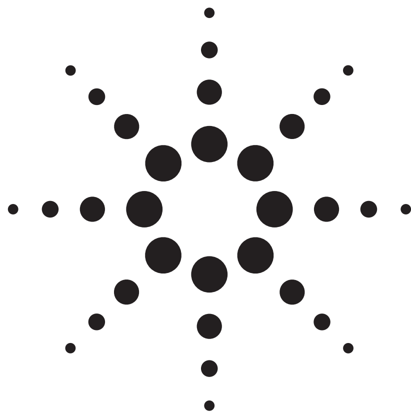

Analysis of small RNAs with the Agilent 2100 bioanalyzer

Application Note

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Abstract

The Total RNA 6000 Nano Kit was used with the Agilent 2100 bioanalyzer to determine the integrity and the concentration of small RNA (i.e., 5.8S, 5S RNAs and tRNA) samples extracted with different protocols from various cell lines. The small RNAs fraction (<200 bp) should also contain microRNAs in their primitive (pri-miRNA), precursor (pre-miRNA) and mature (miRNA) forms. The data analysis software automatically reports the corresponding RNA concentrations for each sample in a range between 5 and 500 ng/µl. Moreover, the performance of Agilent 2100 bioanalyzer was compared to the most commonly used techniques for RNA separation, detection and quantitation. Comparisons between different techniques were based on sensitivity and quantitative accuracy. The advantages of detection sensitivity and accuracy, coupled with a rapid and automated system, indicate that analyses performed by Agilent 2100 bioanalyzer are superior to the leading alternatives.

Agilent Technologies

Introduction

Isolation of pure and intact RNA is essential for several applications but the concentration and integrity of total RNA preparations is significantly affected by the specific purification process used. Applications may include RT-PCR, preparations of targets for microarrays, ribonuclease protection assays, preparations of cDNA libraries and Northern blotting among others. Recently, the importance of low molecular weight RNAs (miRNAs, siRNAs, and snRNAs, etc.) has been outlined in different studies[1]. In particular, the importance of a new class of small RNA, namely microRNAs (miRNAs), has been highlighted. MicroRNAs are small RNA molecules encoded by the genomes of plants and animals. These highly conserved, ~21-mer RNAs regulate the expression of genes binding the 3'-untranslated regions (3'-UTR) of specific mRNAs. Each miRNA is thought to regulate multiple genes, and since hundreds of miRNA genes are predicted to be present in higher eukaryotes the potential regulatory circuitry afforded by them is enormous. It has been hypothesised that in higher eukaryotes, the role of miRNAs in regulating gene expression could be as important as the transcription factors. Therefore, techniques that allow an accurate detection of small RNA quality and quantity are highly important.

Standard protocols for the isolation of total RNA and mRNA actually are not optimized for recovery of small RNA molecules and may lead to the loss of substantial amounts of miRNAs and other small RNAs. For example, if RNA is isolated using the traditional glass fiber filtration, it most likely lacks a complete representation of small RNAs. Therefore, it is of crucial importance to develop an efficient and accurate method to determine the integrity and the concentration of total and small RNAs, prepared with different RNA isolation procedures or when purchasing commercially available purified RNAs. The performance of the Agilent 2100 bioanalyzer to separate, detect and quantitate small RNAs is compared to the most commonly used techniques. Samples extracted with different protocols and from various cell lines were evaluated. Comparisons between techniques were based on sensitivity and quantitative accuracy.

Materials and methods

Total RNA extration and small RNAs enrichment protocols

Total RNA (sample A) was extracted with MirVana™ miRNA Isolation Kit (Ambion) according to manufacturer's instructions. This kit also provides specific procedures to isolate and enrich small RNAs (LMW) (samples 2 and 4) from higher molecular weight (HMW) RNAs (i.e, 28S, 18S and 5.8S) (samples 1 and 3).

Cell lines

Two cell lines, one lymphoblastoid and one human brain tumor (U118), were cultured using standard procedures. Cells were trypsinized and pelleted by centrifugation. Approximately 10^6 cells for each extraction were resuspended in the appropriate lysis solution contained in the kit and treated according to manufacturer's instructions.

Denaturing gels

All samples (500 ng) were resolved into 15 % polyacrylamide TBE-Urea gel and 1 % agarose MOPS-formaldehyde gel stained with ethidium bromide.

