



Validation of Multiple Protein Quantification Kits

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PURPOSE/KITS TESTED

- Validate quantification kit:
 - Reproducible results
 - High throughput
 - Broad dynamic range
 - Small sample volume
- Kits tested:
 - A280
 - Pierce™ BCA Protein Assay Kit
 - Bio-Rad Protein Assay (Bradford)
 - Pierce™ Modified Lowry Protein Reagent
 - Pierce™ 660nm Protein Assay Kit
 - NanoOrange Protein Quantitation Kit
 - Quant-iT™ Protein Assay Kit

DETERMINING CONFIDENCE

- The confidence interval of a sample set allows one to determine the percentage probability that a given value could be taken from that sample set. In this case it was used to help depict the detection limit achieved with each kit.
- Confidence Range = mean +- Confidence Interval
- Confidence Interval Excel Formula =CONFIDENCE(alpha,standard_dev,size)
- The CONFIDENCE function uses the following arguments:
 - Alpha (required argument) – This is the significance level used to compute the confidence level. The significance level is equal to 1– confidence level. So, a significance level of 0.05 is equal to a 95% confidence level.
 - Standard_dev (required argument) – This is the standard deviation for the data range.
 - Size (required argument) – This is the sample size
- Method:
 - 1 .Determine the average concentration of the replicates of the highest concentration tested.
 2. Determine if that average was higher than the upper limit of the 95% confidence range of the next highest concentration.
 3. If so, it can be concluded that there is a 95% chance that the highest concentration average did not come from the same sample set as the next highest concentration.
 4. Repeat the process with incrementally decreasing concentrations until the condition in step 2 is not met. This range is referred to throughout as the 95% confidence range.

DETERMINING CONFIDENCE (CONT.)

Example

G	H	I	J	K	L	M	N	O	P	Q	R	S	T
							avg	stdev	stdev %	95% confid upper limit	lower limit	Y/N	
2000	2.192	2.236	2.21	2.224	2.15	2.187	2.199833	0.030675	1.394432	0.026887	=N2+Q2	2.172946	YES
1500	1.632	1.703	1.682	1.697	1.695	1.661	1.678333	0.027186	1.619808	0.023829	1.702162	1.654504	YES
2000	2.192	2.236	2.21	2.224	2.15	2.187	2.199833	0.030675	1.394432	O2,5)	2.226721	2.172946	YES

G	IF(logical_test, [value_if_true], [value_if_false])					M	N	O	P	Q	R	S	T
							avg	stdev	stdev %	95% confid upper limit	lower limit	Y/N	
2000	2.192	2.236	2.21	2.224	2.15	2.187	2.199833	0.030675	1.394432	0.026887	2.226721	2.172946	"NO")
1500	1.632	1.703	1.682	1.697	1.695	1.661	1.678333	0.027186	1.619808	0.023829	1.702162	1.654504	YES
1000	1.164	1.171	1.133	1.063	1.14	1.139	1.135	0.038382	3.381699	0.033643	1.168643	1.101357	YES

DETERMINING % STANDARD DEVIATION

- Method:
 - Used STDEV() function for replicates at a given concentration to determine stdev.

fx =STDEV(H2,I2,J2,K2,L2,M2)

G	STDEV(number1, [number2], [number3], [number4], [number5], [number6], [number7], ...)						P	Q	R	S	T	
		avg	stdev	stdev %	95% confid	upper limit	lower limit	Y/N				
2000	2.192	2.236	2.21	2.224	2.15	2.187	2.199833	K2,L2,M2)	1.394432	0.026887	2.226721	2.172946 YES
1500	1.632	1.703	1.682	1.697	1.695	1.661	1.678333	0.027186	1.619808	0.023829	1.702162	1.654504 YES

- Performed (stdev/avg) * 100 to determine % stdev.

fx =(O2/N2)*100

G	H	I	J	K	L	M	N	O	P	Q	R	S	T
						avg	stdev	stdev %	95% confid	upper limit	lower limit	Y/N	
2000	2.192	2.236	2.21	2.224	2.15	2.187	2.199833	0.030675	100	0.026887	2.226721	2.172946 YES	
1500	1.632	1.703	1.682	1.697	1.695	1.661	1.678333	0.027186	1.619808	0.023829	1.702162	1.654504 YES	

- Averaged the results within the 95% confidence range

fx =AVERAGE(P2:P10)

G	AVERAGE(number1, [number2], ...)						L	M	N	O	P	Q	R	S	T	U
		avg	stdev	stdev %	95% confid	upper limit	lower limit	Y/N	avg std							
2000	2.192	2.236	2.21	2.224	2.15	2.187	2.199833	0.030675	1.394432	0.026887	2.226721	2.172946 YES	(P2:P10)			
1500	1.632	1.703	1.682	1.697	1.695	1.661	1.678333	0.027186	1.619808	0.023829	1.702162	1.654504 YES				
1000	1.164	1.171	1.133	1.063	1.14	1.139	1.135	0.038382	3.381699	0.033643	1.168643	1.101357 YES				
750	0.798	0.824	0.871	0.88	0.816	0.879	0.844667	0.036187	4.284122	0.031718	0.876385	0.812948 YES				
500	0.594	0.602	0.542	0.551	0.542	0.623	0.575667	0.035058	6.089991	0.030729	0.606396	0.544937 YES				
250	0.345	0.35	0.343	0.343	0.307	0.31	0.333	0.019173	5.757627	0.016805	0.349805	0.316195 YES				
125	0.214	0.197	0.197	0.199	0.2	0.201	0.201333	0.006408	3.182944	0.005617	0.20695	0.195716 YES				
25	0.124	0.124	0.126	0.121	0.128	0.126	0.124833	0.002401	1.923676	0.002105	0.126938	0.122728 YES				
10	0.116	0.118	0.119	0.118	0.118	0.117	0.117667	0.001033	0.87773	0.000905	0.118572	0.116761 YES				

A280

Specs/Experimental results

- Listed Dynamic Range: 50-2000 ug/ml
- Incubation time: 0 min.
- Incubation Temp: RT
- Absorption wavelength: 280 nm
- Reagent cost: \$0.00 per assay

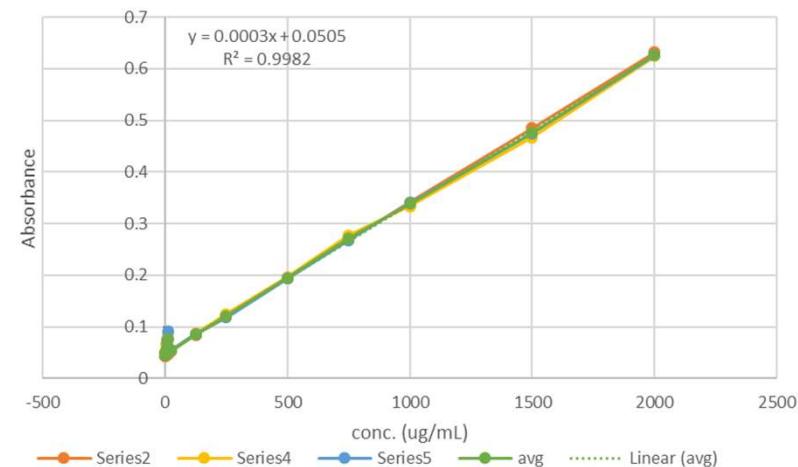
Results:

confirmed dynamic range (95% confidence)	Avg % std error over dynamic range	protein volume (ul)	reagent vol (ul)	total sample volume (ul)	plate type
25-2000 ug/ml	Uncorrected: 1.24% Corrected: 0.62%	50 ul	0	50 ul	96-well half-area microplate
25-2000 ug/ml	Uncorrected: 4.82% Corrected: 2.45%	100 ul	0	100 ul	96-well half-area microplate
25-2000 ug/ml	Uncorrected: 1.38% Corrected: 2.04%	100 ul	0	100 ul	96-well full-area microplate
25-2000 ug/ml	Uncorrected: 1.63% Corrected: 1.38%	200 ul	0	200 ul	96-well full-area microplate

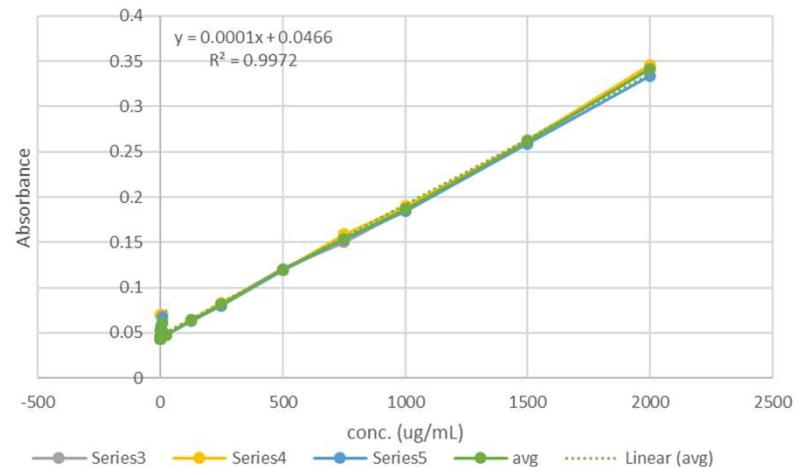
A280

Experimental Results: Uncorrected graphs

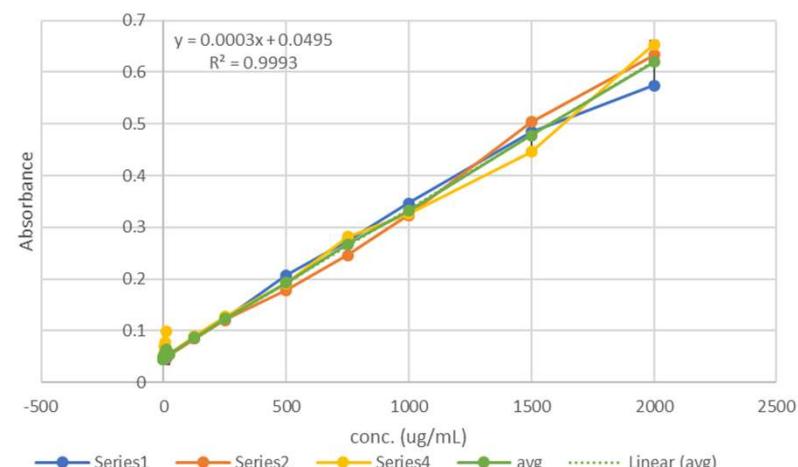
A280 full-area 200 mL (uncorrected)



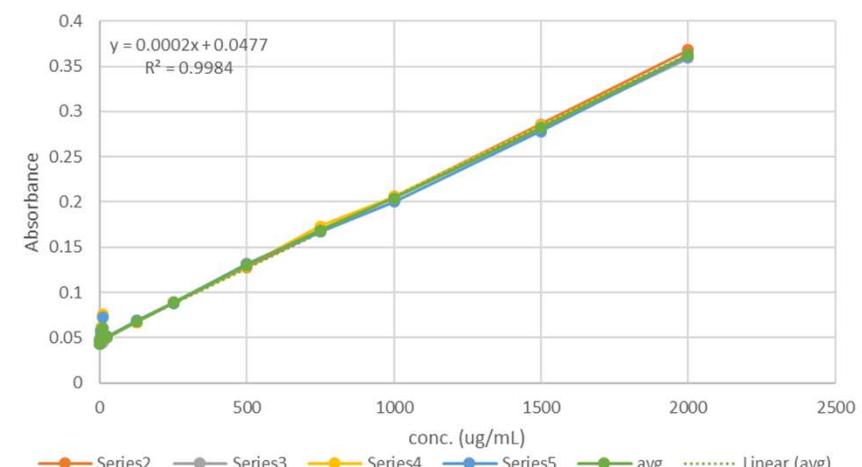
A280 full-area 100 mL (uncorrected)



A280 half-area 100 mL (uncorrected)

