RNA Integrity Number (RIN) – Standardization of RNA Quality Control

Application

Odilo Mueller
Samar Lightfoot
Andreas Schroeder

Abstract

The assessment of RNA integrity is a critical first step in obtaining meaningful gene expression data. Using intact RNA is a key element for successful microarray or RT-PCR analyses. The Agilent 2100 Bioanalyzer System and RNA kits play an important role in assisting researchers in the determination of RNA quality. Profiles generated on the Agilent 2100 Bioanalyzer System yield information on concentration, allow a visual inspection of RNA integrity, and generate ribosomal ratios. This Application Note describes a new software algorithm that has been developed to extract information about RNA sample integrity from a Bioanalyzer electrophoretic trace.

Agilent Technologies

Introduction

Determining the integrity of RNA starting materials is a critical step in gene expression analysis. The Agilent 2100 Bioanalyzer System and associated RNA 6000 Nano and Pico kits have become the standard in RNA quality assessment and quantitation[1,2]. Using electrophoretic separation on microfabricated chips, RNA samples are separated and subsequently detected via laser induced fluorescence detection. The Bioanalyzer software generates an electropherogram and gel-like image and displays results such as sample concentration and the so-called ribosomal ratio. The electropherogram provides a detailed visual assessment of the quality of an RNA sample. However, methods that rely on human visual interpretation of data are intrinsically flawed. Previously, researchers have used the ribosomal ratio in both slab gel analysis and as a feature within the Bioanalyzer software to characterize the state of RNA intactness. Slab gel analysis of total RNA samples using ribosomal ratios often results in an inaccurate assessment of the RNA integrity[3]. The Agilent 2100 Bioanalyzer System provides a better assess-ment of RNA intactness by show-ing a detailed picture of the size distribution of RNA fragments.

RNA degradation is a gradual process. As degradation proceeds (Figure 1), there is a decrease in the 18S to 28S ribosomal band ratio and an increase in the baseline signal between the two ribosomal peaks and the lower marker. The Bioanalyzer software automatically generates the ratio of the 18S to 28S ribosomal subunits. Although ribosomal ratios play an important role in determining the level of sample degradation in gel electrophoresis, the more detailed analysis on the Agilent 2100 Bioanalyzer System reveals that it inadequately describes sample integrity.

In order to standardize the process of RNA integrity interpretation, Agilent Technologies has introduced a new tool for RNA quality assessment. The RNA Integrity Number (RIN), was developed to remove individual interpretation in RNA quality control. It takes the entire electrophoretic trace into account. The RIN software algorithm allows for the classification of eukaryotic total RNA, based on a numbering system from 1 to 10, with 1 being the most degraded profile and 10 being the most intact. In this way, interpretation of an electropherogram is facilitated, comparison of samples is enabled and repeatability of experiments is ensured.

Development of the RIN tool

The RIN software algorithm was developed for samples acquired with the Eukaryote Total RNA Nano assay on the Agilent 2100 Bioanalyzer System. Input data included approximately 1300 total RNA samples from various tissues, three mammalian species (human, mouse and rat), all with varying levels of integrity. Categorization of the RNA samples was done manually by application specialists who classified each total RNA

