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

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Introduction

Quantitative PCR (qPCR) and gene expression microarrays are two complementary approaches for determining quantitative or relative levels of gene expression. In qPCR, RNA is converted to cDNA and its amplification monitored during PCR cycling. Gene transcripts in greater abundance become amplified to detectable levels before transcripts at lower abundance. In the case of gene expression microarrays, absolute transcript levels are not determined. Instead, the relative abundance of gene transcripts from two samples is measured by the signal intensity from a microarray where each element contains the sequence of a specific gene and the two sample probes are labeled with a different color and hybridized concurrently.

RNA quality plays a major role in the generation of accurate quantitative results from gene expression analysis experiments (Bustin and Nolan 2004). cDNA made from RNA that has been degraded will not become amplified or labeled to the same degree as cDNA made from intact, undegraded RNA. However, for many studies the isolation of high-quality, intact RNA is a challenge, particularly with clinical samples whose tissues must be handled with extreme care to keep their RNA intact. The determination of RNA quality is therefore a critical initial step in any quantitative gene expression analysis workflow.

The traditional approach to determining RNA quality is agarose gel electrophoresis. This approach, however, requires relatively large amounts (5–10 µg) of RNA. This requirement can be problematic when the quantity of the source material is limited (such as in tumor and tissue biopsies) and when experimental methods require large amounts of sample for analysis (for example, microarray experiments require 5–10 µg RNA for labeling and hybridization). When only small amounts of RNA are available for experimentation, one viable option is to amplify the RNA (Van Gelder et al. 1990, Pabon et al. 2001, Wang et al. 2000) by reverse-transcription PCR (RT-PCR). However, amplification itself may inadvertently introduce experimental bias into subsequent sample analysis, rendering the relative quantitation of transcript levels from different genes inaccurate (Cole et al. 2006, Dumur et al. 2004, Ji et al. 2004, Spiess et al. 2003). It is therefore frequently preferable, in order to obtain accurate quantitative results, not to amplify sample material.

The Experion™ automated electrophoresis system provides an effective method for determining both the quality and quantity of RNA in gene expression analysis experiments. The Experion system accurately quantitates and evaluates the integrity of a sample using as little as 25 ng total RNA — several hundred times less material than that required for gel electrophoresis. Once RNA quantity and quality have been established using the Experion system, one can prudently determine whether to move forward with additional experiments, knowing to what degree the results may be compromised.

Here we analyze how RNA degradation affects the ability to reliably detect differences in gene expression using qPCR and microarray analysis. First, intact RNA samples were degraded to different degrees, and the extent of degradation was characterized using the Experion system. These RNA samples were then analyzed in qPCR experiments to determine the effect of RNA degradation on quantitation of specific transcripts. Lastly, a differential gene expression experiment was performed using DNA microarrays with RNA probes from two sources, control human liver tissue and a human liver carcinoma cell line. In these experiments, hybridization was performed with probes derived from intact or degraded RNA to examine the effect of degradation on the ability to detect differences in gene expression using DNA microarrays.

Methods

RNA Samples and Experion Analysis

RNA samples (1 mg/ml) prepared from control human liver tissue and from human liver carcinoma cell line HEPG2 were obtained from Ambion, Inc. These samples were diluted to 0.1 mg/ml in TE and incubated at 90°C for up to 5 hr (controls) or up to 7 hr (carcinoma cell line samples). Aliquots were taken at various times during incubation, and the degree of degradation was assessed by analyzing RNA (50 ng) with the Experion system and RNA StdSens analysis kit (according to instructions provided with the kit).

