RNA quality Indicator (RQI) - A New Tool for Assessing RNA Integrity to reliably detect differences in gene expression using qPCR 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 to the same degree as cDNA made from intact, undegraded RNA. The Experion™ automated electrophoresis system (Bio-Rad Laboratories, Inc.) 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 200 pg of total RNA - several thousand times less material than that required for gel electrophoresis.

Here, we describe the development of a method for RNA quality assessment, the RNA quality indicator (RQI), a new feature of the Experion automated electrophoresis system that automatically assesses the integrity of RNA samples. Then, we demonstrate how RNA degradation affects the ability to reliably detect differences in gene expression using qPCR. We show that quantification of RNA using qPCR correlates well with RQI measurements.

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. These samples were diluted to 0.1 mg/ml in TE and incubated at 90°C for up to 12 hr for the degradation standards, 5 hr (controls) or up to 7 hr (carcinoma cell line samples) for the qPCR analysis. RNA were analysed using the Experion RNA StdSens or HighSens analysis kits.

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 of each primer pair for 18S rRNA, β-actin, β-tubulin, HPRT or GADPH using an iCycler iQ® real-time PCR detection system with version 3.1 software (Bio-Rad Laboratories, Inc.).

RESULTS

1 RQI Integrates three important regions of the electropherogram

RQI calculations are based on mapping an RNA sample's electropherogram profile into a set of degradation standards. A set of profile standards — from intact to degraded — constituting a degradation reference scale from 10 (intact) to 1 (fully degraded) has been established (Figure 1). Three regions of the electropherogram are taken into account when mapping a sample for RQI calculations: the 28S region, the 18S region, and the pre-18S region (before the 18S rRNA band) (Figure 2). Differential weighting is used to evaluate components of the electropherogram, based on how the sample maps to the reference standards.

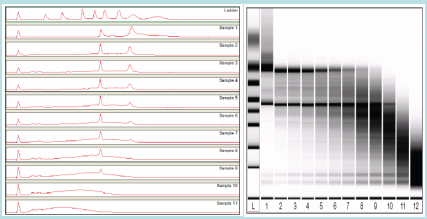

Fig. 1. Electropherograms (left) and virtual gel image (right) of 12 RNA degradation standards. Human liver RNA (100 ng/µl in TE) were degraded by incubation at 90°C for 0, 3, 5, 12, 20, 25, 31, 40, 52, 90, 150, and 270 min (lanes 1–12, respectively) and run on the Experion automated electrophoresis system using an Experion RNA StdSens analysis kit. L, RNA ladder.

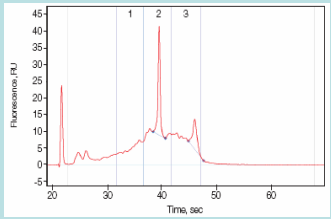

Fig. 2. RQI calculation. Three electropherogram regions used for RQI determination are indicated on an electropherogram of partially degraded RNA. 1, pre-18S peak region; 2, 18S peak region; 3, 28S peak region.

2 Customization of RQI threshold values for downstream applications

The RQI number by itself is not self explanatory enough to decide if a sample can be safely used for downstream application. A validation process is required to correlate RQI values and successful or failed downstream application, using a set of artificially degraded samples. This process allows defining threshold RQI values. The Experion software groups those RQI values into three colour codes, which appear in a summary screen for a quick visualisation (Figure 3). Once the threshold values have been determined, it is possible to customize the range for each colour group.

Well ID	Sample Name	RNA Area	RNA Concentration (pg/µl)	Ratio [28S/18S]	RQI	RQI Classification	RQI Alert
L	Ladder	861.49	1000.00				
1	2089	421.98	489.83	0.99	7.9		
2	2116	549.95	638.37	0.89	2.9		
3	2119	347.18	403.00	1.24	7.9		
4	2120	153.25	177.89	0.64	[6.0]		RNA conc. too low
5	2162	352.20	408.83	1.05	7.3		
6	2163	274.45	318.57	1.54	8.1		
7	2164	457.38	530.92	0.24	4.2		
8	2165	441.91	512.96	1.43	7.9		
9	2168	430.35	499.54	0.96	6.8		
10	2169	472.71	548.71	1.02	3.5		
11	2170	330.35	383.47	1.45	8.9		

Fig. 3. Example of a run summary screen from Experion software: All the data of all the samples analysed within a chip are summarized in a one view table. The customisable RQI colour code allows a quick visualisation and identification of the samples that can be safely used for downstream application. The RQI has been forced to be estimated in well 4 even with a low concentration.

