White Paper

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A Review of RNA Analysis using the Agilent Automated Electrophoresis Portfolio

Abstract

Good-quality RNA is crucial to the success of many downstream applications, including qPCR, microarray analysis, and RNA sequencing. Agilent automated electrophoresis instruments are designed to aid in determining the integrity of RNA. While every instrument offers unique capabilities and throughputs, each assesses RNA by assigning an objective quality metric to every sample. The systems provide necessary integrity, quantity, and size information that can be used to assess RNA from different extraction methods or tissues and in vitro transcription (IVT). Here, we highlight a variety of peer-reviewed publications that use the automated electrophoresis instruments for RNA analysis in their workflows.

Introduction

The Agilent automated electrophoresis instruments are used for fast and accurate assessment of RNA quality and quantity. These instruments include the Agilent 2100 Bioanalyzer system, the Agilent Fragment Analyzer systems, and the Agilent TapeStation systems. Each system has a comparable quality metric that provides an objective measure to assess the integrity of total RNA[1,2,3]. The RNA Integrity Number (RIN) is a well-established quality score, providing a reliable and objective assessment of sample quality using the Bioanalyzer. The Fragment Analyzer uses the RNA Quality Number (RQN). The RNA integrity number equivalent (RIN^e) is the metric used by the TapeStation systems. While different algorithms are used for determining these quality scores, each considers the electrophoretic separation of the RNA sample, including the ribosomal fragments and the presence or absence of degradation products. Samples are automatically provided a score from 1 to 10, where 1 indicates strongly degraded RNA, and 10 indicates the highest possible RNA quality. Knowing the quality of an RNA sample can help researchers decide if a sample is sufficient for downstream analyses. In this paper, we discuss the importance of RNA QC, and highlight the use of the automated electrophoresis instruments during many workflows, including RNA extraction, IVT RNA synthesis, qPCR, microarray analysis, and RNA sequencing.

RNA extraction

The quality of starting material is crucial for any successful downstream experiment. For applications using RNA or cDNA, the first step is to determine the quality of the extracted RNA. Researchers can use the information from this initial QC check to make important decisions for downstream workflows or help ascertain possible improvements to the extraction protocol used in order to generate samples of the highest quality. For example, Ford et al.[4] used the Bioanalyzer to assess RNA extracted from different porcine tissues using different commercially available kits. By identifying the method that yielded the best results in preliminary studies, they were able to preserve their limited clinical human samples by validating the RNA extraction workflow with only one kit. Different tissues can have specific challenges when it comes to nucleic acid extraction, including a low concentration of starting RNA, an abundance of RNases and PCR inhibitors, the size of the tissue, and how fibrous the tissue is. Thus, the authors state "for technically demanding and costly applications such as RNA sequencing or other high-throughput screening methods, it is critical to validate the RNA extraction methods for each sample type, as no single method is optimal for all." Shown in Figure 1 are digital gel images and electropherogram traces generated by the Bioanalyzer, indicating the RIN of RNA from porcine ventricular tissue using different extraction kits. Both total RNA (A-C) and small RNA analyses (D) were performed, as the authors state: "Bioanalyzer results should be treated critically–a high RIN alone may not indicate a successful extraction, as inspection of the electropherograms may indicate problems with the RNA not detected by the RIN calculation. Considering this, we would encourage authors to publish representative Bioanalyzer traces as well as RIN values. Furthermore, if small RNAs are of interest in subsequent analyses, the Small RNA chip should be run, since a high RIN does not necessarily indicate good recovery of small RNAs."[4] Together with other tests, the authors were able to successfully identify a suitable method for RNA extraction of human heart tissue for clinical studies.

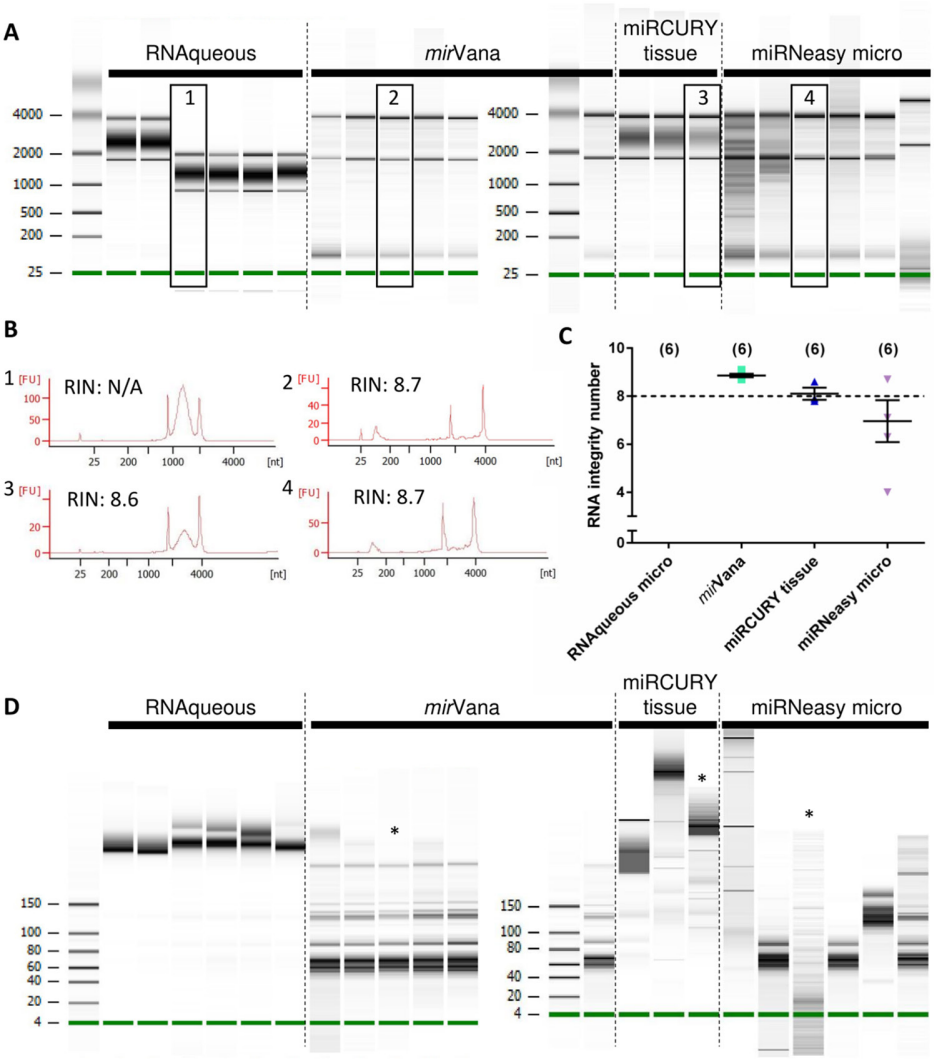

Figure 1. RNA analysis of porcine ventricular tissue using the Agilent 2100 Bioanalyzer system. A-C) Total RNA analysis was performed with the Agilent RNA 6000 Nano kit. Extraction methods 2 and 3 consistently show a high RIN > 8, but method 3 also shows a smear at 2,500-3,500 nt. Method 4 gives variable RINs. With method 1, the RIN was undetectable, and the authors state that an unexplained mobility shift was observed in some samples. However, further examination of the electropherogram image indicates that the lower marker may have been misaligned in some electropherograms, which could explain this shift. D) Analysis of the small RNA with the Agilent Small RNA kit indicated that methods 1 and 3 failed to recover miRNAs, while methods 2 and 4 were more reliable. This figure reproduced from Ford et al.[4] This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license.

