Analysis of small RNAs from *Drosophila* Schneider cells using the Small RNA assay on the Agilent 2100 bioanalyzer

Application Note

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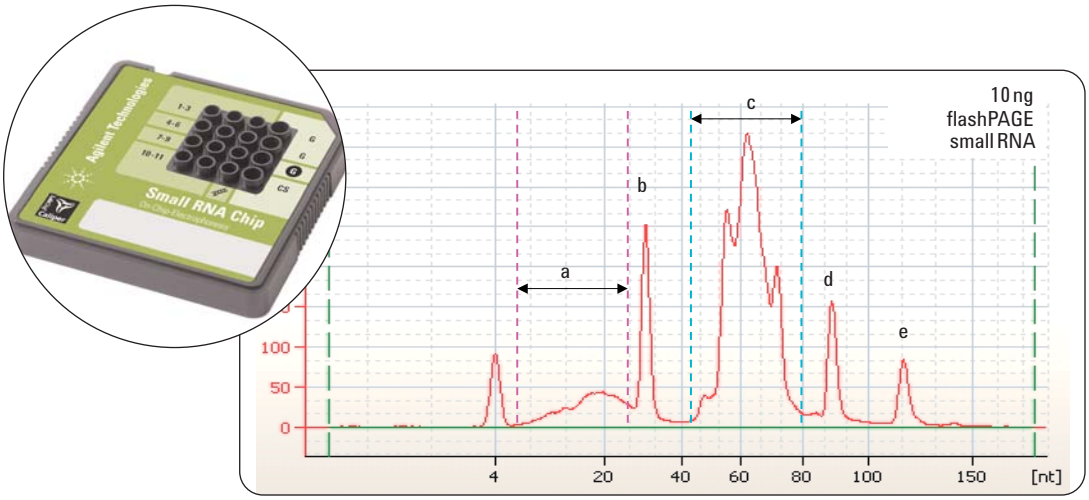

Abstract

Over the past ten years, the importance of diverse classes of small non-coding RNA molecules has increasingly been recognized. To study all these newly discovered regulatory RNAs, it has been necessary to improve existing technologies or to develop new ones. The Agilent 2100 bioanalyzer was used with the recently available Agilent Small RNA assay to analyze and monitor microRNA enrichment in *Drosophila* small RNA preparations. The performance of the Small RNA assay was compared to other techniques like polyacrylamide gel electrophoresis and northern analysis.

Agilent Equipment

- Agilent 2100 bioanalyzer
- Agilent RNA 6000 Nano kit
- Agilent Small RNA kit

Application Area

- Gene expression

Agilent Technologies

Introduction

In the past few years, small noncoding RNAs (ncRNAs) have rapidly emerged as important contributors to gene regulation. All of these RNAs range from 15 to 30 nucleotides in length and have revealed unexpected layers of transcriptional and post-transcriptional mechanisms of gene regulation.[1]

Among these small RNAs, microRNAs (miRNAs) form a particular class of 21- to 24-nucleotide RNAs that negatively regulate the translation and stability of target messenger RNAs (mRNAs). Each miRNA can potentially regulate hundreds of mRNAs. As a consequence, miRNAs play critical roles across various biological processes, including early development, cell growth and proliferation, differentiation, and apoptosis.[2] They have been shown to have unique tissue-specific, developmental stage-specific, or disease-specific patterns. These observations imply that each tissue is characterized by a specific set of miRNAs that might define features of that tissue. For instance, the miRNA profiles reflect the developmental lineage and differentiation state of tumors.[3]

Increased interest in the function of miRNAs has rapidly increased the demand for new technologies or the adaptation of old technologies to study these small molecules. The isolation and recovery of intact small RNAs are essential for many applications, such as microarrays and RT-qPCR. As a consequence, analytical solutions to assess the RNA integrity of small RNA preparations are required. The RNA 6000 Nano assay and the RNA 6000 Pico assay run on the Agilent 2100 bioanalyzer are methods of choice to assess RNA quality and quantity. However, resolution is not optimized for accurate analysis of small RNA populations.[4] To this end, Agilent Technologies recently developed a Small RNA assay.

