Application Note

Biopharmaceuticals

Comparative Assessment of RNA Purity Using Simultaneous UV-Vis Measurements

Evaluating the effects of pH and ionic strength on RNA $A_{260/280}$ using an Agilent Cary 3500 UV-Vis

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Abstract

The standard method for assessing the purity of nucleic acids uses spectrophotometric absorbance measurements at wavelengths of 260 nm (A_{260}) and 280 nm (A_{280}). This application note demonstrates that changes in both the pH and ionic strength of a ribonucleic acid (RNA) sample buffer influences the $A_{260/280}$ ratios. Other results show that the detection of protein contamination is significantly enhanced when RNA is measured in an alkaline solution. Using an Agilent Cary 3500 Multicell Peltier UV-Vis spectrophotometer, this study highlights the advantages of conducting multiple experiments simultaneously and using the in-built equation software features of the Cary 3500 UV-Vis to assess RNA purity.

Introduction

Assessing the integrity and purity of RNA is essential for ensuring RNA quality in downstream experiments and applications. Compromised or contaminated RNA can lead to unreliable gene expression results, potentially impacting the conclusions drawn from these types of analyses.[1] Also, since encapsulated RNA can be used as a drug product, RNA quality is a critical quality attribute in pharmaceutical manufacturing applications.

The use of spectrophotometry for RNA quantification and purity assessment is well established. The $A_{260/280}$ ratio, which is determined from measuring the absorbance of the sample at both 260 and 280 nm, is used to assess the purity of a nucleic acid sample. The absorbance at 260 nm primarily indicates the presence of nucleic acids, while the absorbance at 280 nm indicates the presence of any contaminating proteins. So, a pure RNA sample would have an $A_{260/280}$ ratio of 2.00, while a protein sample would have a ratio of 0.57.[2]

While the $A_{260/280}$ ratio is a useful parameter, Wilfinger *et al.* reported that the pH and concentration of the solution used in spectrophotometric evaluations can significantly affect the ratio.[3] The quantitative assessment of nucleic acid preparations is also impacted by pH and concentration.

In this study, similar experiments to those reported by Wilfinger *et al.* were carried out using a Cary 3500 Multicell Peltier UV-Vis spectrophotometer (Figure 1). The Cary 3500 Multicell Peltier UV-Vis is an innovative system that includes eight cuvette positions to improve measurement efficiency. Simultaneous measurement of UV absorbance for multiple samples by the Cary 3500 avoids unwanted variables, enhancing confidence in the results. The in-built equation function of the Agilent Cary UV Workstation software automatically calculates the $A_{260/280}$ ratio based on the UV scans, further improving the throughput of the analyses.

The effects of pH and ionic strength of the RNA sample solution on the $A_{260/280}$ ratio were also investigated using the Cary 3500. In addition, the impact of pH and ionic strength on the identification of protein contamination in RNA was examined. Comparisons of UV absorbance scans of RNA in two different buffers and water were also carried out.

Figure 1. Agilent Cary 3500 Multicell Peltier UV-Vis spectrophotometer.

Experimental

Instrumentation

An Agilent Cary 3500 Multicell Peltier UV-Vis spectrophotometer was used in this study. Data acquisition was performed using Agilent Cary UV Workstation software (version 1.4.256) with the Cary 3500 Multizone software add-on, using the parameters shown in Table 1. The software includes more than 50 equation functions. Ultra-microvolume rectangular cells with a UV pathlength of 10 mm a 70 µL fill volume (part number 5062-2496) were used. A sample volume of 50 µL was used for each cell. The Cary 3500 allows the measurement of such small sample volumes due to its highly collimated, uniform beam, which is less than 1.5 mm wide, and the permanent optical alignment of its stationary cell holders.

Table 1. Agilent Cary 3500 Multicell Peltier UV-Vis spectrophotometer parameters.

Parameters	Value
X Mode	nm
Y Mode	Absorbance
Collect Mode	Scan
Scan Range Start	400 nm
Scan Range Stop	220 nm
Averaging Time	0.020 s
Data Interval	1.00 nm
Scan Rate	3,000 nm/min
Spectral Bandwidth	2.00 nm
Detector Module	Multicell Peltier UV-Vis

Reagents and materials

The HeLa cell line was bought from American Type Culture Collection (ATCC, Manassas, Virginia, USA). Dulbecco's Modified Eagle Medium (DMEM) was bought from Thermo Fisher Scientific (Waltham, Massachusetts, USA).

