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Drug Discovery

ASSAY TUTORIAL

Simplifying HT RNA Quality & Quantity Analysis

Automated CE System Designed to Improve Rapid Assessment

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Current methods for the determination of RNA quantity/quality/integrity are labor-intensive, costly, and often cannot deliver adequate information or analyze high numbers of samples. New developments in capillary electrophoresis (CE) now provide for automated RNA quality/quantity analysis in a high-throughput environment.

Spectrophotometry is the most common method for RNA quantification, where the absorbance at 260 nm gives quantity, while the ratio of 260 nm/280 nm gives a measure of purity with respect to protein or solvent contaminants. However, the absorbance method does not give any differentiating information related to DNA contamination or the degradation state of the RNA (RNA integrity). Also, fluorometric methods are commonly used to quantify RNA, but lack information on RNA integrity and often miss the presence of contaminants such as genomic DNA (gDNA).

Partially denaturing agarose gel electrophoresis is used for determining the quality of an RNA sample, wherein the ratio or visual presence of the large and small subunit (28S/18S) of the ribosomal RNA is correlated with the integrity of the RNA. However, this method is labor-intensive, requires large amounts of extracted RNA, lacks resolution, and is prone to errors in the interpretation of RNA quality.

Recent improvements to the agarose gel method include the use of microgels sandwiched within laminated tape-like cartridges, but the method still lacks resolution.

Until recently, the benchmark standard for determining RNA integrity has been based on microchannel, chip-based electrophoresis systems that provide faster run times and improved data quality compared to agarose gel electrophoresis, but require hands-on processing for priming and loading of gel, markers, and samples onto the system.

The samples are analyzed sequentially in the same separation channel, without flushing or priming between injections, which can result in sample carryover or trapped particulates and air bubbles that adversely affect subsequent separations.

Capillary electrophoresis offers advantages over both agarose and microchip electrophoresis in that gel filling and sample loading are automated. However, many commercial CE instruments are prohibitively expensive and focus on single-stranded DNA rather than RNA or double-stranded DNA analysis. Other CE instruments lack the sensitivity, dynamic range, and separation quality required for adequate RNA quality/quantity analysis.

Although all techniques can be interfaced to a robotic system, only the most sophisticated core labs can afford both the cost and expertise required to operate such systems.

Advanced Analytical Technologies recently introduced the Fragment Analyzer™ CE System. With the capability of running 12 or 96 samples per run, the Fragment Analyzer bridges the gap between a low-volume stand-alone instrument and one that is interfaced with a robotic system. The instrument features:

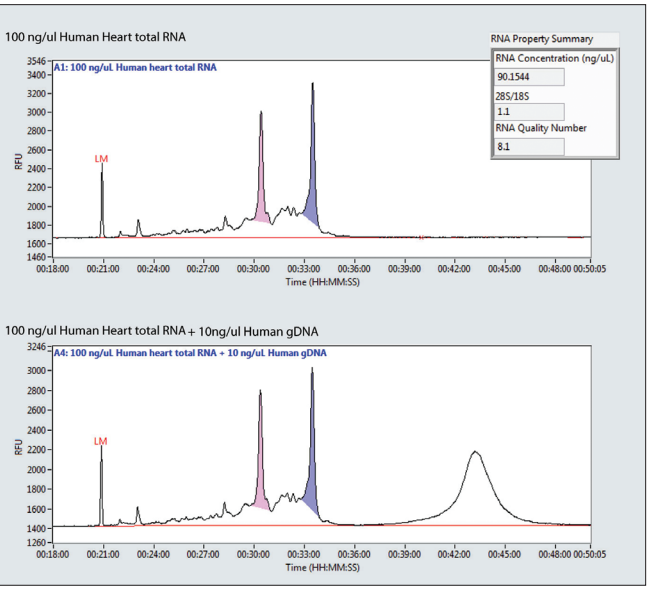

Figure 1. Human heart total RNA electropherograms obtained on the Fragment Analyzer without added gDNA (top panel) and with added gDNA (bottom panel). The blue peak corresponds to the 28S region, while the red peak corresponds to the 18S region.

Quantification of Known RNA Samples

Expected Concentration	Spectro-photometry[1]	Fluorimetry[2]	Chip-Based Electrophoresis[2]		Fragment Analyzer[2] 12-Capillary Array	
Concentration ng/µL	Concentration ng/µL	Concentration ng/µL	Concentration ng/µL	RIN (8.5±0.3)[3]	Concentration ng/µL	RIN (8.5±0.3)[3]
500	528.2 ± 7.7	451.0 ± 7.1	643.0 ± 4.2	8.0 ± 0.1	568.5 ± 0.7	8.7 ± 0.0
250	271.9 ± 1.6	231.0 ± 9.9	329.5 ± 24.7	8.4 ± 0.1	282.5 ± 19.1	8.6 ± 0.1
100	109.8 ± 1.9	91.8 ± 1.8	139.0 ± 9.9	8.9 ± 0.1	101.0 ± 1.4	8.8 ± 0.1
25	26.3 ± 0.2	21.9 ± 0.4	36.5 ± 3.5	8.7 ± 0.4	27.0 ± 4.2	8.5 ± 0.1
5	29.8	4.1 ± 0.1	9.0 ± 1.4	8.7 ± 1.2	5.0 ± 0.0	8.1 ± 0.4

1) Average ± standard deviation from 3 replicates. 2) Average ± standard deviation from 2 replicates. 3) Average from samples of all concentrations.

