APPLICATION NOTE #2

Use of Poly(A)+ RNA with NuGEN's RNA-Seq Solutions

I. Introduction

The Ovation® RNA-Seq System V2 (Part No. 7102) from NuGEN offers enhanced depth of transcriptome coverage, with minimal 5′/3′ positional bias from just 500 pg to 100 ng total RNA. The fast, easy protocol leverages NuGEN's proprietary Ribo-SPIA® technology to produce high-quality, double-stranded cDNA ready for RNA-Seq library construction. It enables researchers to conduct RNA-Seq research using small quantities of total RNA, with uniform read coverage across the full length of poly(A)+ RNA and non-polyadenylated transcripts.

The Ovation RNA-Seq System V2 was designed for use with total RNA; however, some studies may warrant starting with poly(A)+ RNA. This is particularly true for screening or profiling studies examining expression under different biological conditions using a defined set of polyadenylated transcripts. Researchers particularly interested in RefSeq or other well-annotated transcripts or who want to enrich for sequencing reads from mature transcripts may also find it desirable to use poly(A)+ as input. Additionally, enriching for coding RNA transcripts may simplify data analysis. Lastly, researchers engaged in collaborative efforts or working in service laboratories may simply have only isolated poly(A)+ RNA with which to work.

This application note provides data showing that as little as 5 ng to 500 ng of total RNA can be used to isolate an adequate amount of poly(A)+ RNA for input into the Ovation RNA-Seq System V2 or Encore® Complete RNA-Seq Library Systems and provide high-quality sequencing results comparable to those achieved using total RNA as direct input to the system.

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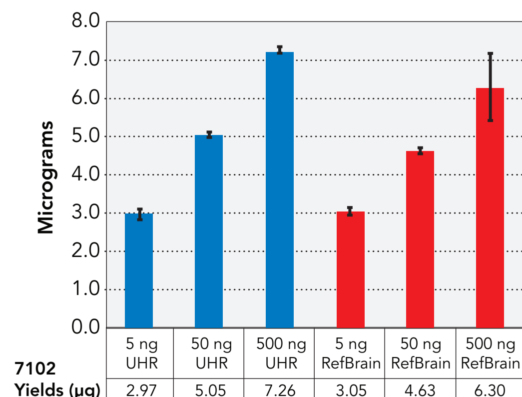
II. Poly(A)+ RNA in the Ovation RNA-Seq System V2

Selecting for poly(A)+ RNA focuses sequencing reads on mature mRNA transcripts rather than sequencing all RNAs present in the sample.

In this study, poly(A)+ RNA was prepared from the MAQC A UHR and MAQC B Human Brain Reference total RNA samples using the MPG mRNA Purification Kit from PureBiotech (Cat. #MRRK 1010). Starting quantities of total RNA ranged from 5 ng to 500 ng. One round of purification was performed. All recovered RNA was used as input into the Ovation RNA-Seq System V2. Library construction for sequencing was performed using the NuGEN Encore NGS Multiplex System I (Part No. 0301 - now obsolete) and sequencing was performed using the Illumina Genome Analyzer IIx platform with 40-base, single end reads.

As illustrated in Figure 1, the amount of cDNA produced from each of the reference samples increased proportionally with the amount of purified poly(A)+ RNA used as input. The

FIGURE 1. cDNA yield when using poly(A)+ RNA as input into the Ovation RNA-Seq System V2.



7102	5 ng UHR	50 ng UHR	500 ng UHR	5 ng RefBrain	50 ng RefBrain	500 ng RefBrain
Yields (µg)	2.97	5.05	7.26	3.05	4.63	6.30

Poly(A)+ RNA was prepared from each reference sample using the MPG mRNA Purification Kit from PureBiotech (Cat. #MRRK 1010). Starting quantities of total RNA were 5 ng, 50 ng and 500 ng. One round of purification was performed. All recovered RNA was used as input into the Ovation RNA-Seq System V2.

yields ranged from 3 µg to 7 µg, in all cases generating sufficient material for downstream library preparation workflows.

As expected, when compared to output from the Ovation RNA-Seq System V2 using total RNA as input, libraries resulting from input of poly(A)+ RNA showed an increase in non-rRNA reads and a reduction in cytoplasmic rRNA, as shown in Table 1.

