Estimation and Reduction of the Bias Caused by RNA Degradation in Microarray Analysis

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Measurements of gene expression are based on the assumption that the analyzed RNA sample closely resembles the amount of transcripts *in vivo*. Established knowledge that transcripts of different genes possess different stabilities suggests that degradation of RNA occurring during the isolation procedure is also non-uniformly distributed among different RNA species. Indeed, comparison of RNA samples of different degrees of degradation shows that up to 75% of microarray-based measurements of differential gene expression can be caused by degradation bias alone[1]. We demonstrate that analysis of capillary-electrophoresis data does allow reproducible characterization of RNA degradation and its differentiation from apoptosis-associated RNA cleavage. Degradometer software for quantification of RNA integrity is available on our website (www.dnaarrays.org). Our results suggest that comparison of RNA samples of similar integrity eliminates skewed results of differential gene expression. Consequently, information about quantification of RNA integrity will help to improve reproducibility of microarray results.

Standard Methods to Measure RNA Integrity

Controls frequently used to measure the integrity of RNA samples applied to microarray expression studies can roughly be divided into 3 groups: 1. No described control of integrity; 2. Estimation of integrity by subjective inspection of agarose gel electrophoresis; and 3. Capillary electrophoresis. Standard agarose gel electrophoresis allows the detection of severe degradation only (Fig. 1a). One of the most effective tools for characterizing RNA integrity is capillary electrophoresis, where determination of the fluorescent intensity ratio of 28 S to 18 S rRNA has been suggested [2] and degraded RNA should be indicated by an altered 28 S/18 S rRNA signal ratio. Quantification of 18 S and 28 S rRNA calculated by Bioanalyzer software is compromised by the fact that this calculation is based on area measurements which are heavily dependent on definitions of start and end points of peaks (Fig 1b). Even accurate determination of this ratio is not sufficient to detect degradation with sufficient sensitivity (Fig. 1c, d).

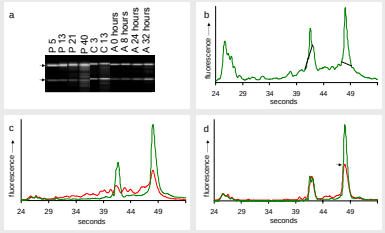


Figure 1 (a) A gel-like image of RNA at various stages of degradation, Samples "P" and"C" represent two RNA samples, both followed by the value of the degradation factor; samples "A" represent RNA from apoptosis experiments, followed by the treatment time of induction of apoptosis; (b) chromatograms of RNA size distribution; the x-axis shows time in seconds, the y-axis shows fluorescence intensities; area measurements for rRNA calculation can be misleading due to incorrect definition of baselines of the areas; a typical example of automatic baseline detection by the Bioanalyzer software; (c) green graph shows intact RNA (degradation factor 5), red graph shows degraded RNA (degradation factor 40); (d) RNA of NB4 cells, mock treated (green) and treated for 32 hours by 5 mM valproic acid (red) to induce apoptosis; fluorescence intensity of 28S rRNA is reduced relative to 18S rRNA (arrow).

Consequences of RNA Degradation on Measurement of Gene Expression

Two RNA samples, each at four levels of integrity, have been utilized in GeneChip expression analysis. Pair-wise comparisons have been performed for each sample pair at each level of integrity. A comparison of one intact RNA and one degraded sample resulted in measurement of up to 75% of differential gene expression being unrelated to biological differences between samples.

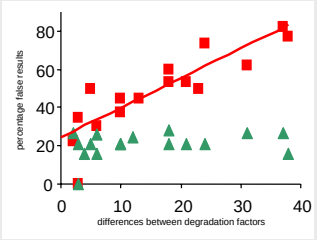
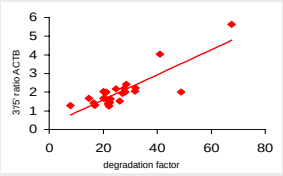


Figure 2 False positive and negative rates of differential gene expression due to degradation. The x-axis shows differences between RNA samples in the degradation factor, the y-axis shows percentage of incorrectly measured differential gene expression in pair-wise comparisons. Red squares represent false positive results and green triangles false negative results of differential gene expression measurements.

The Degradation Factor

About 95% of cellular RNAs consist of non-messenger RNAs which mainly fall into 3 classes of molecular weight: 28S, 18S rRNA and "small RNAs" such as the 5S rRNA and tRNAs. Consequently, a chromatogram of the size distribution of cellular RNAs generally presents itself as a graph containing three distinct peak signals, which correspond to the three predominant RNA classes and a broad distribution of molecular weights with much weaker signals. With increasing degradation, not only are heights of the 18S and 28S peaks gradually reduced, but also additional "degradation peak signals" appear in a molecular weight range between "small RNAs" and the 18S peak (Fig 1c). The ratio of the average of degradation peak signals to the 18 S peak signal is a reproducible parameter for degradation of RNA. This ratio multiplied by 100 is called "degradation factor". It correlates well with another measurement of RNA integrity, namely the 3'/5' ratios measured in microarray experiments (Fig 3).



The Degradometer Software

The Degradometer analyzes CSV files exported from Bioanalyzer software. It compensates for variations in timing by determining the timing of the synchronization peak and the 28S peak, and then generates a "Scaled" spreadsheet for optical comparison of samples (Fig. 3b). The "Degradation" spreadsheet contains the degradation factor and the ratio of signal heights of 28S/18S rRNA calculated for measurement of apoptosis (Fig.1c). For degradation, an alert system divides RNAs into 4 groups of integrity (Table 1). The latest version (Degradometer 1.31) contains several improvements, such as better handling of weak front marker peaks and air bubbles.

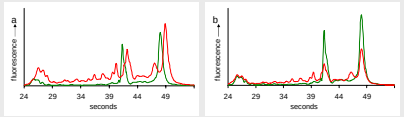


Figure 4 Unscaled (a) and scaled (b) chromatograms of total RNA samples. The Degradometer compensates for differences in migration time and amount of loading for optical comparison.

Alert	Degradation factor (%Deg/18S)
	0-8
Yellow	8-16
Orange	16-24
Red	> 24
Black	18S and/or 28S peaks not reliably detected

References

1. Chipping away at the chip bias: RNA degradation in microarray analysis. Auer H, Liyanarachchi S, Newsom D, Klisovic MI, Marcucci G, Kornacker K (2003) Nature Genetics 35:292-293

2. The principle and promise of Labchip technology. Van de Goor T.A. (2003) PharmaGenomics 3:16-18