Stringent RNA quality control using the Agilent 2100 bioanalyzer

Application Note

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Abstract

Successful application of any RNA technology is dependent on the use of high quality RNA. Therefore, RNA isolation has to be followed by a stringent RNA quality control. The Agilent 2100 bioanalyzer in combination with RNA 6000 Nano and Pico LabChip® kits has become "the method of choice" for high-resolution analysis of small and very small RNA samples. The expected electropherograms vary, depending on species, tissue type and method of RNA isolation. These features are presented and specifically, the importance of heating RNA samples prior to electrophoresis is illustrated.

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Introduction

The isolation of RNA from biological samples is a tedious process, plagued by the presence of ribonucleases within the samples, and introduced as external contaminations. The primary concern in the quality of prepared RNA samples is RNA integrity[1]. Conventionally, this is checked by gel electrophoresis and monitoring of well-defined bands of the predominant rRNA species (rRNAs comprise more than 90 % of the mass of total RNA). Increasing routine applications of gene expression studies based on quantitative, real-time RT-PCR and DNA microarray hybridisations require reliable, highly reproducible technologies.

The Agilent 2100 bioanalyzer is the first commercially available chip-based nucleic acid analysis system. Pre-packed kits, standardized sample preparation and automated analysis reduce manual intervention and yield more accurate and reproducible data. It has become "the method of choice" for high-resolution RNA analysis, and using the RNA 6000 Nano LabChip kit, only about 25 ng of total RNA are required[2]. Recently, the increasing demand for the analysis of well-defined, specific cells was met by the development of laser microdissection technologies. The very low RNA yields obtained in these applications necessitated a substantial increase in the sensitivity of RNA analysis techniques. With the recently introduced RNA 6000 Pico LabChip, a detection limit of about 200 pg was achieved, enabling RNA analysis of laser microdissected samples with approximately 1000 cells[3]. Although the Agilent 2100 bioanalyzer is a reliable tool in RNA quality control, RNA quantitation is strongly influenced by the salt content of RNA samples and requires careful evaluation[4].

In this Application Note, electropherograms of RNA samples are presented with examples from different species and tissues and with variable RNA quality. General features of electropherograms are discussed, with emphasis on the importance of sample heating prior to electrophoresis.

Materials and methods

Human total RNA was prepared from approximately 10^7 Jurkat cells (derived from human T-cell leukemia, ATCC TIB-152, mostly diploid cells), using the RNeasy kit (Qiagen) according to the instructions of the manufacturer. Because a spin column purification with the RNeasy kit was included, the small RNAs (mainly tRNAs, 5S and 5.8S rRNAs) were removed. Fish (rainbow trout) total RNA (courtesy of Dr. Elgar Susanne Quabius, University of Aberdeen) was prepared from fresh liver samples, immediately flash-frozen in liquid nitrogen. Cryosections (10 µm) were prepared at –18 °C and lysed immediately in Trizol (Invitrogen) or RNA Stat-60 (ams Biotechnology), followed by RNA isolation according to the instructions of the manufacturer. Electropherograms of plant RNAs were generously provided by Dr. Peter Hedley, Scottish Crop Research Institute, Dundee. All RNA samples (30 to 100 ng in 1 µl water) were run with RNA 6000 Nano LabChip kits on the Agilent 2100 bioanalyzer according to the manufacturer's instructions.

For the heat treatment, the RNA samples were incubated for 2 minutes at 70 °C in thin-wall 0.5 mL PCR tubes (in a thermocycler with its lid heated to 110 °C). Samples were cooled and evaporated water was collected by centrifuging briefly in a tabletop centrifuge, followed by immediate analysis using the Agilent 2100 bioanalyzer.

Results

In the evaluation of bioanalyzer electropherograms, the first indication of good quality of total RNA samples is the appearance of well-defined, prominent peaks for ribosomal RNAs (rRNAs)[2,3]. Depending on the species, this general appearance can vary substantially, and in table 1, the different sizes and classes of expected rRNA molecules are summarized for a few species and tissue types. For high quality total RNAs, the following general observations are standard:

- Features of the instrument:
  (1) Capillary electrophoresis with the Agilent 2100 bioanalyzer is a native electrophoresis. Therefore, stringent RNA quality control is improved by heating the RNA prior to electrophoresis (see below).
  (2) A fast migrating "reference marker" compound is added to all samples and appears at the start of the electropherogram. It is used for software alignment of all electropherograms within one LabChip run.
  (3) In total RNA, rRNAs comprise more than 90 % of its mass. If the "Eukaryotic Total RNA Nano Assay" is used, the Agilent 2100

bioanalyzer software automatically searches for, and identifies peaks for the two most abundant large rRNAs (always labelled as 18S and 28S), and peak area ratios are calculated (see table 1 for expected species-dependent ratio variations).