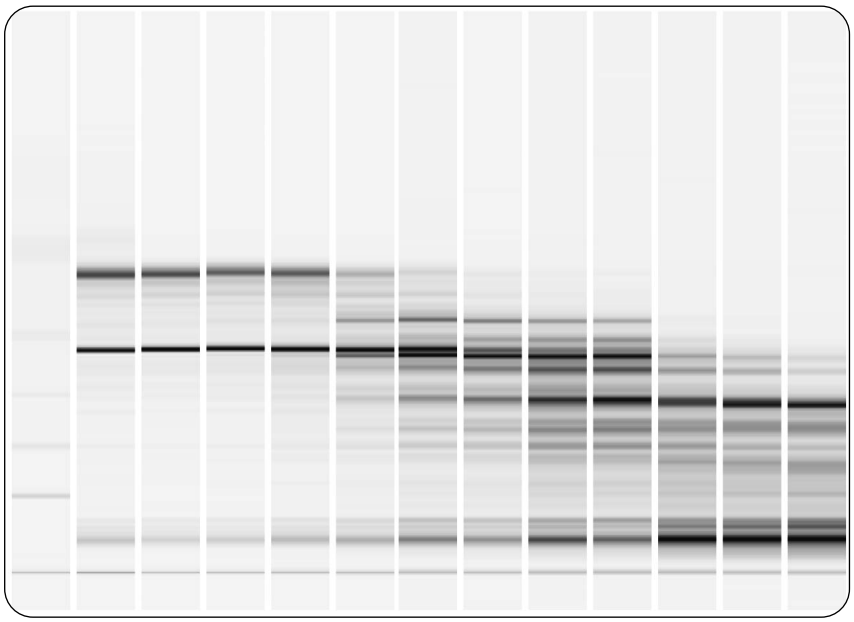

Figure 1
A total RNA sample was degraded for varying times and the resulting samples were analyzed on the Agilent 2100 Bioanalyzer System using the Eukaryote Total RNA Nano assay. A shift towards shorter fragment sizes can be observed with progressing degradation.

sample within a predefined numeric system from 1 through 10. Figure 2 shows representative electropherograms for different RIN classes (10, 6, 3, 2, respectively).

For development of the RIN algorithm, adaptive learning tools, such as neural networks, were employed (tools provided by quantiom bioinformatics). They allowed the determination of critical features that can be extracted from an electrophoretic trace. These features are parts of an electropherogram that can be analyzed using an appropriate integrator. They can be signal areas, intensities, ratios etc. Important elements of an electropherogram are listed in figure 3. They include different regions (pre-, 5S-, fast-, inter-, precursor-, post-region) and peaks (marker, 18S, 28S).

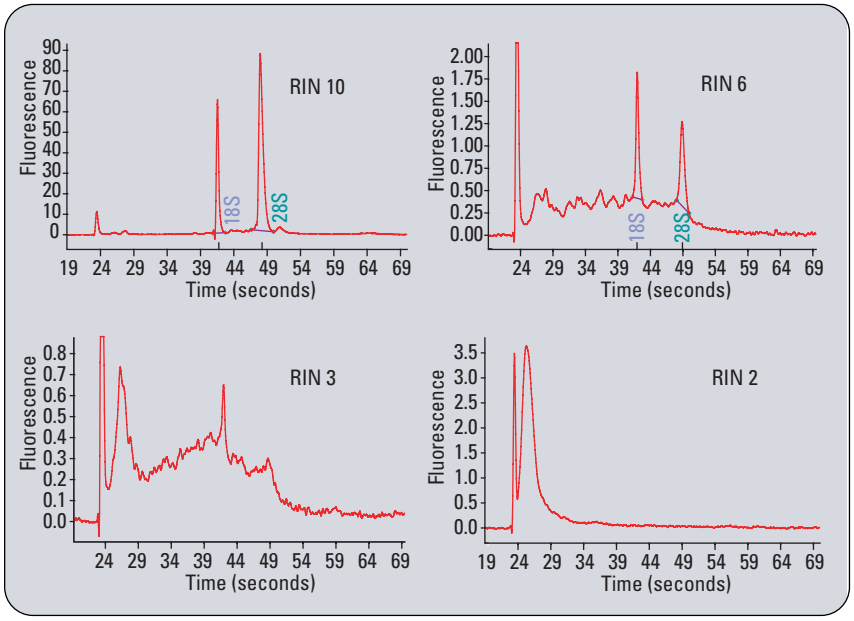

Figure 2
Sample electropherograms used to train the RNA Integrity Number (RIN) software. Samples range from intact (RIN 10), to degraded (RIN 2).

