

■ Advantages of RNA Amplification Prior to Quantitative PCR Analysis

Background

Quantitative real-time PCR (qPCR) is a powerful tool that is being 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 homogeneous samples composed of as few different cell populations as possible.

Technologies such as LCM, cell sorting, cell titration, single cell analysis and other cell population isolation schemes are therefore commonly employed to reduce the complexity of samples. The increased homogeneity 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 homogeneous 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 amount 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 frequently impact quality and integrity of the RNA, presenting a significant obstacle in obtaining high quality data.

NuGEN's Ovation® qPCR System (Part No. 2210-24) meets these challenges and enables analysis of hundreds of genes with qPCR from small and difficult samples for even the lowest abundance transcripts. Ovation qPCR System amplification is initiated both at the 3' end and randomly throughout the transcriptome with little 3' bias. This global amplification makes this system ideal for small and compromised RNA samples.

In this technical report, we demonstrate the advantages of amplification prior to qPCR by comparing results with and without amplification.

Materials and Methods

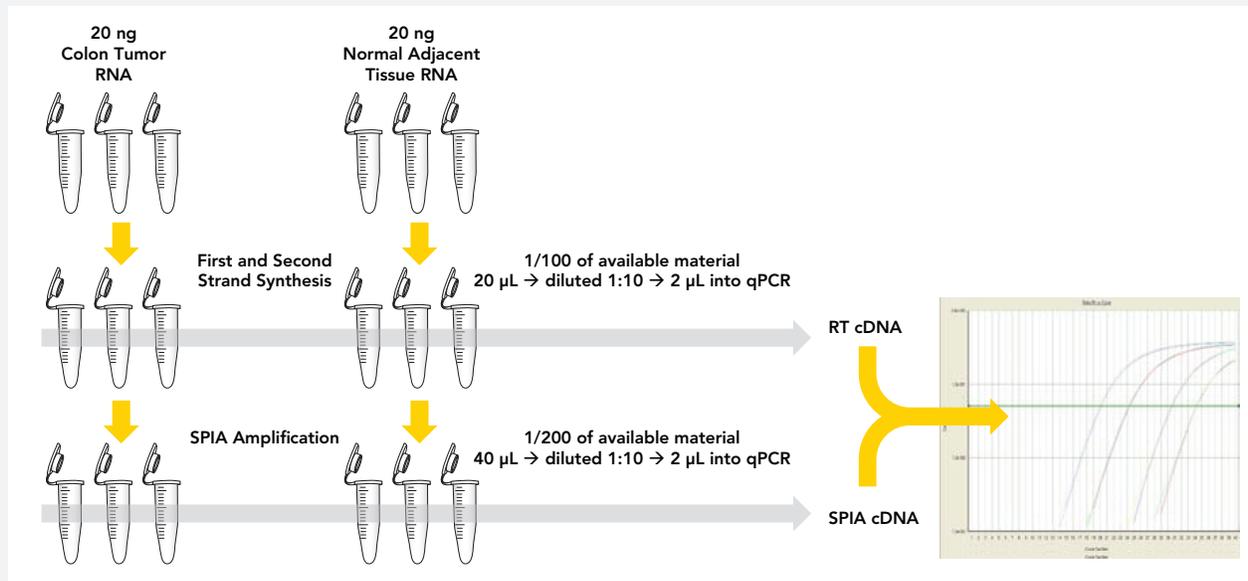
In this report, two separate studies are described. In the first study, we used total RNA from a human colon tumor and the matched normal adjacent tissue (NAT) (Ambion, Cat. #7236). Both differentially expressed genes and invariant transcripts were chosen based on information from literature and results 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 Ovation qPCR System, following standard protocols. The study design is outlined in **Figure 1**. After first and

second strand synthesis, 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 these transcripts are shown in **Figure 2** and **Figure 3**.

