TECHNICAL REPORT

User QC Guidelines: qPCR Assessment of Effective RNA Quantity

Introduction

NuGEN's Ovation whole transcriptome amplification products enable gene expression analysis even when working with limiting or degraded RNA samples. RNA quantity, purity, and integrity are important factors in the success of any gene expression analysis and can affect amplification. Assessing these traits in all RNA samples is often laborious if not impossible, especially in the case of very limited amounts of RNA. NuGEN has developed a series of real-time quantitative PCR (qPCR) assays for assessing the effective amount of RNA in a sample available for amplification using the WT-Ovation Pico RNA Amplification System (replaced by Ovation Pico WTA System Part No. 3302). The qPCR results will allow users to determine if they have enough material to continue their experiment.

The Ovation Pico WTA System V2 is designed for the whole transcriptome amplification of 500 pg – 50 ng of total RNA, yielding 6–10 µg of cDNA ready for qPCR analysis and storage in 5 hours. The resulting cDNA can be fragmented and labeled using the Encore Biotin Module for analysis on GeneChip® arrays.

In this report, an assessment guideline is described, including instructions for cDNA synthesis, sequence information for primers, procedures for running SYBR Green qPCR assays, and a reference data set that allows estimation of the effective amount of RNA in a sample. This approach provides an additional QC check for samples that may be degraded and whose quantity is difficult to determine. Also included are several example cases to demonstrate the application of this approach.

FIGURE 1. **WT-Ovation Pico processing workflow.**

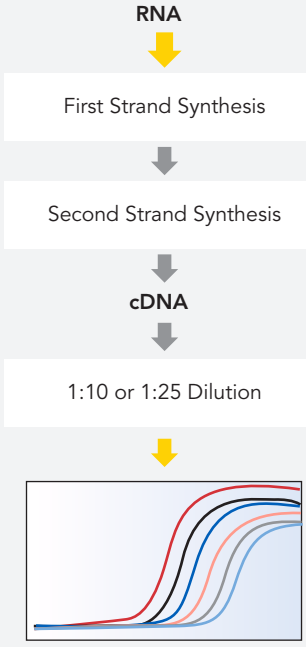

TABLE 1. **RNA tissue sources used in this study.**

Tissue RNA	Ambion Catalog Number
Adipose	7956
Brain	7962
Breast	7952
Colon	7986
Heart	7966
Kidney	7976
Liver	7960
Lung	7968
Prostate	7988
Skeletal Muscle	7982

The primary goal of these guidelines is to save time and cost associated with expensive analysis procedures. This approach can be used to assess whether an RNA sample has sufficient starting material to proceed to array analysis, or to assess if the sample is a good candidate for in-depth and large scale qPCR analysis. Evaluation of a subset of samples representative of a sample collection is recommended. However if the goal is to amplify a set of RNAs and use the amplified cDNA for a very limited number of qPCR assays, then this tool may not save considerable time and effort.

Development of the qPCR RNA Assessment Guidelines

Ten different, high-quality human tissue RNA samples (**Table 1**) were screened using a set of qPCR assays to gene transcripts that are expressed in the majority of human RNA samples (**Table 2**). The transcripts were chosen from the set of Normalization Control Probe Set genes present on Affymetrix GeneChip® Human Genome U133 arrays (described in Affymetrix's Gene Expression Monitoring Technical Note 2). A few longer transcripts were included that allowed the design of