- Depending on the species, the expected number of well-defined, major peaks for rRNAs can vary (see table 1).
- If the RNA sample was prepared without size fractionation, (e.g. without RNeasy spin columns), a peak for small RNAs (tRNAs, 5S and 5.8S rRNAs) occurs close to the reference marker.
- Apart from rRNA peaks, the signal intensities should be very low, between rRNA peaks, and especially in the segment between rRNAs and the reference marker.

Sample results from vertebrates (human and trout) and from plant tissues (barley and *Arabidopsis*) are presented and discussed in more detail.

Heating the RNA sample: How to do it and why is it crucial?

RNAs are highly structured molecules with many intramolecular, base-paired double-stranded segments, especially rRNAs have a very stable and complex secondary structure (see 18S rRNA in figure 1). If the quality of an RNA sample has suffered, several nicks or RNA cleavages occur in sensitive, single-stranded regions. However, the resulting fragments are still held in place by extensive base-pairing in double-stranded regions. A well-known example is the presence of naturally occuring "hidden breaks" in rRNAs. This means, the 28S rRNA is composed of two

Species (mass ratio)	rRNAs	Length [in kb]
Human (2.6)	18S/28S	1.9 / 5.0
Mouse (2.5)	18S/28S	1.9 / 4.7
Teleost fish (Zebrafish, *Danio rerio)* (2.3)	18S/28S	1.9 /4.1
Drosophila melanogaster	18S/28S	2.0 / 4.1*
Plant		
cytoplasmic (1.9)	18S/25S	1.9 / 3.7
chloroplasts ** (1.8)	16S/23S	1.5 / 2.7
Caenorhabditis elegans (2.0)	18S/26S	1.75 / 3.5
Yeast *(Saccharomyces cerevisiae)* (1.9)	18S/26S	2.0 /3.8
Bacteria *(E.coli) (1.9)*	16S/23S	1.5 /2.9

Table 1
Length variations of rRNAs from different species and tissue types.
Species names are followed by rRNA ratios (in brackets). Please note: * Drosophila 28S rRNA is split in 2 fragments, comigrating with 18S rRNA. ** Prominent bands only in RNAs from green tissues with high chloroplast content.