Quantitative RT-PCR (qPCR)

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 µM each primer pair for 18S rRNA, β-actin, β-tubulin, hypoxanthine phosphoribosyltransferase (HPRT), or glyceraldehyde-3-phosphate dehydrogenase (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). The microarrays were prehybridized with 5x SSC, 0.1% SDS, and 0.5 mg/ml BSA at 55°C for 30 min. RNA (10 µg) was converted to aminoallyl cDNA and labeled with reactive Cy3 and Cy5 monoesters (GE Healthcare). Fluorescently labeled cDNA (5 µg each label) was hybridized to the microarrays for 24 hr at 45°C using an Advalytix hybridization station with 25% formamide, 4x SSC, 0.5% SDS, 2x Denhardt's solution, and 13% LNA dT block (Genisphere, Inc.). Following hybridization, the microarrays were first rinsed with 2x SSC, 0.1% SDS for 5 min at 37ºC, and then rinsed twice with 0.2x SSC for 2 min at 37°C. Microarrays were scanned immediately at equivalent laser power and photomultiplier tube (PMT) settings for all arrays.

Results and Discussion

Analysis of RNA With the Experion Automated Electrophoresis System

RNA is more prone to degradation than DNA, and care must be taken during isolation procedures to ensure accurate quantitation of transcript levels. 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 and RNA StdSens analysis kit.

Having closely examined the Experion results, the kinetics of degradation appeared equivalent between the two RNA preparations from control human liver tissue and the liver carcinoma cell line. Therefore, for simplicity, we only show the results from the liver carcinoma RNA preparation (Figure 1). The ratio of the two large ribosomal RNA molecules (28S/18S rRNA) provides a measure of RNA degradation since the 28S rRNA is more susceptible to degradation than the 18S rRNA fragment. Intact eukaryotic RNA samples generally have a 28S/18S rRNA ratio of 2.0–2.2. Experion software automatically determines the 28S/18S rRNA ratio and provides a visual assessment of degradation in electropherograms or virtual gel images. As shown in Figure 1, the 28S/18S rRNA ratio and the size distribution of the smear of mRNA both decreased as degradation progressed.

