Quantitation of DNA and RNA with Absorption and Fluorescence Spectroscopy

Reliable quantitation of nanogram and microgram amounts of DNA and RNA in solution is essential to researchers in molecular biology. In addition to the traditional absorbance measurements at 260 nm (see Basic Protocol), three more sensitive fluorescence techniques (see Alternate Protocols 1 to 3) are presented below. These four procedures cover a range from 5 to 10 ng/ml DNA to 25 pg/ml DNA (see Commentary and Table A.3D.3 therein).

Absorbance measurements are straightforward as long as any contribution from contaminants and the buffer components are taken into account. Fluorescence assays are less prone to interference than \( A_{260} \) measurements and are also simple to perform. As with absorbance measurement, a reading from the reagent blank is taken prior to adding the DNA. In instruments where the readout can be set to indicate concentration, a known concentration is used for calibration and subsequent readings are taken in \( \mu \)g/ml, ng/ml, or pg/ml DNA.

DETECTION OF NUCLEIC ACIDS USING ABSORPTION SPECTROSCOPY

Absorption of the sample is measured at several different wavelengths to assess purity and concentration of nucleic acids. \( A_{260} \) measurements are quantitative for relatively pure nucleic acid preparations in microgram quantities. Absorbance readings cannot discriminate between DNA and RNA; however, the ratio of \( A \) at 260 and 280 nm can be used as an indicator of nucleic acid purity. Proteins, for example, have a peak absorption at 280 nm that will reduce the \( A_{260}/A_{280} \) ratio. Absorbance at 325 nm indicates particulates in the solution or dirty cuvettes; contaminants containing peptide bonds or aromatic moieties such as protein and phenol absorb at 230 nm.

This protocol is designed for a single-beam ultraviolet to visible range (UV-VIS) spectrophotometer. If available, a double-beam spectrophotometer will simplify the measurements, as it will automatically compare the cuvette holding the sample solution to a reference cuvette that contains the blank. In addition, more sophisticated double-beam instruments will scan various wavelengths and report the results automatically.

Materials

1× TNE buffer (see recipe)
DNA sample to be quantitated
Calf thymus DNA standard solutions (see recipe)
Matched quartz semi-micro spectrophotometer cuvettes (1-cm pathlength)
Single- or dual-beam spectrophotometer (ultraviolet to visible)

1. Pipet 1.0 ml of 1× TNE buffer into a quartz cuvette. Place the cuvette in a single- or dual-beam spectrophotometer, read at 325 nm (note contribution of the blank relative to distilled water if necessary), and zero the instrument. Use this blank solution as the reference in double-beam instruments. For single-beam spectrophotometers, remove blank cuvette and insert cuvette containing DNA sample or standard suspended in the same solution as the blank. Take reading. Repeat this process at 280, 260, and 230 nm.

   It is important that the DNA be suspended in the same solution as the blank.
2. To determine the concentration (C) of DNA present, use the $A_{260}$ reading in conjunction with one of the following equations:

**Single-stranded DNA:** \[ C \text{ (pmol/µl)} = \frac{A_{260}}{10 \times S} \]

**Double-stranded DNA:** \[ C \text{ (µg/ml)} = \frac{A_{260}}{0.027} \]

**Single-stranded RNA:** \[ C \text{ (µg/ml)} = \frac{A_{260}}{0.025} \]

**Oligonucleotide:** \[ C \text{ (pmol/µl)} = A_{260} \times \frac{100}{1.5 N_A + 0.71 N_C + 1.20 N_G + 0.84 N_T} \]

where $S$ represents the size of the DNA in kilobases and $N$ is the number or residues of base A, G, C, or T.

For double- or single-stranded DNA and single-stranded RNA: These equations assume a 1-cm-pathlength spectrophotometer cuvette and neutral pH. The calculations are based on the Lambert-Beer law, $A = ECl$, where $A$ is the absorbance at a particular wavelength, $C$ is the concentration of DNA, $l$ is the pathlength of the spectrophotometer cuvette (typically 1 cm), and $E$ is the extinction coefficient. For solution concentrations given in mol/liter and a cuvette of 1-cm pathlength, $E$ is the molar extinction coefficient and has units of M$^{-1}$cm$^{-1}$. If concentration units of µg/ml are used, then $E$ is the specific absorption coefficient and has units of (µg/ml)$^{-1}$cm$^{-1}$. The values of $E$ used here are as follows: ssDNA, 0.027 (µg/ml)$^{-1}$cm$^{-1}$; dsDNA, 0.020 (µg/ml)$^{-1}$cm$^{-1}$; ssRNA, 0.025 (µg/ml)$^{-1}$cm$^{-1}$. Using these calculations, an $A_{260}$ of 1.0 indicates 50 µg/ml double-stranded DNA, ~37 µg/ml single-stranded DNA, or ~40 µg/ml single-stranded RNA (adapted from Applied Biosystems, 1987).

