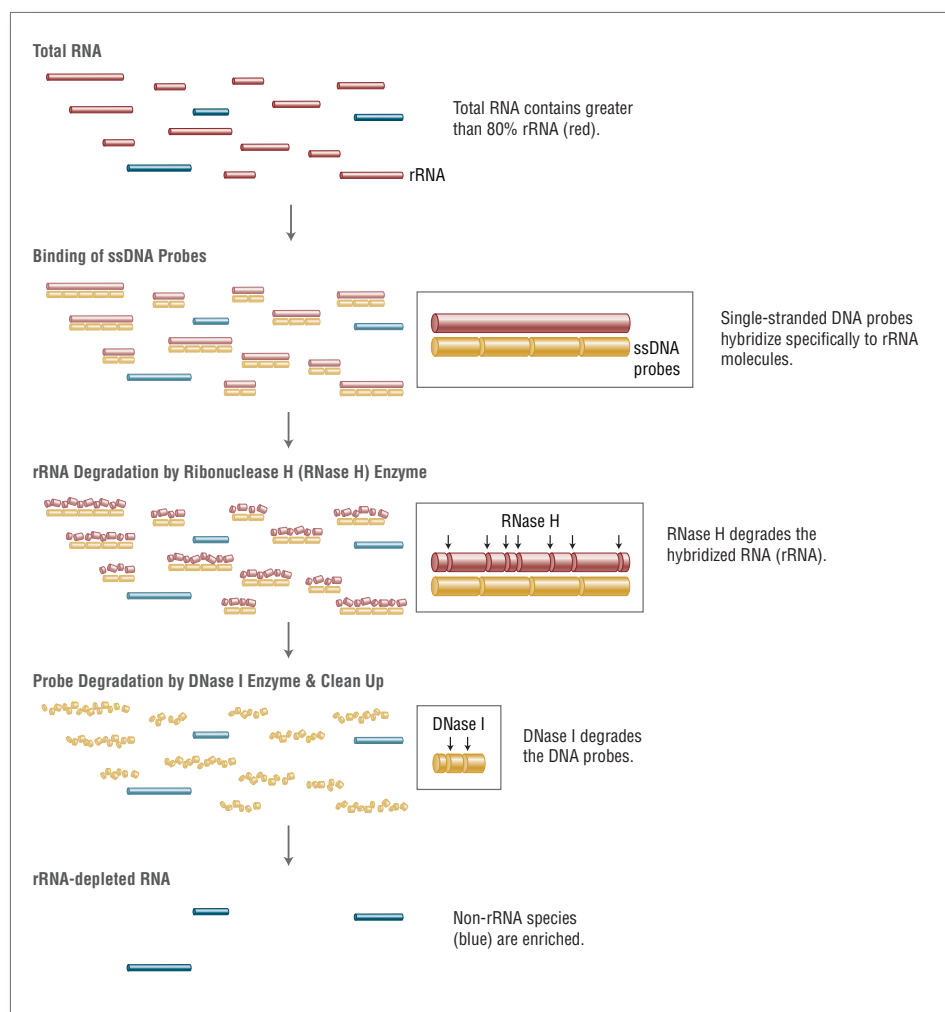
For information on performance in a poly(A) mRNA enrichment workflow, please refer to the separate technical note on that topic.

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 poly(A) mRNA enrichment workflow
- Non-directional NEBNext Ultra II 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



**FIGURE 1:**  
**NEBNext rRNA depletion kit workflow**



# The NEBNext Ultra II Directional RNA Workflow with Ribosomal RNA Depletion

The workflow combines removal of rRNA using the NEBNext rRNA Depletion Kit (Human/Mouse/Rat) (NEB #6310) and library construction using the NEBNext Ultra II Directional RNA Library Prep Kit (NEB #E7760).

The NEBNext rRNA Depletion Kit employs an RNase H-based method (1,2) to deplete both cytoplasmic (5S rRNA, 5.8S rRNA, 18S rRNA and 28S rRNA) and mitochondrial ribosomal RNA (12S rRNA and 16S rRNA) from human, mouse and rat total RNA preparations (Figure 1). The kit is suitable for both intact and degraded RNA (e.g. FFPE RNA).

The library prep kit's new reverse transcriptase master mix improves first strand synthesis, and the inclusion of Actinomycin D in the kit (in the Strand Specificity Reagent) increases the user-friendliness of this step. As in the Ultra II DNA kit, combining the End Repair and dA-Tailing steps and minimizing clean up steps makes the kit fast (~ 6 hours) and easy to use (Figures 2 and 3). The protocol can accommodate 5 ng to 1 µg of total RNA for the rRNA depletion workflow. As little as 1ng of previously isolated rRNA-depleted RNA can be used directly with the NEBNext Ultra II Directional RNA Library Prep Kit. The protocol is compatible with adaptors and primers from the NEBNext product line ("NEBNext Oligos") or from other sources.

1. Adiconis, X., et al. (2013) *Nature Methods* 10, 623–629.  
2. Morlan, J. D., et al. (2012) *PLoS One* 77. e42882.

FIGURE 2:  
NEBNext Ultra II Directional RNA Library Prep Kit workflow

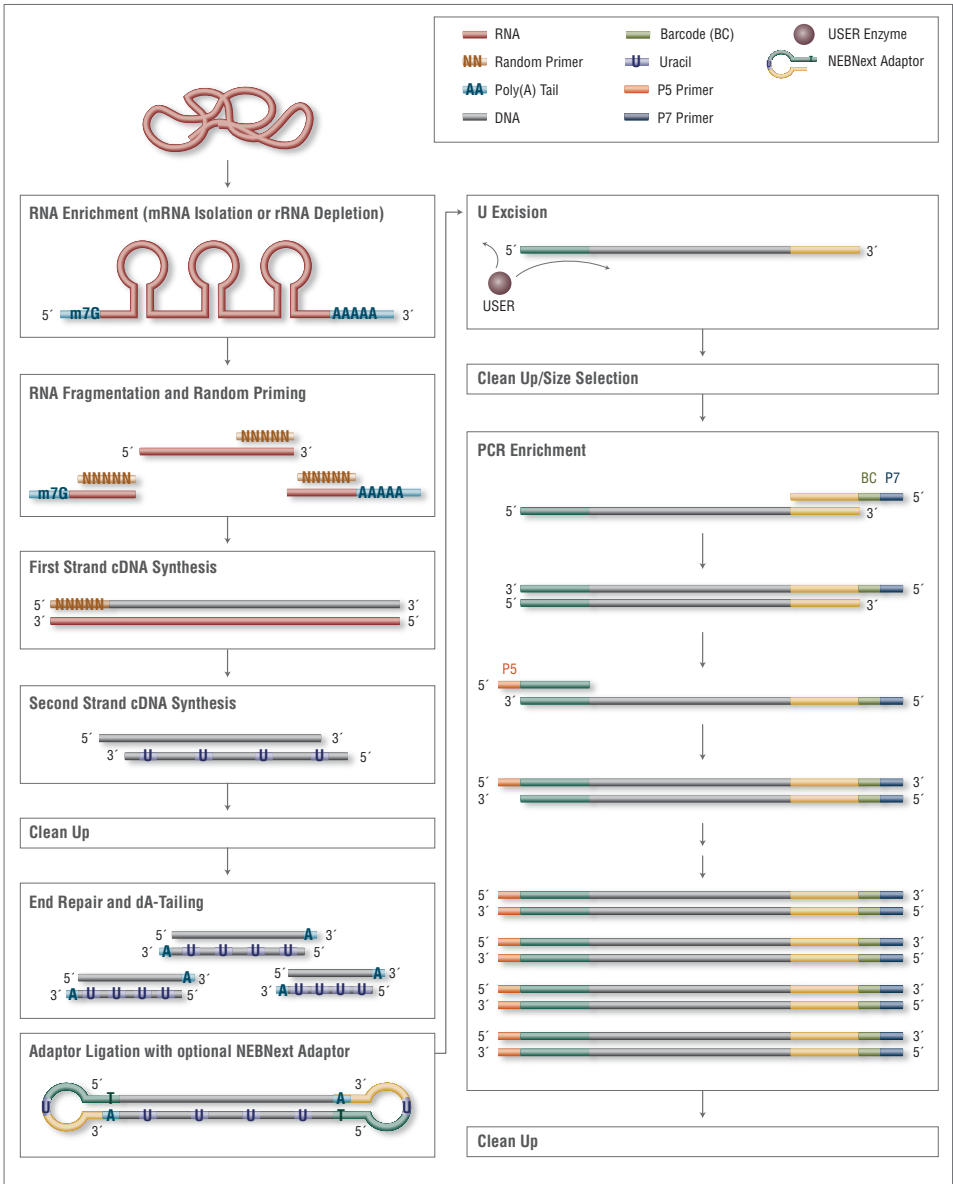


FIGURE 3:  
NEBNext Ultra II Directional RNA workflow with NEBNext rRNA Depletion