In another example, Velada et al.[5] used the Fragment Analyzer to compare the RNA quality they obtained from olive tissues that had been stored using different fixation methods in order to help develop a standard protocol for sample handling. The concentration and RQN of three tissue types were compared after laser microdissection (Figure 2) or paraffin-embedding. The laser microdissection method resulted in higher RQN values than the paraffin-embedding. This fixation method allowed them to limit handling of tissue sections and the amount of time the tissues were at room temperature. Additionally, RNA degradation was minimized, RNA quality was increased, and the RNA yield provided sufficient amounts for downstream gene expression analysis or transcriptomics.

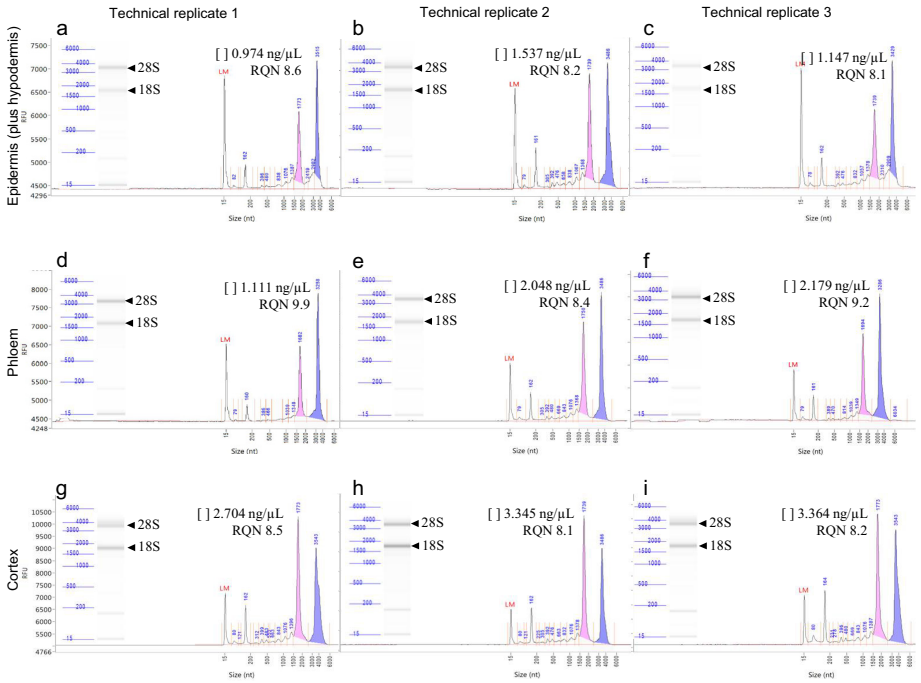

Figure 2. The Agilent Fragment Analyzer system was used to analyze the quality of RNA isolated from triplicate replicates of microdissections from olive A-C) epidermis, D-F) phloem, and G-I) cortex. The concentration and RQN reported by the Fragment Analyzer are shown for each sample. This figure reproduced from Velada et al.[5] This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license.

With a similar goal of identifying an optimal method resulting in the highest quality samples for downstream analysis, Cho et al.[6] used a TapeStation system to examine bacterial RNA quality. For RNA extraction from bacteria, the cell walls must first be lysed to release the nucleic acids. However, the efficiency of known lysis methods (such as bead beating and lysostaphin) can vary based on the cell wall structure. The researchers were interested in seeing if the antimicrobial peptides porcine myeloid antimicrobial peptide 36 (PMAP-36), protegrin-1 (PG-1), melittin, or nisin could be used to optimize the lysis conditions for *Staphylococcus aureus* and other bacterial strains. Analysis with the TapeStation system showed that the RNA from each lysis method resulted in similar yield and RIN^e values (Figure 3), indicating that PMAP-36 can be used as a bacterial lytic agent independently or in combination with other methods. Optimizing the lysis conditions may provide many benefits, including cost effectiveness, decreased bias from sample preparation, simpler methods, and higher-quality RNA.

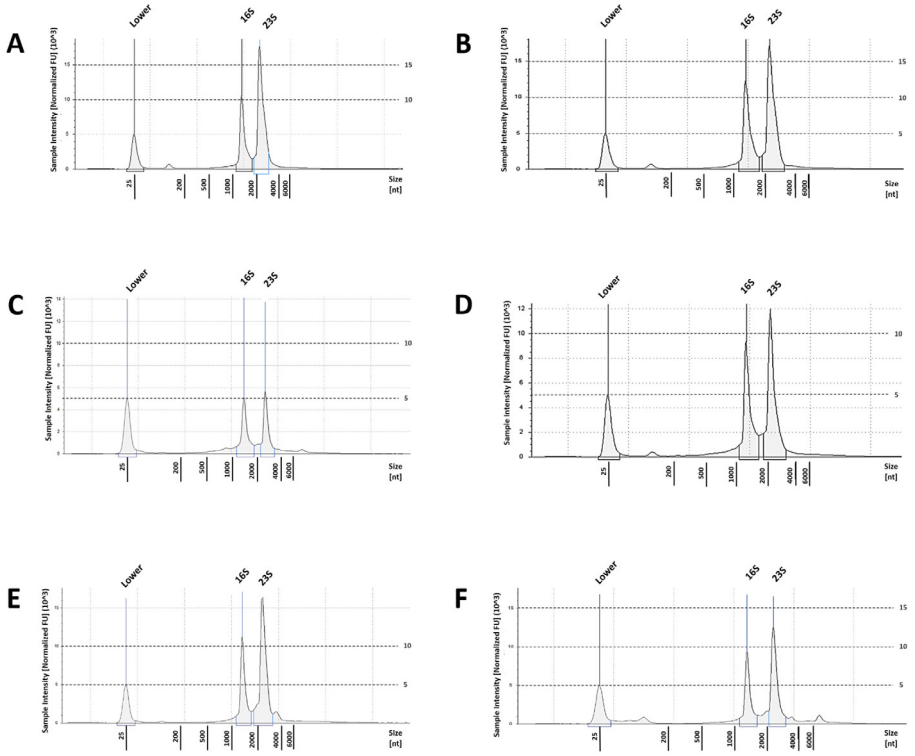

Figure 3. The Agilent TapeStation system was used to examine the quality of RNA extracted from bacteria subjected to different cell lysis conditions. *Staphylococcus aureus* was lysed with A) PMAP-36 and displayed a RIN^e of 9.3, B) Lysostaphin, with RIN^e 9.3, C) bead beating, RIN^e 8.3, D) Lysostaphin + PMAP-36, RIN^e 9.0, E) and PMAP-36 + bead beating, RIN^e 9.3. F) *Staphylococcus typhimurium* was lysed with PMAP-36, with a RIN^e of 9.3. This figure reproduced from Cho et al.[6] This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license.