An Agilent Absolutely RNA microprep kit was used to extract the RNA from the HeLa cells (part number 400805).

Bovine serum albumin (BSA), disodium phosphate (Na_2HPO_4), tris-hydrochloride (tris-HCl), and sodium chloride (NaCl) were bought from Sigma-Aldrich (St. Louis, MO, USA). The EDTA solution was obtained from Merck (Darmstadt, Germany).

Fresh ultrapure water was obtained from a Milli-Q Integral system (Millipak, Merck-Millipore, Billerica, MA, USA) equipped with a 0.22 µm membrane point-of-use cartridge.

Extraction of RNA from HeLa cell pellets

HeLa cells were grown to 100,000 cells in the DMEM cell culture medium. The cells were scraped from the plate into the media and transferred into a 15 mL falcon tube. The cell suspension was centrifuged at 1,200 rpm for five minutes to pellet the cells before RNA extraction using the Absolutely RNA Microprep kit protocol. A quality check of the extracted RNA was conducted using an Agilent 4200 TapeStation system (part number G2991AA) with the RNA ScreenTape (part number 5067-5576), RNA ScreenTape sample buffer (part number 5067-5577), and the RNA ScreenTape ladder (part number 5067-5578).

The RNA was frozen at –80 °C and only thawed before analysis.

Workflow of RNA extraction from HeLa cells and purity assessment

As shown in Figure 2A, the HeLa cells were grown in-house, and total RNA was extracted using the Absolutely RNA microprep kit protocol. The extracted RNA was checked for RNA quality using the 4200 TapeStation system—an automated electrophoresis solution for the quality control of DNA and RNA samples. Using an RNA ScreenTape device for the analysis of total RNA enables the assessment of total RNA quality by identifying the ribosomal peaks and any potential degradation products. An individual RNA quality score, known as the RNA Integrity Number equivalent (RIN^e), is assigned to each sample based on a scale from 1 to 10, where 1 indicates highly degraded RNA and 10 indicates highly intact RNA. The extracted RNA used in this study had an RIN^e value of 10 (Figure 2B). The extracted RNA absorbance was then measured on the Cary 3500 UV-Vis for RNA purity assessment.

