

Application Note

QUANTIFICATION of NUCLEIC ACIDS USING ABSORBANCE

INTRODUCTION

A260 absorbance reading is one of the most common methods for estimating the quantity of nucleic acids in samples. It is an inexpensive method using a spectrophotometer that gives immediate results. While it is very useful technique for estimating quantity (and with the A280 reading, the relative purity) of a sample, it is often misused by researchers. The technique is not very accurate, and a number of issues can further compromise the accuracy of the readings.

This application note will outline the physics of absorbance readings and discuss the proper use of this information. Additionally, there are instructions on how to program Tecan GENios readers to perform A260/A280 readings.

OPTICAL DENSITY READINGS

Optical density units are defined as the amount of substance dissolved in 1mL that will give an absorbance reading of 1.0 with a 1cm pathlength. Nucleic acids absorb light at a wavelength of 260nm, so the absorbance or optical density of DNA and RNA are often reported at this wavelength.

$$\text{O.D.} = \log_{10}(1/\text{Transmittance})$$

Where Transmittance = (light in/light out)

NUCLEIC ACID QUANTIFICATION

Different nucleic acid quantities absorb light differently. When measuring nucleic acids, an A260 value of 1.0 in a 1cm pathlength will represent different amounts of sample.

- Double stranded (ds) DNA = 50ug/mL
- Single stranded (ss) DNA = 33ug/mL
- RNA = 40ug/mL
- Oligonucleotides = 30ug/mL

A derivation of Beer's Law can be used to calculate the quantity of any of these nucleic acids with the A260 reading from the sample (1).

$$\text{ug/mL} = \text{A260} * \text{CF} * 1 \text{ cm pathlength}$$

Where CF = nucleic acid conversion factor listed above



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This calculation depends upon the sample having a 1 cm pathlength. Most microplates will not accommodate a 1 cm column of solution, and therefore it is necessary to measure the actual pathlength of the liquid in the well. This process can be accomplished by measuring the absorbance of water (the diluent) in the sample. Water can be measured at 999nm using a 900 nm reference wavelength, and through a series of calculations the pathlength can be determined. A simpler alternative is to use the half area UV plates available from Costar (Cat# 3679). When 175ul is placed in the well, it produces a 1 cm pathlength and the calculation described above can be applied to directly measure the concentration of DNA without the need to measure the absorbance of water.

RELATIVE PURITY OF THE SAMPLE

In conjunction with an A280 reading, the A260 reading can also be used to estimate the relative purity of a sample as well, as proteins (a common contaminant) absorb light at 280nm.

An A260/A280 ratio will give an approximate evaluation of the relative purity of the sample.

- For DNA, a pure sample will have an A260/A280 value of 1.8.
- For RNA, a pure sample will have an A260/A280 value of 2.0.

Additionally, an A260/A230 ratio can help factor out contamination from carbohydrates, peptides, phenols, and aromatic compounds, which absorb light at the 230nm wavelength. A pure sample should have an A260/A230 ratio of 2.2.

LIMITATIONS OF THE METHOD

While the absorbance method for determining nucleic acid amount and relative purity is quick and easy, it is limited in its accuracy. Since an A260 value of 0.1 corresponds to 5ug/mL, the overall sensitivity of the method is approximately 50-250ng/mL of DNA. A comparable fluorescent method using Pico Green is sensitive down to 300pg/mL of DNA (roughly 100 to 1000 times more sensitive than the A260 reading). (2)

This difference in sensitivities does not make the A260 method of no value. If a "quick and dirty" estimation of sample concentration and purity is all that is needed, this method may be the preferred technique. If more quantitative results are needed, researchers should switch to the more accurate fluorescent techniques for measuring concentrations.

The volume of sample in the well can have a significant effect on readings. The lower the volume, the less accurate the measurement. This is particularly true if the meniscus is deeper in some wells than in others. (3)



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TAKING ABSORBANCE READINGS IN MAGELLAN ON THE GENios

A default method exists in the software for measuring A260/A280 values. The method is called DNApurity-Genions.mth.

The method is set up as a multi-label run, taking the first reading at 260nm and the second at 280nm. The transformation is then run as $x[0]/x[1]$, where $x[0]$ is the raw value of the well for the first read cycle, and $x[1]$ is the raw data of the second read cycle. The final value given is a 260/280 ratio.

TAKING ABSORBANCE READINGS IN XFLUOR

You will want to set up two different reads for your plate, at 260nm and 280nm.

To set the first read at 260nm:

1. From the Xfluo4 menu in Excel, choose Multi-labelling.
2. Select the checkbox for Do Measurement 1, and click the Edit measurement parameter button.
3. In the General tab, select Absorbance.
4. In the Plate tab, select the pdf file for the microtiter plate in use.
5. In the Meas Params tab, select 260nm for the Measurement Wavelength, and 0 for the Reference Wavelength. Leave the number of reads at the default of 3.
6. Set the parameters for the Temperature and Shaking tabs as the protocol calls for.

To set the second read at 280nm:

1. From the Xfluo4 menu in Excel, choose Multi-labelling.
2. Select the checkbox for Do Measurement 2, and click the Edit measurement parameter button.
3. In the General tab, select Absorbance.
4. In the Plate tab, select the pdf file for the microtiter plate in use.
5. In the Meas Params tab, select 280nm for the Measurement Wavelength, and 0 for the Reference Wavelength. Leave the number of reads at the default of 3.
6. Set the parameters for the Temperature and Shaking tabs as the protocol calls for.

To save this setup for use in the future, choose Save measurement parameters in the Xfluo4 menu, and give the set of reading parameters a name. In the future, you can choose Load measurement parameters from the Xfluo4 menu to run this file.

The Excel file will now display 2 sets of measurements in 2 grids. One grid will contain 260 results, the other 280 results. Using Excel's mathematical functions, divide the 260 reading by the 280 reading. The results are the 260/280 ratios for each well.



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