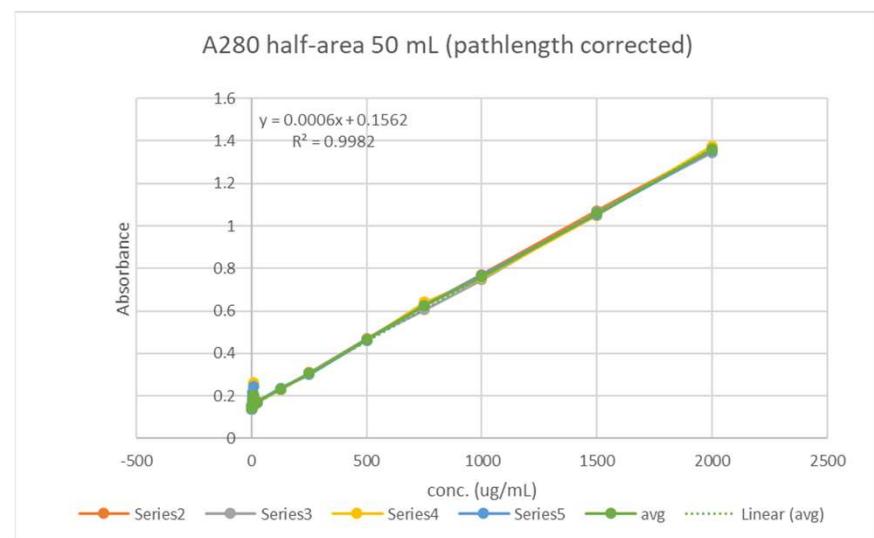
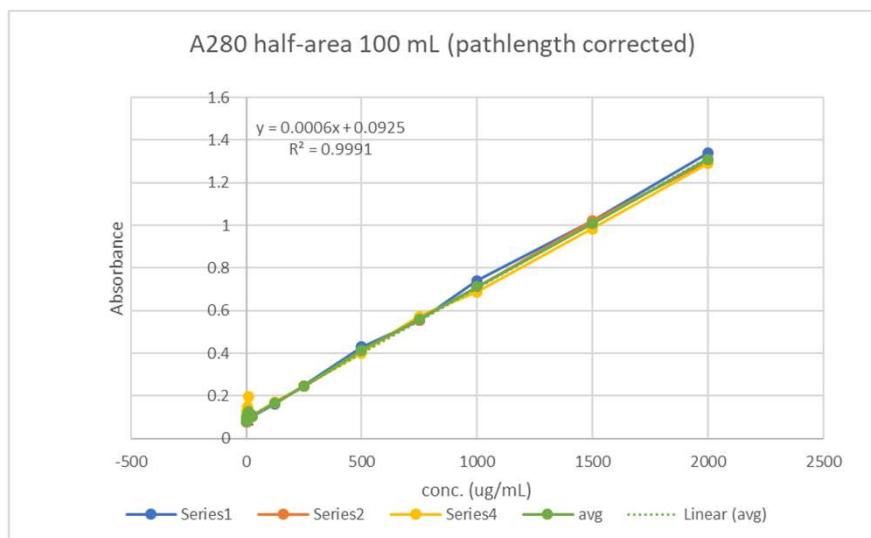
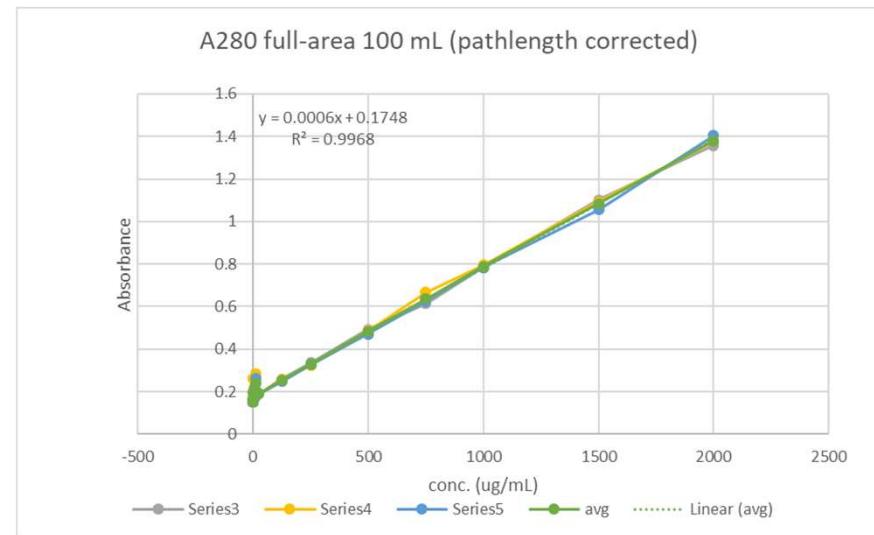
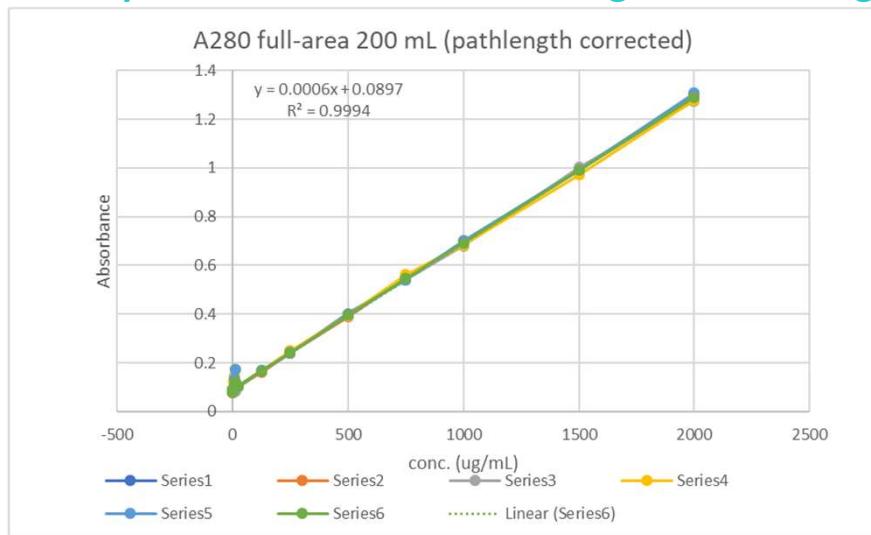


A280 half-area 50 mL (uncorrected)



A280

Experimental Results: Pathlength corrected graphs



A280

Pros & Cons

- Pros:
 - No incubation time
 - No reagents necessary
 - High degree of linearity ($R^2 = 0.9994$)
 - Low % stdev
- Cons:
 - Lacks sensitivity at lower ranges (<25 ug/mL)
 - High sample volume (50-200 uL)
- Possible sources of error:
 - Pipetting technique

PIERCE™ BCA PROTEIN ASSAY KIT – HOW IT WORKS

Copper-based protein assays (biuret reaction)

- Peptides containing three or more amino acid residues form a colored chelate complex with cupric ions (Cu^{2+}) in an alkaline environment containing sodium potassium tartrate. Produces a light blue to violet complex that absorbs light at 540 nm.

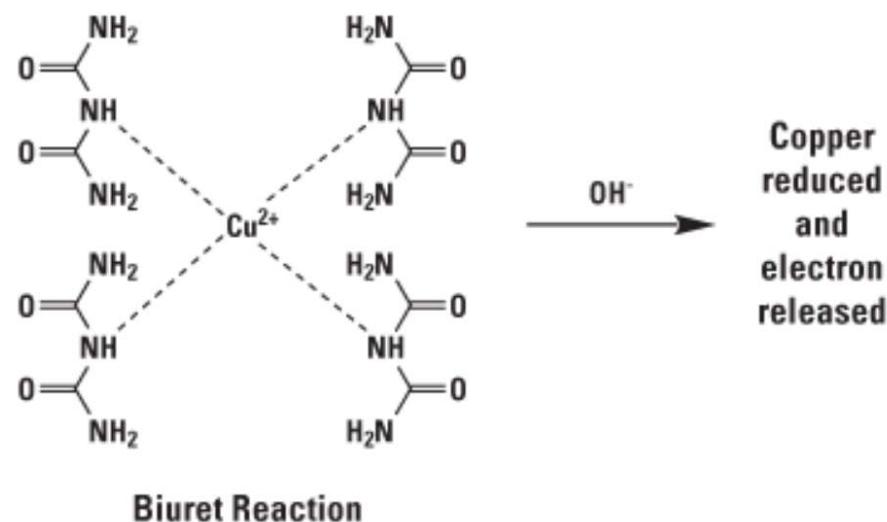
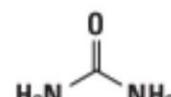


Diagram of the biuret reaction. By reducing the copper ion from cupric to cuprous form, the reaction produces a faint blue-violet color.

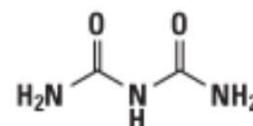
PIERCE™ BCA PROTEIN ASSAY KIT – HOW IT WORKS

Copper-based protein assays (biuret reaction)

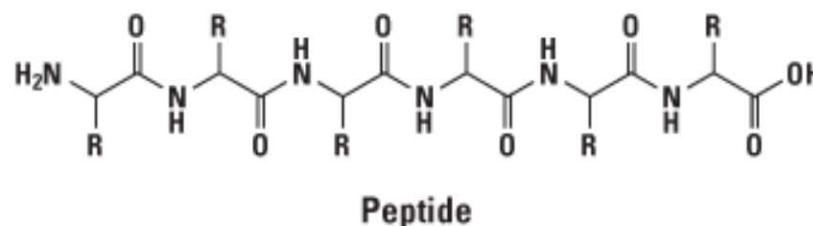
- Known as the biuret reaction because it is chemically similar to a complex that forms with the organic compound biuret and the cupric ion. Biuret, a product of excess urea and heat, reacts with copper to form a light blue tetradentate complex.



Urea



Biuret



Structures of urea, biuret and peptide. Because polypeptides have a structure similar to biuret, they are able to complex with copper by the biuret reaction.

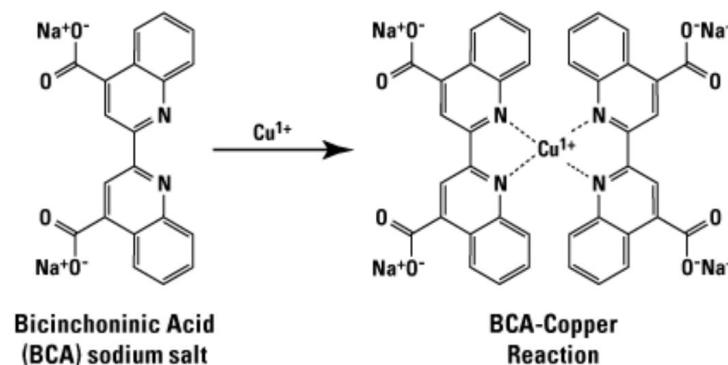
PIERCE™ BCA PROTEIN ASSAY KIT – HOW IT WORKS

Bicinchoninic Acid (BCA) Assays

- Two steps:

1. Biuret reaction. Faint blue color as Cu²⁺ reduced to Cu¹⁺
2. Colorimetric detection of Cu¹⁺ by BCA. Intense purple color as Cu¹⁺ chelates with BCA. BCA/Cu¹⁺ complex absorbs at 562 nm.

BCA Reagent 100 times more sensitive than biuret reagent. Relatively low protein-to-protein variability because peptide backbone also contributes to color formation. Good compatibility with detergents.



The reaction of BCA with cuprous ion. Two molecules of BCA bind to each molecule of copper that had been reduced by a peptide-mediated biuret reaction.

PIERCE™ BCA PROTEIN ASSAY KIT

Setup

Kit Contents:

BCA Reagent A, 500mL, containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide

BCA Reagent B, 25mL, containing 4% cupric sulfate

Storage: RT

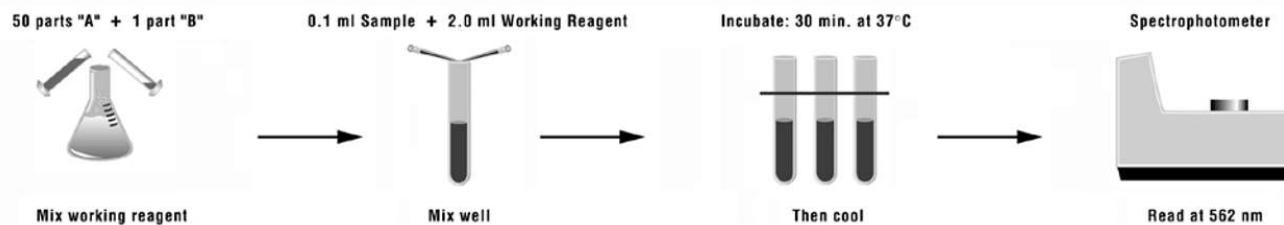
Reagent prep:

Prepare WR by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B).

Microplate Procedure:

1. Pipette 25 μ L of each standard or unknown sample replicate into a microplate well
2. Add 200 μ L of the WR to each well and mix plate thoroughly on a plate shaker for 30 seconds.
3. Cover plate and incubate at 37°C for 30 minutes.
4. Cool plate to RT. Measure the absorbance at or near 562nm on a plate reader.

Procedure Summary (Test-tube Procedure, Standard Protocol)



PIERCE™ BCA PROTEIN ASSAY KIT

Specs/Experimental results

- Listed Dynamic Range: 20-2000 ug/ml
- Incubation time: 30 min.
- Incubation Temp: 37° C
- Absorption wavelength: 562 nm
- Reagent cost: \$0.05 per assay

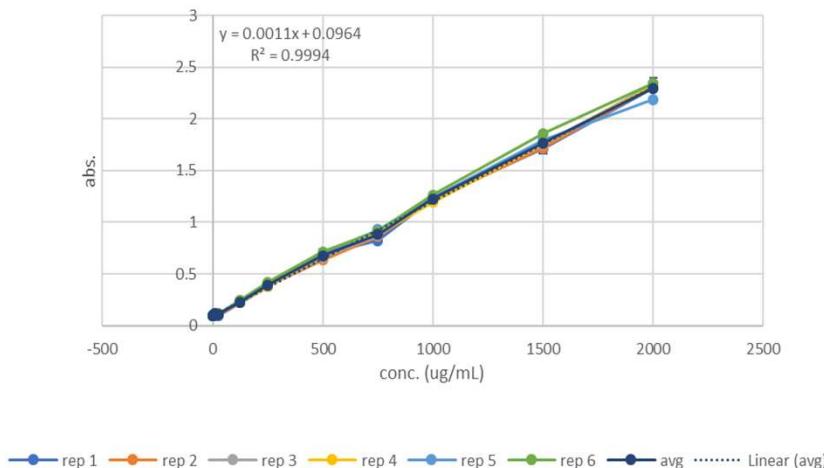
Results:

confirmed dynamic range (95% confidence)	Avg % std error over dynamic range	protein volume (ul)	reagent vol (ul)	total sample volume (ul)	plate type
25-2000 ug/ml	Uncorrected: 4.19% Corrected: 5.20%	6.25	50	56.25	96-well half-area microplate
1-2000 ug/ml	Uncorrected: 3.36% Corrected: 2.86%	12.5	100	112.5	96-well half-area microplate
25-2000 ug/ml	Uncorrected: 4.73% Corrected: 4.05%	12.5	100	112.5	96-well full-area microplate
25-2000 ug/ml	Uncorrected: 3.39% Corrected: 3.25%	25	200	225	96-well full-area microplate

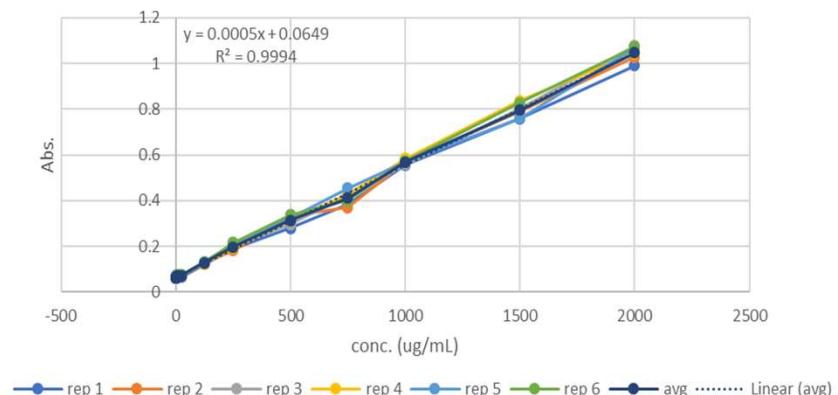
PIERCE™ BCA PROTEIN ASSAY KIT

Experimental Results: Uncorrected graphs

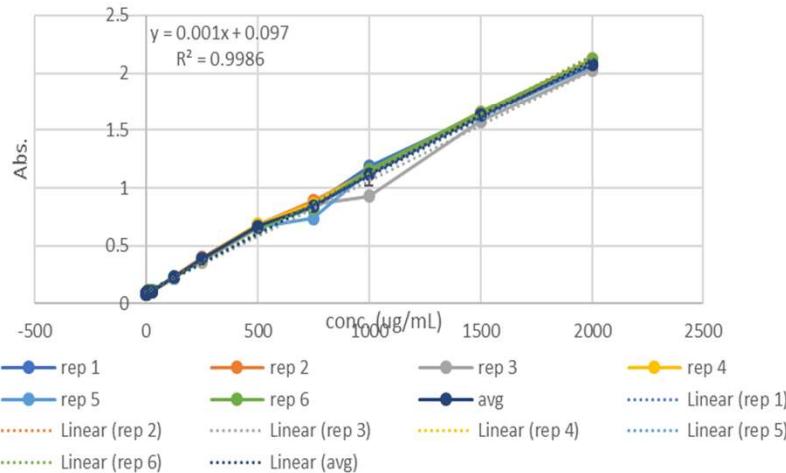
BCA Kit Absorbance at 562 nm, full-area 225 mL (uncorrected)