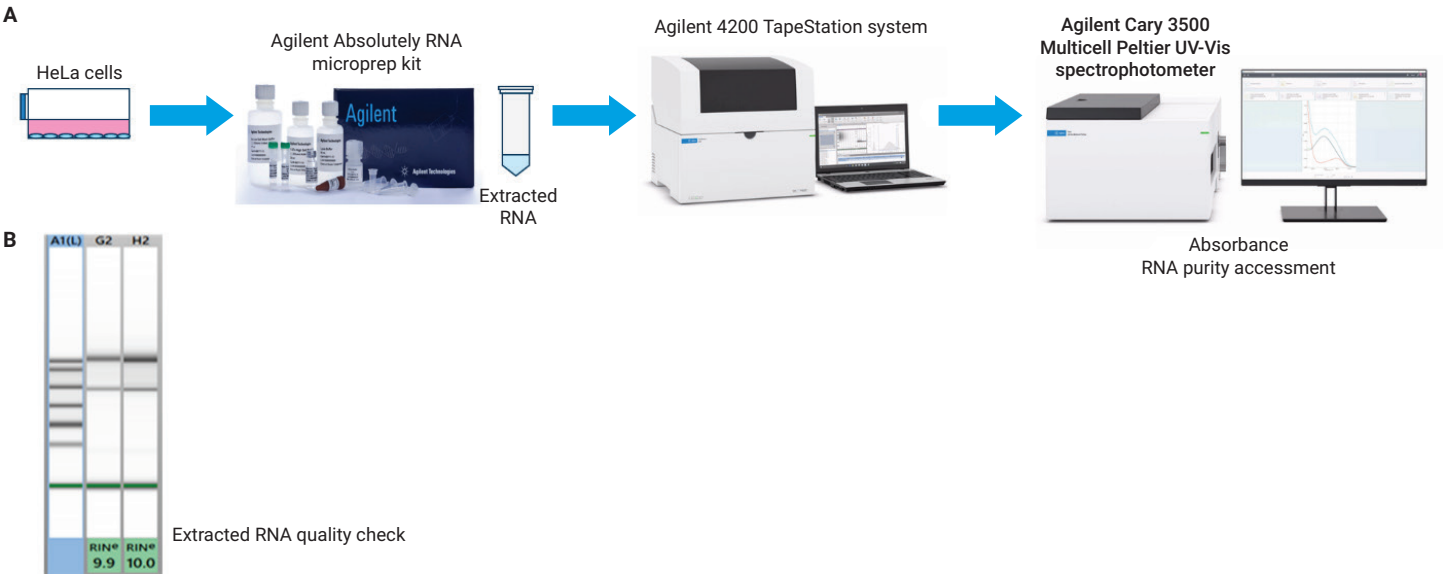

Figure 2. (A) Workflow diagram showing the use of Agilent consumables to extract RNA, and the Agilent 4200 TapeStation system and Agilent Cary 3500 UV-Vis spectrophotometer to assess its quality and purity. (B) The RNA extracted from HeLa cells was analyzed on the 4200 TapeStation, yielding an RIN^e value of 10.

Experimental

The following three experiments were conducted in this work:

1. **Studying the effects of pH and Na_2HPO_4 concentration on the $A_{260/280}$ ratio and absorbance of RNA**

   Na_2HPO_4 was diluted with ultrapure water to the following concentrations: 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, and 10 mM. Ultrapure water was included in the experiment as a control. All pH measurements were performed using a SevenCompact pH meter S220 from Mettler Toledo (Greifensee, Switzerland). RNA was diluted to 0.0062 µg/µL with the respective concentrations of Na_2HPO_4 solutions.

2. **Studying the effects of pH and ionic strength on the $A_{260/280}$ ratio of RNA in the presence of solubilized BSA protein**

   Na_2HPO_4 was diluted to the following concentrations: 0.01, 0.1, 1 and 10 mM. Water was included in the experiment as a control. RNA was dissolved in the respective buffer solutions to a final concentration of 0.0062 µg/µL. RNA spiked with protein (containing both RNA at a final concentration of 0.0062 µg/µL and BSA at a final concentration of 0.1 µg/µL) was dissolved in the respective buffers. BSA was also prepared in the respective buffers to a concentration of 0.1 µg/µL.

3. **UV absorbance scans of HeLa RNA analyzed in 1 mM Na_2HPO_4, TNE buffer, and water**

   A TNE buffer containing 10 mM tris, 1 mM EDTA, and 0.2 mM NaCl at pH 7.4 was prepared. RNA was diluted to a final concentration of 0.0062 µg/µL in the TNE buffer, in 1 mM Na_2HPO_4, and in water.

Absorbance measurements

Absorbance values of all the experimental samples were measured using the Cary 3500 Multicell Peltier UV-Vis spectrophotometer. An appropriate blank solution was used to zero the spectrophotometer for each experiment and baseline correction was used during the analysis.

The capability of the Cary 3500 Multicell Peltier UV-Vis to measure multiple samples simultaneously makes spectrophotometric measurements more efficient, while also maintaining consistent measurement conditions. With eight cell positions available within four zones, the instrument can perform analyses on samples in a single zone (eight cuvettes), two zones (four cuvettes in each zone), or four zones (two cuvettes in each zone). Each zone has its own reference channel.

The two-zones feature of the Cary 3500 UV-Vis was used for the protein contamination study. This configuration allowed two buffers to be studied in one measurement (Figure 3). The four-zones feature of the instrument was used for the concurrent measurement of RNA in three different solutions (TNE buffer, 1 mM Na_2HPO_4, or water), as shown in Figure 4. Following each measurement, the in-built equation function of the Cary UV Workstation software automatically calculated and reported the $A_{260/280}$ ratios (Figure 5). These features of the Cary 3500 increase the throughput of UV absorbance scans compared to using a single cuvette system.

