A STUDY OF OVATION™ RNA AMPLIFICATION SYSTEM V2 PERFORMANCE

INTRODUCTION

NuGEN's RNA amplification solutions have generated excellent gene expression results from challenging samples that yield limited amounts of RNA. Many such studies have been performed using the Ovation™ Biotin RNA Amplification and labeling System (Cat.# 2300), which included the amplification and fragmentation and labeling components to complete target preparation for Affymetrix GeneChip® arrays. Using this product, researchers have created amplified cDNA for analysis via microarrays and by qPCR for validation of array results.

The same advantages in consistency of results and reduced time and costs of preparation are now available with NuGEN's more flexible modular products. The same 3'-focused amplification reagent set is now offered in the product, **Ovation™ RNA Amplification System V2 (Cat.# 3100-12, -60)**, without fragmentation and labeling reagents. This product generates amplified cDNA that can be used directly for qPCR, storage and archiving, and can be processed using NuGEN's **FL-Ovation™ cDNA Biotin Module V2 (Cat.# 4200-12, -60)** to fragment and label (F&L) the cDNA for GeneChip® array analysis. This F&L module is a simple and robust method for target preparation in less than 2 hours and requiring no purification steps. Elimination of purification columns allows for easier, more automation-friendly sample processing and reduces both costs and hands-on time, lowering error rates.

These products are examples of NuGEN's overall modular product approach that provides significant flexibility and choice for scientists who want to perform qPCR today but also want to maximize their options by storing their amplified cDNA for microarray studies at a later time on the same amplified sample. NuGEN's modular approach allows researchers to purchase only what they need today, while retaining the option of using F&L approaches on their samples in the future.

Specific study designs that will realize significant benefits from this approach include large collaborative group projects and consortia. Collaborative investigators at various sites often need to study the same sample using a variety of gene expression platforms and analytical approaches. Since maintaining the homogeneity of the originating sample is a strict technical requirement for such studies, starting from the same Ovation™-amplified cDNA ensures consistency, sample stability, and input standardization. Furthermore, small and limited samples can be more readily considered for such collaborative approaches due to the Ovation™ System's ability to amplify limited amounts of RNA to produce the large amounts of amplified cDNA.

In this report we demonstrate the performance of the Ovation™ RNA Amplification System V2 (Cat.# 3100) through yields, qPCR data, and GeneChip array analysis using the FL-Ovation™ cDNA Biotin Module V2 (Cat.# 4200).

MATERIALS AND METHODS

The studies described here utilized purchased total HeLa cell line RNA, (Ambion, Cat. # 7852) as well as purchased UHR total RNA, (Stratagene, Cat.# 740000). Spleen and Placental total RNAs were also purchased for the tissue mixing experiment (Ambion, Cat. # 7970 and 7950, respectively).
All RNAs were amplified using the Ovation™ RNA Amplification System V2 (Cat.# 3100), at various RNA inputs, as indicated, following the procedure outlined in the product user guides.

Real time qPCR assays were designed using the Universal ProbeLibrary™ and primer design software. Primers were ordered from Integrated DNA Technologies (Coralville, IA). Multiple assays were designed for each gene spanning the 5 prime, middle and the 3 prime end. Assays were screened for good efficiency as close to 100% as possible with a slope of 1 $\pm$ 0.1. Further primer and probe information and accession numbers are provided upon request.

The qPCR reactions were set up using unpurified amplified cDNA diluted 1/10 into 1 X TE (pH 8.0) or 20 ng of purified cDNA into TaqMan® Fast Universal PCR master mix with 5 nM each of the forward and reverse primers and 100 nM ProbeLibrary™ probe or with ABI's Assays-on-Demand™ primer and probe mix following vendor's instructions in 20 µL final volume. The assays were analyzed using the ABI 7500 with the Fast block installation using the default settings. For GeneChip array

analysis of the amplified cDNA, fragmentation and labeling was performed using the FL-Ovation™ cDNA Biotin Module V2 (Cat.# 4200), with 3.75 µg of cDNA in 25 µl. Array analysis was performed on HG-U133A 2.0. GeneChip arrays (Affymetrix, Cat.# 900469), with a final cDNA target concentration of 17 ng/µl. Array data was analyzed by Affymetrix GCOS software (GeneChip Operating System, 1.4.0.036).

The quantity of total RNA was assessed using the Nanodrop ND-1000 spectrophotometer (Wilmington, DE). Agilent Bioanalyzer was used to obtain traces for the amplified cDNA with an RNA 6000 Nano LabChip® (Agilent Cat. #5065-4476) and the Eukaryotic Total RNA Nano program (Nano assay in the Expert 2100 software) were used, according to the manufacturer's instructions.