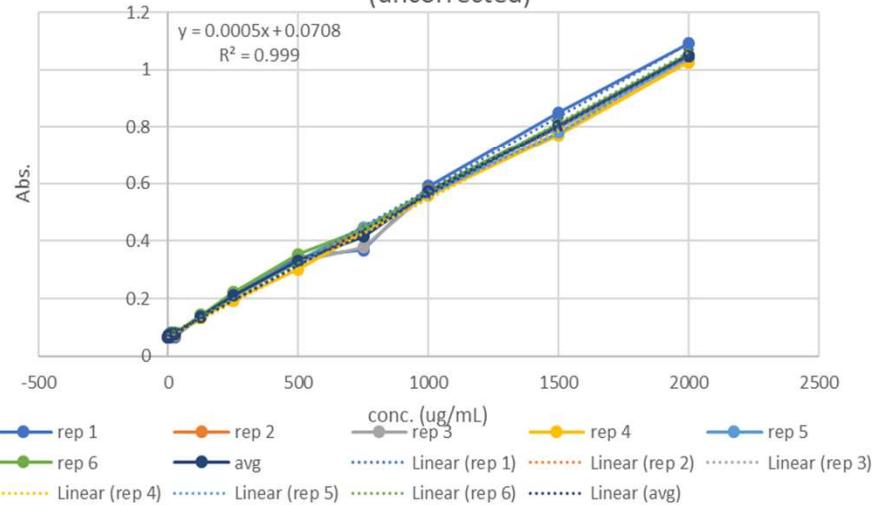
BCA Kit Absorbance at 562 nm, full-area 112.5 mL (uncorrected)



BCA Kit Absorbance at 562 nm, half-area 112.5 mL (uncorrected)



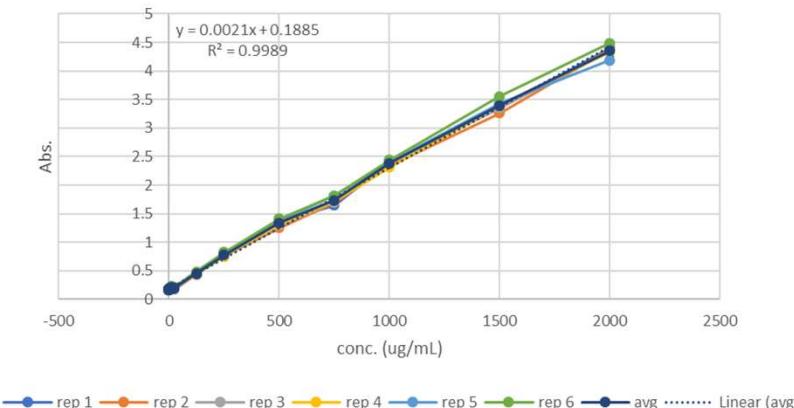
BCA Kit Absorbance at 562 nm, half-area 56.25 mL (uncorrected)



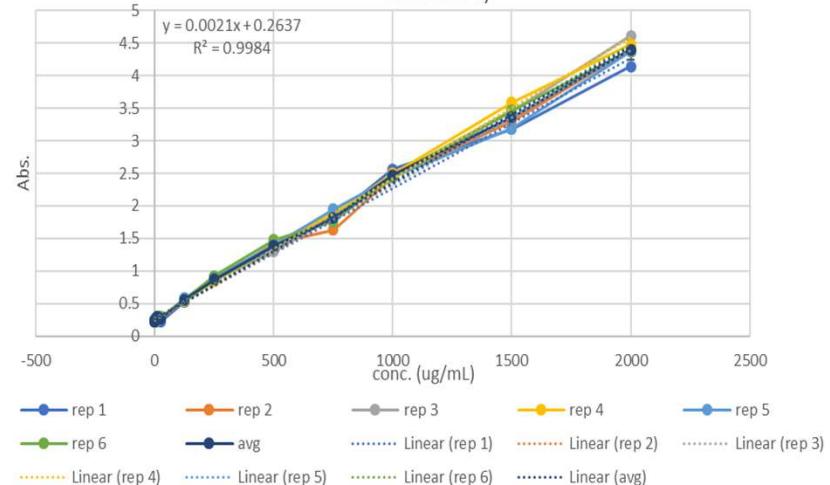
PIERCE™ BCA PROTEIN ASSAY KIT

Experimental Results: Pathlength corrected graphs

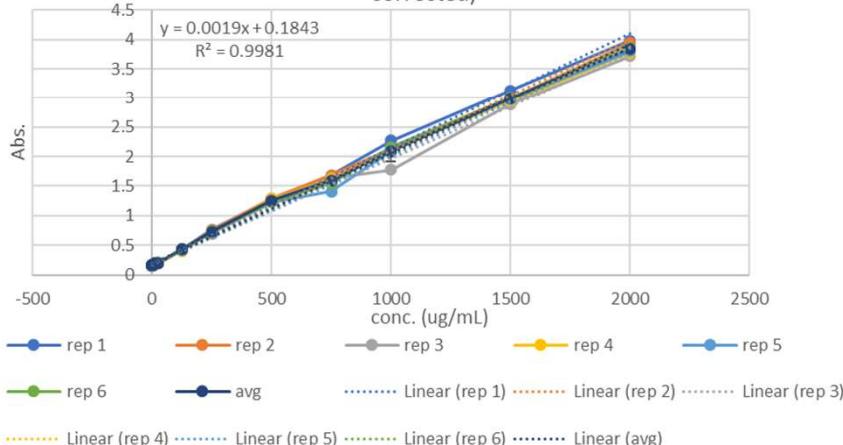
BCA Kit Absorbance at 562 nm, full-area 225 mL (pathlength corrected)



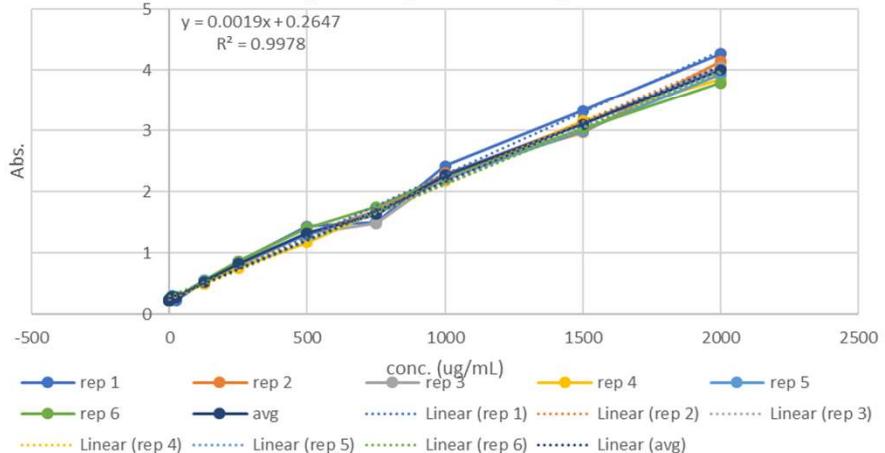
BCA Kit Absorbance at 562 nm, full-area 112.5 mL (pathlength corrected)



BCA Kit Absorbance at 562 nm, half-area 112.5 mL (pathlength corrected)



BCA Kit Absorbance at 562 nm, half-area 56.25 mL (pathlength corrected)



PIERCE™ BCA PROTEIN ASSAY KIT

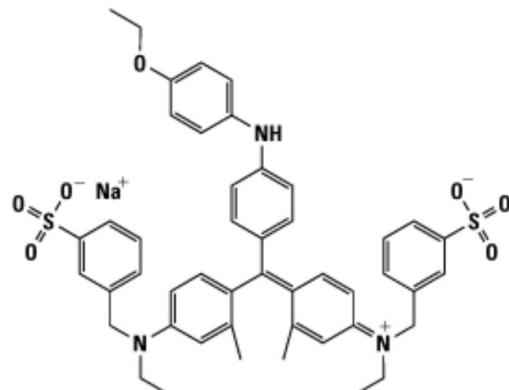
Pros & Cons

- Pros:
 - Low sample volume (6.25 – 25 uL)
 - High degree of linearity ($R^2 = 0.999$)
 - Low % stdev
 - Reagents stored at room temp, quick setup
 - Low protein-to-protein variation
- Cons:
 - Long incubation period (30 min.)
 - Lacks sensitivity at lower ranges (<25 ug/mL, one anomalous 1-2000 ug/mL)
- Possible sources of error:
 - Pipetting technique
- Future steps:
 - Attempt with different reagent/sample volume ratios
 - Test with 384-well plates

BIO-RAD BRADFORD ASSAY – HOW IT WORKS

Coomassie Dye (Bradford) Protein Assays

- In the acidic environment of the reagent (dye is protonated), protein binds to the Coomassie dye (dye becomes deprotonated). This results in a spectral shift from the reddish/brown form of the dye (absorbance maximum at 465 nm) to the blue form of the dye (absorbance maximum at 610 nm)
- Development of color in Bradford protein assays is associated with the presence of certain basic amino acids (primarily arginine, lysine and histidine) in the protein. Two times more protein-to-protein variation than copper-based assays.
- Dye-based assays fastest and easiest to perform



Coomassie Brilliant Blue G-250 Dye

$\text{C}_{47}\text{H}_{48}\text{N}_3\text{NaO}_7\text{S}_2$

MW 854.02

Chemical structure of Coomassie dye. Formulated in a low-pH phosphoric acid buffer, this colloidal form of Coomassie dye is the basis for Bradford protein assay reagents.

BIO-RAD PROTEIN ASSAY (BRADFORD)

Setup

Kit Contents:

1x Dye Reagent: 1 L of dye solution containing methanol and phosphoric acid.

Storage: 4° C

Reagent prep:

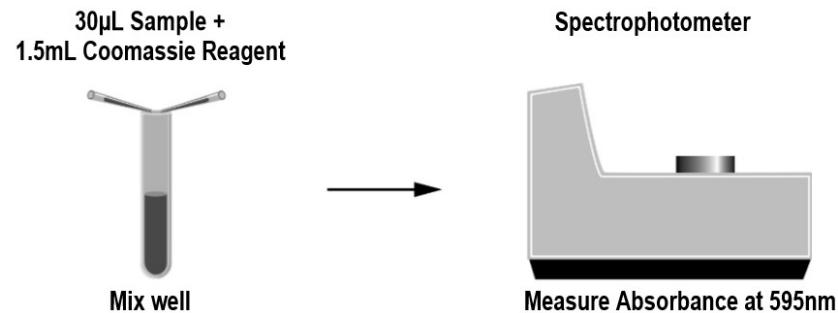
Let reagent warm to RT

Microassay protocol:

1. Pipet 150 μ L of each standard and unknown sample solution into microplate wells.
2. Use a multichannel pipet to dispense 150 μ L of the 1x dye reagent. Depress and release the plunger repeatedly to mix the sample and reagent in the wells. Replace with clean tips and add reagent to the next set of wells.
3. Incubate at room temperature for at least 5 min. Samples should not be incubated longer than 1 hr at room temperature.
4. Measure absorbance at 595 nm

Procedure Summary

(Standard Test Tube Protocol):



BIO-RAD PROTEIN ASSAY (BRADFORD)

Specs/Experimental results

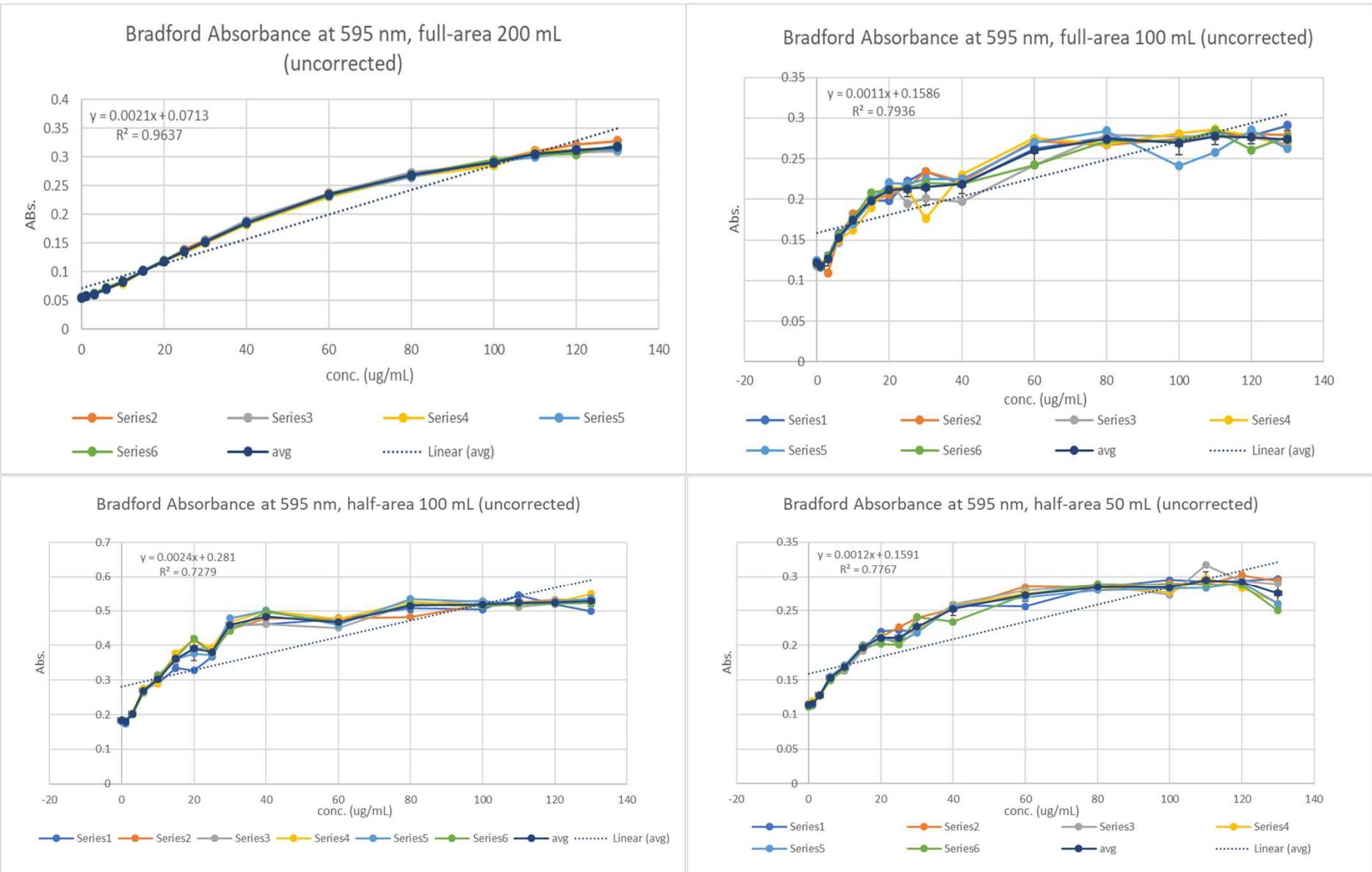
- Listed Dynamic Range: 125-1500 ug/ml (at 1:1 ratio)
- Incubation time: 5 min.
- Incubation Temp: RT
- Absorption wavelength: 595 nm
- Reagent cost: \$0.03 per assay