	rRNA Depletion	mRNA Fragmentation	First Strand cDNA Synthesis	Second Strand cDNA Synthesis	End Repair/ dA Tailing	Adaptor Ligation	Size Selection	PCR Enrichment	Clean Up	Total Workflow
Ultra II Directional Kits	<b>rRNA Depletion Kit (Human/Mouse/Rat) (NEB #6310)</b>	<b>NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB #E7760)</b>								
		<b>NEBNext Ultra II Directional RNA Library Prep with Sample Purification Beads (NEB #E7765)</b>								
	<ul style="list-style-type: none"> <li>• RNase H/RNase H Reaction Buffer</li> <li>• rRNA Depletion Solution</li> <li>• Probe Hybridization Buffer</li> <li>• DNase I/DNase I Reaction Buffer</li> <li>• Nuclease-free Water</li> </ul>	<ul style="list-style-type: none"> <li>• First Strand Synthesis Reaction Buffer</li> <li>• First Strand Synthesis Enzyme Mix</li> <li>• Random Primers</li> <li>• Strand Specificity Reagent</li> </ul>	<ul style="list-style-type: none"> <li>• Second Strand Synthesis Reaction Buffer with dUTP Mix</li> <li>• Second Strand Synthesis Enzyme Mix</li> <li>• Nuclease-free Water</li> </ul>	<ul style="list-style-type: none"> <li>• End Prep Enzyme Mix</li> <li>• End Repair Reaction Buffer</li> </ul>	<ul style="list-style-type: none"> <li>• Ultra II Ligation Master Mix</li> <li>• Ligation Enhancer</li> <li>• Adaptor Dilution Buffer</li> </ul>	<ul style="list-style-type: none"> <li>• NEBNext Sample Purification Beads (SPRIselect) – NEB #E7765 only</li> </ul>	<ul style="list-style-type: none"> <li>• NEBNext Ultra II Q5 Master Mix</li> </ul>	<ul style="list-style-type: none"> <li>• NEBNext Sample Purification Beads (SPRIselect) – NEB #E7765 only</li> </ul>		<b>Hands-On</b> 27 min <b>Total</b> 6.6 – 6.8 hrs

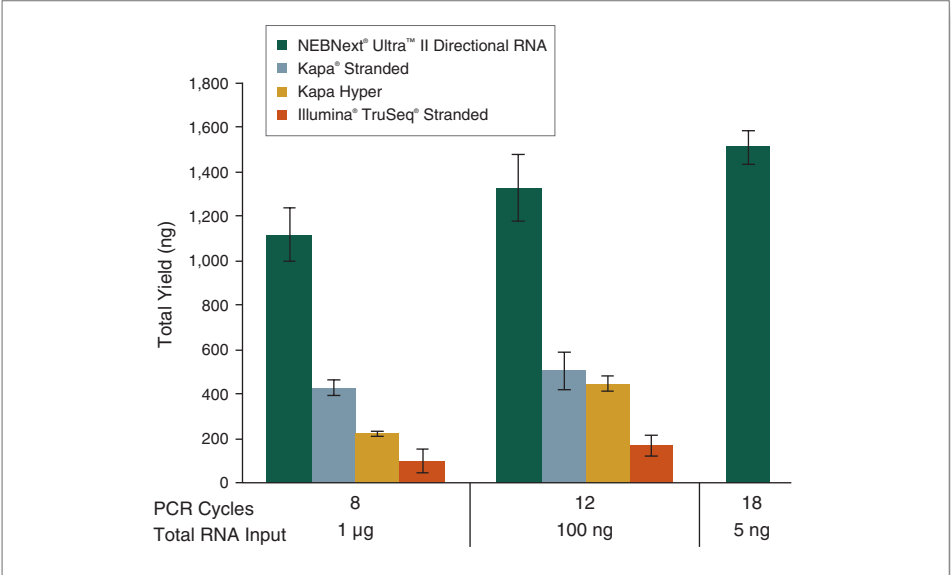
### Library Yields

One measure of the success of library preparation is the yield of the final library. The NEBNext Ultra II Directional RNA Kit produces substantially higher yields compared to other commercially available kits (Figures 4 and 5), and compared to NEB's original Ultra Directional RNA kit (Figure 6). 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.



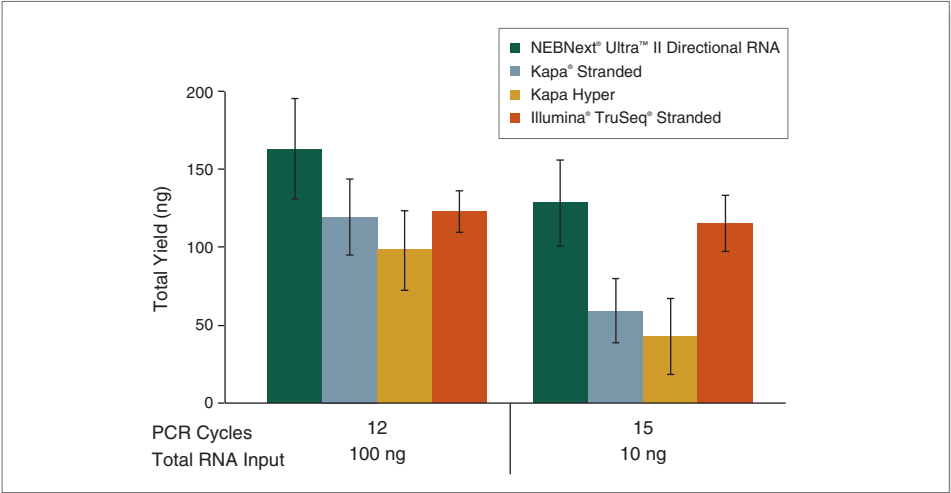
**FIGURE 4: NEBNext Ultra II Directional RNA produces the highest yields, from a range of input amounts**

Ribosomal RNA (rRNA) was depleted from Human Universal Reference RNA (Agilent® #740000) and libraries were made using 1 µg, 100 ng and 5 ng input with NEBNext Ultra II Directional RNA Kit (plus the NEBNext rRNA Depletion Kit (Human/Mouse/Rat)), and using 1 µg and 100 ng input with Kapa Stranded RNA-Seq Kit with RiboErase, Kapa HyperPrep Kit with RiboErase, and Illumina TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero™ Gold. The input RNA amount and number of PCR cycles are indicated. Library yields from an average of three replicates are shown.



**FIGURE 5: NEBNext Ultra II Directional RNA produces the highest yields with FFPE RNA**

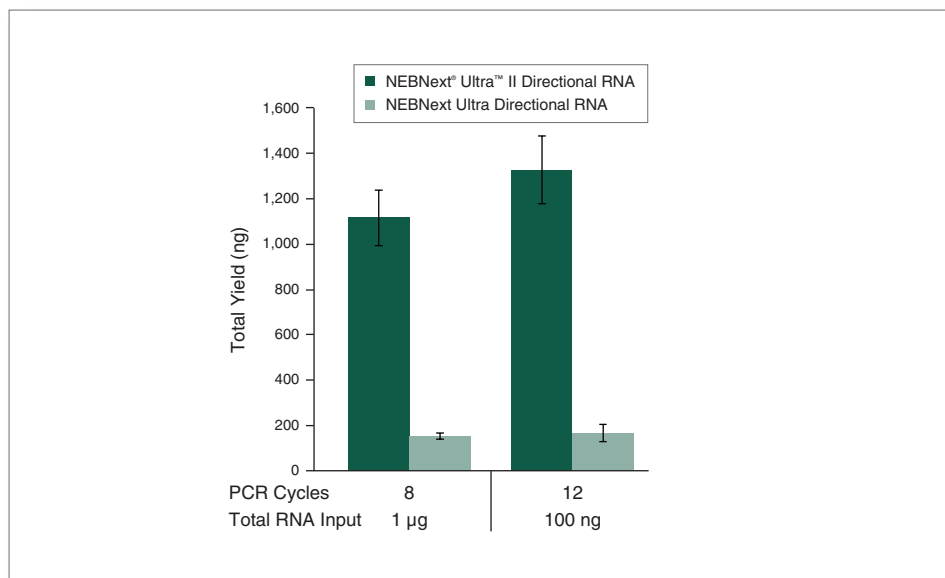
Ribosomal RNA was depleted from human adult normal liver tissue FFPE Total RNA (Biochain # R2234149, RIN 2.5) and libraries were made using NEBNext Ultra II Directional RNA Kit (plus the NEBNext rRNA Depletion Kit (Human/Mouse/Rat)), Kapa Stranded RNA-Seq Kit with RiboErase, Kapa HyperPrep Kit with RiboErase, and Illumina TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero Gold. The input RNA amount and number of PCR cycles are indicated. Library yields from an average of three replicates are shown.





**FIGURE 6: NEBNext Ultra II Directional RNA produces yields several fold higher than the original Ultra Directional RNA Kit**

Ribosomal RNA was depleted from Human Universal Reference RNA (Agilent #740000) using the NEBNext rRNA Depletion Kit (Human/Mouse/Rat) and libraries were made using either the Ultra II Directional RNA Kit or the original Ultra Directional RNA Kit. The input RNA amount and number of PCR cycles are indicated. Significantly higher yields were achieved with the Ultra II Directional RNA Kit than with the original Ultra Directional RNA Kit. Library yields from an average of three replicates are shown.



