INSTRUCTIONS

Pierce™ 660nm Protein Assay

22660 22662

2070.3

Number	Description
22660	**Pierce 660nm Protein Assay Reagent,** 750mL, contains sufficient reagents for 500 test tube or 5000 microplate assays
22662	**Pierce 660nm Protein Assay Kit,** sufficient reagents for 300 test tube or 3000 microplate assays
	Kit Contents:
	Pierce 660nm Protein Assay Reagent, 450mL
	Pre-diluted Protein Assay Standards: Bovine Serum Albumin (BSA) Set, 7 × 3.5mL, contains standardized BSA solutions at a specific concentration from 125 to 2000µg/mL in 0.9% saline and 0.05% sodium azide
	Storage: Upon receipt store the assay reagent at room temperature and the pre-diluted BSA standards at 4ºC. Product is shipped at ambient temperature.

Introduction

The Thermo Scientific™ Pierce™ 660nm Protein Assay is a quick, ready-to-use colorimetric method for total protein quantitation. The assay is reproducible, rapid and more linear compared to coomassie-based Bradford assays and compatible with high concentrations of most detergents, reducing agents and other commonly used reagents. The assay has a moderate protein-to-protein variation.

This simple assay is performed in either test tube or a microplate. Protein concentrations are estimated by reference to absorbances obtained for a series of standard protein dilutions assayed alongside the unknown samples. The best relative standard to use gives a color response similar to that of the protein being assayed. The two most common protein standards for protein assays are BSA and BGG. The BSA standard is an appropriate standard if the sample contains primarily albumin. The BGG standard is an appropriate standard if the sample contains primarily globulins.

Procedure for the Pierce 660nm Protein Assay

Note: Certain substances interfere with the Pierce 660nm Protein Assay. Please see the Interfering Substances Section for more information.

Sample Preparation

- For samples containing > 0.0125% SDS, add one pack of Ionic Detergent Compatibility Reagent (IDCR, Product No. 22663) to 20mL of the Pierce 660nm Protein Assay Reagent before performing the assay. The IDCR Solution is stable for 24 hours at room temperature. Mix the solution before each use.
- For cells lysed in Laemmli sample buffer, dilute the lysate from 1:10 to 1:20 in Laemmli buffer. Also add one pack of IDCR to 20mL of the Pierce 660nm Protein Assay Reagent before performing the assay (see above bullet point).
- For cell lysates prepared in RIPA buffer, add Triton™ X-100 to a final concentration of 0.8% to the sample before performing the assay. For example, to 46µL of control RIPA buffer and diluted lysates (1:10, 1:20 etc), add 4µL of 10% Triton X-100 and mix. Perform the assay as described in the protocol and multiply the protein concentration of the sample by 1.087 (i.e., the dilution factor).

Test Tube Procedure (working range 25-2000µg/mL)

1. Prepare a standard curve within the assay's working range. If using the pre-diluted standards and want a 25µg/mL standard, mix 10µL of the 1000µg/mL standard with 390µL of 0.9% saline and 0.05% of sodium azide.
2. Add 0.1mL of each replicate of standard, unknown sample and the appropriate blank sample into an appropriately labeled test tube.

   Note: A smaller sample volume may be used if the sample to Assay Reagent ratio is maintained at 1:15.
3. Add 1.5mL of the Protein Assay Reagent to each tube and vortex to mix well.
4. Cover and incubate tubes for 5 minutes at room temperature.
5. With the spectrophotometer set to 660nm, zero the instrument on a cuvette filled with only water. Subsequently, measure the absorbance of all the samples.

   Note: If a 660nm filter is not available, measure the assay at any wavelength from 645 to 670nm; however, the assay linear range is 25-2000µg/mL and occurs only when the absorbance is measured at 660nm. Measuring the absorbance at another wavelength will decrease the assay's linear range and might increase the minimum detection level (i.e., decrease sensitivity).
6. Subtract the average 660nm absorbance measurement of the Blank standard replicates from the 660nm absorbance measurement of all other individual standard and unknown sample replicates.
7. Prepare a standard curve by plotting the average Blank-corrected 660nm measurement for each BSA standard vs. its concentration in µg/mL. Use the standard curve to determine the protein concentration of each unknown sample.