Results:

confirmed dynamic range (95% confidence)	Avg % std error over dynamic range	protein volume (ul)	reagent vol (ul)	total sample volume (ul)	plate type
3-20 ug/ml	Uncorrected: 1.76% Corrected: 2.76%	40	10	50	96-well half-area microplate
3-20 ug/ml	Uncorrected: 3.97% Corrected: 3.66%	80	20	100	96-well half-area microplate
3-20 ug/ml	Uncorrected: 4.05% Corrected: 3.14%	80	20	100	96-well full-area microplate
1-120 ug/mL	Uncorrected: 1.63% Corrected: 1.59%	160	40	200	96-well full-area microplate

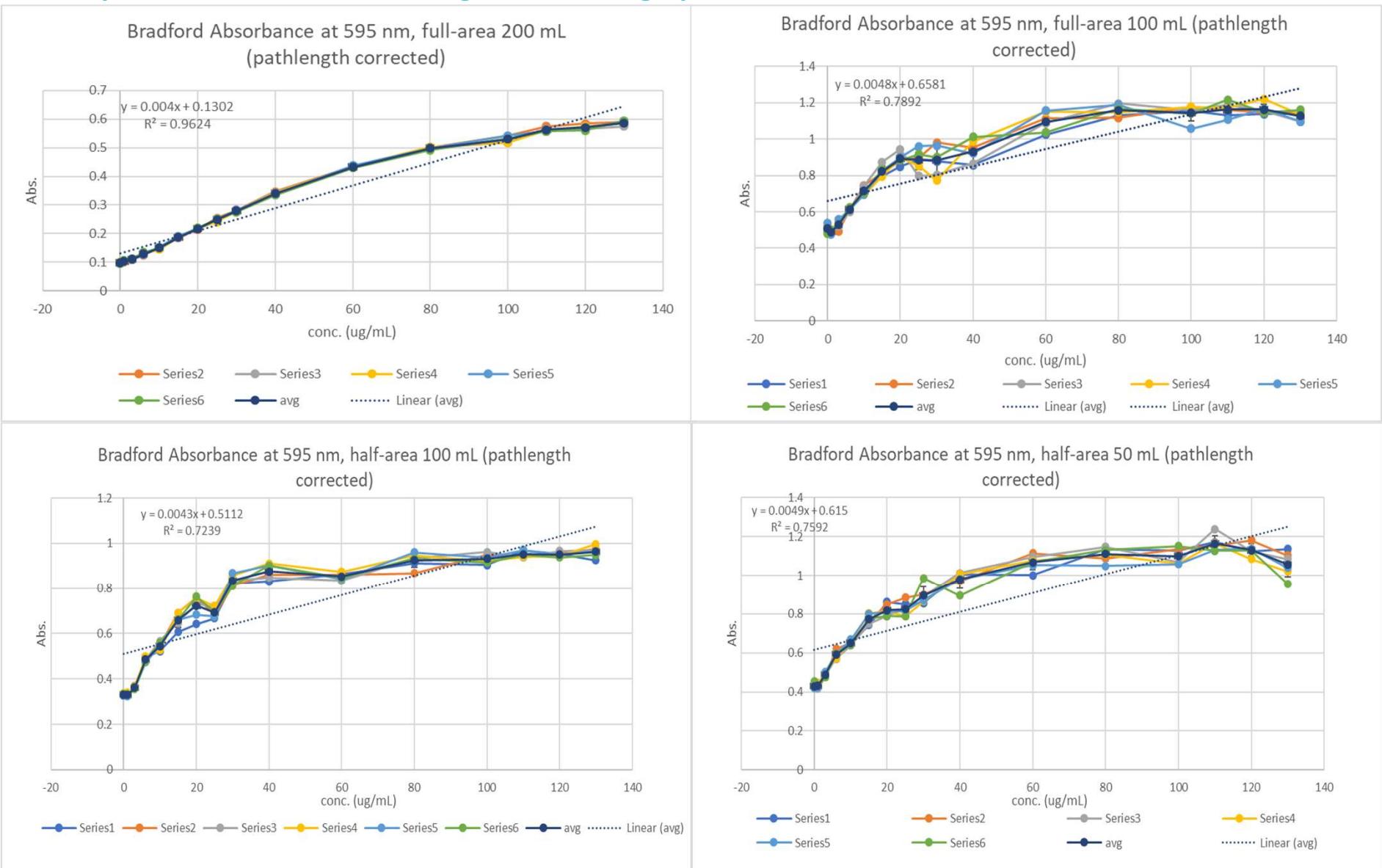
BIO-RAD PROTEIN ASSAY (BRADFORD)

Experimental Results: Uncorrected graphs



BIO-RAD PROTEIN ASSAY (BRADFORD)

Experimental Results: Pathlength corrected graphs



BIO-RAD PROTEIN ASSAY (BRADFORD)

Pros & Cons

- Pros:
 - Sensitivity at lower ranges (1-3 µg/mL)
 - Short incubation period (5 min.)
- Cons:
 - Low degree of linearity ($R^2 = 0.9637$)
 - High sample volume (40-160 µL)
 - Narrow dynamic range (~10x)
 - Must ensure reagent reaches RT (WR may be stored 2 weeks at RT)
- Possible sources of error:
 - Pipetting technique
 - **Low reagent/sample volume ratio (lack of sensitivity at certain ranges)**
- Future steps:
 - Attempt with different reagent/sample volume ratios (1:1 instead of 1:4, should get different conc. range)
 - Test with 384-well plates

PIERCE™ MODIFIED LOWRY PROTEIN REAGENT – HOW IT WORKS

Lowry Protein Assays

- Copper-based protein assay, based on biuret reaction
- Color-forming mechanism still poorly understood
- Two steps
 1. “Biuret reaction” - Protein reacted with alkaline cupric sulfate in the presence of tartrate for 10 min. at RT. Tetradeinate copper complex forms from four peptide bonds and one atom of copper.
 2. Folin-Phenol reagent added, becomes reduced and produces blue color which intensifies for 30 min. incubation at RT. Can be measured at 750 nm.

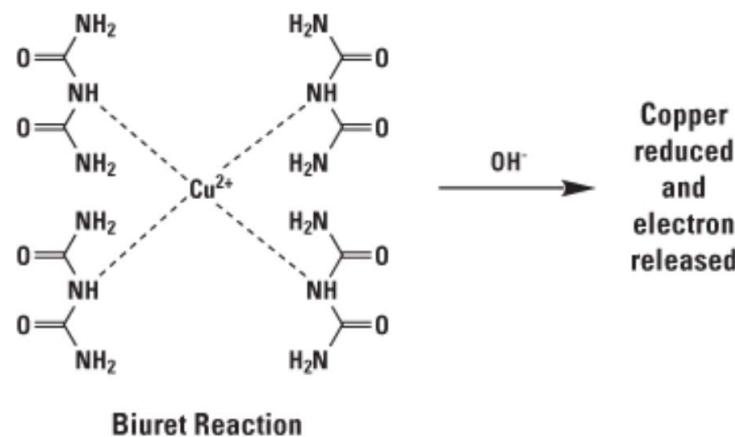


Diagram of the biuret reaction. By reducing the copper ion from cupric to cuprous form, the reaction produces a faint blue-violet color.

PIERCE™ MODIFIED LOWRY PROTEIN REAGENT

Setup

Kit Contents:

Modified Lowry Protein Assay Reagent, 480mL, containing cupric sulfate, potassium iodide, and sodium tartrate in an alkaline sodium carbonate buffer

2N Folin-Ciocalteu Reagent, 50mL

Storage: 4° C

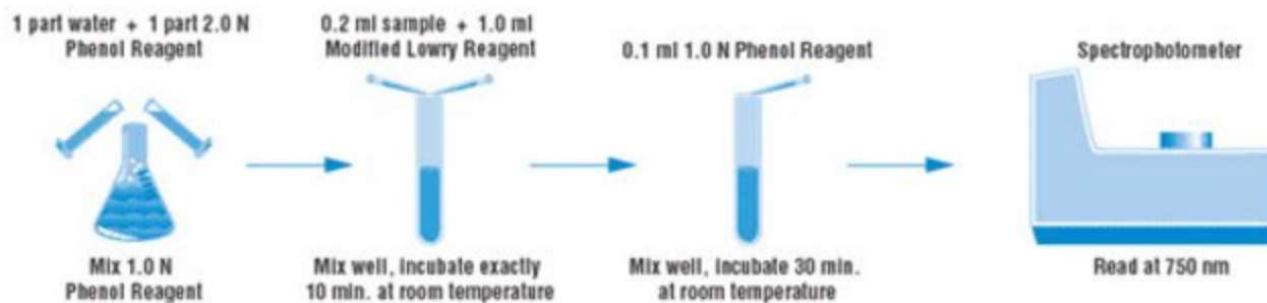
Reagent prep:

Modified Lowry Protein Assay Reagent: Let warm to RT

2N Folin-Ciocalteu Reagent: Dilute 1:1 with ultrapure water. Unstable, must use on same day as preparation.

Microplate protocol:

1. Pipette 40 μ L of each standard and unknown sample replicate into a microplate well
2. Add 200 μ L of Modified Lowry Reagent to each well. Mix microplate on plate mixer for 30 seconds.
3. Cover and incubate microplate at RT for 10 minutes.
4. Add 20 μ L of 1X Folin-Ciocalteu Reagent to each well. Mix microplate on plate mixer for 30 seconds.
5. Cover and incubate microplate at RT for 30 minutes.
6. Measure the absorbance at or near 750nm on a plate reader.



PIERCE™ MODIFIED LOWRY PROTEIN REAGENT

Specs/Experimental results

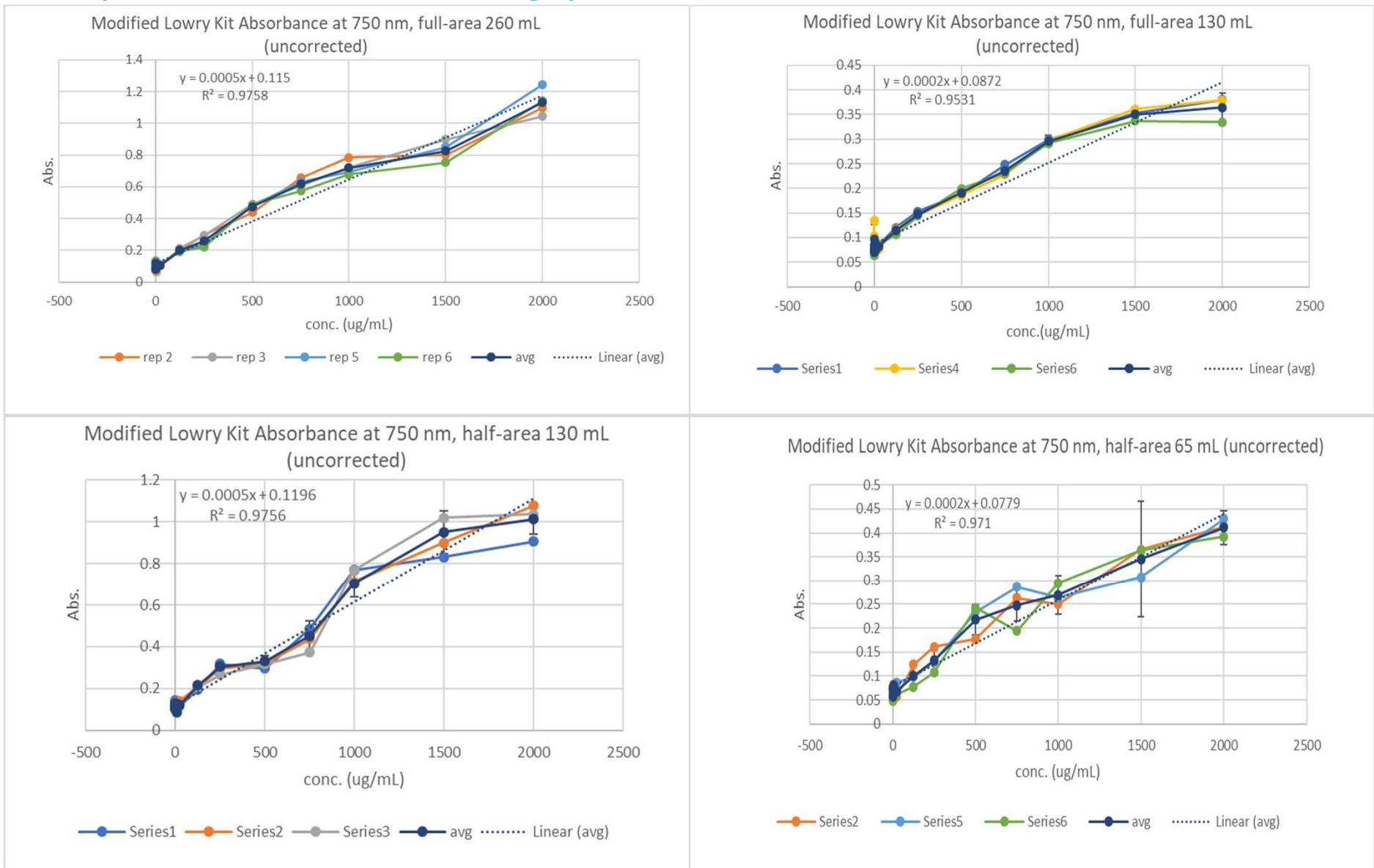
- Listed Dynamic Range: 1-1500 ug/ml
- Incubation time: 10 + 30 min = 40 min
- Incubation Temp: RT
- Absorption wavelength: 750 nm
- Reagent cost: \$0.07 per assay