While all of the automated electrophoresis instruments are capable of total RNA analysis, the Bioanalyzer and Fragment Analyzer systems are also compatible with specialized kits for the analysis of small RNA, such as microRNA (miRNA). These noncoding miRNAs are known to regulate mRNA transcription and translation, and many may be used as biomarkers for cancer and other diseases. Unger et al.[7] used both the Bioanalyzer and the Fragment Analyzer systems to compare methods for miRNA extraction from equine serum, and fresh and archived blood samples. The electropherograms generated by the Bioanalyzer show an increased amount of miRNA in one of the two commercially available kits tested (Figure 4 A-B). RNA extracted with this method was further tested to assess the effect of storage time and sample handling on sample quality. Samples with a phase separation, indicative of prolonged storage at room temperature before freezing, showed severe RNA degradation (Figure 4C). miRNA was successfully extracted from archived samples in the absence of phase separation of the sample, albeit with a moderate decrease in RNA quality as reported by a low RQN on the Fragment Analyzer (Figure 4D). Together, the results from the Bioanalyzer and Fragment Analyzer helped the researchers validate a method for sample handling and miRNA extraction to aid in obtaining high-quality samples for downstream analysis.

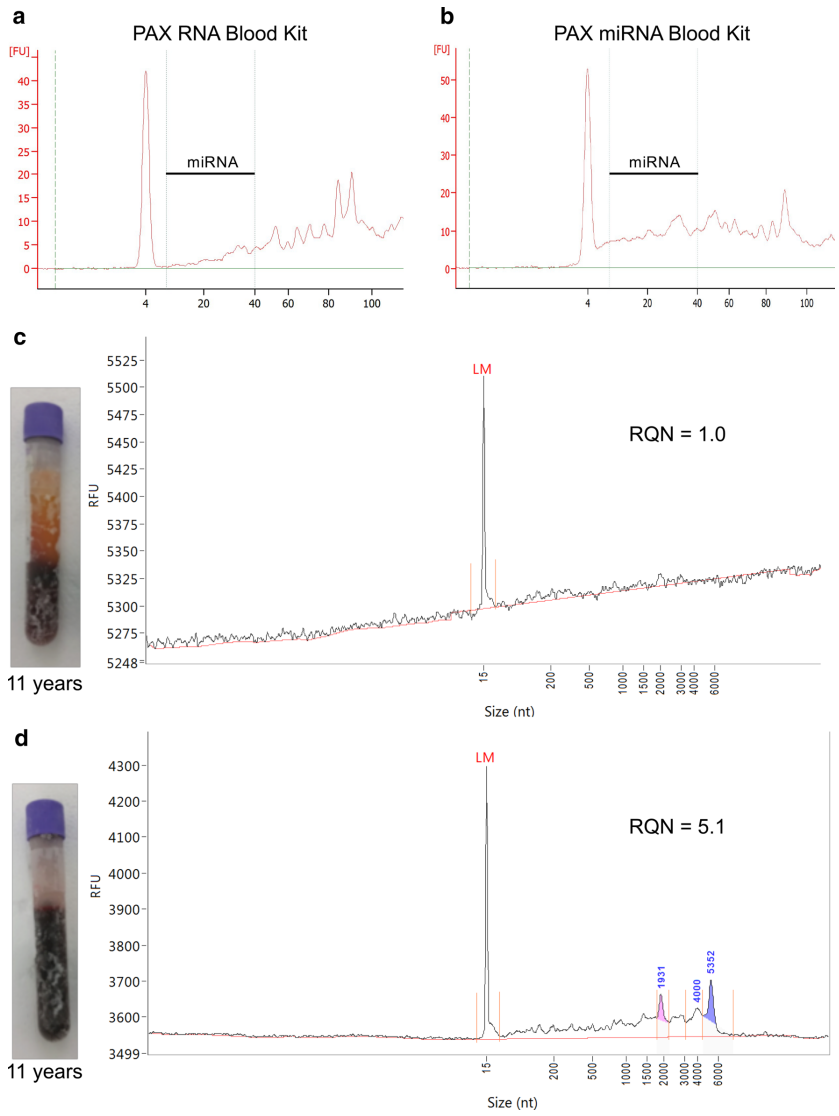

Figure 4. A-B) miRNA from equine serum was isolated from two different extraction kits and analyzed on the Agilent Bioanalyzer system with the Agilent Small RNA kit. The PAX miRNA Blood kit showed an increase in the amount of miRNA present in the small RNA region of the electropherogram. C-D) Archived samples were analyzed on the Agilent Fragment Analyzer system for RNA quality following extraction with the PAX miRNA Blood kit. Samples that showed a phase separation were further degraded (C) than samples without a phase separation (D). This figure reproduced from Unger et al.[7] This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license.

RNA from in vitro transcription (IVT)

One way to prepare synthetic RNA is through a process called in vitro transcription (IVT). The Fragment Analyzer and Bioanalyzer systems can both be used for the analysis of IVT RNA in QC steps throughout the synthesis workflow to ensure the material is of sufficient quality for downstream use. Additionally, the instruments can be used to help make improvements to the synthesis process, to reduce costs and improve the quality of the IVT RNA. Specialized methods are available for the Fragment Analyzer systems[8,9], making them uniquely capable of analyzing longer IVT RNAs such as those used in vaccine development.

Recently, IVT RNA is becoming widespread in biochemical and molecular biology research and has gained popularity in the field of vaccine development with the onset of the SARS-CoV-2 pandemic. Vogel et al.[10] discuss their process for "preclinical development of two vaccine candidates (BNT162b1 and BNT162b2) that contain nucleoside-modified messenger RNA that encodes immunogens derived from the spike glycoprotein (S) of SARS-CoV-2, formulated in lipid nanoparticles."[10] Part of the QC workflow includes analysis of the vaccines using the Fragment Analyzer to confirm the length of the synthetic RNA, as well as to determine the purity and integrity of the sample (Figure 5).

In addition to QC, the automated electrophoresis instruments can be utilized to improve protocols for preparing synthetic IVT RNAs. For instance, in a study by Hadas et al.[11], researchers sought to make an enhanced and more cost-efficient synthesis protocol by modifying the nucleotide composition of the RNAs (modRNA). By evaluating different modRNAs with the Bioanalyzer, they identified a new modRNA (nucleotide composition 5, ARCA 10) that generated a higher yield than the current composition (nucleotide composition 1, ARCA 5), while other compositions showed incomplete IVT products (Figure 6).

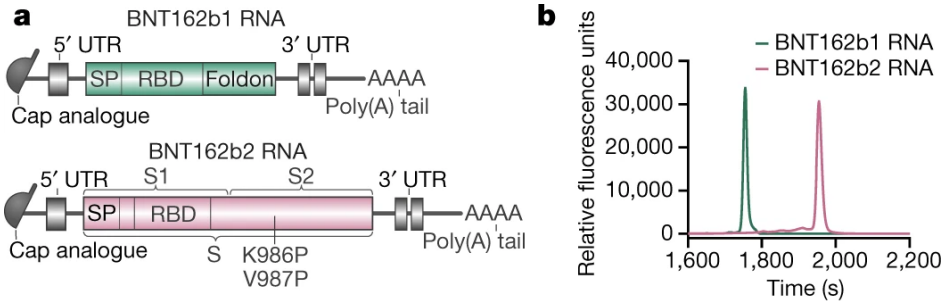

Figure 5. A) The structure of two candidate vaccines for SARS-CoV-2: BNT162b1 and BNT162b2 RNA. UTR, untranslated region; SP, signal peptide. The proline substitutions of S(P2) (K986P and V897P) are indicated. B) Analysis of the vaccine candidates with the Agilent Fragment Analyzer systems shows that "profiles of both of the RNAs show single sharp peaks that are consistent with their calculated lengths, indicating high purity and integrity." This figure reproduced from Vogel et al.[10] Permission has been obtained from the publisher.