3 Linear dynamic range of RNA concentrations for RQI calculations

The effect of RNA concentration on RQI precision was determined by analyzing RNA samples with different levels of integrity. RNA samples were diluted to cover the entire dynamic range of the standard and high-sensitivity RNA kits. The results in Table1 indicate that the RQI value is accurately reported above 200 pg/µl with the Experion RNA HighSens analysis kit.

Experion RNA Analysis Kit	Qualitative LLOD* of RNA	**Lower limit for RQI determination**	Quantitative LLOD of RNA
StdSens	5 ng/µl	**≥10 ng/µl**	25 ng/µl
HighSens	100 pg/µl	**≥200 pg/µl**	200 pg/µl

*Lower limit of detection

Table 1. Lower limits of RNA concentrations for RNA detection and RQI determination.

4 Application of RQI to different tissues and organisms and correlation between RQI and RIN

The RQI calculation algorithm was established using human liver RNA samples for standards and is intended to be used on eukaryotic samples. To test the applicability of the RQI feature to different RNA sample types and the correspondence between RQI and RIN values, a variety of different sample tissues and sources were evaluated. These included a series of 60 data points generated using the 20 human RNA samples from the FirstChoice total RNA human survey panel (Ambion) (100 ng), either intact or degraded, for 10 or 30 min. Each sample was analyzed on both the Experion system (using the Experion RNA StdSens analysis kit) and the Agilent 2100 bioanalyzer (using an Agilent RNA nano kit), and the measured RQI and RIN values were plotted (Figure 4). The best-fit line through the 60 data points shows a tight correlation with an R2 value of 0.9449, and within each case, the RQI and RIN values are within one unit of each other. Therefore, the RQI value has been shown to be functionally equivalent to the RIN value in reliably determining RNA integrity.

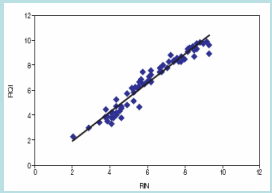

Fig. 4. Correlation between RQI and RIN values. A total of 60 samples were analyzed on the Experion with the RNA StdSens analysis kit and on the 2100 bioanalyzer with the RNA nano kit. The 60 data points were generated using the 20 human RNA samples from the FirstChoice total RNA human survey panel (100 ng), either intact or heat degraded, for 10 or 30 min. Calculated R^2 is 0.9449 for a slope of y = 1.0542x – 0.258.

5 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 5, for each qPCR reaction, the detection of amplified product was seen at successively later cycles as the RNA was degraded over time. In qPCR experiments, the CT 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 CT values of the qPCR reactions from the five gene transcripts are shown in Table 2.

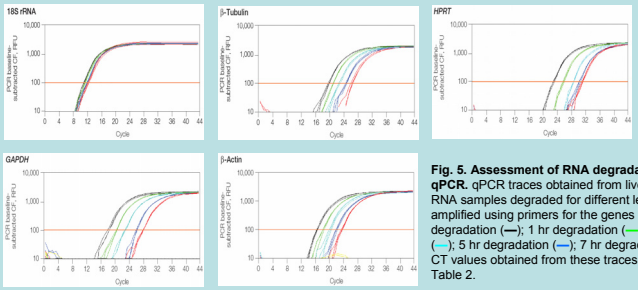

Fig. 5. Assessment of RNA degradation by real-time qPCR. qPCR traces obtained from liver carcinoma total RNA samples degraded for different lengths of time and amplified using primers for the genes indicated. No degradation (—); 1 hr degradation (—); 3 hr degradation (—); 5 hr degradation (—); 7 hr degradation (—). Mean CT values obtained from these traces are shown in Table 2.