Results:

confirmed dynamic range (95% confidence)	Avg % std error over dynamic range	protein volume (ul)	reagent vol (ul)	total sample volume (ul)	plate type
125-2000 ug/ml	Uncorrected: 19.68% Corrected: 18.95%	10	55	65	96-well half-area microplate
125-2000 ug/ml	Uncorrected: 7.80% Corrected: 0.123%	20	110	130	96-well half-area microplate
10-2000 ug/ml	Uncorrected: 4.53% Corrected: 5.55%	20	110	130	96-well full-area microplate
25-2000 ug/ml	Uncorrected: 6.36% Corrected: 15.49%	40	220	260	96-well full-area microplate

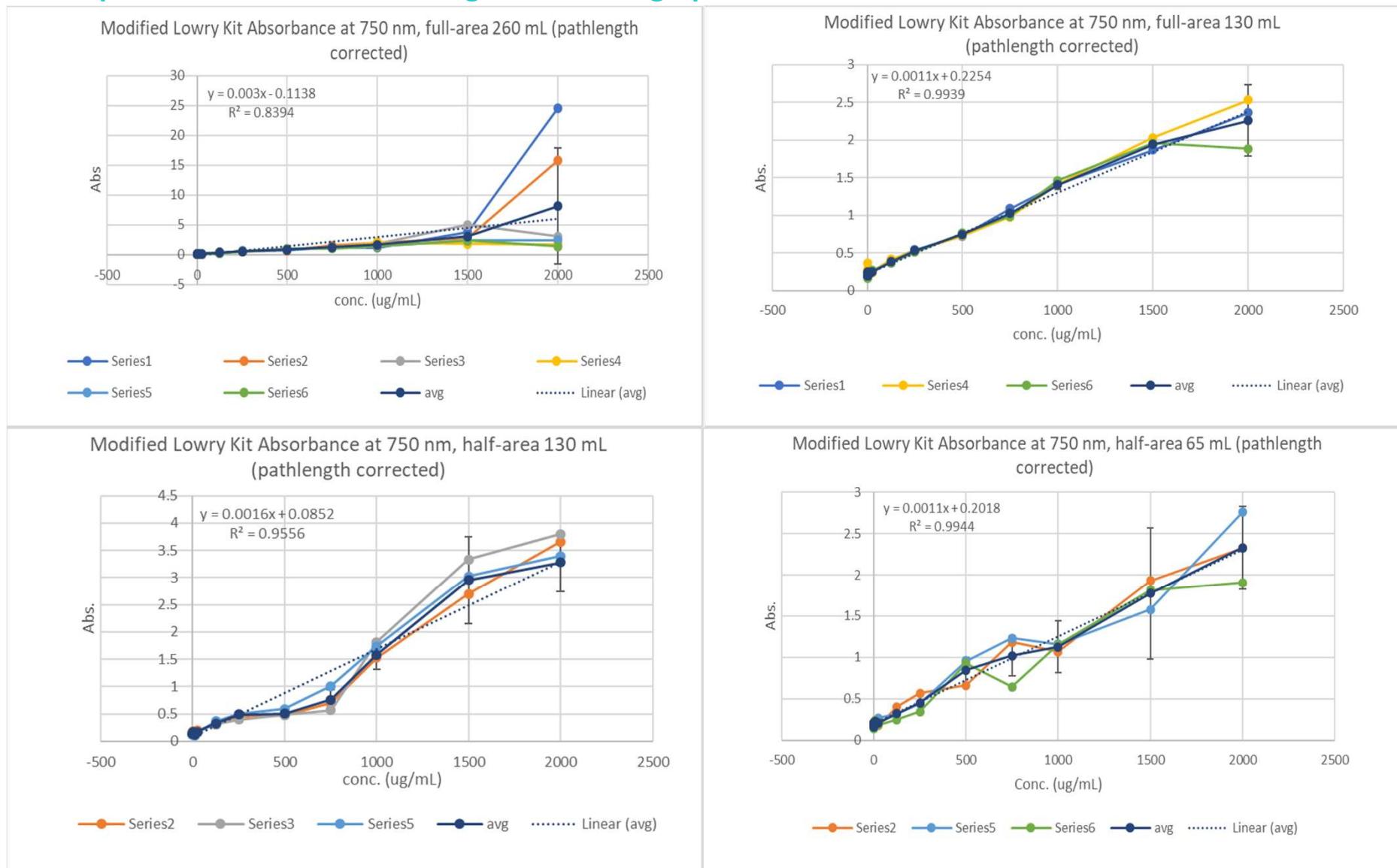
PIERCE™ MODIFIED LOWRY PROTEIN REAGENT

Experimental Results: Uncorrected graphs



PIERCE™ MODIFIED LOWRY PROTEIN REAGENT

Experimental Results: Pathlength corrected graphs



PIERCE™ MODIFIED LOWRY PROTEIN REAGENT

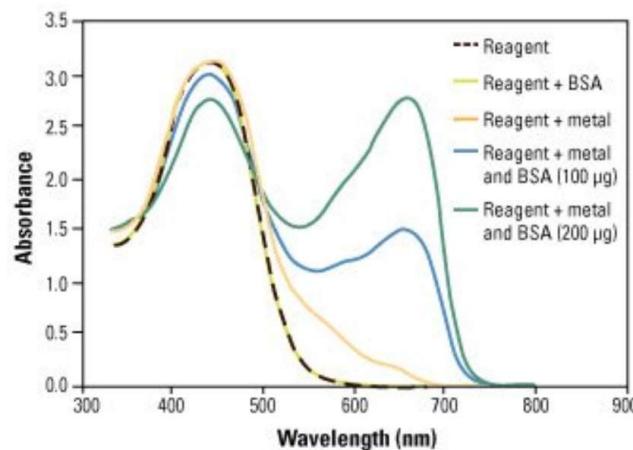
Pros & Cons

- Pros:
- Cons:
 - Low degree of linearity ($R^2 = 0.9758$)
 - Inconsistent results
 - Long incubation period (40 min. total)
 - Lacks sensitivity at lower concentration ranges (<10 ug/mL)
 - Must ensure reagent reaches RT (reagent unstable when mixed, must be prepared on same day as use)
- Possible sources of error:
 - Pipetting technique
 - Does 750 nm absorption bleed into 900 nm pathlength measurements?
- Future steps:
 - Attempt with different reagent/sample volume ratios
 - Test with 384-well plates

PIERCE™ 660NM PROTEIN ASSAY KIT – HOW IT WORKS

Dye-based Reagent

- Dye-based reagent that offers the same convenience as Coomassie-based assays while overcoming several of their disadvantages. In particular, the Pierce 660 nm Assay is compatible with most detergents and produces a more linear response curve.
- The detailed assay chemistry is proprietary, but the essential mechanism can be summarized as follows: The reagent contains a proprietary dye-metal complex in an acidic buffer. The dye-metal complex binds to protein in the acidic condition, causing a shift in the dye's absorption maximum, which is measured at 660nm. The reagent is reddish-brown and changes to green upon protein binding.
- Similar protein-to-protein variability to Coomassie (Bradford) assay methods.



Absorption maximum of the 660 nm Assay Reagent-metal complex shifts proportionally upon binding to BSA. The absorption spectra were recorded for the Pierce 660 nm Protein Assay Reagent from 340 to 800 nm using a spectrophotometer. Protein in the presence of the reagent-metal complex produces a significant absorbance shift at a wavelength of 660 nm.

PIERCE™ 660NM PROTEIN ASSAY KIT

Setup

Kit Contents:

Pierce 660nm Protein Assay Reagent, 450 mL

Storage: RT

Reagent prep:

None

Microplate protocol:

1. Add 10 μ L of each replicate of standard/unknown sample into a microplate well
2. Add 150 μ L of the Protein Assay Reagent to each well.
3. Cover plate and mix on a plate shaker at medium speed for 1 minute. Incubate at room temperature for 5 minutes.
4. Measure absorbance at 660nm.

PIERCE™ 660NM PROTEIN ASSAY KIT

Specs/Experimental results

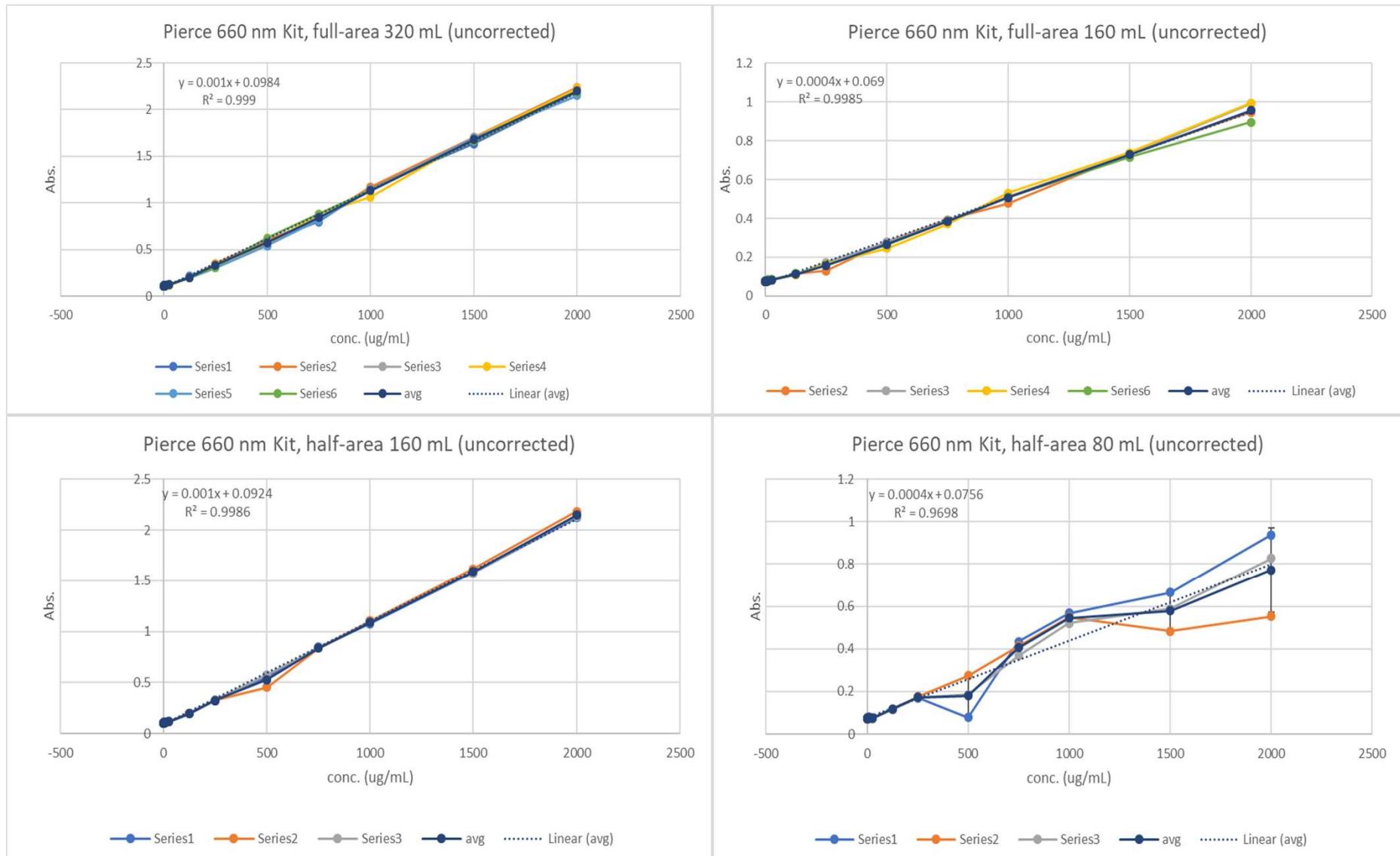
- Listed Dynamic Range: 50-2000 ug/ml
- Incubation time: 5 min
- Incubation Temp: RT
- Absorption wavelength: 660 nm
- Reagent cost: \$0.03 per assay

Results:

confirmed dynamic range (95% confidence)	Avg % std error over dynamic range	protein volume (ul)	reagent vol (ul)	total sample volume (ul)	plate type
125-2000 ug/ml	Uncorrected: 15.97% Corrected: 15.90%	5	75	80	96-well half-area microplate
3-2000 ug/ml	Uncorrected: 1.90% Corrected: 1.85%	10	150	160	96-well half-area microplate
6-2000 ug/ml	Uncorrected: 4.00% Corrected: 1.12%	10	150	160	96-well full-area microplate
10-2000 ug/ml	Uncorrected: 3.17% Corrected: 2.95%	20	300	320	96-well full-area microplate

PIERCE™ 660NM PROTEIN ASSAY KIT

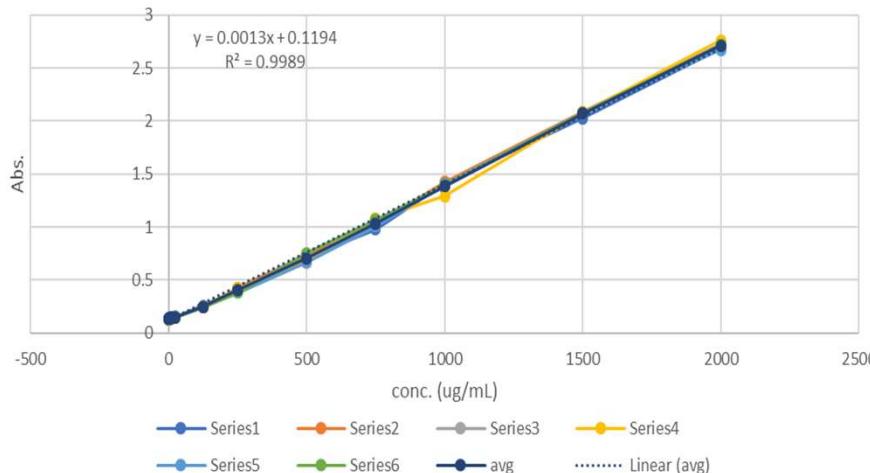
Experimental Results: Uncorrected graphs