## 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.

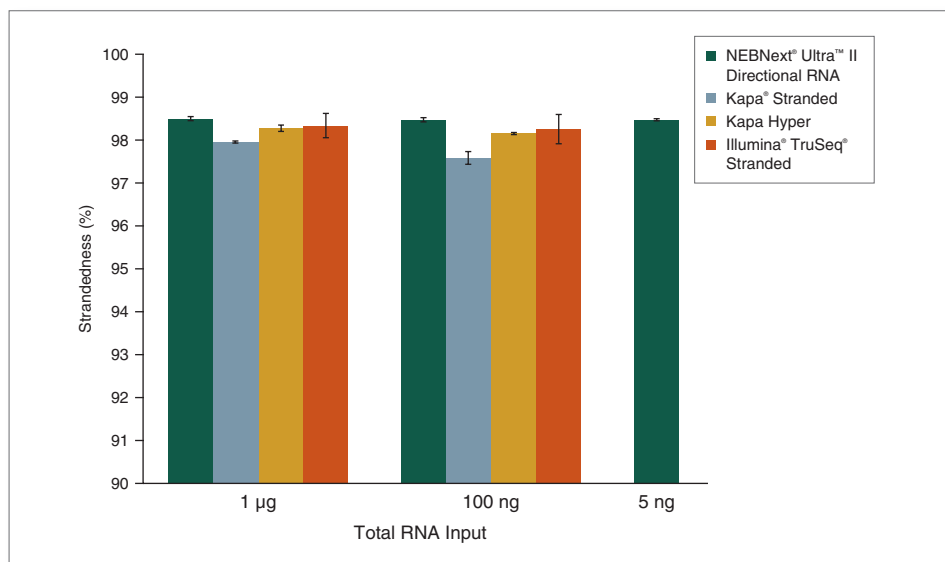
## Library Directionality/Strand-specificity

The NEBNext Ultra II Directional RNA Library Prep Kit derives its directionality from the "dUTP" method for strand-specificity, the proven gold standard method for this application (3,4). Labeling of the second strand cDNA using dUTP enables subsequent selective destruction of that strand, with the result that only one strand is incorporated into the final library, thus providing directionality (Figures 7 and 8).



**FIGURE 7: NEBNext Ultra II Directional RNA libraries have high directionality/strandedness**

Ribosomal RNA (rRNA) was depleted from Human Universal Reference RNA (Agilent #740000) and libraries were made using 1 µg, 100 ng and 5 ng input with NEBNext Ultra II Directional RNA Kit (plus the NEBNext rRNA Depletion Kit (Human/Mouse/Rat)), and using 1 µg and 100 ng input with Kapa Stranded RNA-Seq Kit with RiboErase, Kapa HyperPrep Kit with RiboErase, and Illumina TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero Gold. 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 and directionality was calculated using the infer experiment tool from the RSeQC Quality Control Package. Ultra II Directional RNA retains high directionality at low ng input amounts.



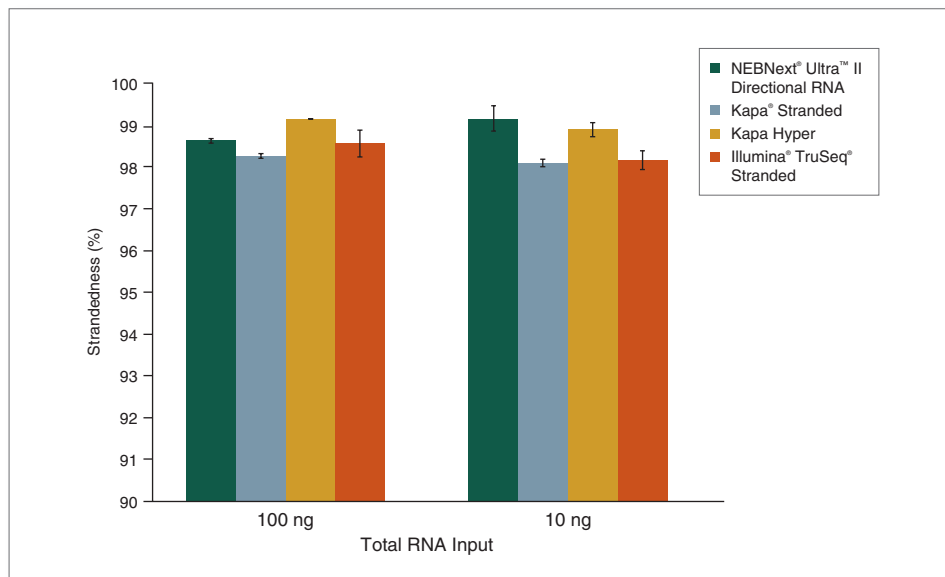
3. Parkhomchuk, D., et al. (2009) *Nucleic Acids Res.* 37, e123.

4. Levin, J. Z., et al. (2010) *Nature Methods* 7, 709–715.



**FIGURE 8: NEBNext Ultra II Directional RNA libraries have high directionality/strand specificity with FFPE samples**

Ribosomal RNA was depleted from human adult normal liver tissue FFPE Total RNA (Biochain # R2234149, RIN 2.5) and libraries were made using NEBNext Ultra II Directional RNA Kit (plus the NEBNext rRNA Depletion Kit (Human/Mouse/Rat)), Kapa Stranded RNA-Seq Kit with RiboErase, Kapa HyperPrep Kit with RiboErase, and Illumina TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero Gold. 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 and directionality was calculated using the infer experiment tool from the RSeQC Quality Control Package.



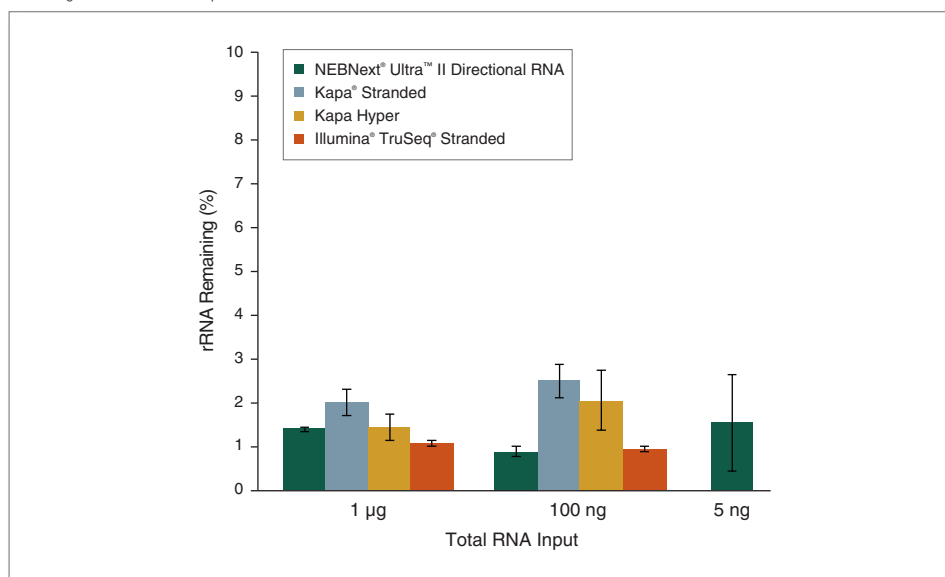
### Levels of Ribosomal RNA Remaining After rRNA Depletion

Ribosomal RNAs (rRNAs) are extremely abundant, constituting 80–90% of total RNA. Efficient removal of rRNA is critical to enable cost-effective sequencing of RNA samples, but this can be especially challenging with low quality RNA and with low input amounts. The NEBNext rRNA Depletion kit employs the efficient RNase H method, as well as complete probe tiling of rRNA, thereby ensuring that even degraded rRNA is hybridized and subsequently removed (Figures 1, 9 and 10).