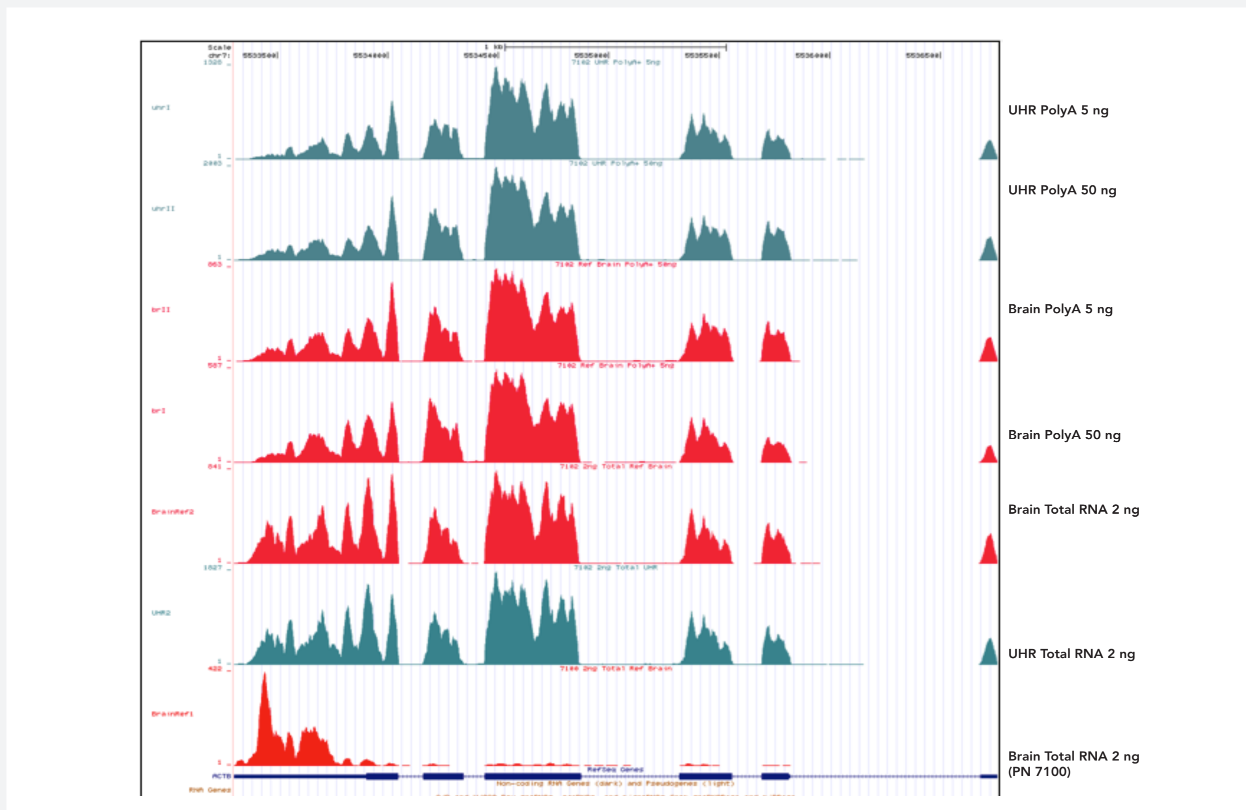
Figure 2 illustrates that sequencing reads were distributed uniformly across the exons regardless of input amount (between 2 ng and 50 ng) or the type of RNA input into the Ovation RNA-Seq System V2.

TABLE 1. Comparison of sequencing reads from UHR and Brain Reference samples using either total RNA or poly(A)+ RNA as input into Ovation RNA-Seq System V2.

	Total RNA (Brain)	Total RNA (UHR)	Poly(A) (Brain)	Poly(A) (UHR)
Total Aligned Reads	91.2%	90.4%	88.1%	88.9%
Total Not Aligned	8.8%	9.6%	11.9%	11.1%
% Mito rRNA	31.0%	23.2%	28.5%	21.7%
% Cyto rRNA	17.5%	18.0%	4.9%	6.6%
% Non-rRNA	50.2%	56.7%	64.8%	69.5%

Poly(A) results were obtained with poly(A)+ RNA isolated from 5 ng of total RNA—all recovered material input to Ovation RNA-Seq System V2. Assuming mRNA is 5% of total RNA in human cells, this represents input of ~250– 300 pg poly(A)+ RNA. Total RNA results were obtained with 2.0 ng input using the standard Ovation RNA-Seq System V2 workflow.

FIGURE 2. Sequencing read distribution for ACTB.



Total RNA or poly(A)+ RNA from MAQC B (human brain) and MAQC A UHR was amplified using the Ovation RNA-Seq System V2 (Part No. 7102). The resulting double-stranded cDNA was input into the Encore NGS Multiplex System I (Part No. 0301) to construct NGS libraries. Sequencing results were obtained using the Illumina Genome Analyzer IIx platform with 40-base, single end reads.

III. Poly(A)+ RNA in the Encore Complete RNA-Seq Library Systems

The use of poly(A)+ RNA as input into NuGEN's Encore Complete RNA-Seq Library Systems was investigated with results similar to those for the Ovation RNA-Seq System V2. The poly(A)+ fraction was isolated from total RNA in the same fashion as described for Ovation RNA-Seq System V2. In this case, duplicate MAQC A UHR samples were run for each of four starting total RNA amounts (100 ng, 150 ng, 300 ng, 600 ng). As described in Table 2, high-quality libraries were produced from poly(A)+ RNA input, with only 3%–5% of mapped reads aligned to rRNA. As expected, the percentage of rRNA content is lower in these libraries than in libraries prepared using total RNA as input (see Table 3). Interestingly, a comparison of the percentage of sense reads in sense orientation from Tables 2 and 3 show that the use of poly(A)+ selected material as input slightly reduced the strand retention capability of the Encore Complete RNA-Seq Library Systems, indicated by a small reduction in strandedness in the exon regions.