Microplate Procedure (working range 50-2000µg/mL)

1. Prepare a standard curve within the assay's working range. If using the pre-diluted standards and want a 50µg/mL standard, mix 10µL of the 1000µg/mL standard with 190µL of 0.9% saline and 0.05% of sodium azide.
2. Add 10µL of each replicate of standard, unknown sample and the appropriate blank sample into a microplate well (e.g., Thermo Scientific™ Pierce™ 96-Well Plates, Product No. 15041).
3. Add 150µL of the Protein Assay Reagent to each well.
4. Cover plate and mix on a plate shaker at medium speed for 1 minute. Incubate at room temperature for 5 minutes.
5. Use the blank wells to zero the plate reader. Measure the absorbance of the standards and unknown samples at 660nm.

   Note: If a 660nm filter is not available, measure the assay at any wavelength from 645 to 670nm; however, the assay linear range is 50-2000µg/mL and occurs only when the absorbance is measured at 660nm. Measuring the absorbance at another wavelength will decrease the assay's linear range and might increase the minimum detection level (i.e., decrease sensitivity).
6. Prepare a standard curve by plotting the average Blank-corrected 660nm measurement for each BSA standard vs. its concentration in µg/mL. Use the standard curve to determine the protein concentration of each unknown sample.

   Note: If using curve-fitting algorithms associated with a microplate reader, a four-parameter (quadratic) curve produces more accurate results than a linear fit.

Troubleshooting

Problem	Possible Cause	Solution
Standards and samples yield lower values than expected	Absorbance measured at incorrect wavelength	Measure absorbance at 660nm
A precipitate forms in some tubes	Samples left to stand for extended time, allowing aggregates to form with the dye	Mix samples by pipetting up and down immediately before measuring absorbance
	Sample contains RNA/DNA	Add a final concentration of 0.8% Triton X-100 to samples
Blank is > 0.25	Sample contains an interfering substance	Refer to Table 1 for more information
	Assay reagent is stored at 4°C	Store the assay reagent at room temperature
Color of samples appear darker than expected	Protein concentration is too high	Dilute sample

Interfering Substances

Certain substances are known to interfere with the Pierce 660nm Protein Assay. Maximum compatible concentrations for many substances are listed in Table 1. Substances were considered compatible in the assay if the error in protein concentration estimation caused by the presence of the substance was ≤ 10%. Blank-corrected 660nm absorbance values for 1mg/mL of BSA plus interfering substance were compared to the net 660nm values of the same standard prepared in water.[§]

Table 1. Maximum compatible substance concentrations in the Thermo Scientific Pierce 660nm Protein Assay.

Substances	Maximum Compatible Concentration
Detergents	
Tween-20	10%
Triton X-114	0.5%
Triton X-100	1%
Octylthioglucopyranoside	10%
CHAPS	5%
CHAPSO	4%
NP-40	5%
Octyl-β-glucoside	5%
Brij-35	5%
SDS	0.0125%, 5%*
Sodium deoxycholate	0.25%
Zwittergent 3-14	0.05%
CTAB*	2.5%
Cetylpyridinium chloride*	2.5%
DTAB*	2%
Reducing Agents	
DTT	500mM
2-Mercaptoethanol	1M
L-Cysteine	350mM
Ascorbic acid	500mM
TCEP	40mM
Glutathione (reduced)	100mM
Buffers	
PBS	Undiluted
HEPES, pH 7.5	100mM
Tris•HCl, pH 8.0	250mM
Glycine buffer, pH 2.8	100mM
Carbonate-bicarbonate, pH 9.4	diluted 3-fold
Imidazole pH 7.0	200mM
MOPS, pH 7.2	125mM
MES, pH 6.1	125mM
PIPES, pH 6.8	100mM
Sodium acetate, pH 4.8	100mM
Borate buffer, pH 8.5	Undiluted (# 28384)

