

USER QC GUIDELINES: QPCR ASSESSMENT OF EFFECTIVE RNA QUANTITY FOR USE WITH THE WT-OVATION™ PICO SYSTEM

INTRODUCTION

NuGEN's Ovation™ whole transcriptome amplification products continue to enable gene expression analysis even when working with limiting or degraded RNA samples. The 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. The qPCR results will allow users to determine if they have enough material to continue their experiment.

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

In this report, we describe this assessment guideline, 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 those samples that may be degraded and whose quantity is difficult to determine. We also include several example cases to demonstrate the application of this approach.

The primary goal of these guidelines is to save you time and costs associated with expensive analysis procedures. This approach can be to assess if 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. We recommend evaluating a subset of samples that are representative of your sample collection. However if your goals are 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 you considerable time and effort.

DEVELOPMENT OF THE QPCR RNA ASSESSMENT GUIDELINES

Initially, we screened ten different, high-quality Human tissue RNA samples (Table 1) 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 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 without the purification step. 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 thirteen qPCR assays described in Table 3. The transcripts analyzed have different patterns of expression in the different tissues tested. In Table 2, we list the lowest RNA input mass into the cDNA synthesis reaction 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 with the WT-Ovation™ Pico RNA Amplification System.

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 your sample are graphically represented in Figure 1, and listed below.

1. Determine which type of RNA in Table 1 is most similar to your experimental sample.
2. Choose at least three qPCR assays from Table 2 that show detection at the lowest amounts of input RNA, of the type most similar to your experimental samples.
3. Order the primers for the qPCR assays you have chosen from Table 3.

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4. Choose one or more RNA sample(s) that best represents the general quality of your sample set and that you believe have sufficient quantity for multiple cDNA reactions.
5. Process these samples following the WT-Ovation™ Pico System protocol through primer annealing, first, and second strand cDNA synthesis, to just prior to the bead purification.
6. Remove 2 µl from the 20-µl reaction. Make sure 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 you obtain

to the levels of detection listed in Table 2 and determine the minimum amount of amplifiable RNA present in your sample.

10. Use these results to determine if your samples as a group are likely to be good candidates for amplification with the WT-Ovation™ Pico System.

EXAMPLES

Example 1. If you have a colon tumor tissue sample, you might 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. Your 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 which suggests that the sample has 0.2 ng or more. You can estimate 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. If you have a lung small cell carcinoma sample, you might 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, you can estimate that the RNA amount is less than 0.2 ng and probably will not amplify well.

Example 3. If you have a hippocampus sample, you would 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.

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

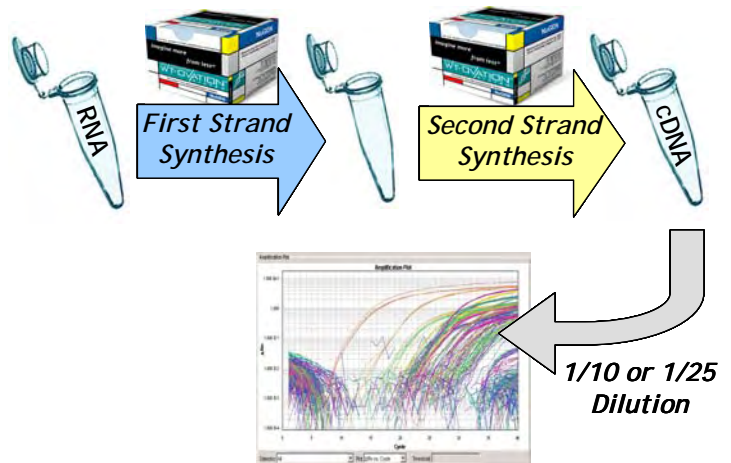


Figure 1. Sample processing flowchart

Table 2. Detection limits of qPCR assays in different tissues. RNAs from different tissues at varying input levels were converted to cDNA using the WT-Ovation™ Pico RNA Amplification System. The unamplified cDNA was then diluted and assayed by qPCR. The lowest level of input at which the gene was detected is shown.