Incubation time, hr	C_T RQI	18S rRNA	β-Actin	GAPDH	HPRT	β-Tubulin
0	9.9	11.2	16.5	18.1	22.5	19.7
1	6.9	11.6	18.2	20.5	25.1	20.9
3	3.5	12.0	20.1	22.9	27.9	22.7
5	1.8	12.1	22.0	26.1	29.5	24.6
7	1.5	12.5	23.5	28.0	30.0	26.5
ΔC_T	-	**1.3**	**7.0**	**9.9**	**7.8**	**6.8**

Table 2. Impact of RNA degradation on real-time qPCR CT. Real-time qPCR was performed on liver carcinoma RNA samples that were degraded for different lengths of time by incubation at 90°C. Mean CT values of five transcripts obtained from triplicate reactions were determined at a threshold of 100 relative fluorescence units (RFU) using the iCycler iQ real-time PCR detection system with version 3.1 software. ΔCT indicates the change in CT value over the 7 hr degradation period. Traces for the qPCR reactions from which these data were derived are shown in Figure 5.

6 Time course of transcript degradation.

To graphically present the data from Figure 5, the proportion of RNA that could be amplified was plotted as a function of degradation time (Figure 6). This data indicates that the transcripts do not degrade at the same rate within the same RNA sample. It is clear that comparing qPCR results derived from RNA in different states of degradation will generate very different quantitative conclusions. This can be as great as 1000 fold, as seen in Figure 5 with samples subjected to 7 hr of heat degradation.

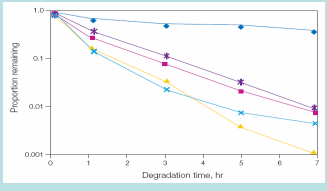

Fig. 6. Time course of degradation of specific liver carcinoma transcripts. The proportion of remaining RNA capable of being amplified is shown as a function of time of incubation at 90°C. The plot is based on the assumption that 100% PCR effi*ciency was achieved and that for each successive PCR cycle, the difference in RNA remaining is equivalent to half that of the previous cycle.
18S (—); β-actin (—); *GAPDH* (—); *HPRT* (—); β-tubulin (—).

7 Correlation of RQI values with qPCR quantitation of RNA degradation

To correlate the relative amount of remaining RNA of the five different gene transcripts with the measured RQI of the RNA samples, values were plotted as shown in Figure 7. The graph shows that the transcript levels for all five genes decrease logarithmically relative to the RQI measurements down to an RQI value of 3.

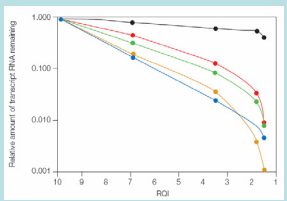

Fig. 7. Correlation between RQI and the relative amount of specific transcript RNA remaining. An arbitrary value of 1 was assigned to the transcript levels corresponding to an RQI of 10. All other values were calculated from the CT values shown in Table 1, assuming that the number of transcripts is reduced by a factor of 2 for each CT increase of 1.0. 18S (—); β-actin (—); *GAPDH* (—); *HPRT* (—); β-tubulin (—).

CONCLUSION

1. The measurement of RNA degradation is critical to reliable real-time qPCR results.

2. 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.

3. The RQI method developed for the Experion automated electrophoresis system offers a robust assessment of RNA integrity, using an algorithm that compares three regions of an electrophoretic profile to a series of degradation standards. The simple 3 region concept behind the RQI gives results that are comparable to the RIN. The RQI is accurately calculated over a wide range of RNA concentrations (200 pg to 500 ng), is very reproducible (%CV <3), and is applicable to a wide range of mammalian tissues.

4. By providing an RQI score and electropherogram, the Experion automated electrophoresis system allows even the most inexperienced user to quickly and effectively quantitate the level of degradation of an RNA sample prior to gene expression analysis. We propose that RQI can be used as a standardized measure of RNA degradation in mammalian samples, in order to reliably detect differences in gene expression using real-time qPCR experiments.