NUCLEIC ACID AND PROTEIN QUANTITATION METHODS

Prepared by: Bree Mitchell, Ph.D.

Keywords:

Nucleic Acid, Protein Quantitation, PicoGreen®, Hoechst 33258 Dye, Quant-iT™ dsDNA Assay, Quant-iT™ ssDNA Assay, RiboGreen®, BCA™ Assay, Quant-iT™ Protein Assay, BSA, DNA, RNA, Double-Stranded DNA, Single-Stranded DNA, Clinical Research, Macromolecule Concentration, Protein Purification, Electrophoresis, Biochemistry, Cell Biology, Molecular Biology, Microplate Readers, Multimode Readers, Single Tube Readers, Fluorometer, Photometer, Luminometer, Modulus™, 2020n, Picofluor™.

INTRODUCTION

Quantitation of nucleic acids (DNA/RNA) and proteins are common measurements in basic science and clinical research. Estimation of macromolecule concentration is necessary in protein purification, electrophoresis, biochemistry, cell biology, molecular biology and clinical research applications. Quantitation of nucleic acids or proteins can be separated into two groups: direct quantitation or quantitation through an intermediate signal - generally a fluorophore or colorimetric molecule. Both nucleic acid and protein quantitation can be performed in single tube or microplate assays.

Table 1
Comparison of common DNA, RNA and protein quantitation assays

Method	Detection Range	Wavelength Abs/Em (nm)	Interferences/Precautions
DNA Quantitation			
Absorbance at 260 nm	1-100 µg/ml	260/280	RNA, protein
Hoechst 33258 Dye	10 ng/ml - 20 µg/ml	352/461	Mutagen, wear protective clothing and work under a fume hood
Quant-iT™ PicoGreen® Assay Reagent	0.2-200 ng/ml	480/520	NaCl, $MgCl_2$, possible mutagen - wear protective clothing
Quant-iT™ DNA Assay Kit, Broad Range	2-1000 ng/ml	480/520	NaCl, $MgCl_2$, possible mutagen - wear protective clothing
Quant-iT™ DNA Assay Kit, High Sensitivity	0.2 - 100 ng/ml	480/520	NaCl, $MgCl_2$, possible mutagen - wear protective clothing
Quant-iT™ ssDNA Assay Kit	1 pg/ml - 1 µg/ml	480/520	Salt, detergent, protein, other phenolic compounds, possible mutagen - wear protective clothing
RNA Quantitation			
Absorbance at 260 nm	1-80 µg/ml	260/280	DNA, protein
Quant-iT™ RiboGreen® RNA Assay Kit	1 – 200 ng/ml	485/530	SDS, mutagen wear protective clothing
Quant-iT™ RNA Broad Range Assay Kit	1 ng/µl -1 µg/µl	485/530	SDS, mutagen wear protective clothing
Protein Quantitation			
Absorbance at 280 nm	0.1 - 5 mg/ml	280	Nucleic Acid, detergent, cofactors, phenolic compounds, pigments, reducing agents and aromatic compounds.
Bradford Assay	1 - 1500 µg/ml	600	Detergent
Lowry Assay	1 - 1500 µg/ml	750	Reducing agents, chelating agents, strong acids or bases
BCA™ Protein Assays	1 - 1500 µg/ml	560	Reducing agents, chelating agents, strong acids or bases
Quant-iT™ Protein Assay	25 – 500 ng/µl	485/590	Detergent
NanoOrange®	10ng/ml – 10µg/ml	485/590	Detergent

As Table 1 demonstrates, there are many reagent or detection methods available, each with different detection ranges, assay time and interfering compounds. All of these assays can be performed in both single tube and microplate format. Turner BioSystems provides a variety of instruments capable of detecting DNA, RNA and proteins. Table 2 illustrates which assays are compatible with different instruments in the Turner BioSystems instrument line.

