Application Note 116

RNA Chromatography Under Thermally Denaturing Conditions: Analysis and Quality Determination of RNA

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Chromatography of RNA at elevated denaturing temperatures using the WAVE™ Nucleic Acid Fragment Analysis System and an RNASep™ Cartridge is presented as a new method for the analysis of RNA. This strategy provides a safe and reliable alternative to the classical methods of RNA quality determination.

Introduction

In cells, genetic information flows from DNA to RNA to protein. RNA is therefore the key intermediary containing critical information pertaining to genetics and proteomics. RNA is also the starting material in numerous molecular investigations involving the characterization of known messenger RNA (mRNA), the identification of unknown genes and the assignment of functions to various proteins. A major factor determining the rate of success in applications such as cDNA library construction, Northern blot analysis, reverse-transcription (RT), and *in situ* hybridization analysis is the quality of the RNA starting material. A number of labor-intensive, hazardous and lengthy techniques are currently available for RNA analysis. Quality determination is generally performed by gel electrophoresis followed by ethidium bromide staining and visualization by fluorescence (1), or by Northern blotting and hybridization using specific probes. The quality of the RNA analyzed by these classical procedures is jeopardized by the presence of ribonucleases in the analysis buffer, the gel and the electrophoresis apparatus. The RNA analyzed cannot be recovered and used in downstream applications.

Here, we present a faster, safer, less labor intensive and highly accurate alternative method for RNA analysis. RNA chromatography using the WAVE Nucleic Acid Fragment Analysis System equipped with an RNASep Cartridge provides a reliable means for determining RNA integrity. Our results indicate that RNA purified with the WAVE System is more stable than the original RNA sample applied.

Materials and Methods

The WAVE System with the RNASep Cartridge (internal diameter of 7.8 mm and a length of 50 mm) and triethylammonium acetate (TEAA) were provided by Transgenomic, Inc. (Omaha, NE). The RNA ladder was purchased from Roche Molecular Biochemicals (Indianapolis, IN). Total RNA and mRNA samples were obtained from Clontech (Palo Alto, CA). Standard buffers used were: (A) 0.1 M TEAA and (B) 0.1 M TEAA, 25% acetonitrile (ACN). *In vitro* transcription kits for RNA synthesis were purchased from Ambion (Austin, TX).

RNA chromatography was carried out under denaturing conditions at 75°C using the WAVE System and the RNASep Cartridge. The stationary phase in the RNASep Cartridge consists of a nonporous alkylated poly(styrene-divinylbenzene) bead matrix. The mobile phase consists of a two-eluant buffer system composed of buffer A and buffer B. Buffer A consists of 0.1 M triethylammonium acetate (TEAA), pH 7.0, and buffer B consists of 0.1 M TEAA at pH 7.0 containing 25% acetonitrile (ACN) by volume.

RNA eluting from the column was detected with a UV detector at 260 nm, and the data were recorded and displayed as chromatograms by the WAVE System's WAVEMAKER™ Software. Gradient conditions applicable to Figures 1 and 4 are presented in Table 1. Gradient conditions applicable to Figures 2 and 3 are presented in Table 2.

Results and Discussion

RNA samples, size markers, total RNA and mRNA can be analyzed on the WAVE System using an RNASep Cartridge. RNA, which readily undergoes degradation by ribonucleases or chemical hydrolysis, is remarkably stable during analysis on the WAVE System. In addition, this system provides a high-resolution capability for RNA analysis. This is exemplified in Figure 1 in which well-resolved RNA peaks were observed for an RNA ladder containing five fragments ranging in size from 310 to 1517 nts.

The WAVE System can also be used to determine the integrity of RNA transcripts. To demonstrate this application, different plasmid vectors carrying an RNA polymerase promoter were linearized with restriction endonucleases and transcribed *in vitro* to create single RNA transcripts of a specific size. Each transcript was then analyzed individually using the WAVE System in order to determine its quality. In Figure 2, the chromatograms of individual transcripts were superimposed. The purity and stability of each of these transcripts is clearly demonstrated by the presence of a single peak. In Figure 3, a 2876-nt transcript was analyzed on the WAVE System and its chromatogram reveals the presence of smaller size transcripts and/or degraded RNA in the sample. When RNA undergoes degradation it is broken down into smaller fragments which have a weaker interaction with the

RNASep Cartridge and thus elute faster. This is shown by the presence of smaller peaks to the left of the 2876-nt peak.

