Low-volume, High Throughput Workflow for Analysis of Nucleic Acid Samples for Biobanking

Peter J. Brescia, Jr., Chris Wilson, and Peter Banks, BioTek Instruments, Inc., Winooski, VT

Accurate quantification of nucleic acids remains a critical step prior to performing downstream processes such as sequencing, mutagenesis, and qPCR. The most common method of analysis are fluorometric and spectrophotometric methods where the former is used for applications requiring high sensitivity; the latter for quantification of purity and yield from nucleic acid extraction procedures resulting in larger volumes and samples of higher concentration. Quantification is commonly performed in a cuvette or microplate based format. Increasingly, there has been interest in sample conservation and higher throughput analyses more amenable to a microplate format given the ability to analyze smaller volumes, increased sample capabilities, and incorporate automation.

Key Words:

Nucleic Acid Quantification

$A_{260/280}$ Ratio

UV-Star

µClear

Low-volume Microplate

Low-volume DNA Quantification

Biobanking

DNA Contamination

Protein Quantification

Fluorescence

RNA

DNA

Introduction

Nucleic acids provide genetic information in all living things, from plants and animals to bacteria and viruses. In many instances it is important to characterize purified RNA and DNA samples prior to downstream processes such as sequencing, restriction enzyme digestion for molecular cloning, ligation, PCR and qPCR, among others.

Both fluorometric and spectrophotometric methods are routinely used for nucleic acid quantification. The former is used for applications requiring high sensitivity, while the latter is more traditionally used for analysis of purity and yield from common nucleic acid purification methods. Nucleic acid quantification is commonly performed in a cuvette spectrophotometer, where a monochromator based optical system provides light at 260 nm (the absorbance maxima for nucleic acids). Increasingly, researchers are using microplate spectrophotometers in place of cuvette based instruments for higher throughput and sample conservation.

Background

Biomolecules absorb ultra-violet (UV) light which directly correlates to the concentration of molecules present in the sample. Nucleic acid samples are routinely analyzed at several wavelengths of light including 230, 260 and 280 nm, where 260 and 280 nm represent peak absorbance of nucleic acids and proteins, respectively (Figure 1). The ratio of the 260 nm to 280 nm measurement provides an estimation of the purity of the nucleic acid, with a ratio of ~1.8-

2.0 indicative of a nucleic acid sample of high purity (Table 1).

Additionally, measurements at 230 nm can be used to assess residual contaminants following nucleic acid extraction from samples. A depressed 260/230 ratio can be indicative of residual phenol and/or guanidine contamination from the extraction process. Figure 2 demonstrates the effect of increasing amounts of phenol on a spectral scan of dsDNA. Note the significantly higher absorbance at 230 nm from the phenol contaminant.
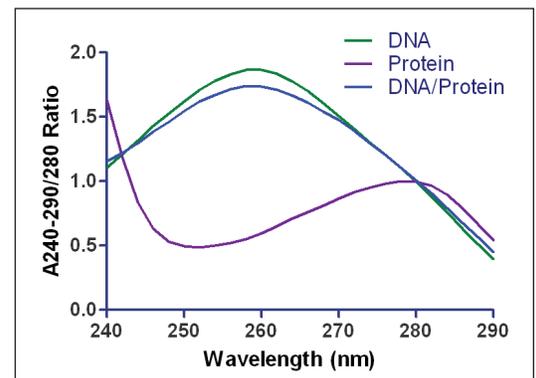

Figure 1. Typical absorbance spectrum for DNA, RNA, and protein, indicating peak at about 260 nm for nucleic acids and 280 nm for protein.

Nucleic Acid Type	Approximate A_{260}/A_{280} Ratio
Pure DNA	1.8
Pure RNA	2.0
Pure Protein	0.57

Table 1. Purity assessment based on A_{260}/A_{280} ratio. Note the various forms of nucleic acids are not distinguished from each other using this ratio.
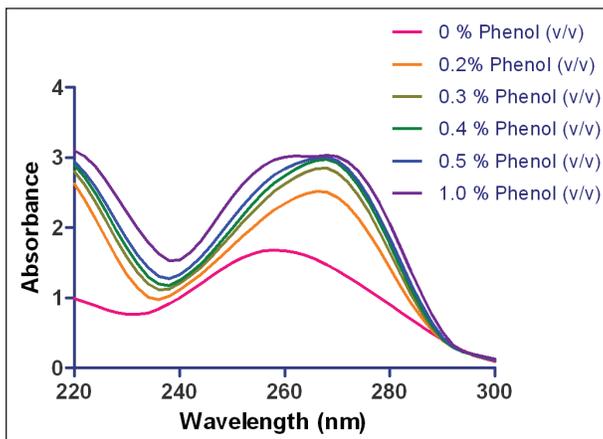

Figure 2. Spectral analysis of herring sperm dsDNA spiked with increasing concentrations of phenol. Data normalized to a 1 cm pathlength.

