A STUDY OF WT-OVATION™ AMPLIFICATION PERFORMANCE

BACKGROUND

The utility of quantitative PCR (QPCR) analysis for differential gene expression studies has increased in recent years with the availability of large QPCR assay collections and commercial primer probe sets. This has facilitated efficient, non-array based interrogation of large numbers of transcripts in samples.

Systems such as ABI's Assays-on-Demand™, Roche's Exiqon Universal Human ProbeLibrary™ or the TaqMan® Low Density Immune Profiling Arrays allow efficient QPCR analysis of samples with large collections of genes and various loci of interest across the transcript length. A significant limitation to the quantification of a large number of gene transcripts in a given sample is the increased requirement of RNA input. This limitation is critical when analyzing small samples such as those obtained by laser capture microscopy (LCM), sorted cell populations, and other valuable clinical samples, especially for analysis of very low abundance transcripts of interest.

NuGEN's Ovation™ family of products are based on the isothermal linear amplification process, Ribo-SPIA™, which affords a rapid, simple and highly efficient amplification of RNA from very small input RNA samples. The method has now been implemented in a novel system, WT-Ovation™ RNA Amplification System, for the amplification of RNA across the entire transcriptome with a high level of sensitivity, and without the 3'-bias commonly associated with poly(A) initiated amplification approaches. Herein we describe the performance of the WT-Ovation™ RNA Amplification System for non-array based gene expression profiling and quantification of very small RNA samples in the range of 5 – 50 ng. These results demonstrate the linearity, sensitivity and reproducibility of WT-Ovation™ RNA Amplification System across the entire transcript length.

MATERIALS AND METHODS

The biological model chosen for this study is Colon Cancer. Total RNA from a human Colon Tumor and the matched Normal Adjacent Tissue (NAT) were purchased from Ambion, Inc (cat #7236, Austin TX). Three replicate amplifications were performed on 20 ng (unless otherwise stated) of each RNA type using the WT-Ovation™ RNA Amplification System, following the manufacturer's instructions.

The QPCR reactions were set up using the amplified cDNA diluted 1/10 into 1 X TE (pH 8.0) 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.

A set of differentially expressed genes and other invariant transcripts were chosen based on information from the literature and results from arrays run previously on this model system. All or a subset of these transcripts were used in the various experiments discussed here. For some samples, the amplified cDNA product was purified using the Zymo clean and concentrate-25 columns (Zymo Research, Orange , CA) and quantiated using a NanoDrop -1000 (NanoDrop Tech, Wilmington, DE).

Real time QPCR assays were designed using the 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.

In addition, 2 ABI Assays-on-Demand™ were purchased for Myosin heavy polypeptide 11 gene (MYH11 NM-022844), designated by an asterisk in Figure 5.

Nine more 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 = $Ct_{\text{test gene}} - Ct_{\text{PGK1}}$. To measure differential expression, the delta Ct for the tumor total RNA sample was compared to the delta Ct for the NAT sample by calculating the delta delta Ct = delta Ct_{tumor} – delta Ct_{normal}.

The dynamic range of amplification was determined using Affymetrix GeneChip® Eukaryotic Poly-A RNA Control Kit (Cat# 900433).

ProbeLibrary™ assays were designed to the 5 prime region of the THR,

DAP, LYS, PHE RNA spike sequences. The RNA spike mixture containing RNA spikes at different concentrations was diluted 1 to 20 into 1X TE (pH 8.0). Two microliters of this mixture was reverse transcribed using the cDNA synthesis reagents of the WT-Ovation™ System. This cDNA was used to prepare standard curves for QPCR analysis. The RNA spike mixture was diluted 1:1000 into RNase-free water then serially diluted 1:10 six times. The 7 spike dilutions and a water control were diluted one to two into HeLa RNA at 20 ng/µL (BioChain, cat# R1255811-50). Two microliters of the RNA mixtures were amplified using the WT-Ovation™ System following manufacturer's instructions. The amplified cDNA was diluted 1 to 10 into 1X TE and assayed by QPCR as described above.