RESULTS

Reproducible and robust amplification across RNA input range and transcript abundance: HeLa RNA was amplified with 50, 20, and 5 ng total RNA input, the superimposed Bioanalyzer traces shown in **Figure 1**. are highly similar across inputs. Using both purified and unpurified cDNA for qPCR input the qPCR Ct results for a set of 7 transcripts also show that amplification is highly sensitive and reproducible across a wide range of transcript abundance as well as input RNA, shown in **Figure 2**.
In Figure 2 and 3 cDNA yield data from UHR and HeLa RNAs are shown; in addition a panel of RNA isolated from other tissues consistently result in amplification yields of 4 - 8 µg. Even for difficult tissue sources of RNA such as Placenta and whole blood, minimum yields of 4 – 5 µg of amplified cDNA are generated.

Reproducible and robust amplification across multiple product lots: Three lots of the Ovation™ RNA Amplification System V2 were compared for consistency in performance by amplifying 5 ng of UHR RNA in duplicates and performing qPCR assays for 5 transcripts. In a comparison of yields and qPCR data the 3 lots were highly consistent and reproducible in performance, shown in **Figure 3**.

Amplified cDNA generates expected and consistent fold change results: Using a Placenta/Spleen tissue mixing model, described in detail in the Ovation™ RNA Amplification System V2 Technical Report #2, multiple mixed RNAs were prepared. Amplifications were preformed with 5 ng of a 100% Placenta RNA as well as a mix of 50%:50% Placenta/Spleen RNA. The cDNA was interrogated with a panel of qPCR assays for 18 Placenta-specific transcripts to determine fold change after amplification. A 2-fold change is expected when comparing a sample containing only Placenta RNA to one with equal amounts of Spleen and Placenta RNA. The qPCR Ct results show that approximately a 2-fold change is observed across the different transcripts, as shown in **Figure 4**. The high and low confidence intervals shown as error bars demonstrate extremely consistent and accurate fold change detection in amplified samples. RPL35-1 was used as a normalizer gene for the Ct data and M4-2, DAD1-1, and SEPT2-1 were all included as housekeeping genes, which showed consistent expression at a slightly higher level in Spleen.

Duplicate samples show high quality array metrics and high signal correlation and call concordance: Duplicate amplifications of 5 ng of HeLa RNA were performed, cDNAs were fragmented and labeled and hybridized to GeneChip arrays as described in the Methods section. The table in **Figure 5** shows the high quality array performance metrics obtained, with scaling factors, % present calls, background and 3'/5' ratios well within acceptable ranges. **Figure 6** shows the signal correlation of 0.994 and call concordance of 93.2% obtained from a set of duplicate amplifications from 5 ng of HeLa total RNA and analyzed on GeneChip arrays, demonstrating the high level of reproducibility and consistency in results obtained from amplified cDNA targets.

CONCLUSIONS

The data presented here demonstrates the robust, reproducible amplification obtained using the Ovation™ RNA Amplification System V2, using as little as 5 ng of total RNA input. These low input RNA requirements enable utilization of only a fraction of minute and irreplaceable specimens, allowing researchers to conserve large portions of their original RNA samples. Robust cDNA yields from very small amounts of total RNA are critical for analysis of limiting clinical samples which are difficult to obtain, costly, and often irreplaceable.
The Ovation's simple, fast, and easy to automate process is ideal for high throughput discovery and clinical research applications, while the high level of sensitivity and specificity offered by the cDNA target yields excellent array results.

Systems Specifications

Cat No.: 3100-12, 12 reactions
3100-60, 60 reactions

Input: 5-100 ng total RNA

Yield: 4-7 µg single stranded cDNA

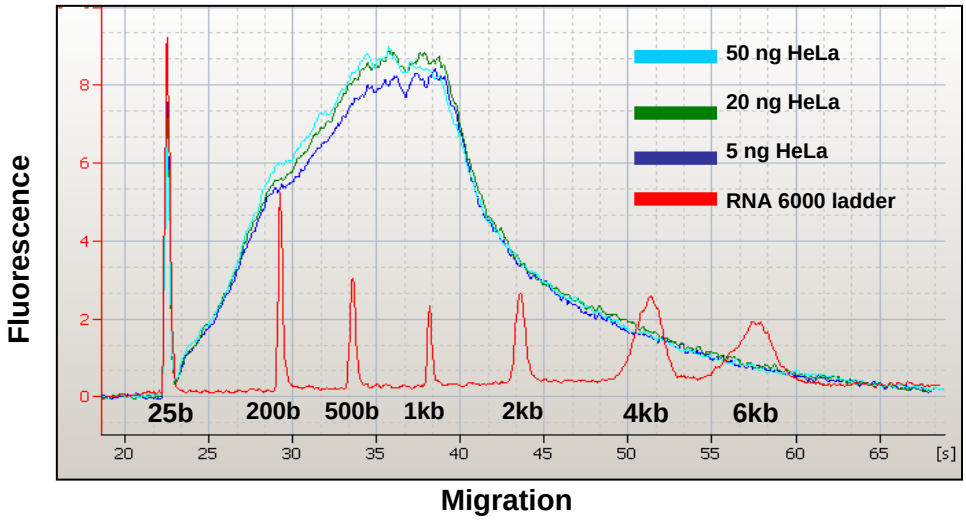


Figure 1. Size distribution of amplified cDNA across total RNA input. Amplified cDNA using 50, 20, and 5 ng of total HeLa RNA show consistent profile and size distribution across RNA input range upon analysis of Agilent Bioanalyzer traces.