Substances	Maximum Compatible Concentration
Chelating Agents	
EDTA	20mM
EGTA	20mM
Sodium citrate	12.5mM
Misc Reagents/Solvents	
NaCl	1.25M
GuHCl	2.5M
Urea	8M
Thiourea	2M
Ammonium sulfate	125mM
Glycerol	50%
NaOH	125mM
HCl	125mM
Sucrose	50%
Methanol	50%
Ethanol	50%
DMF	50%
DMSO	50%
Acetone	50%
Acetonitrile	50%
Phenol Red	0.5mg/mL
Calcium chloride in TBS, pH 7.2	40mM
Cobalt chloride in TBS, pH 7.2	20mM
Ferric chloride in TBS, pH 7.2	5mM
Nickel chloride in TBS, pH 7.2	10mM
Zinc chloride in TBS, pH 7.2	10mM
Y-PER™ Reagent	Not compatible
B-PER™ Reagent	diluted 2-fold
M-PER™ Reagent	diluted 2-fold
P-PER™ Reagent	diluted 2-fold
T-PER™ Reagent	diluted 2-fold
MEM-PER™ Reagent	Compatible (1:1:2 of Reagent A:Reagent B:Reagent C)
NE-PER™ Reagent	Compatible (400µL CER I, 22µL CER II and 200µL NER)
RIPA buffer	50mM Tris•HCl, 150Mm NaCl, 0.25% deoxycholic acid, 1% NP-40, 1mM EDTA
2-D Sample Buffer for soluble and insoluble proteins	8M urea, 4% CHAPS, and 7M urea, 2M thiourea, 4% CHAPS
Laemmli SDS sample buffer*	65mM Tris•HCl, 10% glycerol, 2% SDS, 0.0025% bromophenol blue

[§]For a more extensive list of substances, download Tech Tip # 68: Protein Assay Compatibility Table from our website. This Tech Tip includes compatible substances for all of our protein assays and enables easy comparisons.

*In the presence of 50mM Ionic Detergent Compatible Reagent (IDCR).

Additional Information

A. Response Characteristics for Different Proteins

Each total protein assay method exhibits some degree of varying response toward different proteins. These differences relate to amino acid sequence, isoelectric point, structure and the presence of certain side chains or prosthetic groups that can dramatically alter the protein's color response. The ideal protein to use as a standard in any protein assay is a purified preparation of the protein being assayed. In the absence of a reference protein, use another protein that produces a similar color response to that of the protein being assayed. Most protein methods use BSA or BGG as the standard against which the concentration of protein in the sample is determined (Figure 1). The BSA standard is an appropriate standard if the sample contains primarily albumin. The BGG standard is an appropriate standard if the sample contains gamma globulins.

Typical protein-to-protein variations in color response are listed in Table 2. All proteins were tested at 1mg/mL using the test-tube protocol. The average net color response for BSA was normalized to 1.00 and the average net color response of the other proteins is expressed as a ratio to the response of BSA.

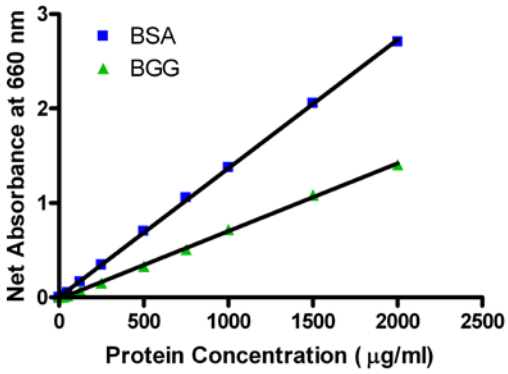

Figure 1. Typical color response curves for BSA and BGG using the test tube procedure.

Table 2. Protein-to-protein variation.

Protein Tested	Ratio
Albumin, bovine serum	1.00
Gamma globulin, bovine	0.51
IgG, human	0.57
IgG, rabbit	0.38
IgG, mouse	0.48
Insulin, bovine pancreas	0.81
Cytochrome c, horse heart	1.22
α-Lactalbumin	0.82
Lysozyme	0.79
Myoglobin, horse heart	1.18
Trypsin inhibitor, soybean	0.38
Ovalbumin	0.54
Transferin, human	0.8
Aldolase	0.83
Average Ratio	**0.7364**
Standard Deviation	**0.2725**
Coefficient of Variation	**37%**

Related Thermo Scientific Products

15041	**Pierce 96-Well Plates – CornerNotch,** 100/pkg
22663	**Ionic Detergent Compatibility Reagent,** 5 × 1g
23208	**Pre-Diluted Protein Assay Standards: Bovine Serum Albumin (BSA) Set**, 7 × 3.5mL
23209	**Albumin Standard Ampules, 2mg/mL,** 10 × 1mL ampules
23212	**Bovine Gamma Globulin Standard Ampules**, 2mg/mL, 10 × 1mL
23213	**Pre-Diluted Protein Assay Standards: Bovine Gamma Globulin Fraction II**, 7 × 3.5mL

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Quantitation of proteins using a dye–metal-based colorimetric protein assay