RESULTS AND CONCLUSIONS

Yields are reproducible and proportional to input across 5-50 ng RNA input range. In Figure 1, results show the amplification yield is consistent and dependent upon RNA input. The Normal Adjacent Tissue (NAT) total RNA was titrated into the WT-Ovation amplification with 5, 10, 20, 30, and 50 ng of total RNA input, and then the product was purified and quantitated. Results show reproducible yields across the RNA input range.

Ct results are highly reproducible and consistent across 5-50 ng RNA input range. Shown in Figure 2, 10 ng of the amplified cDNA product from the RNA input titration was analyzed in QPCR with primer probe sets at various distances from 3' of 4 different genes. The uniform and highly reproducible Ct results among all RNA input levels show that even the 5 ng RNA input does not compromise amplification fidelity, sensitivity or transcript coverage.

Differential expression is reproducibly maintained by 3 operators. 3 first time users of the WT-Ovation™ System performed triplicate amplifications each of Tumor, and NAT total RNA. The amplified product was analyzed with QPCR assays for genes previously shown to be differentially expressed in this model system. Results show high inter-operator correlation in differential expression as measured by delta-delta Ct between Tumor and NAT demonstrating amplification fidelity and reproducibility.

The dynamic range is linear across 6 orders of magnitude. Using bacterial spikes, THR, DAP, LYS, and PHE, the sensitivity of WT-Ovation™ System is demonstrated in Figure 4 showing a linear dynamic range of amplification over 6 logs with detection sensitivity of down to 20 copies of mRNA present in the sample.

Amplification is uniform across the entire transcript. In Figure 5 cDNA amplification product for both Tumor and NAT RNA were analyzed with 9 QPCR assays distributed across the length of the MYH11 gene, with the furthest assay at 6,310 nt from the 3' end. The results show consistent and uniform amplification and coverage of the entire transcript. 6 other genes analyzed similarly showed uniform coverage from ~750 to 5900 nt from 3' ends (data not shown). The small Ct variation between the MYH11 assays shows a similar pattern between Tumor and NAT demonstrating that differential expression analysis is consistent across the length of transcript.

Amplicon length is not prohibitive. WT-Ovation™ System amplified cDNA was tested with various QPCR amplicon lengths. Tumor and NAT were amplified in duplicates with 20 ng total RNA input. Nine GAPDH assays were designed with varying amplicon lengths shown in Figure 6. The results show that Ct values were uniform for amplicon lengths of up to 400 bases.

In conclusion the study results described here demonstrate that WT-Ovation™ RNA Amplification System is a sensitive, reproducible, and reliable method for amplifying the entire transcriptome of 5-50 ng total RNA into micrograms of cDNA across a wide range of transcript abundance and without a 3' bias, while maintaining differential expression.