Agilent chip preparation

RNA samples were analyzed with the Total RNA 6000 Nano Kit, specifically optimized for the analysis of total RNA with the Agilent 2100 bioanalyzer. The chips were loaded and run on the Agilent 2100 bioanalyzer according to manufacturer's instructions .

Agilent 2100 bioanalyzer instrument and software

The Agilent 2100 expert B.02.02 software includes data collection, presentation and interpretation functions. Data can be displayed as a gel-like image and/or as electropherogram(s). The RNA profile is automatically displayed as individual electropherograms. An additional data evaluation tool is available for data comparison that allows to compare files from multiple chips. The Agilent 2100 bioanalyzer is equipped with independent high voltage power supplies connected to 16 platinum electrodes, which allow multiple and precisely controlled injections and separations. The instrument uses fluorescence detection, by monitoring the emission between 670 nm and 700 nm.

Results and discussion

We purified RNA from different samples, using two different RNA extraction procedures according to MirVana™ miRNA Isolation Kit instructions. The first is designed to recover total RNA (sample A) and the other one, specific for small RNA enrichment, is able to efficiently separate RNA molecules of approximately 200 nt from the larger ones (samples 1 and 3). Following the latter procedure the resulting RNA preparations (samples 2 and 4) are highly enriched for low molecular weight (LMW) RNAs (5.8S, 5S, tRNA, miRNAs, siRNAs, and/or snRNAs both single- and double-stranded species). This enrichment protocol can be useful in all the experiments requiring the removal of predominant large RNAs, for example, for accurate analysis of miRNA expression by QPCR or microarray analysis. Moreover, an accurate quantitation is important for a lot of procedures including the determination of the correct amount of RNA template needed for RT-PCR and microarray labeling reactions or the minimum amount needed in a Northern blot.

All RNA samples, obtained following these two protocols, were separated on a denaturing agarose and polyacrilamide gels. Briefly, each sample (500 ng) were loaded on 1 % agarose gel and stained with ethidium bromide in order to estimate the quality and quantity of RNA sample. Results were compared to the Agilent 2100 Bioanalyzer digital gel (figure 1A) and to the corresponding electropherograms. (figure 1B). Comparing the agarose gel sample electrophore-

