Effect of RNA Degradation on Data Quality in Quantitative PCR and Microarray Experiments

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Introduction

RNA quality plays a major role in the generation of accurate quantitative results from gene expression analysis experiments. cDNA made from RNA that has been degraded will not become amplified or labeled to the same degree as cDNA derived from intact, undegraded RNA. The Experion™ automated electrophoresis system provides an effective method for determining both the quality and quantity of RNA to be used in gene expression analysis experiments using as little as 25 ng total RNA. Here we purposely degrade RNA to varying extents and demonstrate the effects of RNA degradation on quantifying gene expression levels using quantitative PCR (qPCR) and microarray analysis.

Methods

RNA samples (1 mg/ml) prepared from control human liver tissue and from human liver carcinoma cell line HEPG2 were obtained from Ambion, Inc. (now Applied Biosystems, Inc.). RNA was analyzed using the Experion system and the RNA StdSens analysis kit.

Quantitative RT-PCR

RNA (500 ng) was converted to cDNA using the iScript™ cDNA synthesis kit. The cDNA (10 ng) was then amplified in triplicate reactions with iQ™ SYBR® Green supermix and 0.5 mM each primer pair for 18S rRNA, β-actin, β-tubulin, HPRT, or GAPDH using an iCycler iQ® real-time PCR detection system with v. 3.1 software.

Microarray Analysis

Microarrays consisting of long oligonucleotides (MWG-Biotech Inc., Illumina, Inc., and Qiagen Inc.) to detect ~2,000 human genes and a series of control spots were fabricated as described in Karlak et al. (2006). Fluorescently labeled cDNA (5 µg each of Cy3 and Cy5 label) was hybridized to the microarrays as described in Gingrich et al. (2006).

Results

Analysis of RNA With the Experion Automated Electrophoresis System

RNA is susceptible to degradation by endogenous cellular RNases as well as by chemical or heat treatment. To mimic and accelerate these natural processes, we degraded intact commercial RNA preparations over time by incubating them at 90°C in TE buffer. The degree of degradation was monitored using the Experion system (Figure 1). As shown, the 28S/18S rRNA ratio and the size distribution of the smear of mRNA both decreased as degradation progressed.

Effects of RNA Degradation on qPCR

To examine the effects of RNA degradation on quantitation of specific gene transcripts, qPCR was performed on equivalent amounts of RNA that had been degraded to various extents. As seen in Figure 2, for each qPCR reaction, the detection of amplified product is seen at successively later cycles as the RNA is degraded over time. In qPCR experiments, the C_T (threshold cycle) number is used to compare the difference in quantity of starting transcript, with a difference of 1 cycle reflecting a 2-fold difference in starting transcript level (assuming 100% amplification efficiency). The C_T values of the qPCR reactions from the five gene transcripts degraded at different points in time are shown in Table 1.

In order to graphically present these data, the proportion of amplifiable RNA remaining was plotted as a function of degradation time (Figure 3). It is clear from the results that comparing qPCR results derived from RNA in different states of degradation will result in very different quantitative conclusions. These differences can be as great as 1,000-fold, as seen in Figure 3 with samples subjected to 7 hr of heat degradation.

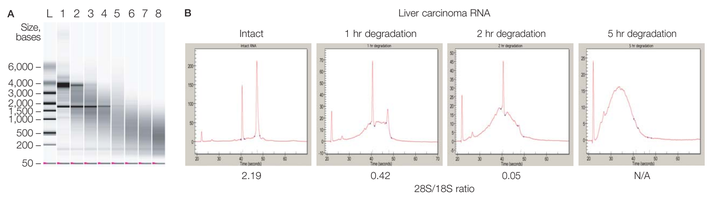

Fig. 1. Time course of degradation of liver carcinoma RNA. Samples of human liver carcinoma total RNA were incubated at 90°C in TE buffer in 1 hr increments from 0 (lane 1) to 7 hr (lane 8). Aliquots (50 ng) were then separated with the Experion RNA StdSens analysis kit. **A**, simulated gel view showing separation of the RNA samples with the RNA sizing ladder indicated in lane L; **B**, electropherograms of samples collected at selected time points and indicating the positions of the 18S and 28S rRNA peaks and the 28S/18S rRNA ratios. The peak at the far left of the electropherograms, corresponds to the lower marker used for alignment of the sample wells.