Drosophila is one of the first species in which miRNAs have been cloned and characterized, and it provides a powerful model organism to study miRNA functions through genetic and genomic approaches.[5] This Application Note evaluates the use of the Agilent 2100 bioanalyzer, in conjunction with the Small RNA assay, to analyze *Drosophila* small RNAs. Small RNA samples were prepared from *Drosophila* Schneider cells according to different protocols and were analyzed using polyacrylamide gel electrophoresis (PAGE), northern blot, or Agilent RNA 6000 Nano and Small RNA assays.

Materials and methods

Cell culture and RNA preparation

Drosophila melanogaster S2 Schneider cells were grown at 23 °C in Schneider medium (Invitrogen) containing 10 % fetal calf serum. Total RNA was extracted from S2 cells using TRIzol Reagent (Invitrogen). RNA highly enriched for small RNA species was extracted from total RNA using the *mir*Vana miRNA Isolation Kit (Ambion).

Alternatively, a flashPAGE Fractionator (Ambion) was used to enrich small RNA. This fractionator is a miniaturized electrophoresis apparatus that was specifically designed to isolate small single-stranded nucleic acids. Total RNA sample was run for 15 minutes at 80 V on a flashPAGE Pre-Cast Gel. The small RNAs that were contained in the lower-running electrophoresis buffer were ethanol-precipitated overnight at -20 °C in the presence of 0.3 M sodium acetate and 5 µg of linear acrylamide, according to the manufacturer's protocol.

RNA was resuspended in RNase-free water. RNA concentration was determined by spectrophotometry at 260 nm using a Nanodrop ND-1000 Spectrophotometer.

Lab-on-a-Chip technology

RNA samples were analyzed with the Agilent 2100 bioanalyzer using the RNA 6000 Nano kit or the new Small RNA kit according to the manufacturer's instructions. All analyses were performed with the Agilent 2100 expert software B.02.05.

Polyacrylamide Gel Electrophoresis (PAGE) and northern blots

RNA samples were fractionated on denaturing 15 % polyacrylamide gel containing 8 M urea and 1X TBE. Gels were stained with ethidium bromide or SYBR Green II RNA gel stain (Molecular Probes). Stained gels were excited using 300 nm transillumination.

For northern analysis, fractionated RNAs were transferred to a Hybond NX nylon membrane (Amersham Biosciences) in 0.5X TBE and fixed twice by UV-crosslinking (0.24 J). Oligonucleotide probes were 5'-end-labeled using T4 polynu-

