

REPRODUCIBILITY OF MICROARRAY AND QUANTITATIVE PCR RESULTS FROM NANOGRAM SAMPLES OF RNA AMPLIFIED USING THE OVATION™ AMINOALLYL SYSTEM

BACKGROUND

Messenger RNA from nanogram amounts of total cellular RNA must be amplified before differential gene expression analysis can be performed on microarrays. The amplification process must be exceptionally reproducible; otherwise signal intensity differences observed after microarray hybridization may be due to amplification-to-amplification variation, rather than from real differences in gene expression.

The Ovation™ Aminoallyl System uses the Ribo-SPIA™ process, an extraordinarily fast and simple linear amplification method for generating micrograms of cDNA from a few nanograms of total RNA. (For more information, visit the NuGEN website at www.nugeninc.com). This study examines the reproducibility of the Ribo-SPIA™ process by measuring the outcome of parallel amplification of independent biological replicates followed by microarray hybridization.

MATERIALS AND METHODS

Eight separate 20 ng total RNA samples were amplified with the Ribo-SPIA™ process using the reagents and protocols provided in the Ovation™ Aminoallyl System kit (NuGEN cat. #2101-12). Four samples were Universal Human Reference RNA (UHR, Stratagene cat. #74000), a mixture of total RNA from ten cancer cell lines, and four samples were a mixture of colon, heart, liver, and skeletal muscle (CHLS) RNA, obtained as individual RNAs from BD Biosciences-Clontech.

To assess amplification-to-amplification consistency, the yield of amplified cDNA product from each of the

eight samples was determined spectrophotometrically. Then a portion of each amplified cDNA product was diluted 1:10 and duplicate TaqMan assays were performed for the GAPDH gene according to the Applied Biosystems User Guide [Forward Primer: GAGTCAACGGATTTGGTCGTATT; Probe: CCTGGTCACCAGGGCTGCTT; Reverse Primer: GAATTTGCCATGGTGAAT; amplicon size 143 bp located 1,040 bp from the poly (A) tail at the 3' end].

Two micrograms of each of the four UHR amplified cDNA samples were coupled to Cy5 and the four amplified CHLS cDNA samples to Cy3 (Cy3 Mono-Reactive Dye Pack, Amersham, cat. #PA23001; Cy5 Mono-Reactive Dye Pack, Amersham, cat. #PA25001) following the recommendations in the Ovation™ User Guide. The UHR and CHLS cDNA samples were then paired

and mixed together to create a total of four UHR-CHLS samples. Labeled UHR-CHLS samples were co-purified over QIAquick columns (Qiagen, cat. #28104) and concentrated using Microcon YM-30 columns (Millipore, cat. #4241).

To evaluate microarray result reproducibility between samples, an amount equivalent to 2 µg of each of the four cye dye-labeled UHR-CHLS cDNA samples was hybridized to a 22,000-feature, 60-mer oligonucleotide microarray (Agilent cat. #G4110A) at 60°C for 16 to 18 hours and washed using protocols provided by the array manufacturer.

Hybridized arrays were scanned on a GenePix 4000B Scanner (Axon Instruments) and fluorescent intensities determined using GenePix 4.1 software. Correlation of the average log intensities