While cuvette based measurements provide simple, precise determinations, throughput is limited to single sample measurements, higher concentration samples must be diluted, and larger sample volumes are required. In contrast, microplate spectrophotometers, and in some cases micro-volume systems, afford end-users increased sample throughput freedom from dilution and require significantly smaller sample volumes while maintaining precision and accuracy.

By convention, the extinction coefficients for nucleic acids are based on 1 cm pathlength measurements (Table 2). Therefore, in a microplate spectrophotometer, where the sample pathlength is dependent upon the volume in the microplate well, it is necessary to correct the absorbance measurement to a 1 cm pathlength equivalent in order to properly quantify the sample.

Nucleic Acid Type	Avg. Extinction Coefficient $(\mu g/mL)^{-1}cm^{-1}$	Concentration $(\mu g/mL)$ if OD=1*
dsDNA	0.02	50
ssDNA	0.027	37
ssRNA	0.025	40

Table 2. Commonly accepted extinction coefficients. *Based on a 1cm pathlength.

A cuvette-based spectrophotometer uses a horizontal light path where light of a specific wavelength is focused perpendicular to the vessel. Vessels typically have a fixed pathlength of 1 cm although a variety of fixed pathlengths are available. In contrast, a microplate spectrophometer measures samples in a microplate well using a vertical pathlength which varies with sample volume (Figure 3).
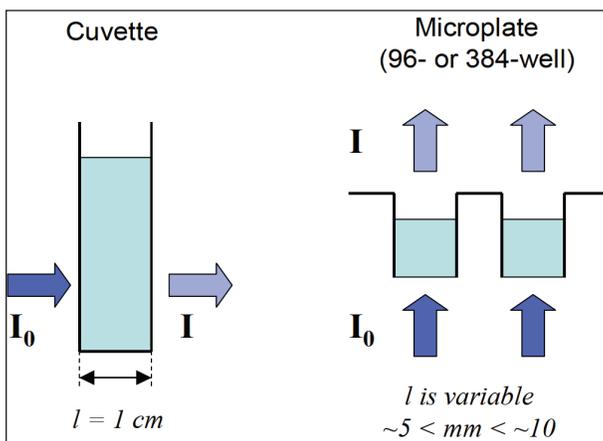

Figure 3. Comparison of the fixed 1 cm pathlength using a vertically orientated cuvette based system and the variable vertical light path of a microplate based system. Pathlength is dependent on sample volume and microplate well dimensions.

Nucleic acid calculations are based on the Beer-Lambert Law according to this equation:

$$A = \varepsilon cl$$

the absorbance (A) of the sample can be determined by the product of the extinction coefficient (ε) and concentration (c) of the sample and thickness (pathlength) (l) through which the sample is being measured.

Gen5™ Data Analysis software has built-in, automated methods for determination of pathlength for samples diluted in aqueous buffers. Gen5 uses the absorbance peak of water at room temperature (977 nm) and a background measurement at 900 nm. The 977 nm – 900 nm optical density difference is divided by 0.18, which represents the optical density of water at 1 cm. The result of the above calculation is the pathlength of the sample, which can then be used to accurately determine the concentration of nucleic acid in the microplate well. Gen5 software provides the following automated data analysis steps in a defined experimental protocol:

1. Measurement of the OD of the solution at 977 nm – 900 nm and divide by the known OD of water at 1 cm:

   $A_{977} - A_{900}$ sample / 0.18 OD = sample pathlength (cm)

2. Measurement of the sample at 260 nm, subtraction of blank, and division by the pathlength:

A_{260} sample – A_{260} blank / sample pathlength = OD corrected to 1 cm

3. Calculation of the of DNA concentration in the sample wells: multiplication of the corrected OD value by the appropriate extinction coefficient:

OD corrected to 1 cm * 50 = concentration of DNA in the well (µg/mL)

Along with accurate nucleic acid quantification, it is important to determine the purity of the sample prior to use in downstream applications. Proteins and other contaminants like phenol and salts associated with the extraction process may interfere with accurate absorbance readings skewing the results. Purity of nucleic acids is estimated by finding the ratio of the nucleic acid peak absorbance at 260 nm to measurements at 280 nm and 230 nm. To correct for background, a measurement at ~320 nm can be taken; since protein and nucleic acid do not absorb at this wavelength. The background subtraction and ratio calculations can be automated with Gen5 software.

