A STUDY OF WT-OVATION™ PICO RNA AMPLIFICATION SYSTEM PERFORMANCE

BACKGROUND

As the need for more in-depth gene expression analysis increases, so do the requirements placed on sample size and quality. But since clinical specimens are routinely limited in both size and quality, some large clinical sample sets have become challenging candidates for gene expression studies.

NuGEN's amplification products have long set the standard for small sample RNA amplification and labeling. Expanded RNA sample availability and higher quality analyses are now easily attained with the new WT-Ovation™ Pico RNA Amplification System. This kit allows whole transcriptome (WT) amplification from picogram quantities of total RNA, yielding 6-10 µg of cDNA in 5 hours. The amplified cDNA can be used for qPCR analysis and storage for future use.

The amplified cDNA can also be analyzed on arrays following fragmentation and labeling with the FL-Ovation™ cDNA Biotin Module V2 (cat# 4200-12). NuGEN's simple and well-established Ovation™ amplification method combined with the low input requirements and the WT approach uniquely equips this product for small, challenging clinical samples. The System is especially well suited to samples whose integrity may have been compromised during the sample acquisition and storage, for example by loss of the 3' polyA tail. Because this amplification system provides both 3'-initiated and random primed amplification, it offers the benefit of whole transcriptome amplification for compromised samples and flexibility in qPCR assay design. In addition, 3' initiated amplification provides cDNA appropriate for fragmentation, labeling, and analysis on the current 3' focused GeneChip® arrays and potentially for WT array designs.

MATERIALS AND METHODS

Two different total RNAs were used to study performance metrics of the the WT-Ovation™ Pico RNA Amplification System. Total HeLa cell line RNA and total RNA from a human Colon Normal Adjacent Tissue (NAT) were purchased from Ambion, Inc (cat #7852, #7236, respectively, Austin TX). Three replicate amplifications were performed on 500 pg of each RNA type (unless otherwise stated) using the WT-Ovation™ Pico RNA Amplification System, following the procedure outlined in the user guide. Amplified cDNA product was purified using Zymo clean and concentrate-25 columns (Zymo Research, Orange , CA) and quantitated using a NanoDrop -1000 (NanoDrop Tech, Wilmington, DE). Real time qPCR assays were designed using the Universal ProbeLibrary™ and primer design software (Exiqon/Roche). Primers were ordered from Integrated DNA Technologies (Coralville, IA). Multiple assays were designed for each gene spanning the 5', middle, and the 3' regions. Assays were screened for good efficiency as close to 100% as possible with a slope of 1 $\pm$ 0.1.

qPCR reactions were set up using the purified amplified cDNA at 20 ng/reaction into TaqMan® Fast Universal PCR master mix with 500 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. Several assays were designed for the human glyceraldehyde- 3-phosphate dehydrogenase gene (GAPDH NM-BC029340.1) with varying amplicon sizes. The ProbeLibrary™ primer design software was used to design an assay to GAPDH with the TaqMan® probe Human #9 positioned at ~250 nt from the 5' end of the gene. The primer design software Primer3 was then used to design primers around this probe site of increasing amplicon length and anchoring either the 3' or the 5' primer. Further primer and probe information and accession numbers are provided upon request.

The assay for the phosphoglycerate kinase 1 gene (PGK1 NM-000291) was run on each plate and used to normalize data for each experiment. This is expressed as

delta Ct = Ct test gene – Ct PGK1.

Fragmentation and labeling was performed using the FL-Ovation™ cDNA Biotin Module V2 according to the product user guide. 5 µg of amplified cDNA was fragmented and labeled and each U133A 2.0 GeneChip® array was hybridized with 3.4 µg of labeled cDNA. Hybridization, washing and staining protocols outlined in the FL-Ovation™ user guide were followed.

RESULTS AND CONCLUSIONS

The basic performance parameters of the WT-Ovation™ Pico System are presented in terms of cDNA yield, product size distribution, qPCR and array performance and reproducibility.

Figure 1 shows that the amplified cDNA yields are reproducible, robust, and proportional to the total RNA input. The yields increase as input RNA quantities increase, with yields of about 6 µg with 500 pg of input RNA and 10 µg with 50 ng of RNA. Even with input RNA amounts below the recommend input (gray histograms) amplified cDNA yields are consistent and well above that required for further fragmentation and labeling for analysis on GeneChip® arrays in addition to qPCR analysis.

Figure 2 shows Agilent Bioanalyzer traces of amplified cDNA with inputs of 500 pg and 10 ng of Colon total RNA demonstrate the consistent cDNA size profile. **Figure 3** demonstrates the consistency of qPCR results across a wide input range, generated from Colon total RNA amplified in triplicate with the WT-Ovation™ Pico RNA Amplification System. Five qPCR assays were designed and performed for three transcripts and Ct values were normalized to the PGK transcript. The graph shows the Delta Ct plotted against the RNA input. Results show that the Ct values from a set of transcripts remain consistent across a wide range (100 pg to 50 ng) of total RNA input for all interrogated transcripts.