Table 2
DNA, RNA or protein quantitation assay compatibility of Turner BioSystems instruments

Method	Turner BioSystems Instrument					
	Modulus™ II Microplate	Modulus™ Microplate	Modulus™ Single Tube	20/20n	TBS-380	Picofluor™
DNA Quantitation						
Absorbance at 260 nm	X					
Hoechst 33258 Dye	X	X	X	X	X	X
Quant-iT™ PicoGreen® Reagent	X	X	X	X	X	X
Quant-iT™ DNA Assay Kit, Broad Range	X	X	X	X	X	X
Quant-iT™ DNA Assay Kit, High Sensitivity	X	X	X	X	X	X
Quant-iT™ ssDNA Assay Kit	X	X	X	X	X	X
RNA Quantitation						
Absorbance at 260 nm	X					
Quant-iT™ RiboGreen® RNA Assay Kit	X	X	X	X	X	X
Quant-iT™ RNA Broad Range Assay Kit	X	X	X	X	X	X
Protein Quantitation						
Absorbance at 280 nm	X					
Bradford Assay	X	X	X			
Lowry Assay	X	X	X			
BCA™ Protein Assay	X	X	X			
Quant-iT™ Protein Assay	X	X	X	X	X	X

NUCLEIC ACID QUANTITATION

The quantitation of nucleic acids can be accomplished either by directly converting absorbance to concentration, or by fluorescence assays. The gold standard of DNA and RNA quantitation remains UV absorbance measurements at 260 nm. The absorbance assay is very simple to set up and run, and requires no other assay reagents. However, the sensitivity is limited and it requires UV transparent cuvettes or microplates, which can be very expensive. Direct measurements of nucleic acid samples can be converted to concentration using the Beer-Lambert law which relates absorbance to concentration using the pathlength of the measurement and an extinction coefficient (Table 3) [1].

Table 3
Standard Coefficients for Nucleic Acids

Molecule	Standard Coefficient (µg/ml)
Double Stranded DNA	50
RNA	40
Single Stranded DNA	33

For single tube instruments, the standard light pathlength is 1 cm. Absorbance measurements at 1 cm pathlength have been correlated with specific nucleic acid concentrations; for example an OD of 1.0 at 260 nm correlates to 50 µg of double stranded DNA (dsDNA) (Table 3). However, when using a microplate instrument, the light pathlength varies depending on the volume of liquid in the plate. Therefore, a pathlength correction value must be used to calculate concentration. Pathlength values can be calculated using the sample volume and the diameter of the sample plate. Pathlength values for 25 to 250 µl sample volumes, depending on plate type, have been calculated for common microplates [2] (Table 4). Because pathlength values are proportional to the volume of liquid used, a linear regression has been calculated and can be used to determine the pathlength of any volume between 25 and 250 µl where x = volume used and y = pathlength. (Note: recommended volumes for 96 well microplates are between 100 and 250 µl) Once the pathlength correction is determined, DNA concentration in a microplate is calculated by dividing the OD absorbance value by the pathlength corresponding to the volume used and multiplying by the standard coefficient and dilution values.

$$\text{Nucleic Acid concentration} = \frac{\text{OD260}}{\text{Pathlength}} \times \text{Extinction coefficient} \times \text{Sample Dilution}$$

For example, if an OD260 value was measured at 1.2 OD in a 200 µl (undiluted) volume in a corning 96 well plate, the dsDNA concentration would be $\left(\frac{1.2}{0.58}\right) * 50$ or 103 µg/ml. Alternatively, a standard curve can be created using a nucleic acid standard and concentration of unknown samples can be calculated using the standard curve (Figure 1b). One caveat of using absorbance based measurements of nucleic acid samples is that proteins and reagents commonly used in the preparation of nucleic acids also absorb light at 260 nm and can lead to falsely elevated concentration results. Most reagents that can contaminate a sample also absorb light at 280 nm where nucleic

acids absorb little light. This provides a method of gauging DNA or RNA purity using the ratio of measurements at OD260/OD280. Generally an OD260/OD280 ratio ≥1.8 indicates "pure" DNA and an OD ratio of ~2.0 indicates "pure" RNA. A ratio below 1.8 indicates a DNA or RNA sample that is contaminated by protein, phenol, or other aromatic compounds. The OD260/OD280 ratio does not necessarily indicate the absence of other nucleotides or single stranded nucleic acids.