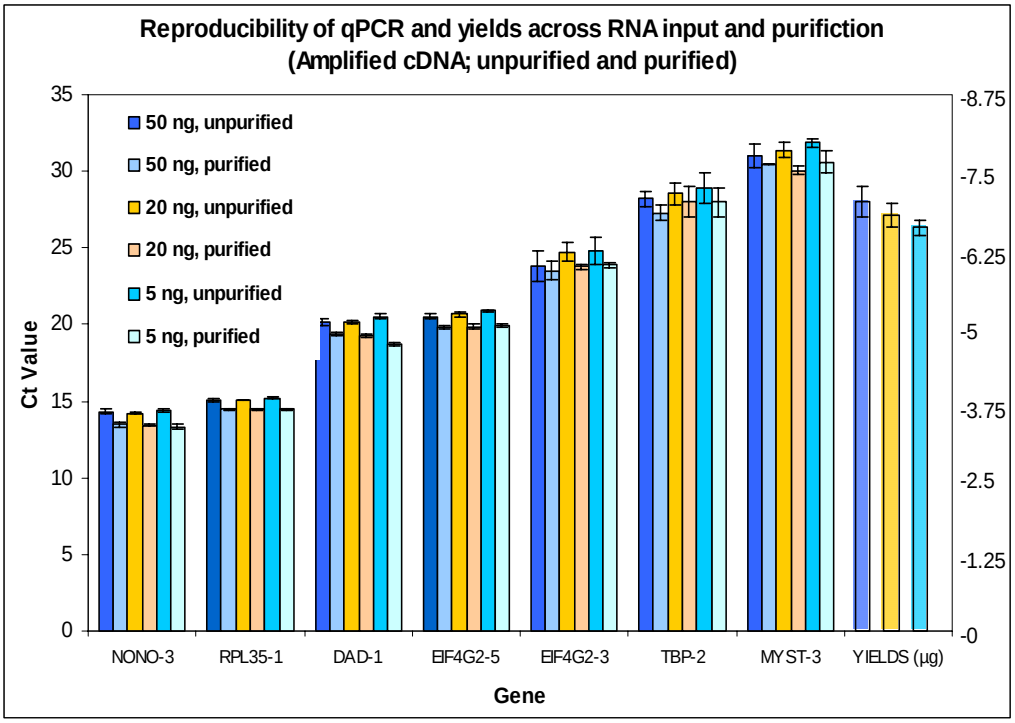


Figure 2. qPCR and yields reproducibility across total RNA input and transcript abundance. 50, 20, and 5 ng of HeLa total RNA were amplified in triplicate. qPCR results for 7 transcripts show reproducible and consistent results with and without purification of amplified cDNA. Yields, shown on the left axis of the graph, also show highly reproducible degrees of amplification across inputs.

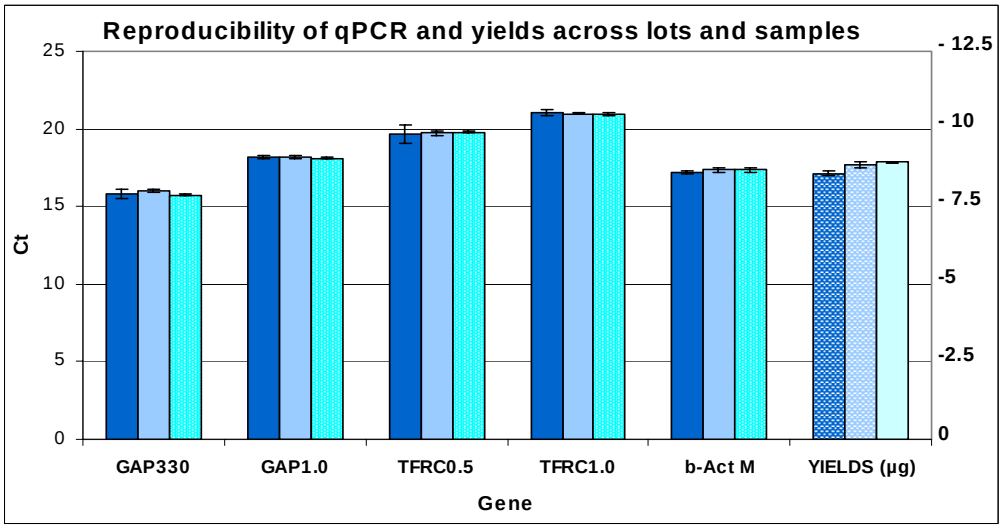


Figure 3. qPCR and yields reproducibility across multiple product lots. 5 ng of UHR total RNA was amplified in triplicate for 3 different lots of the Ovation™ RNA Amplification System V2. qPCR results for 5 genes show reproducible and consistent amplification. Amplified cDNA yields, shown on the left axis of the graph, also show very consistent amplification across multiple lots.

