Application Brief

Molecular Biology and Biochemical

Agilent

Trusted Answers

Microvolume Nucleic Acid Analysis Using Common Assay Methods

Author

Peter J. Brescia, MSc.
Agilent Technologies, Inc.

Introduction

The manipulation of nucleic acids has become standard protocol for many laboratories. While the range of uses in downstream applications is wide, one central requirement is accurate determination of concentration after isolation. Standard methods of nucleic acid quantification, postisolation, include spectrophotometric absorption at 260 nm and the use of fluorescent probes specific for the target biomolecule. Spectrophotometry remains the most common method used due to cost and ease of use, however fluorescent probes provide greater sensitivity and can alleviate inaccurate measurements due to contaminants in crude samples. Additionally, methods have evolved from 1 cm path length measurements to microvolume measurements that conserve sample and often preclude the need for dilution.

Methods

UV absorption

All double-stranded DNA (dsDNA) and RNA standards were created by preparing a 1:2 serial dilution series of a concentrated stock of the respective nucleic acid in TE buffer (tris-EDTA, pH = 7.0). The Beer-Lambert Law relates the absorption and concentration via the following equation where A = log Io/I = εlc. ε refers to the extinction coefficient of the analyte, l is the path length (cm) and c is the concentration of the analyte (ng/µL). For microvolume determinants the path length is defined by the vessels and therefore can be used for normalization to a 1 cm path length measurement.

Fluorescence assays

DNA

Herring sperm DNA (part number D6898) was purchased from Sigma-Aldrich (St. Louis, MO). Quant-iT PicoGreen Reagent (part number P11495) was purchased from Thermo Fisher Scientific (Waltham, MA). The Agilent BioTek Take 3 microvolume plate was used for all determinants. Herring sperm DNA was diluted to 2 µg/mL with TE (10 mM Tris, 1 mM EDTA, pH 7.5) as the diluent. The final concentration was confirmed using 260 nm absorbance. A series of dilutions ranging from 0.0 to 2 µg or 2,000 ng/mL was made using TE as a diluent and 2 µL aliquots pipetted onto the Take 3 spots. Equal amounts (2 µL) of working PicoGreen quantitation reagent were added and incubated for 10 minutes at room temperature, protected from light. Working PicoGreen reagent was prepared by diluting the concentrated DMSO-PicoGreen stock solution 1:133 with TE. Fluorescence was determined using an Agilent BioTek Synergy LX multimode reader with a GFP filter cube (Ex 485/20 nm, Em 528/25 nm and 510 nm cutoff dichroic mirror).

RNA

The Quant-iT RiboGreen RNA Reagent and Kit (part number R11490) was purchased from Thermo Fisher Scientific (Waltham, MA). The Take 3 plate was used for all determinants. Ribosomal RNA was diluted to 10 µg/mL with TE (10 mM Tris, 1 mM EDTA, pH 7.5) as the diluent. The final concentration was confirmed using 260 nm absorbance. A series of dilutions ranging from 0 to 5,000 ng/mL or 0 to 100 ng/mL of purified ribosomal RNA was made using TE and 2 µL aliquots pipetted onto the Take 3 spots. Equal amounts (2 µL) of working RiboGreen quantitation reagent were added and incubated for 10 minutes at room temperature, protected from light. Working RiboGreen reagent was prepared by diluting the concentrated DMSO-RiboGreen stock solution 1:100x with TE. Fluorescence was determined using an Agilent BioTek Synergy LX multimode reader with a GFP filter cube (Ex 485/20 nm, Em 528/25 nm and 510 nm cutoff dichroic mirror).

Take 3 microvolume plate

The Agilent BioTek Take 3 and Take 3 Trio microvolume plates allow measurement of multiple undiluted samples with volumes as low as 2 µL, as well as standard 1 cm path length measurements. When samples are place on the microspots, and the vessel lid closed, a 0.5 mm nominal path length through the sample results. Path length calibration values for each microspot location are stored in the Agilent BioTek Gen5 microplate reader and imager software and measurements can be normalized to 1 cm path length determinants.

Results and discussion

By combining both absorbance and fluorescence methods for quantification of nucleic acids, a wide range of concentrations can be analyzed, and qualitative information such as purity assessment is captured. UV absorbance is a nondestructive method allowing a native sample to be measured with no further reagent requirements. However, the limit of detection may be insufficient or concentration overstated when contaminating absorbing species are present, especially when working with samples of very low concentration. This becomes more evident when working with microvolume analyses with very short path lengths. RNA and DNA from a variety of sources were analyzed in a microvolume format requiring just 2 µL of sample (Figures 1A and 1B). Good linearity of RNA and DNA over a range of concentrations is evident.

