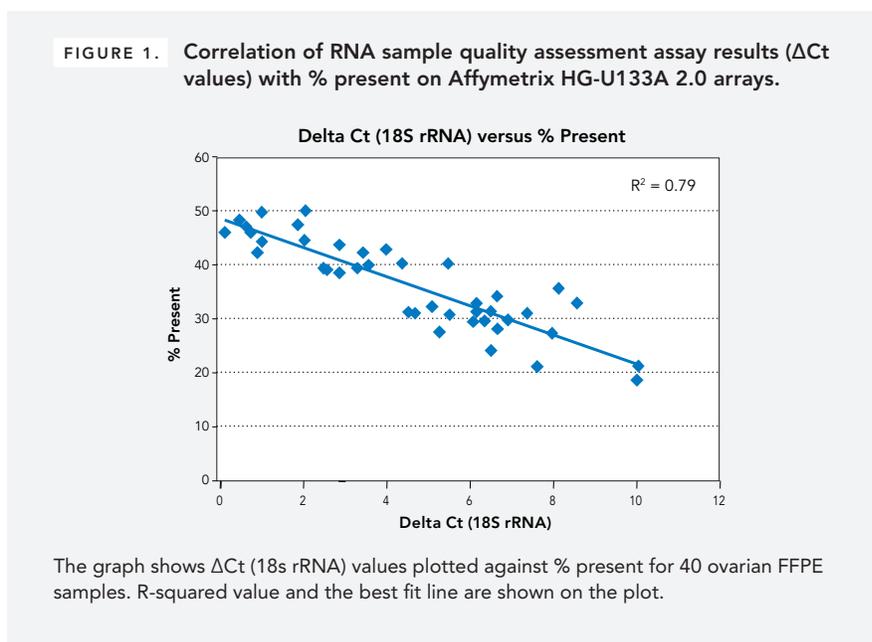# ■ WT-Ovation® FFPE System Validation Guidelines and RNA Sample Quality Assessment Tool Pilot Study

Introduction

Due to the highly variable quality of RNA isolated from FFPE sample sources, it is critical to assess RNA quality within a particular sample set before performing a larger scale gene expression study. This Technical Report describes NuGEN's RNA Sample Quality Assessment Tool and a pilot study that can be incorporated into a gene expression analysis workflow using FFPE-derived RNA samples. This tool is described in more detail with instructions and recommendations in the RNA Sample Quality Assessment Test for the WT-Ovation FFPE System Technical Report. This validation guideline document is meant to be used in conjunction with the technical report mentioned above and describes in general terms the recommended validation process and the appropriate application of the quality assessment tool.

The validation guideline involves performing a small pilot study, which provides significant information on:

• The general quality of data achievable with a particular FFPE sample set

• The selection of samples that will be required to generate high quality data from a large FFPE sample set

• The level of variability in performance within a sample set in order to determine an appropriate quality assessment workflow

FIGURE 1. Correlation of RNA sample quality assessment assay results (ΔCt values) with % present on Affymetrix HG-U133A 2.0 arrays.

The graph shows ΔCt (18s rRNA) values plotted against % present for 40 ovarian FFPE samples. R-squared value and the best fit line are shown on the plot.

This information will allow researchers to establish an effective, cost-efficient workflow and set metrics which yield the desired results. Of course the performance of a set of FFPE samples cannot be guaranteed; however, using these validation guidelines and the pilot study described here will help predict the quality of the data achievable with any given set of FFPE-derived RNA samples.

RNA Sample Quality Assessment Application

Depending on the researchers' experimental design and workflow expectations, validation studies and sample quality assessment may be done in a variety of ways. It is recommended that for every new study in which a significant number of samples will be used, the pilot study described here be performed on a subset of the samples. This subset of samples should be selected to reflect the distribution of the entire sample population for known variables that contribute to RNA quality, such as age. It is preferable to run at least 10% of the total number of samples anticipated for the final study. It is highly recommended that sample sets be separated based on the source tissue and the type of arrays planned for use in the study.