TABLE 2. **Detection limits of qPCR assays in different tissues.**

		Detectable Input RNA Amount (ng)									
qPCR Assay	cDNA Dilution	Adipose	Brain	Breast	Colon	Heart	Kidney	Liver	Lung	Prostate	Skeletal Muscle
RPL35-1	1/25	0.05	0.2	0.2	0.2	0.2	0.05	0.05	0.05	0.2	0.01
ARF3-2	1/10	0.2	0.01	0.05	0.05	0.05	0.2	0.5	0.05	0.2	0.2
NARS-2	1/10	0.2	0.5	0.5	0.2	0.2	0.2	0.2	0.2	0.5	0.2
NARS-3	1/10	0.05	0.05	0.05	0.2	0.5	0.05	0.05	0.05	0.05	0.05
HSP90B-2	1/10	0.2	0.01	0.05	5	0.01	0.05	0.05	0.2	0.2	0.2
HSP90B-4	1/25	0.05	0.2	0.05	0.05	0.05	0.2	0.2	0.05	0.2	0.05
EIF4G2-3	1/25	0.5	0.2	5	0.2	0.2	0.05	0.5	0.5	0.2	0.2
EIF4G2-5	1/25	5	0.5	0.2	5	0.2	0.5	5	0.2	0.5	0.2
NONO-2	1/10	0.2	0.5	0.2	5	0.5	0.2	0.5	0.2	0.2	0.5
NONO-3	1/10	0.2	0.2	0.05	0.2	0.2	0.5	0.2	0.05	0.2	0.5
GDIS-4	1/10	5	0.5	0.5	0.2	0.2	0.2	0.2	0.2	0.5	0.5
DAD1	1/10	5	0.5	5	5	0.5	0.5	0.2	0.2	5	5
MYST2-1	1/10	5	5	5	5	0.5	50	50	5	5	5

RNAs from different tissues at varying input levels were converted to cDNA using the WT-Ovation Pico RNA Amplification System (replaced by the Ovation Pico WTA System V2, Part No. 3302). The unamplified cDNA was then diluted and assayed by qPCR. The lowest level of input at which the gene was detected is shown.

multiple qPCR assays spanning the lengths of the transcripts (**Table 3**). RNA was converted to cDNA using the first two steps of the WT-Ovation Pico RNA Amplification System protocol without the purification step (note: the Ovation Pico WTA System V2, Part No. 3302, has replaced the WT-Ovation Pico RNA Amplification System). A wide range of total RNA input quantities were tested: 0, 0.001, 0.01, 0.05, 0.2, 0.5, 5, and 50 nanograms. These cDNA samples were then analyzed using the 13 qPCR assays described in **Table 3**. The analyzed transcripts displayed different patterns of expression in the different tissues tested. **Table 2** lists the lowest RNA input that allowed for detection of the transcript in that RNA. This table can be used to estimate the amount of RNA present in the experimental sample after cDNA synthesis.

Instructions for qPCR

The general approach of this tool is to use the cDNA prior to amplification to estimate the amount of amplifiable RNA present in a sample using qPCR. The steps involved in using this approach to assess the sample are graphically represented in **Figure 1** and listed below.

1. Determine which type of RNA in **Table 1** is most similar to the experimental sample.
2. Choose at least three qPCR assays from **Table 2** that show detection at the lowest amounts of input RNA.
3. Order primers from **Table 3** for the qPCR assays chosen.
4. Choose one or more RNA samples that best represent the general quality of the sample set and that have sufficient quantity for multiple cDNA reactions.
5. Process these samples following the Ovation Pico WTA System V2 (this kit has replaced the WT-Ovation Pico RNA Amplification System) protocol through primer annealing, first-

TABLE 3. **Description of the Genes, qPCR assay positions, and primer sequences.**