The global aspect of Whole Transcriptome amplification is demonstrated in **Figure 4**, where qPCR Ct are shown for the FGFR2 transcript, using 6 assays designed at various distances from the 3' end, (from ~1700 to 4200 nts from the 3'). Results are shown for two different input levels of Colon total RNA into the amplification reaction. The amplification covers the entire transcript, resulting in consistent Ct values as far as 4200 nts from the 3' end. This not only provides flexibility for qPCR assay design location, but demonstrates the WT-ness of the amplification, ensuring that even when some portion of the transcript population has compromised polyA ends, transcripts are still detected and consistently represented independent of the distance of the qPCR assay from the 3' end.

Amplicon length was also tested to demonstrate flexibility in qPCR primer design. HeLa and Colon RNA were amplified and the cDNA was used in 4 qPCR assays for the GAPDH transcript that yielded amplicon lengths between 65 to 390 bases. In **Figure 5**, HeLa RNA at two input levels as well as Colon RNA were amplified then analyzed via qPCR; results show uniform Ct values for amplicon lengths of up to 390 bases.

The amplified cDNA from the WT-Ovation™ Pico RNA Amplification System was also analyzed on Affymetrix GeneChip® arrays following fragmentation and labeling using the FL-Ovation™ cDNA Biotin Module V2 (cat.# 4200-12).
Table 1 shows standard array metrics for triplicate amplifications of HeLa RNA at 5 ng and 500 pg input. Results show robust array performance across all standard array metrics.
Array reproducibility is demonstrated in **Figure 6**. Amplifications were performed for two independent reactions each with 5 ng and 500 pg of HeLa RNA. cDNAs were processed with the FL-Ovation™ cDNA Biotin Module V2 and analyzed on GeneChip® arrays. The results for 5 ng input show a high degree of signal correlation, an R^2 value of 0.990 and a call concordance of 92%. For amplifications starting with 500 pg of RNA, reproducibility and other array performance metrics are slightly lower, consistent with the expected stochastic nature of low copy number amplifications. With these samples signal correlations show an R^2 value of 0.975 and call concordance rates were 89%.

In conclusion, these results demonstrate that the WT-Ovation™ Pico RNA Amplification System is a reproducible, highly sensitive, and robust method for whole transcriptome amplification, starting with 500 pg to 50 ng total RNA, it yields 6 to 10 micrograms of cDNA, enabling the detection of a wide range of transcript abundances.

Systems Specifications

Cat No.: 3300-12, 12 reactions
Input: 500 pg - 50 ng total RNA
Yield: 6 -10 µg single stranded cDNA

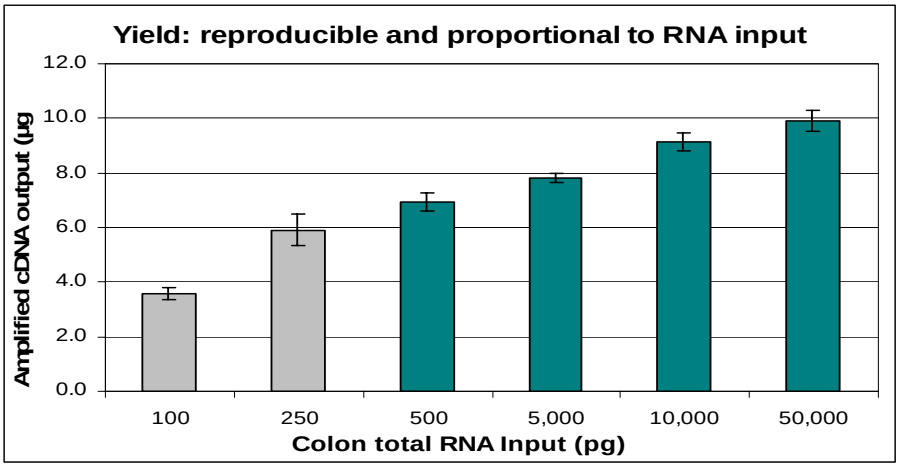


Figure 1. cDNA yields are reproducible, robust, and proportional to the total RNA input, increasing as input RNA quantities increase. Even at RNA amounts below the recommend input (gray histograms), cDNA yields are sufficient for further fragmentation and labeling for analysis on GeneChip® arrays in addition to qPCR analysis.