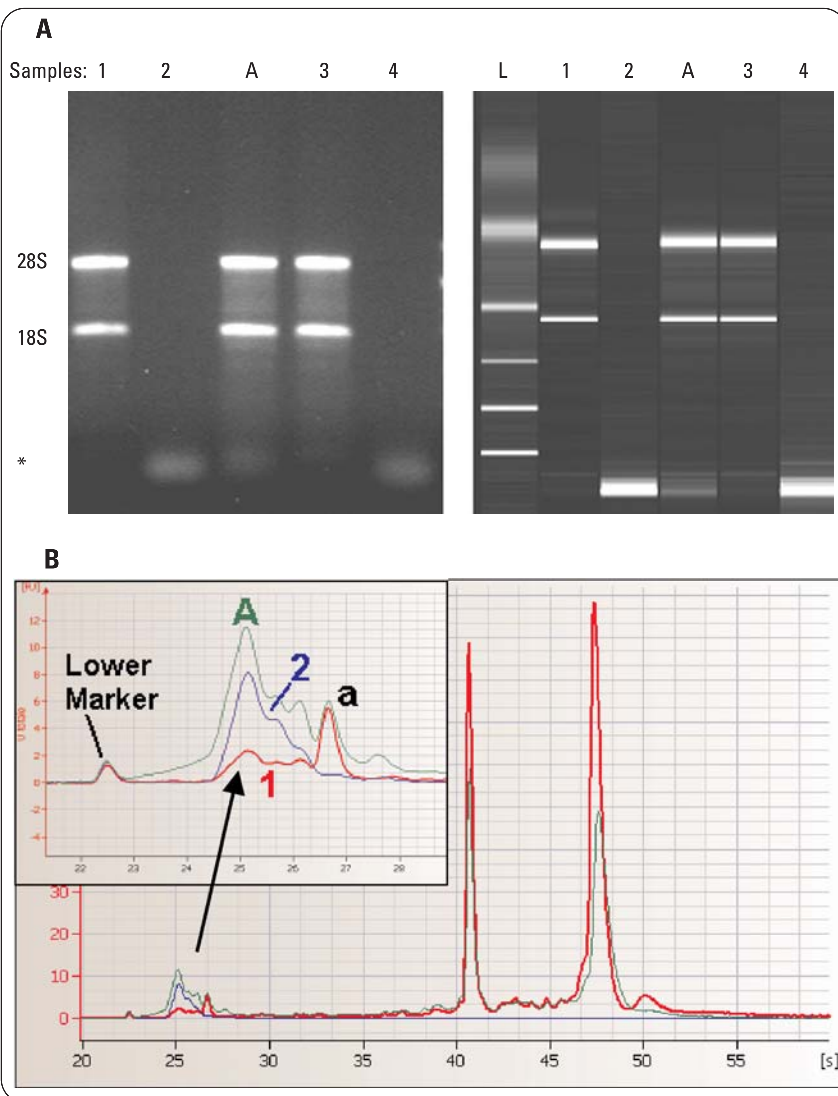

Figure 1
A) Agarose gel (1 %, left) of total RNA (sample A), small RNAs (LMW) (samples 2 and 4) and higher molecular weight (HMW) RNAs (samples 1 and 3) (500 ng per sample) extracted with MirVana™ miRNA Isolation Kit (Ambion) compared to the digital gel (right) obtained with Agilent 2100 bioanalyzer (L=ladder) (500 ng per sample).
B) Comparison of Agilent 2100 bioanalyzer electropherograms of sample A (total RNA), 1 (HMW RNA) and 2 (LMW RNA). Peak "a" (insert) was detected only in total and HMW RNA samples (A and 1) but not in LMW RNA (2) enriched sample.

ses (figure 1A) we were able to efficiently detect 18S and 28S rRNA bands while LMW RNA were visualized as faint, smeary bands. These evidences suggest that agarose gel does not represent an efficient system to detect the quality and quantity of small RNA and to verify the enrichment protocol efficiency. On the contrary, the Agilent 2100 bioanalyzer can easily detect an intense peak at 100-175 bp due to small RNA fragments located under the 0.2 kb band of the ladder. Taking into account the electropherograms of samples A, 1 and 2, and by examining the early electropherogram region (from 25 to 200 bp) in more detail, it was possible to identify different profiles (figure 1B insert). In particular, sample A shows a more defined profile due to small RNAs, even if it was not possible to completely resolve the various species (5.8S, 5S, miRNA and tRNA). sample 1 shows a profile similar to sample A in the later part (18S and 28S RNAs) of the electropherogram but not in the first part (small RNAs) where only one peak is retained with comparable concentrations (peak “a” in the insert of figure 1B). Interestingly, the two extraction protocols seem to similarly retain the HMW RNAs but not the smaller ones with the same efficiency. The small RNAs enriched sample (sample 2) has no HMW RNAs detectable in the electropherogram but presents a higher concentration of small RNAs, if compared to sample 1. In LMW RNA samples (samples 2 and 4) peak "a" is undetectable.

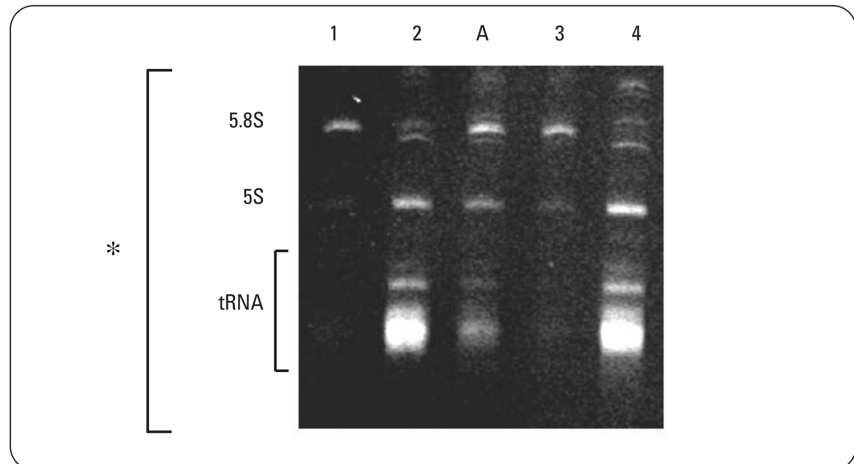

Figure 2
Polyacrylamide gel (15%) of total RNA (sample A), small RNAs (LMW) (samples 2 and 4) and higher molecular weight (HMW) RNAs (samples 1 and 3) (500 ng per sample) extracted with MirVana™ miRNA Isolation Kit (Ambion) stained with ethidum bromide.