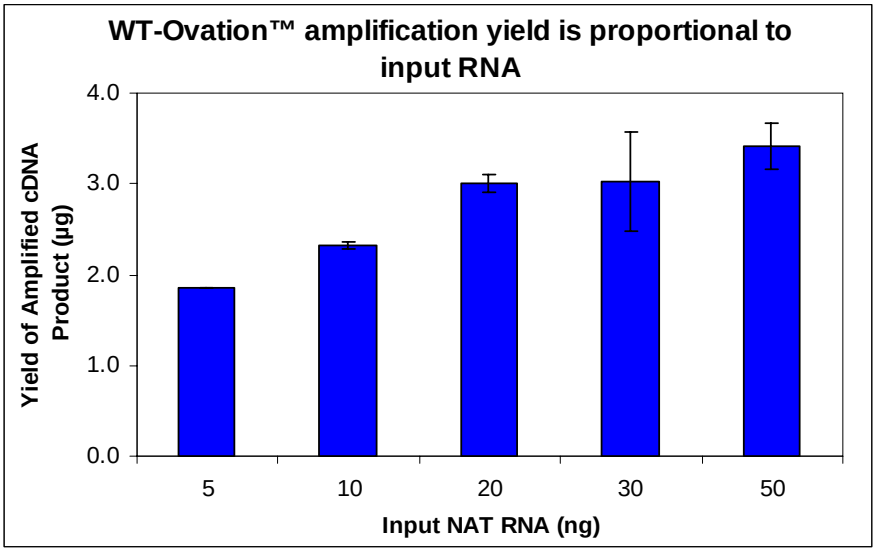


Figure 1. Yields are reproducible and proportional to input across the 5-50 ng RNA input range. Performed in triplicate the NAT total RNA was titrated into the WT-Ovation™ reaction with 5, 10, 20, 30, and 50 ng of total RNA input. Results show reproducible and yields across the RNA input range.

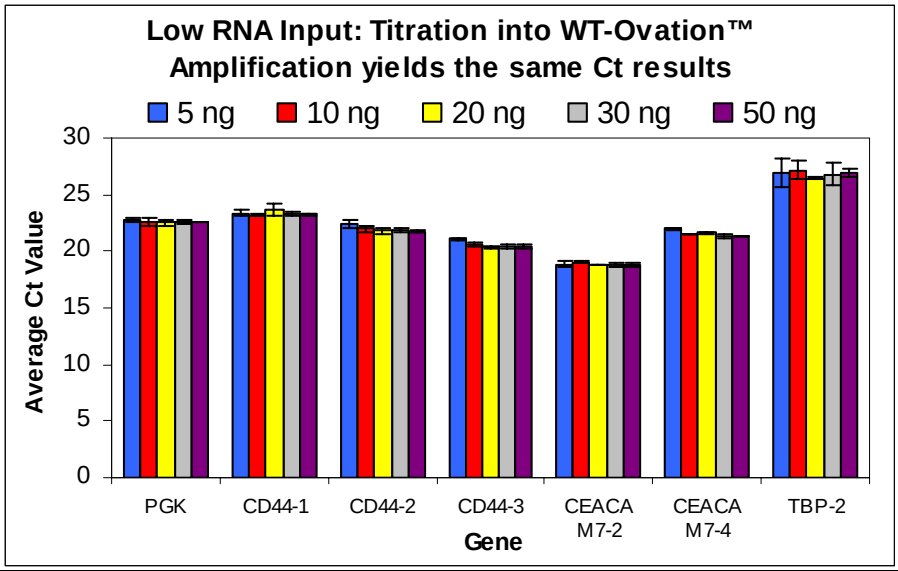


Figure 2. Ct results are highly reproducible and consistent across 5-50 ng RNA input range
The WT-Ovation™ System amplified product was analyzed with 10 ng of amplified cDNA input into QPCR reactions for 2 housekeeping and 2 test genes, using 7 assays. The gene designation and distance of assay from the 3' end are listed here: PGK (1364 nt), CD44 (1:5319 nt, 2:3309 nt, 3:174 nt), CEACAM7 (2:1598 nt, 4:290 nt), and TBP (1817 nt). Cts are consistent and uniform across amplification input range. Accession numbers provided upon request.