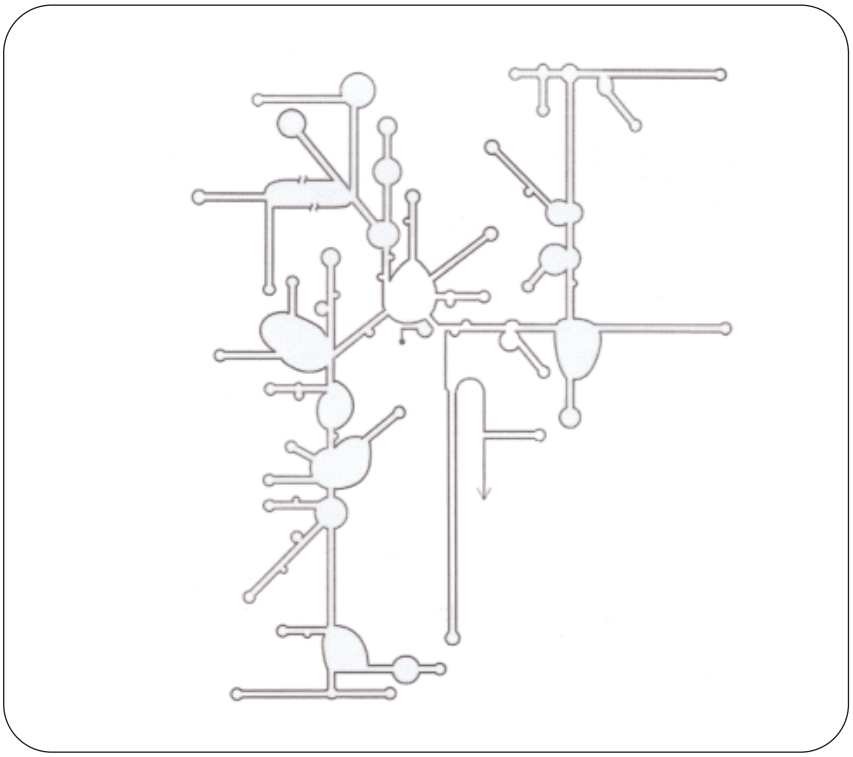


Figure 1
Complex secondary structure of 18S rRNA, maintained by multiple, large double-stranded sections (redrawn according to reference [1]).

smaller fragments that are united in vivo to form a functional 28S rRNA. The isolated RNA migrates as one band in native gel electrophoresis conditions, whereas the two fragments migrate as two bands in denaturing gels[5]. If such aggregrates of RNA fragments are analyzed by native electrophoresis (e.g. with the Agilent 2100 bioanalyzer), they migrate just like intact rRNAs and the resulting peaks in the electropherogram mimic intact rRNAs. This misleading result can be avoided by heating RNA samples prior to electrophoresis. Base-pairing is destabilized and aggregates of RNA fragments fall apart.

To achieve this, RNAs should be incubated for 2 min at 70 °C. During this "tempering step", fragmented rRNAs and RNA aggregates will dissociate, whereas RNA secondary structures within intact RNA molecules are desta-

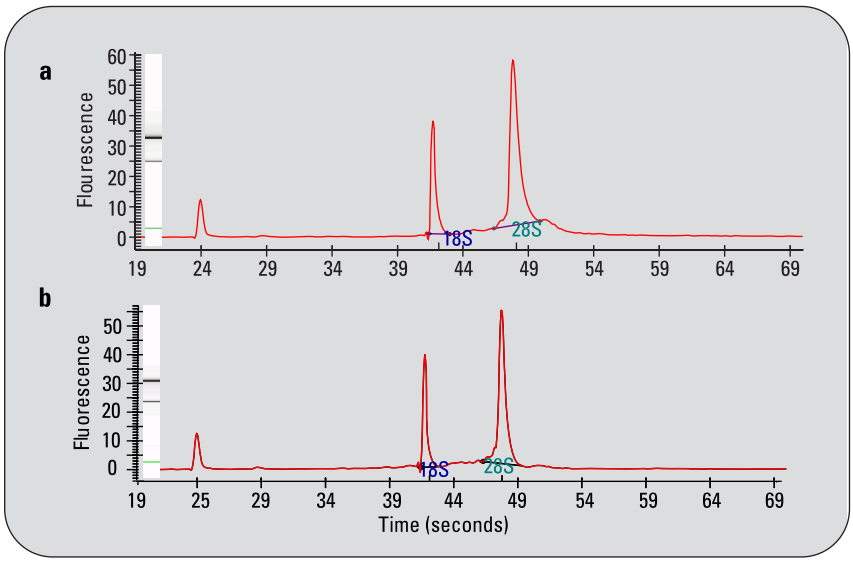
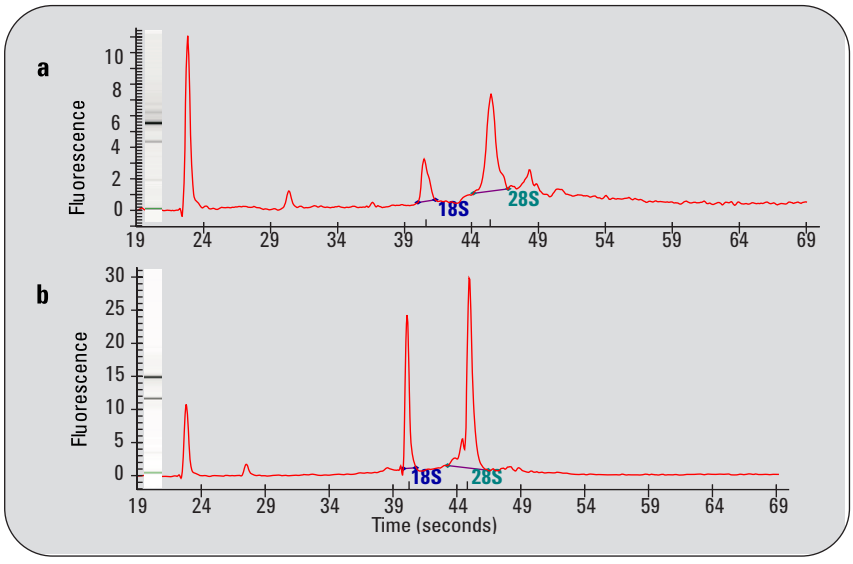