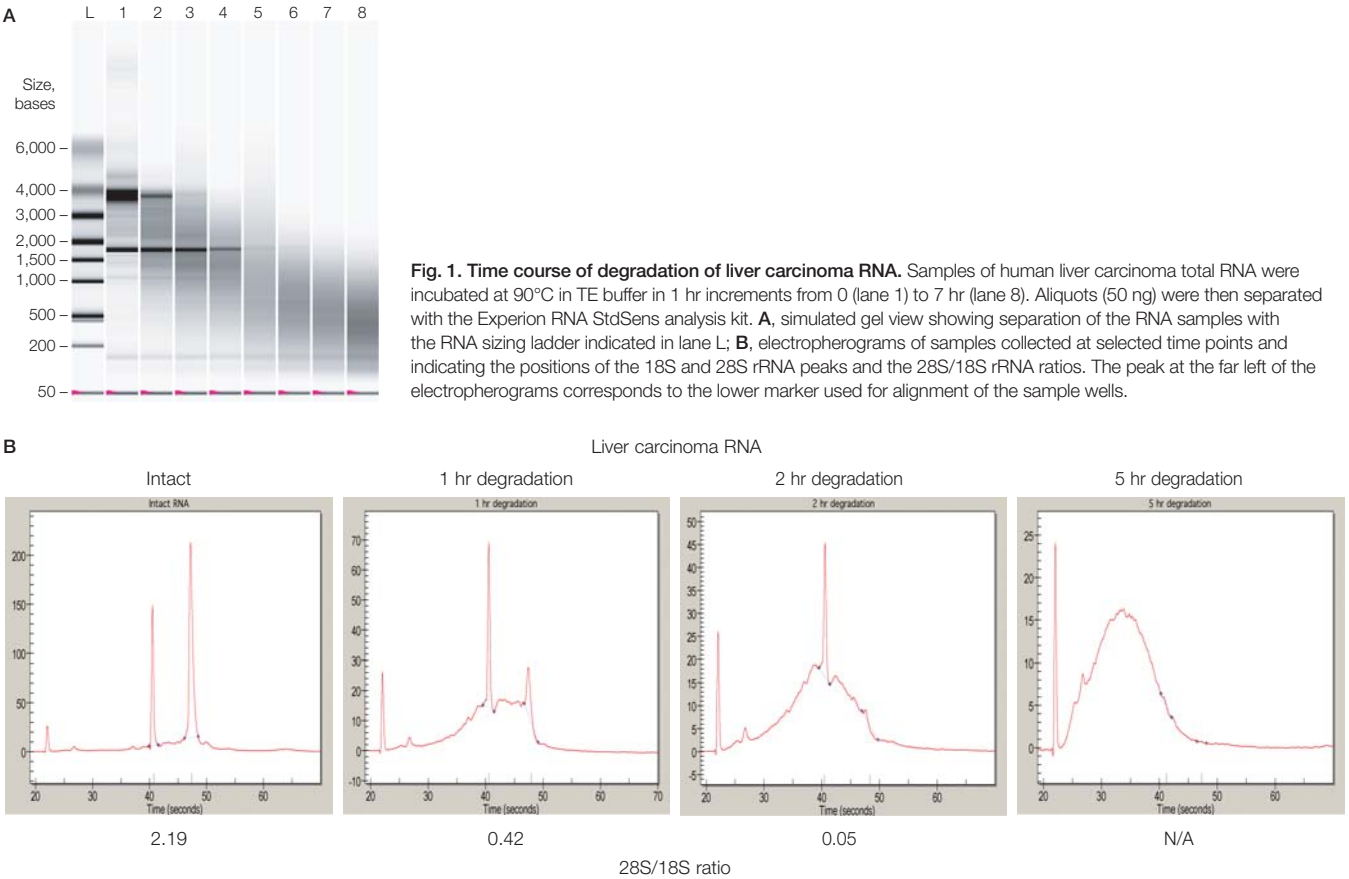

Fig. 1. Time course of degradation of liver carcinoma RNA. Samples of human liver carcinoma total RNA were incubated at 90°C in TE buffer in 1 hr increments from 0 (lane 1) to 7 hr (lane 8). Aliquots (50 ng) were then separated with the Experion RNA StdSens analysis kit. **A**, simulated gel view showing 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.

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. Following conversion of the RNA to cDNA, primers for the 18S rRNA and four selected protein-encoding gene transcripts were used in qPCR reactions to quantitate the relative abundance of transcripts in the samples.

Figure 2 shows the effect of RNA degradation on qPCR. For each qPCR reaction, the detection of amplified product was seen at successively later cycles as the RNA was degraded over time. Figure 2 also shows that the 18S rRNA transcript degraded much more slowly than the four protein gene transcripts. This is indicated by the small differences in cycle number versus degradation time in which the 18S rRNA transcript was detected. Higher degradation rates for the protein-encoding genes are reflected in the greater differences in cycle numbers in which the protein gene transcripts started to become amplified. This correlates with data generated by the Experion system that confirmed the 18S rRNA transcript was less susceptible to degradation by heat than were other gene transcripts (not shown).

In qPCR experiments, the C_T (threshold cycle) number is the cycle number at which fluorescence from amplified DNA passes a set fluorescence threshold. The C_T 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 are shown in Table 1. The initial, undegraded concentration of 18S rRNA transcript was significantly higher than the other transcripts, while the β-actin transcript was present at a higher level than GAPDH, β-tubulin, and HPRT, respectively. Over 7 hr of degradation time, the 18S rRNA was degraded to a much lower degree than the protein gene transcripts, as seen by the ΔC_T of 1.3 (equal to $2^{1.3}$, or 2.5-fold) for the rRNA transcript and a ΔC_T of 6.8 to 9.9 (equal to $2^{6.8}$ to $2^{9.9}$, or 128- to 1,000-fold) for the protein gene transcripts.