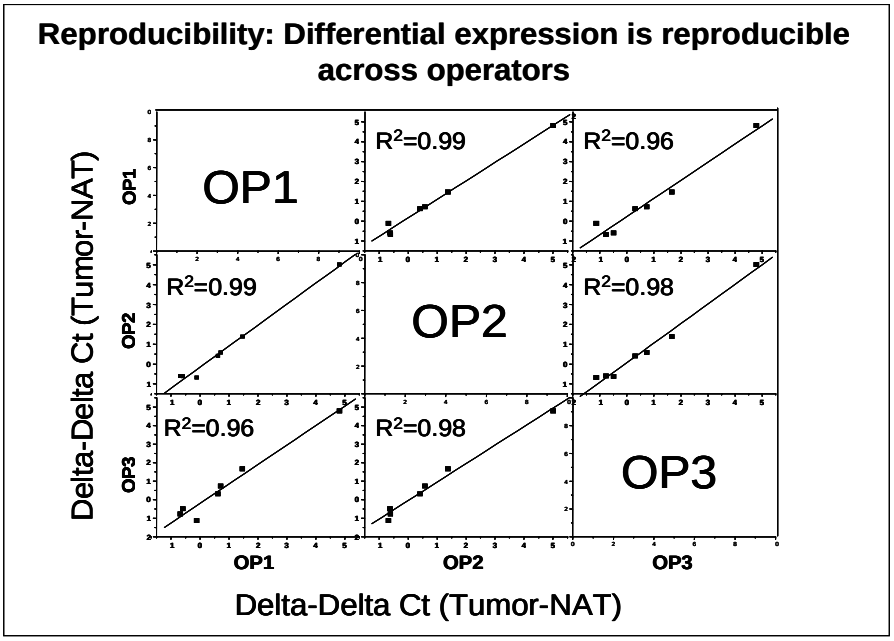


Figure 3. Differential expression is reproducibly maintained by 3 operators. 3 first time users of the WT-Ovation™ System performed amplifications in triplicate with 20 ng each of Tumor, and NAT total RNA. The products were analyzed with QPCR reactions performed for 7 of the 15 genes described earlier. Results shows high inter operator correlations of differential expression as measured by delta-delta Ct between Tumor and NAT.

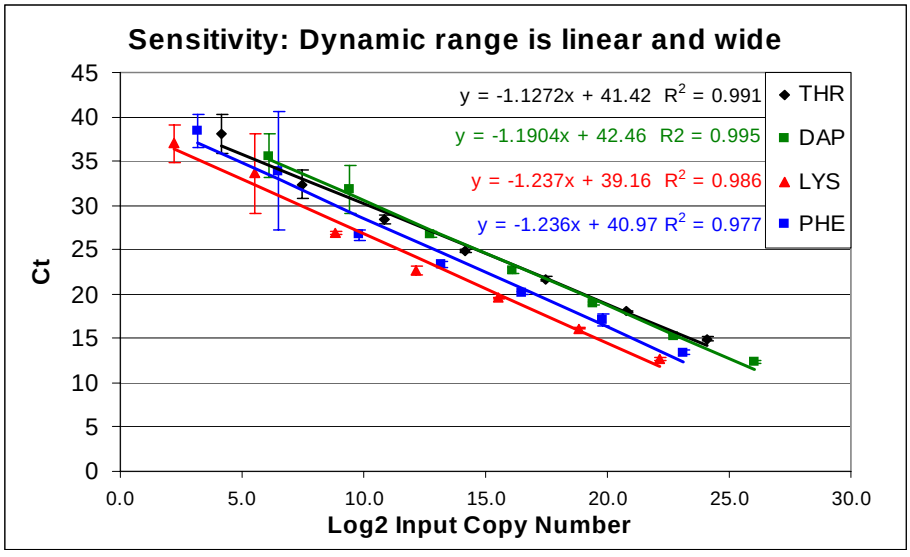


Figure 4. The dynamic range is linear across 6 orders of magnitude.
The sensitivity of WT-Ovation™ System is demonstrated by using bacterial spikes. The THR, DAP, LYS, and PHE spikes show that the dynamic range of amplification is linear over 6 logs with detection sensitivity of down to 20 copies of mRNA present in the sample.