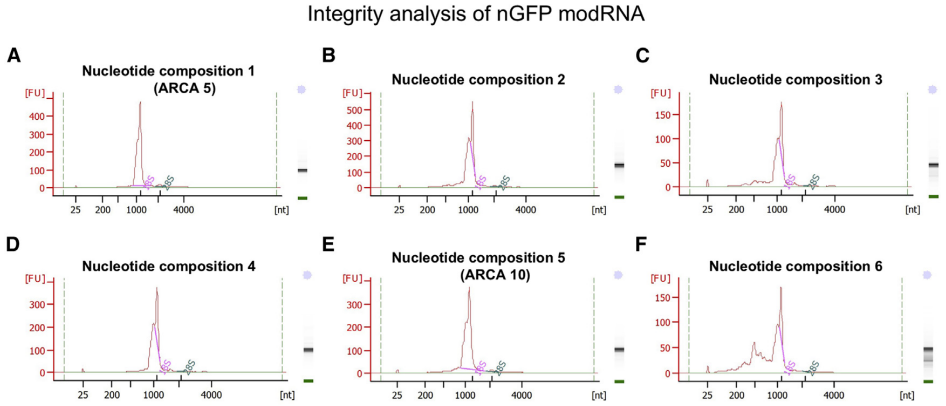

Figure 6. The Agilent Bioanalyzer system was utilized to evaluate the integrity of a variety of IVT RNAs with different nucleotide compositions. This figure reproduced from Hadas et al.[11] Permission has been obtained from the publisher.

qPCR

qPCR is used for characterization and quantification of nucleic acids. When the input material is RNA, the RNA transcripts are first reverse-transcribed into cDNA, which then undergoes PCR. The Agilent automated electrophoresis systems can help ensure successful qPCR reactions by providing information about the quality of the input RNA and helping researchers make decisions regarding their experiments.

In a study by Wylezinski et al.[12], sample handling methods for whole blood, such as long-term storage and multiple freeze-thaw cycles, were examined to investigate the effects of storage on expression profiles obtained by qPCR. RNA extracted from whole blood samples was analyzed on the Bioanalyzer for RNA quality (Figure 7) and by qPCR to examine the expression of long noncoding RNAs. Neither long-term storage nor multiple freeze thaw cycles caused degradation of the RNA, nor did the synthesized cDNA exhibit significant differences in lncRNA expression values, validating the group's sample handling methods.

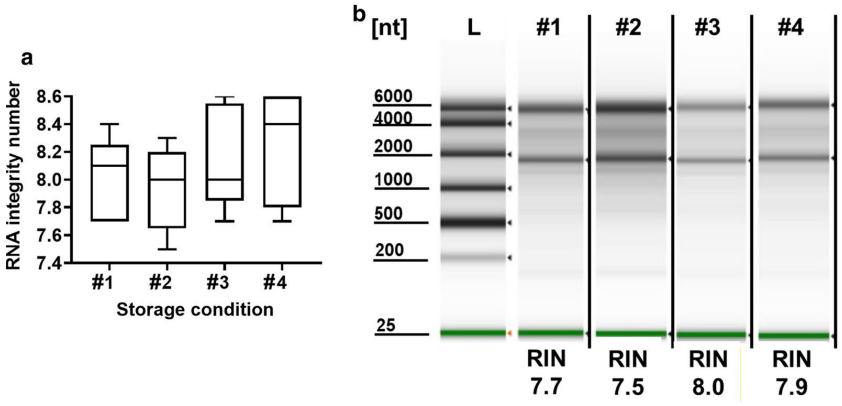

Figure 7. The effects of long-term storage on RNA integrity and impact of multiple freeze-thaw cycles on total RNA were investigated using the Agilent Bioanalyzer system with the Agilent RNA 6000 Nano kit. A) Mean RIN values reported for five individuals from each storage condition. B) Representative digital gel image for each storage condition. Storage condition 1: RNA collected from fresh blood, 2: RNA stored at -80 °C for one year, 3: RNA after five freeze-thaw cycles, 4: RNA after ten freeze-thaw cycles. No significant difference in RNA integrity was seen after 1 year of storage at -80 °C, nor over 5-10 freeze-thaw cycles. This figure reproduced from Wylezinski et al.[12] This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license.

Another group, Esteva-Socias et al.[13], further examined the effects of long-term storage and stabilization on sample integrity. They compared four different stabilization methods used for preserving lung tissue by evaluating the quality of the isolated RNA from each, using both the Bioanalyzer and RT-qPCR. Preservation via Snap Freezing (SF) and FFPE showed lower integrity (RIN average 5.2 and 1.4) than RNAlater (RNL) and snap freezing using Optimal Cutting Tissue compound (SF-OCT) (RIN average 7.6 and 8.1) (n=104 tissue samples from 20 patients). The group used qPCR to examine different amplicon lengths of the HRPT1 gene, as well as expression levels of three housekeeping genes, HPRT1, SNRPD3, and Jun. This allowed the group to "determine differences between the conservation procedures tested and their possible effect on amplification outcome of the extracted RNA in terms of C_q values."[13] The tissues with the lowest RIN scores (SF and FFPE) displayed the highest C_q values, indicating low signal and loss of amplification during the qPCR (Figure 8). Based upon the results achieved by both the Bioanalyzer and the qPCR tests, they conclude that RNL and SF-OCT preserve lung tissue quality and integrity most efficiently.

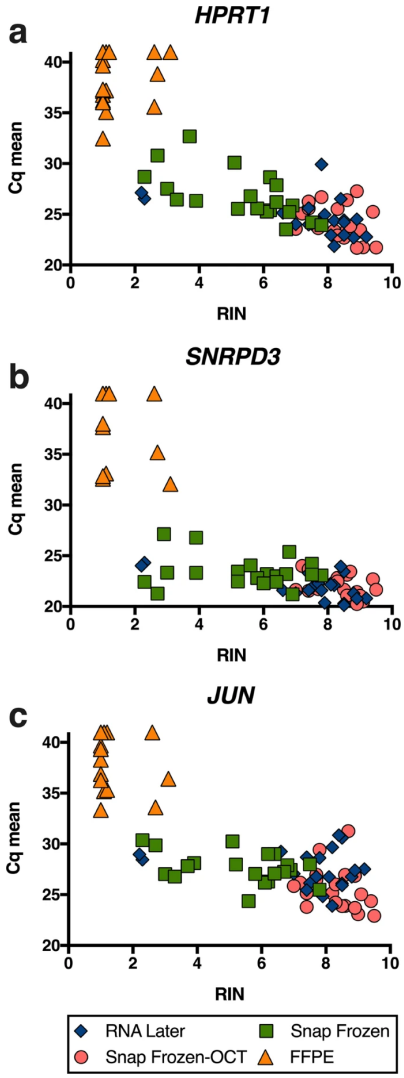

Figure 8. Comparison of RNA quality and expression levels. RNA was extracted from human lung tissue after preservation with four different methods (RNA Later, Snap Frozen, Snap Frozen-OCT, and FFPE). The quality of the RNA as determined by the RIN values from the Agilent Bioanalyzer system with the Agilent RNA 6000 Nano assay was compared to the Cq score from qPCR for three housekeeping genes: A) HPRT1, B) SNRPD3, C) JUN. "Samples with higher C_q values were those that also have lower integrity according to their RIN value," indicating that low-quality RNA samples did not perform well in qPCR assays. This figure reproduced from Esteva-Socias et al.[13] This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license.