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ABSTRACT

We describe a dye–metal (polyhydroxybenzenesulfonephthalein-type dye and a transition metal) complex-based total protein determination method. The binding of the complex to protein causes a shift in the absorption maximum of the dye–metal complex from 450 to 660 nm. The dye–metal complex has a reddish brown color that changes to green on binding to protein. The color produced from this reaction is stable and increases in a proportional manner over a broad range of protein concentrations. The new Pierce 660 nm Protein Assay is very reproducible, rapid, and more linear compared with the Coomassie dye-based Bradford assay. The assay reagent is room temperature stable, and the assay is a simple and convenient mix-and-read format. The assay has a moderate protein-to-protein variation and is compatible with most detergents, reducing agents, and other commonly used reagents. This is an added advantage for researchers needing to determine protein concentrations in samples containing both detergents and reducing agents.

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Accurate measurement of the amount of protein in solution is required to study many biochemical processes. There are currently many methods available for determining the amount of protein in solution. For the determination of relative protein concentration in solution, colorimetric or chromogenic methods are widely used because of their relative simplicity and speed. Currently available methods for the colorimetric determination of protein concentration in solution include the Biuret method [1], the Lowry method [2], the bicinchoninic acid (BCA)[1] assay [3], the Coomassie Blue G-250 dye-binding assay [4], and the colloidal gold protein assay [5].

The Biuret method is based on the complex formation of cupric ions with proteins. Under alkaline conditions, copper(II) ion is bound to peptide nitrogen in proteins and peptides to produce a purple color with an absorption maximum at 550 nm. The sensitivity is 1 to 6 mg protein/ml. So, the Biuret method is somewhat insensitive compared with other methods of colorimetric protein determination. Low amounts of reducing agents and detergents interfere with the assay.

The Lowry method for protein determination relies on the Folin–Ciocalteu reagent to enhance the sensitivity of the Biuret reaction. Under alkaline conditions, Cu^{2+} forms a complex with the peptide bonds of proteins followed by the Folin–Ciocalteu phosphomolybdic–phosphotungstic reduction to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic amino acids. The absorption maximum is at 750 nm, and the method is more sensitive than the Biuret method. The linear sensitivity is 0.1 to 1.5 mg protein/ml of bovine serum albumin (BSA). The disadvantage of the assay is the fact that many reagents (amino acids, detergents, reducing agents, lipids, sugars, and nucleic acids) interfere with the reaction. In addition, the reaction is extremely pH dependent and should be maintained between pH 10.0 and 10.5.

The BCA assay is related to the Lowry assay in that peptide bonds of protein first reduce cupric ion (Cu^{2+}) to produce tetradentate–cuprous ion (Cu^{+}) complex in an alkaline medium. The cuprous ion complex then reacts with BCA (2 molecules per Cu^{+}) to form an intense purple color that can be measured at 562 nm. The BCA assay has many advantages over the Lowry assay. Because BCA is stable in alkaline medium, this assay can be carried out in one step compared with the two steps needed with the Lowry assay. Another advantage of the BCA assay is that it offers more tolerance toward the compounds that interfere with the Lowry assay. In particular, detergents (e.g., sodium dodecyl sulfate [SDS], Triton X-100, Tween 20) up to 5% concentration do not interfere with the assay. The BCA assay also offers increased sensitivity and an expanded linear working range compared with the Lowry method. The disadvantage of the assay is the fact that reducing agents interfere with the assay.

The colloidal gold protein assay is most sensitive (2–20 μg/ml) among colorimetric protein determination methods. Protein binding to the colloidal gold causes a shift in its absorbance that is proportional to the amount of protein added to the assay. Although colloidal gold protein assay is sensitive, the assay has significant protein-to-protein variation. Most common reagents except thiols and SDS are compatible with the assay.

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[1] *Abbreviations used:* BCA, bicinchoninic acid; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; BGG, bovine gamma globulin; IgG, immunoglobulin G; IDCR, ionic detergent compatibility reagent.

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The Coomassie Blue G-250 dye-binding assay is based on the immediate absorbance shift 470 to 595 nm that occurs when Coomassie Brilliant Blue G-250 binds to protein in an acidic medium. The advantage of this assay is that color development is rapid and the assay can be performed in 10 min. Moreover, this assay is comparatively free from interference by common reagents except detergents. However, the assay is prone to inaccuracy in that it typically yields nonlinear standard curves over 1 mg/ml protein concentration and displays more protein-to-protein variability compared with the Lowry and BCA protein assays.