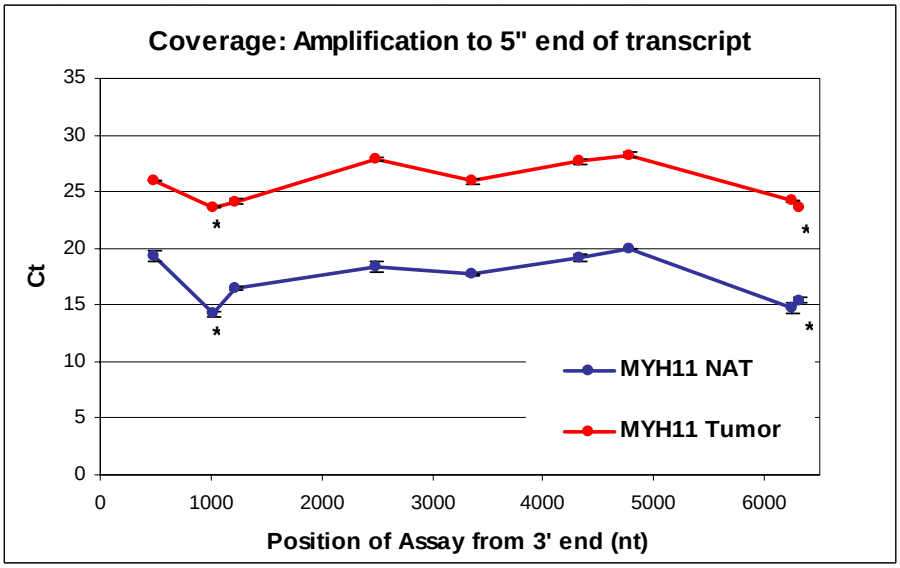
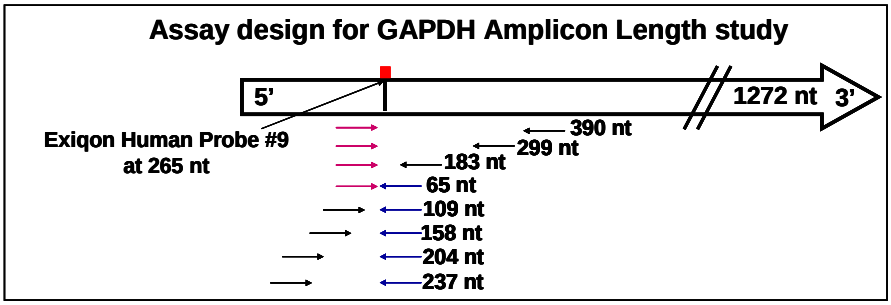


Figure 5. Amplification is uniform across the entire transcript. Tumor and NAT were amplified in duplicate with 20 ng total RNA input. For the MYH11 transcript, assays were designed at 489, 1019, 1220, 2490, 3352, 4334, 4771, 6251, and 6310 nt from the 3' end. Results show that the WT-Ovation™ System yields uniform amplification across the transcript in both Tumor and NAT. The two Assay-on-Demand™ assays for the MYH11 transcript are designated by asterisks.



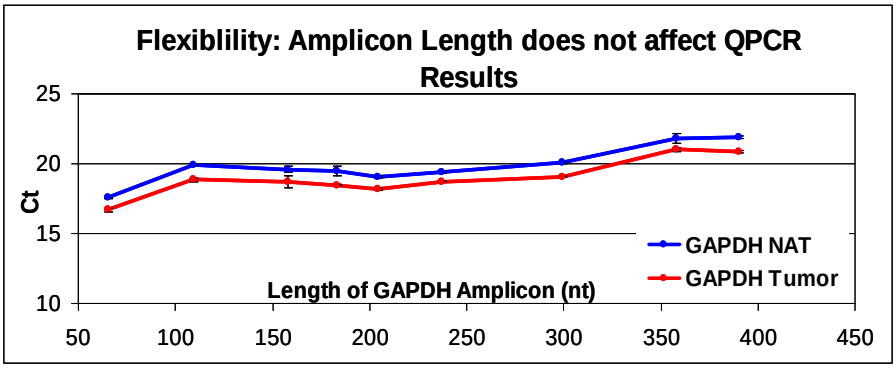


Figure 6. Amplicon length is not prohibitive. Assays were designed utilizing a total of nine different primers resulting in 9 different sizes of amplicons.

Tumor and NAT were amplified in duplicate with 20 ng total RNA input. The GAPDH assays, designed at varying amplicon lengths, all showed uniform Cts.

WT-Ovation™ RNA Amplification System Specifications

Cat No.: 2210-24, 24 reactions

Input: 5 - 50 ng total RNA

Yield: 1.5 - 4 μg single stranded cDNA

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