

Quantitative UV spectral analysis of aqueous samples as small as 1 μL

BY WILLIAM H. CHAPMAN, JR.

MANY MODERN METHODS applied by scientists to the analysis of biological molecules require the prior knowledge of the concentration (or mass) of the species of interest. Problems with analysis are encountered when the volume of sample obtainable is smaller than is compatible with current protocols used to measure concentrations. While it may be possible to vary the volume of sample used for analysis until the procedure yields a result that seems appropriate, data generated with this "method of judicial guessing" are not usually deemed acceptable, especially in the areas of clinical or forensic science. It follows that the development of methods for the rapid and quantitative analysis of the concentration of biological molecules contained in small volumes should help to expedite many experimental procedures and make the results obtained more precise. This application note presents the results of a performance study of the Gene Spec I™ UV absorbance spectrometer (Hitachi Genetic Systems, Alameda, CA) (Figure 1), which allows for the routine determination of the concentrations of biological molecules contained in volumes as small as 1 μL . The results of this study show that the precision and ease of use of the instrument make it an attractive addition to the life scientist's arsenal of techniques.

The Gene Spec I is a UV absorbance spectrometer that is designed to be useful for the measurement of the concentration of solutions of nucleic acids and proteins contained in samples with a volume as small as 1 μL . The probe light beam, produced by a deuterium lamp, is focused through the sample with a concave mirror and is analyzed with a fixed grating monochromator and a 512-element photodiode array (see Figure 2). The light beam that exits the cuvet is collimated with a 0.2-mm slit, making it possible for the



Figure 1 Gene Spec I UV absorbance spectrometer.

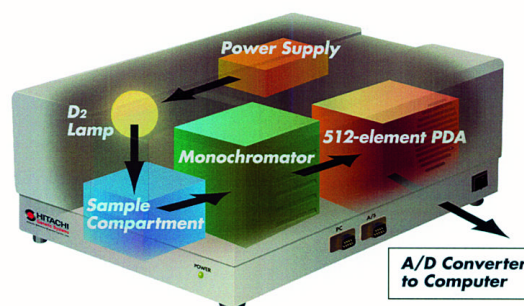


Figure 2 Arrangement of optical components within the Gene Spec I.

monochromator to analyze light that passes through a very small area without the interference of scattered light.

Quartz glass cuvetts of six different sizes (1, 2, 5, 10, 20, and 50 μL) have been validated with the system and are currently available. The instrument will also accept a standard 4-mL cuvet. While the available larger-volume cuvetts (10, 20, and 50 μL) have a 10-mm pathlength, the pathlengths of the smaller-volume cuvetts decrease with the volume (5 μL , 5 mm; 2 μL , 2 mm; 1 μL , 1 mm). The variable pathlength is useful for measuring the absorbance of very concentrated samples without dilution.

The spectrometer is controlled by a PC running Windows 95, 98, or NT (Microsoft Corp., Redmond, WA) and is supplied with software that calculates the concentration of nucleic acid or protein from the spectral data and user-defined parameters. All software operations can be controlled from pull-down menus (or buttons), all of which are accessible from the program's single window (see Figure 3). Up to 20 spectra can be read into the software and displayed separately or overlaid. The results of the calculations are displayed in spreadsheet format directly below the spectral data for comparison. The contents of the spreadsheet can also be exported to Microsoft Excel for further analysis.

The Gene Spec I includes a wavelength calibration routine that uses the UV absorbance spectrum of holmium oxide doped glass as a standard. An absorbance spectrum of the holmium glass is recorded, and the wavelength of several absorbance maxima is recorded, and the wavelength of several absorbance maxima are compared to the true values (stored in the software). If the measured wavelengths differ from the true values, the software will calibrate the wavelength axis

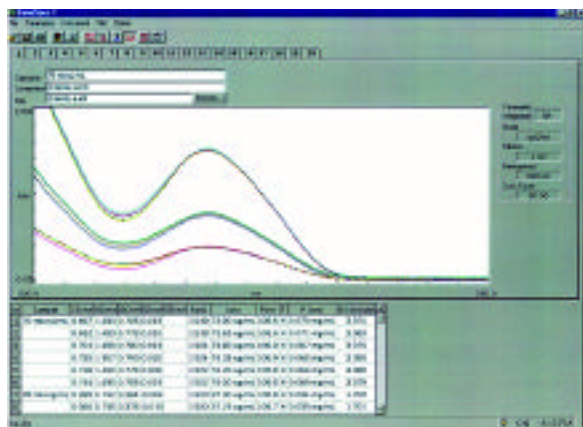


Figure 3 Gene Spec I software displays both the full UV spectra and the values calculated from the spectral data within a single, easy-to-view window.

