Technical Overview

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Nucleic Acid Quantitation

Detection limits

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Abstract

Today's biomedical testing has resulted in sample sizes becoming smaller and smaller, driving the need to measure samples with ever-lower detection limits. The quantitation of nucleic acids is no exception. While other methods may be more sensitive, the ease, simplicity, and noninvasiveness of direct UV absorbance measurements has made it the most popular method of nucleic acid quantitation. The use of UV absorbance measurement to quantitate nucleic acids is the *de facto* standard by which all other methods are measured. This technical overview describes the limit of detection (LOD) for nucleic acid quantitation using Agilent BioTek UV-Vis microplate spectrophotometers.

Limit of detection

There are several different ways to define the limit of detection for an assay-instrument combination. Regardless of the method, the intent is to provide a means to reliably insure a differentiation of a sample containing an analyte and the buffer only control. One method used is interpolating a concentration curve with the value of the blank mean plus three times the standard deviation of the blank. The resultant interpolated concentration is said to be the limit of detection. Samples with signals greater than the interpolated value can be detected reliably. Another related method is known as a signal-to-noise ratio: the signal of a known standard minus the blank divided by the total standard deviation of the standard and the blank. Concentrations that result in a signal-to-noise ratio of 2.0 or greater are considered to be significantly different from the blank. This is the method that Agilent BioTek uses to identify detection limits for fluorescent compounds. A less stringent test often used is the Student's T-test, which compares the probability that two populations are different. This test also uses the means and standard deviation of blanks and samples to determine if the populations of data are different. Probabilities of 0.05 ($p < 0.05$) or less are considered being different.

Theoretical lowest concentration

The smallest absorbance unit capable of being reported by the Agilent BioTek microplate spectrophotometers (such as the PowerWave HT, PowerWave XS, Synergy HT, and µQuant) is 0.001 ODs. If the Agilent BioTek 1 cm quartz Bio-Cell is used to make this measurement, then 0.001 would also be the smallest measurable absorbance reading for a 1 cm path length. A sample in the Bio-Cell that absorbs 0.001 OD above a blanked value and multiply by the extinction value of 50 (1 OD equals 50 µg/mL), the concentration becomes 0.050 µg/mL or 50 ng/mL. However, in a well, the path length is usually less than 1 cm, but 0.001 OD is still the minimum value to be reported. For example, if a 200 µL sample is used, the path length is approximately 0.5 cm so the conversion to a 1 cm path length would result in a corrected absorbance two times the raw value. The minimum reported value is still 0.001 regardless so the path length corrected value would be 0.002 and the concentration would be 0.002 × 50 or 0.100 µg/mL or 100 ng/mL, which equates to 20 ng/well (100 ng/mL × 0.2 mL). However, these calculations assume that each measurement is perfect, that there are no errors inherent with the measurements, and each measurement is completely repeatable.

Concentration versus content

A common misconception occurs when comparing detection limits. This misconception revolves around the differences between concentration and well content. Concentration is an amount of absorbing material per unit of volume (e.g. µg/mL or ng/µL). Content is an amount (weight) in the well (ng/well) and is independent of volume. The two are often referred to interchangeably when they actually are not interchangeable. The subtle difference between the two is that content will change with an increasing volume of a constant concentration. For example, a well that has 200 µL of a 1,000 ng/mL solution has a content of 200 ng/well, while the same well that has 100 µL of a 1,000 ng/mL solution will have a content of 100 ng (Table 1). Note that the raw ODs for each well will be different, while the path length corrected absorbance and concentration calculation will theoretically return the same values, as path length correction normalizes everything to a common 1 cm path length. Two wells that have different concentrations, but have the same content, would have the same raw absorbance. A well that has a content of 500 ng/well in 300 µL (1,667 ng/mL) might have an absorbance of 0.025 ODs and another well also has a content of 500 ng/well, but in 200 µL would also return an absorbance measurement of 0.025 ODs, but the calculated concentration would be 2,500 ng/mL (Table 1).

Table 1. Hypothetical DNA measurements from different well contents using various fluid volumes.