TABLE 2. **Sequencing metrics for cDNA libraries produced by the Encore Complete RNA-Seq Library Systems with poly(A)+ RNA as input.**

	UHR Input into poly(A)+ Selection			
Starting Amount	100 ng	150 ng	300 ng	600 ng
% of Total Reads				
Not aligned	12%	16%	9%	10%
Aligned	88%	84%	91%	90%
% of Mapped Reads				
All rRNA	5.1%	3.0%	3.3%	3.1%
All non-rRNA	94.9%	97.0%	96.7%	96.9%
Non-rRNA single site	51%	46%	52%	53%
Non-rRNA multiple site	32%	32%	37%	37%

	UHR Input into poly(A)+ Selection			
Starting Amount	100 ng	150 ng	300 ng	600 ng
Distribution of RefSeq Reads				
Exons	50.5%	53.4%	56.3%	58.0%
Introns	22.2%	20.3%	19.3%	18.5%
Intergenic	27.2%	26.3%	24.4%	23.5%
RefSeq Strand Retention (% of Reads in Sense Orientation)				
Exons	87.2%	82.5%	79.4%	85.9%
5′ UTR	92.3%	89.3%	87.3%	91.7%
3′ UTR	88.0%	84.4%	80.7%	85.8%

These data were generated using the indicated amounts of MAQC A UHR as starting material for poly(A)+ RNA selection. 71% (5 µl out of 7 µl) of the poly(A)+ selected material was later used as input for the Encore Complete RNA-Seq IL Multiplex System 9–16. The resulting libraries were sequenced on two lanes of the Illumina Genome Analyzer IIx using 4 libraries per lane (4-plex) with 40-base, single end reads. The percentage of non-rRNA single site ranges between 46%–53% depending on the amount of input, and represents the percentage of reads mapping to non-rRNA sequences in the reference genome in a single location. The percentage of non-rRNA multiple sites was 32% for 100 ng and 150 ng inputs, and 37% for 300 ng and 600 ng inputs, representing the percentage of reads mapping to non-rRNA sequences in the reference genome in two or more locations.

TABLE 3. Sequencing metrics for cDNA libraries produced by the Encore Complete RNA-Seq Library Systems with total RNA as input.

	Human MAQC B	Human MAQC A
% of Total Reads		
Not Aligned	4%	5%
Aligned	96%	95%
% of Mapped Reads		
All rRNA	30.9%	23.2%
All non-rRNA	69.1%	76.8%
Non-rRNA single site	50%	51%
Non-rRNA multiple site	18%	25%
Distribution of RefSeq Reads		
Exons	44%	38%
Introns	36%	36%
Intergenic	16%	24%
RefSeq Strand Retention (% of Reads in Sense Orientation)		
Exons	98.2%	95.7%
5′ UTR	96.3%	96.1%
3′ UTR	96.9%	93.6%

These data were generated using 100 ng of total RNA from the indicated sources as input to the Encore Complete RNA-Seq IL Multiplex System 1–8. The resulting libraries were sequenced on the Illumina Genome Analyzer IIx using 4 libraries per lane (4-plex) with 40 bp single-read sequencing. Non-rRNA single site = % for reads mapping to non-rRNA sequences in the reference genome in a single location. Non-rRNA multiple site = % for reads mapping to non-rRNA sequences in the reference genome in two or more locations.

IV. Conclusion

When using poly(A)+ RNA, rather than total RNA, as input into the Ovation RNA-Seq System V2 or Encore Complete RNA-Seq Library Systems, users can expect results comparable to those obtained using total RNA as input. Using the methods described, at least 5 ng total RNA is required to isolate sufficient poly(A)+ RNA for input into the Ovation RNA-Seq System V2. Similarly, a minimum of 100 ng total RNA is recommended to isolate the poly(A)+ fraction that will be used as input for the Encore Complete RNA-Seq Library Systems. Data generated using MAQC A samples suggest that using poly(A)+ RNA as input can increase the percentage of non-rRNA reads and decrease the percentage of cytoplasmic rRNA reads at sequencing, relative to numbers generated using total RNA as input (Tables 2 and 3). Sequencing reads across the transcriptome and across exons show uniformity consistent with that found when using total RNA as input.

While there may be additional time and cost implications, and potential for bias introduced when isolating poly(A)+ RNA for input into the Ovation RNA-Seq System V2 or Encore Complete RNA-Seq Library Systems, these systems produce high-quality cDNA libraries using either total RNA or poly(A)+ RNA as starting material.

NuGEN Technologies, Inc.

Headquarters USA

201 Industrial Road, Suite 310
San Carlos, CA 94070 USA
Toll Free Tel: 888.654.6544
Toll Free Fax: 888.296.6544
custserv@nugen.com
techserv@nugen.com

Europe

P.O. Box 149
6680 AC Bemmel
The Netherlands
Tel: +31-13-5780215
Fax: +31-13-5780216
europe@nugen.com

For our international distributors contact information, visit our website **www.nugen.com**

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