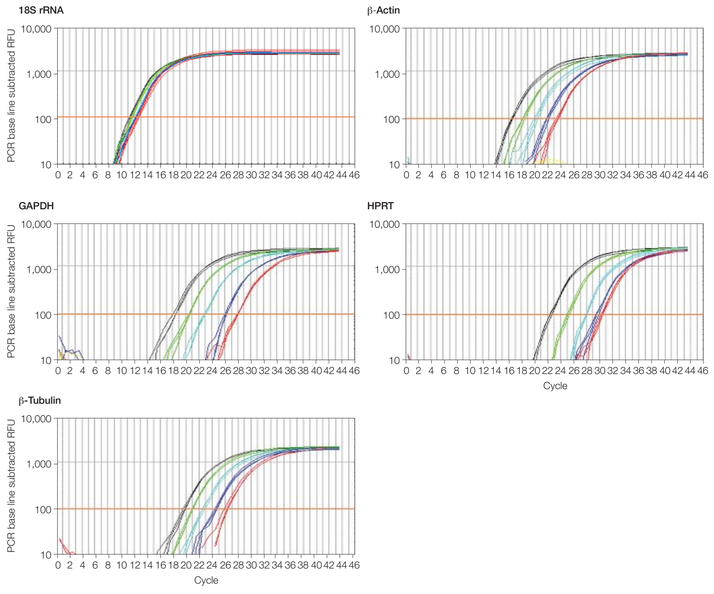

Fig. 2. Impact of RNA degradation on qPCR. qPCR traces obtained from liver carcinoma RNA samples that were degraded for different lengths of time and amplified using primers for the genes indicated. Black traces, no degradation; green traces, 1 hr degradation; light blue traces, 3 hr degradation; dark blue traces, 5 hr degradation; red traces, 7 hr degradation. Average C_T values obtained from these traces are shown in Table 1.

Table 1. Impact of RNA degradation on qPCR. qPCR was performed using liver carcinoma RNA samples that were degraded for different lengths of time and amplified using primers for the genes indicated. Shown are the average C_T values obtained from three reactions and determined for each amplified gene by the software at 100 relative fluorescence units. The ΔC_T value is the change in C_T values over the 7 hr of degradation. Traces for the qPCR reactions from which these data were derived are shown in Figure 2. Gene abbreviations are defined in Table 2.

	C_T				
Degradation Time (hr)	18S rRNA	β-Actin	GAPDH	HPRT	β-Tubulin
0	11.2	16.5	18.1	22.5	19.7
1	11.6	18.2	20.5	25.1	20.9
3	12.0	20.1	22.9	27.9	22.7
5	12.1	22.0	26.1	29.5	24.6
7	12.5	23.5	28.0	30.3	26.5
ΔC_T	1.3	7.0	9.9	7.8	6.8

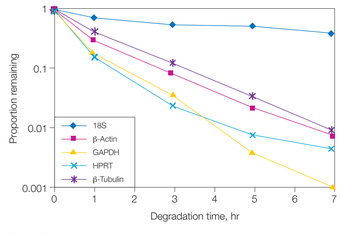

Fig. 3. Time course of degradation of specific liver carcinoma transcripts. The proportion of remaining RNA capable of being amplified is shown as a function of time of incubation at 90°C. The plot is based on the assumption that 100% PCR efficiency was achieved and that for each successive PCR cycle, the difference in RNA remaining is equivalent to 1/2 that of the previous cycle.

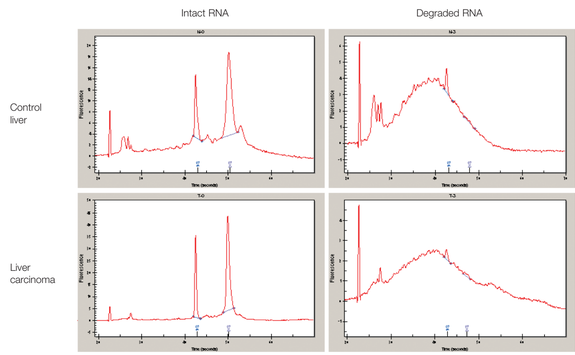

Fig. 4. Assessment of degradation of control liver and liver carcinoma RNA used in gene expression microarrays. Samples of control human liver and human liver carcinoma total RNA were incubated at 90°C in TE buffer for 3 hr. Aliquots (50 ng) of intact and degraded RNA were then separated with the Experion RNA StdSens analysis kit to generate electropherograms.

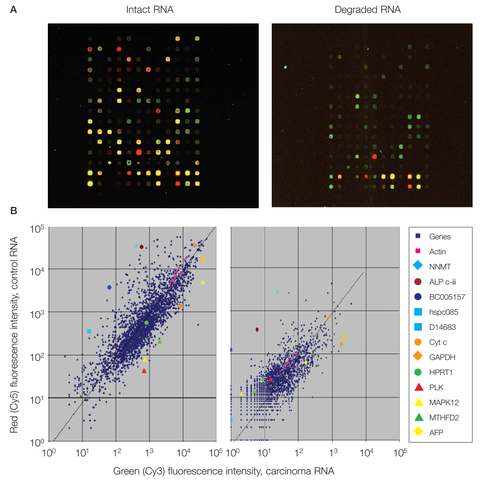

Fig. 5. Impact of RNA degradation on the ability to detect changes in gene expression using microarrays. Microarrays comparing gene expression levels in control liver with those in liver carcinoma. Shown at left is the microarray hybridized with probes derived from intact RNA, and shown at right is the microarray hybridized with probes derived from RNA samples degraded by incubation at 90°C for 3 hr. The microarrays were scanned at the same instrument settings. Individual colored spots are array elements containing the specific gene indicated in the legend. **A**, subarrays showing positions of control sequences (bottom two rows of array elements), including 18S and actin sequences (pairs of spots on the far left and far right of the two rows); **B**, scatter plots of hybridization signals from genes within the entire array. Gene abbreviations are defined in Table 2.