Microarray analysis

A gene expression microarray can be used to detect the expression of thousands of genes simultaneously. A microscope slide is printed with thousands of different oligonucleotide sequences immobilized on the surface, each one corresponding to a known sequence or gene. These oligonucleotides then act as probes to detect gene expression, by allowing labeled cDNA to bind to them and elicit specific color-changing reactions. Following this hybridization, the slide is scanned to measure the expression of each gene, and an expression profile is generated. These profiles can be compared to examine changes in gene expression across different sample treatments or conditions.

For a successful downstream experiment, researchers often use a threshold of RNA quality determined by one of the quality metrics. Many publications, including Ibberson et al.[14], recommend the use of RNA that has an RIN equal to or above seven, for microarrays. To demonstrate this, Ibberson examined the effect of RNA degradation on microarray expression profiles through an RNA degradation series. RNA of varying degrees of degradation was analyzed on the Bioanalyzer for RIN, and then used for microarray analysis. Examples of mouse liver and duodenum RNA with various RIN scores are shown in Figure 9. Correlation plots of the samples indicate that the miRNA expression declines across sampling times, in accordance with the decrease in RIN (Figure 10). The researchers conclude that "MicroRNA expression cannot be reliably profiled in degraded total RNA. For the profiling of microRNAs we recommend use of RNA samples with a RNA integrity number equal to or above seven."[14]

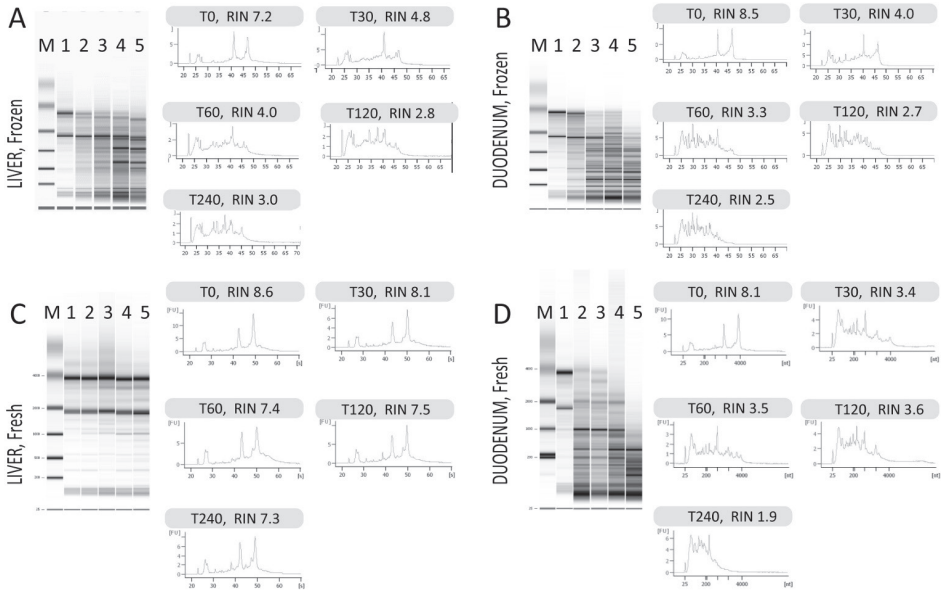

Figure 9. RNA integrity was assessed using an Agilent Bioanalyzer system to calculate the RIN of frozen mouse A) liver and B) duodenum tissues over multiple thawing times to provoke degradation. At T0, the RIN of both samples was greater than seven. After T30, the RIN decreased. Similarly, freshly collected tissues were kept on ice for set time periods prior to RNA extraction, and then RNA integrity was assessed. (C) Fresh liver tissue maintained sample integrity over 4 hours, while (D) the RIN of the duodenum, a tissue rich in RNases, indicated significant degradation over time. This figure reproduced from Ibberson et al.[14] This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license.

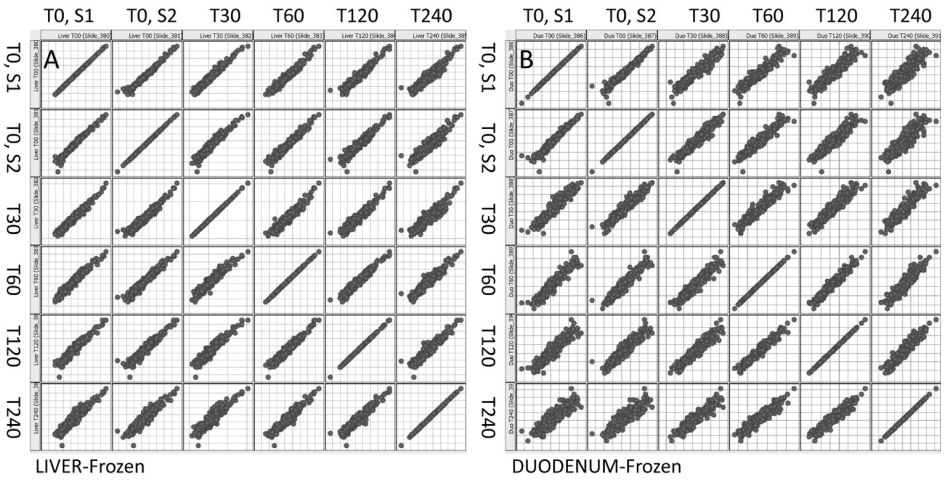

Figure 10. The expression profiles of samples with varying RINs were organized in a series of scatterplots. The correlation of miRNA expression for both A) mouse liver and B) duodenum declines across sampling times, similar to how the RIN values decrease over time, making it difficult to discern miRNAs that are differentially expressed. This figure reproduced from Ibberson et al.[14] This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license.

To understand how the integrity of their samples could affect downstream analyses, Thompson et al.[15] examined how RNA quality affects microarray performance in rat liver tissues with different RNA integrities using a temperature degradation series of fresh tissues (FT) over multiple thawing times (37 °C for 60-300 minutes).
The samples were analyzed on the Bioanalyzer for RNA quality prior to microarray, and the authors stated that "the advantage to RIN as a metric is that it is an automated measurement made prior to performing expensive in vitro transcription (IVT) assays and array hybridizations".[15] The results of the Bioanalyzer and microarray data combined showed that thawing tissue rapidly decreased RNA quality, while incubating fresh samples at 37 °C did not impact the RNA integrity but did induce changes in the transcript levels of stress response genes and immune cell markers (Figure 11).