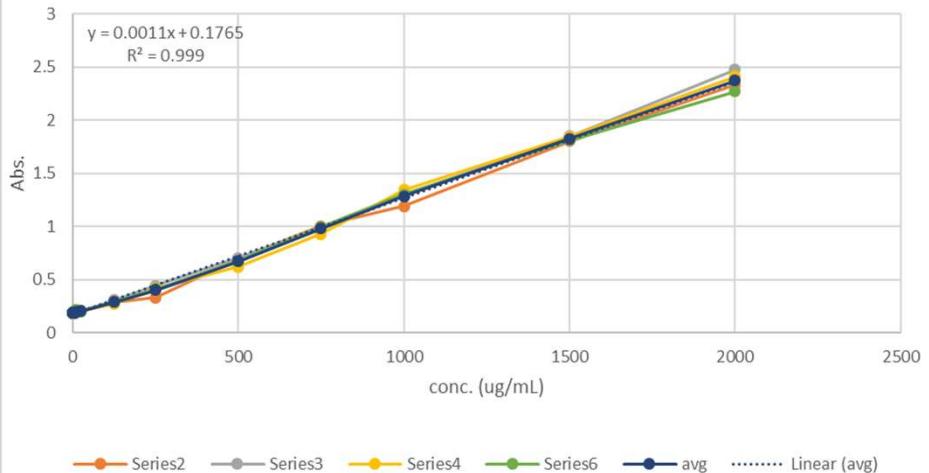
PIERCE™ 660NM PROTEIN ASSAY KIT

Experimental Results: Pathlength corrected graphs

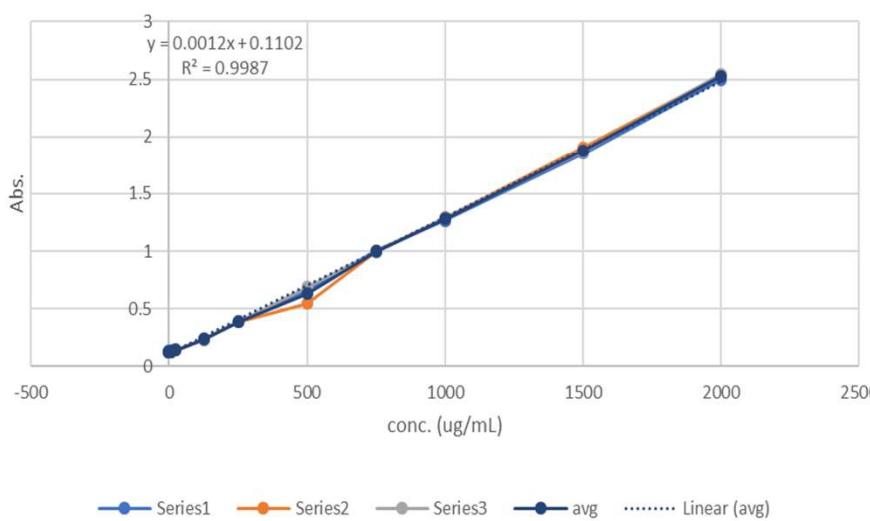
Pierce 660 nm Kit, full-area 320 mL (pathlength corrected)



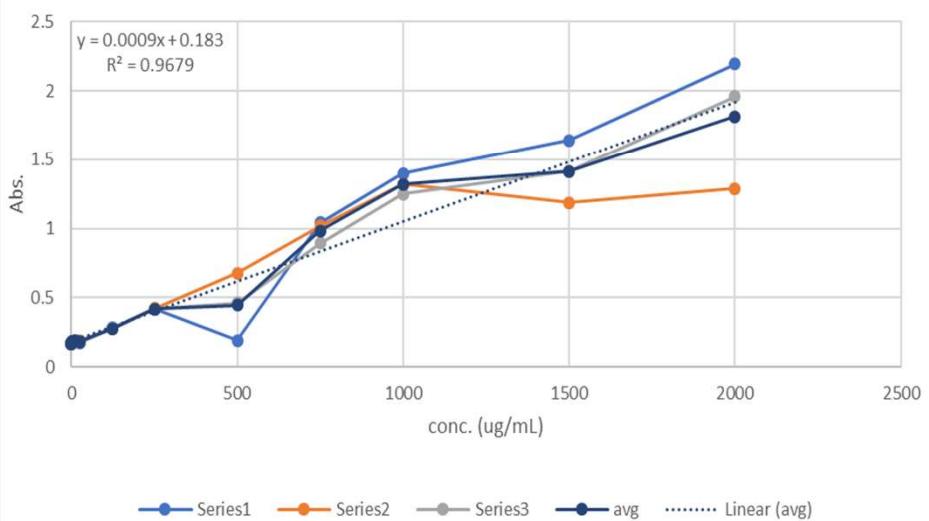
Pierce 660 nm Kit, full-area 160 mL (pathlength corrected)



Pierce 660 nm Kit, half-area 160 mL (pathlength corrected)



Pierce 660 nm Kit, half-area 80 mL (pathlength corrected)



PIERCE™ 660NM PROTEIN ASSAY KIT

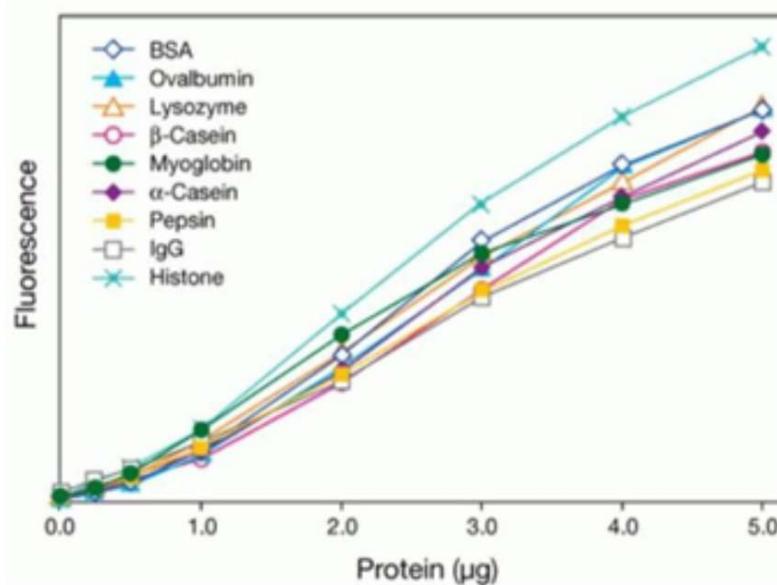
Pros & Cons

- Pros:
 - High degree of linearity ($R^2 = 0.999$)
 - Short incubation period (5 min.)
 - Low % stdev
 - Low sample volume (5-20 uL)
 - Broad dynamic range (200 - ~600x)
 - Reagent stored at room temp, quick setup
 - Works with great range of detergents and reducing agents
- Cons:
 - Lacks sensitivity at low concentration ranges (<10-3 ug/mL)
- Possible sources of error:
 - Pipetting technique
- Future steps:
 - Attempt with different reagent/sample volume ratios
 - Test in 384-well plates

NANOORANGE PROTEIN QUANTITATION KIT – HOW IT WORKS

Fluorescent Protein Assays

- Fluorescence-based protein quantification detection methods provide superior sensitivity.
- Dye molecule binds to detergent coating on proteins and hydrophobic regions of proteins, resulting in fluorescence.
- Excitation and emission wavelengths are measured (rather than absorbance wavelength)



Low protein-to-protein variation in the Qubit® Protein Assay.

NANOORANGE PROTEIN QUANTITATION KIT

Setup

Kit Contents:

NanoOrange® protein quantitation reagent (Component A), 1 mL.

Storage: ≤2–6°C, protect from light. Short term storage up to one week at RT.

NanoOrange® protein quantitation diluent

Storage: 18–25°C

Reagent prep:

Let reagent (component A) vial warm to RT before opening. Briefly centrifuge in a microcentrifuge to deposit the DMSO solution at the bottom of the vial.

Dilute quantitation diluent 10-fold in distilled water.

Dilute the reagent (component A) 500-fold into the diluted protein quantitation diluent.

Microplate protocol:

1. Make standard dilutions with working solution (table and steps indicated on next slide)
2. Incubate samples at 90°C to 96°C for 10 minutes, protected from light
3. Cool to room temperature for 20 minutes, protected from light
4. Measure fluorescence at about 485/590 nm

NANOORANGE PROTEIN QUANTITATION KIT

Setup cont.

Preparing standards

1. Prepare a 10 µg/mL solution of BSA: Dilute 2 mg/mL BSA standard 1:200 into the working solution.
2. Dilute the 10 µg/mL BSA solution to make 0, 1, 3, 6, and 10 µg/mL standards.
3. Prepare 1 µg/mL BSA solution using 10 µg/mL BSA solution and working solution. Use 1 µg/mL BSA solution to prepare 0.1, 0.3, and 0.6 µg/mL standards.

Volume (µL) of BSA Solution*	Volume of 1X NanoOrange® Working Solution	Final BSA Concentration
0 mL	2.50 mL	0 µg/mL
2.50 mL of 10 µg/mL	0 mL	10 µg/mL
1.50 mL of 10 µg/mL	1.00 mL	6 µg/mL
0.75 mL of 10 µg/mL	1.75 mL	3 µg/mL
0.25 mL of 10 µg/mL	2.25 mL	1 µg/mL
1.50 mL of 1 µg/mL	1.00 mL	0.6 µg/mL
0.75 mL of 1 µg/mL	1.75 mL	0.3 µg/mL
0.25 mL of 1 µg/mL	2.25 mL	0.1 µg/mL
1.50 mL of 0.1 µg/mL	1.00 mL	0.06 µg/mL
0.75 mL of 0.1 µg/mL	1.75 mL	0.03 µg/mL
0.25 mL of 0.1 µg/mL	2.25 mL	0.01 µg/mL

* The BSA solutions must be made up in 1X NanoOrange® working solution, as described in the text.

NANOORANGE PROTEIN QUANTITATION KIT

Specs/Experimental results

- Listed Dynamic Range: 0.01 - 10 ug/mL
- Incubation time: 10 min heat + 20 min cool = 30 min
- Incubation Temp: 95° C
- Excitation/Emission: 470/570 nm
- Reagent cost: \$0.17 per assay

Results:

confirmed dynamic range (95% confidence)	Avg % std error over dynamic range	protein volume (ul)	reagent vol (ul)	total sample volume (ul)	plate type
1-100 ug/mL	2.90%	NA*	NA*	80	96-well black plate
0.3-16 ug/mL	5.16%	NA*	NA*	180	96-well black plate

*Requires 25/1 reagent/sample ratio for non-calibration standard samples

NANOORANGE PROTEIN QUANTITATION KIT

Sample graphs

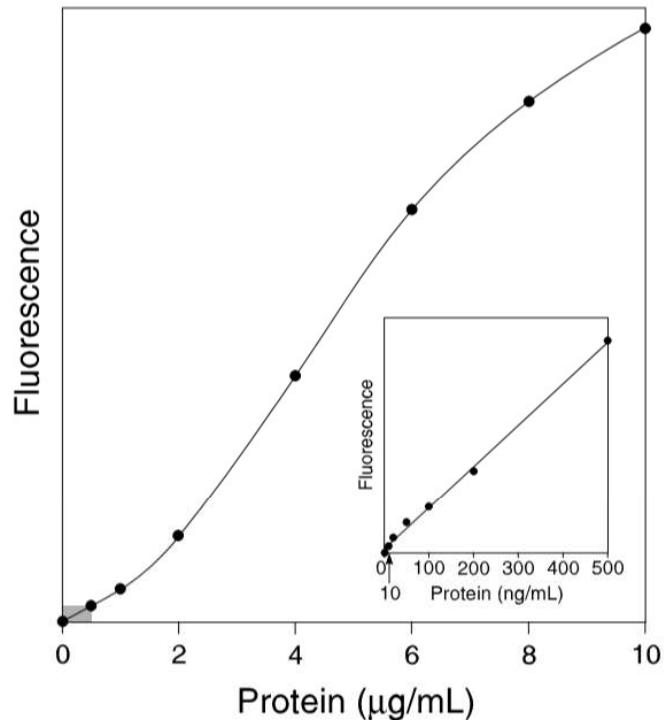


Figure 1. Quantitative analysis of bovine serum albumin (BSA) using the NanoOrange® Protein Quantitation Kit. The inset corresponds to the shaded area in the lower left corner of the plot (0 to 500 ng protein per mL) and illustrates the detection limit of 10 ng/mL. Fluorescence measurements were carried out on a fluorometer using excitation/emission wavelengths of 485/590 nm.

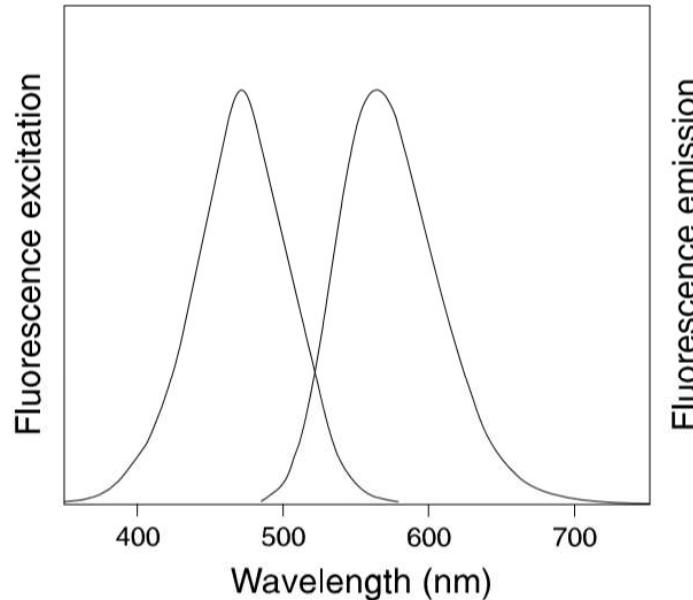
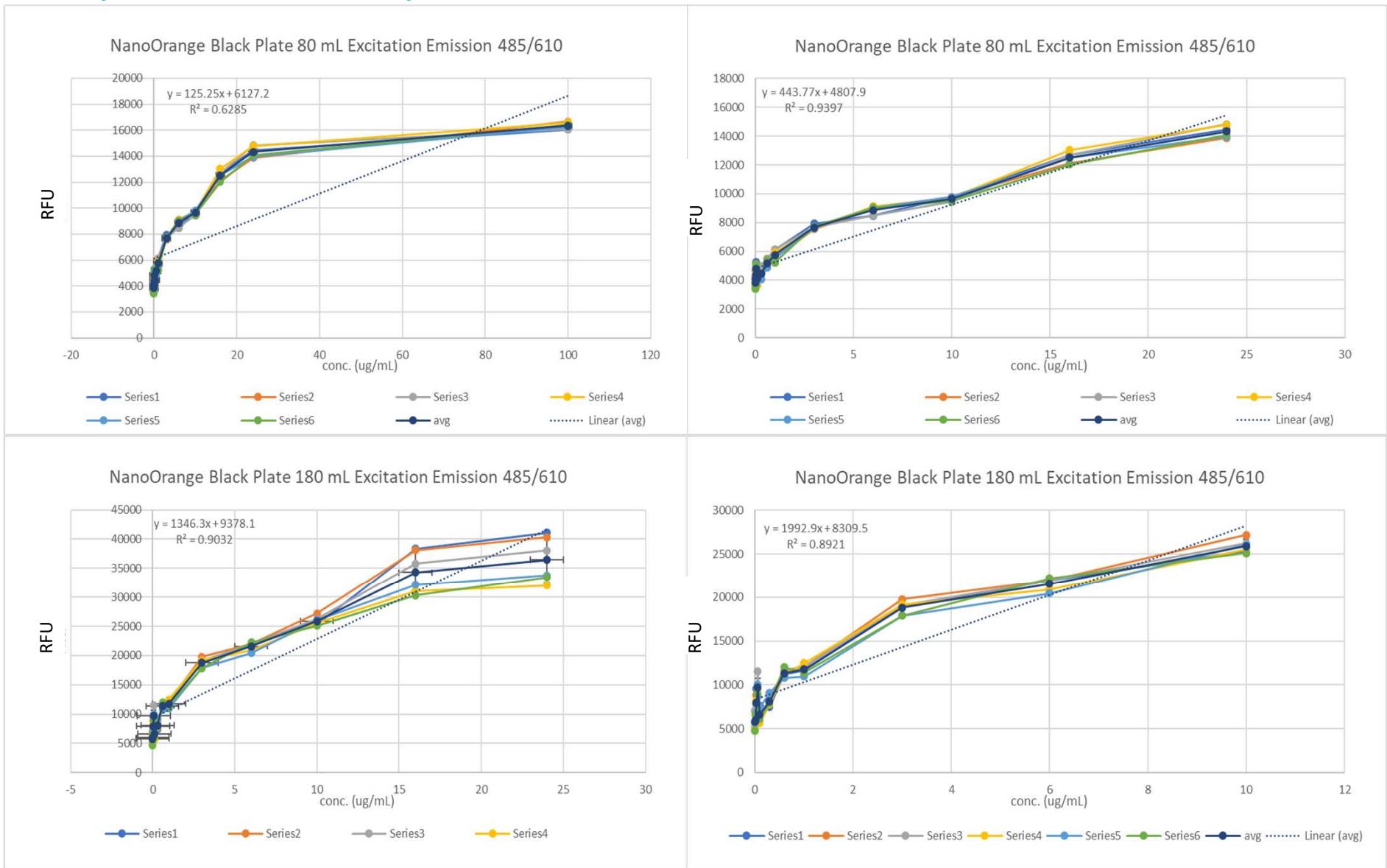