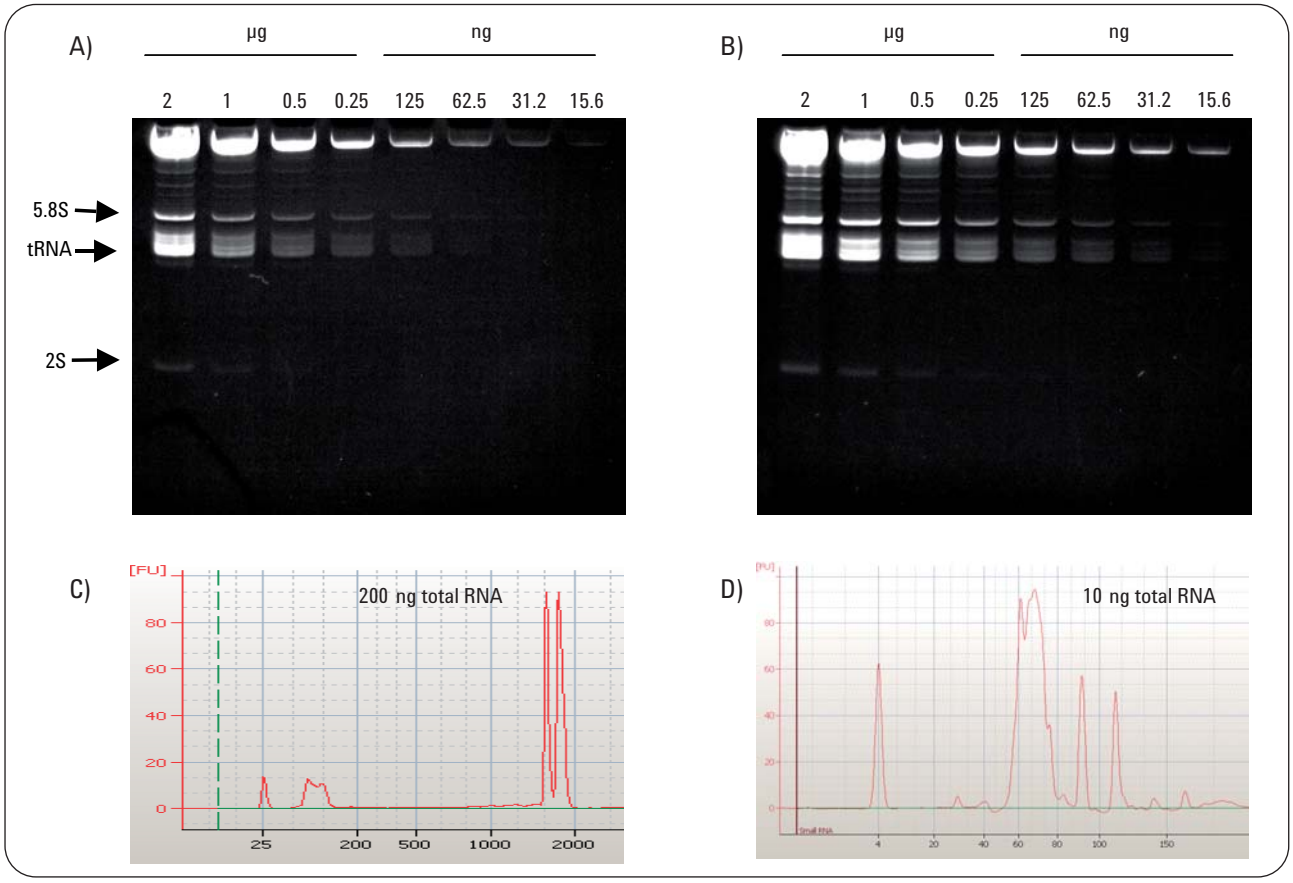

Figure 1
Analysis of total RNA from S2 cells. An identical twofold dilution series of total RNA was resolved on denaturing 15 % acrylamide gel and stained with ethidium bromide (A) or SYBR Green II (B). The same RNA sample was analyzed using RNA 6000 Nano kit (C) or Small RNA kit (D).

cleotide kinase and gamma-32P-ATP. Hybridization was performed overnight at 39 °C in PerfectHyb-Plus Hybridization Buffer (Sigma-Aldrich). The membrane was then washed twice at low stringency in 2X SSC, 0.1 % SDS at 39 °C for 15 minutes.

Results and discussion

Analysis of small RNAs species in total RNA

Total RNA was extracted from S2 *Drosophila* cells using the TRIzol Reagent method. The resulting preparation was analyzed with the Agilent RNA 6000 Nano kit to monitor the quality of the RNA sample. The bioanalyzer electropherogram showed two sharp ribosomal peaks that migrate close to each other (figure 1C). This typical *Drosophila* RNA profile results from the processing of the 28S ribosomal RNA (rRNA) into 28Sa and 28Sb mature forms that migrate in a similar manner to the 18S rRNA. A third RNA peak was also visible below 200 nucleotides (nt) in length, providing evidence that the total RNA preparation still contained small RNAs. The presence of these three individualized peaks with a flat baseline in between attested there was no trace of degradation of the RNA sample. However, with the RNA 6000 Nano kit, resolution of small RNA was not sufficient for accurate analysis.

To assess the small RNA species, an identical twofold serial dilution of total RNA was run on two separate denaturing 15 % polyacrylamide gels that were stained either with ethidium bromide (figure 1A) or SYBR Green II (figure 1B). SYBR Green II staining appeared significantly more sensitive. For instance, 2S RNA, a small RNA of 30 nucleotides resulting from the late steps in the maturation of *Drosophila* 28S rRNA, could be detected when 125 ng of total RNA was loaded on the SYBR Green II stained gel (figure 1B), while detection

required 500 ng on the ethidium bromide stained gel (figure 1A).