**FIGURE 9: NEBNext Ultra II Directional RNA with NEBNext rRNA Depletion results in low remaining ribosomal RNA levels**

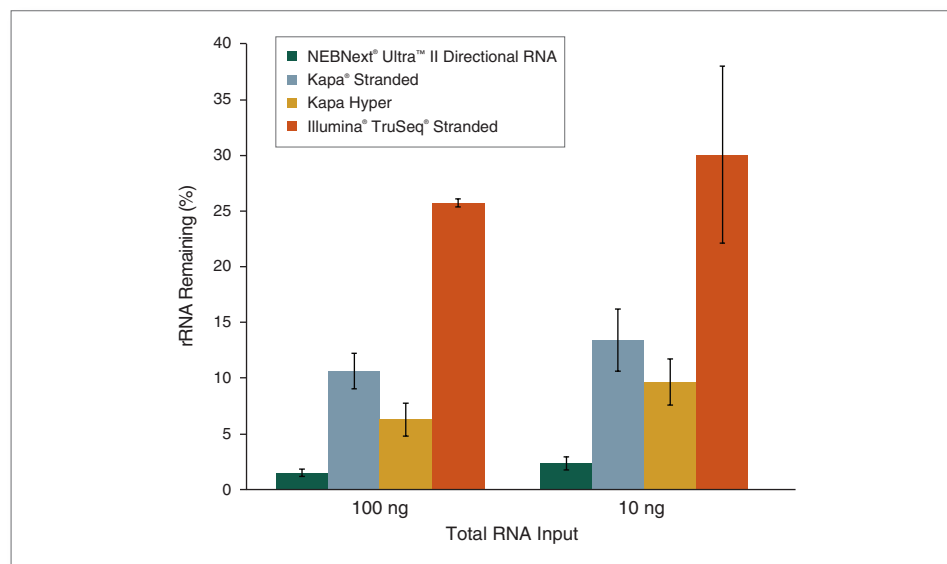
Ribosomal RNA (rRNA) was depleted from Human Universal Reference RNA (Agilent #740000) and libraries were made using 1 µg, 100 ng and 5 ng input with NEBNext Ultra II Directional RNA Kit (plus the NEBNext rRNA Depletion Kit (Human/Mouse/Rat)), and using 1 µg and 100 ng input with Kapa Stranded RNA-Seq Kit with RiboErase, Kapa HyperPrep Kit with RiboErase, and Illumina TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero Gold. Libraries were sequenced on an Illumina NextSeq 500 using paired-end mode (2x76 bp). Read pairs were assessed to be rRNA 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. Average percent rRNA remaining is shown for three replicates.





**FIGURE 10: NEBNext Ultra II Directional RNA with NEBNext rRNA Depletion results in the lowest remaining ribosomal RNA levels with FFPE samples**

Ribosomal RNA was depleted from human adult normal liver tissue FFPE Total RNA (Biochain # R2234149, RIN 2.5) and libraries were made using NEBNext Ultra II Directional RNA Kit (plus the NEBNext rRNA Depletion Kit (Human/Mouse/Rat)), Kapa Stranded RNA-Seq Kit with RiboErase, Kapa HyperPrep Kit with RiboErase, and Illumina TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero Gold. Libraries were sequenced on an Illumina NextSeq 500 using paired-end mode (2x76 bp). Read pairs were assessed to be rRNA 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. Average percent rRNA remaining is shown for three replicates. Error bars indicate standard deviation. The NEBNext rRNA Depletion Ultra II Directional RNA workflow is the most efficient in removing rRNA from total FFPE RNA.



## Duplication Rates

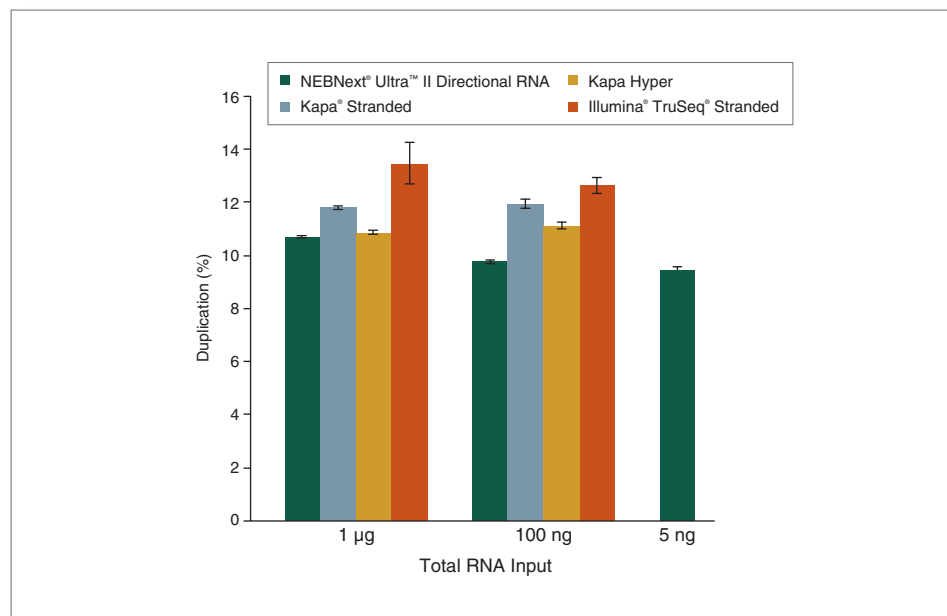
“Duplication rates” refers to the percentage of sequence reads that have identical start and end sites. These have most often arisen from preferential PCR amplification of the same molecule. Low quality libraries can result in high duplication rates, and duplication rates are often greater with libraries constructed from very low input amounts.

The low duplication rates (Figures 11 and 12) achieved with the NEBNext Ultra II Directional RNA Library Prep Kit, even with the high yields obtained (see Figures 4, 5 and 6) indicate the high quality of the libraries produced, and the opportunity to minimize PCR cycles.



**FIGURE 11: NEBNext Ultra II Directional RNA with NEBNext rRNA depletion results in lower duplication rates**

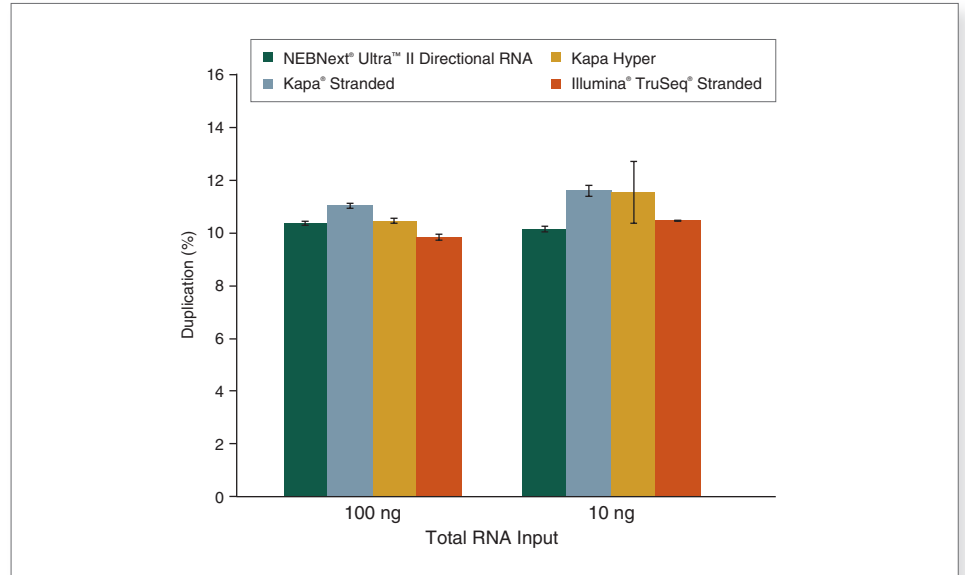
Ribosomal RNA (rRNA) was depleted from Human Universal Reference RNA (Agilent #740000) and libraries were made using 1 µg, 100 ng and 5 ng input with NEBNext Ultra II Directional RNA Kit (plus the NEBNext rRNA Depletion Kit (Human/Mouse/Rat)), and using 1 µg and 100 ng input with Kapa Stranded RNA-Seq Kit with RiboErase, Kapa HyperPrep Kit with RiboErase, and Illumina TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero Gold. 5ng input was tested only with the NEBNext kits. Libraries were sequenced on an Illumina NextSeq 500 using paired-end mode (2x76 bp). Reads were down sampled to 10 million read pairs and mapped to the hg19 reference genome. Duplication rates were calculated as a fraction of uniquely mapped reads using the ‘Read Duplication’ tool of RSeQC where reads mapping to the same genomic location are regarded as duplicated reads.





**FIGURE 12: NEBNext Ultra II Directional RNA with NEBNext rRNA depletion results in lower duplication rates with FFPE RNA**

Ribosomal RNA was depleted from human adult normal liver tissue FFPE Total RNA (Biochain # R2234149, RIN 2.5) and libraries were made using NEBNext Ultra II Directional RNA Kit (plus the NEBNext rRNA Depletion Kit (Human/Mouse/Rat)), Kapa Stranded RNA-Seq Kit with RiboErase, Kapa HyperPrep Kit with RiboErase, and Illumina TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero Gold. Libraries were sequenced on an Illumina NextSeq 500 using paired-end mode (2x76 bp). Reads were down sampled to 10 million read pairs and mapped to the hg19 reference genome. Duplication rates were calculated as a fraction of uniquely mapped reads using the 'Read Duplication' tool of RSeQC where reads mapping to the same genomic location are regarded as duplicated reads.