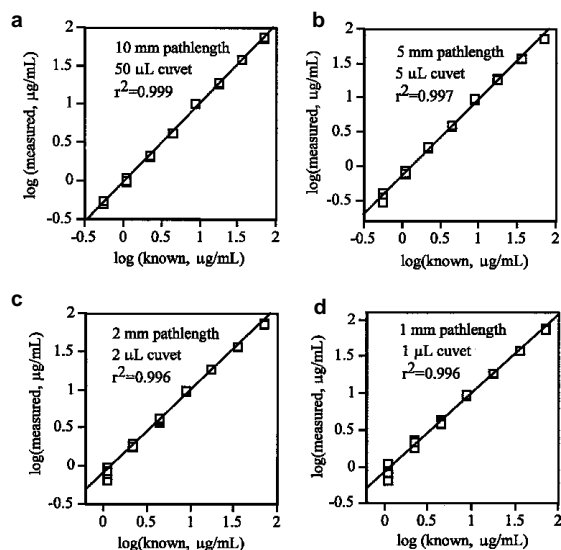


Figure 4 Concentrations measured with the Gene Spec I are plotted against the corresponding known DNA concentration for a serial diluted series of calf thymus DNA samples. Data were collected in cuvettes with the following pathlengths and volumes: a) 10 mm, 50 μ L; b) 5 mm, 5 μ L; c) 2 mm, 2 μ L; and d) 1 mm, 1 μ L. The correlation coefficient from linear regression analysis is shown together with the data, and is greater than 0.99 in all cases.

and apply this calibration to all measurements made until the wavelength calibration function is reactivated.

The linearity of response and sensitivity of the spectrometer have been determined with standard DNA samples (calf thymus DNA, **Sigma Chemicals**, St. Louis, MO) diluted in Tris buffer (10 mM, pH 8.5). Cuvettes of all four pathlengths (1, 2, 5, and 10 mm) were tested (six independent readings at each concentration, see *Figure 4a-d*). The coefficient of variation (CV) in the concentration calculation was determined from the six replicates ($CV = \sigma_{n-1}/\text{mean average}$) and the relationship between the CV and known concentration of DNA was fitted to an equation of the type $y = mx^b$ (*Figure 5*). From this equation, the minimum mass of DNA that can be measured at a chosen error, reported as CV% ($CV \times 100$), can be calculated (see *Figure 6*).

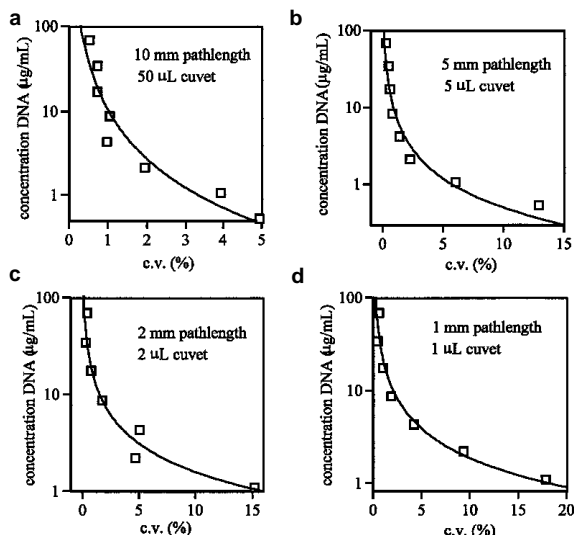


Figure 5 CV calculated from the concentration measurements shown in *Figure 3* are plotted versus the known concentration of each sample. The data calculated for each cuvette were fitted to an equation of the type $y = mx^b$. From the equations, the concentration measured at a defined CV value can be calculated for each cuvette (see *Figure 6*).

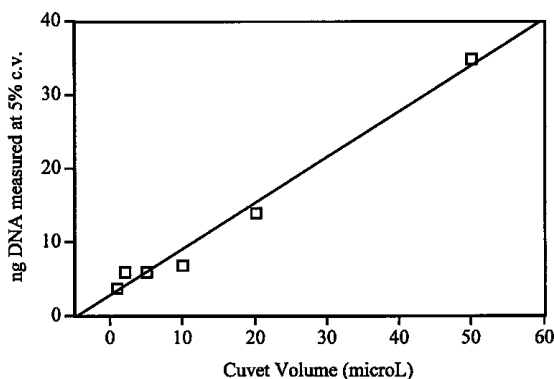


Figure 6 Relationship between the cuvette volume and the mass of DNA measured at constant CV (5%). The values plotted are calculated from the relationship between the CV and concentration of calf thymus DNA described in *Figure 5*.

It is clear from the data presented in *Figure 5* that the CV in the measurement increases as the concentration of the calf thymus DNA decreases in all cuvettes studied. It is also clear that the CV of the measurements is somewhat dependent on the pathlength of the cuvette, increasing with decreases in the pathlength at a constant DNA concentration. What is not so obvious, but can be seen from a plot of the mass of DNA calculated at constant CV (5%) versus the cuvette volume, is that as the volume of the cuvette decreases, the mass of DNA measured at a defined CV also decreases (see *Figure 6*). The sensitivity advantage gained in decreasing the volume of the cuvette outweighs the decrease in sensitivity brought about by the shorter pathlength.

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