The total RNA preparation was then analyzed using the Agilent 2100 bioanalyzer, together with the Small RNA assay. Only 10 ng of total RNA was run on the Small RNA chip (figure 1D). Results were compared to the PAGE profiles obtained previously. Two sharp peaks, corresponding to 5.8S and 5S RNA, were clearly resolved. Transfer RNA (tRNA) formed a third large peak. A small well-defined peak at 30 nucleotides indicated that 2S RNA could be detected with only 10 ng of total RNA. Therefore, under the conditions we used, the Small RNA assay was at least 50-fold or 12-fold more sensitive than a denaturing 15 % polyacrylamide gel stained with ethidium bromide or SYBR Green II, respectively. Hence, the Small RNA assay appeared to be the method of choice to assess small RNA species contained in a total RNA preparation.

Analysis of small RNAs enriched samples

In the past few years, interest in the identification, detection, and use of small RNA molecules, particularly miRNAs, has rapidly expanded. Small RNA enrichment procedures are useful for applications, such as array analysis, where extraneous ribosomal RNA and mRNA might add to background signal. We investigated whether the quality and characteristics of different small RNA-enriched samples could be assessed using the Agilent 2100 bioanalyzer and the Small RNA assay.

We evaluated two different commercially available methods to prepare RNA enriched for small RNA species. One was a column-based isolation method (*mir*Vana miRNA Isolation Kit) and the other was a specialized electrophoresis system (flashPAGE Fractionator).

The small RNA preparations were first compared to total RNA using PAGE and northern blot. Small RNAs derived from 5 µg of total RNA were run in parallel with total RNA on a denaturing 15 % acrylamide gel that was stained with SYBR Green II (figure 2A).