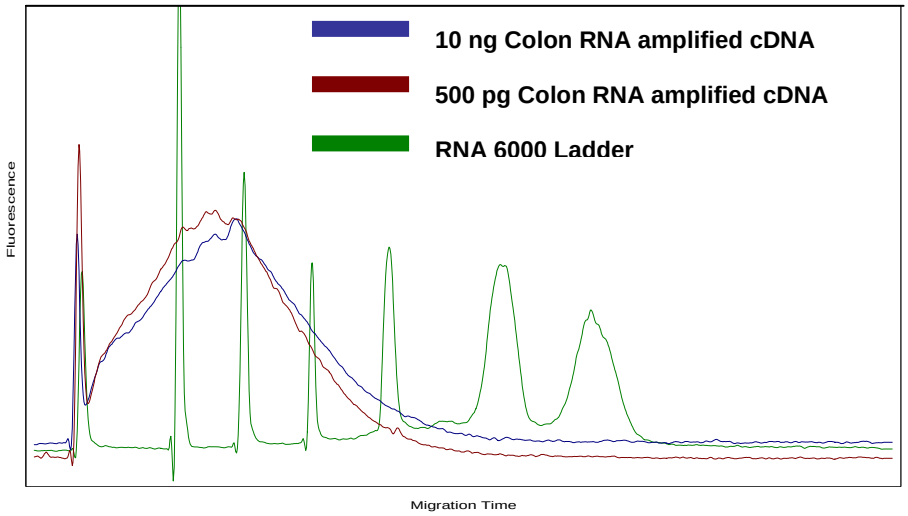


Figure 2. Agilent Bioanalyzer traces of amplified cDNA demonstrate the consistent cDNA size profile across a wide input range. The cDNA product median length for HeLa RNA is approximately 380 bases.

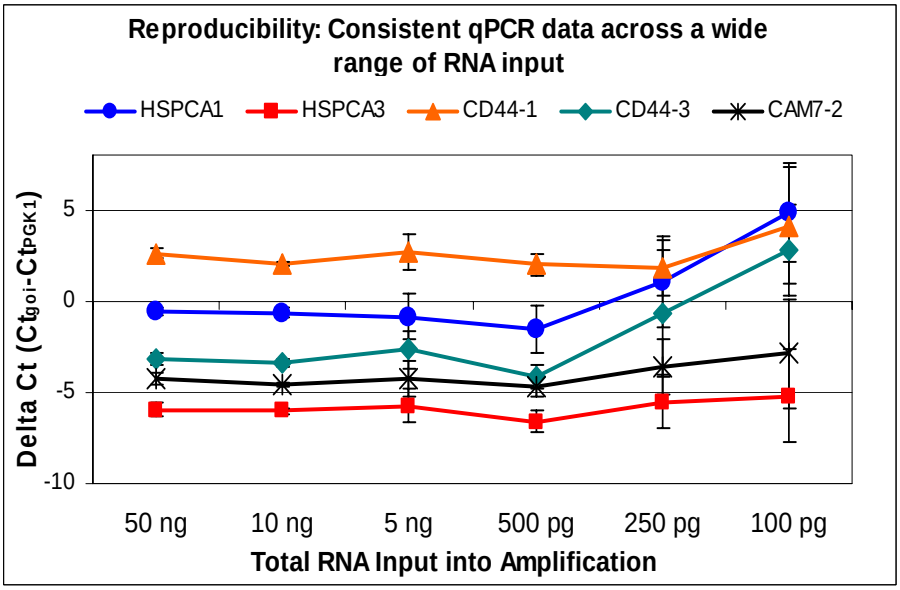


Figure 3. qPCR results show high level of reproducibility in Ct values (normalized to PGK), for multiple transcripts, within each set of replicate amplifications, and throughout the RNA input range tested.

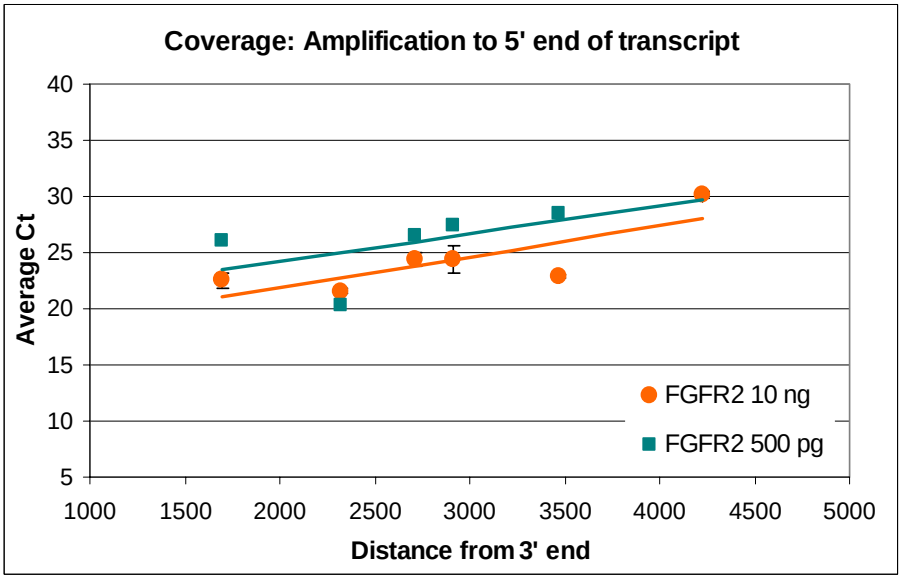


Figure 4. qPCR results for 6 assays designed for the FGFR2 transcript at various distances from the 3' end, show that amplification is relatively consistent across the transcript so that qPCR assays may be designed at distances at least as far as 4200 nucleotides from the 3' end without loss of detection, and regardless of input amounts.