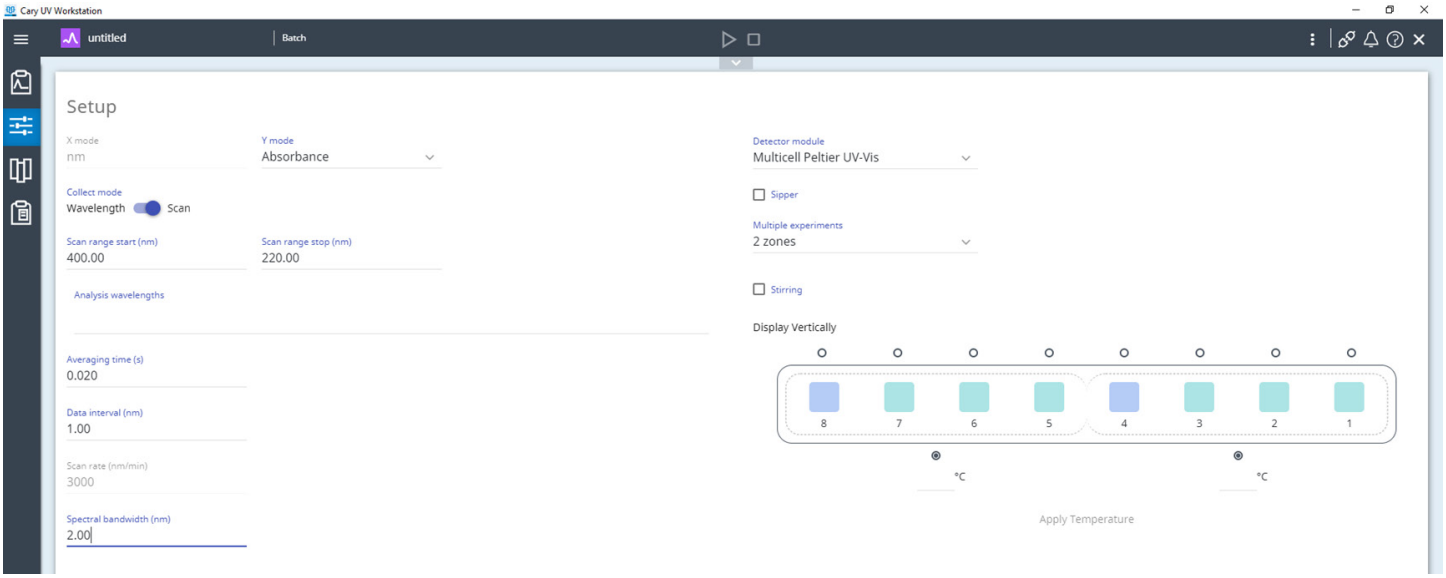

Figure 3. The "two-zones" multiple experiment feature of the Agilent Cary 3500 Multicell Peltier UV-Vis. The green and purple positions represent the samples and reference solutions, respectively.

