TECHNICAL REPORT

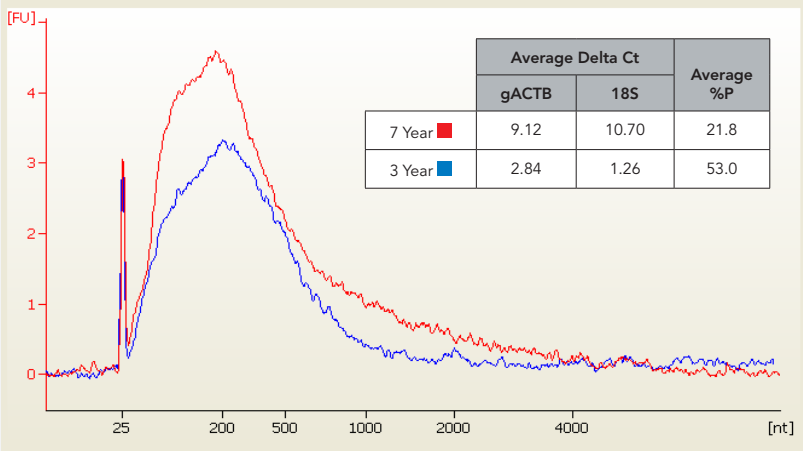
RNA Sample Quality Assessment Test for the WT-Ovation® FFPE System

Introduction

Gene expression analysis of clinical samples is challenging due to sample availability, amount, and integrity. Working with nucleic acids isolated from formalin-fixed, paraffin-embedded (FFPE) tissue samples is difficult due to the extensive degradation and modification resulting from long-term storage and fixation methods. These samples, therefore, have been unlikely candidates for global gene expression analysis using microarrays until now.

The whole transcriptome approach used in the WT-Ovation® FFPE System enables amplification of FFPE samples through a robust and simple solution for RNA amplification and target preparation from as little as 50 ng of total RNA for analysis on Affymetrix GeneChip® arrays when used with NuGEN's Encore® Biotin Module (Part No. 4200). Note: WT-Ovation FFPE System has been replaced by Ovation® FFPE WTA System (Part No. 3403). While the WT-Ovation FFPE System is expected to allow the amplification of a large number of samples previously thought to be too degraded for microarray analysis, there still exists a subset of samples that, due to age and handling, will not yield informative microarray results. The expected wide range of quality and integrity of FFPE-derived RNA produces a pressing need for tools that allow assessment of sample quality so researchers can determine the level of performance expected with the samples under study. To facilitate the use of FFPE RNAs for generating biologically relevant data, a simple qPCR-based test has been developed that assesses the relative quality of FFPE RNA and can be used to predict subsequent performance of FFPE RNA samples on Affymetrix 3' expression arrays.

FIGURE 1. **Agilent Bioanalyzer traces of amplified cDNA from two FFPE samples.**



	Average Delta Ct		Average %P
	gACTB	18S	
7 Year	9.12	10.70	21.8
3 Year	2.84	1.26	53.0

The Bioanalyzer traces look very similar to each other but perform very differently on arrays, as measured by the % Present Calls in the inset table. The average delta Cts used in the quality assessment test are strikingly different and appear to be good predictors for array performance.

Development of a qPCR-Based RNA Quality Assessment Test

Standard methods used to assess RNA quality prior to microarray analysis have proven insufficient for FFPE samples. For example, BioAnalyzer™ traces of amplified cDNA from two FFPE samples are shown in **Figure 1**. Both samples are highly degraded as demonstrated by this analysis (cDNA products are short) and yet after amplification and hybridization to microarrays, one of the samples produced a significantly greater percentage of present calls (shown in **Figure 1** inset). The general assumption for this quality assessment assay is that RNA from a highly-expressed and ubiquitous gene could serve as a marker for overall RNA integrity in a qPCR assay. Highly abundant transcripts have the inherent advantage that they will provide relatively robust Ct values even though the RNA has been degraded. However, if these genes are greatly impacted (high Ct values obtained compared to a reference RNA sample), one can assume that the RNA will generate microarray results that are not as high quality as those generated from less degraded samples.