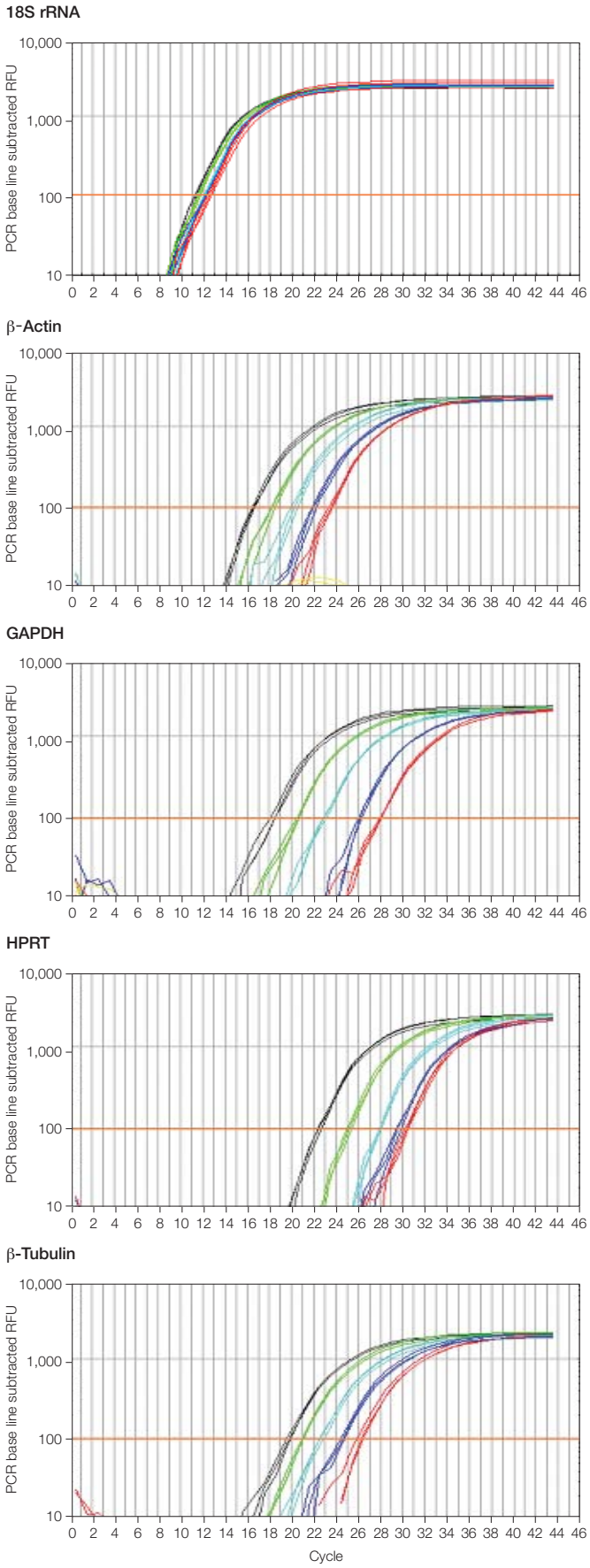

Fig. 2. Impact of RNA degradation on qPCR. qPCR traces obtained from liver carcinoma RNA samples that were degraded for different lengths of time and amplified using primers for the genes indicated. Black traces, no degradation; green traces, 1 hr degradation; light blue traces, 3 hr degradation; dark 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.

	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

In order to graphically present these data, the proportion of RNA that could be amplified was plotted as a function of degradation time (Figure 3). The proportion of RNA that remained was calculated based on the assumption that RNA amplified in each successive cycle of PCR is equivalent to half the RNA that was amplified in the previous cycle. The data in Figure 3 show that the protein gene transcripts (β-actin, GAPDH, HPRT, and β-tubulin) were degraded at roughly similar rates, all of which were significantly faster than the rate at which the 18S rRNA was degraded. It is worth noting that the amplification plot of the least abundant transcript, HPRT, starts to level off at degradation times beyond 3 hr. This may be due to the fact that, since so little HPRT transcript remained intact after degradation, its amplification was limited.

These results demonstrate that comparisons of qPCR results derived from RNA in different states of degradation will generate 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.