Furthermore, all samples (500 ng) were run on 15% polyacrylamide gel and stained with ethidium bromide (figure 2). From the comparison of sample electrophoreses we were able to efficiently detect 5.8S, 5S rRNA and tRNA bands in total RNA (sample A) and in small RNAs enriched preparations (samples 2 and 4). Although 15 % acrylamide gel electrophoresis provides highly resolved profiles of LMW RNAs (5.8S, 5S and tRNA) it is not a feasible method to obtain information about the quality and quantity of high molecular weight RNA (i.e. 28S and 18S) and low molecular weight RNAs in the same run. We may hence conclude that the Agilent 2100 bioanalyzer is able to detect the quality and quantity not only of high[2-4] but also of low molecular weight RNAs and offers information about the efficiency of small RNAs enrichment protocols.

The sensitivity and accuracy of the Agilent 2100 bioanalyzer in detecting the presence of small RNAs in a total RNA sample were also evaluated using six dilutions of sample A, (with the total RNA concentration ranging from 1 µg/µL to 100 ng/µL and the corresponding small RNA fractions ranging from 117 to 5 ng) and comparing the electropherograms to 1 % agarose gel runs (figure 3 and table 1).

Comparison of the gel images of small ribosomal RNAs has clearly shown the higher sensitivity of the Agilent 2100 bioanalyzer to detect small RNAs bands that cannot be visualized on the agarose gel. The bioanalyzer was able to consistently detect as little as 5 ng of LMW RNA out 100 ng of total RNA. When an agarose gel was stained with ethidium bromide, at least 500 ng of total RNA were necessary for a consistent detection of small RNA fraction. In the case of RNA extraction from limited tissue sources (i.e, biopsies), most of the RNA sample is consumed in running the gel. Due to the high sensitivity of the bioanalyzer, only small quantities of a precious sample are needed to obtain meaningful information about the integrity and quantity of different molecular weight RNAs. Moreover, with minimal amount of RNA sample the new Series II RNA 6000 Pico kit provides a sensitivity enhancement in a factor of 100-200x.