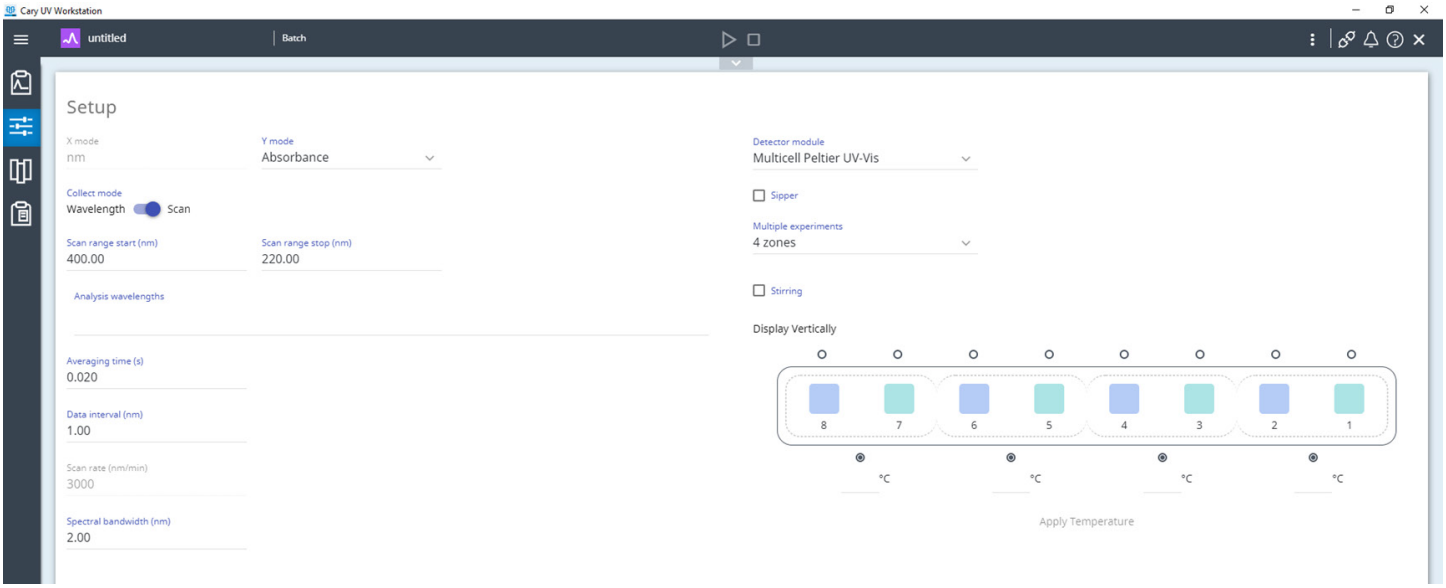

Figure 4. The "four-zones" multiple experiment feature of the Agilent Cary 3500 Multicell Peltier UV-Vis. The green and purple positions represent the samples and reference solutions, respectively.

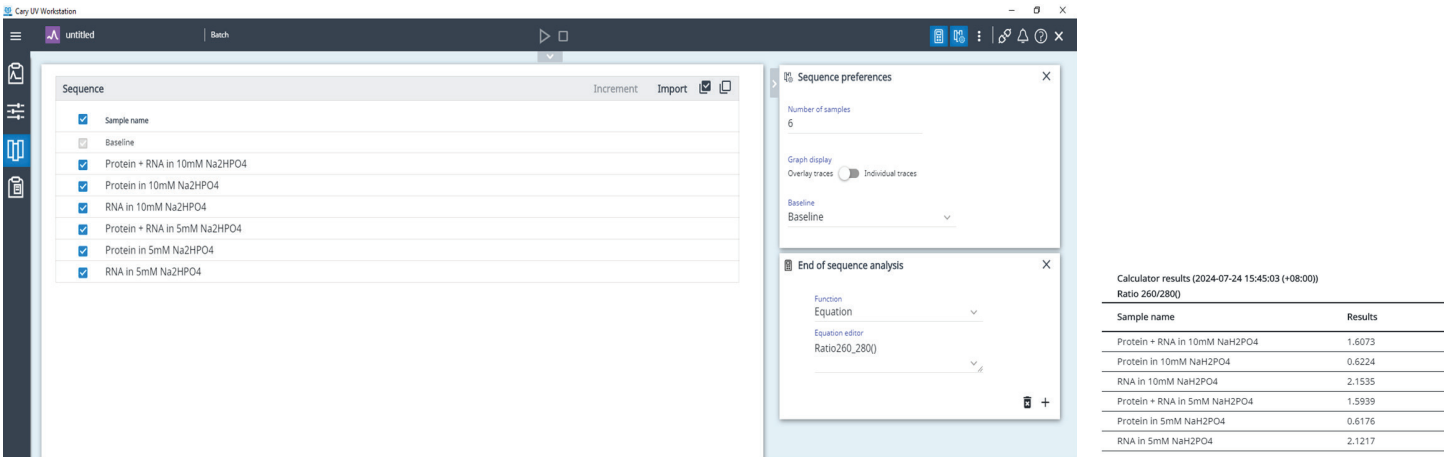

Figure 5. Automatic calculation and reporting of the A260/280 ratios using the in-built equation function in the Agilent Cary UV Workstation software.

Results and discussion

The effects of pH and Na_2HPO_4 concentration on the $A_{260/280}$ ratio and absorbance of RNA

To evaluate the effects of pH and Na_2HPO_4 concentration on the $A_{260/280}$ ratio and absorbance of RNA, 11 Na_2HPO_4 solutions with concentrations ranging from 0.1 to 10 mM were used as a buffering agent.[3] The increase of the $A_{260/280}$ ratio was directly proportional to the increase of pH and Na_2HPO_4 concentration. A steep increase occurred between pH values of 7.2 and 8.6 and Na_2HPO_4 concentrations of 0.02 and 1 mM (Figure 6A).

