Comparison of the TLDA with the Nanodrop and the reference Qubit system

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Abstract. The TLDA (transmitted light drop analyser) is a new microvolume UV-visible drop spectrophotometer new to the market. Assays were compared between a TLDA, NanoDrop and the Qubit system which is based on the gold standard for DNA assay, PicoGreen. The evaluation was carried out by the Trinity Biobank in St. James Hospital, Dublin on Buccal swabs and Blood samples. The data is discussed in this paper. The Qubit system is seen as the reference method in most studies as this is believed to provide more accurate results than UV absorbance methods because it distinguishes between DNA, RNA, free nucleotides, and other contaminants. The Qubit system uses fluorescent dyes to measure the concentration of the molecule of interest. The results compare both the spectrophotometric methods against the Qubit fluorescence technique.

1. Introduction
Correct quantitation of small amounts of DNA is very important for a wide variety of molecular biology applications. Recent review of methods is given by Maquat et al.¹. Quantitation of DNA is simple in theory but can be difficult in practice. Three methods of quantitation are assessed in this study – TLDA, NanoDrop and Qubit.

A relevant study has been conducted to demonstrate the value of the Qubit system as the reference system in DNA and protein studies². The Qubit fluorometer is a device from Invitrogen for quantifying proteins, RNA, or DNA. It uses various assays, which contain sensitive dyes that fluoresce in proportion to the amount of protein, RNA or DNA respectively. PicoGreen dye was used in this study. PicoGreen is a fluorescent nucleic acid stain for quantitating double-stranded DNA (dsDNA). It has an excitation maximum at 480 nm (lesser peaks in the short-wave UV range) and an emission peak at 520 nm. When bound to dsDNA, fluorescence enhancement of PicoGreen is exceptionally high and little background occurs since the unbound dye has virtually no fluorescence. The problem of protein contamination of DNA is highlighted in many studies such as that of Wieczorek³. The first paragraph after a heading is not indented (Bodytext style).

The TLDA⁴ and NanoDrop are both UV/Vis spectrophotometric instruments. Drop UV spectrophotometry is one of the most common analytical methods for nucleic acids analysis. NanoDrop has been used in several BioBank studies such as that of a Spanish team led by R. Palmiotron⁵. The instrument has wide applications also for protein assays such as those of Desjardins et
The two UV instruments have been compared against PicoGreen, seen as the gold standard for DNA analysis. A UV/Vis spectrophotometer measures the intensity of light passing through a sample ($I$) and compares it to the intensity of light before it passes through the sample ($I_0$). The ratio of $I$ to $I_0$ is the transmittance. The absorbance, $A$, is based on the transmittance and is derived using the following formula:

$$A = -\log(\%T)$$  \hspace{1cm} (1)

The absorbance of UV light as measured in a spectrophotometer can be combined with the concentration factor to enable calculation of the concentration and purity of dsDNA. One 260nm absorbance unit of double-stranded DNA = 50 μg/mL.

Even after DNA is isolated from an organism, protein frequently remains present in the DNA solution. Protein is tightly bound to DNA and complete removal is not always possible. Both protein and DNA of course absorb UV light, but have different absorbance spectra. Figure 1 below shows the typical UV absorption spectra for both protein (BSA) and DNA.

![Absorption spectra for BSA and DNA](image)

Figure 1. Absorption spectra for BSA and DNA

The peak of light absorption for DNA is at 260 nm, while protein absorbs at 280 nm, mainly due to tryptophan and tyrosine side chains. When a solution contains both protein and DNA, absorbance at 260 nm is primarily due to the DNA present, but a small amount is due to the protein. At 280 nm, the absorbance is predominantly due to the protein present. Thus, the purity of the DNA can be calculated by examining the ratio of the two absorbance values. A ratio of the absorbance values at 260/280 nm of approximately 1.7 to 1.8 predict “clean or pure” DNA, whereas lower values may be indicative of significant protein contamination. However, the 260/280 absorbance ratio is not always an accurate representation of DNA purity. Certain samples may be very difficult to evaluate at 280nm due to interference. Therefore, peptide bonds, which absorb at 228 nm, are often a more constant indicator of the presence of protein in a sample. Thus, absorbance readings measured both at 230 nm and at 280 nm provide a more accurate estimate of proteins or peptides that may be present in nucleic acid samples. The ratio of the 260/230 absorbance values should be >1.5 since nucleic acids have an absorbance minima at 230 nm. Both the TLDA and NanoDrop provide absorbance readings at 230, 260 and 280nm, and examine both the 260/280 and 230/280 ratios for every sample. Buffer salts can contribute significantly to absorbance readings, especially below 260 nm. Thus, it is imperative to blank the spectrophotometer in the same buffer in which your DNA sample is diluted. The Blank used in these experimental trails was TE Buffer.