Figure 2
High quality human RNA. Isolated from the human Jurkat cell line (ATCC TIB152). Approximately 200 ng total RNA were analyzed on an RNA 6000 Nano LabChip, using the eukaryotic total RNA Nano software. A: without, B: with heat treatment. Both electropherograms feature well defined peaks for 18S and 28S rRNAs, with a ratio of > 2 (maximal theoretical ratio is 2.6; see Table 1); minor amounts of RNA migrating faster than or between both rRNA peaks. Differences after heating: rRNA ratio (about 2.3 without and 2.1 with heating) and abundance of other, "non-ribosmal RNAs" are essentially unchanged; peak of "pre-RNAs" (aggregates that migrate more slowly than 28S rRNA) disappears after heating. Slight sharpening of peaks after heating (widths slightly decrease from 1.6 to 1.4 sec for 18S and 3.5 to 3.0 sec for 28S).

Figure 3
Dramatic change of electropherogram with good quality fish RNA. RNA was isolated from a 10-µm thick cryosection of rainbow trout liver, using trizol. Various general features: this lysis procedure without size fractionation results in high amounts of small RNAs (tRNAs, 5S and 5.8S rRNAs; between 24 and 31 seconds migration time, close to marker peak), and sheared genomic DNA (slightly elevated general background signal). As a dramatic effect, the expected appearance of total RNA with two rRNA peaks was observed after heat treatment only. The increased formation of double stranded RNA structure is concomitant with significantly increased fluorescence intensity. In comparison with human RNA, note the reduced size difference (see table 1) and a corresponding lower migration difference between 18S and 28S rRNAs (about 5.6 sec for the heated human RNA samples, and about 5.2 sec for the heated fish RNA).



bilised but not completely disrupted. This means, unfavored structures in intact RNAs are destabilized and RNAs will refold into their thermodynamically favored structures. As a consequence, misleading peaks for fragmented rRNAs and other RNA aggregates disappear, and peaks for intact rRNAs sharpen. These effects will be demonstrated with a few examples.

Observations with good quality RNA samples

Standard appearance

In general, the heat treatment has only modest effects on good quality RNA. In the following examples, the effects of heating are illustrated by comparing electropherograms without and with heat treatment. Without heating (figure 2a), the electropherogram of a human RNA sample shows only the two distinct peaks for 18S and 28S rRNAs; with a ratio of about 2. Since the RNA was purified using a spin column step, low molecular weight RNAs are absent and the only additional peak is the fast migrating reference marker. A shoulder next to the 28S rRNA indicates the presence of RNA aggregates – sometimes referred to as "pre-RNAs". Apart from these peaks, the signals due to mRNAs are broad, their intensities are low and barely visible above background.

After heating (figure 2b), the general appearance is only slightly altered. As discussed above, sharper peaks for rRNAs occur and "pre-RNA" aggregates essentially disappear.