While UV absorbance methods for quantification of nucleic acids in solution can provide information about their overall abundance and purity, it is impossible to discern the relative abundance of DNA and RNA present in the sample. Since detecting and accurately quantifying DNA is important for a range of downstream applications, more precise and discriminatory methods are often employed. PicoGreen is an extremely sensitive, asymmetrical cyanine dye that can be used for dsDNA quantification. Unbound dye exhibits no intrinsic fluorescence, but upon binding to dsDNA the dye fluoresces strongly with a maximal emission of ~530 nm. Up to 10,000-fold more sensitive than UV absorbance methods, it is highly selective for dsDNA over ssDNA and RNA (Figure 4).
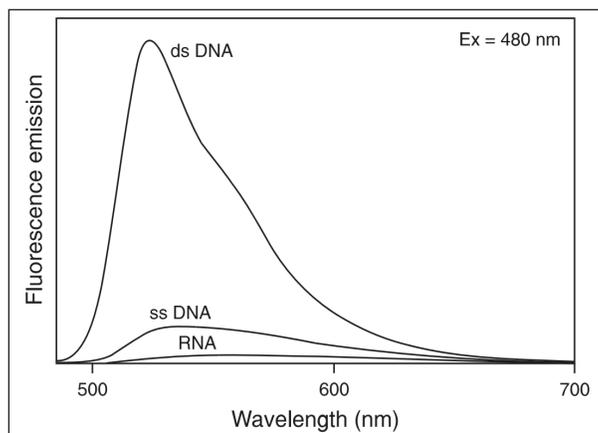

Figure 4. Fluorescent enhancement of PicoGreen dye upon binding to dsDNA

The use of a dsDNA standard curve allows accurate quantification of unknown samples by measurement of the fluorescent emission from samples and interpolation from a standard curve. This approach provides a precise estimate of the dsDNA in the sample. Multiplexing absorbance characterization methods with fluorescent determinants can provide a measure of the quality of the sample as well as the ability to accurately quantify dsDNA using a high throughput approach in a 384-well microplate format.

Materials and Methods

A PicoGreen dsDNA Kit, catalog number P-7589, was a gift from Life Technologies (Grand Island, NY). UV-Star®, µClear®, black, 384-well microplates, catalog number 788876, were a gift from Greiner Bio-One North America, Inc. (Monroe, NC). UV transparent 96-well plates were a gift from Corning, Inc. (Tewksbury, MA). Stock solutions of herring sperm dsDNA (Sigma Aldrich, St. Louis, MO) were prepared in TE buffer and dispensed into cuvettes and/or microplates for quantification as required. A Synergy™ Neo2 Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, VT) or a PharmaSpec Spectrophotometer (Shimadzu America, Inc., Columbia, MD) were used for data acquisition. Gen5 Data Analysis Software was used for Synergy Neo2 instrument control, data collection, and analysis. Data was also exported to Excel (Microsoft, Inc., Redmond, CA) for additional analysis. UVProbe software was used for PharmaSpec instrument control and data acquisition. Data was exported from UVProbe in ASCII format and imported into Microsoft Excel for analysis.

Results

Automated quantification of nucleic acids in microplates has been performed for decades. Yet the challenge presented here was to develop a higher throughput miniaturized assay of dsDNA using a low-volume microplate. The UV-Star®, µClear®, 384-well microplate from Greiner Bio-One was selected. This plate is clear-bottom, black-walled and free of detectable DNase, RNase, and human DNA. The high density and shallow well feature (20 µL maximum volume) offers the ability to significantly reduce analysis volumes while maintaining sufficient path length for accurate and precise measurements of dsDNA.

To determine the minimal volume required for precise optical density measurements in the UV-Star®, µClear®, 384-well microplate, a 1:3 serial dilution of 10 mg/mL dsDNA was performed and pipetted at either 2 or 4 µL in replicate wells. Table 3 demonstrates Gen5 calculated dsDNA concentrations from OD measurements in addition to precision data.