qPCR Assay	cDNA Dilution	Detectable Input RNA Amount (ng)									
		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

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	AACAGCTGGACG ACCTGAAG	AATGGATTCCG GACGACT
HSP90B	chaperone protein HSP90 beta	AF275719	HSP90B-2	2017	1200	200064_at	130 nt	AGCTGTGATGAG TTGATACCAGAG	TGTTTTTGCGAAT GACTTTCA
HSP90B	chaperone protein HSP90 beta	AF275719	HSP90B-4	2017	261	200064_at	88 nt	AACCGCATCTAT CGCATGA	CATCAGGAAGT CAGCATTG
EIF4G2	eukaryotic translation initiation factor 4 gamma, 2	NM_001418.1	EIF4G2-3	3820	2121	200004_at	113 nt	GGACAGCTTAAT GCAGATGAGA	TGTGCACTAGGA GGAATCATAGTT A
EIF4G2	eukaryotic translation initiation factor 4 gamma, 2	NM_001418.1	EIF4G2-5	3820	873	200004_at	96 nt	GGCAAGGCTTTG TTCCAG	TTGGCTGGTTCT TTAGTCAGC
ARF3	ADP-ribosylation factor 3	NM_001659	ARF3-2	3595	2974	200011_s_at	98 nt	CGGGAAGAGCTG ATGAGAAT	GCGTTCATAGCA TTAGGCAGA
NARS	asparaginyl-tRNA synthetase	NM_004539.2	NARS-2	2714	2092	200027_at	69 nt	AATGCTAAATCTT ACCCCAAAGG	TCCCAGAAGTCA CAACTCAGC
NARS	asparaginyl-tRNA synthetase	NM_004539.2	NARS-3	2714	1745	200027_at	128 nt	CCTCTCAGTTGT ACTTGGAGACC	CTCAGCTCCAC GTGAGTGT
GD12	GDP dissociation inhibitor 2	NM_001494.2	GDIS-4	2274	1011	200009_at	75 nt	GAGCTCTGGAA CCAATTGAA	TTCTGTTCCCAA GTCTTTTGG
MYST2	MYST histone acetyltransferase 2	NM_007067.1	MYST2-1	3504	3352	200049_at	73 nt	GACGATCTGCTC GAGTCACC	TTTCGAACAGGA CTGGAATCTT
DAD1	defender against cell death 1	NM_001344.1	DAD1-1	699	513	200088_x_at	77 nt	AGCGTCTGAAGT TGCTGGAC	GAGGAGACAGTA ACCGAAGTGC
NONO	non-POU domain containing, octamer-binding	NM_007363.2	NONO-2	2690	1291	200057_s_at	110 nt	GGATGGAAGTTT GGGATTGA	CTGCACGGTTGA ATGCAG
NONO	non-POU domain containing, octamer-binding	NM_007363.2	NONO-3	2690	706	200057_s_at	77 nt	GCTGGAGTGTAG TGGCATGA	TCAAGATCAACC TGGACAAGC

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

cDNA SYNTHESIS AND QPCR

RNA from ten different human tissues was converted to cDNA using the first two steps of the WT-Ovation™ Pico RNA Amplification System following manufacturer's instructions. 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 1 X TE prior to qPCR. qPCR was performed as described in the PRIMER SELECTION section. Two 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 of less than 35 was used to classify detected transcripts.

The NuGEN technical services team is available to review and assist in the utilization of this tool. Contact us at techserv@nugeninc.com, in the US and europa@nugeninc.com, in Europe. For our international distributors' technical services contacts, visit our website.

Systems Specifications

Cat No.: 3300-12, 12 reactions

Input: 500 pg - 50 ng total RNA

Yield: 6 -10 µg single-stranded cDNA



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