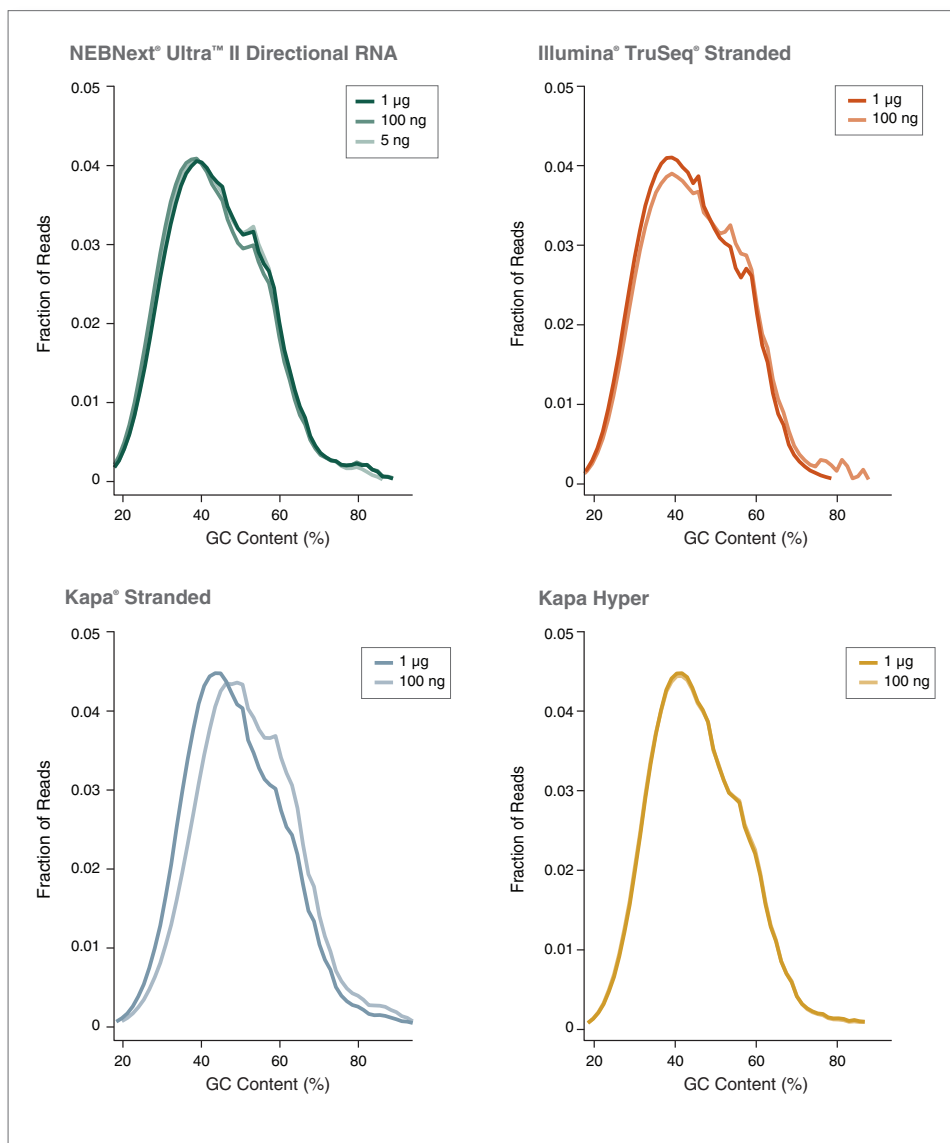
## Uniformity of GC Content Distribution

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, and low quality FFPE RNA, as is demonstrated in the figures below. However, the NEBNext Ultra II Directional RNA Kit maintains uniformity of GC coverage from 1 µg input libraries down to 5 ng input libraries Figures 13 and 14).



**FIGURE 13: NEBNext Ultra II Directional RNA libraries provide uniform GC content distribution, at a broad range of input amounts**

Ribosomal RNA (rRNA) was depleted from Human Universal Reference RNA (Agilent #740000) with recommended amounts of ERCC RNA Spike-In Mix I (Thermo Fisher Scientific #4456740) and libraries were made using 5 ng, 100 ng and 1 µg input with NEBNext Ultra II Directional RNA Kit (plus the NEBNext rRNA Depletion Kit (Human/Mouse/Rat)), and using 1 µg and 100 ng input with Illumina TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero Gold, Kapa Stranded RNA-Seq Kit with RiboErase and Kapa HyperPrep Kit with RiboErase. Libraries were sequenced on an Illumina NextSeq 500 using paired-end mode (2x76 bp). Reads were mapped to the hg19 reference genome. GC content distribution for each library was calculated using mapped reads. Ultra II Directional RNA libraries had uniform GC content distribution across a range of input amounts.

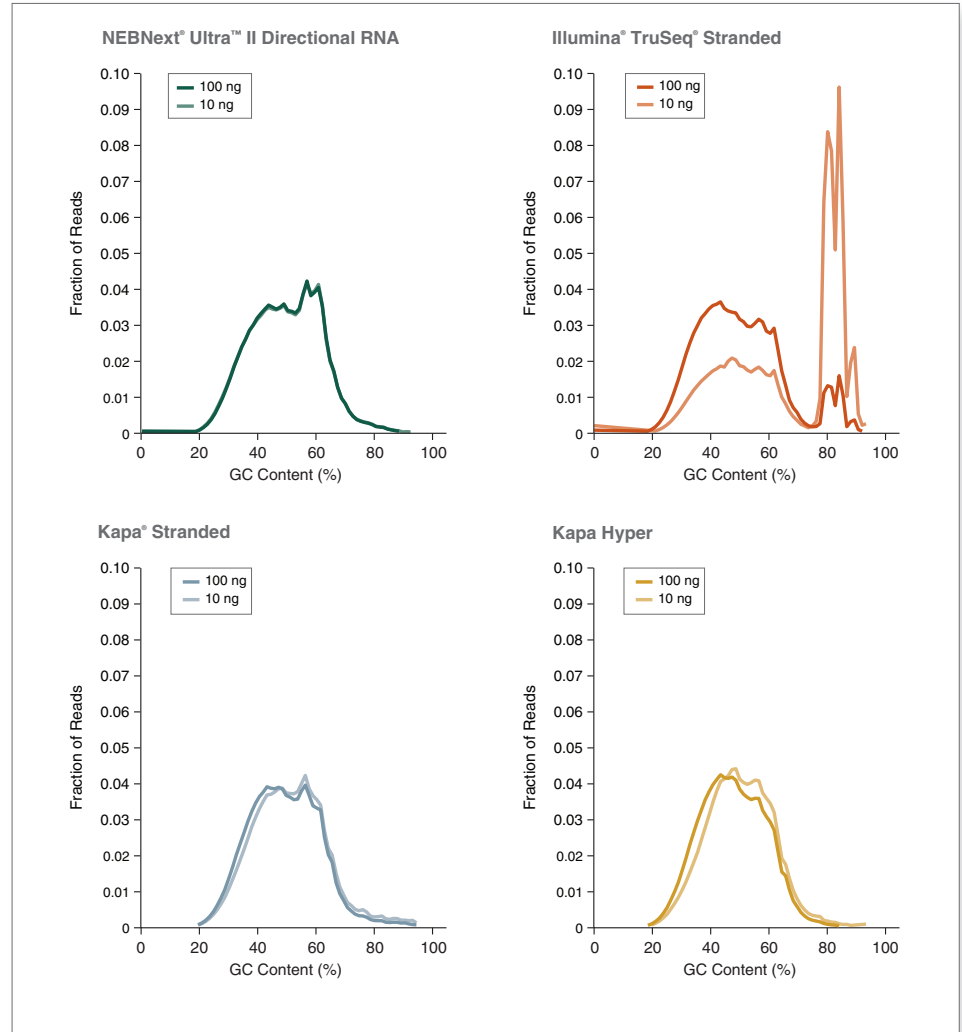






**FIGURE 14: NEBNext Ultra II Directional RNA libraries provide uniform GC content distribution with FFPE samples**

Ribosomal RNA was depleted from human adult normal liver tissue FFPE Total RNA (Biochain # R2234149, RIN 2.5) and libraries were made using NEBNext Ultra II Directional RNA Kit (plus the NEBNext rRNA Depletion Kit (Human/Mouse/Rat)), Illumina TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero Gold, Kapa Stranded RNA-Seq Kit with RiboErase and Kapa HyperPrep Kit with RiboErase. Libraries were sequenced on an Illumina NextSeq 500 using paired-end mode (2x76 bp). Reads were mapped to the hg19 reference genome. GC content distribution for each library was calculated using mapped reads. Ultra II Directional RNA libraries had uniform GC content distribution for both input amounts.