Figure 2. The normalized fluorescence excitation and emission spectra of the NanoOrange® reagent in the presence of protein quantitation diluent and 150 $\mu\text{g/mL}$ BSA.

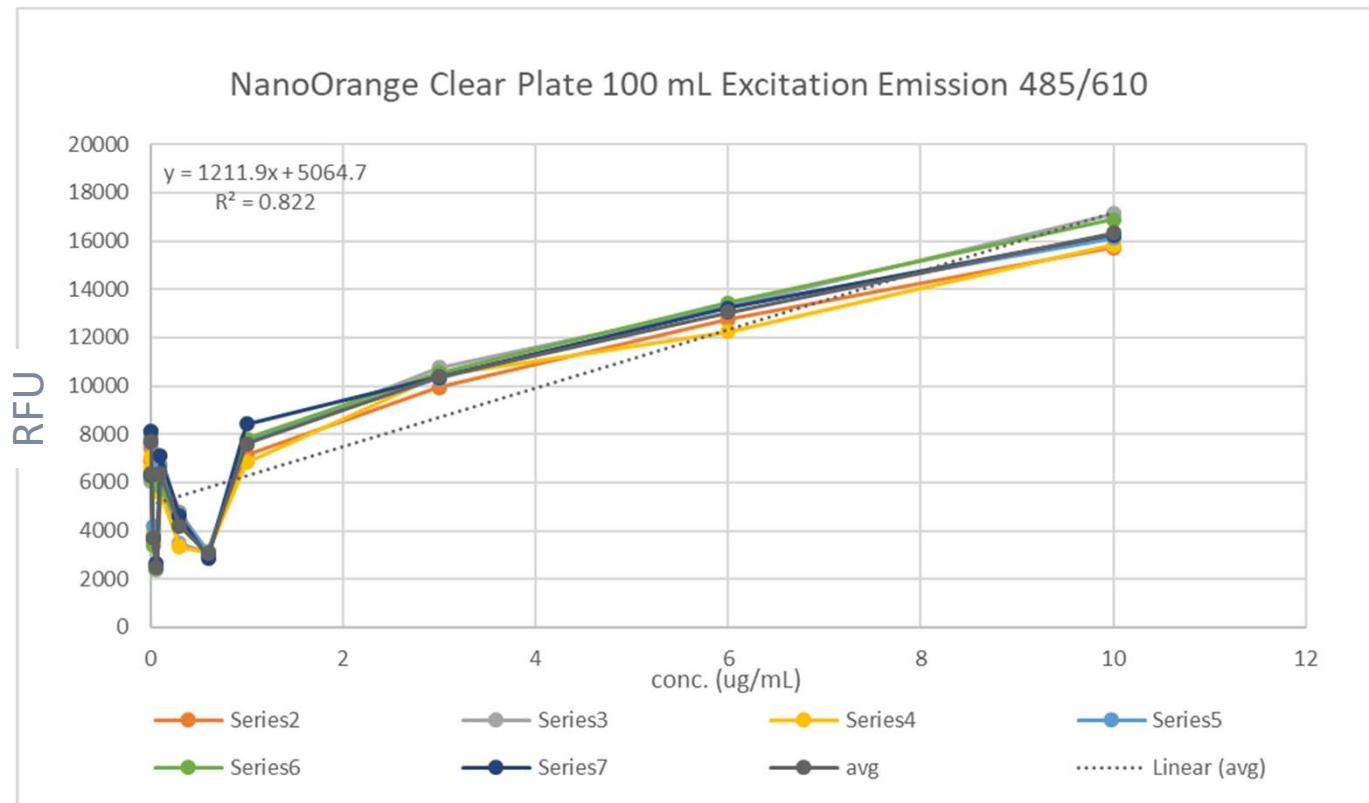
NANOORANGE PROTEIN QUANTITATION KIT

Experimental Results: Graphs



NANOORANGE PROTEIN QUANTITATION KIT

Experimental Results: Graphs (cont.)



NANOORANGE PROTEIN QUANTITATION KIT

Pros & Cons

- Pros:
 - Sensitivity at lower concentration ranges (0.3 ug/mL)
- Cons:
 - Long incubation period (30 min.)
 - Low degree of linearity ($R^2 = 0.9397$)
 - Narrow dynamic range
 - Difficult to work with: Must wait for reagent to reach RT, then microcentrifuge reagent to deposit DMSO. Must protect from light. 95° C incubation temp requires heating in PCR tubes (loss of sample volume during transfer). Preparing standards requires multiple separate dilutions w/ WR (difficult to adjust standard concentrations & reagent/sample ratio). Must wait 3 min. for bulb to heat in plate reader for fluorescence measurements.
- Possible sources of error:
 - Pipetting error during dilution, light exposure, pipetting error during sample transfer to/from PCR tubes, gain needs adjustment, **incorrect filter sizes (Ex 485/10 Em 610/10 nm).**
- Future steps:
 - **Attempt with correct filter sizes (470/570 nm)**
 - Test with 384-well plates

QUANT-IT™ PROTEIN ASSAY KIT

Setup

Kit Contents:

Quant-iT™ protein reagent (Component A), 1 mL.

Storage: RT, protect from light.

Quant-iT™ protein buffer (Component B), 250 mL

Storage: ≤6°C, protect from light. May store for a few days at RT.

Reagent prep:

1. Let components reach RT.
2. Make working solution (WS) by diluting Quant-iT™ protein reagent 1:200 in Quant-iT™ protein buffer. WS stable for 3 hours.

Microplate protocol:

1. Load 200 µL of the WS into each microplate well. WS stable for at least 3 hrs at RT, protected from light
2. Add 10 µL of each BSA standard to separate wells and mix well.
3. Measure the fluorescence using a microplate reader (excitation/emission maxima are 470/570 nm)

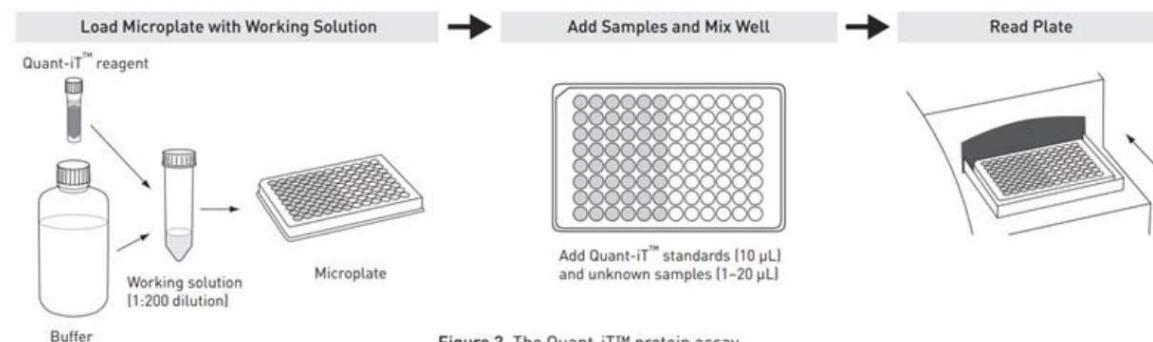


Figure 3. The Quant-iT™ protein assay.

QUANT-IT™ PROTEIN ASSAY KIT

Sample graphs

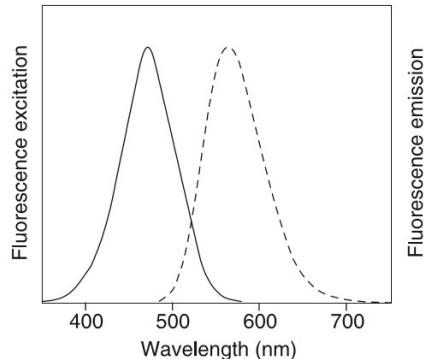


Figure 1. Excitation and emission maxima for the Quant-iT™ protein reagent bound to BSA.

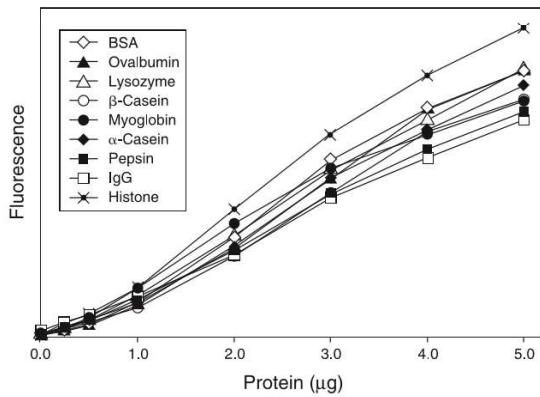


Figure 2. Low protein-to-protein variation in the Quant-iT™ protein assay. Solutions of the following proteins were prepared, diluted, and assayed in the Quant-iT™ protein assay: bovine serum albumin (BSA), chicken-egg ovalbumin, chicken-egg lysozyme, bovine-milk β -casein, equine myoglobin, bovine-milk α -casein, porcine pepsin, mouse immunoglobulin (IgG), and calf-thymus histone. Fluorescence was measured at 485/590 nm and plotted versus the mass of the protein sample. At 3 μ g, the fluorescence variation was 12.4%, or 8.7% excluding the basic histone protein. Background fluorescence has not been subtracted.

QUANT-IT™ PROTEIN ASSAY KIT

Specs/Experimental results

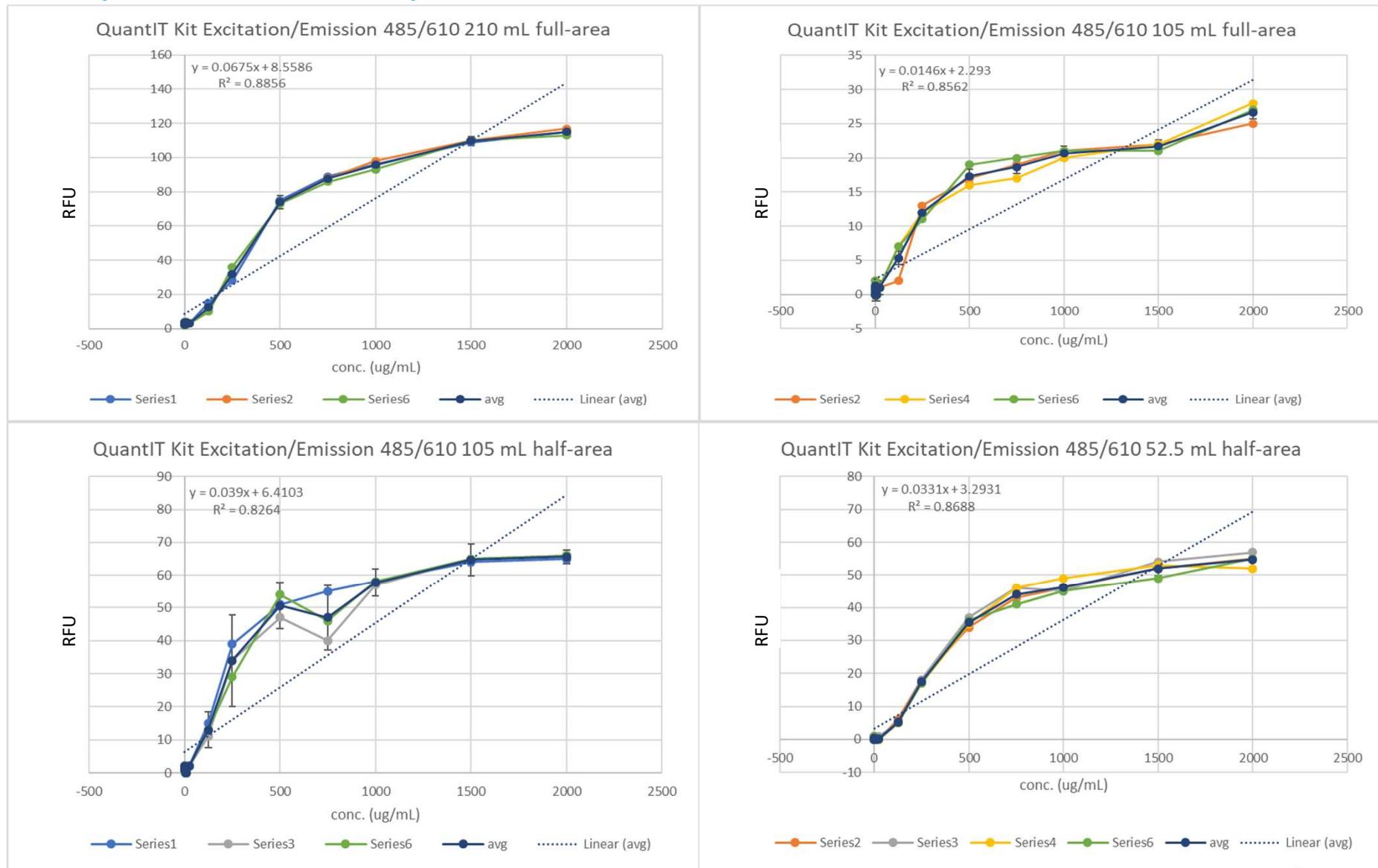
- Listed Dynamic Range: 0.25-5 ug/mL
- Incubation time: 0 min
- Incubation Temp: RT
- Excitation/Emission: 470/570 nm
- Reagent cost: \$0.45 per assay