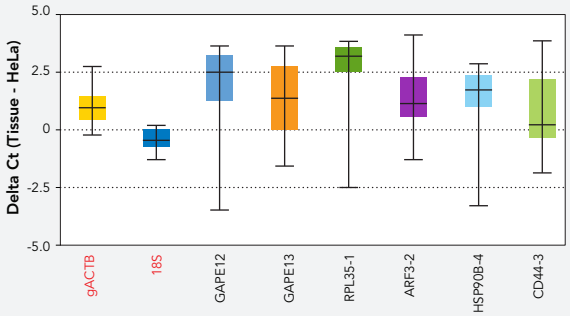
To identify candidate genes for assessing RNA quality, several qPCR amplicons were evaluated for their expression across a panel of tissues and normalized to a reference RNA sample (HeLa RNA). In order to develop a generally applicable test, the expression of the test genes must be detectable and stable across multiple tissues. Of the eight amplicons tested, two (beta actin and 18S rRNA) exhibited the desired expression pattern. As shown in the

NuGEN®

box and whiskers plot in **Figure 2**, the mean ΔCt for both assays was close to zero and the standard deviation across the 12 tissues tested (whiskers) was low. Other gene amplicons showed a high degree of variability across the tissue panel. Note that the study contains other genes commonly used as housekeeping controls, glyceraldehyde-3-phosphate dehydrogenase (GAPE12 and 13) and ribosomal protein L35 (RPL35). Ultimately, these multi-copy, highly expressed transcripts (amplicons gACTB and 18S) were chosen as surrogate markers for RNA integrity based on their robust performance across the tissue panel and for their high correlation to array performance in 63 FFPE RNAs tested (described below).

It was observed that even when comparing samples from the same tissue type, qPCR results for cDNA made from RNA isolated from FFPE tissue tended to vary widely. In general, higher Ct values for specific amplicons appeared to correlate with poorer performance on Affymetrix HG-U133 2.0 GeneChip arrays, as defined by percent present calls (%P). As previously demonstrated, there is a strong relationship between RNA degradation (RIN) and %P on arrays using degraded HeLa RNA, and therefore %P can be used as a metric for array performance. Because raw Ct values can vary from lab to lab and day to day, a delta Ct (ΔCt) was calculated by subtracting the Ct of a high quality commercial control RNA from the Ct of the FFPE test sample ($\Delta Ct = Ct_{FFPE\ RNA} - Ct_{Control\ RNA}$). Ten ng of HeLa total RNA was used as the control sample, however, Universal Human Reference RNA can also be used. Three sets of data were used to test the assay: 21 lymphoma samples, 40 ovarian cancer samples and 2 breast tumor samples. The RNAs in these sets varied widely in quality. In all three sample sets, a comparison of the calculated ΔCt to the %P achieved on arrays demonstrated very good correlation for both the beta actin (gACTB) and 18S rRNA qPCR assays (**Figure 3**). In contrast, the qPCR results

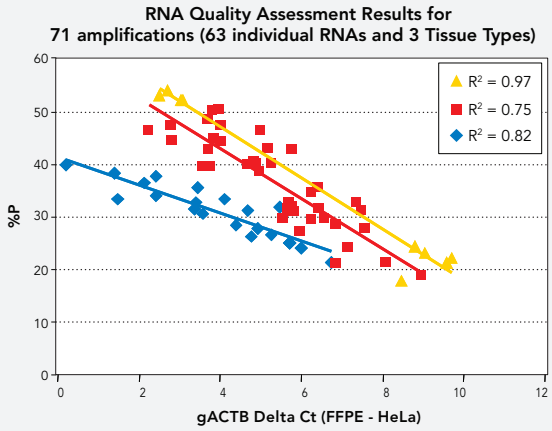
FIGURE 2. **Beta actin and 18S rRNA expression is stable across multiple tissues.**



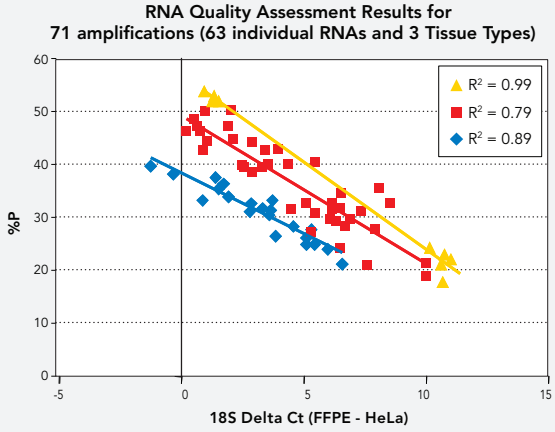
Each box in the graph represents the delta Ct (ΔCt) values for each gene calculated across the entire tissue panel in reference to HeLa RNA. The bars bisecting the boxes show the mean ΔCt and the whiskers define the bounds of the standard deviations. All amplicons except gACTB and 18S show a high degree of variability across the tissue panel, making them unsuitable for use in RNA quality assessment.

