ADVANTAGES OF RNA AMPLIFICATION PRIOR TO QUANTITATIVE PCR ANALYSIS

BACKGROUND

Quantitative real-time PCR (QPCR) is a powerful tool increasingly used in gene expression studies focused on screening and discovery as well as target validation. A critical requirement for these studies is the use of homogenous samples composed of as few different cell populations as possible.

Technologies such as LCM, cell sorting, cell titration schemes, single cell analysis, and other cell population isolation schemes are therefore commonly employed to reduce the complexity of samples. This allows investigators to make more definitive and unambiguous correlations between observed characteristics (i.e. pathology) and expression patterns in specific cell types. Gene expression results obtained with more homogenous cell populations can be more specifically and reliably confirmed by biological relevance and pathway analysis.

A major challenge in high resolution sample isolation, however, is that the amounts of available RNA is reduced to prohibitive quantities. This limitation can often affect study design and the total number of transcripts that can be interrogated using QPCR. This is more restrictive for low to medium abundance genes of interest that require much higher input of sample for QPCR analysis.

Another challenge posed by small RNA samples is that the isolation methods used can frequently impact quality and integrity of the RNA, presenting a significant obstacle in obtaining high quality data.

NuGEN's WT-Ovation™ RNA Amplification System meets these challenges and enables analysis of hundreds of genes with QPCR from small and difficult samples for even the lowest abundant transcripts. WT-Ovation RNA amplification is initiated both at the 3' end and randomly throughout the whole transcriptome with little 3' bias. It is this global amplification that makes this an ideal system for small and compromised RNA samples. Here we demonstrate the advantages of amplification prior to QPCR by comparing results with and without amplification.

MATERIALS AND METHODS

In this report, 2 separate studies are described.

In the first study we used total RNA from a human Colon Tumor and the matched Normal Adjacent Tissue (NAT) (Ambion, cat. #7236). Both differentially expressed genes and invariant transcripts were chosen based on information from literature and results from arrays run previously on this model system. All sample preparations for this experiment were performed in triplicate. Amplified and unamplified cDNAs were prepared from 20 ng of each RNA type using the WT-Ovation RNA Amplification System, following standard protocols. The study design is outlined in Figure 1: After 1st and 2nd strand, an aliquot of unamplified cDNA was removed for QPCR to determine the baseline prior to amplification. Following SPIA™ amplification, a panel of amplicons across multiple genes was interrogated by QPCR; results from some of the transcripts are shown in Figure 2 and 3.

QPCR reactions were performed in 20 µl volumes using the ABI 7500 real-time PCR System and the ABI Fast mix (Applied Biosystems, cat. # 4352042). The un-amplified products were in 20 µl volume, diluted 1:10, and 2 µl (approximately 0.2 ng) was used per QPCR reaction, yielding 100 reactions. The amplified cDNA in 40 µl, were diluted 1:10 in TE, then 2 µl (approximately 15-20 ng) was added per QPCR reaction, yielding 200 reactions. Exiqon ProbeLibrary™ and ABI Assays-on-Demand™ were used for all QPCR assays, further details about primer probe design and sequences are available upon request.

Note that in this study the QPCR input is determined based on the availability of material for amplified vs. unamplified samples, so the same dilutions and input volumes are used, not the same quantity. This design simulates the challenges of limiting RNA samples and the choices and compromises an investigator needs to make between the number of assays vs. the sensetivity to low copy number transcripts. Here we demonstrate the affect of this choice on the quality of gene expresion results obtianed.

In an independent study designed and implemented at a leading biomedical core facility, cDNA was prepared from Human Jurkat Cell Total RNA (Stratagene, cat. # 540107), for interrogation by QPCR. Unamplified cDNA was prepared in triplicate (RT) directly from 5 µg of total RNA with the SuperScript™ III First-Strand Synthesis System for RT-PCR using random hexamers according to the vendor's instructions (Invitrogen, cat. #18080-051). WT-Ovation System was used to linearly amplify cDNA in triplicate (WT) from 20 ng of total RNA. cDNA equivalents of 100 ng for each method were loaded per sample port as input into the TaqMan® Low Density Immune Profiling Arrays [LDA] (Applied Biosystems, cat. #4342510) and the QPCR reactions are run in 4 replicates. Genes with RT-PCR C_T of 36 or lower were defined as detectable.