Table 2. Signal levels and ratios of genes expressed to different levels in microarrays. I, intact; D, degraded.

Probe	RNA Quality	532 nm (Green) Signal	635 nm (Red) Signal	532/635 nm Ratio
Moderate to High Expression				
Actin	I	6,892 (avg)	7,665 (avg)	0.94 (avg)
	D	60 (avg)	61 (avg)	0.96 (avg)
GAPDH (glyceraldehyde-3-phosphate dehydrogenase)	I	22,061	34,766	0.63
	D	748	673	1.11
D14683 (glycine cleavage system t-protein)	I	40,772	16,253	2.51
	D	2379	245	9.71
AFP (α-fetoprotein)	I	39,219	4,794	8.18
	D	166	62	2.68
NNMT (nicotinamide N-methyltransferase)	I	403	33,997	0.012
	D	24	2,744	0.009
ALP c-iii (apolipoprotein c-iii)	I	593	31,719	0.019
	D	6	357	0.017
Cyt c (cytochrome c)	I	8,167	1,339	6.10
	D	1,913	158	12.11
BC005157 (t-cell leukemia translocation altered gene)	I	64	3,619	0.018
	D	1	125	0.008
Low Expression				
MTHFD2 (methylene tetrahydrofolate dehydrogenase 2)	I	2,072	208	9.96
	D	5	14	0.36
HPRT1 (hypoxanthine phosphoribosyltransferase 1)	I	833	549	1.52
	D	9	25	0.36
PLK (polo-like kinase)	I	703	44	15.98
	D	15	26	0.58
MAPK12 (mitogen-activated protein kinase 12)	I	744	79	9.42
	D	2	12	0.17
hspc085 (unknown transcript)	I	16	345	0.05
	D	1	3	0.33

Effects of RNA Degradation on the Ability to Detect Changes in Gene Expression Using Microarrays

We further examined the effect of RNA degradation on differential gene expression data derived from spotted oligonucleotide microarrays. In these experiments, differential gene expression was demonstrated from a pair of microarrays in which gene expression levels were compared from control human liver tissue to those from a human liver carcinoma cell line. In one microarray, the RNA from both sources was intact. In the other microarray, both RNA samples were degraded for 3 hr by heating to 90°C in TE. The RNA degradation level was first evaluated using the Experion system (Figure 4).

The gene expression microarray results obtained from these RNA samples are shown in Figure 5. Equivalent microarray subarrays (top) show that, excluding the control spots on the bottom two rows of each subarray, the signal in the microarray hybridized with probe derived from intact RNA was uniformly higher than that of the microarray hybridized with probe derived from the degraded RNA. This difference in hybridization signal is reflected in the scatter plots for the entire microarray (bottom) and correlate with the decrease in amplifiable transcripts expected from the degraded RNA sample as evaluated on the Experion system (see Figure 3).

A number of specific gene array elements are highlighted in the scatter plots to demonstrate the utility of data derived from degraded RNA. For example, there are a number of array elements that contain the actin transcript (shown in pink, Figure 5, bottom). The data indicate that for a highly expressed gene such as actin, the relative expression levels between two samples can still be determined with degraded RNA, but with significantly higher variability.

Also indicated in Figure 5 (bottom) are signal levels from other transcripts present in only a single array element. The signal levels and data ratio for these genes are shown in Table 2. For actin and other genes that are highly expressed, the array data still show a comparable level of differential expression. In contrast, for genes expressed at lower levels, it is no longer possible to detect the transcript when the RNA is degraded, as the signals generated by these spots are close to the background noise level of the microarray. For genes with low expression levels, the relative level of expression for intact and degraded RNA was quite different, indicative of poor data derived from microarrays hybridized with probes derived from degraded RNA.

Conclusions

The Experion automated electrophoresis system provides a quick and effective way to characterize RNA samples prior to gene expression analysis. As RNA becomes degraded, quantitative expression levels determined by qPCR decrease. This can lead to erroneous conclusions regarding levels of gene expression when comparing samples that are degraded to different extents. When degraded RNA is used in gene expression experiments employing microarrays, genes with high levels of expression can still be characterized with respect to relative expression levels. However, degradation compromises the ability to detect differences in expression of genes expressed at low levels.

References

Karlak CC et al., DNA microarrays: optimization and fabrication parameters and control plate design, Bio-Rad bulletin 5308 (2006)

Gingrich J et al., Effect of RNA degradation on data quality in quantitative PCR and microarray experiments, Bio-Rad bulletin 5452 (2006)