Gene Name	Description	Accession #	Assay Name	Gene Length	Distance from 3' End	Affy ID#	Amplicon Length	Forward Primer	Reverse Primer
RPL35	Ribosomal protein L35	NM_007209.1	RPL35-1	475	282	200046_at	107 nt	AACAGCTGGA CGACCTGAAG	AATGGATTTC CGGACGACT
HSP90B	Chaperone protein HSP90 beta	AF275719	HSP90B-2	2017	1200	200064_at	130 nt	AGCTGTGAT GAGTTGATA CCAGAG	TGTTTTTGCGA ATGACTTTCA
HSP90B	Chaperone protein HSP90 beta	AF275719	HSP90B-4	2017	261	200064_at	88 nt	AACCGCATCT ATCGCATGA	CATCAGGAAC TGCAGCATTG
EIF4G2	Eukaryotic translation initiation factor 4 gamma, 2	NM_001418.1	EIF4G2-3	3820	2121	200004_at	113 nt	GGACAGC TTAATGCAG ATGAGA	TGTGCACT AGGAGGAA TCATAGTTA
EIF4G2	Eukaryotic translation initiation factor 4 gamma, 2	NM_001418.1	EIF4G2-5	3820	873	200004_at	96 nt	GGCAAGGCT TTGTTCCAG	TTGGCTGGTT CTTTAGTCAGC
ARF3	ADP-ribosylation factor 3	NM_001659	ARF3-2	3595	2974	200011_s_at	98 nt	CGGGAAGAG CTGATGAGAAT	GCGTTCATAGC ATTAGGCAGA
NARS	asparaginyl-tRNA synthetase	NM_004539.2	NARS-2	2714	2092	200027_at	69 nt	AATGCTA AATCTTACCC CAAAGG	TCCCAGAA GTCACAAC TCAGC
NARS	Asparaginyl-tRNA synthetase	NM_004539.2	NARS-3	2714	1745	200027_at	128 nt	CCTCTCAGT TGTACTTGG AGACC	CTCAGCTTCC ACGTGAGTGT
GDI2	GDP dissociation inhibitor 2	NM_001494.2	GDIS-4	2274	1011	200009_at	75 nt	GAGCTCTT GGAACCAA TTGAA	TTCTGTTCCCA AGTCTTTTGG
MYST2	MYST histone acetyltransferase 2	NM_007067.1	MYST2-1	3504	3352	200049_at	73 nt	GACGATCTGC TCGAGTCACC	TTTCGAA CAGGACT GGAATCTT
DAD1	Defender against cell death 1	NM_001344.1	DAD1-1	699	513	200088_x_at	77 nt	AGCGTCTGAA GTTGCTGGAC	GAGGAGA CAGTAACC GAACTGC
NONO	Non-POU domain containing, octamer-binding	NM_007363.2	NONO-2	2690	1291	200057_s_at	110 nt	GGATGGAACT TTGGGATTGA	CTGCACGGT TGAATGCAG
NONO	Non-POU domain containing, octamer-binding	NM_007363.2	NONO-3	2690	706	200057_s_at	77 nt	GCTGGAGTGT AGTGGCATGA	TCAAGATCAAC CTGGACAAGC

and second-strand cDNA synthesis, to just prior to the bead purification.

6. Remove 2 µL from the 20-µl reaction. Ensure that the cDNA aliquot for this test is removed from the sample prior to the purification step. Proceed with the purification and amplification exactly as listed in the user guide. Store amplified cDNA at –20°C. The loss of the small aliquot of cDNA has a minimal effect on the final yields of amplification.
7. Based on the input levels for qPCR detection of this RNA type and assay listed in **Table 2**, determine the appropriate dilution level. For a 1:10 dilution, add 18 µL of TE to the 2 µL of cDNA. If necessary, make a 1:25 dilution, by taking 5 µL of the 1:10 dilution and adding 7.5 µL of TE.
8. Perform SYBR Green qPCR assays using the primers for the genes selected from **Table 2**.
9. Compare the Ct data obtained to the levels of detection listed in **Table 2** and determine the minimum amount of amplifiable RNA present in the sample.
10. Use these results to determine if the samples are likely to be good candidates for amplification with the Ovation Pico WTA System V2.

Examples

Example 1. For colon tumor tissue samples, use the assays that detect the colon RNA over a range of concentrations, such as ARF3-2, NARS-2, and HSP90B-2. cDNA is diluted 1/10 into 1X TE for all three QPCR assays. Results show that ARF3-2 and NARS-2 are detected but HSP90B-2 is not. From **Table 2**, ARF3-2 is detected at 0.05 ng, NARS-2 is detected at 0.2 ng, and HSP90B-2 is detected at 5 ng. Because the sample does not detect HSP90B-2, it means that there is less than 5 ng in the sample.