Table 4
Calculated pathlength values for common 96 and 384 well UV compatible microplates

		Pathlength (cm)					
UV compatible plates	**Part Number**	**25 µl**	**50 µl**	**100 µl**	**200 µl**	**250 µl**	**Linear Regression**
BD Falcon 96 well UV plate	353261	n/a	n/a	0.28	0.56	0.70	y=0.0028x-3E-16
BD Falcon 384 well UV plate	353262	0.19	0.39	0.77	n/a	n/a	y=0.0077x-4E-16
Corning 96 well UV plate	3635	n/a	n/a	0.29	0.58	0.73	y=0.0029x+3E-16
Corning 384 well UV plate	3675	0.25	0.50	1.01	n/a	n/a	y=.0101x+4E-16
Corning 96 well half volume UV plate	3679	0.14	0.28	0.56	n/a	n/a	y=0.0056x
Greiner 96 well UV Star (also Thermo Scientific/Nunc)	655801	n/a	n/a	0.28	0.56	0.69	y=0.0028x
Greiner 384 well UV Star (also Thermo Scientific/Nunc)	781801	0.20	0.41	0.82	n/a	n/a	y=0.0082x
Greiner 96 well half volume UV star (also Thermo Scientific/Nunc)	675801	0.14	0.29	0.58	n/a	n/a	y=0.0058x

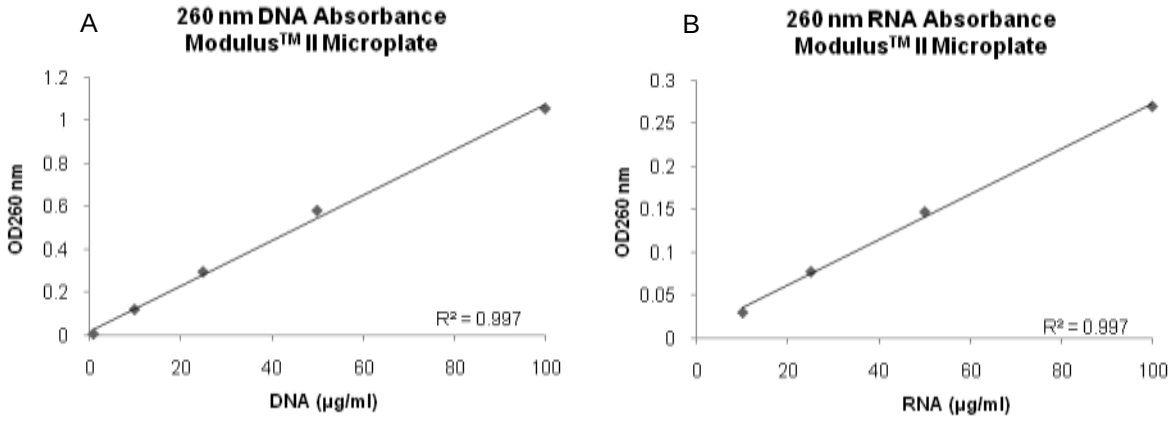

Figure 1. DNA and RNA standard curves determined using the Modulus™ II Microplate UV-Visible Absorbance module A) DNA standard curve of Lambda DNA standard created using 260 nm absorbance measurements. B) RNA standard curve of Ribosomal RNA created using 260 nm absorbance measurements