The procedure used for NanoDrop measurements is well-established; the sample is pipetted directly onto the measurement surface. No cuvette is used. Using a patented retention system, a column is drawn between the ends of two optical fibers to establish the measurement path. The TLDA from Drop Technology uses a patented system which uses surface tension to hold the small volume sample
drop in place on the quartz measurement surface. The computer model of the drop spectroscopy system is given by McMillan et al. The theory derived in this study for a pendant drop is relevant to the sessile drop TLDA system. The TLDA sample is a 1.5-3µl sample droplet onto the quartz optical surface that is pipetted onto the drophead rotating instrument loading system. A quartz plinth is necessary as quartz is transparent in the UV. Surface tension alone holds the sample in place for the spectral measurement. With the rotating camber closes the fiber light source is aligned directly over the detector. The light passes through an aligned sessile drop from a source fiber to the detector. The pathlength is determined mathematically from the drop size selected in the software.

2. Experimental

2.1. Measurement Details

DNA and most of the common contaminants found in DNA absorb in the 230nm to 320nm region. Hence an absorbance measurement in this region allows the DNA concentration to be calculated and provides information about the contaminant levels. The most important wavelengths to note are:

**230nm:** Phenol and guanidium salts absorbs strongly at 230nm. Therefore, high absorbances at this wavelength can be an indication of carry-over of either of these compounds into the test sample.

**260nm:** DNA absorbs light most strongly at 260nm. The absorbance value at this wavelength can be used to estimate the DNA concentration using the equation Concentration (µg/ml) = (A$_{260}$ reading – A$_{120}$ reading) x 50, which is derived from Beer’s Law.

**280nm:** Tyrosine and tryptophan absorb strongly at this wavelength. Hence, the absorbance at 280nm is used as an indicator of protein contamination.

**320nm:** The absorbance at 320nm provides a measurement of the turbidity of the sample. It is normally subtracted from the A$_{260}$ value as a background reading for the calculation of DNA concentration. Excessive values here may indicate non-specific contamination.

A good quality DNA sample should have a 260/280 absorbance ratio of 1.7-2.0 and a 260/320 absorbance ratio of greater than 1.5. For an accurate measurement, realistically the absorbance value at 260nm must lie between 0.1 and 1, so dilution of concentrated samples may be required.

For pure DNA, the observed 260/280 nm ratio will be near 1.8. Ratios above this usually indicate the presence of RNA. Pure RNA will have a 260/280 ratio of approximately 2.2-2.3. 260/280 ratios below 1.8 often signal the presence of a contaminating protein or phenol. Alternatively, protein or phenol contamination is indicated by 230/260 ratios greater than 0.5. Where DNA, RNA and protein is present, a 260/280 ratio greater than 1.8 and a 230/260 ratio greater than 0.5 is expected.

The capability of the TLDA to measure DNA was tested with Buccal swabs and Blood samples. The same samples were run on the Nanodrop and Qubit systems. As the TLDA and NanoDrop are both UV systems a direct comparison wanted to be made with these. The findings were directly compared with the standard PicoGreen assay using the Qubit system. Double replicates read within acceptable correlation of variance between samples, 10% allowance.

2.2. Buccal swabs.

The comparison data from the Buccal swabs is shown in Figure 2 below.