Figure 6B shows a decrease in the A_{260} and A_{280} values with increasing Na_2HPO_4 concentration or pH. The percent decrease in the absorbances for the respective Na_2HPO_4 concentrations was determined relative to water. The slope of the percent decrease curve of A_{280} is steeper than the slope of the A_{260} curve, as shown by the respective dotted lines in Figure 6C. These results indicate that the increase in the $A_{260/280}$ ratio is due to a pH- or ionic strength-dependent decrease in absorbance at 280 nm.

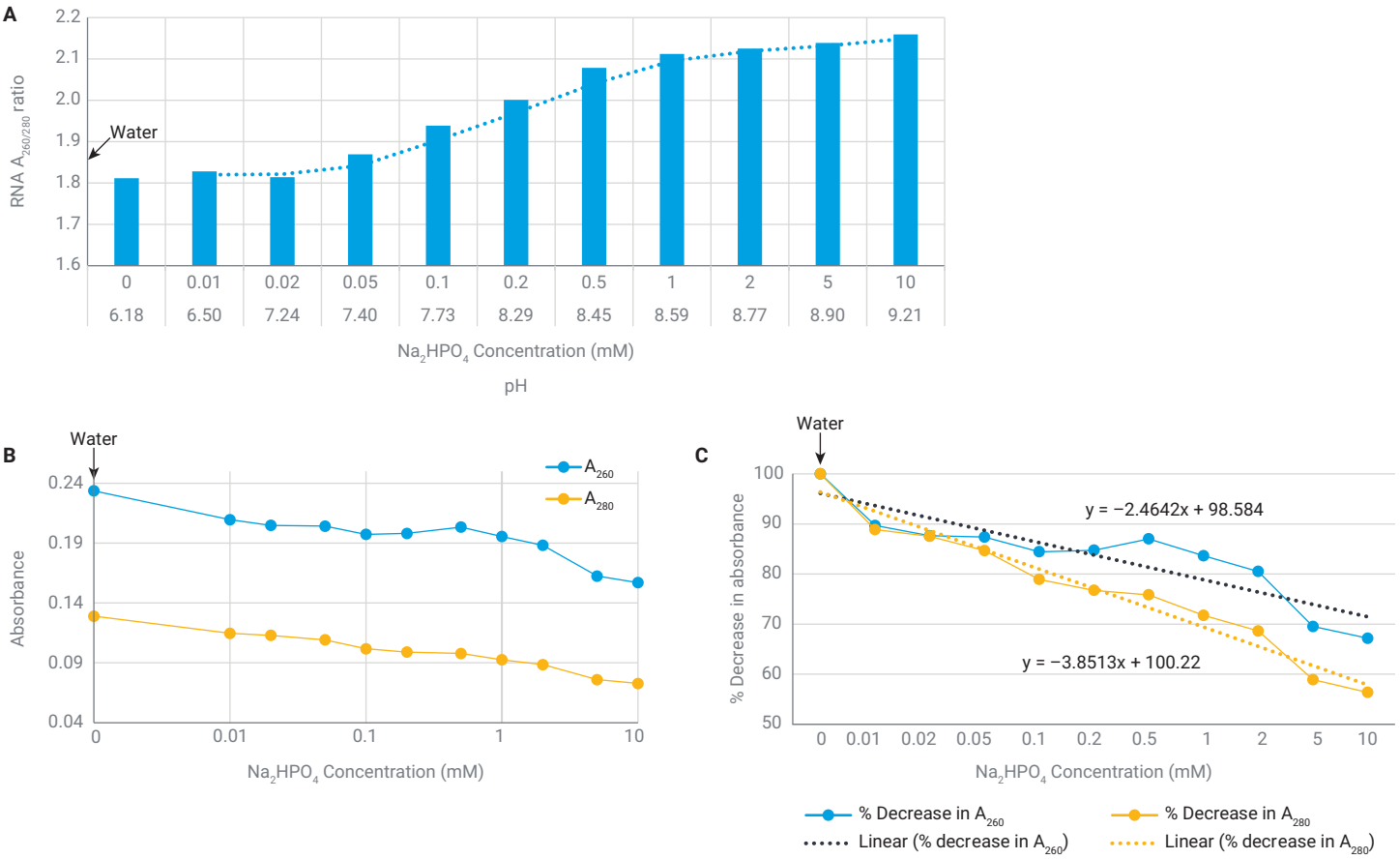

Figure 6. The effects of pH and Na_2HPO_4 concentration on (A) the $A_{260/280}$ ratio and (B) absorbance. (C) Percentage change in absorbance at 260 and 280 nm.