Total RNA and mRNA samples can also be analyzed on the WAVE System. Figures 4a and 4b demonstrate the high degree of stability of these samples in our system. Peaks corresponding to ribosomal and mRNA samples provide a highly accurate visual way of determining the quality of RNA samples. For example, the quality of oligo(dT)-cellulose purified mRNA is shown in Figure 4b in which, even though it contains some ribosomal RNA contamination, no degradation is observed.

In these experiments, analysis of large RNA samples is achieved in less than 18 minutes and results are highly reproducible. In addition, each RNA peak can be quantitated by peak integration (performed automatically by WAVE-MAKER™ Software), purified by peak-capture and directly used in downstream applications (2). Since RNA detection is performed by UV spectroscopy at 260 nm, there is no need for the use of harmful chemicals such as ethidium bromide. The UV detector provides a powerful detection limit as low as 1 ng per peak. No signs of RNA degradation were observed during analysis on the WAVE System. Furthermore, RNA purified by peak capture showed an increase in stability. This is demonstrated by the fact that the purified RNA could be stored for days at room temperature, or for months at -20°C or -70°C with no sign of degradation. The observed increase in stability of the collected RNA is believed to be due to the complete removal of RNases during the chromatographic process. The collected RNA samples can be precipitated or used directly (in the collection buffer) in downstream applications such as RT-PCR (3). The WAVE System, therefore, allows for a fast, accurate, safe, and automated alternative to the classical methods of RNA quality assessment.

Conclusions

Here, we have shown that RNA can be analyzed quickly and easily by RNA chromatography carried out on the WAVE System at an elevated temperature. RNA is very stable under the analysis conditions and in the collection buffers used.

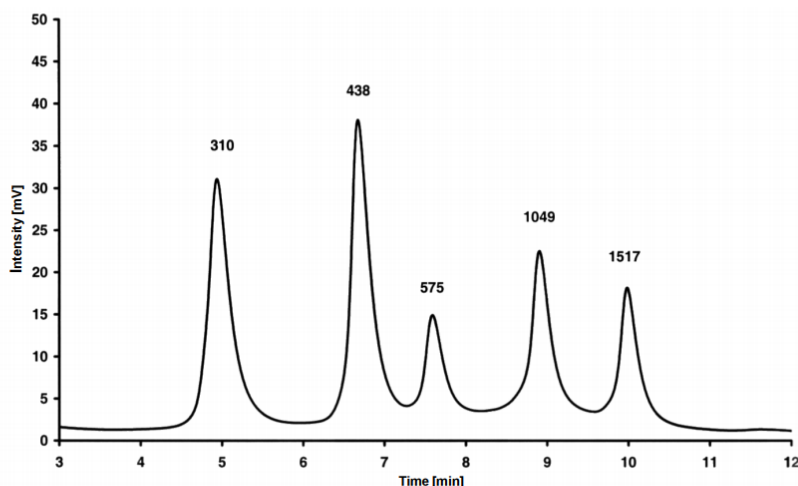


Figure 1: RNA ladder (1 µg.) analyzed on the WAVE System.
An RNA ladder containing five fragments ranging in size from 310 to 1517 nts was analyzed on the WAVE System to demonstrate the high-resolution capability of the RNASep Cartridge.

Table 1

Gradient Conditions for Figures 1, 4a, and 4b				
Time (minutes)	**Buffer A (%)**	**Buffer B (%)**	**Flow Rate (ml/min)**	**Temperature (°C)**
0	62	38	0.90	75
1	60	40	---	---
16	40	60	---	---
22	34	66	---	---
22.5	30	70	---	---
23	0	100	---	---
24	0	100	---	---
25	62	38	---	---
27	62	38	---	---