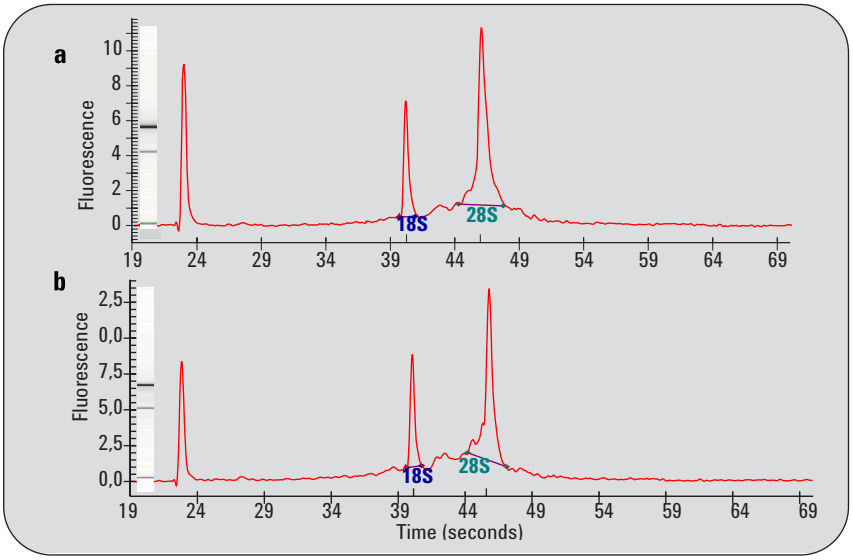


Figure 4
Good quality human RNA with minor degradation. Same source of RNA as in Figure 1. General features are similar to Figure 1; note the higher amount of "non-ribosomal RNAs" and a further increase after heating; rRNA ratio remains high (about 2.6 without and 2.2 with heating). Slight sharpening of peaks after heating (widths slightly decrease from 1.3 to 1.1 sec for 18S and 3.5 to 2.9 sec for 28S).

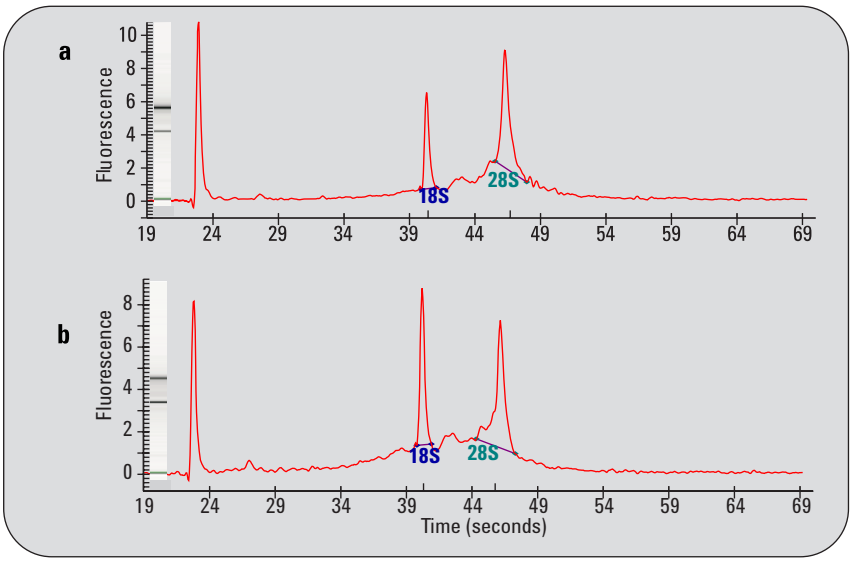


Figure 5
Human RNA with reduced quality. Same source of RNA as in figure 1. Already the general features are different from RNA in figure 1. The rRNA ratio is lower (about 1.9 without, and drops to 1.4 with heating). Note the high amount of "non-ribosomal RNAs" which further increases after heating.

Unusual observations with trout RNA

Without heating, this RNA sample from rainbow trout resulted in an electropherogram with numerous peaks (figure 3a). With this non-standard electropherogram, an evaluation of the RNA quality is difficult. After heating, the abnormalities are resolved and the typical electropherogram of a good quality RNA is obtained (figure. 3b). This extreme observation illustrates that rRNAs can fold into a number of different secondary/tertiary structures, and with heat treatment they can be converted into their thermodynamically favored native structures.

Observations with only slightly reduced and significantly reduced quality RNA samples

In general, prominent rRNA peaks are still observed, but ratios drop below 2, and the presence of RNA fragments is revealed by higher signals in the region between rRNAs and between 18S rRNA and the reference marker. Slightly reduced RNA quality was observed with the sample shown in figure 4. Without heating, the rRNA ratio is above 2, and the signal between both rRNAs is quite low. After heating the rRNA peaks narrow (maintaining a ratio of >2), and additional signals become only slightly more prominent. With the sample shown in figure 5, further reduced quality is evident. Without heating the rRNA ratio is about 2, but significant signal