RESULTS AND CONCLUSIONS

Maintain differential expression after amplification:
In Figure 2, a direct comparison of unamplified vs. amplified Tumor and NAT RNA samples, shows an approximately 10 cycle shift in C_T values for the ***CEACAM7*** transcript (Human carcinoembryonic antigen-related cell adhesion molecule 7), for each sample, simply showing the magnitude of amplification. Here the QPCR C_T curves show that the differential expression between the Tumor and NAT is maintained after amplification.

With amplification find differentially expressed genes even at the lowest abundance ranges with limited samples size: In Figure 3, C_T data is shown for the Tumor and NAT RNA samples for multiple assays across the length of three different genes. These results demonstrate the advantages of amplification prior to QPCR with limiting starting material.
All assays along the length of the ***SDCCAG33*** transcript (serologically defined colon cancer antigen 33) can detect it for both Tumor and NAT samples. However, without amplification, the QPCR assay designed closest to the 3' end shows highly variable C_T results leading to an ambiguous expression difference between Tumor and NAT (note large error bars). With amplification, not only is the same assay informative and reproducible, the C_Ts highly correlated with the results of the other assays for the same gene.
The ***Col11A1*** transcript (collagen, type XI, alpha 1), without amplification, is virtually undetectable in the NAT sample with all assays, while it does show consistent detectable C_Ts in the Tumor sample. This data set is still unusable for analysis of differential expression due to the missing data for the NAT sample. Although this may hint at the over-expression of this gene in Tumor, there is no way to determine the fold change or amount of up regulation of the transcript without the NAT C_Ts. These missing data points render the entire experiment inconclusive. However after amplification with WT-Ovation, C_Ts are reliably measurable for both samples with high reproducibility, allowing the definitive determination of fold change in expression. For the ***CA4*** transcript (carbonic anhydrase IV), the unamplified sample shows variable and inconsistent C_T values. The same QPCR assay for the amplified cDNA, shows robust, reproducible, and consistent C_T results across the entire transcript for both samples, identifying this transcript as having significant differential expression between Tumor and NAT samples.

With amplification, detect more genes with more sensitivity and reproducibility: In this study the QPCR reaction input were equivalent between amplified and non-amplified samples, so the advantages of amplification are demonstrated very well. Figure 4 shows the Heat map representation of a portion of the Low Density Array data. Here the 4 replicate QPCR C_Ts are stacked in bars of color for each of the 17 genes shown. The pale yellow color represents effectively undetectable C_Ts and the dark blue represents the lower C_Ts values obtained for higher abundance genes. Four specific examples are annotated A, B, C and D, where amplification using WT-Ovation has brought genes that were undetectable or barely detectable without amplification into much lower C_T ranges allowing for more genes to be detected reliably.
Example A is the ***Fas*** gene transcript that is barely detectable in unamplified cDNA (RT). With WT Ovation the gene is easily detected with a consistently low C_T.
Example B and C show very low abundance and sporadically detected genes, ***GZMB*** and ***HO-1***, both made more reproducibly detectable by amplification. WT-Ovation amplified sample shows better agreement between C_Ts of the 4 QPCR replicates. This is critically valuable in investigating low abundance genes where comparative expression analysis must be reliable and reproducible.
Example D is the ***Interleukin IL-10*** gene, completely undetectable without amplification, but reproducibly detected at very low levels after WT-Ovation amplification.
In this comparison of unamplified vs. amplified samples, with equivalent input into the LDA card, 20 percent more genes were detected with WT-Ovation amplified cDNA vs. unamplified cDNA, see Figure 5.
These results confirm that a significantly higher proportion of expressed transcripts can be reliably and reproducibly detected and quantified using the WT-Ovation RNA Amplification System than can be detected using standard reverse transcriptase.

In Conclusion the WT-Ovation System global amplification of small and limiting samples prior to QPCR enables analysis of very low abundant genes that are below the detection levels in unamplified samples. This system allows sensitive and accurate interrogation of hundreds of more genes with QPCR, while more of the original precious RNA sample can be archived and stored for later applications.