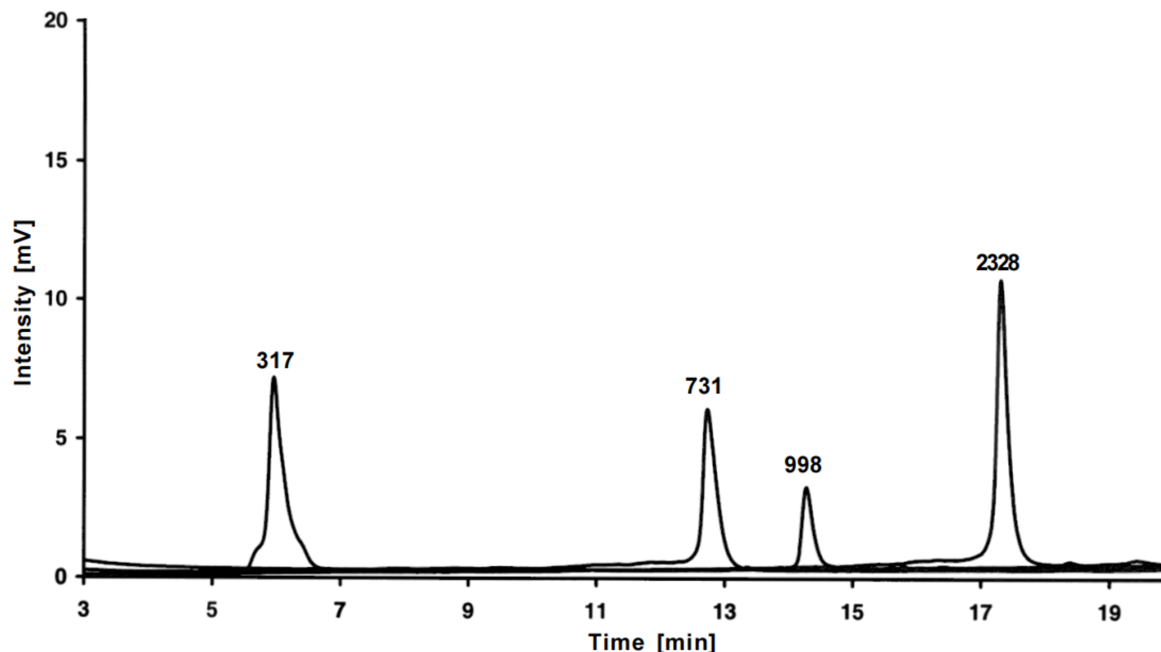


Figure 2: RNA transcripts analyzed on the WAVE System.

The plasmid pALTER-Ex1 (Promega, Madison, WI) was linearized with Pvu I and transcribed with SP6 RNA polymerase to yield a 317-nt transcript. The other transcripts were obtained by transcribing the linearized plasmid pNGGM (kindly provided by Maryam M. Matin, University of Sheffield, UK). Acc I, Sma I and Xba I digestion yielded the 731-, 998- and 2328- nucleotide transcripts, respectively. Each transcript was obtained and analyzed separately. Single chromatograms are superimposed and show the degree of purity and stability of each transcript produced.