Comparison of the Fragment Analyzer to other methods for measurement of RNA quantity and quality: A dilution series of rat liver total RNA was prepared and analyzed concurrently by all four methods.

- An automated capillary flush with fresh gel between every injection cycle to prevent sample carryover.
- The ability to run up to three 96-well sample plates unattended, with a capacity of over 1,500 samples per day.
- The ability to switch between two different gel types automatically between injections, enabling both RNA and DNA to be run on the same instrument without having to manually load gels between runs.
- The ability to analyze total RNA and messenger RNA (mRNA) quantity/quality (integrity), DNA fragments, DNA smears, gDNA, and next-generation sequencing DNA library samples.
- High separation resolution.
- Two different kits for RNA; standard sensitivity with an input sample concentration range of 5 ng/µL to 500 ng/µL and a high sensitivity kit with a range of 50 pg/µL to 5,000 pg/µL.
- Highly reproducible injections between instruments and between runs.

RNA Analysis

The Fragment Analyzer enables the simultaneous analysis of both the quantity and integrity of RNA in a single measurement. *Figure 1* (top panel) shows a typical electropherogram output of the Fragment Analyzer, displaying a measured concentration of human heart RNA of 90 ng/µL (98 ng/µL as measured by spectrophotometry).

One of the key benefits of the Fragment Analyzer is the ability to give an indication of contamination of gDNA, whereas other methods may give misleading results or not measure the presence of gDNA. For a sample with gDNA contamination, spectrophotometry reports anomalously high RNA concentration values, while flourometry or chip-based electrophoresis may report the accurate quantity of RNA, but entirely miss the presence of gDNA.

Figure 1 (top panel) shows human heart RNA without gDNA contamination. *Figure 1* (bottom panel) shows the same sample with gDNA added, using the Fragment Analyzer. This same sample analyzed on a chip-based electrophoresis system does not show the presence of gDNA.

The *Table* shows the quantification of known RNA samples using four different techniques: spectrophotometry, fluorometry, chip-based electrophoresis, and CE with the Fragment Analyzer. The results show that the measured RNA concentration by the Fragment Analyzer is within 15% of the expected values. The chip-based electrophoresis method exhibited high deviations from expected values, with an error of between 30–80% in some cases.

The Fragment Analyzer reports the quality or integrity of RNA as a calculated RQN (RNA quality number).

The RQN is equivalent to the broadly accepted RIN and is based on a proprietary algorithm that uses three areas of the electropherogram: (A) the area before the 18S peak, (B) the total area of the 18S and 28S peaks, and (C) the ratio of the 28S and 18S peaks.

In the *Table*, the equivalence of the Fragment Analyzer RQN vs. the chip electrophoresis RIN number can be observed, with an agreement of better than 10%. The equivalence of the Fragment Analyzer RQN to RIN is further validated in *Figure 2*.

A correlation between the Fragment Analyzer RQN and the RIN, based on the analysis of over 100 samples from various origins with various states of degradation at different concentrations, demonstrates excellent agreement and equivalency between the two methods. GEN

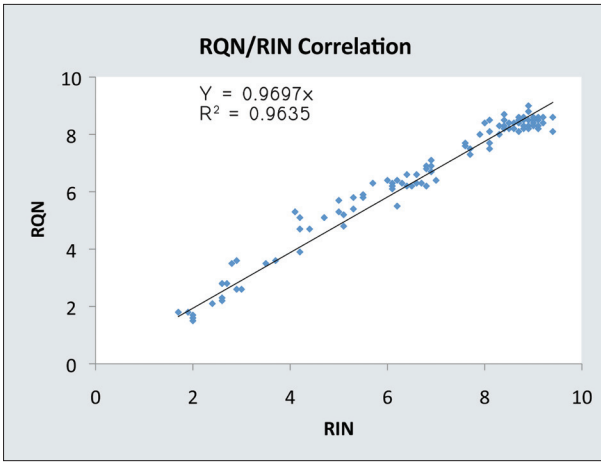

Figure 2. A correlation between the Fragment Analyzer RQN and the well-established chip-based electrophoresis RIN, showing that the Fragment Analyzer RQN is equivvalent to RIN. The dataset includes over 100 samples from various origins with various states of degradation at different concentrations.

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