Since this approach helps determine the extent of performance variability within a larger sample set, the results can assist in determining whether an in-process quality assessment test for all samples will be required.

Assessment Study Design

The RNA sample assessment tool consists of defined qPCR assays for which the results correlate to array results. For instructions and details of this assay refer to the RNA Sample Quality Assessment Test for the WT-Ovation

ΔCt (18S rRNA)	25% Present				≥30% Present			
	True Pos. +QC +Arrays	False Pos. +QC −Arrays	False Neg. −QC +Arrays	True Neg. −QC −Arrays	True Pos. +QC +Arrays	False Pos. +QC −Arrays	False Neg. −QC +Arrays	True Neg. −QC −Arrays
0.9	5	0	31	4	5	0	25	10
1.9	8	0	28	4	8	0	22	10
2.9	14	0	22	4	14	0	16	10
3.9	17	0	19	4	17	0	13	10
4.9	20	0	16	4	20	0	10	10
5.9	24	0	12	4	23	1	7	9
6.9	32	1	4	3	27	6	3	4
7.9	33	2	3	2	28	7	2	3
8.9	36	2	0	2	30	8	0	2
>9.0	36	4	0	0	30	10	0	0

The table shows the number of samples that fall above and below a defined array performance criteria (25% Present left, and 30% Present right). The highlighted row in the table represent the application of a cut-off value ΔCt of 7.

FFPE System Technical Report, but in brief, here is a summary.

A control RNA sample such as HeLa or UHR RNA along with the selected subset of FFPE sample RNAs are processed through the standard WT-Ovation FFPE System protocol (note: the Ovation FFPE WTA System, Part No. 3403, has replaced the WT-Ovation FFPE System). After the second strand cDNA synthesis a small aliquot of the mixture (2 μL) is removed from each of the reactions prior to the purification and amplification step. The samples are then processed as described in the user guide through to through to amplification. This second strand cDNA aliquot from all samples is then used at a consistent dilution in two qPCR assays with amplicons corresponding to two abundant, multi-copy transcripts (18s rRNA, beta-actin). All amplified FFPE samples are then processed using the Encore® Biotin Module V2 (Part No. 4200), and analyzed on Affymetrix 3' GeneChip expression arrays. Basic array metrics including %P are determined.

The difference between Cts in the control RNA and FFPE RNA (termed $\Delta Ct = Ct_{FFPE} - Ct_{HeLa}$) can be determined and correlated to the sensitivity of the sample on arrays (%P). A graph of such an example is shown in **Figure 1**. For a set of 40 ovarian FFPE samples ranging in age from 2–15 years, the ΔCt values of the 18s rRNA assay correlates with % present on Affymetrix HG-U133A 2.0 arrays. By generating this plot with pilot study samples a researcher can determine whether the qPCR reaction should be performed on each sample used in the full study, whether it appears that all samples will yield desirable results and therefore quality assessment of the remainder of the samples is not required, or whether the RNAs collected from the samples are generally poor and will not yield sufficient array results. In cases where it is desirable to run the quality assessment assay on the larger sample set, an appropriate ΔCt cut-off value will need to be selected.

Assessment Data Analysis: Establishing Quality Cut-off

In order to establish an appropriate cut-off value for the quality assessment assay, it is important for a researcher to quantitatively assess the effects of any particular cut-off value in terms of the number of arrays run that yield successful array results (True

Positives), the number of arrays run that will yield poor array results (False Positives), the number of samples eliminated that would have yielded poor array results (true negatives) and the number of samples eliminated that would have yielded good array results (false negatives). The quantitative analysis of the ovarian cancer samples is shown in **Table 1**.

This quantitative analysis can now be further analyzed to select an appropriate cut-off value. The specific value chosen is based on the needs of the researcher in terms of tolerance to the number of arrays run that yield poor results, and the total number of arrays run yielding good results. Each researcher will have their own requirements regarding these factors.