FIGURE 3. **Delta Cts between FFPE and control RNAs show high correlation to array performance (%P).**

A



B



Delta Cts for 3 different sample sets were calculated and plotted versus the %P for the corresponding arrays. A correlation for the gACTB amplicon is shown in Figure 3A and for the 18S rRNA amplicon in 3B. Blue represents a 21 sample lymphoma set, red represents a 40 sample ovarian tumor set, and yellow represents a 2 sample breast tumor set (with 5 replicates of each plotted).

for GAPDH had very poor correlation to %P especially at the higher ΔCt values (R^2 values of 0.26 and 0.54 for the lymphoma and ovarian data sets, respectively—data not shown). The strong correlation between the ΔCt value and the %P suggested that the calculation of the ΔCt value for either 18S rRNA or beta actin would serve as a reasonable qualification test for samples prior to array hybridization.

Instructions for the Quality Assessment Test

The general approach is to compare qPCR results with unamplified cDNA using the 18S and beta actin qPCR assays to %P obtained from microarray analysis of amplified cDNA from a subset of samples (pilot study) representative of a large sample set.

Once the relative quality of the starting RNA samples has been assessed, QC metrics can be determined and applied to the large sample set (see WT-Ovation FFPE System Validation Guidelines and RNA Sample Quality Assessment Technical Report). The steps involved in using this approach are listed below.

1. Process the pilot study samples following the WT-Ovation FFPE System protocol through primer annealing, first and second strand cDNA synthesis, to just prior to the bead purification. Process 10 ng of HeLa or UHR RNA as the control.
2. Remove 2 µl from the 20 µl reaction. Make sure that the cDNA aliquot for this test is removed prior to the purification step. Proceed with the purification and amplification exactly as instructed in the user guide. The loss of the small aliquot of cDNA has a minimal effect on the final yields of the amplification.
3. Dilute the cDNA 10-fold by adding 18 µL of TE to the 2 µL aliquot.
4. Perform SYBR Green qPCR assays using the primers listed in **Table 1**.

TABLE 1. **Forward and Reverse primer sequences for gACTB and 18S amplicons.**

Assay Name	Description	Forward Primer	Reverse Primer	Amplicon Length (nt)
gACTB	Beta Actin	CAGCAGATGTG-GATCAGCAAG[1]	GCATTTGCG-GTGGACGAT[1]	67
18S	18S ribosomal RNA	CGAAGACGAT-CAGATACCGT	GGT-CATGGGAATA-ACGCCG	78

[1] Reference available, please contact NuGEN Technical Support.

5. For each RNA calculate the ΔCt between that sample and the control sample ($\Delta Ct = Ct_{FFPE\ RNA} - Ct_{control\ RNA}$)
6. The magnitude of the ΔCt predicts the relative level of performance that can be expected from array analysis (the larger the ΔCt, the lower the expected %P).
7. Fragment and label 5 µg of amplified cDNA using the Encore Biotin Module and hybridize on Affymetrix GeneChip arrays.
8. Plot ΔCt values versus %P to determine overall RNA quality and choose ΔCt cut-offs (if required).
9. If necessary, repeat steps 1–6 with the large study sample set through ΔCt calculations. Use the ΔCt cut-offs to select samples for further processing on microarrays.

Performance Analysis of the Quality Assessment Test

The qPCR-based RNA Quality Assessment Test described above is meant to be used as a method to screen large FFPE sample sets to choose those samples most likely to yield usable data in microarray studies. %P was chosen as the metric for array performance because of the relationship between %P and the amount of degradation in an RNA sample. Therefore, each researcher must choose a threshold for %P that will reflect the desired quality of the RNA sample based on known gene expression signatures. This threshold will most likely vary for the different sample types, disease, and arrays. Specific recommendations regarding employing this quality assessment assay for a specific gene expression study are described in the WT-Ovation FFPE System Validation Guidelines and RNA Sample Quality Assessment Technical Report.