To combat the weaknesses of the traditional absorbance nucleic acid concentration method, a variety of fluorescent molecules can also be used for nucleic acid quantitation. The most common of those are Hoechst 33258 and the Life Technologies (Invitrogen) Quant-iT™ kits. These assays are based on dyes that intercalate into the nucleic acid chain. Different dyes interact with different nucleic acids under different assay conditions allowing for specificity of DNA, RNA and single stranded DNA depending on which dye is used. In addition to improved specificity, fluorescence based nucleic acid quantitation is generally much more sensitive than absorbance based

detection. For dsDNA, the common methods include Hoechst, and Invitrogen Quant-iT™ PicoGreen®, Broad Range, and High Sensitivity dsDNA kits. These dyes have different excitation/emission profiles (Table 1) which may be more or less convenient depending on the individual application. Hoechst can be less expensive than the Invitrogen kits but is a known mutagen so precautions must be taken when using the dye. The Invitrogen Kits have lower detection limits but Hoechst has a wider detection range; again making the choice of dye specific to the application. In addition to kits for dsDNA, Invitrogen has developed reagent kits specifically for ssDNA and RNA. Fluorescence based nucleic acid quantitation can be performed using any of the Turner BioSystems single tube instruments or the Modulus™ and Modulus™ II Microplate instruments. The assay for fluorescent dye detection is similar regardless of which dye is used. In brief, the assay protocols consist of three steps 1) diluting standards to create a standard curve 2) mixing the dye with the standard and unknown samples, generally at a 1:1 dye:sample ratio 3) reading the sample in a fluorometer using the appropriate filters (see Table 1 for fluorophore wavelengths). Figure 2 shows examples of a PicoGreen® standard curve from the Modulus™ Microplate instrument and a Hoechst 33258 standard curve from the Modulus™ Single Tube instrument. The detection limits of each assay type are given in Table 1. The actual detection limit may vary depending on the instrument used. Refer to the specific instrument specifications at www.turnerbiosystems.com for more detail.

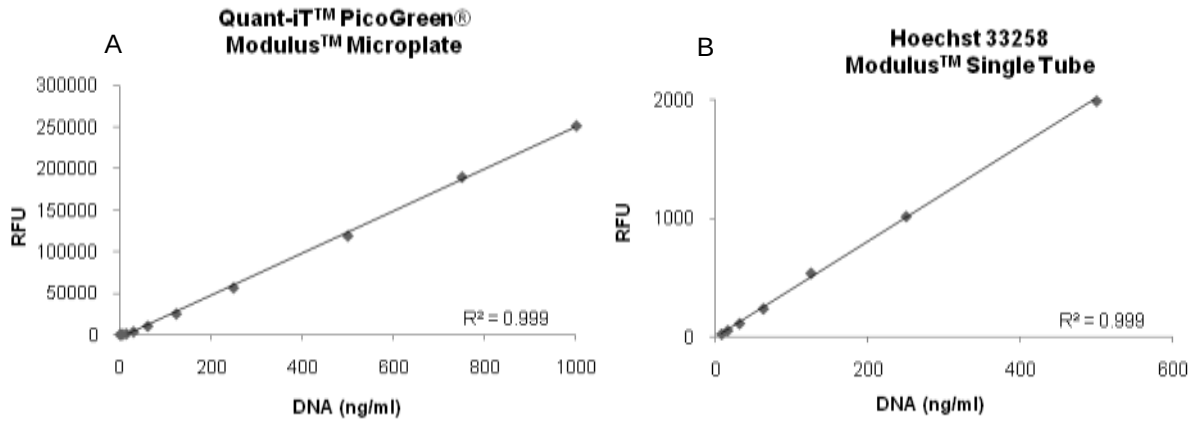

Figure 2: A) PicoGreen® DNA quantitation standard curve using the Modulus™ Microplate instrument and using Lambda DNA Standard B) Hoechst 33258 DNA quantitation standard curve generated using the Modulus™ Single Tube instrument and using Lambda DNA standard