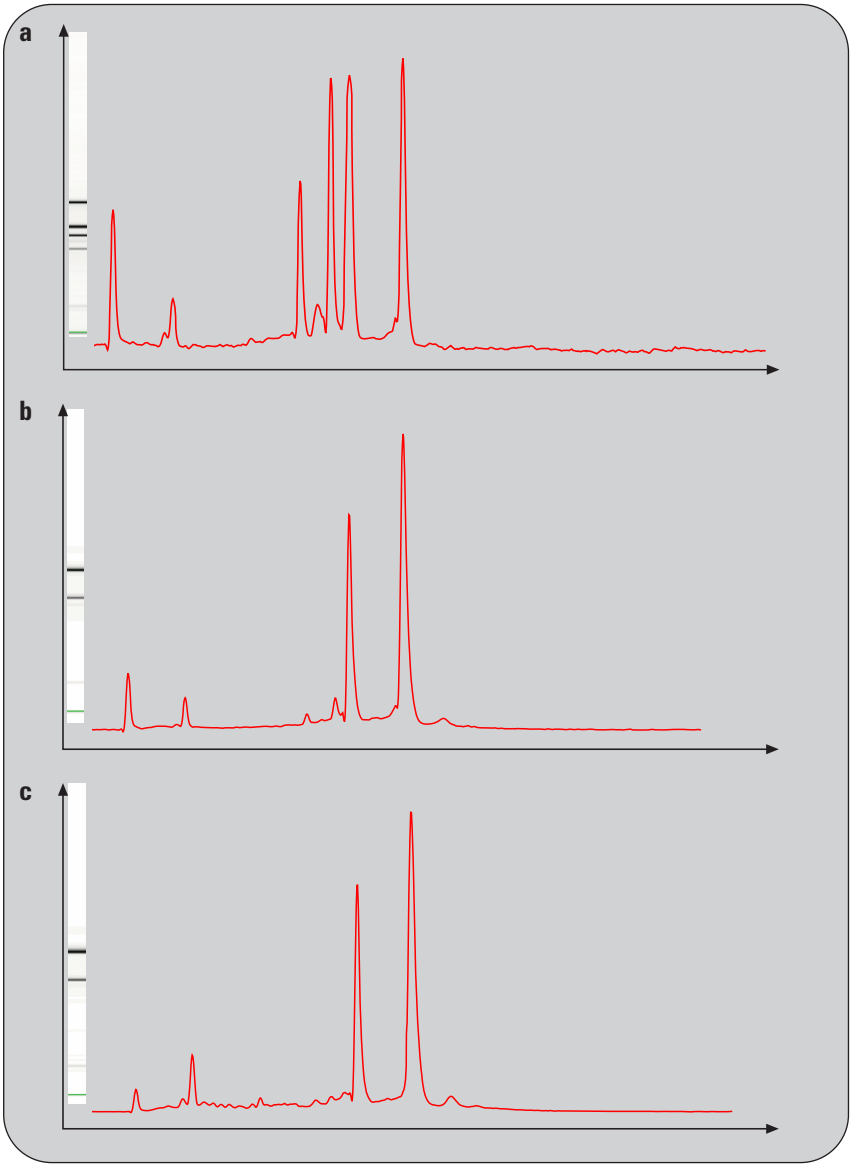


Figure 6

RNAs from barley. The samples were heated prior to electrophoresis. The comparison of RNAs from green leaves (a) with those from shoots (b) and roots (c) reveals two additional prominent peaks in RNA from the green tissue (a). Both migrate faster than cytoplasmic 18S rRNA, indicating an abnormal migration of 16S and 23S rRNAs from chloroplasts, presumably due to secondary structures effects that are not resolved by heat treatment and non-denaturing capillary electrophoresis.

intensities occur between rRNAs. After heating, the rRNA ratio drops below 1.5, and prominent RNA fragments occur which originate at the 28S rRNA peak and are distributed in the low molecular weight section down to the reference marker. The quality of this RNA sample is significantly reduced and careful data evaluation is advised in gene expression studies.

Special features of plant RNAs

In addition to cytoplasmic 18S and 25S rRNAs, total RNA in green leaves also contains 16S and 23S rRNAs from chloroplasts (see table 1, figures. 6 and 7). Using RNA from *Hordeum vulgare* (barley) leaf, the expected four major peaks are visible (figure. 6a). However, an abnormal migration is observed for chloroplast 23S rRNA (2.7 kb), since it migrates slightly faster than 18S rRNA (1.9 kb). The chloroplast content of barley shoots is very low, and even lower in roots. Accordingly, the chloroplast rRNAs are essentially absent in RNA samples from these two plant tissues (figures. 6b and c). In RNA from *Arabidopsis thaliana* leaves, three peaks can be assigned to 16S and 23S rRNAs from chloroplasts (figure 7), since they are almost absent in RNA samples from tissues with low chloroplast content, *Arabidopsis* seed pods and flowers (figures 7b and c).