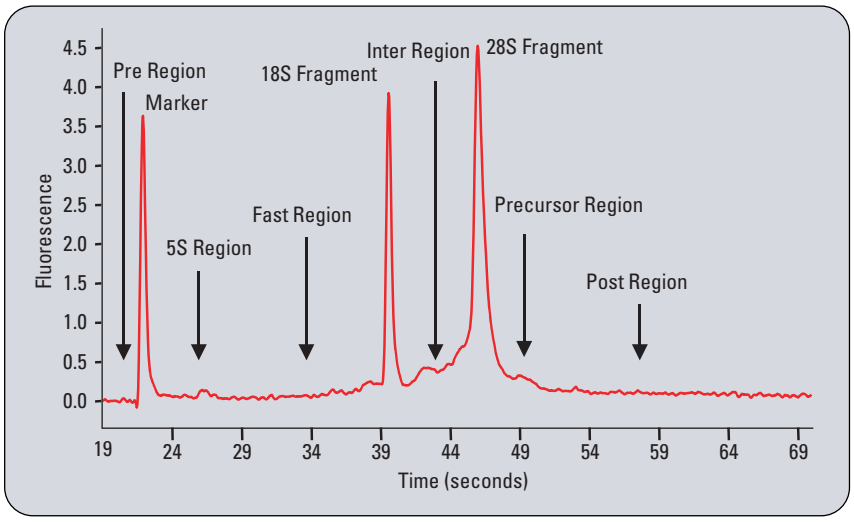

Figure 3
Electropherogram detailing the regions that are indicative of RNA quality.

RIN visualization

RIN will be part of the Agilent 2100 expert software. Data found in previous versions of the biosizing software can also be found in the next expert software version, for example, RNA area, RNA concentration, rRNA ratios. The RIN software includes the RIN number (Figure 4), which can be expressed either as a decimal or integer. The RIN value can be changed from a decimal to an integer in the Assay Properties tab, in the Set Point Explorer under Global Advanced settings. RIN values may not be computed if the software finds an unexpected peak or signal in certain regions. This will result in an error message indicating that an

anomaly has been detected (listed in the error tab of the software). Anomalies include genomic DNA contamination, ghost peaks, spikes, and wavy baselines. Anomalies can be divided into two classes: critical and non-critical. Non-critical anomalies, for example, a spike in the post region, will result in the computation of a RIN number while critical anomalies, for example, spikes in the fast region, will result in no RIN computation. If an anomaly is not deemed to be critical (such as genomic contamination, where a DNase digest should be performed to obtain meaningful data), a RIN value can still be computed by increasing the anomaly threshold settings found in the advanced settings in the Set Point Explorer for the sample that has been flagged (Figure 5). The maximum value for anomaly threshold detection is 1. Description of the error message will correspond to an appropriate threshold number.

Results obtained with RIN

The RIN software was developed to remove user-dependent interpretation of RNA quality. Characterization of total RNA samples is largely independent of the instrument, sample concentration, and the operator allowing for the comparison of samples across different laboratories.

RNA integrity

Figure 6 shows three RNA samples at varying stages of intactness. The RIN tool gave three different designations representing their respective intactness. During a large validation study using different samples from the ones that

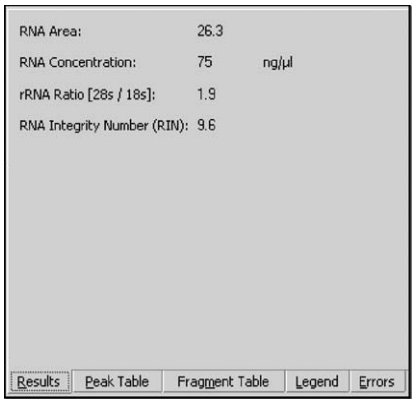

Figure 4
RIN visualization in the Agilent 2100 Bioanalyzer System expert software. RIN numbers are found in the results tab, while the error tab will contain useful information in cases where the RIN was not computed.