PROTEIN QUANTITATION

Protein, like nucleic acids, can be directly quantitated using UV absorbance (280 nm), by colorimetric, or by fluorescent assay. Because each protein has a different amino acid structure, a direct association between 280 nm absorbance and protein concentration is generally an approximation. A very rough protein concentration can be obtained by making the assumption that the protein sample has an extinction coefficient of 1, so 1 OD = 1 mg/ml protein. For better accuracy, an extinction coefficient should be used. Some standard protein extinction coefficients have been published, see Table 5 for a few selected extinction coefficients or the Practical Handbook of Biochemistry and Molecular Biology for a more extensive table [3]. Finally, if the protein sequence of the protein

to be measured is known, the theoretical extinction coefficient can be calculated using the question ε = 5690(#Tryptophans) + 1280(#Tyrosines) + 60(#Cysteines) [4] or online tools such as ExPASy Protparam. In a microplate instrument, the OD must also be divided by the pathlength correction to account for the shorter pathlength of the sample in the microplate (Table 4). The equation for calculating protein concentration using OD280 measurements is the following:

$$\text{Protein Concentration} = \frac{\text{OD}_{280}}{\text{Extinction coefficient} \times \text{Pathlength}} \times \text{Sample Dilution}$$

Table 5
Calculated Extinction Coefficients for proteins measured in a 1 cm cuvette

Molecule	Calculated Extinction Coefficient (mg/ml) -1 cm
BSA	.66
IgG	1.35
IgM	1.2

Protein concentration can also be calculated by creating a standard curve of known protein concentrations and using the linear regression line to calculate unknown concentrations. Figure 3 shows a standard curve of BSA measured at 280 nm on the Modulus™ II Microplate instrument

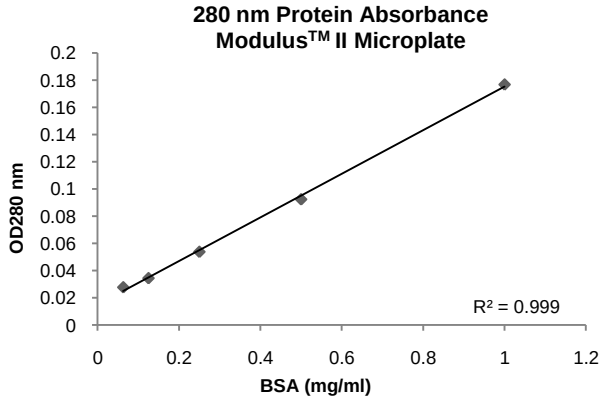

Figure 3. 280 nm Absorbance measurements of BSA using the Modulus™ II Microplate instrument.

More commonly, protein quantitation is determined by one of several colorimetric quantitation methods. The most frequently cited total protein quantitation assay is the method described by Lowry et al [5]. This method is based on a biuret test for detecting the presence of peptide bonds. In a positive test, a Cu^{+2} ion is reduced to Cu^{+1} which forms a complex with the nitrogen and carbon of the peptide bonds resulting in a color change of the solution from green to violet. In the modified Lowry assay commonly used, Folin Ciocalteau regent is added to improve sensitivity. The resulting color development is then measured at 750 nm. The Lowry assay is the most time consuming to perform but also has the largest total protein concentration range. Alternatively, the Bradford

assay uses the color change that occurs when Coomassie Blue G-250 dye binds to peptides in an acidic medium [6]. The color change of this assay is measured at 595 or 600 nm. The Bradford assay takes only 10 minutes but is sensitive to detergent which is often found in protein samples. Finally, the bicinchoninic acid (BCA™) method was developed as an improvement to the Lowry assay [7]. The BCA™ method is measured at 560 nm and has improved reagent stability and a broader tolerance to interfering compounds such as detergents and denaturing agents but does require a 30 minute incubation at 37°C. It is important to note that all three assays are not purely linear so the standard curve should be plotted using a 4 parameter or best-fit curve to provide more accurate results. Any of these assays are compatible with the Turner BioSystems Modulus™ line of instruments including Modulus™ Single Tube, Modulus™ Microplate, and Modulus™ II Microplate. Figure 4 shows an example of standard curves measured using the Lowry (Modulus™ II Microplate), BCA™ (Modulus™ Microplate) and Bradford assays (Modulus™ Single Tube).