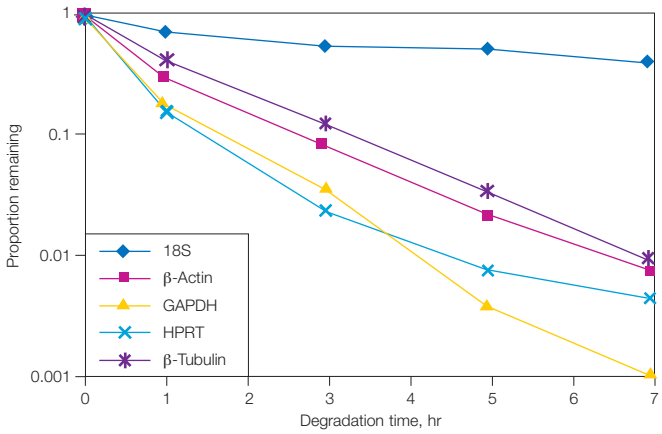

Fig. 3. Time course of degradation of specific liver carcinoma transcripts. The proportion of remaining RNA capable of being amplified is shown as a function of time of incubation at 90°C. The plot is based on the assumption that 100% PCR efficiency was achieved and that for each successive PCR cycle,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

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. The RNA degradation level was first evaluated using the Experion system (Figure 4).

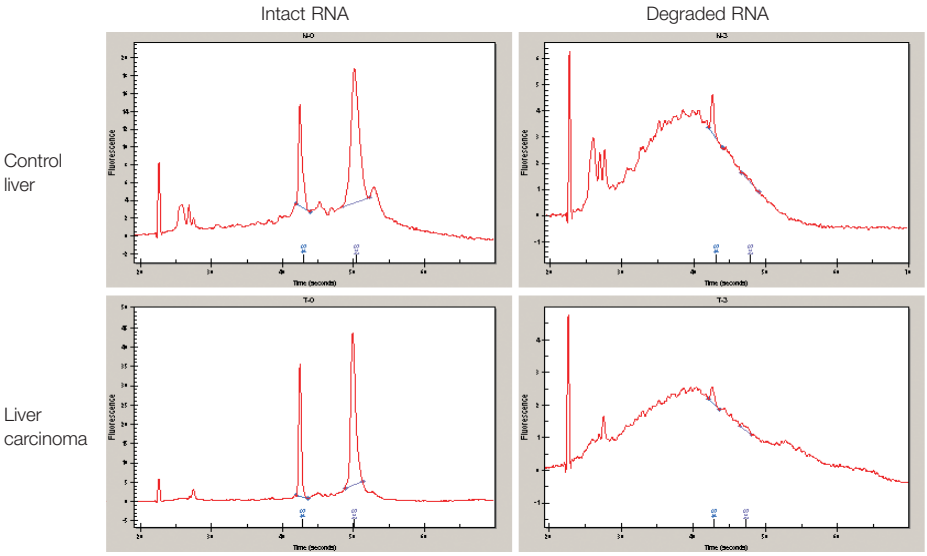

Fig. 4. Assessment of degradation of control liver and liver carcinoma RNA used in gene expression microarrays. Samples of control human liver and human liver carcinoma total RNA were incubated at 90°C in TE buffer for 3 hr. Aliquots (50 ng) of intact and degraded RNA were then separated with the Experion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

Based on the qPCR results shown in Figure 3, 10–50 times fewer transcripts could be amplified from samples degraded for 3 hr than from intact RNA samples. This suggests that the signal intensity from the microarrays should also be 10–50 times lower when using the fluorescently labeled degraded RNA as probe. However, it is unclear whether there is a direct relationship between the ability of a transcript to be amplified and its ability to be labeled and hybridized to a microarray element.