[Approx.] (µg/mL)	2µL/well			4µL/well		
	[Mean] (µg/mL)	Std Dev	CV (%)	[Mean] (µg/mL)	Std Dev	CV (%)
10000.0	OD > 4	N/A	N/A	OD > 4	N/A	N/A
3333.3	3618.5	73.5	2.0	OD > 4	N/A	N/A
1111.1	1156.9	61.5	5.3	1065.0	16.9	1.6
370.4	393.0	20.7	5.3	362.0	1.3	0.4
123.5	148.5	8.8	5.9	126.0	2.6	2.1
41.2	62.3	3.0	4.7	55.0	1.0	1.9
13.7	20.5	1.5	7.3	18.2	0.3	1.5
4.6	6.8	0.8	11.0	6.0	0.1	1.5
1.5	1.8	0.5	29.2	1.8	0.2	8.7
0.5	0.8	0.6	77.2	0.4	0.2	49.0

Table 3. Determination of dsDNA concentrations and precision at 2 and 4 µL total volume using Synergy™ Neo2 and Greiner Bio-One UV-Star®, µClear®, 384-well microplate.

The path length using 2 µL total volume was determined to be 0.6 mm; for 4 µL it was 1.2 mm. It is evident from the precision data in Table 3 that significantly improved precision can be obtained by the use of 4 µL due to the increased path length. This improved precision is comparable to larger sample volume determinations in 96-well microplates to [dsDNA] of about 1.5 µg/mL.

Import Samples ID's to Gen5 Data Analysis Software

Blank empty plate

Pipette 4 µL of dsDNA standard and/or sample into 384-well plate

Read plate at 230 nm, 260 nm, 280 nm, and 320 nm

Add 20 µL of PicoGreen reagent to all wells

Read plate with 488 nm excitation and 530 nm emission
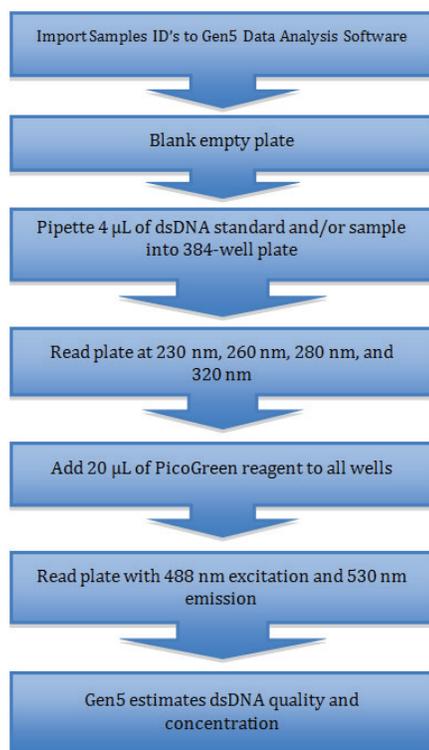
Gen5 estimates dsDNA quality and concentration

Figure 5. Workflow diagram for high throughput analysis of nucleic acid samples.

Following determination of the minimum sample volume required for analysis, a series of experiments were performed to validate the method. A dilution series of dsDNA was created and quantified via cuvette and the UV-Star®, µClear®, 384-well microplate for direct comparison.

Quality assessment of the dsDNA by cuvette using the Shimadzu Pharmaspec revealed that the A_{260}/A_{280} and A_{260}/A_{230} ratios were consistent over much of the dilution series and met the commonly accepted values for pure nucleic acid (Table 4). At concentrations below ~50 µg/mL the A_{260}/A_{280} ratios decline below the commonly associated value indicative of a pure sample (~1.8); a common observation for dilute nucleic acid samples.

Sample ID	Microwell		Cuvette		% Acc
	A260/A280	[dsDNA] (ng/µL)	A260/A280	[dsDNA] (ng/µL)	
1	2.0	953.9	1.9	997.0	-4.3
2	2.0	460.4	1.8	513.0	-10.3
3	2.0	228.9	1.7	261.0	-12.3
4	1.9	119.3	1.9	110.4	8.1
5	1.8	62.4	1.9	60.9	2.5
6	1.7	30.3	1.7	32.2	-5.9

Table 4. Typical concentrations for biobanking applications.