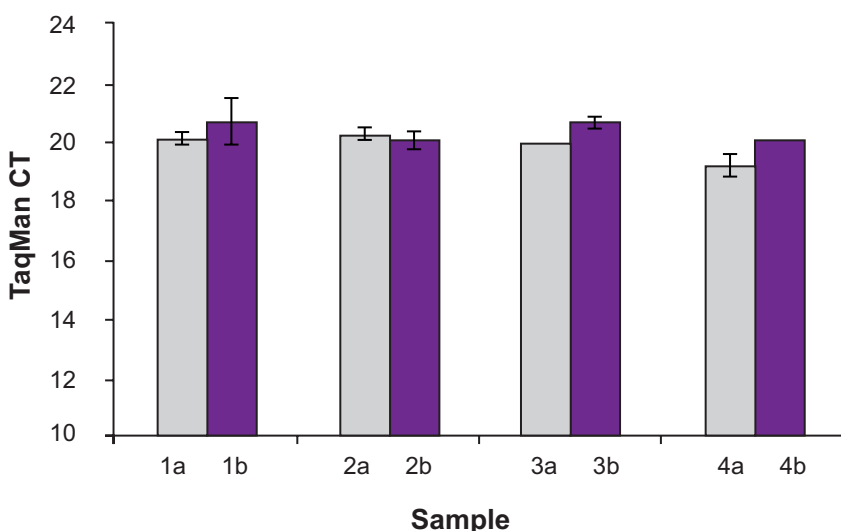


Figure 1. Sample-to-Sample Amplification Reproducibility Measured by TaqMan Analysis. The amount of an amplified sequence of GAPDH gene was measured in each of eight amplified cDNA samples generated from 20 ng total RNA [four UHR (a) and four CHLS (b)]. The average cycle threshold (CT) of two replicates per sample is shown. Bars indicate one standard deviation. The average cDNA yield from all samples was 5.3 µg ± 0.5.

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[Log₁₀(R + G)/2] was determined by pair-wise comparisons of each of the four UHR-CHLS hybridizations.

RESULTS AND CONCLUSIONS

The average cDNA yield, after amplification of each of the eight 20 ng total RNA samples, was 5.3 µg + 0.5, demonstrating consistent amplification among samples.

When the same amount of amplified cDNA product for each of the eight samples was assayed for GAPDH gene expression levels, using realtime quantitative PCR, very uniform cycle

thresholds near 20 were observed, both between and within UHR and CHLS samples (Figure 1).

When the independently amplified UHR-CHLS samples were hybridized to four oligonucleotide DNA microarrays, the signal intensity appeared very consistent among all arrays upon visual inspection (Figure 2).

In addition, high correlation coefficients with an average R² = 0.95 ± 0.01 (Table 1) were demonstrated using pair-wise comparison of log signal intensities of the four arrays.

The high correlation between microarray results and uniformity in quantitative

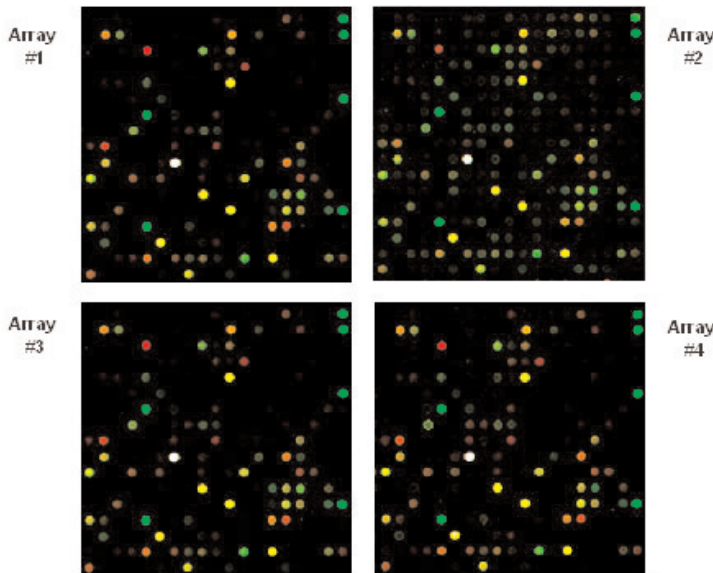
PCR results for independently amplified nanogram samples of RNA reveals minimal sample- to-sample variation when using the Ribo-SPIA™ amplification process. Such reproducibility is a prerequisite for reliable gene expression analysis of small samples.

System Specifications

Cat No.: 2101-12, 12 reactions

Input: 5-100 ng total RNA

Yield: 5-10 µg single stranded cDNA



Array	# 1	# 2	# 3	# 4
# 1		0.94	0.96	0.95
# 2	0.94		0.93	0.94
# 3	0.96	0.93		0.96
# 4	0.95	0.94	0.96	

Table 1. R² Values Between Replicate Arrays. Results of pair-wise comparisons of log signal intensities between the four arrays shown in Figure 2. Average R² = 0.95 ± 0.01

Figure 2. Correlation of Microarray Data Between Independently Amplified Samples. Four UHR cDNA samples were coupled to Cy5 and four CHLS cDNA samples to Cy3, mixed, then hybridized to four Agilent human oligo arrays as described. The same region of four arrays is shown.

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