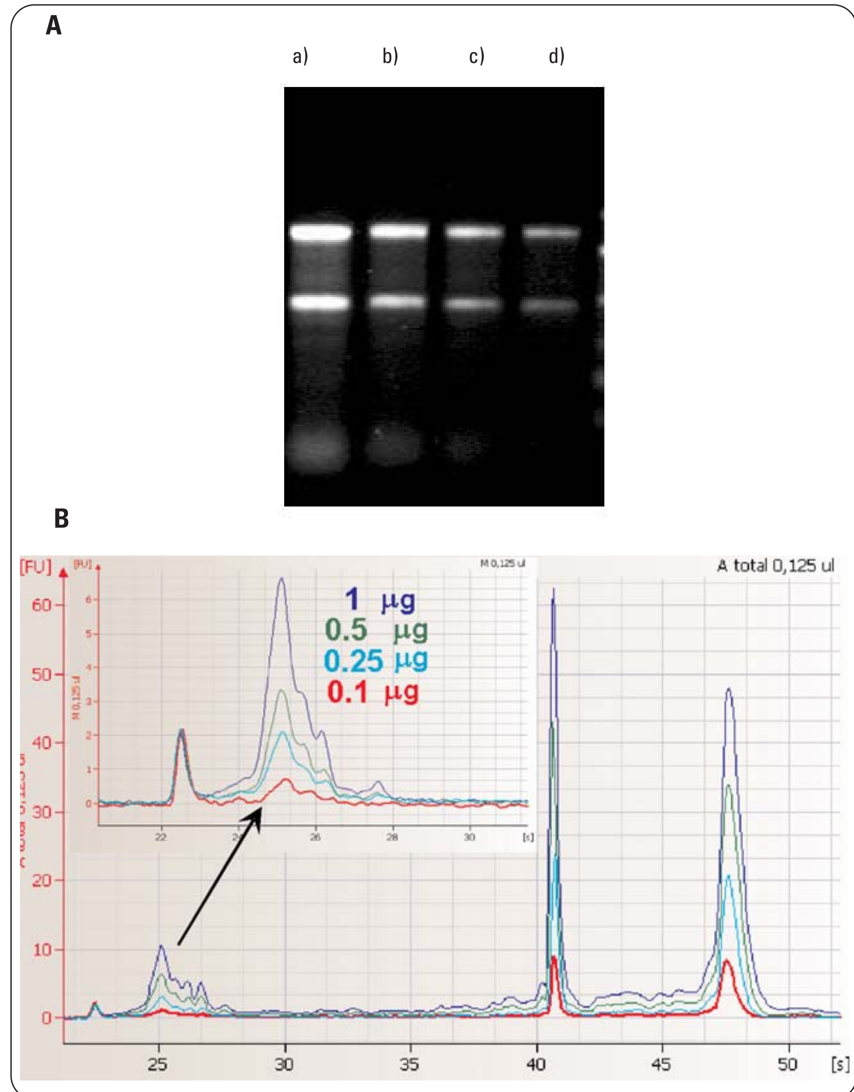

Figure 3
A) Agarose gel (1 %) of 1 µg (a), 0.5 µg (b), 0.25 µg (c) and 0.125 µg (d) of sample A.
B) Electropherograms of 1 µg (blue), 0.5 µg (green), 0.25 µg (cyan), and 0.125 µg (red) of sample A. Inset: Expansion of the small RNAs fraction.

Total RNA (Sample A)	1 µg	0.5 µg	0.25 µg	0.1 µg
Small RNAs	117 ng	70 ng	29 ng	5 ng

Table 1
Total RNA (sample A) dilutions and relative small RNAs concentrations obtained from the bioanalyzer software result table.

Conclusions

With the increased interest in expression profiling applications, there has been a steadily growing demand for faster, more automated analysis tools consuming minimal sample amounts. Lab-on-a-Chip technology is particularly well suited for the rapid analysis of nucleic acids because it integrates multiple experimental steps. With the introduction of the Agilent 2100 bioanalyzer, sample handling, separation and analysis have been integrated in a more automated manner. Chip-based analysis offers several benefits over existing technology including reduced sample consumption, minimal manual intervention, increased analysis speed and data precision, and minimized exposure to hazardous materials. The Agilent 2100 bioanalyzer shows excellent performance for quantitative and qualitative analysis also of low molecular weight RNA samples. As the separations are performed, RNA samples (high and low molecular weight) are detected and analyzed in real-time, and the digital output facilitates easy data exchange. There are no additional staining, destaining or imaging steps before data extraction. The ability to accurately determine sample concentration, while simultaneously checking integrity and purity with the Agilent 2100 bioanalyzer, is a valuable advantage over current competitive technologies. Automation of both the separation and data analysis makes the Agilent 2100 bioanalyzer easy to use, while the superior performance makes it an ideal tool for the analysis of nucleic acids. Moreover, significant advantages are represented by the data analysis and comparison functions, which are embedded in the software. No additional time consuming steps, such as scanning or densitometric analysis are required.

The RNA 6000 Nano LabChip kit can be used with the Agilent 2100 bioanalyzer to measure both the quantity and the integrity/purity also of small RNAs. The integration of these two measurements provides a simple and quick assay that consumes minimal sample amounts, has a high sensitivity, accuracy, reproducibility and a better resolution compared to the agarose gel. The combination of the intercalating dye used in the assay and the fluorescent detection used by the system enables detection of small concentration differences between samples that may not be detected using traditional gel electrophoresis. The systems provides a more precise quantitation of different molecular weight RNA compared to the rough estimate obtained from gel electrophoresis.

At the moment, identification of small RNA species obtained with the RNA 6000 Nano kit is better than that achieved using agarose gels but not comparable to that of acrylamide gels in terms of resolution. However, unlike acrylamide gel electrophoresis it provides simultaneous assessment of high and low molecular weight RNAs which is a useful feature to estimate high molecular weight contaminations of low molecular weight RNA samples.

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Published July 1, 2006
Publication Number 5989-5215EN

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