The gene expression microarray results are shown in Figure 5. The microarray subgrids (Figure 5A) show that, excluding the control spots on the bottom two rows of each array, the signal in the microarray hybridized with probe derived from intact RNA was uniformly higher than that in 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 (Figure 5B). In the microarray hybridized with intact RNA, the signal ranges from 10 to over 10,000 counts, with most spots showing signal in the 100–5,000 count range. In contrast, the microarray hybridized with probe derived from degraded RNA shows signal ranging from only a small number of counts to approximately 1,000 counts, with the bulk of the spots having signal in the 10–100 count range. These differences correlate with the decrease in amplifiable transcripts expected from the degraded RNA sample as evaluated on the Experion system (see Figure 4).

A number of specific gene array elements are highlighted in the scatter plots to demonstrate the usefulness of data derived from degraded RNA. As an example, there are a number of array elements that contain the actin transcript (shown in pink, Figure 5B). In the microarray hybridized with probes derived from intact RNA, these spots cluster at around 7,000 counts from each cell type, indicating that actin is highly expressed in relatively equivalent amounts in both cell types. In the microarray hybridized with degraded RNA, the actin array elements continue to show equivalent expression in both cell types; however, the individual data points are more spread out and reveal a substantially decreased signal level of ~7,000 counts with a coefficient of variation (CV) of ~35% for the intact probe array, and 60 counts with a CV of ~60% in the degraded probe array. These data indicate relative expression levels can still be determined with degraded RNA, but with significantly higher variability.

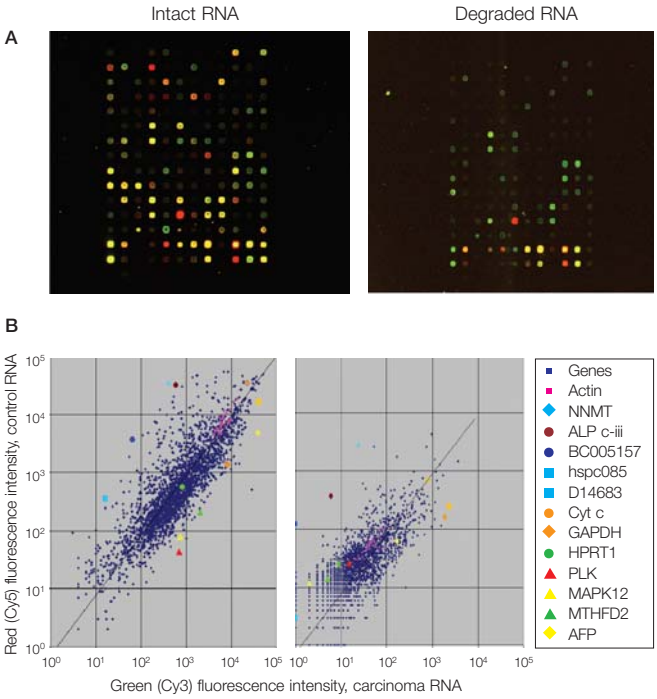

Fig. 5. Impact of RNA degradation on the ability to detect changes in gene expression using microarrays. Microarrays comparing gene expression levels in control liver with those in liver carcinoma. Shown at left is the microarray hybridized with probes derived from intact RNA, and shown at right is the 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.

Also indicated in Figure 5B are other transcripts present in only a single array element. The signal levels and data ratios for these genes are shown in Table 2. For actin and other genes that are highly expressed (for example, spots labeled NNMT, ALP c-iii, D14683, cytochrome c, and GAPDH), the array data still show a comparable level of differential expression; that is, differences in relative expression levels can still be determined when using degraded RNA. 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. Examples are seen in spots labeled HPRT1, PLK, MAPK12, MTHFD2, and hspc085. For these and other genes with low expression levels, the relative level of expression for intact and degraded RNA was quite different, as indicated by the different ratio of signal at 532 versus 635 nm (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	2,379	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

Conclusions

The Experion automated electrophoresis system provides a quick and effective way to characterize an RNA sample prior to gene expression analysis. As RNA becomes degraded, quantitative expression levels determined by qPCR decrease with increasing levels of degradation. 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 in two samples. However, degradation compromises the ability to detect differences in expression of genes that are expressed at low levels.

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