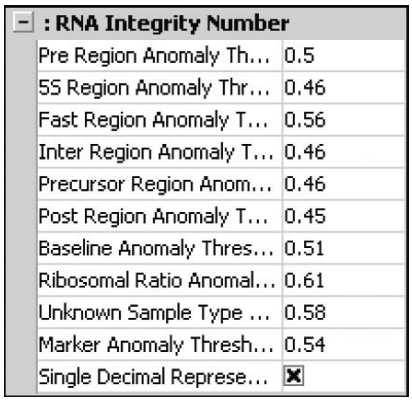

Figure 5
Changing anomaly thresholds and single decimal RIN representation. If critical anomalies have been detected during the analysis, in many cases RIN values can still be computed by increasing the thresholds (max. = 1). Information regarding anomalies can be found in the error tab.

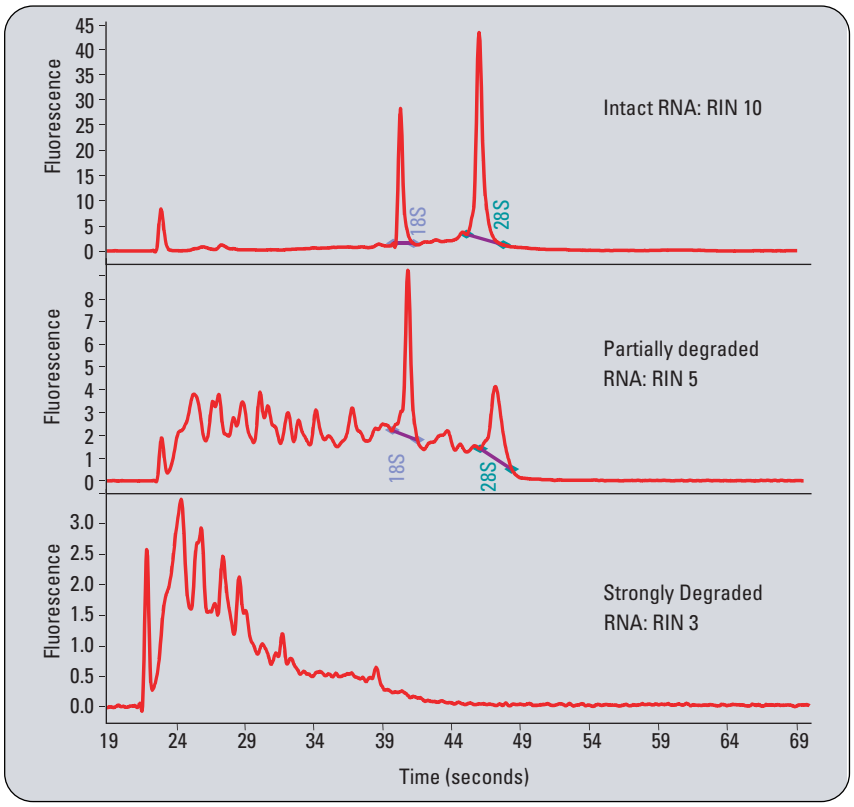

Figure 6
The RNA integrity number was tested on samples of varying levels of intactness. The RIN software algorithm was able to accurately classify the samples.

were used to train the algorithm, reliable sample classification was obtained.

Ribosomal ratios

Figure 7 shows the same sample human brain (Ambion, Inc.) total RNA that was run on three different instruments and representative electropherograms of instrument 1 and 3 are shown. Ribosomal ratios as generated with the Bioanalyzer software are compared with RIN values. For the 36 samples there is a larger degree of variability when using ribosomal ratios as compared to RIN values. Calculated RIN values were at 1.4 % coefficient of variance while ribosomal ratios had a 5.1 % CV. Keep in mind that these CV values refer to identical samples. When including samples from different species and tissues, significantly larger CV values are found for the ribosomal ratio.