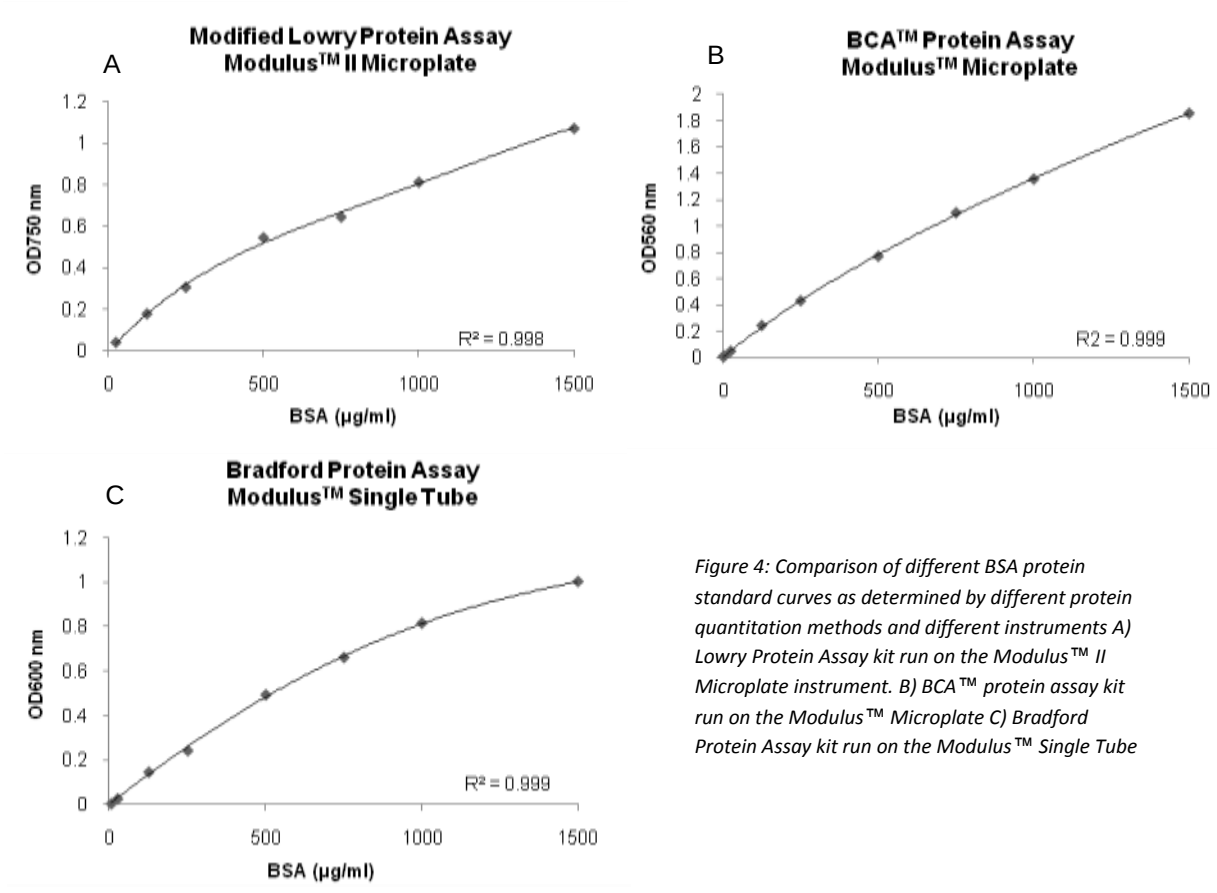

Figure 4: Comparison of different BSA protein standard curves as determined by different protein quantitation methods and different instruments A) Lowry Protein Assay kit run on the Modulus™ II Microplate instrument. B) BCA™ protein assay kit run on the Modulus™ Microplate C) Bradford Protein Assay kit run on the Modulus™ Single Tube