Content	300 µL Volume			200 µL Volume			100 µL Volume		
(ng/well)	Concentration (ng/mL)	Raw OD	Correction* OD	Concentration (ng/mL)	Raw OD	Correction* OD	Concentration (ng/mL)	Raw OD	Correction* OD
500 ng	1,667	0.025	0.033	2,500	0.025	0.050	5,000	0.025	0.100
200 ng	666.7	0.010	0.013	1,000	0.010	0.020	2,000	0.010	0.040
100 ng	333.0	0.005	0.007	500	0.005	0.010	1,000	0.005	0.020
50 ng	166.7	0.003	0.003	250	0.003	0.005	500	0.003	0.010

*Based on an estimated path length of 0.75, 0.5, and 0.25 cm for 300, 200, and 100 µL well volumes, respectively.

Larger volume per well equals greater path length

When trying to measure low concentrations, the longer the path length the better the measurement will be. Longer path lengths result in a larger signal if the concentration is the same. In microplates the path length of the sample depends on the volume of fluid in the well. This means that a well with 300 µL of a DNA solution will have three times the absorbance as a well with 100 µL of the same solution after background subtraction. Besides increasing the volume of the well, one can reduce the size of the well to increase path length. Half area 96-well plates or 384-well plates will provide a greater fluid path length than a standard plate with the same fluid volume.

Blanking: preread versus identified blanks

Due to the background absorbance of the microplate, it is necessary to perform some sort of blanking on the raw data at 260 nm in order to achieve accurate results. With regard to blanking the plate there are two options: preread an empty microplate (preferably the plate about to be used in the assay) before measuring samples experimentally or define one or more wells as blanks using the plate layout. While it is more convenient to define one or more wells as a blank, this method assumes that the background for each well is the same. In reality, there may be as much as a 0.005 difference between the highest and lowest well. In general, the commonly used diluents used in conjunction with nucleic acids do not have any appreciable absorbance at 260 nm, with all the background absorbance the result of the microplate itself. As a matter of practice using a blank well or two will provide acceptable results regarding nucleic acid quantitation. If possible it is recommended that both a preread and a blank well or wells be defined. This combination will accommodate variations in plate background as well as any influence that the sample buffer may provide.

Errors can add up

While the theoretical limit to the measurement is 0.001 OD units, there are several errors inherent to the measurement. These errors have the potential to add up. Reader specification for repeatability is ±1% ±0.010 ODs, which at very low ODs means that the 1% component essentially drops out of the equation, which leaves an error of ±0.010 ODs. Typically, Agilent BioTek readers perform much better than specification and can estimate accuracy at ±0.002 for this paper. In addition to the reader variability, well-to-well variability in the plate is approximately ±0.002 ODs, which represents a total variation of 0.004 OD across the plate. The meniscus at the air-liquid interface has the potential for a variability of ±0.001 ODs as well. Note that the error caused by the well-to-well variability and the meniscus can be corrected for using the preread functionality and blank well subtraction respectively. This effectively limits the error to the error of the reader, which has been estimated at ±0.002 ODs.

Path length, sample volume, and detection limits

Relationship between sample volume and path length can confuse the issue regarding detection limits as measured by concentration. Because of this, the preferential method is to use well content (ng/well) as a means by which detection limits can be defined. With vertical photometry the absorbance of a well content is the same regardless of the volume. In other words, the absorbance of 500 ng of DNA in a well is the same if it is in 100, 200, or 300 µL. Note that the DNA concentration would be 5 µg/mL, 2.5 µg/mL, and 1.67 µg/mL, respectively. As demonstrated in Figure 1, when various concentrations of DNA are quantitated, 300 µL samples can be distinguished from the buffer only blank at lower concentrations than the 100 µL samples. Please note the use of concentration in this example. Again, this relates to the total DNA content on a per-well basis, as large volumes of a fixed concentration will result in a larger content of absorbing material.