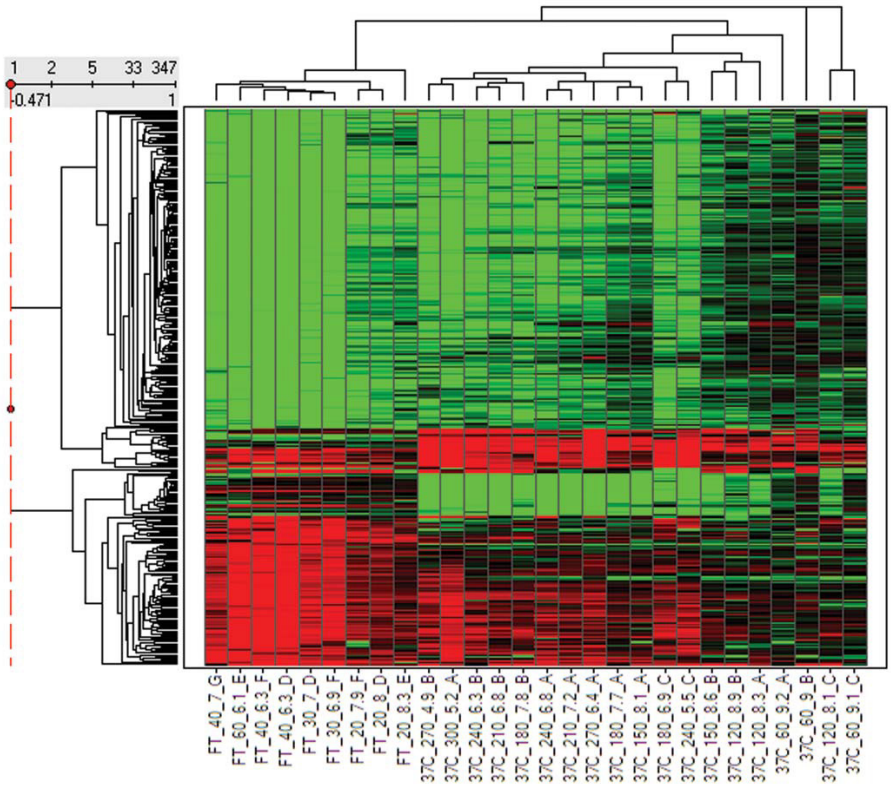

Figure 11. Individual probe-set signals vary in sensitivity to RNA degradation. Samples are clustered by handling condition (FT, 37C), then by degree of degradation. The majority of probe sets showed a decrease in signal induced by degradation, independent of handling conditions. This figure reproduced from Thompson et al.[15] This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license.

To produce a transcriptome, or genome-wide transcriptional profile, from microarray data, Kucharski et al.[16] state that it is necessary to have total RNA extracts that are "representative of the exact abundance of all messenger RNA as well as other RNA species in the cell. The RNA isolations must be of sufficient quality to support subsequent transcriptome wide amplifications (TWA) and readout platforms thus allowing genuine measurements of RNA abundance in a particular cellular/biological system."[16] The group aims to produce a reliable transcriptome for malaria *(P. falciparum)*, a challenging feat, as samples often need to be transported from remote field locations to research laboratories, making it difficult to obtain high-quality RNA. Thus, they first tested a variety of storage temperatures, storage times, preservation media, and extraction methods to determine the optimal conditions for RNA extraction. Once a method was established, they tested if the RNA extraction method performed equally across a range of parasitaemia, or the presence of parasites in blood. At parasitaemia levels as low as 0.05%, the Bioanalyzer was able to distinguish separate human and parasite 18S rRNA peaks that migrate approximately one second apart (Figure 12A). The ratio of the parasite to human 18S peaks (18S-Pf/18S-Hs) correlates linearly with parasitaemia levels in laboratory-created samples, as well as a weaker (but still significant) correlation in the field samples (Figure 12C). Microarray analysis showed that satisfactory transcriptome coverage was achieved with a low threshold of 0.05% parasitaemia, and that the main factor affecting the generation of the parasite transcriptome is the percentage of uninfected human RNA material in the starting RNA sample.

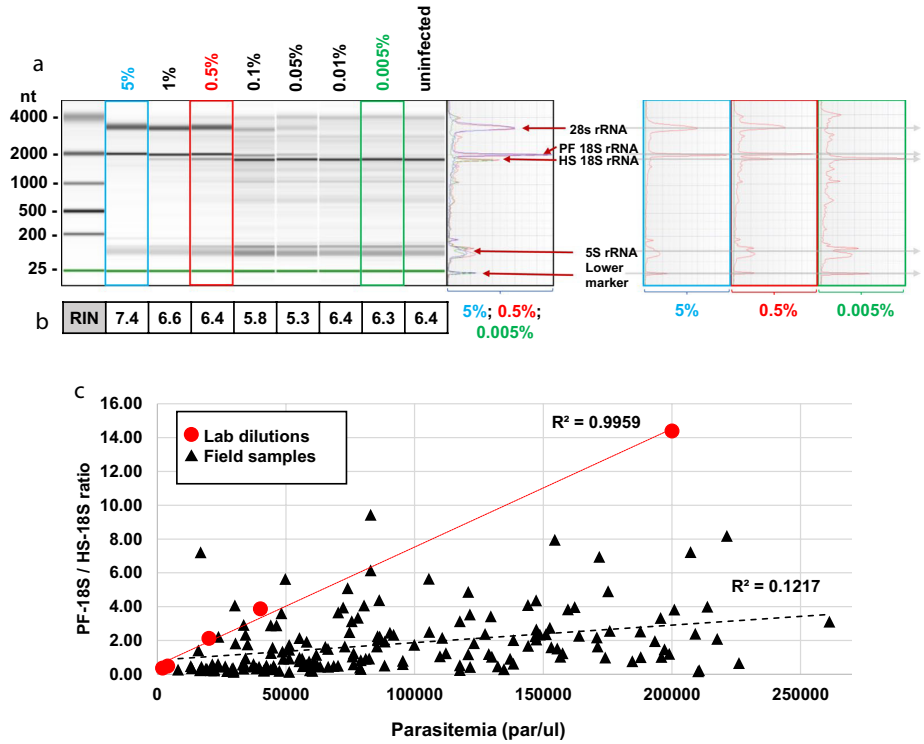

Figure 12. A-B) To make sure that the RNA extraction method works across a range of parasitaemia, samples containing a range of parasites from 5 to 0.005% were analyzed with the Agilent Bioanalyzer system using the Agilent RNA 6000 Nano kit. High parasitaemia levels (1 to 5%) display an electropherogram which contains peaks that correspond to the *P. falciparum* 28S, 18S, and 5S rRNA transcripts. At lower levels (0.5 to 0.005%), parasite-specific peak heights are diminished, especially for the *P. falciparum* and human 18S rRNA peaks. At the lowest levels of parasitaemia (0.1 to 0.005%), the *P. falciparum* peaks are below the detection limit, while the human 18S rRNA peak is more prevalent. The presence of both *P. falciparum* and human peaks in the medium parasitaemia samples (0.1-0.05%) decreases the RIN compared to the high and low parasitaemia samples, which show only one peak, and therefore have a higher RIN. C) The distinct parasite and human 18S peaks made it possible to estimate the iRBC (infected red blood cell) concentration within a sample by measuring the ratio of the 18S *P. falciparum* peak height to 18S human peak height (18S-Pf/18S-Hs). This figure reproduced from Kucharski et al.[16] This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license.

RNA sequencing

RNA sequencing (RNA-seq) is a specific type of next-generation sequencing (NGS) used to analyze the transcriptome of a genome in order to examine gene expression. To prepare an RNA-seq library, RNA is first converted to cDNA, which is then made into a sequencing library. As with any sequencing application, the quality of the input material (in this case, RNA), is of utmost importance for a successful experiment. The Agilent automated electrophoresis instruments are well-suited for the recommended QC steps throughout library preparation, including not only the input RNA, but also the resulting cDNA and the final NGS library.