Results:

confirmed dynamic range (95% confidence)	Avg % std error over dynamic range	protein volume (ul)	reagent vol (ul)	total sample volume (ul)	plate type
125-2000 ug/ml	4.86%	2.5	50	52.5	96-well half-area microplate
125-1500 ug/ml	6.75%	5	100	105	96-well half-area microplate
1000-2000 ug/ml	3.73%	5	100	105	96-well full-area microplate
250-2000 ug/ml	3.41%	10	200	210	96-well full-area microplate

QUANT-IT™ PROTEIN ASSAY KIT

Experimental Results: Graphs

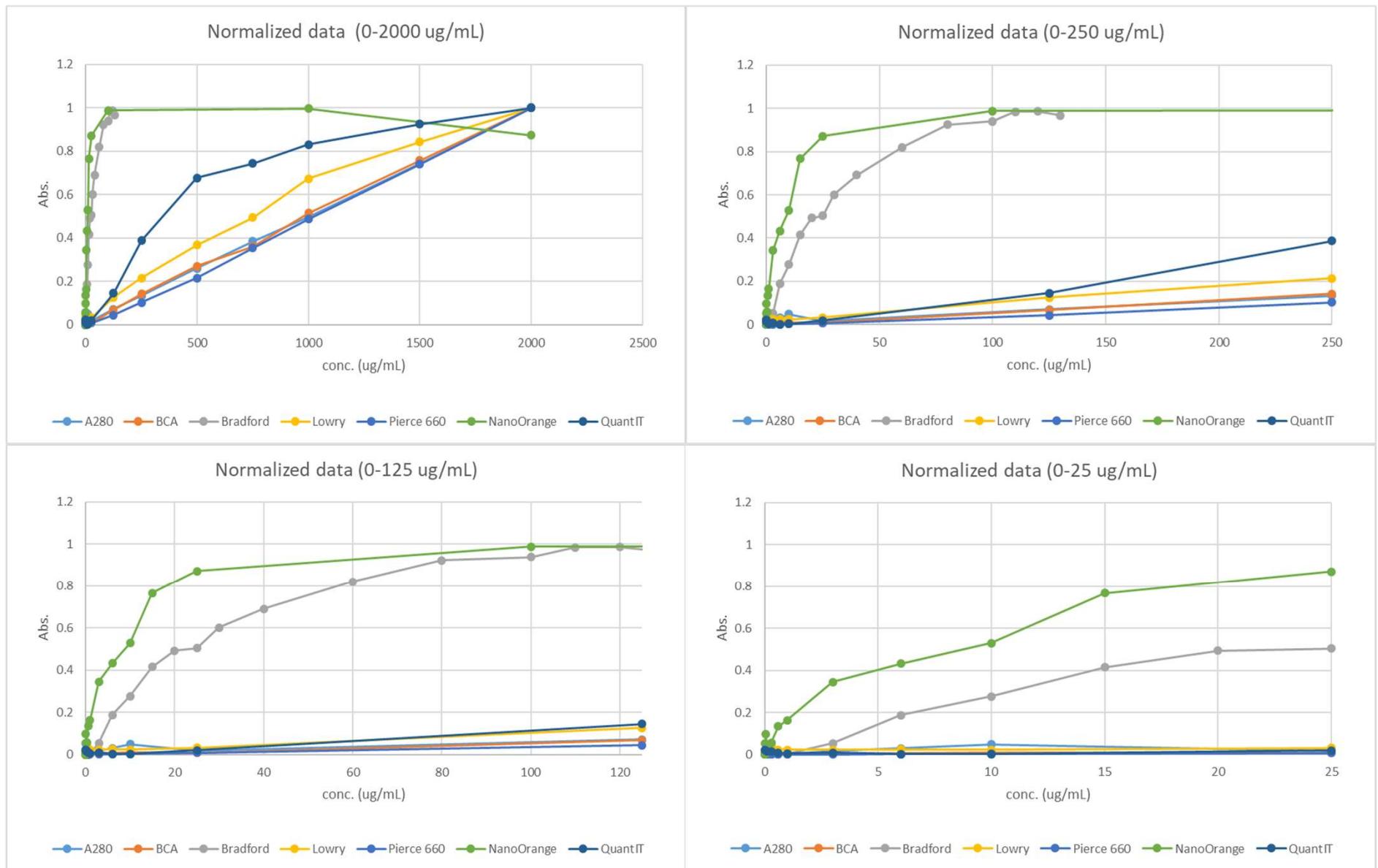


QUANT-IT™ PROTEIN ASSAY KIT

Pros & Cons

- Pros:
 - Low sample volume (2.5-10 uL)
 - No incubation time
- Cons:
 - Inconsistent results
 - Calibration curve lacks linearity ($R^2 = 0.8856$)
 - Narrow dynamic range (<10x)
 - Must wait for reagent to reach RT, protect from light
- Possible sources of error:
 - Pipetting technique, light exposure, gain needs adjustment, **incorrect filter sizes (485/610 nm)**
- Future steps:
 - **Attempt with correct filter sizes (470/570 nm)**
 - Attempt with different sample/reagent ratios
 - Test with 384-well plates

NORMALIZED GRAPHS - ALL KITS

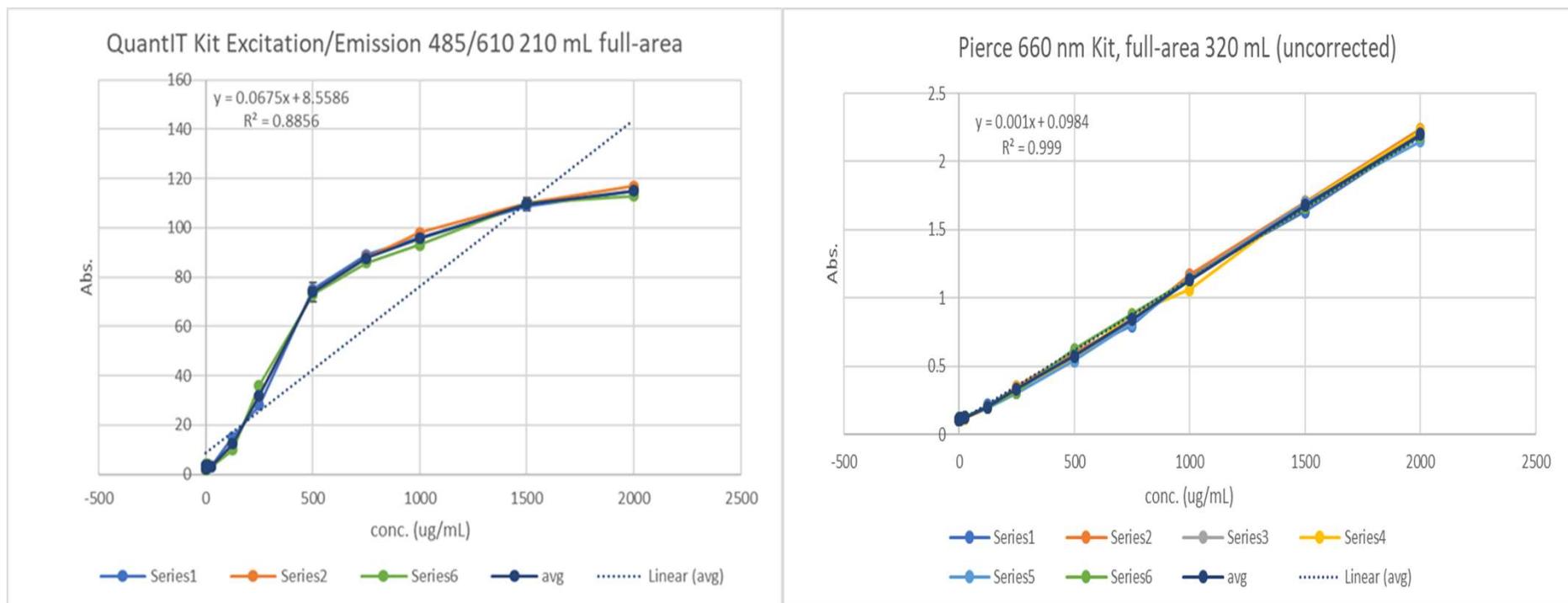


CONCLUSIONS

Detection Range

Narrowest range of detection thus far:
Quant-iT (~8-16x)

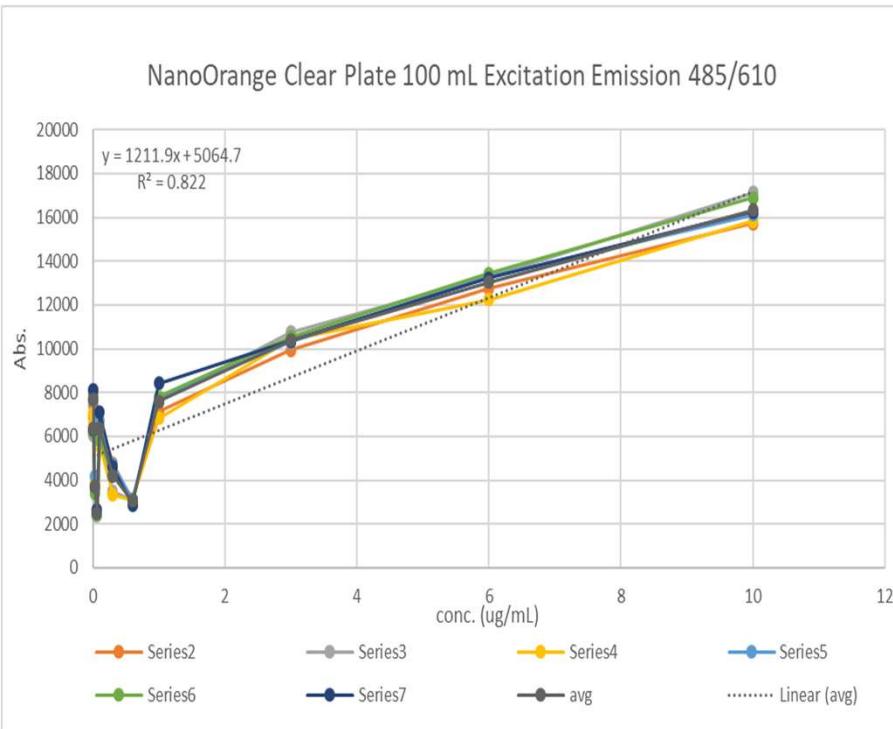
Broadest range of detection thus far:
Pierce 660 nm (~200x – 666x)



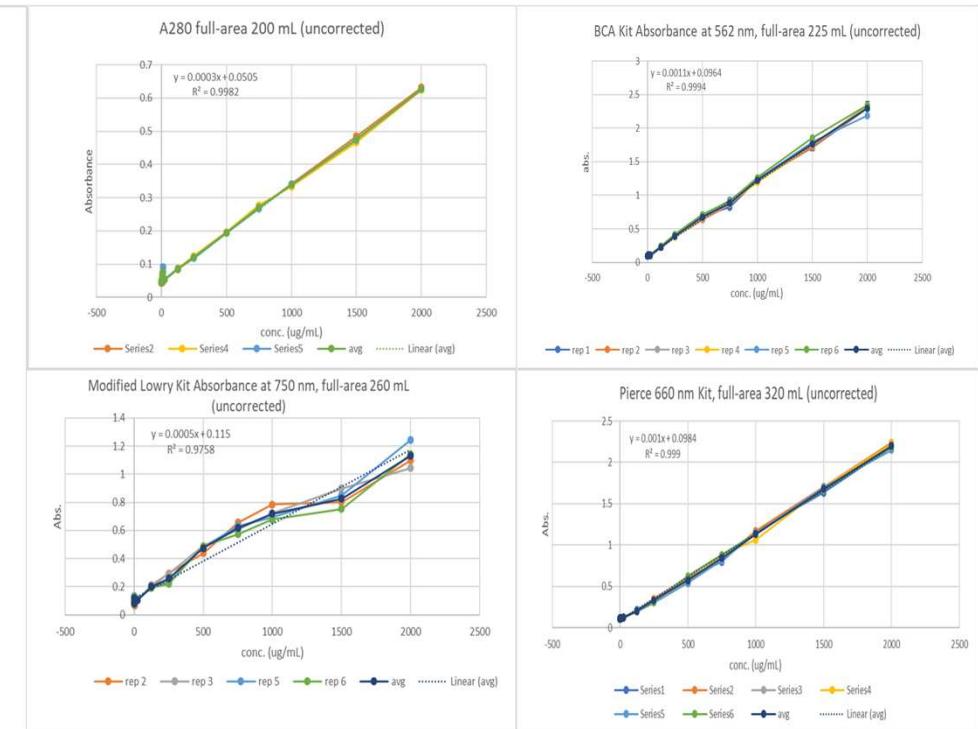
CONCLUSIONS

Lower Limit of Detection (LLOD)

Lowest LLOD: NanoOrange



Highest LLOD: A280, BCA, Lowry, Pierce



CONCLUSIONS

Cont.

- **Conclusions (cont.)**
 - Most difficult/time consuming setup: NanoOrange,
 - Easiest/fastest setup: A280, Pierce 660 nm
 - \$\$\$ Highest reagent cost: Quant-iT (\$0.45 per assay)
 - \$\$\$ Lowest reagent cost: Pierce 660 nm, Bio-Rad Bradford (\$0.03 per assay)
 - Least favorable kit thus far: Modified Lowry
 - Most favorable kit thus far: Pierce 660 nm
 - Most difficult aspect was preparing dilution standards; Standard preparation for NanoOrange was particularly time consuming.
 - Half-area plates tend to give similar results with half of the sample volume
 - NanoOrange/Quant-iT inconclusive but still promising for lower range
- **Future steps**
 - Retest Quant-iT and NanoOrange with new filters
 - Retest Bio-Rad Bradford with proper reagent/sample ratio
 - Utilize 384-well plate format (Gilson, Echo, etc.)



THANK YOU.