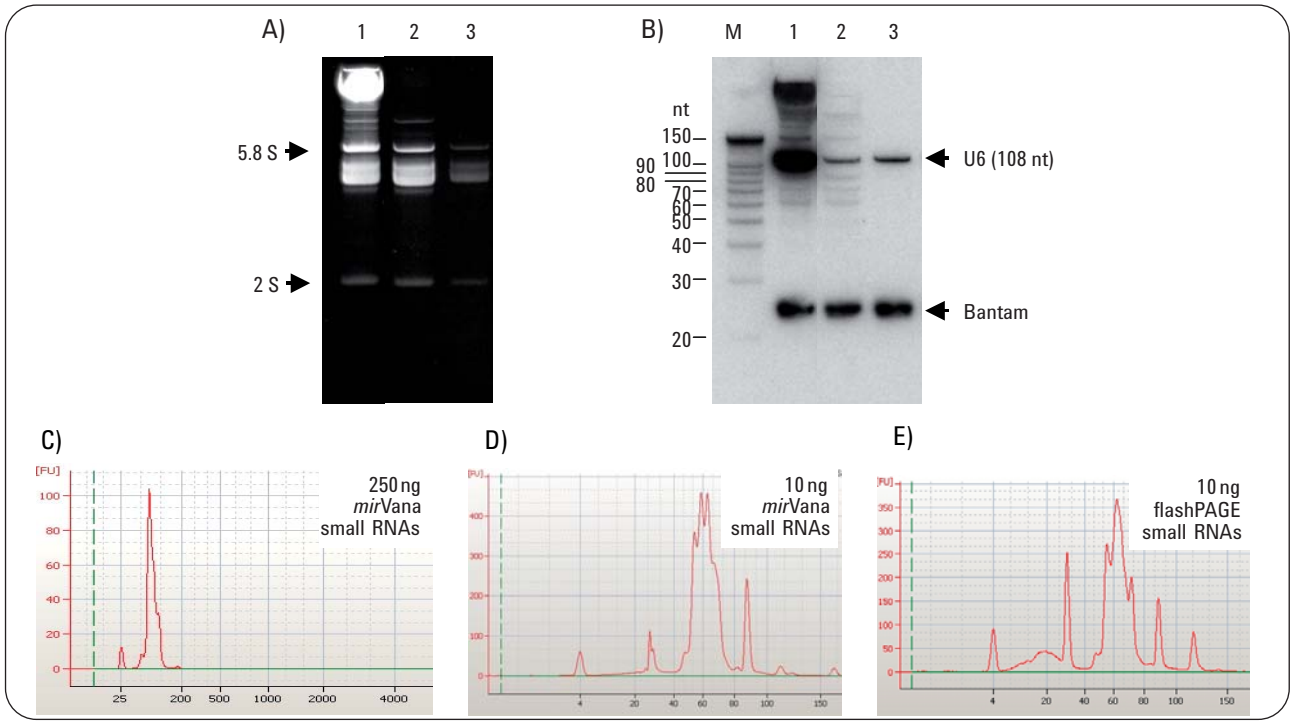

Figure 2
Analysis of RNA enriched for small RNA species. Panels A and B: 5 µg of total RNA extracted using TRIzol Reagent (lane 1) were compared with 731 ng of *mir*Vana small RNAs (lane 2) and 76 ng of flashPAGE small RNAs (lane 3). Panel A shows results on PAGE and panel B shows results on northern blot hybridized with probes for the RNA indicated. Ambion Decade Markers were used as the RNA ladder (M). Panels C to E: Electropherograms of small RNA preparations analyzed on the Agilent 2100 bioanalyzer. *Mir*Vana small RNA run on the RNA 6000 Nano chip (C) and Small RNA chip (D). FlashPAGE small RNA analyzed on the Small RNA chip (E).

The gel was transferred onto a nylon membrane that was subsequently hybridized with oligonucleotide probes directed against U6 RNA and bantam miRNA, respectively (figure 2B). In small RNA samples, high-molecular-weight RNA was totally removed (figure 2A) and the signal for U6 RNA was significantly lowered (figure 2B). Nevertheless, the amount of bantam miRNA appeared equivalent in the three RNA preparations. Considering the amount of RNA loaded onto the gel, we could estimate a relative enrichment in bantam miRNA of 7- and 65-fold in *mir*Vana and flashPAGE small RNA, respectively.

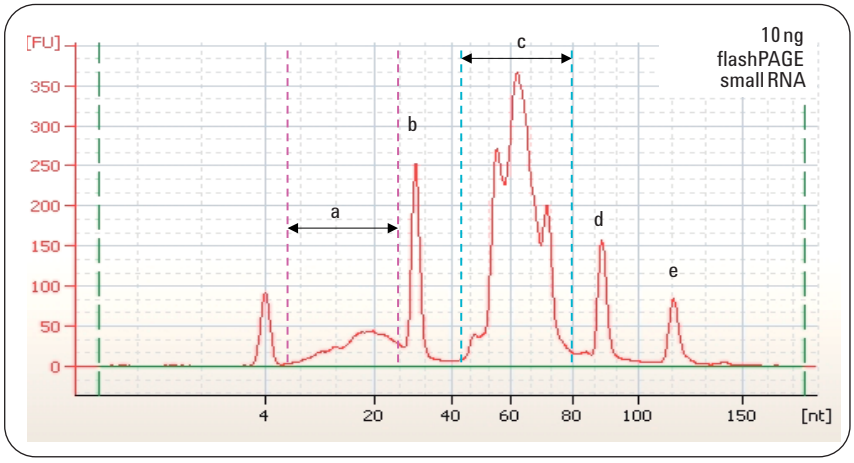

Figure 3
Electropherogram of flashPAGE small RNA analyzed using the Agilent Small RNA assay. Various RNA populations were resolved: a) small non-coding RNAs including miRNA, b) 2S RNA, c) transfer RNA, d) 5S RNA, (e) 5.8S RNA.

Small RNA preparations were further assessed on the Agilent 2100 bioanalyzer. *Mir*Vana small RNA was first analyzed with the RNA 6000 Nano kit. The electropherogram exhibited a single peak, indicating that resolution was not sufficient for accurate analysis (figure 2C). Conversely, on the Small RNA chip, tRNAs and 5S RNA were efficiently resolved and 2S RNA formed a clear individual peak (figure 2D).

Interestingly, flashPAGE small RNA exhibited a significantly different pattern on the Small RNA chip, with a relative amount of 2S RNA considerably increased. Moreover, the small non-coding RNA (ncRNA) region (including small interfering RNA, repeat-associated small-interfering RNA, and miRNAs) was noticeably separated from the 2S RNA (figures 2E and 3).