Like nucleic acids, proteins can also be quantitated using fluorescent molecules. Initial protein detection dyes were amino acids specific, water insoluble, or required harsh chemicals to induce binding. However, more recently introduced fluorophores, including Invitrogen's NanoOrange® and Quant-iT™ Protein kit, address many of these issues. NanoOrange® is water soluble and is not amino acid specific resulting in greater sensitivity and less protein-to-protein variability in the assay. However, NanoOrange® requires that the protein be heat denatured, making the choice of microplate important; many polystyrene microplates warp when subjected to heat. Invitrogen has

also introduced the Quant-iT™ Protein assay kit which is less sensitive than NanoOrange® but does not require incubation at high heat. Both the Quant-iT™ Protein kit and the NanoOrange® fluorophore can be used by any Turner BioSystems instrument with fluorescence detection including the Picofluor™, TBS-380, 20/20n, Modulus™ Single Tube, Modulus™ Microplate and Modulus™ II Microplate. Figure 5 shows a standard curve from the Invitrogen Quant-iT™ Protein assay calculated using five of the compatible Turner BioSystems instruments.

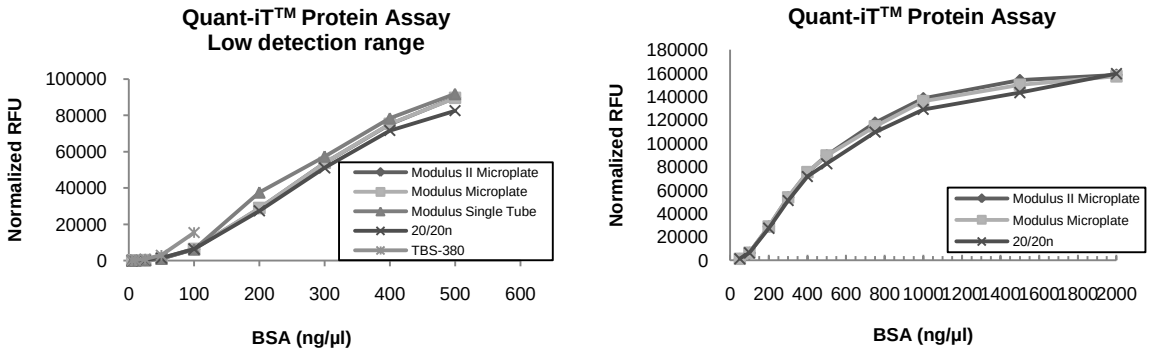

Figure 5. Comparison of BSA standard curve generated using the Invitrogen Quant-iT™ Protein kit and five different Turner BioSystems instruments.

CONCLUSIONS

There are many options for quantitating nucleic acids and proteins. Each option has pros and cons, many of which have been described here. Deciding on the right assay depends upon which instrument will be used for detection, the concentration range to be measured, the interfering compounds in the samples and the preferences of the individual. Turner BioSystems offers a wide range of instrument options for nucleic acid and protein quantitation that meet the needs for all types of quantitation assays. For more information about these assays and Turner BioSystems instruments, please visit www.turnerbiosystems.com

REFERENCES

1. Maniatis T, Fritsch EF, Sambrook J. Molecular cloning a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor NY (1982).
2. Mitchell, BL. Calculating nucleic acid or protein concentration using the Modulus II Microplate. Turner BioSystems Technical Note (2009).
3. Fasman G. Ed. Practical handbook of biochemistry and molecular biology. CRC press, Boston (1992)
4. Gill, SC and von Hippel, PH. Calculation of protein extinction coefficients from amino acid sequence data. (1989) Anal. Biochem 182:319-26.
5. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J. Biol Chem (1951). 193:265-275
6. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem (1976). 72:248-254.

7. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. Anal. Biochem. (1985) 150:76-85

TRADEMARKS

TURNER BIOSYSTEMS

For more information, visit www.turnerbiosystems.com or contact us at:

Turner BioSystems, Inc.
645 N Mary Avenue
Sunnyvale, CA 94085
Phone: 408.636.2400
Toll Free: 888.636.2401
Fax: 408.737.7919

Enabling People. Enabling Science.