When analyzing a sample at various dilutions a similar picture is found (figure 8). Mouse brain total RNA was diluted into three different concentrations, 25 ng/µL, 100 ng/µL and 500 ng/µL. For the 108 samples tested it is apparent that the RIN value outperforms the ribosomal ratio by a large margin. RIN CVs were at 3 % versus 22 % for the ribosomal ratio. It should be noted that below 25 ng/µL, no accurate RIN values can be obtained. Best results are obtained for concentration values above 50 ng/µL.

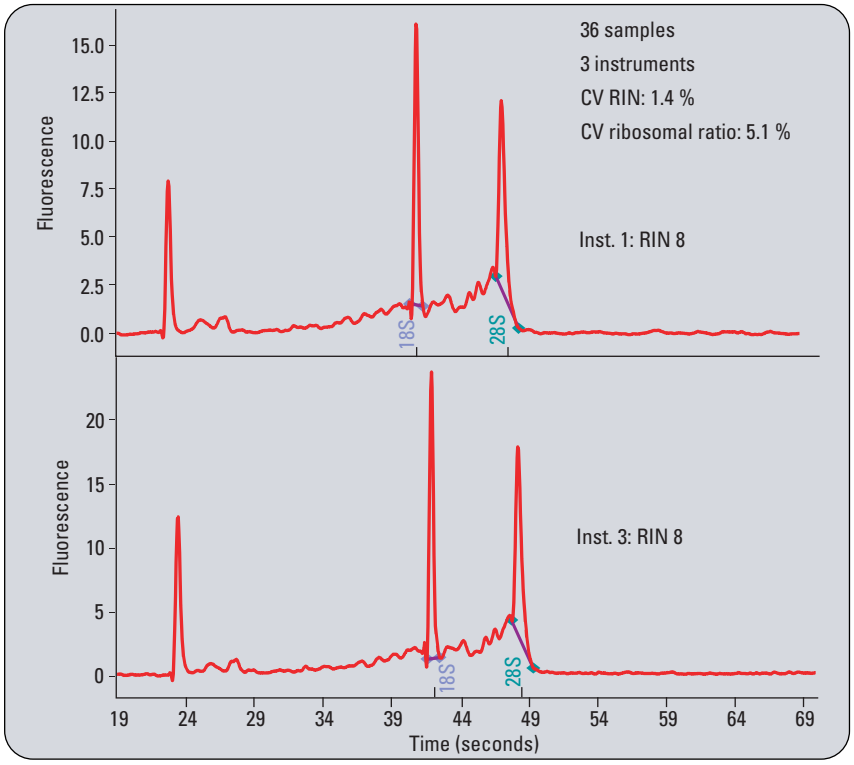

Figure 7
36 total RNA samples were analyzed on three different instruments. The RIN was compared with the ribosomal ratio value. CV's for the RIN tool were significantly lower than for the ribosomal ratios.