## 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'.

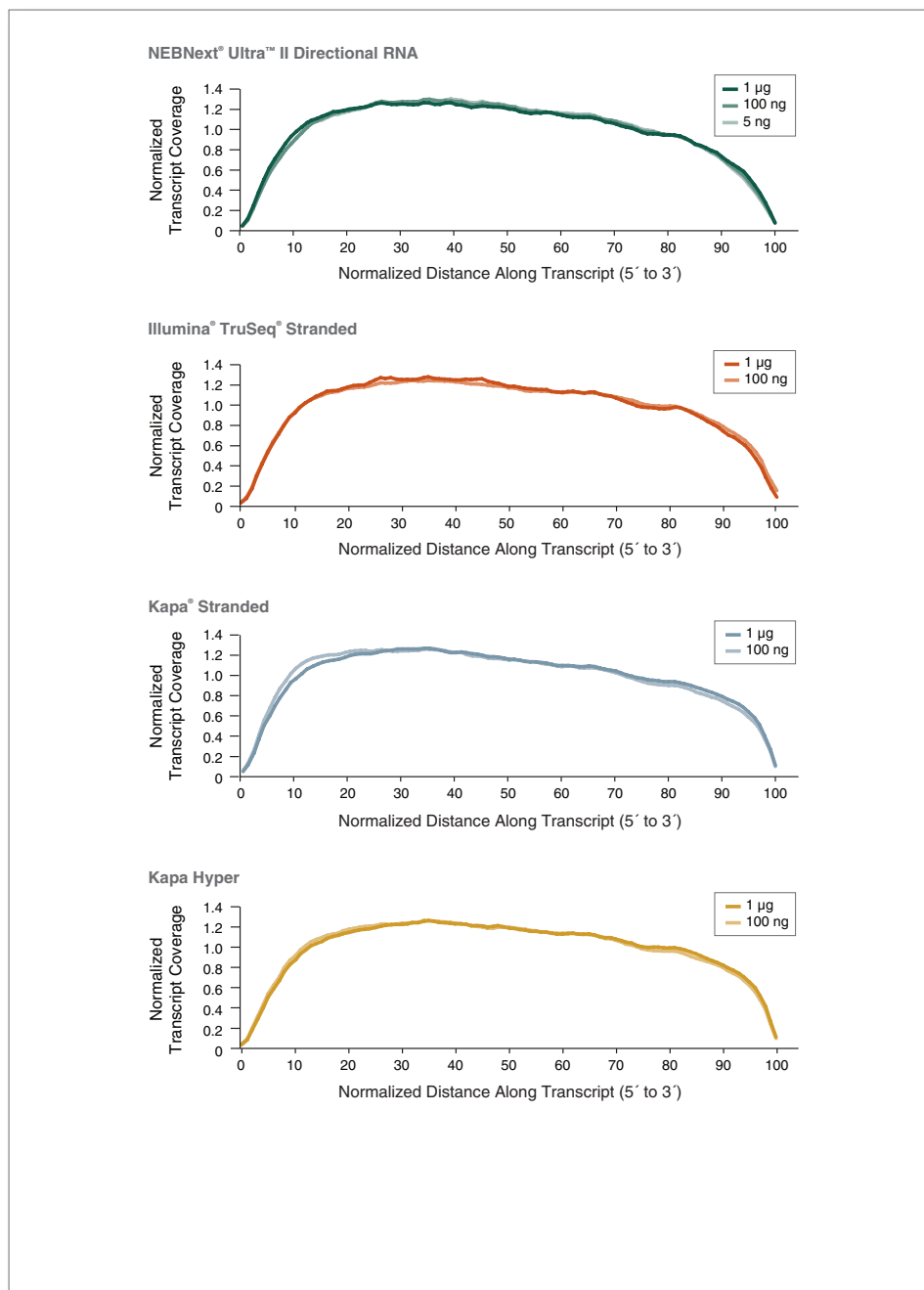
Transcript coverage can be examined on a global basis, and by looking at individual transcripts.

This can highlight differences between transcript coverage at different input amounts, and between different library kits. The use of ERCC standards, a set of RNA controls developed by the External RNA Controls Consortium (ERCC) and consisting of known, unlabeled, polyadenylated transcripts, is another useful tool in this type of experiment.



**FIGURE 15: NEBNext Ultra II Directional RNA libraries provide uniform coverage across the gene body of transcripts or coverage across the length of transcripts**

Ribosomal RNA (rRNA) was depleted from Human Universal Reference RNA (Agilent #740000) with recommended amounts of ERCC RNA Spike-In Mix I (Thermo Fisher Scientific #4456740) and libraries were made using 1 µg, 100 ng and 5 ng input with NEBNext Ultra II Directional RNA Kit (plus the NEBNext rRNA Depletion Kit (Human/Mouse/Rat)), and using 1 µg and 100 ng input with Illumina TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero Gold, Kapa Stranded RNA-Seq Kit with RiboErase and Kapa HyperPrep Kit with RiboErase. Libraries were sequenced on an Illumina NextSeq 500 using paired-end mode (2x76 bp). This view of the 5' to 3' coverage of RefSeq (5) transcripts reveals consistent coverage for Ultra II Directional RNA libraries as input RNA is decreased from 1 µg to 5 ng.