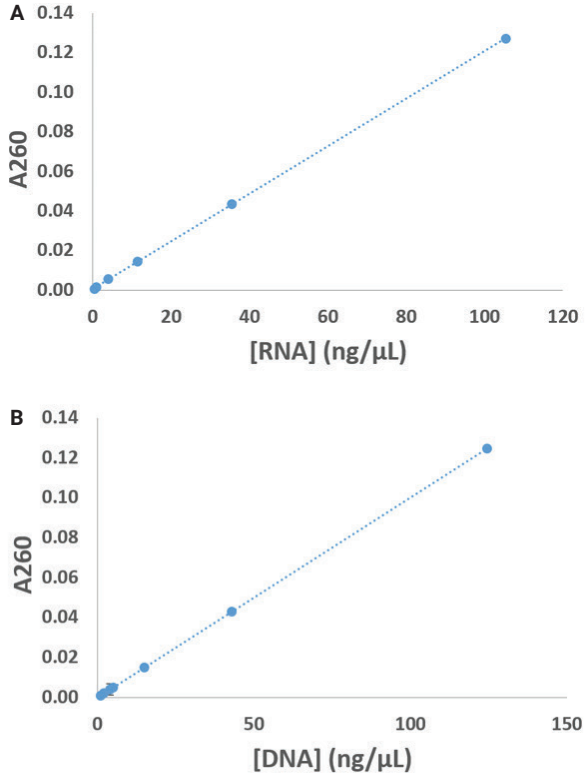

Figure 1. Nucleic acid absorbance at 260 nm. A dilution series of ribosomal RNA or herring sperm DNA was prepared ranging from ~0 to 120 ng/mL in TE buffer. Standards were analyzed in duplicate on an Agilent BioTek Take 3 microvolume plate.

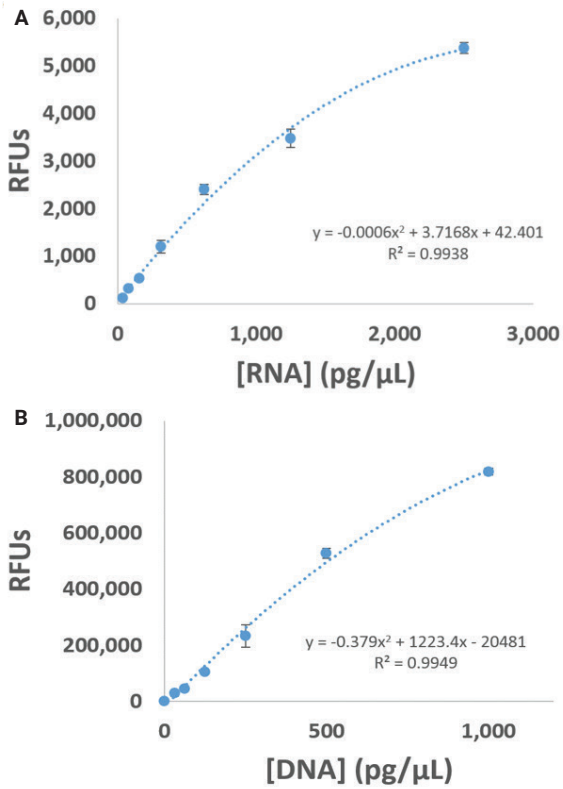

Figure 2. Nucleic acid microvolume fluorescent assay. A dilution series of ribosomal RNA or herring sperm DNA was prepared ranging from ~0 to 2,500 or 1,000 ng/mL in TE buffer. Standards were analyzed in duplicate on an Agilent BioTek Take 3 microvolume plate.

Fluorescent probes can provide increased sensitivity and dynamic range. These methods are capable of quantification to tens of picogram per microliter concentrations in a microvolume assay format and lower in a standard microplate format, where path length is increased. Assay reagents are developed specifically for either RNA or DNA with minimal interference from other molecular entities. RNA quantification shows excellent correlation using a second order polynomial fit to low pg/µL concentrations with a calculated LOD of ~35 picograms per microliter or ~71 picograms of RNA in a 2 µL sample volume (Figure 2A). DNA quantification was performed in a manner similar to the RNA fluorescence assay. The assay shows excellent correlation using a second order polynomial fit to low pg/µL concentrations with a calculated LOD of ~67 picograms per microliter or ~134 picograms DNA in a 2 µL sample volume (Figure 2B).

Conclusion

The Agilent BioTek Synergy LX multimode reader provides the most common detection technologies used in biological research including absorbance, fluorescence, and luminescence detection. This application brief demonstrates its utility to quantify nucleic acids in a microvolume assay format using UV detection and fluorescence methods. The use of fluorescence methods ensure specificity when contaminating species may be present while the microvolume assay format minimizes sample volume requirements and provide higher sensitivity.

www.agilent.com/lifesciences/biotek

DE44314.2324884259

Printed in the USA, March 17, 2022
5994-2765EN

Agilent
Trusted Answers