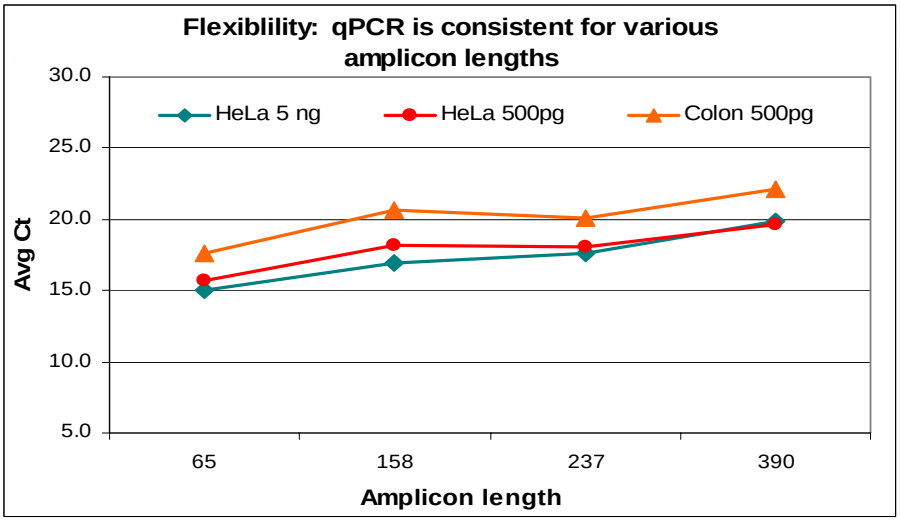
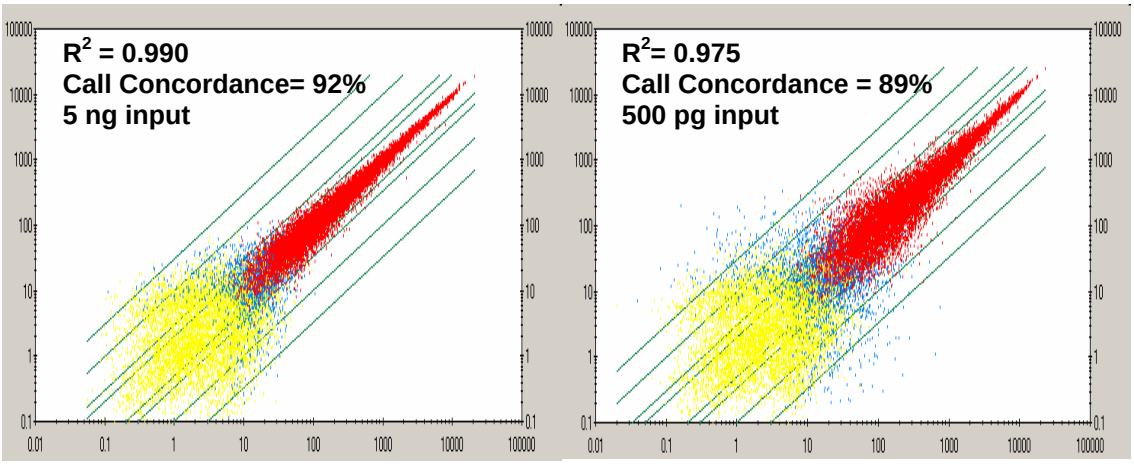


Figure 5. qPCR results for 4 assays designed for the GAPDH transcript but with various amplicon lengths are similar between longer and shorter qPCR amplicons. They are also similar for various RNA input levels.

RNA input	Scaling Factor	Back-ground	%P	3'-5' GAP	3'-5' Actin	Raw Q
5 ng	1.44	30.2	62.0	1.25	5.94	0.88
500 pg	1.72	29.6	58.0	1.25	4.63	0.85

Table 1. Standard array metrics for triplicate amplifications of HeLa RNA at 5 ng and 500 pg input show robust and comparable array performance.

Figure 6. Signal Correlation and Call Concordance for 2 independent WT-Ovation™ Pico System amplifications each with 5 ng and 500 pg HeLa input RNA show good reproducibility and call concordance.



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