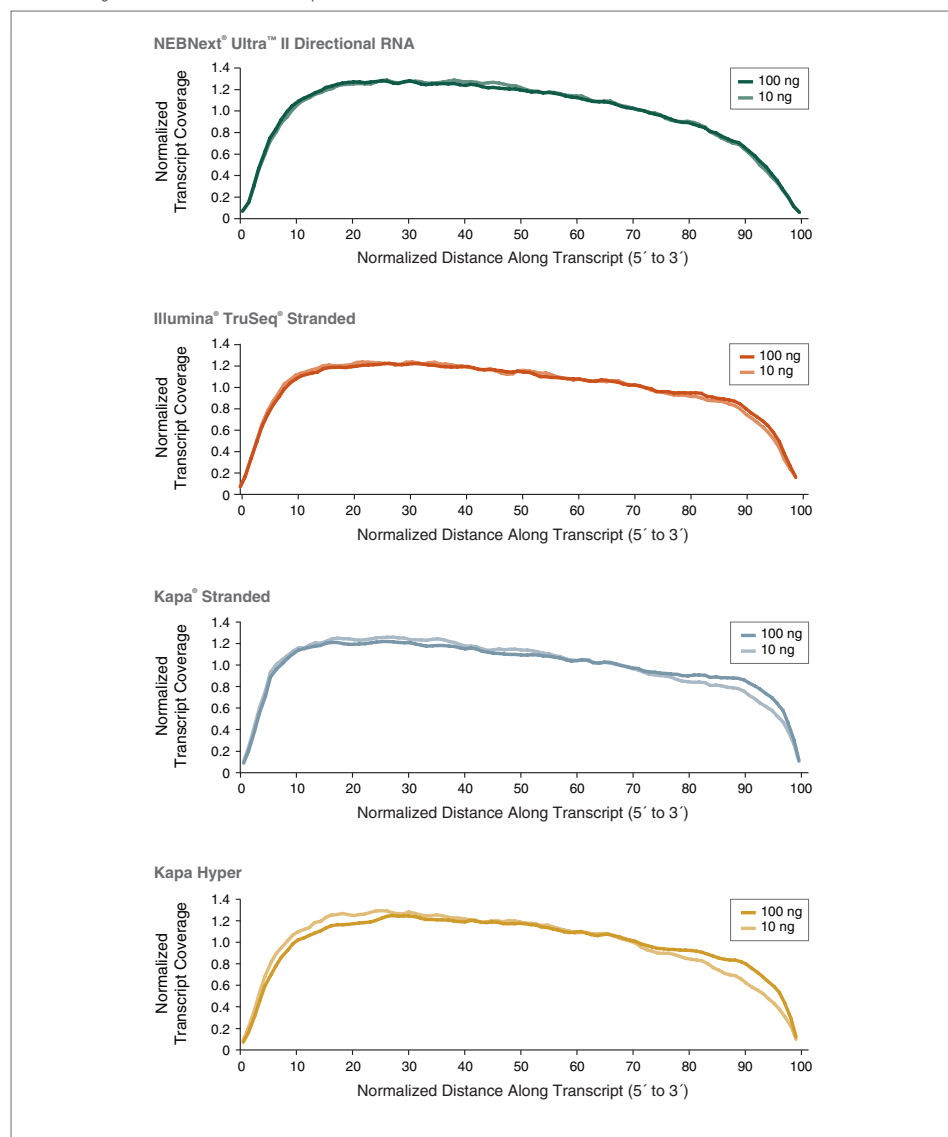


5. O'Leary, N. A., et al. (2016) *Nucleic Acids Res.* 44 (D1).



**FIGURE 16: NEBNext Ultra II Directional RNA libraries provide uniform coverage across the gene body for FFPE samples**

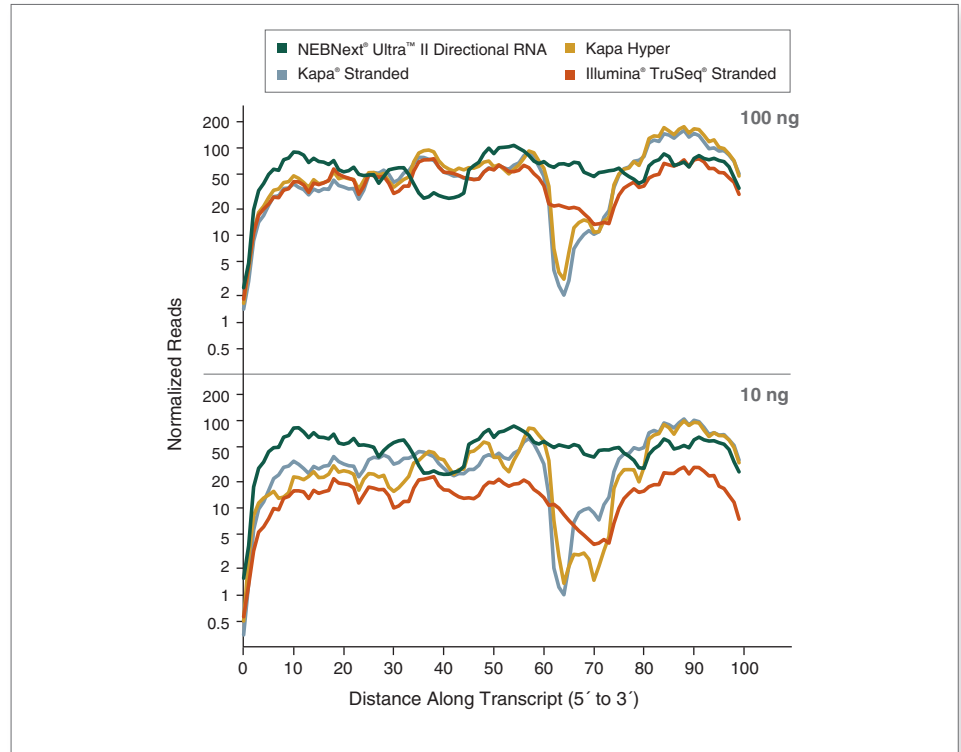
Ribosomal RNA was depleted from human adult normal liver tissue FFPE Total RNA (Biochain # R2234149, RIN 2.5) and libraries were made using NEBNext Ultra II Directional RNA Kit (plus the NEBNext rRNA Depletion Kit (Human/Mouse/Rat)), Illumina TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero Gold, Kapa Stranded RNA-Seq Kit with RiboErase, Kapa HyperPrep Kit with RiboErase. Libraries were sequenced on an Illumina NextSeq 500 using paired-end mode (2x76 bp). This view of the 5' to 3' coverage of RefSeq transcripts reveals consistent coverage for Ultra II Directional RNA libraries as input RNA is decreased from 100 ng to 10 ng. The changes apparent in other kits result from loss of coverage at the 3' end of some transcripts.





**FIGURE 17: Uniformity of Coverage across the AP000769.1-201 transcript**

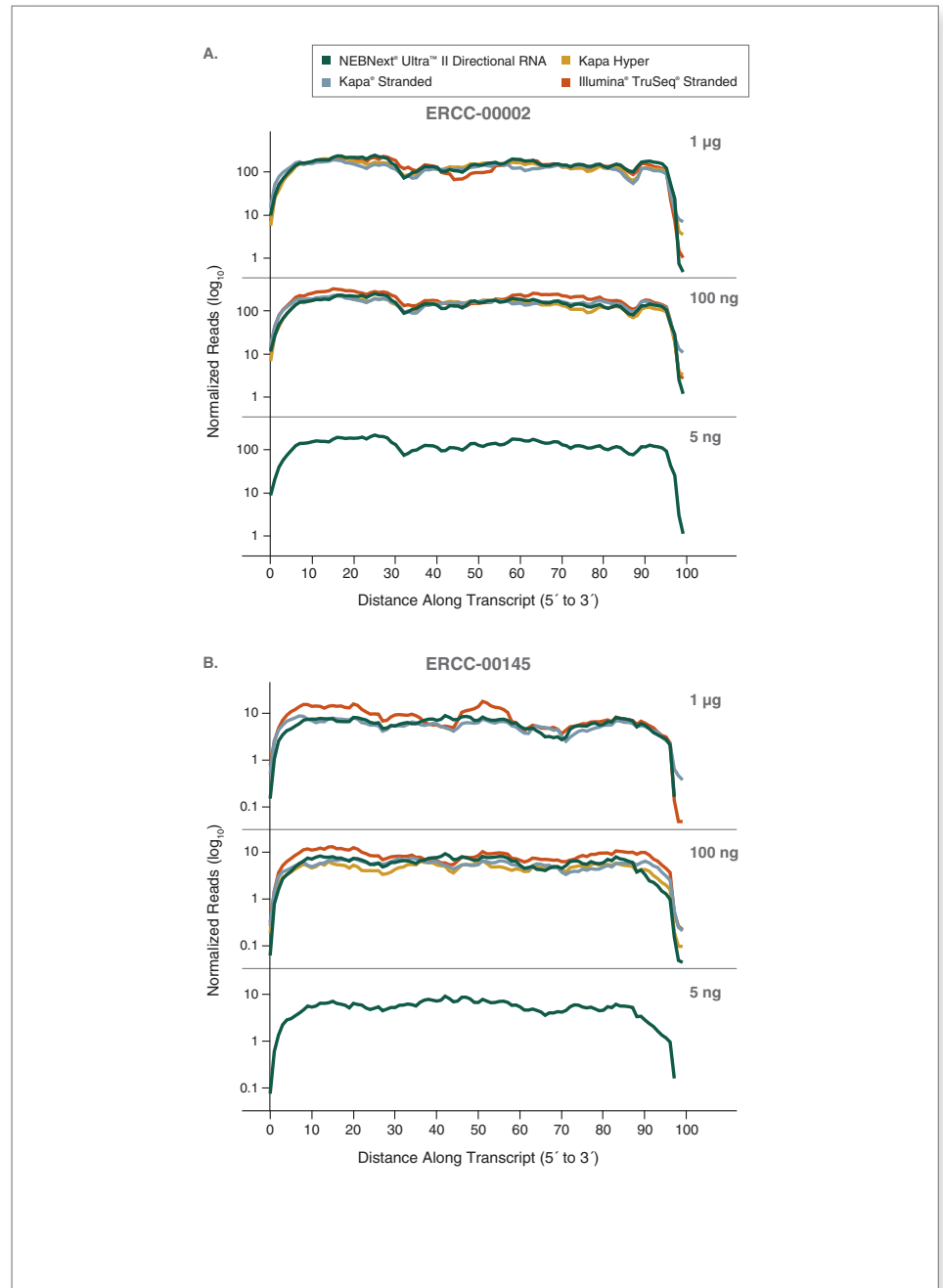
Ribosomal RNA was depleted from human adult normal liver tissue FFPE Total RNA (Biochain # R2234149, RIN 2.5), and libraries were made using NEBNext Ultra II Directional RNA Kit (plus the NEBNext rRNA Depletion Kit (Human/Mouse/Rat)), Kapa Stranded RNA-Seq Kit with RiboErase, Kapa HyperPrep Kit with RiboErase, and Illumina TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero Gold. Libraries were sequenced on an Illumina NextSeq 500 using paired-end mode (2x76 bp). Coverage across the length of this individual transcript (ENST00000625158.1; AP000769.1-201) was assessed by mapping reads directly to the GENCODE v25 transcripts and examining 100 bins along the transcript length. NEBNext Ultra II Directional RNA libraries provided coverage across the entire length of the transcript even as input was decreased from 100 ng to 10 ng.





**FIGURE 18: Uniformity of Coverage across ERCC-00002 and ERCC-00145**

Ribosomal RNA (rRNA) was depleted from Human Universal Reference RNA (Agilent #740000) with recommended amounts of ERCC RNA Spike-In Mix I (Thermo Fisher Scientific #4456740) and libraries were made using 1  $\mu$ g, 100 ng and 5 ng input with NEBNext Ultra II Directional RNA Kit (plus the NEBNext rRNA Depletion Kit (Human/Mouse/Rat)), and using 1  $\mu$ g and 100 ng input with Illumina TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero Gold, Kapa Stranded RNA-Seq Kit with RiboErase and Kapa HyperPrep Kit with RiboErase. Libraries were sequenced on an Illumina NextSeq 500 using paired-end mode (2x76 bp). Coverage across transcripts ERCC-00002 (A) and ERCC-00145 (B) were assessed by mapping reads directly to the ERCC sequences and assessing coverage using bedtools in 100 bins along the transcript length. Libraries prepared using the NEBNext Ultra II Directional RNA kit provided good coverage across the transcripts at all input amounts.