For example, Pisu et al.[17], present a protocol for dual RNA-sequencing as a way to assess both bacterial and host transcriptomes simultaneously. As a part of the library preparation protocol, they recommend QC of the extracted RNA and of the final library using the Fragment Analyzer system. They emphasize that high-quality RNA is important for the workflow and give example electropherograms of intact and degraded RNA of varying RQN scores (Figure 13A). According to their data, samples with an RQN of less than six decrease the efficiency of the rRNA depletion step, and so it is recommended to use RNA with an RQN of seven or above. The protocol also recommends QC of the final library to ensure the size distribution is sufficient for sequencing (Figure 13B). As Pisu[17] demonstrates, the ability of the automated electrophoresis platforms to run both RNA and DNA samples make them well-suited for QC throughout the entire NGS library preparation protocol.

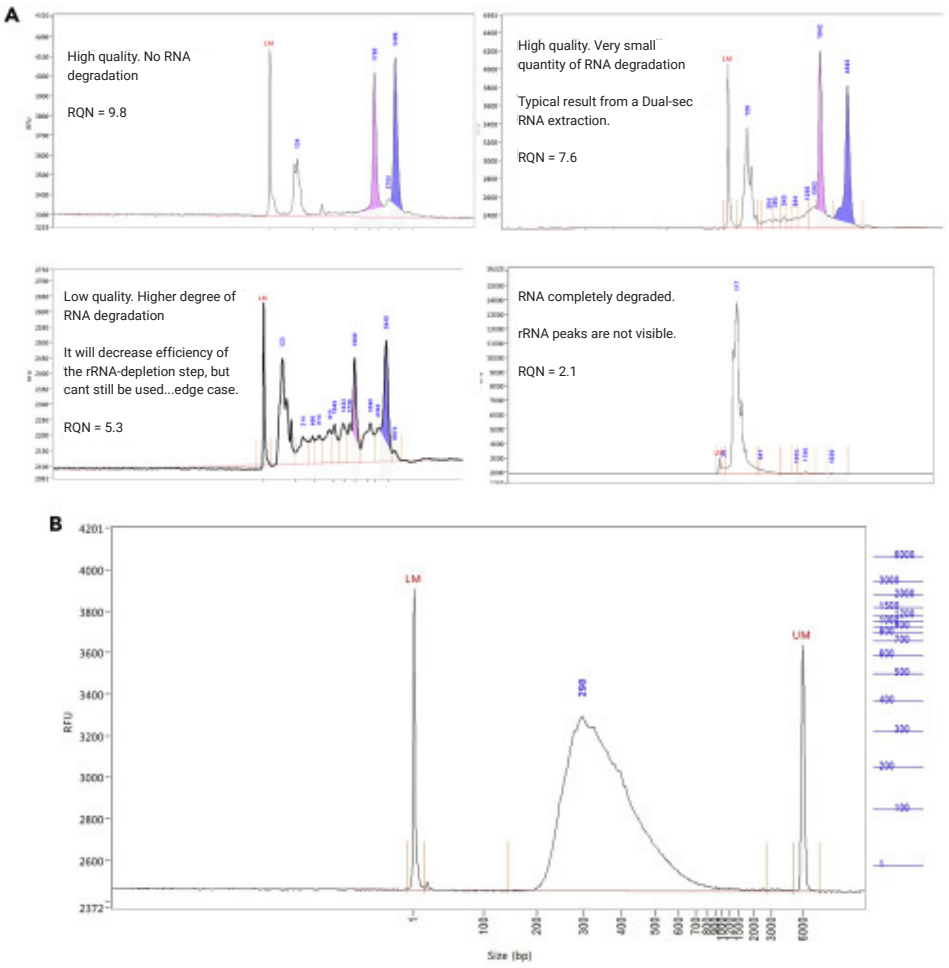

Figure 13. Quality control analysis throughout the RNA-seq library preparation protocol can be performed with the Agilent Fragment Analyzer system. A) The input RNA is assessed for integrity using the RQN score. Samples with an RQN above seven are considered high quality for sequencing, while those with an RQN of six or less display signs of degradation and will result in decreased sequencing capacity. B) An example electropherogram of a final NGS library. This figure reproduced from Pisu et al.[17] Permission has been obtained from the publisher.

Agilent automated electrophoresis instruments can also be used to help make improvements to existing methods to generate better sequencing results, as well as to save time and costs. For example, Khnouf et al.[18] explored various methods of cell lysis to help improve their protocol for single-cell small RNA sequencing (sc-sRNA-seq). Of the methods shown, only two were able to be used for successful libraries, as shown in the Bioanalyzer traces in Figure 14. Sequencing of both libraries showed that the method using Triton-X for cell lysis yielded 17% on-target reads, while the microfluidics method yielded only 4%. Using both the Bioanalyzer and the sequencing results, the researchers were able to identify a more efficient workflow and achieve higher-quality data. In another example, Mildrum et al.[19] provide an automated and miniaturized RNA-seq protocol, including bead-based poly(T) mRNA enrichment and enzymatic rRNA depletion steps, in order to reduce sequencing costs while still preserving library quality. The integrity of the input RNA and the final NGS libraries prepared with both the manual and automated methods were compared using a Fragment Analyzer system (Figure 15). Ultimately, the "miniaturization scale for each stage was based upon the lowest reproducible transfer volume of the liquid handlers used in this work that provided successful reactions based on Fragment Analyzer traces."[19]

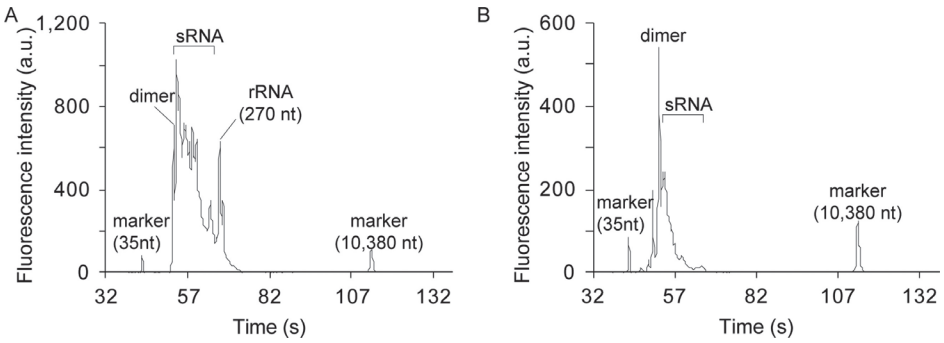

Figure 14. RNA-seq libraries generated with Triton-X with A) an overnight -80 °C incubation and B) a microfluidic approach to cell lysis were analyzed using an Agilent Bioanalyzer system. Successful library preparation was achieved using both methods, with a variety of RNA products detected including miRNA around 140 nt and adapter dimers around 120 nt. This figure reproduced from Khnouf et al.[18] Permission has been obtained from the publisher.