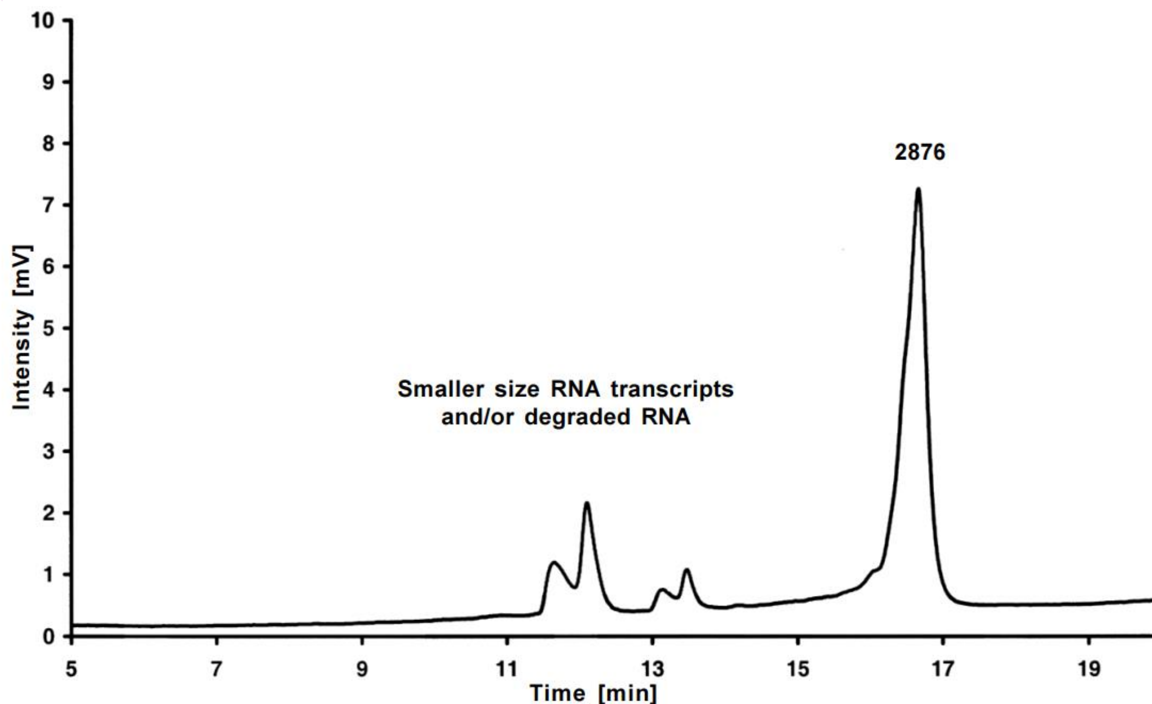
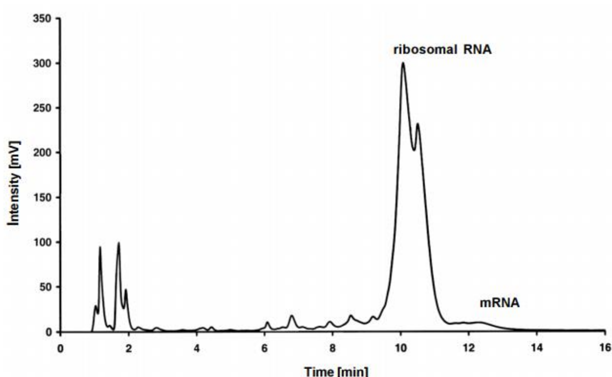


Figure 3: RNA transcript analyzed on the WAVE System.

A plasmid vector was specifically digested and transcribed in vitro to create a single RNA transcript. This transcript was analyzed on the WAVE System; the chromatogram reveals the presence of impurities in the RNA created. Smaller size transcripts and/or degraded RNA peaks are shown to the left of the 2876-nt transcript peak.

Table 2

Gradient Conditions for Figures 2 and 3				
Time (minutes)	**Buffer A (%)**	**Buffer B (%)**	**Flow Rate (ml/min)**	**Temperature (°C)**
0	62	38	0.90	75
30	40	60	---	---
32	0	100	---	---
35	62	38	---	---



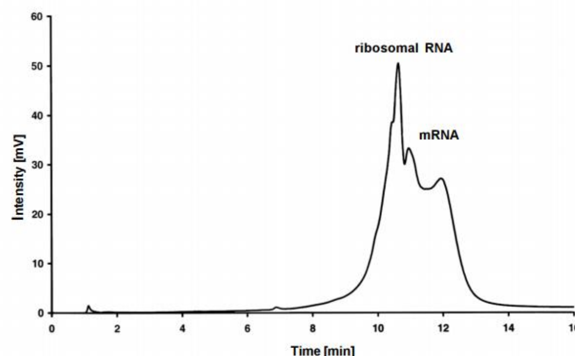


Figure 4a: Mouse brain total RNA analyzed on the WAVE System.

Figure 4b: Oligo(dT)-cellulose purified mouse brain mRNA analyzed on the WAVE System.

Mouse brain total RNA (20 µg) and mouse brain mRNA (5 µg) were analyzed on the WAVE System. The mouse brain mRNA (Clontech, Palo Alto, CA) was isolated by two rounds of poly(A)$^+$ RNA selection.

References

1. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd ed., chapter 7. CSH Laboratory Press, Cold Spring Harbor, NY.
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ADS Biotec is currently the sole producer of the Transgenomic-designed WAVE Nucleic Acid Fragment Analysis System, RNASep™ columns (Part #: RNA-99-3810), and the referenced HPLC Buffers used as the eluents in this work.

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