WT-Ovation™ RNA Amplification System

System Specifications

Cat No.: 2210-24, 24 reactions

Input: 5 - 50 ng total RNA

Yield: 1.5 - 4 μg single stranded cDNA

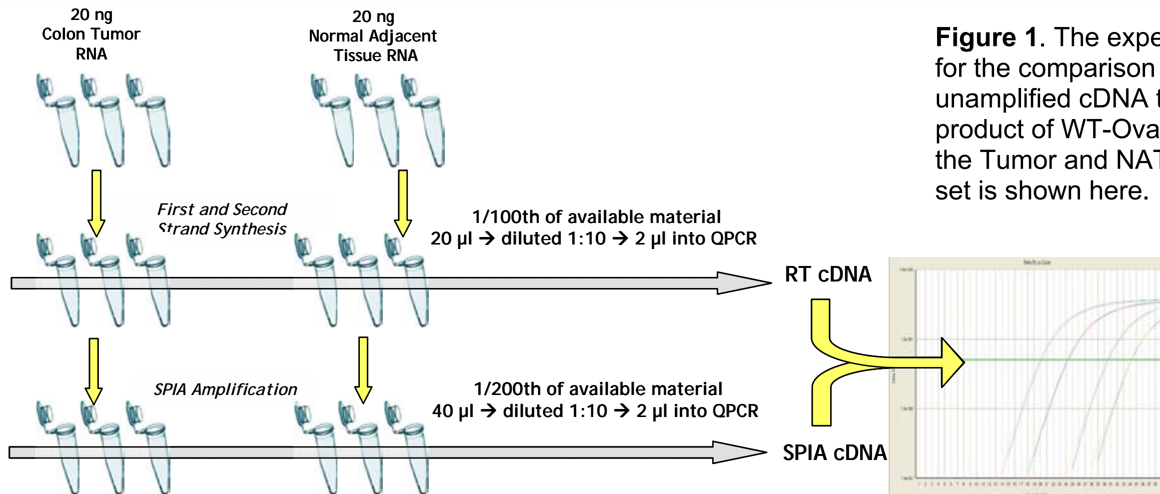

Figure 1. The experimental design for the comparison of the unamplified cDNA to the amplified product of WT-Ovation System for the Tumor and NAT RNA sample set is shown here.

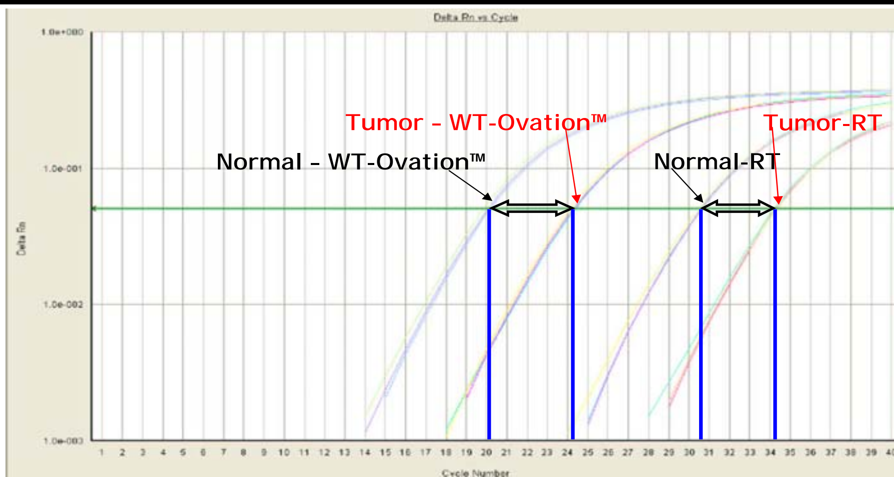

Figure 2. Maintain differential expression after amplification. The differential expression of the CEACAM7 transcript between Tumor and NAT samples observed before amplification is maintained after amplification, while the 10 cycle difference in C_Ts for each sample demonstrates the extent of amplification.

