DNA and RNA Measurements in SPECTRAmax® Microplate Spectrophotometers

Evelyn McGown, Ph.D.
Molecular Devices Corporation, 7/99

Ultraviolet (UV) measurements in microplates became possible 5 years ago when Molecular Devices introduced the first UV–capable microplate spectrophotometer and UV–transparent microplates. Since then, microplate measurements of DNA, RNA and proteins have become very popular. However, microplate assays require awareness of the optical properties of the microplate materials and more attention to technique than do traditional cuvettes in order to get accurate absorbance results, especially in the UV range. Lack of attention to these details is the most frequent cause of difficulty in adapting assays to microplates.

Absorbance measurements made through microplates are subject to pathlength variability and are vulnerable to interference from surface effects at the air/liquid interface (Maxline App. Note #32). Modern microplate spectrophotometers, with smaller optical beams than older wide–beam plate readers, are much more vulnerable to spurious readings due to dust. Particles in the light beam at the time of a read can cause artifactual absorbance spikes of up to 0.3 OD. Therefore it is especially important that sample solutions be free of particles. All of the above factors must be kept in mind in order to get accurate and reproducible absorbance results in microplates. The purpose of this Application Note is to provide guidelines for optimizing DNA/RNA absorbance measurements in SPECTRAmax microplate spectrophotometers.

MATERIALS AND METHODS

1. SPECTRAmax microplate spectrophotometer (Molecular Devices Corp.)

2. UV–transparent microplates; e.g.
   - UV–Plate™ (Corning–Costar Cat. No. 3635, Tel: 1–800–492–1110)
   - UV–Star® (Greiner Cat. No. 655801, E & K Scientific Products, Tel: 408–378–2013)
   - UVMax™ (Polyfiltronics) from Whatman LabSales or distributors
   - Quartz microplate (Hellma Cells, Inc., Tel: 718–544–9534, –9166)
RECOMMENDATIONS FOR BEST QUALITY DNA ABSORBANCE VALUES

For details on optimizing UV measurements in general, see Maxline App. Note # 32.

1 **Use clean microplates and particle–free solutions.** Filter buffers (e.g. pore size ~ ≤ 5 µ) to remove particulates and, for best results, do not re–use disposable microplates. Cover microplate if not read immediately.

2 **Note absorbance values of buffer blanks** (include blanks at least in triplicate). If the blank values do not fall into the expected range, the most likely cause is a dirty microplate, particulates in the wells or a defective microplate. Plate background OD values of disposable UV microplates vary slightly between lots. You should determine the expected mean OD value by reading a clean water–filled plate from each new lot. Table 1 shows approximate OD<sub>260</sub> and OD<sub>280</sub> and standard deviation (SD) values for water–filled UV–transparent microplates. Costar UV and Greiner UV plates have background ODS almost as low as a quartz microplate. Polyfiltronics UV microplates have a higher background and SD, but good results can be obtained by pre–reading the plates and subtracting the background on a well–by–well basis (automatically performed by SOFTmax® PRO, see Option 2, below).

<table>
<thead>
<tr>
<th></th>
<th>OD 260 nm Mean</th>
<th>SD</th>
<th>OD 280 nm Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quartz</td>
<td>0.034</td>
<td>0.002</td>
<td>0.032</td>
<td>0.002</td>
</tr>
<tr>
<td>Costar UV*</td>
<td>0.050</td>
<td>0.001</td>
<td>0.039</td>
<td>0.001</td>
</tr>
<tr>
<td>Greiner UV**</td>
<td>0.062</td>
<td>0.002</td>
<td>0.043</td>
<td>0.001</td>
</tr>
<tr>
<td>Polyfiltronics UV</td>
<td>0.166</td>
<td>0.006</td>
<td>0.281</td>
<td>0.009</td>
</tr>
</tbody>
</table>

Table 1: Optical densities of microplates at 260 nm and 280 nm. Values are averages from 5 clean plates, each filled with 200 µL water/well. *For Costar UV plates, mean values at 260 nm ranged from 0.046 to 0.057, depending on lot number. **Pre-production samples

3 **For low–absorbing samples use maximum possible volume** (250–300 µL) to get maximum optical pathlength (and therefore maximum absorbance). Include duplicates or triplicates if possible. Automix plate briefly to encourage symmetrical meniscus formation.

4 If your procedure calls for a small sample volume (1–20 µL) plus a diluent, **pipet the small volume into the well first**, followed by the larger volume. Automix in the SPECTRAmax instrument for 10 to 20 seconds to complete the mixing. Results may be highly variable if the small sample is added last, due to incomplete mixing.

5 Be aware that **increasing ionic strength decreases absorptivity**<sup>1,2</sup>. DNA has approximately 23% less absorbance when dissolved in TE-buffered saline rather than water (See Figure 2and discussion on page 5 of this Application Note). For highest sensitivity, DNA samples should be in deionized water.