The qPCR reactions were performed in 20 μ L volumes using the ABI 7500 real-time PCR System and the Thermo Fisher TaqMan® Fast Universal PCR Master Mix (Thermo Fisher, Part No. 4352042). The unamplified products were in 20 μ L volume, diluted 1:10 in TE, and 2 μ L (approximately 0.2 ng) was used per qPCR reaction, yielding 100 reactions. The amplified cDNA in 40 μ L, was diluted 1:10 in TE, then 2 μ L (approximately 15–20 ng) was added per qPCR reaction, yielding 200 reactions. Exiqon ProbeLibrary™ and Thermo Fisher 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 and the sensitivity to low copy number transcripts. Here we demonstrate the effect of

FIGURE 1 Experimental design.



The experimental design for the comparison of the unamplified cDNA to the amplified product of the Ovation qPCR System for the tumor and NAT RNA sample sets.

this choice on the quality of the gene expression results we obtained.

In an independent study designed and implemented at a leading biomedical core facility, cDNA was prepared from Human Jurkat Cell total RNA (Agilent, Cat. #540107), for interrogation by qPCR. Unamplified cDNA was prepared in triplicate 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 (Thermo Fisher, Cat. #18080-051). The Ovation qPCR System was used to amplify cDNA in triplicate 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) (Thermo Fisher, Cat. #4342510) and four replicate qPCR reactions were run. Genes with an RT-PCR C_T of 36 or lower were defined as detectable.

Results

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. In this figure, the qPCR C_T curves show that the differential expression between the tumor and NAT is maintained after amplification.

Find differentially expressed genes even at the lowest abundance ranges with limited sample 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 (serologi-

cally defined colon cancer antigen 33) showed detectable differences between 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 the large error bars). With amplification, not only is the same assay informative and reproducible, the C_T s are highly correlated with the results of the other assays for the same gene.

Without amplification, the Col11A1 transcript (collagen, type XI, alpha 1) is virtually undetectable in the NAT sample with all assays, while it shows consistent, detectable C_T s 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 result may hint at the over-expression of the gene in tumor cells, there is no way to determine the fold change or amount of upregulation of the transcript without the NAT C_T s. These missing data points

render the entire experiment inconclusive. However after amplification with the Ovation qPCR System, C_T s 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.

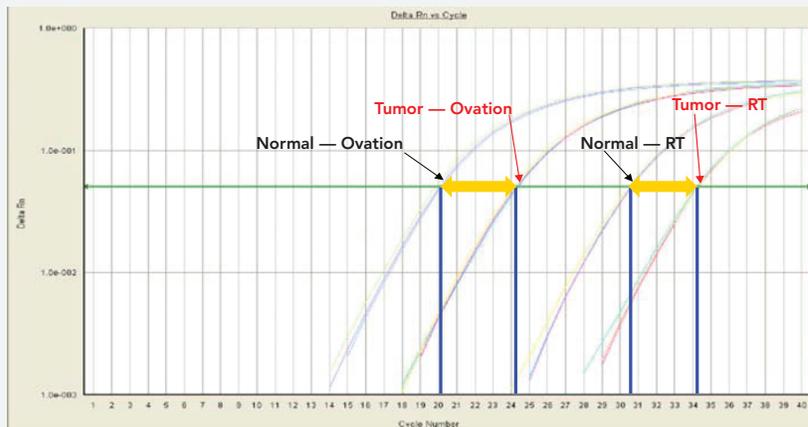
With amplification, detect more genes with more sensitivity and reproducibility

In this study, the qPCR reaction input was equivalent between amplified and non-amplified samples, so the advantages of amplification are evident. **Figure 4** shows the heat map representation of a portion of the Low Density Array data. Here the four replicate qPCR C_T s are stacked in bars of color for each of the 17 genes shown. Pale yellow represents effectively undetectable C_T s and dark blue represents the lower C_T values obtained for higher abundance genes. Four specific examples are annotated A, B, C and D, in which amplification using the Ovation qPCR System has brought genes that were undetectable or barely detectable without amplification into much lower C_T ranges allowing more genes to be detected reliably.