In this case, a ΔCt of 7 was selected (highlighted row in table) resulting in a balance of the number of arrays passing the QC and giving good array data (defined either as 25% P on left, 30% P on right) versus the number of arrays passing QC and failing minimum array metrics. At this cut-off value and a minimum of 25% P, 32/40 (80%) of samples will pass the QC and give good array data, 1/40 (2.5%) of samples would pass the QC test but fail array performance metrics, 4/40 (10%) of the samples would fail the QC test, but pass array performance criteria, and 3/40 (7.5%) of the samples would fail the QC test and fail array performance criteria. Assuming that the 40 FFPE samples represent the overall quality and performance of the entire FFPE sample set to be used in the final study, 82% of the samples will be expected to pass QC with less than 3% of all arrays run generating data below the 25% P threshold, and 18% of the samples available for the study will be eliminated by the QC test.

'Acceptable' array performance needs to be defined by the researcher in order to obtain the highest quality results, while considering the quality and quantity of samples available for the study. For example, if the minimum desired array performance is 30% present, the analysis can be done at that threshold (**Table 1**, right side). If available samples are not limiting and they are of generally high quality, then a more stringent QC cut-off can be chosen to further reduce the number of samples going onto arrays thereby minimizing time and cost. In this example, a QC cut-off at the more stringent ΔCt value of 6 would reduce the number of arrays that would yield %P below 30%. On the other hand, if samples are limiting and an investigator desires to get the most array data possible, then a more relaxed QC cut-off can and %P threshold be selected such that more of the samples will yield acceptable array data.

Developing a Custom QC Test

The QC test described in this report and in the RNA Sample Quality Assessment Test for the WT-Ovation FFPE System Technical Report was designed to be generally applicable to a wide variety of samples and experiments. The performance metric chosen for array performance was simply a surrogate for overall sensitivity on the array (%P). The transcripts chosen for the quality assessment assay were selected to assess the degree of degradation in RNA samples because they are stably expressed in a wide variety of tissues and are high expressers from multi-copy genes (see the RNA Sample Quality Assessment Test for the WT-Ovation FFPE System Technical Report). If a researcher intends to run a large study to validate particular signatures of interest, it may be beneficial to generate a custom qPCR array to be run in addition to or in place of this test. The paradigm described here can be applied to particular transcripts of interest and alternate performance metrics on arrays.

In generating custom QC assays, the recommendation is to use a qPCR-based approach similar to that described here. When validating existing signatures, it may be beneficial to develop the qPCR QC assay against known relevant transcripts to the signature. It is recommended that several transcripts of interest are chosen, generating multiple amplicons (primer and probe sets) against each transcript, and testing the correlation of the qPCR results against a desired array performance metric. When testing novel amplicons, it is important to perform enough replication in order to assure correlation between the qPCR Ct data and results on arrays. Be aware that not all transcripts, or amplicons against those transcripts will correlate to array performance. In this study, high abundance, multi-copy transcripts were chosen as they are the most robust indicators of RNA quality. Low abundance transcripts may yield less reproducible results and may not be indicative of overall RNA quality (see the RNA Sample Quality Assessment Test for the WT-Ovation FFPE System Technical Report for more details).

Array performance can be defined in any way deemed appropriate for a particular study. In other words, if a limited set of transcripts are used in a signature, then the appropriate array performance metric may be the percentage of transcripts in the signature detected by the array. In this case, the pilot study would generate the plot shown in **Figure 1** with ΔCt for transcript of interest (X-axis) versus % of transcripts in signature detected (Y-axis). Then the same analysis shown in **Table 1**, above, can be applied to the resulting data set to select the appropriate transcripts and cut-off values.

Conclusions

Recommendations are described for applying an RNA sample quality assessment tool to an experimental design using FFPE samples. This approach correlates qPCR assay results with general performance on arrays (% present). Also included is the description of a pilot study to

assess the general performance of a sample set, allowing researchers to decide whether an in-line QC step is appropriate for their samples and to set the appropriate QC cut-off value if desired. Alternatively, investigators can generate their own custom QC assays, using the same model described here. The generation of this pilot study data, and the thoughtful application of appropriate QC test and cut-off values will assure the highest quality results possible from FFPE gene expression experiments and allow the full utilization of these valuable samples.

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

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