Two other dye-binding protein assays have been reported using Pyrogallol Red [6] and Pyrocatechol Violet [7]. Like the Coomassie Blue G-250 dye-binding assay, both of these dye-binding protein assays have the disadvantage of the difference in sensitivity to various proteins and sensitivity to detergents.

Here we describe a new colorimetric protein assay, the Pierce 660 nm Protein Assay, which is rapid, sensitive, and free from interference by detergents, reducing agents, and other common reagents. The assay is based on a dye–metal (polyhydroxybenzenesulfonephthalein-type dye and a transition metal) complex binding to mainly basic amino acid residues in proteins.

Materials and methods

BSA, bovine gamma globulin (BGG), lysozyme, immunoglobulin G (IgG: rabbit, mouse, and human), ionic detergent compatibility reagent (IDCR), the Coomassie protein assay kit, and the Pierce 660 nm Protein Assay reagent were obtained from Thermo Fisher Scientific (Rockford, IL, USA). Human transferrin, α-lactalbumin (bovine milk), cytochrome *c* (horse heart), ovalbumin, aldolase (rabbit muscle), insulin (bovine pancreas), trypsin inhibitor (soybean), myoglobin (horse heart), poly-L-glutamic acid, poly-L-aspartic acid, poly-L-histidine, and poly-L-arginine were obtained from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were of analytical reagent standard or the best grade available. Standard protein solutions were prepared in saline containing 0.05% sodium azide.

Standard assay procedure

To each test tube containing 0.1 ml of each protein standard replicate (25, 50, 125, 250, 500, 750, 1000, 1500, and 2000 μg/ml, 2.5–200 μg) in saline, 1.5 ml of assay reagent solution (as received from Thermo Fisher Scientific) was added, mixed well, and incubated at room temperature (22 °C) for 5 min. The absorbance values of all the samples and controls were measured at 660 nm against water in a Varian Cary spectrophotometer. The average absorbance at 660 nm for the control was subtracted from absorbance at 660 nm for all other individual standard replicates. A standard curve was generated by plotting the average blank-corrected 660 nm measurement for each standard versus its concentration in micrograms (μg).

The Coomassie-based Bradford protein assay was performed using 0.03 ml of protein standard and 1.5 ml of dye reagent. The absorbance values of all the samples and controls were measured at 595 nm following 10 min incubation at room temperature.

Interfering substances evaluation

Each substance was assayed in duplicate using the standard assay procedure. In addition to adding the substance to a sample containing 1000 μg/ml BSA (100 μg total protein), a blank sample containing only the substance was tested. The blank-corrected 660 nm absorbance measurements for the 1 mg/ml BSA (100 μg) plus the interfering substance were compared with the net absorbance of the 1 mg/ml BSA prepared in water. To evaluate the compatibility of ionic detergents such as SDS, solid IDCR (an oligosaccharide) was added to the assay reagent to a final concentration of 50 mM and used in the assay. Substances are considered as compatible in the assay if the error in protein concentration estimation of BSA at 1 mg/ml caused by the presence of the substance is $\leqslant$ 10%.

Protein-to-protein variability evaluation

All proteins evaluated were prepared in sets of concentrations from 125 to 2000 μg/ml and were assayed using the standard assay procedure. The average response for BSA at 1 mg/ml was normalized to 1, and the average response to other proteins at 1 mg/ml was expressed as a ratio to the response with BSA.

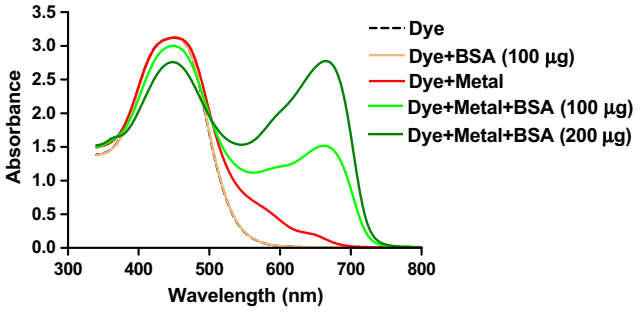

Fig. 1. Spectral analysis. Assay reagent (1.5 ml) was added to 0.1 ml of saline (with and without metal as well as with and without BSA), and the spectra were recorded against water from 340 to 800 nm in a Varian Cary spectrophotometer. (For interpretation of the references to color in the key within this figure, the reader is referred to the Web version of this article).