Triplicate measurements were taken for each of the 11 Na_2HPO_4 solutions. Using the two-zones multiple experiments feature of the Cary 3500, measurements of triplicate samples of Na_2HPO_4 at two concentrations were conducted. Using this methodology, it was possible to analyze Na_2HPO_4 at all 11 concentrations in 60 minutes (six measurements of 33 samples and 11 reference solutions). This multicell approach increased the throughput of the analysis by six times compared with a single cuvette system.

The effects of pH and ionic strength on the detection of protein in RNA

To investigate the effects of the pH and ionic strength of the buffer on the detection of protein contamination[3], RNA absorbance was assessed in the 11 Na_2HPO_4 solutions in the presence of a BSA protein.

Figure 7A shows the absorbance profiles of Na_2HPO_4 with and without added protein. RNA in 10 mM Na_2HPO_4 yielded an $A_{260/280}$ ratio of 2.15; however, in the presence of protein, the $A_{260/280}$ ratio dropped to 1.61. The figure clearly shows how protein changes the absorbance profile of RNA.

The decrease in the $A_{260/280}$ ratio between RNA and protein with RNA was 19.1% in water compared to a 26.6% decrease observed in 1.0 mM Na_2HPO_4 (Figure 7B). This finding indicates that protein contamination is more readily detected under alkaline conditions.