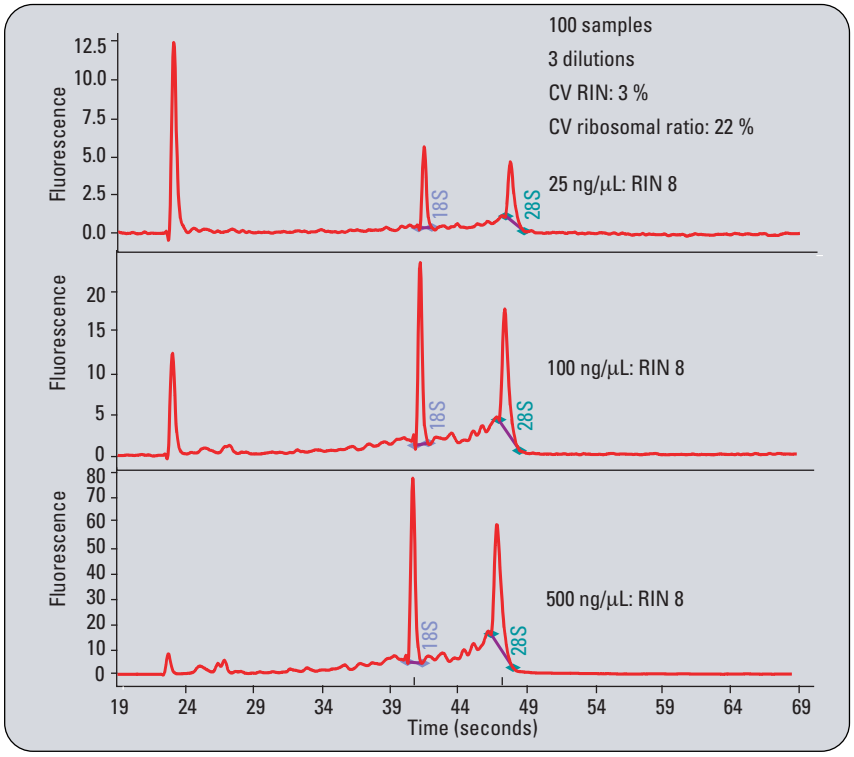

Figure 8
When testing an identical RNA sample in various dilutions, identical RINs are obtained, within narrow limits, whereas ribosomal ratios show a much lesser degree of reproducibility.

Using RIN

RIN is a powerful new tool in RNA integrity measurement. The schematic representation in Figure 9 proposes a best practices use-case for RIN. As a first step the RIN values have to be validated (Figure 9A). This can be done by correlating RIN numbers with a specific downstream experiment such as microarray analysis or RT-PCR. This correlation step can be used to establish a RIN threshold value between successful down-stream experiments and failed experiments. It can be performed on sets of existing Bioanalyzer data, if available. After the thresh-old value has been established, this value can be used in the standard RNA QC procedure (Figure 9B). All samples with a RIN higher than the threshold value pass the QC test, while samples below the threshold value are discarded. The correlation step of validating the RIN has to be repeated if a significant experimental parameter is changed (for example, different organism investigated, different type of microarray used, using different probe sets, etc.). So far the RIN algorithm has been tested using the eukaryote total RNA Nano assay.