USING PATHCHECK TO NORMALIZE ABSORBANCE VALUES TO A 1 CM PATHLENGTH

SPECTRAmax instruments are equipped with PathCheck™ sensor, which measures the pathlength in each well of a microplate and normalizes the absorbance values to a 1 cm pathlength so that they are the same as values obtained in a 1 cm cuvette.

\[ A_{\text{Sample per cm}} = \frac{A_{\text{Sample}}}{\text{pathlength}_{\text{Sample}} (\text{cm})} \quad (1) \]
Before normalizing for pathlength, the OD of the microplate itself must be taken into account. The raw OD of a sample in a microplate is:

\[ OD_{\text{Total}} = A_{\text{Sample}} + A_{\text{Blank}} + OD_{\text{microplate}} \]  

Both \( A_{\text{Sample}} \) and \( A_{\text{Blank}} \) are pathlength-dependent. The \( OD_{\text{microplate}} \) is due to the OD of the microplate material and to reflectances from surfaces, and is pathlength-independent. Therefore, it must be subtracted first, before calculating pathlength-normalized absorbance values by Equation (1) above. SOFTmax® PRO offers 3 options for subtracting \( OD_{\text{microplate}} \). Options 1 and 2 are recommended if sample volumes are variable. If all well volumes are identical, the \( OD_{\text{microplate}} \) may be included in the ordinary reagent blank (Option 3).

### OPTIONS FOR ELIMINATING MICROPLATE OD IN PATHCHECK CALCULATIONS

**Option 1: Use Plate Background Constant(s).** *(Recommended for microplates with a low and uniform background.)*

For each new lot of disposable UV microplates measure the \( OD_{\text{microplate}} \) (“Plate Background Constant”) at each wavelength intended to be used with PathCheck. Fill a clean plate with 100–200 µL water per well (volume is not critical because water does not absorb light appreciably between 190 and 900 nm) and read the plate. Do not use a dry plate because air has a different refractive index than water and thus gives a different OD value. The mean OD at a given wavelength is the Plate Background Constant for that wavelength. \( OD_{\text{microplate}} \) values at 260 nm and 280 nm should be similar to those shown in Table 1. If the SD is greater than 0.003 at 260 nm, you probably have a dirty or defective microplate.

When you have determined the Plate Background Constant, enter it in the PathCheck section of the Instrument Setup dialog box in SOFTmax PRO. Designate locations of samples, standards and blanks as usual and fill the microplate accordingly. When the plate is read, SOFTmax PRO automatically:

1. Subtracts the Plate Background constant from the raw well absorbance values.
2. Applies PathCheck to all samples, standards and blanks (unless you have deselected it under Reduction†).
3. Subtracts the reagent blank(s).

**Option 2: Pre–read the microplate.** *Use this option for Polyfiltronics UV plates and any other plate with a higher, more variable background. The plate OD is subtracted on a well–by–well basis. (See Maxline Application Note # 25.)*

In the ‘Automix and Blanking’ section of the Instrument Setup dialog box, select “Pre–Read Plate”. (Do not use the Plate Background Constant.) Fill a clean microplate with 100–200 µL water (volume not critical). Pre–read the plate. Empty the plate, put in the samples, standards and blanks into their designated wells and read again (the “normal” read). SOFTmax PRO automatically:

1. Subtracts the Pre–Read value from the \( OD_{\text{Total}} \) of each well.
2. Applies PathCheck to all samples, standards and blanks (unless you have deselected it under Reduction).
3. Subtracts the reagent blank(s).

† If you de-select PathCheck, the plate background subtraction is also de-selected.
Option 3: Include $OD_{\text{microplate}}$ as part of the Plate Blank. Use this option when all well volumes (and pathlengths) are identical and all samples are blanked with the same solution.

By design, SOFTmax PRO subtracts the reagent blank(s) after pathlength normalization. The $OD_{\text{microplate}}$ can be subtracted as a component of the reagent blank if (and only if) all sample and blank volumes are identical.

Select three or more wells in the microplate to contain the blanking solution (typically water or buffer). In SOFTmax PRO’s Template Editor, assign those wells as “Blank”. Designate the remaining wells as samples and standards and prepare the plate accordingly. When the plate is read, SOFTmax PRO automatically:

1. Applies PathCheck to all samples and blanks (unless you have deselected it.)
2. Subtracts the average of the Blanks from each well of the microplate. Provided that all sample and blank pathlengths are identical, potential error from applying pathlength normalization to $OD_{\text{microplate}}$ is cancelled out.

EXAMPLE OF DNA MEASUREMENTS IN SPECTRAMAX INSTRUMENTS

Aliquots of calf thymus DNA (0.1 - 2.0 µg/mL in water) were read 260 nm in a Costar UV microplate with PathCheck applied (Figure 1). The calculated limit of detection (amount producing an absorbance value higher than 3 positive SDs of the blank values above zero) was approximately 25 ng/well. The calculated limit of quantitation was approximately 75 ng/well, by using a very conservative criterion (amount producing an absorbance value higher than 10 positive SDs of the reagent blank above zero). In wells containing 300 µL, this limit translates 250 ng/mL. Lower limits can be achieved with a less conservative criterion, but require strict attention to technique. The results above were obtained with pre–diluted solutions under optimal conditions. If the protocol instead involves a small sacrificial DNA sample plus a diluent in each well, the limit of quantitation will be somewhat higher.