To understand the overall performance of the assay across multiple tissue types, analysis was performed on the data set at two different %P threshold values: 25 and 30%. 73 out of 86 samples exceeded the 25% P threshold, while 63 samples exceeded the 30% P threshold. In **Figure 4**, the data is presented as a histogram where the x-axis represents different bins categorized by ΔCt values generated with the beta actin qPCR assay. The samples exceeding 25 or 30% P clearly cluster around the lower ΔCt values. Conversely, samples with less than 25 or 30 %P cluster towards higher ΔCt values. Samples generating intermediate ΔCt values (6–8 for 25% P threshold, 5–8 for the 30% P threshold) either pass or fail the minimum %P threshold. The percentage of false positives

TABLE 2. **Results of the assay at various ΔCt values.**

Beta actin ΔCts assuming 25% Present Calls				
ΔCt Cut-off	True Positive (ΔCt +, ≥ 25% P)	False Positive (ΔCt +, <25% P)	False Negative (ΔCt -, ≥25% P)	True Negative (ΔCt -, <25% P)
5	48 (66%)	0 (0%)	25 (34%)	13 (100%)
6	64 (88%)	2 (15%)	9 (12%)	11 (85%)
7	70 (96%)	5 (38%)	3 (4%)	8 (62%)
8	73 (100%)	6 (46%)	0 (0%)	7 (54%)
9	73 (100%)	10 (77%)	0 (0%)	3 (23%)

Beta actin ΔCts assuming 30% Present Calls				
ΔCt Cut-off	True Positive (ΔCt +, ≥30% P)	False Positive (ΔCt +, <30% P)	False Negative (ΔCt -, ≥30% P)	True Negative (ΔCt -, <30% P)
4	33 (52%)	0 (0%)	30 (48%)	23 (100%)
5	45 (71%)	3 (13%)	18 (29%)	20 (87%)
6	58 (92%)	8 (35%)	5 (8%)	15 (65%)
7	61 (97%)	14 (61%)	2 (3%)	9 (39%)
8	63 (100%)	16 (70%)	0 (0%)	7 (30%)
9	63 (100%)	20 (87%)	0 (0%)	3 (13%)

The data in the table are from Figure 4. ΔCt + or - refers to samples with ΔCt values less than or greater than the ΔCt value on the left, respectively.

(samples that have low ΔCt values yet do not achieve minimum array % present calls) and false negatives (samples that have high ΔCt values yet do achieve minimum % present calls) generated at different ΔCt values are shown in **Table 2**.

For example, consider the data at a ΔCt value of 6 or less with a %P threshold of 25% (**Table 2**). The sensitivity rate, defined as the percentage of those arrays with ≥25% P that pass the ΔCt cut-off (true positives) is 88%. The specificity rate — the percentage of those arrays with ≤25% P that fail the ΔCt cut-off (true negatives) — is 85%. The corresponding false negative and false positive rates for this threshold value are 12 and 15% respectively. In this case, 12% of the samples that yielded good array results would not pass the ΔCt cut-off (false negatives) and 15% of the samples that had less than 25% P would have passed the ΔCt cut-off (false positives).

Utilizing the same ΔCt value for the 30% present call, data yields a sensitivity of 92% and a specificity of 65%. The corresponding false negative and false positive rates for this threshold value are 8 and 35%, respectively. This data set illustrates the trade-off between running samples giving poor array results versus losing samples that potentially could generate valuable array results depends on the number of samples available to the researcher and the resources available for completing the study. Note that this data set encompassed multiple sample types, storage times, and RNA purification as well as two different GeneChip array types. This analysis describes the overall performance of the assay and is not meant as a guideline for setting ΔCt cut-off values for other studies.

For a description of how to set an appropriate ΔCt value for a pilot study performed within a single tissue and array type see the WT-Ovation FFPE System Validation Guidelines and RNA Sample Quality Assessment Technical Report.