For oligonucleotides: Concentrations are calculated in the more convenient units of pmol/µl. The base composition of the oligonucleotide has significant effects on absorbance, because the total absorbance is the sum of the individual contributions of each base (Table A.3D.1).

3. Use the $A_{260}/A_{280}$ ratio and readings at $A_{230}$ and $A_{325}$ to estimate the purity of the nucleic acid sample.

### Table A.3D.1 Molar Extinction Coefficients of DNA Bases$^a$

<table>
<thead>
<tr>
<th>Base</th>
<th>$\varepsilon_{260\text{nm}}^{1\text{M}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>15,200</td>
</tr>
<tr>
<td>Cytosine</td>
<td>7,050</td>
</tr>
<tr>
<td>Guanosine</td>
<td>12,010</td>
</tr>
<tr>
<td>Thymine</td>
<td>8,400</td>
</tr>
</tbody>
</table>

$^a$Measured at 260 nm; see Wallace and Miyada, 1987. Detailed spectrophotometric properties of nucleoside triphosphates are listed in UNIT 3.4.
Table A.3D.2  Spectrophotometric Measurements of Purified DNA$^a$

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>Absorbance</th>
<th>$A_{260}/A_{280}$</th>
<th>Conc. (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>325</td>
<td>0.01</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>280</td>
<td>0.28</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>260</td>
<td>0.56</td>
<td>2.0</td>
<td>28</td>
</tr>
<tr>
<td>230</td>
<td>0.30</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

$^a$Typical absorbancy readings of highly purified calf thymus DNA suspended in 1× TNE buffer. The concentration of DNA was nominally 25 µg/ml.

Ratios of 1.8 to 1.9 and 1.9 to 2.0 indicate highly purified preparations of DNA and RNA, respectively. Contaminants that absorb at 280 nm (e.g., protein) will lower this ratio.

Absorbance at 230 nm reflects contamination of the sample by phenol or urea, whereas absorbance at 325 nm suggests contamination by particulates and dirty cuvettes. Light scatter at 325 nm can be magnified 5-fold at 260 nm (K. Hardy, pers. comm.).

Typical values at the four wavelengths for a highly purified preparation are shown in Table A.3D.2.

**DNA DETECTION USING THE DNA-BINDING FLUOROCHROME HOECHST 33258**

Use of fluorometry to measure DNA concentration has gained popularity because it is simple and much more sensitive than spectrophotometric measurements. Specific for nanogram amounts of DNA, the Hoechst 33258 fluorochrome has little affinity for RNA and works equally well with either whole-cell homogenates or purified preparations of DNA. The fluorochrome is, however, sensitive to changes in DNA composition, with preferential binding to AT-rich regions. A fluorometer capable of an excitation wavelength of 365 nm and an emission wavelength of 460 nm is required for this assay.

### Additional Materials (also see Basic Protocol)
- Hoechst 33258 assay solution (working solution; see recipe)
- Dedicated filter fluorometer (Hoefer TKO100) or scanning fluorescence spectrophotometer (Shimadzu model RF-5000 or Perkin-Elmer model LS-5B or LS-3B)
- Fluorometric square glass cuvettes or disposable acrylic cuvettes (Sarstedt)
- Teflon stir rod

1. Prepare the scanning fluorescence spectrophotometer by setting the excitation wavelength to 365 nm and the emission wavelength to 460 nm.
   
   *The dedicated filter fluorometer has fixed wavelengths at 365 and 460 nm and does not need adjustment.*

2. Pipet 2.0 ml Hoechst 33258 assay solution into cuvette and place in sample chamber. Take a reading without DNA and use as background.
   
   *If the fluorometer has a concentration readout mode or is capable of creating a standard curve, set instrument to read 0 with the blank solution. Otherwise note the readings in relative fluorescence units. Be sure to take a blank reading for each cuvette used, as slight variations can cause changes in the background reading.*

3. With the cuvette still in the sample chamber, add 2 µl DNA standard to the blank Hoechst 33258 assay solution. Mix in the cuvette with a Teflon stir rod or by capping and inverting the cuvette. Read emission in relative fluorescence units or set the commony used techniques

**A.3D.3**