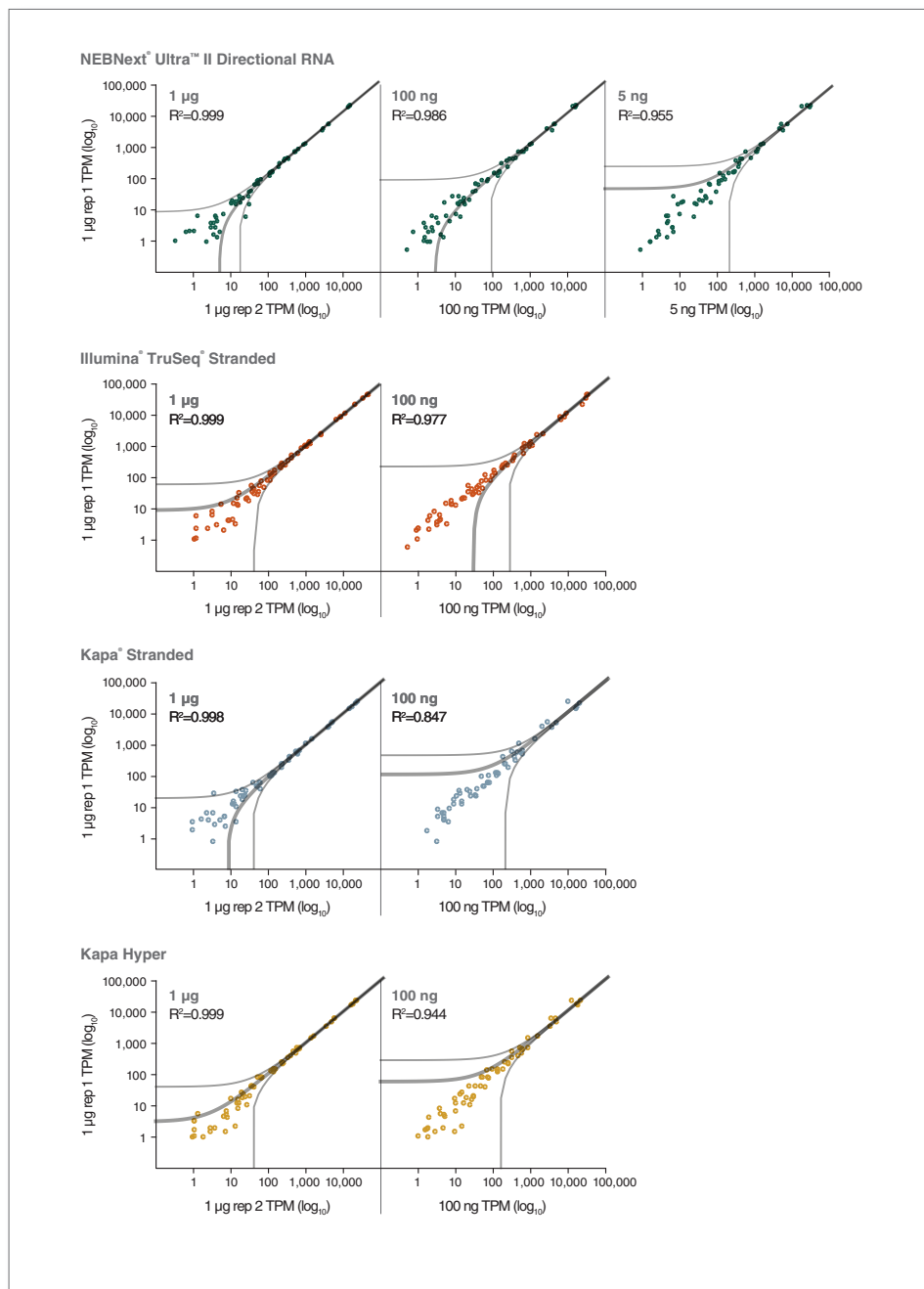
## Superior Library Complexity

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 Directional RNA library workflow improves the consistency of the composition of a library as input amounts are decreased from 1  $\mu$ g to 100 ng and 5 ng, for Universal Human Reference RNA (Figure 19) and 100 ng to 10 ng for FFPE RNA (Figure 20).



**FIGURE 19: Low input NEBNext Ultra II Directional RNA libraries retain superior complexity**

Ribosomal RNA (rRNA) was depleted from Human Universal Reference RNA (Agilent #740000) with recommended amounts of ERCC RNA Spike-In Mix I (Thermo Fisher Scientific #4456740) and libraries were made using 1  $\mu$ g, 100 ng and 5 ng input with NEBNext Ultra II Directional RNA Kit (plus the NEBNext rRNA Depletion Kit (Human/Mouse/Rat)), and using 1  $\mu$ g and 100 ng input with Illumina TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero Gold, Kapa Stranded RNA-Seq Kit with RiboErase and Kapa HyperPrep Kit with RiboErase. Libraries were sequenced on an Illumina NextSeq 500 using paired-end mode (2x76 bp). Salmon 0.4.0 was used for read mapping and quantification of all ERCC transcripts.  $R^2$  values for linear fit are shown. TPM (Transcripts Per Kilobase Million) correlation analysis of the synthetic ERCC spike-in indicates superior transcript expression correlation between the different inputs for Ultra II Directional RNA libraries.



## Conclusion

The NEBNext Ultra II Directional RNA Library Prep Kit for Illumina represents a substantial advance in strand-specific library preparation for RNA sequencing in conjunction with rRNA depletion, and with low quality samples such as FFPE RNA. Improved reagents and protocol increase the 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 Directional RNA Library Prep Kit in poly(A) mRNA enrichment workflows, see the separate application note.

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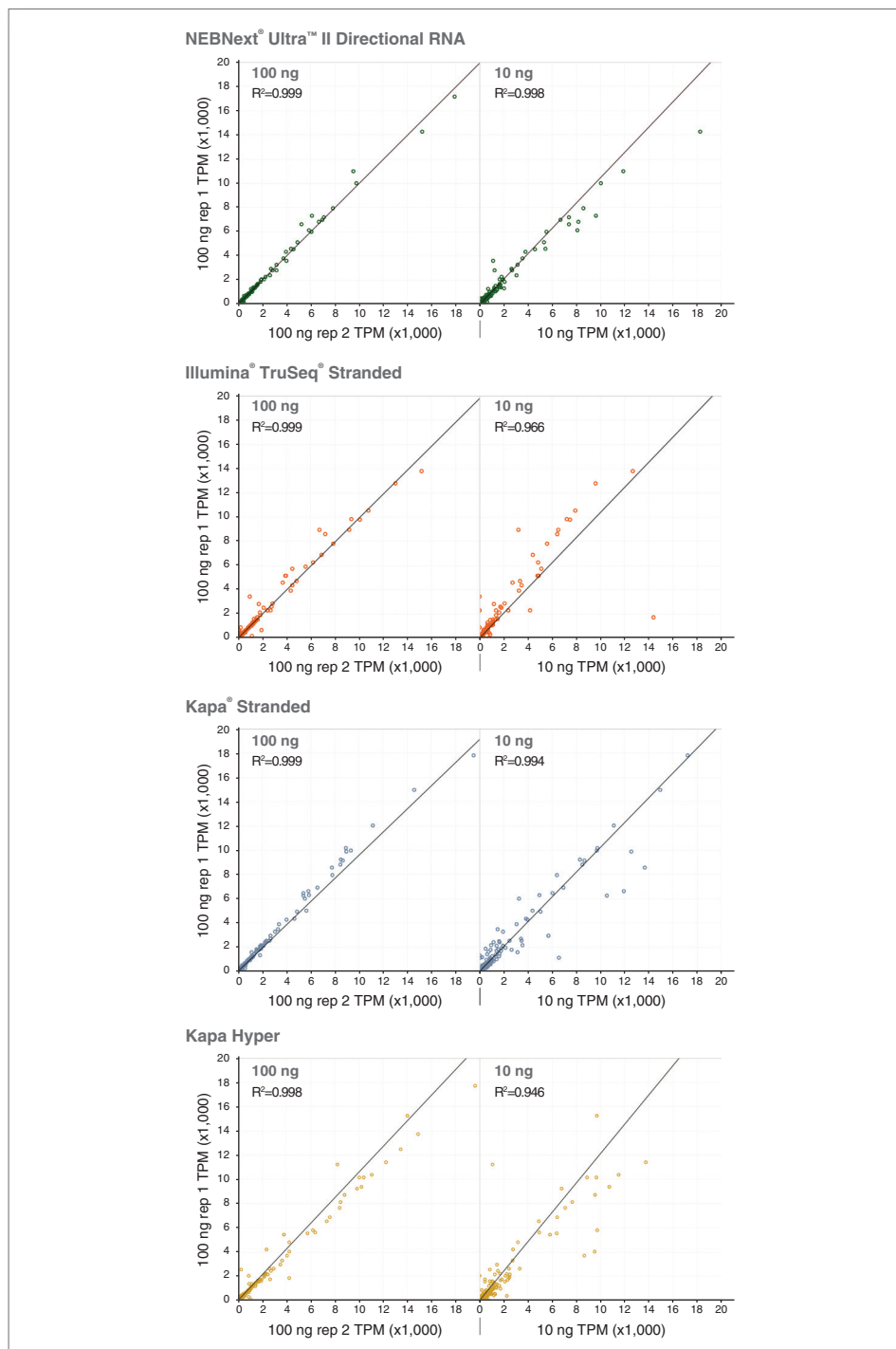
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**FIGURE 20: Low input FFPE NEBNext Ultra II Directional RNA libraries retain superior complexity**

Ribosomal RNA was depleted from human adult normal liver tissue FFPE Total RNA (Biochain # R2234149, RIN 2.5), and libraries were made using NEBNext Ultra II Directional RNA Kit (plus the NEBNext rRNA Depletion Kit (Human/Mouse/Rat)), Illumina TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero Gold, Kapa Stranded RNA-Seq Kit with RiboErase and Kapa HyperPrep Kit with RiboErase. Libraries were sequenced on an Illumina NextSeq 500 using paired-end mode (2x76 bp). Salmon 0.4.0 was used for read mapping and quantification of all GENCODE v25 transcripts. TPM (Transcripts Per Kilobase Million) correlation analysis of the transcripts indicates superior transcript expression correlation between the different inputs for Ultra II Directional RNA libraries.



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