![Figure 2](image-url)

Figure 2. Buccal swabs data measured with the Picogreen Assay, Nanodrop and TLDA. Also shown are the 260/280 and 230/260 absorbance ratios.
Comment:
Most of these samples have a 260/280nm value below 1.8 (average = 1.5) and a 230/260nm value greater than 0.5 (average = 0.68), which signifies the presence of a contaminating protein. However, the deviation between the concentration results from both the Nanodrop and the TLDA with the Qubit (Picogreen) are shown in Figure 3 below.

The average deviation value for NanoDrop was found to be 41 whereas with the TLDA it was 36 ng/µl.

2.3. Blood swabs
The comparison data from the Blood swabs is shown in Figure 4 below.

Comment:
The graphical results above indicate that there was only a small trace or perhaps in some cases no protein or phenol contamination as the 260/280nm value is above 1.8 (average = 1.93) and the 230/260nm value is around 0.5 (average = 0.54). There may also be RNA present as the 260/280 value averages to be 1.93.

However, the deviation between the concentration results from both the Nanodrop and the TLDA with the Qubit (Picogreen) are shown in Figure 5 below.
Figure 5. Deviation of the Nanodrop and TLDA concentration results from the Picogreen values for the blood data.

The average deviation value for NanoDrop was found to be 175 whereas the TLDA was slightly lower with a value of 139 ng/µl.

3. Discussion
Due to the dye-based quantification of the Qubit system the dyes are quite specific, allowing you to quantify only either the RNA or DNA. This is a big advantage of the Qubit system over the TLDA or NanoDrop (spectrophotometric instruments) as they calculate the total amount of RNA or DNA into one quantity. The spectrophotometric instruments count anything that absorbs light at 260nm. This could be DNA, RNA, single stranded or double stranded, proteins or contaminant. The Qubit uses PicoGreen technology, which fluoresces only when it binds to double stranded DNA etc., making it a more accurate method of quantification where the molecules of the sample are unknown.

The use of the TLDA and the NanoDrop is faster than setting up the Qubit system, because you have to prepare the dye and buffer for each sample, you have to prepare two standards for each set of samples, and you have to place each sample and standard in its own tube for quantification. The TLDA and the NanoDrop requires less sample volume. For concentrations the Qubit requires 10-20µl of sample in comparison to typically 2µl with the two spectrophotometric instruments. A big disadvantage with the Qubit system is that you need to have a rough estimate of your sample concentration to know which dye to use and to know what amount of sample to use in the quantification, otherwise you will get an out of error range. Also the Qubit system does not tell you the purity of the sample.

Both spectrophotometric methods are simple to use, very sensitive, require a small sample volume and no cuvettes or tubes are required. Also, a major advantage is that no standard curve needs to be generated prior to measurements. As an added benefit, the samples are also recoverable after quantitation.

4. Conclusion
Approximately, 60 buccal swab DNA samples and a similar number of Blood derived DNA samples have been assayed and analysed. It was found that for buccal samples both the spectrophotometric methods gave much higher results than the Qubit standard. This was expected, and is traditionally interpreted as resulting from contamination in the sample (protein, etc) as judged by the 260/280 ratio.
of all of these samples (1.4-1.6). Much better comparisons can be made between all three assay with DNA derived from blood where this ratio was invariably 1.8-2.0.

Overall, any fluorescence based quantification is not going to be as straightforward as the spectrophotometric methods, but the extra effort produces improved results. The methods are measuring different things. The Qubit (PicoGreen) is measuring the amount of dsDNA in the sample, while the spectrophotometric (TLDA & NanoDrop) instruments is measuring anything in the sample that scatters or absorbs light at the wavelength used for the measurement. The correlation of the results above is not always great. The TLDA and NanoDrop readings are usually higher that the PicoGreen, indicating that the DNA sample may contain a mixture of double and single stranded DNA, contaminants which scatter light, or UV-absorbing materials that are not nucleic acids. Therefore, the main comparison should be made between the TLDA and the NanoDrop.

Regarding practical issues, it was found that the TLDA is more user friendly than NanoDrop. It had about the same 'repeat analysis' incidence (i.e. when duplicates did not agree within 10% for blood samples). It would have been preferable to reverse-pipette each applied sample to obtain better results, but it was not possible as the wastage of samples was not permitted.

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