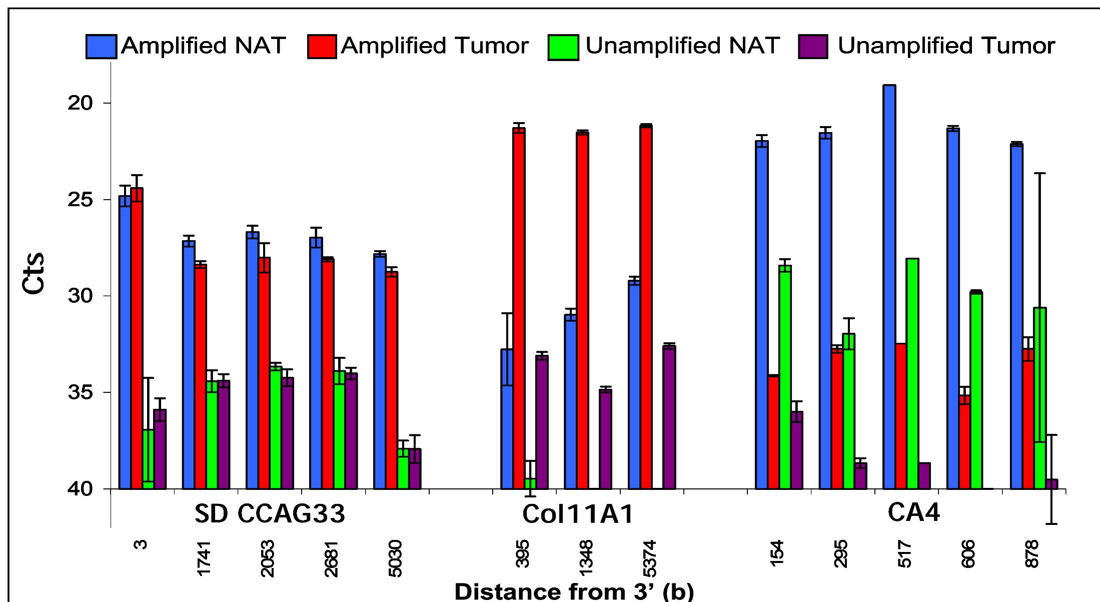

Figure 3. Find differentially expressed genes even at the lowest abundance ranges. A number of QPCR assays performed on unamplified samples are completely undetectable or show highly variable C_Ts. In the lower abundance range even for those genes detected in the unamplified sample, the low reproducability can make it difficult to determine conclusively if some genes are differentially expressed. Upon amplification, however, multiple QPCR assays yield robust and reproducible C_T results that show maintenance and detection of differential expression in the Tumor and NAT system.

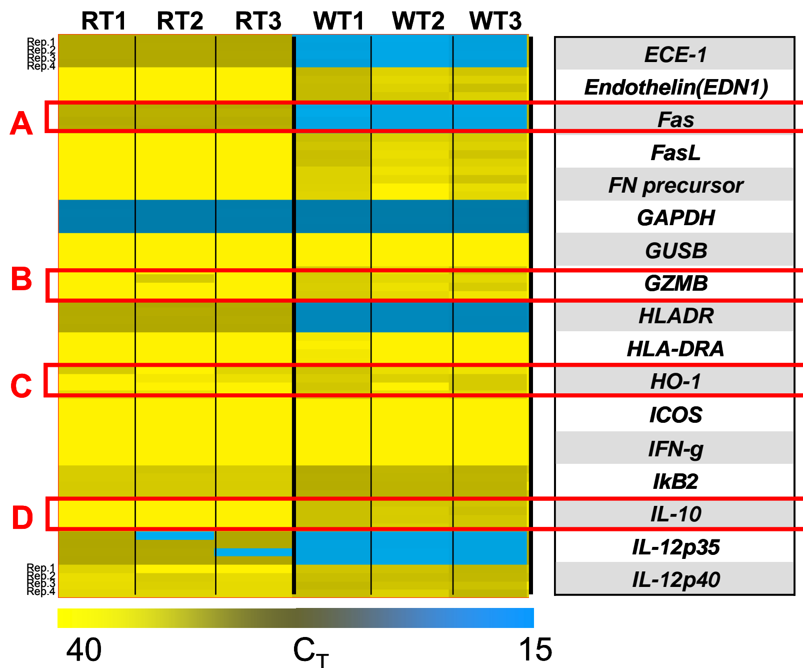

Figure 4. Detect genes with more sensitivity and reproducibility with amplification.
Results show a number of genes that are typically "missed" (in pale yellow) because they fall under the detectable levels with standard RT apporaches. With WT-Ovation amplification (WT) these genes are detected reliably, demonstrating that global amplification, yields more informative data from QPCR interrogation, especially for genes in the low abundance range.

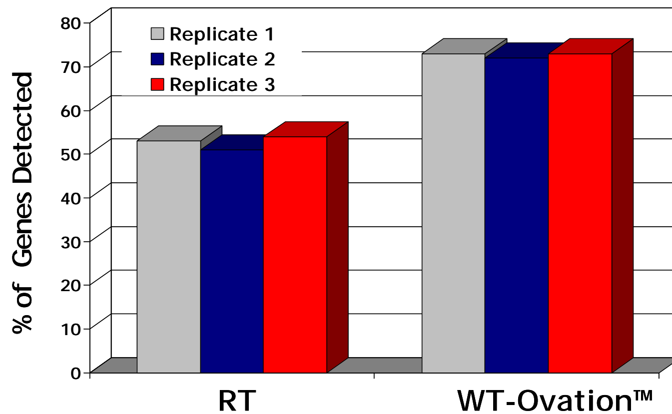

Figure 5. Detect more genes with amplification.
In this study 20 % more genes were detected in the WT-Ovation amplified cDNA compared to the RT samples, allowing more effective utilization of small and valuable samples.

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