NARS-2 is detected, suggesting that the sample has 0.2 ng or more. Thus an estimate can be made that the amount of RNA in the sample is between 0.2 and 5 ng and is probably going to amplify well but may have a slightly lower yield than expected if the amount of RNA is below 0.5 ng.

Example 2. For a lung small cell carcinoma sample, use the assays that detect lung RNA over a range of concentrations, such as ARF3-2, NARS-2, and EIF4G2-3. The cDNA is diluted 1/10 into 1X TE for ARF3-2 and NARS-2 and a further 1/2.5 (1/25 final) for EIF4G2-3. The results show that only ARF3-2 is detected. From **Table 2**, ARF3-2 is detected at 0.05 ng, NARS-2 is detected at 0.2 ng, and EIF4G2-3 is detected at 0.5 ng. Because the sample has no detectable levels of EIF4G2-3 and NARS-2, an estimate can be made that the RNA amount is less than 0.2 ng and probably will not amplify well.

Example 3. For hippocampus samples, use the assays that detect brain RNA over a range of concentrations, such as NARS-3, EIF4G2-3 and EIF4G2-5. The cDNA is diluted 1/10 into 1X TE for NARS-3 and a further 1/2.5 (1/25 final) for EIF4G2-3 and EIF4G2-5. The results show that all three are detected. From **Table 2**, NARS-3 is detected at 0.05 ng, EIF4G2-3 is detected at 0.2 ng, and EIF4G2-5 is detected at 0.5 ng. One can estimate that the amount of RNA is greater than 0.5 ng and will amplify well.

Materials and Methods

Materials

First Choice™ Total RNA from human adipose, brain, breast, colon, heart, kidney, liver, lung, prostate, and skeletal muscle were purchased from Ambion, Inc (Austin TX), see **Table 1**. Universal Human Reference RNA was purchased from Stratagene (La Jolla, CA). Primers were purchased from Integrated DNA Technologies (Coralville, IA). iQ™ SYBR® Green Supermix was purchased from BIO-RAD (Hercules, CA). 50X TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) was purchased from USB Corporation (Cleveland, OH).

Primer Selection

Primers were designed using the ProbeFinder software available on the Roche Applied Science website (http://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp) for both SYBR Green and Taqman qPCR assays. Assays were chosen that span exon junctions with a few exceptions. Multiple assays were designed to ensure coverage of the transcript sequences (**Table 3**).

To test the efficiencies of the different primer and probe sets designed above, cDNA from Univeral Human Reference RNA was prepared. Of this, 5 µg of RNA was reverse transcribed using the RETROscript® Kit from Ambion (Austin, TX) with the random primers following manufacturer's instructions. The cDNA was pooled from four reverse transcription reactions and diluted serially.

First, the primers were tested using SYBR Green with and without template on the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, CA) using a modified protocol without the ROX reference dye to ensure that no product was made by the primer pairs. The default fast protocol of the Applied Biosystems 7500 Fast Real-Time PCR System was modified to extend the first heat denaturation step from the original 30 seconds to 3 minutes. Next, the Ct values calculated by the 7500 Fast System Sequence Detection Software 1.3 were plotted versus the Log_2 of the input cDNA amounts. Assays that could detect the transcripts in 30 pg of cDNA and had slopes with an absolute value of 1±0.1 were chosen (**Table 3**).

cDNA Synthesis and QPCR

RNA from 10 different human tissues was converted to cDNA using the first two steps of the WT-Ovation Pico RNA Amplification System, following the manufacturer's instructions (this kit has been replaced by Ovation Pico WTA System V2, Part No. 3302). The cDNA purification step was not performed.

The total RNA input into amplification was tested across a wide range from 0 to 50 ng. cDNA products described in the amplification section above were diluted into 1X TE prior to qPCR. qPCR was performed as described in the Primer Selection section, above. Of this cDNA product, 2 µL were analyzed by qPCR with 500 nM each of forward and reverse primers in a 20-µL final volume. The assays were analyzed using a standard threshold of 45,000 and a Ct value <35 to classify detected transcripts.

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