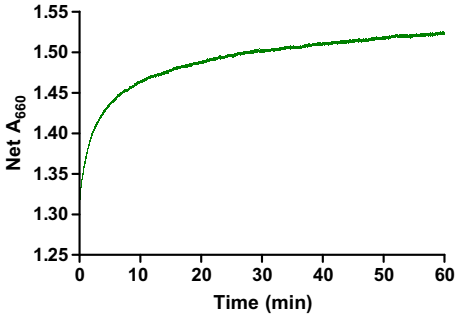

Fig. 2. Kinetics of color development. Protein sample (100 μg of BSA in saline, 0.05% sodium azide) was mixed with 1.5 ml of assay reagent, and the color development was followed at 660 nm.

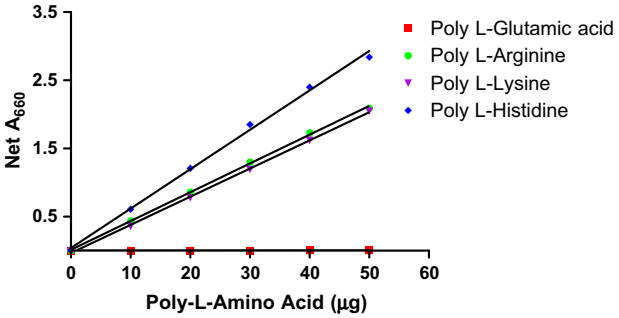

Fig. 3. Response of poly-L-amino acids. Each poly-L-amino acid (10–50 μg) in 0.1 ml of saline, 0.05% sodium azide was mixed with 1.5 ml of assay reagent, and the assay was performed as described in Materials and methods.

Table 1
Protein-to-protein variation.

Protein tested	Pierce 660 nm Protein Assay (ratio)	Coomassie-based Bradford protein assay (ratio)
BSA	1.00	1.00
BGG	0.51	0.56
Human IgG	0.57	0.63
Rabbit IgG	0.38	0.37
Mouse IgG	0.48	0.59
Bovine pancreas insulin	0.81	0.60
Horse heart cytochrome *c*	1.22	1.07
α-Lactalbumin	0.82	1.01
Lysozyme	0.79	0.25
Horse heart myoglobin	1.18	1.19
Soybean trypsin inhibitor	0.38	0.39
Ovalbumin	0.54	0.32
Human transferrin	0.80	0.84
Aldolase	0.83	0.76
Average ratio	0.7364	0.6842
Standard deviation	0.2725	0.3014
Coefficient of variation	37%	44%

Note. All of the proteins assayed were prepared in sets of standard concentrations from125 to 2000 μg/ml using the standard assay procedure. The average response for BSA at 1 mg/ml was normalized to 1, and the average response to other proteins at 1 mg/ml was expressed as a ratio to the response with BSA.

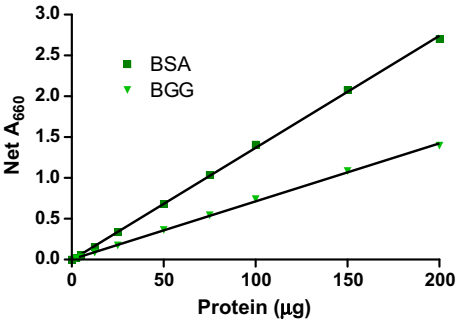

Fig. 4. Typical color response curves using the standard assay procedure. Protein stock solutions (25, 50, 125, 250, 500, 750, 1000, 1500, and 2000 μg/ml) were prepared in saline, 0.05% sodium azide. To each test tube containing 2.5 to 200 μg of each protein standard replicate in 0.1 ml of saline, 1.5 ml of assay reagent solution was added and the assay was performed as described in Materials and methods.

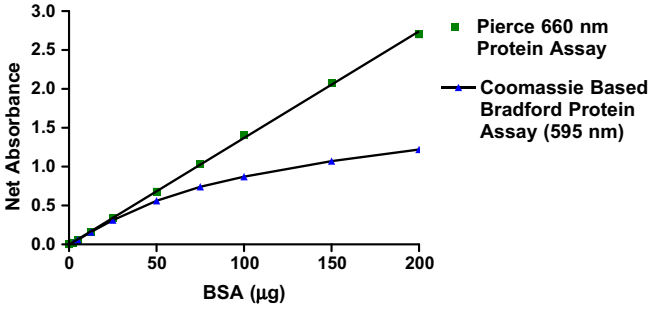

Fig. 5. Comparison between Pierce 660 nm Protein Assay and Coomassie-based Bradford protein assay. Protein stock solutions (25, 50, 125, 250, 500, 750, 1000, 1500, and 2000 μg/ml) were prepared in saline, 0.05% sodium azide. To each test tube containing 2.5 to 200 μg of each protein standard replicate in 0.1 ml or 0.03 ml (for Coomassie assay) of saline, 1.5 ml of assay reagent solution was added and the assay was performed as described in Materials and Methods.