Example A is the *Fas* gene transcript that is barely detectable in unamplified cDNA (RT). Using the Ovation qPCR System, the gene is easily detected with a consistently low C_T .

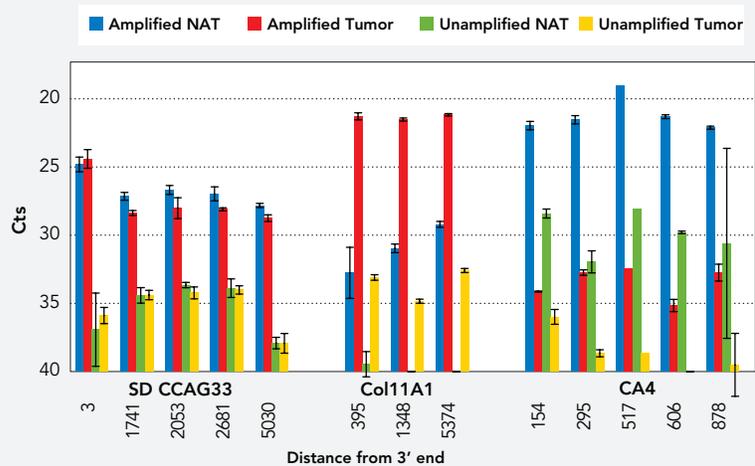
Examples B and C show very low abundance and sporadically detected genes, *GZMB* and *HO-1*, both made more reproducibly detectable by amplification. The Ovation qPCR System-amplified sample shows better agreement between C_T s of the four

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_T s 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 CTs. In the lower abundance range, even for those genes detected in the unamplified sample, the low reproducibility can make it difficult to determine conclusively whether some genes are differentially expressed. Upon amplification, however, multiple qPCR assays yield robust and reproducible CT results that show maintenance and detection of differential expression in the tumor and NAT system.

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 Ovation qPCR System amplification.

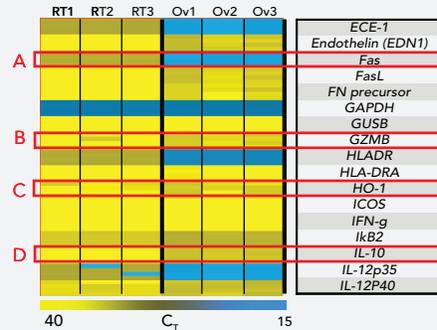
In this comparison of unamplified vs. amplified samples with equivalent input into the LDA card, 20 percent more genes were detected using Ovation qPCR System-amplified cDNA than using unamplified cDNA, see **Figure 5**.

These results confirm that a significantly higher proportion of expressed transcripts can be reliably and reproducibly detected and quantified using the Ovation qPCR System than can be detected using standard reverse transcriptase.

Conclusion

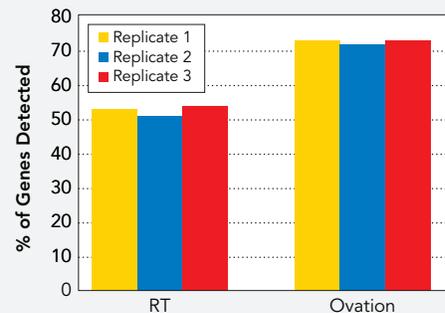
The Ovation qPCR System global amplification of small and limiting samples prior to qPCR enables analysis of very low abundance genes that are below detection levels in unamplified samples. This system allows sensitive and accurate interrogation of hundreds more genes with qPCR, while more of the original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

FIGURE 4 Detect genes with more sensitivity and reproducibility with amplification.



Results show a number of genes that are typically “missed” (pale yellow) because they fall under the detectable levels with standard RT approaches. With Ovation qPCR System amplification 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 Ovation qPCR System-amplified cDNA than in the RT samples, suggesting more effective utilization of small and valuable samples with amplification.



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