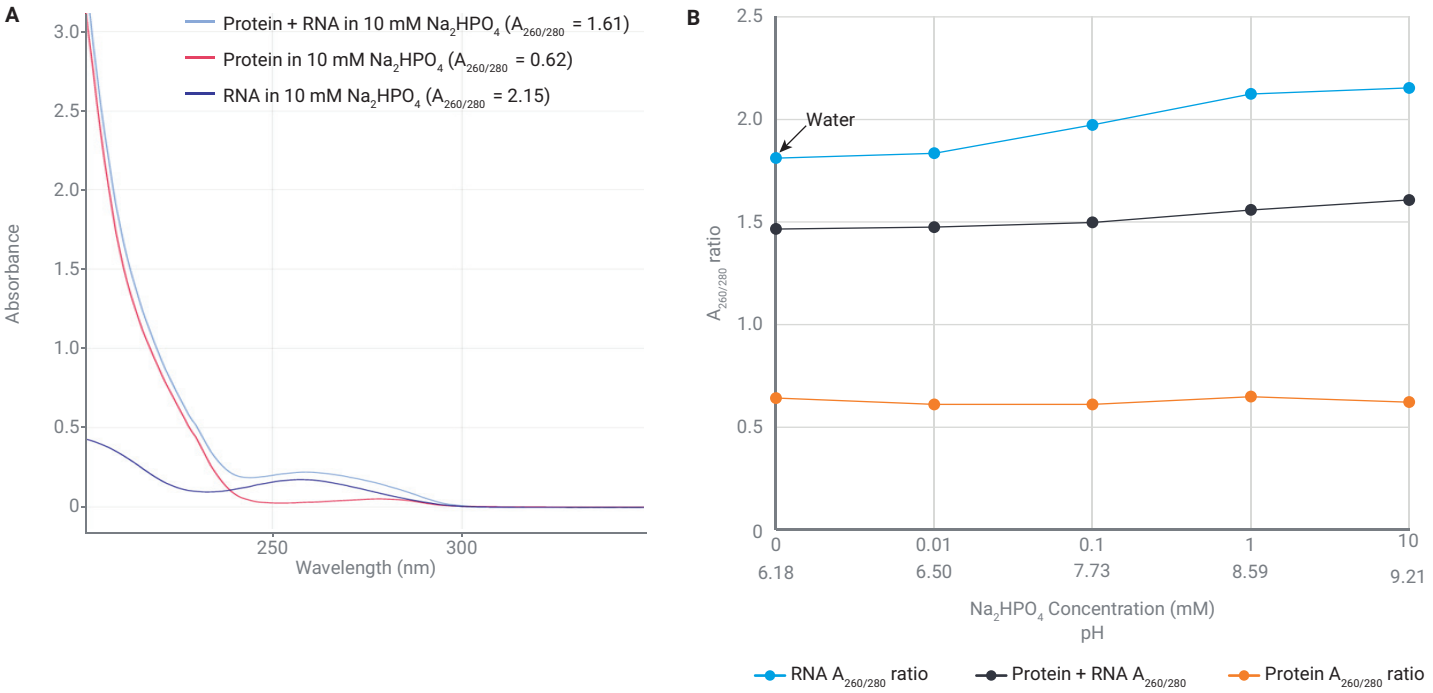

Figure 7. The effects of pH and ionic strength on the RNA $A_{260/280}$ ratio in the presence and absence of protein. (A) Absorbance profiles of RNA, protein, and RNA spiked with protein samples in 10 mM Na_2HPO_4. (B) $A_{260/280}$ ratio plots of RNA, protein, and RNA spiked with protein in 0.01 to 10 mM Na_2HPO_4.

HeLa cell total RNA measurements in different buffer solutions

To investigate the effects of different buffers on the UV absorbance spectra of RNA, analyses were carried out in water (pH 6.18), 1 mM Na_2HPO_4 (pH 8.59), and a buffer solution (TNE). The TNE buffer was included in this experiment because it is frequently recommended for nucleic acid spectrophotometric analysis.[3]

As shown in Figure 8, the UV absorbance spectra of RNA in the two buffer solutions (1 mM Na_2HPO_4 and TNE) are similar, while the UV profile of RNA in water has shifted to a higher wavelength. The results show that an RNA sample dissolved in a buffer solution provides a different UV absorbance trace compared to RNA dissolved in water. The alkaline buffered solution offers a better estimation of RNA purity than the RNA sample dissolved in pure water. It is therefore recommended to use an alkaline buffered solution to dissolve extracted RNA.

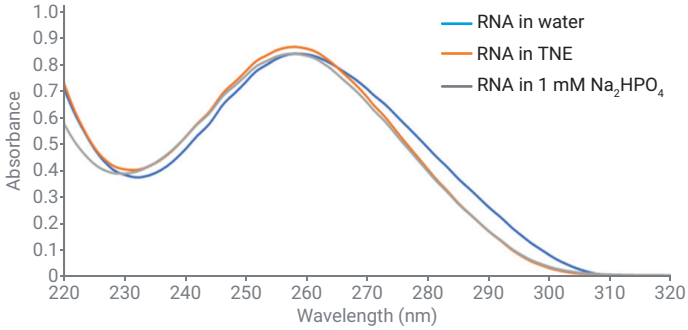

Figure 8. UV absorbance profiles of HeLa RNA analyzed in water, TNE buffer, and 1 mM Na_2HPO_4. The results align with the findings reported by Wilfinger *et al.*[3], demonstrating consistency in the outcomes across the studies.

Conclusion

This application note demonstrates an effective method for assessing RNA purity based on the $A_{260/280}$ ratio using an Agilent Cary 3500 Multicell Peltier UV-Vis spectrophotometer. The Cary 3500 allowed multiple experimental conditions to be tested simultaneously, avoiding unwanted experimental variables.

The data showed that the pH and ionic strength of RNA solutions significantly affect the $A_{260/280}$ ratio and that protein contamination of RNA samples is better estimated under alkaline conditions. The built-in equation functions of the Cary 3500 software allowed the calculation of $A_{260/280}$ to be calculated and reported automatically. These features enabled the accurate and efficient assessment of RNA samples under different buffer conditions. The software is compatible with the Agilent OpenLab software suite for use in regulated environments. OpenLab provides technical controls to securely acquire and store data in laboratories that must comply with FDA 21 CFR Part 11, EU Annex 11, and similar regulations in other countries.

Agilent provides the required consumables (e.g., Absolutely RNA Microprep kit), instrumentation (e.g., the 4200 TapeStation system and Cary 3500 UV-Vis spectrophotometer), and workflow solutions for carrying out RNA quality determinations.

References

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2. Brescia, P. Microvolume Purity Assessment of Nucleic Acids Using A_{260}/A_{280} Ratio and Spectral Scanning. *Agilent Technologies application note*, publication number 5994-2538EN, **2021**.
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Printed in the USA, November 14, 2024
5994-7892EN
PR7001-3461

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