The same dilution series was quantified in 4 µL volumes in a Greiner UV-Star®, µClear®, 384-well microplate using the Synergy™ Neo2. Dilutions of highly concentrated nucleic acid were prepared as necessary for comparison to microvolume determinants. Blanking and pathlength correction of the data generated on the Synergy Neo2 resulted in ratios that are consistent with pure nucleic acid (Table 4). A plot of the A_{260}/A_{230} and A_{260}/A_{280} ratios (Figure 6) indicated acceptable values indicative of highly purified dsDNA within the typical concentration range of sample prepared using nucleic acid extraction methods, ~50-1,000 µg/mL.
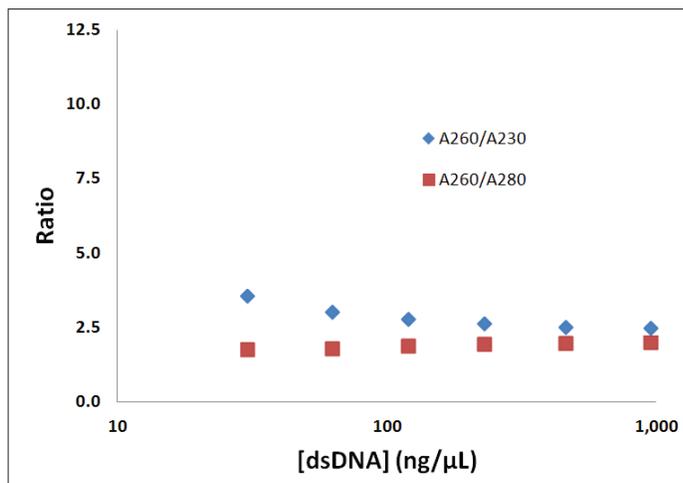

Figure 6. Representative A_{260}/A_{230} and A_{260}/A_{280} ratios derived from data collected on a Synergy Neo2 using 4 µl samples in a Greiner UV-Star®, µClear®, 384-well microplate.

The accuracy of the A_{260} concentration determinants from 4 µL dsDNA samples were compared to cuvette based determinants. Focusing on the working range of the typical dsDNA preparation samples seen in Table 4, the percent accuracy of the nucleic acid determinants range from -12.3% to 2.6%.

sample #	PicoGreen			Microwell	Cuvette
	Mean	Std Dev	CV (%)	[dsDNA] (ng/µL)	[dsDNA] (ng/µL)
1	72804	1264	1.7	**886.7**	**997.0**
2	61310	1058	1.7	**445.1**	**513.0**
3	46600	398	0.9	**221.9**	**261.0**
4	30247	933	3.1	**113.8**	**110.4**
5	20658	569	2.8	**61.1**	**60.9**
6	13721	854	6.2	**29.5**	**32.2**

Table 5. PicoGreen fluorescence from 4uL dsDNA samples using Synergy Neo Multi-Mode Microplate reader following absorbance measurements and reagent addition.

Accurate estimation of dsDNA concentration by fluorescent intensity in the proposed workflow is based on the selective binding of PicoGreen dye to dsDNA. Following UV/Visible analysis of the dsDNA in the 384-well microplates 20 µL of PicoGreen working reagent was added to all wells. Subsequent analysis of the plate revealed that there is a dynamic range of >~60,000 RFU in the typical working range of sample concentrations. Furthermore, coefficient of variance (CV's) are low, ranging from ~0.9% to 6% (Table 5) indicative of good correlation between replicate measurements. A plot of the raw RFU data indicates excellent linearity in the typical working range (Figure 7).
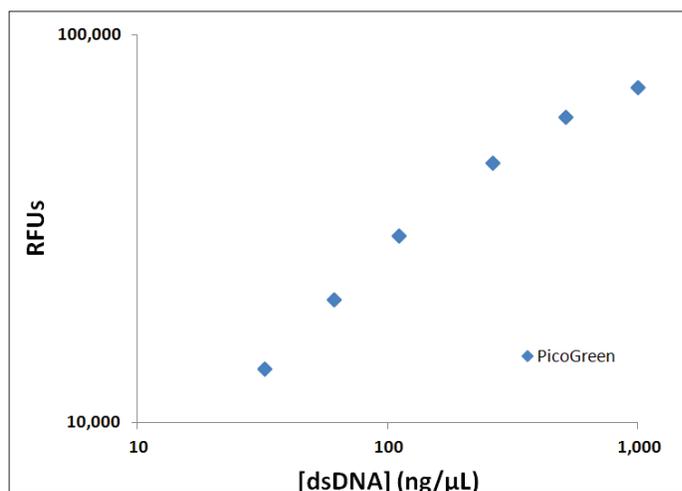

Figure 7. Plot of dsDNA concentration versus raw RFU's using BioTek's Synergy Neo2 Multi-Mode Microplate Reader.

Conclusion

The above data indicate that the proposed multiplexed workflow that combines UV/Visible absorbance quality assessment and fluorescence (PicoGreen) quantification of dsDNA in a 384-well plate is a viable solution for higher-throughput quantification and QC of dsDNA preparations. The results indicate that within the typical yield range between 50 and 150 µg/mL of nucleic acid, the quality and quantity of a 4 µL sample can be analyzed and conforms to typical QC requirements. In addition, the workflow as outlined above will realize additional cost savings given the reduced reagent usage as well as significant time savings when large sample numbers are being analyzed such as in a biobanking facility.