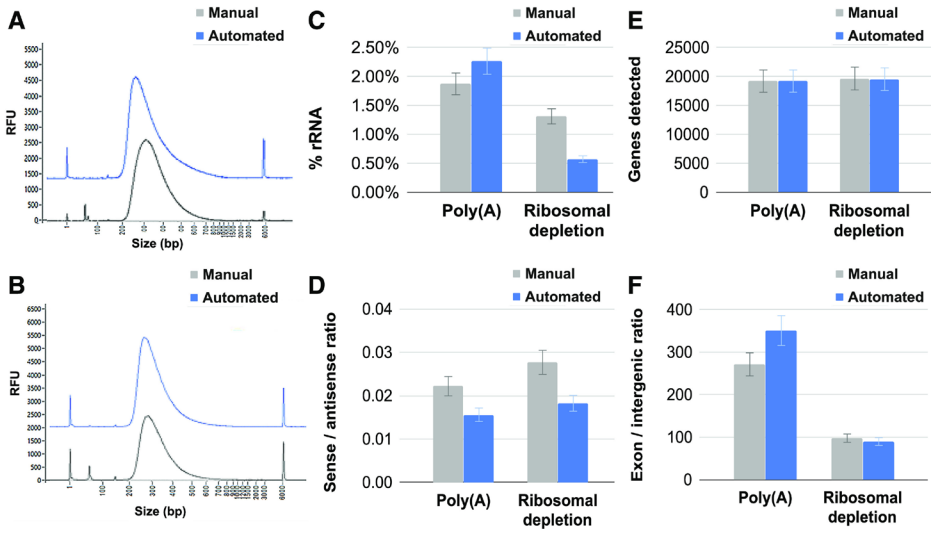

Figure 15. A-B) The Agilent Fragment Analyzer system was used to analyze libraries from a full-volume, manual method and compare them to a miniaturized, automated protocol. Both showed consistent size distributions with minimal primer and adaptor dimer contamination. C-F) Sequencing quality metrics were evaluated for each of the libraries, with the miniaturized and full-scale methods highly similar. This figure reproduced from Mildrum et al.[19] Permission has been obtained from the publisher.

While many RNA-seq experiments utilize the total cellular content of RNA, including rRNA, mRNA, and tRNA, it can be beneficial to examine only subunits of the RNA. For example, Fleming et al.[20] examined the integrity of mRNA in dried soybean seeds. Previous studies showed that the integrity of total RNA in dry seeds is strongly and positively correlated with germination. mRNA persists in the seed until germination occurs, but any potential damage to the RNA can compromise germination, making it a potentially more sensitive marker for aging, and therefore, germination potential. Bioanalyzer traces of seeds stored for two years (2015; 100% germination rate) and 23 years (1994; 61% germination rate) showed that the total RNA from each was similar, with RIN values of 6.72 and 7.86 respectively, and only slight signs of degradation were present in the older seeds (Figure 16A). However, examination of the small RNA showed that the length of the poly(A)-selected mRNA from the older samples was 1,000-2,000 nt, compared to the two-year-old seeds at 1,000-3,000 nt, "indicating a shift towards lower molecular mass molecules" [20] (Figure 16B). Analysis of the RNA-sequencing data corresponded with the Bioanalyzer results, demonstrating that there was substantially more tissue damage in the older seeds, and that "damage occurred mostly by fragmentation, as seen in changes in electropherogram profiles, length of transcript coverage, and depth of sequencing…The continuous distribution of fragment sizes in degraded transcripts and positive correlation between transcript length and degradation support the hypothesis that mRNA degradation occurs by random fragmentation." [20]

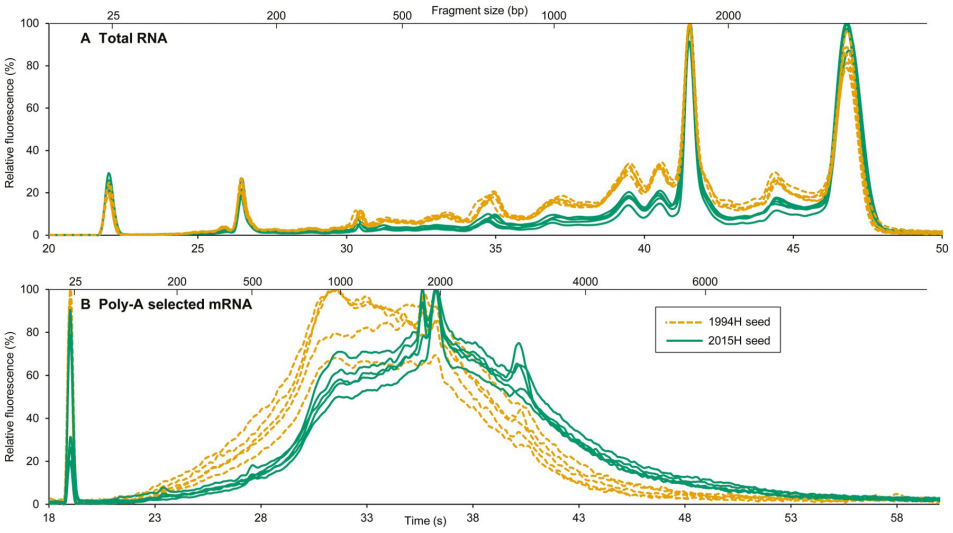

Figure 16. RNA was obtained from seeds harvested and dried in 1994 (stored 23 years) and 2015 (stored 2 years). A) Total RNA was analyzed on the Agilent Bioanalyzer system with the RNA 6000 Pico kit using the Plant RNA assay, with similar RINs for each sample, but with slightly higher peaks in the 1994 seeds, indicative of slight degradation. B) Analysis of the Poly-A-selected mRNA using the RNA 6000 Pico kit with the mRNA Pico Series II Assay indicated a shift in size, with the 1994 seeds having a greater abundance of smaller mRNAs than the 2015 seeds. This figure reproduced from Fleming et al.[20] This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license.

Summary

Agilent automated electrophoresis systems can help ensure successful downstream applications by providing information regarding the quality of the input RNA and helping researchers make informed decisions regarding their experiments. In this white paper, we have highlighted the use of the automated electrophoresis instruments in a variety of workflows, as reported by researchers in the genomic analysis community. The instruments were used to optimize a variety of RNA extraction protocols from various origins including porcine heart, olive, and bacteria, as well as identifying sample handling and storage protocols for future work. For IVT RNA, the automated electrophoresis instruments can be utilized for QC, for example, in the analysis of two SARS-CoV-2 vaccine candidates, as well as to optimize the nucleotide composition of synthetic RNA and to identify more efficient and cost-effective synthetic RNA strands. RNA integrity scores and qPCR expression profiles were examined to evaluate the effects of long-term sample storage on qPCR experiments. The automated electrophoresis systems were used to monitor a degradation series and demonstrate that microarrays are most reliable using intact RNA with a RIN of seven or above. Further, the systems allowed researchers to determine ideal storage conditions and optimize extraction methods for RNA to be used for microarray analyses. The automated electrophoresis instruments are vital QC tools for NGS workflows. For RNA-seq, at a minimum QC of both the input RNA and the final library should be performed, as demonstrated in a dual RNA-sequencing protocol. Additionally, the instruments were used to explore various cell lysis methods to obtain higher-quality RNA and subsequently improve single-cell small RNA-seq results. In a final example, the fragmentation pattern from dried soybean seeds was correlated with degradation seen in RNA-seq results, allowing the researchers to improve storage conditions for the samples in future work. Together, the automated electrophoresis systems encompass a versatile assay portfolio suitable for a broad range of molecular biology applications. They provide the ability to monitor preanalytical parameters that are important to the success of these applications, including sample processing, RNA extraction, and storage. Additionally, the quality metrics allow researchers to establish a threshold for eliminating samples that do not meet the requirements necessary for time consuming and costly downstream applications, allowing them to save time and money and leading to better results.

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