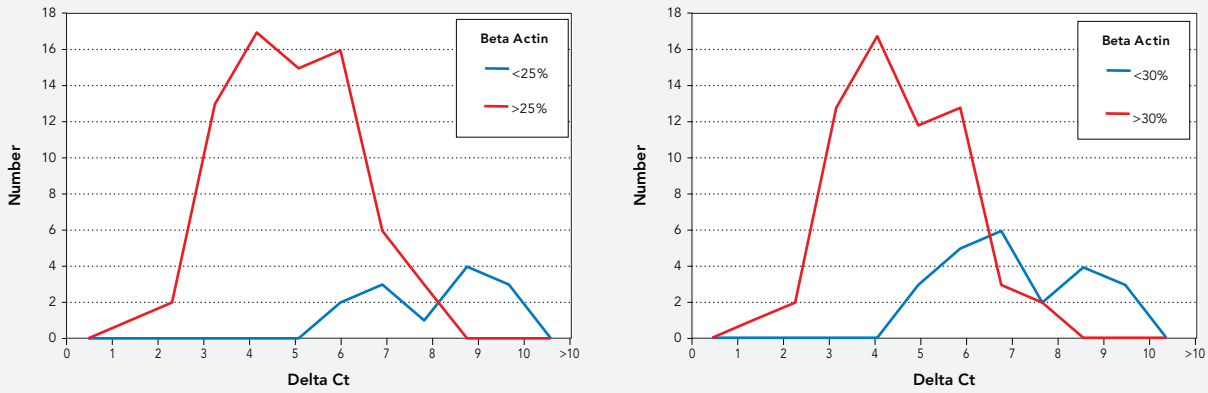
Pilot Study Recommendations

It is recommended that data be generated for a subset of samples representing the RNA quality range expected in the larger experimental sample set. This assay has a higher predictive capability when samples from a single tissue type are used (see the WT-Ovation FFPE System Validation Guidelines and RNA Sample Quality Assessment Technical Report for an example). The qPCR and %P data generated from the pilot study can be used to select a ΔCt cut-off appropriate for that specific sample set, as demonstrated above. In some cases this quality assessment assay may reveal that further testing of the sample set prior to hybridization is not necessary, if the vast majority of samples tested consistently show low ΔCt and high %P values. It is possible that there may be data sets for which the ΔCts for the amplicons we have chosen do not correlate with array performance. In these cases a new custom qPCR test may need to be developed and employed.

Materials and Methods

Materials: FirstChoice™ Total RNAs from human adipose, brain, breast, colon, heart, kidney, liver, lung, prostate, skeletal muscle, breast tumor, and colon normal adjacent tissue were purchased from Ambion. Universal Human Reference RNA (UHR, Stratagene, cat. #740000) and HeLa total RNA (Ambion Cat.# 7852) were used as controls. FFPE RNA samples were kindly provided by collaborators. Primers were purchased from Integrated DNA Technologies (Coralville, IA), **Table 1** lists primer sequences and amplicon lengths. iQ SYBR Green Supermix (BIORAD, Hercules, CA), and 50X TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA) (USB, Cleveland, OH) were also used.

FIGURE 4. **Histograms showing the number of arrays with %P values above and below 25% (A) or 30% (B) versus the beta actin ΔCt value.**



The red lines denote the number of arrays with % P values exceeding the threshold values: 25% (A) and 30% (B). The blue lines denote the number of arrays with %P values below the threshold values. The ΔCt values on the x axis represent bins where the number shown is the maximum value for a given bin.

cDNA Synthesis and qPCR: RNA from 12 different human tissues was converted to cDNA using the first step of the WT-Ovation FFPE System following the user guide. Note: the WT-Ovation FFPE System has been replaced by the Ovation FFPE WTA System (Part No. 3403). The total RNA input was 50 ng for FFPE samples and 10 ng for control RNA samples (HeLa or UHR). Both FFPE and control RNAs were converted to cDNA using the first two steps of the WT-Ovation FFPE System, and the cDNA purification step was not perfomed. cDNA products were diluted in 1X TE prior to qPCR. SYBR Green qPCR was performed on the Applied Biosystems 7500 Fast Real-Time PCR System with a 60 °C extension temperature without the ROX reference dye. Fast protocol's first heat denaturation step was modified to conform to the SYBR mix manufacturer's specifications. 2 µl of diluted cDNA were analyzed by qPCR with 500 nM each of forward and reverse primers in a 20-µL final volume. A standard threshold of 35,000 was used to analyze assays. The delta Cts between FFPE and control samples were calculated and plotted versus the %P attained from the microarray analysis.

Conclusions

The NuGEN WT-Ovation FFPE System enables gene expression analysis of FFPE-derived RNA. Since traditional RNA quality assessment approaches are uninformative for the highly degraded FFPE RNA, a novel RNA sample quality assessment assay has been developed that correlates qPCR results to the quality of microarray results generated with these samples. This in-process quality assessment assay, in conjunction with a pilot study approach described in the FFPE System Validation Guidelines Technical Report, can predict the performance of a given set of FFPE samples, allowing researchers to utilize these valuable samples to their fullest potential.

NuGEN Technologies, Inc.

Headquarters USA
201 Industrial Road, Suite 310
San Carlos, CA 94070 USA
Toll Free Tel: 888.654.6544
Toll Free Fax: 888.296.6544
custserv@nugen.com
techserv@nugen.com

Europe
P.O. Box 109
9350 AC Leek
The Netherlands
Tel: +31-13-5780215
Fax: +31-13-5780216
europe@nugen.com

Worldwide
For our international distributors contact information, visit our website

www.nugen.com

M01057 v2.1