Table 2
Substances compatible with the Pierce 660 nm Protein Assay.

Substance	Maximum compatible concentration
Detergents	
Tween 20	10%
Triton X-100	1%
Octyl-β-thioglucopyranoside	10%
CHAPS	5%
CHAPSO	4%
NP-40	5%
Octyl-β-glucoside	5%
Brij-35	5%
Sodium deoxycholate	0.25%
SDS	0.0125 and 5%[a]
Cetyltrimethylammonium bromide	2.5%[a]
Cetylpyridinium chloride	2.5%[a]
Dodecyltrimethylammonium bromide	2%[a]
Reducing agents	
Dithiothreitol	500 mM
β-Mercaptoethanol	1 M
L-Cysteine	350 mM
Ascorbic acid	500 mM
Tris(2-carboxyethyl)phosphine	40 mM
Glutathione (reduced)	100 mM
Buffers	
Phosphate-buffered saline	0.1 M sodium phosphate and 0.15 M NaCl (pH 7.2)
HEPES (pH 7.5)	100 mM
Tris–HCl (pH 8.0)	250 mM
Imidazole (pH 7.0)	200 mM
Glycine Buffer (pH 2.8)	100 mM
Mops (pH 7.2)	125 mM
MES (pH 6.1)	125 mM
Chelating agents	
Ethylenediaminetetraacetic acid	20 mM
Ethyleneglycoltetraacetic acid	20 mM
Salts/Solvents	
NaCl	1.25 M
GuHCl	2.5 M
Urea	8 M
Thiourea	2 M
Glycerol	50%
Sucrose	50%
Acetonitrile	50%
Methanol	50%
Phenol red	0.5 mg/ml
NaOH	125 mM
HCl	125 mM
Lysis buffers	
Two-dimensional sample buffer for soluble and insoluble proteins	8 M urea, 4% Chaps, and 7 M urea, 2 M thiourea, 4% Chaps
Radioimmunoprecipitation buffer	50 mM Tris–HCl, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, and 1 mM ethylenediaminetetraacetic acid
Laemmli SDS sample buffer	63 mM Tris–HCl, 10% glycerol, 2% SDS, and 0.0025% bromophenol blue

Note. Each substance was assayed in duplicate using the standard procedure. In addition to adding the substance to a sample containing 1000 μg/ml (100 μg total protein) BSA, a blank sample containing only the substance was tested. Substances are considered as compatible if the substance produced a concentration estimation error of ⩽10%. The blank-corrected 660 nm absorbance measurements for 1 mg/ml BSA (100 μg) standard with the substance were compared with the net 660 nm absorbance of 1 mg/ml BSA standard prepared in water.

[a] In the presence of 50 mM IDCR. Solid IDCR was added to the assay reagent to a final concentration of 50 mM and used in the assay.

Results and discussion

Every protein assay has limitations depending on the application and the specific protein sample analyzed. The most useful fea-

tures to consider when choosing a protein assay are sensitivity (lower detection limit), compatibility with common substances in samples (e.g., detergents, reducing agents, chaotropic agents, inhibitors, salts, buffers), standard curve linearity, and protein-to-protein variation. The new Pierce 660 nm Protein Assay is based on the binding of a polyhydroxybenzenesulfonephthalein-type dye and a transition metal complex to proteins in acidic conditions that causes a shift in the dye–metal's absorption maximum from 450 to 660 nm. The dye–metal complex is reddish brown and changes to green on protein binding. The color produced in the assay is stable and increases in proportion to a broad range of increasing protein concentrations. To demonstrate the effect of protein binding on the dye–metal complex, we performed spectral analysis of the dye with and without metal and in the presence and absence of BSA. As shown in Fig. 1, the absorption maximum of the dye–metal complex shifts proportionally on binding to BSA. The absorbance at 660 nm was found to be optimal for the measurement of protein concentrations. The color development is fast, taking approximately 5 min. The end-point color stability of the assay was assessed by performing the assay and observing any further absorbance changes at 660 nm. Fig. 2 shows the rate of formation of protein–dye–metal complex in the assay system and the stability of the color complex. As seen from the graph, the color development is essentially complete at 5 min and showed a very little upward drift of 0.12% per minute. This indicates that the color developed is fairly stable over a period of 1 h at room temperature. The color change in the assay is produced by deprotonation of dye at low pH facilitated by protein-binding interactions through positively charged amino acid groups and the negatively charged dissociated dye–metal complex. As shown in Fig. 3, the assay reagent showed a significant response to basic amino acids in proteins with little or no response to acidic amino acids, indicating that the interaction is electrostatic.