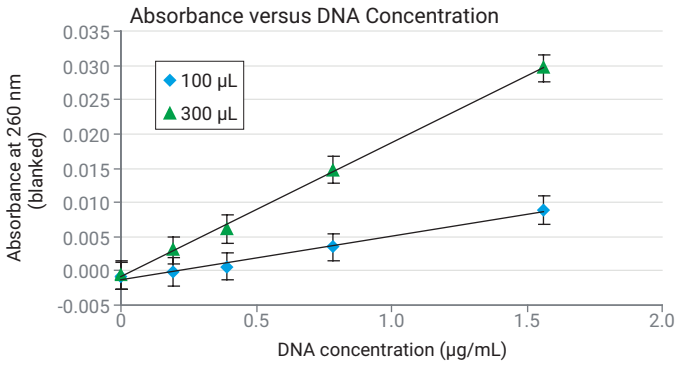

Figure 1. Absorbance of various DNA concentrations with different sample volumes. The absorbance at 260 nm (after blanking) of various DNA concentrations was plotted and linear regression analysis performed for two different sample volumes. Error bars represent a ±0.002 ODs from the mean at each determination.

Conclusion

As seen in Figure 2, when the absorbance was plotted against the total DNA content on a per-well basis, a linear relationship between content and absorbance is observed. Using the estimated error of measurement of ±0.002 OD one observes that the upward error bars from the blank do not overlap the downward error bars of the 100 ng/well samples. Therefore, regarding detecting DNA in solution using absorbance, the minimum amount that can be detected is approximately 100 ng per well under the best of circumstances. While samples with lower per well content have a mean absorbance that is higher than that observed with the blanks, the difference is not outside the error of measurement. Nucleic acids, such as RNA or ssDNA can be measured using absorbance in the same manner as dsDNA. Because these compounds have a higher extinction coefficient than dsDNA, the detection limits would be expected to be lower. RNA has an extinction coefficient 20% higher than dsDNA. This suggests that the detection limit for RNA would be approximately 20% lower than dsDNA or 80 ng/well. ssDNA, such as oligonucleotides, have an even higher extinction coefficient. The detection for these molecules can be calculated to be approximately 67 ng/well.

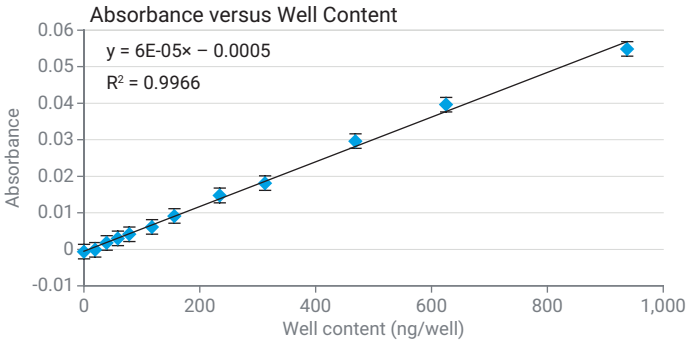

Figure 2. Absorbance of wells with different total DNA content. Data from wells with differing volumes and DNA concentrations were pooled to provide a plot of DNA content versus absorbance. Error bars represent ±0.002 ODs from the mean of each observation.

There are various factors that can prevent one from achieving these detection limits. High-quality DNA is essential for good detection limits. DNA solutions and buffers containing protein contamination or particulates will often result in a greater variability of replicates, which is observed as higher standard deviation or %CV values. Using sufficient volumes for the well type being used is also critical. Volumes that do not completely fill and cover the bottom of the well will result in aberrant results. Standard 96-well plate wells require a minimum of 100 µL. Half area 96-well plates can be used with as little as 50 µL and samples in 384-well plates can be measured using 25 µL. Dirty or scratched microplates can affect the variability of samples as well, particularly if the plate preread feature of the PC software (KC4 or KCjunior) is not used. Proper storage of the unused plates can minimize this potential problem. Inaccurate pipetting of samples can lead to unintended inaccuracies that cannot always be corrected using path length correction. Good laboratory practices, such as maintaining buffers and solutions contaminate free, are also essential for obtaining accurate and precise nucleic acid quantitation. This is important at very low concentration levels.

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