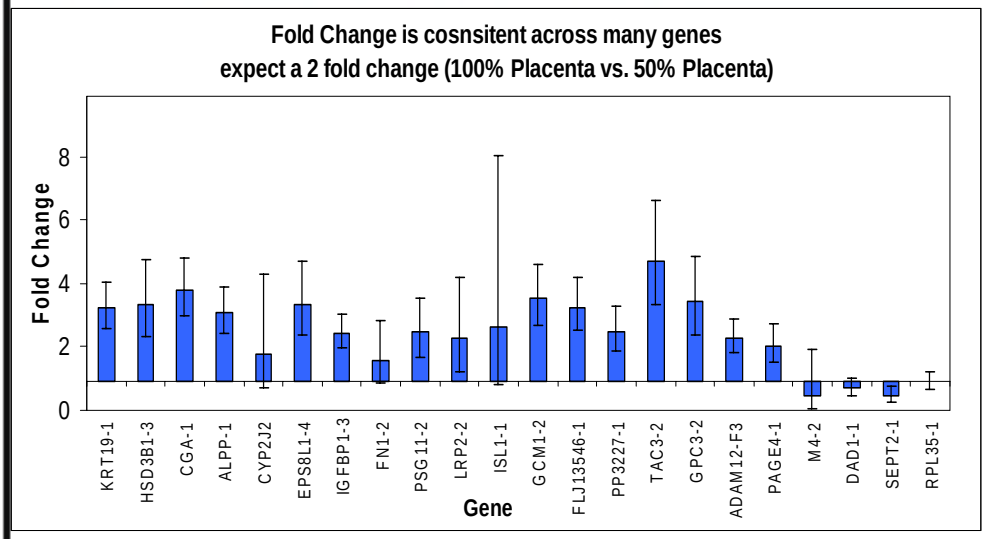
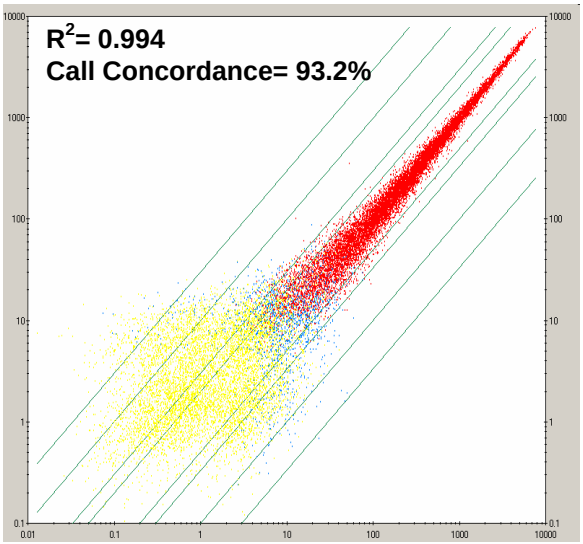


Figure 4. qPCR data shows expected and consistent fold change: Using a tissue mixing model, amplification of 5 ng of a 100% Placenta RNA sample vs. a 50% mix of Placental and Spleen RNA and consequent qPCR assays of a panel of 18 Placenta-specific genes showed that the expected 2 fold change is observed and maintained across the different transcripts. The rightmost 4 genes in the graph are housekeeping genes, and the very rightmost gene, RPL35-1 was used as a normalization gene.



Raw Q	Scaling Factor	Back-ground	% Present	(3'/5') GAPDH	3'/5' Actin
0.97	0.69	34.5	64.1	1.30	4.1
0.98	0.59	34.4	65.6	1.21	3.8

Figure 5. Array metrics for duplicate samples: 5 ng of HeLa RNA, amplified, fragmented and labeled in duplicate, and hybridized to HG-U133A 2.0 GeneChip ® arrays, show robust and reproducible array metrics.

Figure 6. High reproducibility demonstrated by signal correlation and call concordance: 5 ng of HeLa RNA, amplified, fragmented and labeled in duplicate, as previously described show high signal correlations of R^2 of 0.994 and call concordance of 93.2% demonstrating very high level of reproducibility between independently processed samples.

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