Dye-based protein determination assays are widely used to estimate protein concentration; however, various reports suggest that the response is dependent on the composition and sequence of the protein, limiting confidence in the resulting concentration estimates [8,9]. Still, all of these assays are useful to determine the relative amount of protein in different solutions. These assays are also useful for mixtures of proteins such as whole cell lysates where individual differences in protein levels are averaged. Each protein in a complex sample is unique, and this is manifested in protein assays as variation in the color response. Such protein-to-protein variation refers to differences in the amount of color (absorbance) obtained when the same mass of various proteins is assayed. These differences in color response relate to differences in amino acid sequence, isoelectric point, secondary structure, and the presence of certain side chains or prosthetic groups. The color response for 14 different proteins was tested using the standard assay procedure, and the results indicate that the assay has moderate protein-to-protein variation similar to Coomassie dye-based assays (Table 1). Color development is significantly greater with BSA than with most other proteins, including BGG. Therefore, BSA is a suitable standard if the sample contains primarily albumin or if the protein being assayed has a response to the dye similar to that of BSA. For color response that is typical of globulins, BGG is an appropriate standard protein. The linear detection ranges are 25 to 2000 μg/ml and 50 to 2000 μg/ml using BSA and BGG proteins, respectively (Fig. 4). The assay can also be done in the microplate format using 10 μl of sample and 150 μl of assay reagent (data not shown). As shown in Fig. 5, the assay is more linear compared with the Coomassie dye-based Bradford protein assay.

The potentially interfering compounds selected for inclusion in this study were chosen on the basis of being frequently encountered during the purification and isolation of proteins. As shown in Table 2, the Pierce 660 nm Protein Assay is compatible with high concentrations of most detergents, reducing agents, and other commonly used reagents. In addition, by simply adding IDCR (an oligosaccharide with the ability to bind and mask hydrophobic molecules) to the assay reagent to a final concentration of 50 mM, the assay is compatible with samples containing Laemmli SDS sample buffer and many other common ionic detergents. The addition of IDCR to the assay reagent increased the compatible concentration of SDS from 0.0125 to 5%. IDCR itself does not have any effect on the assay (data not shown). Because detergents and reducing agents are frequently used for solubilizing and stabilizing proteins, this assay has a significant advantage over the Coomassie dye-based assay in that this assay is compatible with detergents at concentrations commonly used by researchers.

In conclusion, the Pierce 660 nm Protein Assay is a detergent- and reducing agent-compatible protein assay that is linear over a wide range of concentrations. The simple mix-and-read format is easy to use, providing researchers with a fast and reliable method for protein quantitation.

References

[1] A.G. Gornall, Determination of serum proteins by means of the biuret reaction, J. Biol. Chem. 177 (1949) 751–766.
[2] O.H. Lowry, Protein measurement with folin–phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[3] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, Anal. Biochem. 150 (1985) 76–85.
[4] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. Biochem. 72 (1976) 248–254.
[5] C.M. Stoscheck, Protein assay sensitive at nanogram levels, Anal. Biochem. 160 (1987) 301–305.
[6] N. Watanabe, S. Kamel, A. Ohkubo, M. Yamanaka, S. Ohsawa, K. Makino, K. Tokuda, Urinary protein as measured with a pyrogallol red–molybdate complex manually and in a Hitachi 726 automated analyzer, Clin. Chem. 32 (1986) 1551–1554.
[7] Y. Fujita, I. Mori, S. Kitano, Determination of proteins by using the color reaction with pyrocatechol violet–molybdenum (VI) complex, Chem. Pharm. Bull. 32 (1984) 4161–4164.
[8] R. I. Krohn, The colorimetric detection and quantitation of total protein, in: Current Protocols in Cell Biology, appendix 3H, John Wiley, New York, 2003, pp. B1.1.1–B1.1.28.
[9] C.M. Stoscheck, Quantitation of protein, Methods Enzymol. 182 (1990) 50–68.