Compared to PAGE and northern analysis, the Small RNA assay provides a quick, sensitive, high-resolution analytical method to monitor small RNA content in different RNA preparations. The presence of sharp individualized 5S and 2S peaks can be used to directly evaluate the quality of small RNA samples, just like 18S and 28S profiles are indicative of total RNA samples' quality on the RNA 6000 Nano assay. PAGE and northern analysis allow one to evaluate small RNA preparations and to assess a specific miRNA like bantam, but this analysis is time-consuming and requires a large amount of RNA.

Monitoring of small non-coding RNA enrichment

The Agilent Small RNA assay proved to be very efficient in resolving small RNA species. We used it in conjunction with the Agilent 2100 expert software to calculate the small ncRNA enrichment in small RNA preparations. Regions or individualized peaks corresponding to the various small RNA species were manually defined as indicated in figure 3. The software automatically calculates the total amount of material that migrates in the size range between 1 and 150 nucleotides, and allows manual quantification of each individualized peak or region with high precision.

*Mir*Vana and flashPAGE small RNA preparations were compared. The ratio of each small RNA species was calculated as a percentage of the total small RNA fraction. Results of this quantification are displayed in figure 4. Calculations showed that the relative amount of tRNA was significantly decreased in flashPAGE small RNA, while 2S RNA and small non-coding RNA were highly enriched. Because miRNA are included in the small ncRNA fraction, we could extrapolate a 20-fold enrichment in miRNA from flashPAGE relative to *mir*Vana small RNA.

It is noteworthy that the presence of the 2S RNA in *Drosophila* small RNA samples offers an invaluable advantage to assess small RNA integrity, and can provide an internal control for quantification of small non-coding RNA. These data show that the Small RNA assay is a powerful technology to monitor small RNA preparations and miRNA enrichment.

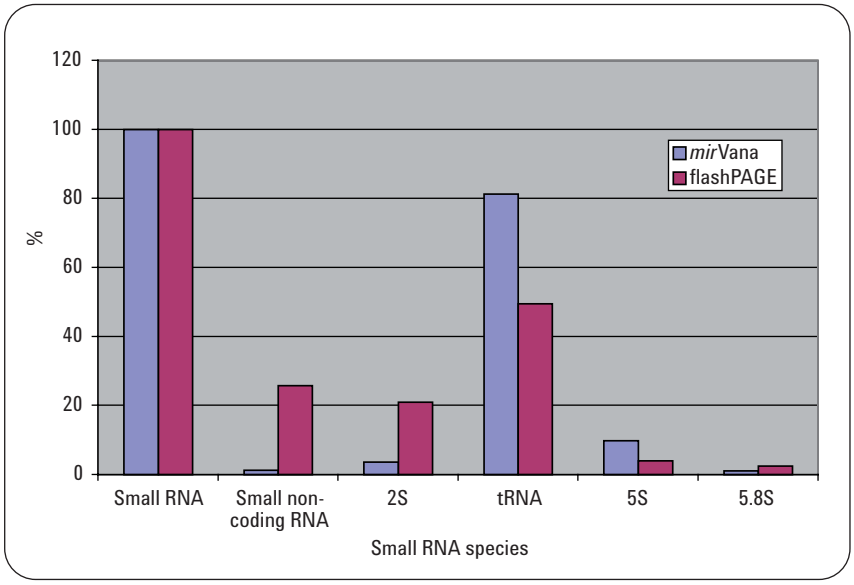

Figure 4
Comparison of *mir*Vana and flashPAGE small RNA preparations analyzed using the Agilent Small RNA assay. The relative amounts of the various small RNA species were compared, and the measured percentages are indicated.

Conclusion

The data show that the Agilent 2100 bioanalyzer with the Small RNA assay is a compelling technology to analyze small RNAs. Small RNA enrichment procedures can be necessary for some applications, such as array analysis. The Small RNA assay provides a simple and rapid way to monitor small RNA enrichment with reduced sample consumption. The sensitivity and accuracy of the procedure enable the quantification of small non-coding RNAs, including miRNAs, in small RNA preparations.

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