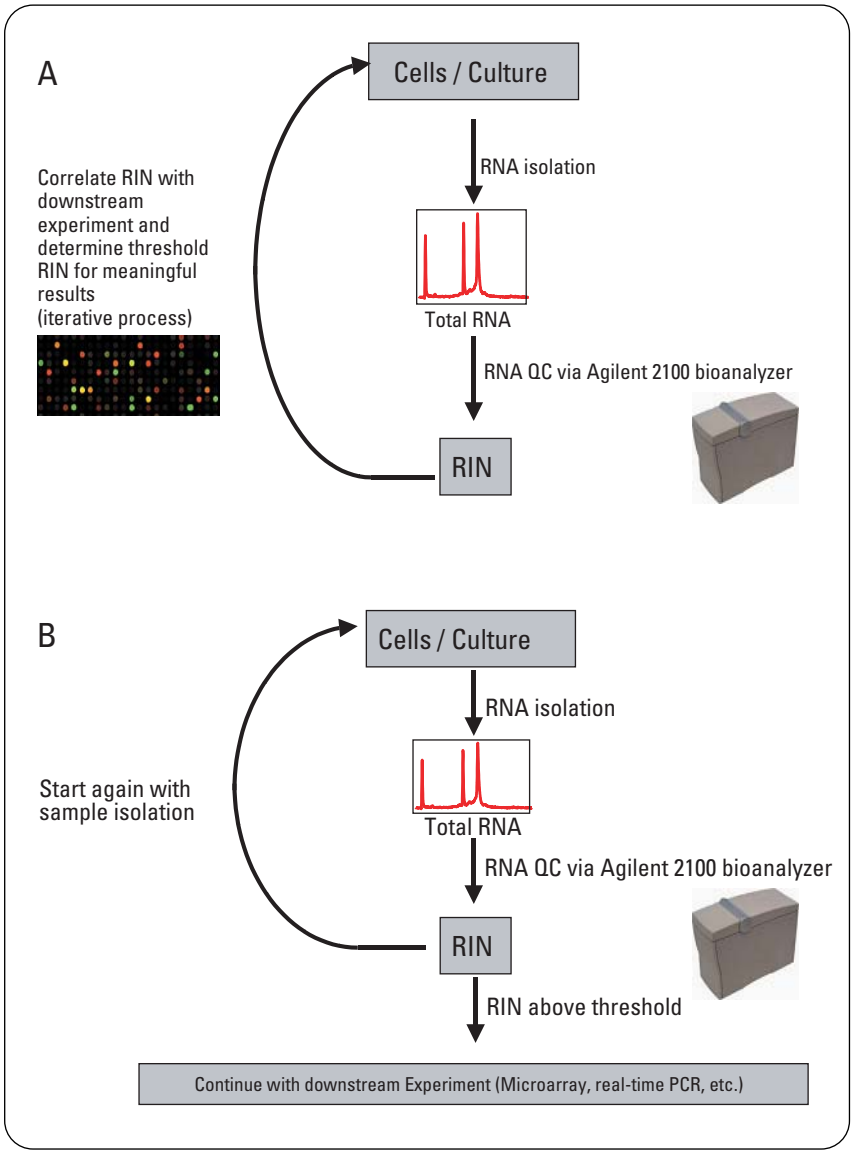

Figure 9
A. Correlate RIN values with downstream experiments (e.g. microarray or RT-PCR) and determine threshold value for obtaining meaningful gene expression results.
B. Once initial correlation experiment has been performed, and data thresholds have been set, RIN values can be used to discard samples that do not pass the sample QC on the Agilent 2100 Bioanalyzer System (RIN below threshold).

RIN limitations

RIN has been designed to provide unambiguous assessment of RNA integrity. A measure of sample integrity is given, which can be used to directly compare samples before and after shipment, and for the comparison of samples from one lab to another lab. Most importantly, it can be used to ensure the repeatability of gene expression experiments as far as the sample extraction step is concerned. However, RIN cannot predict the usefulness of gene expression data without prior validation work. As an example, a sample might be too degraded to perform a genome-wide microarray experiment, but might deliver good RT-PCR data. It is essential that the necessary correlative work be done in order to effectively use RIN.

Conclusion

We have designed a software algorithm that is capable of assessing RNA quality better than ribosomal ratios. The RIN tool is a major step in the standardization of RNA integrity assessment. The researcher is no longer tied to arbitrary classification of total RNA. By performing correlative experiments, thresholds can be created to ensure repeatability of experiments. The RIN tool has been found to be largely free from instrument and concentration variability, thereby facilitating the comparison of samples from instrument to instrument and lab to lab.

References

1.
"Quantitation comparison of total RNA using the Agilent 2100 Bioanalyzer, ribogreen analysis, and UV spectrometry", *Agilent Application Note*, *Publication Number 5988-7650EN*, **2002**.

2.
"A microfluidic system for high-speed reproducible DNA sizing and quantitation", *Electrophoresis*, *21(1)*, *128-34*, **2000.**

3.
"Advancing the quality control methodology to asses isolated total RNA and generated fragmented cRNA", *Agilent Application Note*, *Publication Number 5988-9861EN*, **2003.**

Acknowledgements

We would like to extend special thanks to our collaboration partners Ambion Inc. and the German Resource Center for Genome Research RZPD as well as to Quantiom Bioinformatics. We would also like to acknowledge with special gratitude all the researchers who alpha-tested the RIN algorithm and provided valuable inputs.

Odilo Mueller is Assay Manager Nucleic Acid LabChip kits, Samar Lightfoot is Assay Support Biochemcist for Nucleic Acids and Andreas Schroeder is Software Specialist, all at Agilent Technologies, Waldbronn, Germany.

www.agilent.com/chem/labonachip

Published January 21, 2016
Publication Number 5989-1165EN

Agilent Technologies