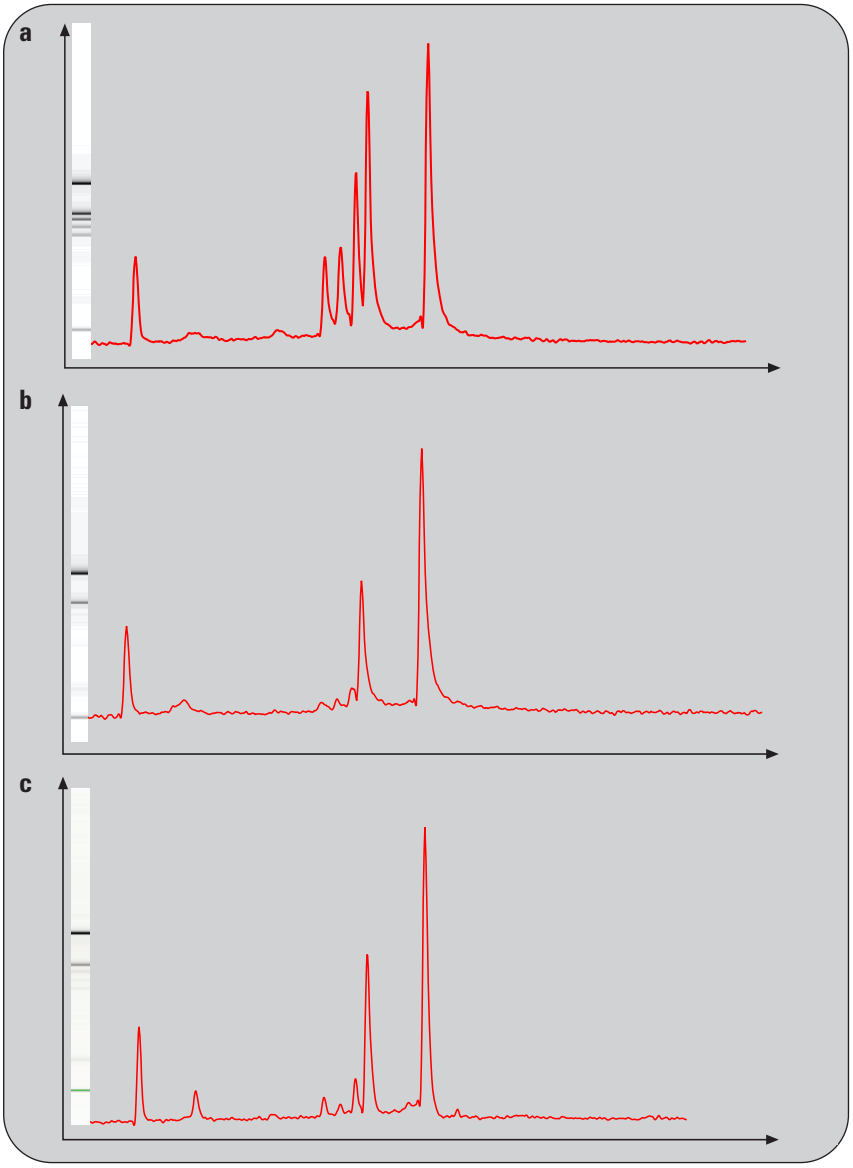


Figure 7
RNAs from *Arabidopsis thaliana*. The samples were heated prior to electrophoresis. The comparison of RNAs from green leaves (a) with those from seed pods (b) and flowers (c) reveals three additional peaks in RNA from the green tissue (a). This is a further abnormality in the migration of 16S and 23S rRNAs from chloroplasts.

Conclusions

The Agilent 2100 bioanalyzer is well suited to detect differences in RNA quality. It can be used to optimize methods for RNA isolation and to increase success rates, reliability and reproducibility of subsequent gene expression studies. In this application note, a few examples were shown to illustrate species variations and to demonstrate that evaluation of RNA quality is can be further impoved by heat treatment of RNA samples prior to electrophoresis.

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