Figure 1: Standard curve obtained with calf thymus DNA dissolved in water. Triplicate 300 µL aliquots were placed in a Costar UV microplate and read at 260 nm with PathCheck applied.

††Select “Blank” from the Group pop-up menu on the left side of the Template editor. This is called the “Plate blank” because SOFTmax PRO subtracts its average absorbance value from each well of the microplate.
The microplate results agree favorably with published limits of detection of DNA using \(A_{260}\) measurements in spectrophotometers\(^3,4\). This is not unexpected, in view of the high quality of UV–transparent microplates available and the fact that the optical pathlength in a full microplate well is approximately 1 cm. If the samples contain lower DNA concentrations, they cannot be measured by UV absorbance, and can only be measured with another technique, such as reaction with a fluorescent dye.

**DNA/RNA ESTIMATION USING ABSORPTIVITY**

DNA (RNA) concentration is commonly estimated by dividing the \(A_{260}\) value by the 1-cm absorptivity value (or multiplying by its reciprocal). By using PathCheck, SPECTRAmax instruments can automatically normalize sample absorbance values to a 1-cm pathlength and calculate the concentrations. The \(1/\text{absorptivity}\) value for double stranded DNA at 260 nm is commonly assumed to be \(~50\ \mu\text{g/ML}\) for a 1-cm pathlength\(^3,4\). However, this value appears to be correct only when the solution has a relatively high salt concentration (Figure 2). DNA has 15% lower absorbance in TE buffer compared to water, and 23% lower absorbance in TE plus saline (TES). The calculated \(1/\text{absorptivity}\) values are 38, 45 and 50 \(\mu\text{g/ML}\) for DNA in water, TE and TES, respectively (Figure 2 inset).

**Figure 2**: Effect of ionic strength on DNA absorption at 260 nm. The DNA (Sigma Type I “Highly polymerized”, Cat. No. 1501) was dissolved in deionized water or TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4) or TES (TE buffer + 0.9% NaCl). Insert: \(1/\text{absorptivity}\) values; Average of 7 concentrations (2.5–50 \(\mu\text{g/mL}\)), 4 replicates each.

**USING SOFTMAX PRO TO CALCULATE DNA CONCENTRATION**

DNA/RNA assay protocols often call for \(A_{260}\) measurements of small (2 - 10 \(\mu\text{L}\)) samples to which a diluent is added to get a workable sample volume. An example is the extraction of DNA from blood or tissue samples prior to determining nucleotide sequences. Before proceeding to the sequencer, the DNA concentration of a small sacrificial aliquot of the extract is determined and the result is used to calculate the aliquot size needed in order to transfer the desired amount of DNA to the sequencer. SOFTmax PRO can automatically normalize \(A_{260}\) values from SPECTRAmax instruments, determine the concentrations and
calculate the desired aliquot size. SOFTmax PRO can also round off to the nearest 0.5 µL to accommodate liquid handling systems which are settable in 0.5 µL increments. Figure 3 gives an example of such a case, where the sacrificial aliquot was 5 µL and the target amount for the sequencer was 0.4 µg. The nominal aliquot volume was calculated for each sample and also that volume rounded off to the nearest 0.5 µL. These calculated results can easily be exported for use by external data handling systems.

<table>
<thead>
<tr>
<th>Sample</th>
<th>wells</th>
<th>OD 260nm</th>
<th>ng/µL</th>
<th>Target Aliquot*</th>
<th>Aliquot**</th>
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<tbody>
<tr>
<td>1</td>
<td>B1</td>
<td>0.125</td>
<td>250.9</td>
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<tr>
<td>2</td>
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<tr>
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<tr>
<td></td>
<td>H2</td>
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</table>

*Aliquot size needed to transfer 400 ng
**Aliquot size rounded to nearest 0.5 µL

Figure 3: Example of report generated by SOFTmax PRO listing results for DNA extracts and the calculated aliquot volumes needed to contain a specified amount of DNA, for example to transfer to a sequencer.

**SUMMARY**

With attention to technique, accurate and reproducible DNA/RNA measurements can be made easily in microplates. The lower limits of quantitation are comparable to those obtained in conventional UV–VIS spectrophotometers. Results can be normalized automatically to a 1 cm pathlength by using the PathCheck sensor to produce values equivalent to those obtained in a standard 1-cm pathlength cuvette. SOFTmax PRO can calculate aliquot volumes needed to contain a specific amount of DNA(RNA), as well as report the results in customized formats.

**REFERENCES**


