Frederick National Laboratory for
Cancer Research, Frederick, MD

Biopharmaceutical Development Program

Standard Operating Procedure

Title: Determination of Protein Concentration by the Pierce Coomassie Plus Assay

SOP Number: 22164

Revision Number: 05

Supersedes: Revision 04

Effective Date: APR 16 2020

Originator/Date: [redacted]

Approval/Date:

Approval/Date:

Approval/Date:

Table of Contents

1.0 Purpose

This procedure determines the concentration of a protein solution based upon the absorbance shift from 465 to 595 nm that occurs when the Coomassie® Plus reagent binds to proteins in acidic solution.

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted].

2.0 Scope

This SOP applies to Process Analytics/Quality Control (PA/QC) and Manufacturing Personnel who perform the Pierce Coomassie Plus Assay to determine Protein Concentration.

3.0 Authority and Responsibility

3.1 The Director, Process Analytics/Quality Control (PA/QC) has the authority to define this procedure.

3.2 The Director, PA/QC and Associate Director, Manufacturing Operations are responsible for training personnel in this procedure and for documenting this training to Biopharmaceutical Quality Assurance (BQA).

3.3 PA/QC and Biopharmaceutical Development Program (BDP) Manufacturing Operations personnel are responsible for the implementation of this procedure.

3.4 BQA is responsible quality oversight of this procedure.

4.0 Equipment and Materials

4.1 Coomassie® Plus Protein Assay Reagent, Pierce, BDP PN 30055.

4.2 2 mg/mL Bovine Serum Albumin Standard, Pierce, BDP PN 30060.

NOTE: For analysis of monoclonal antibodies, use 2 mg/mL Bovine Gamma Globulin (Pierce, BDP PN 30059) as the standard.

4.3 Phosphate Buffered Saline, BDP PN 30007 or other appropriate diluent solution, i.e., the formulation buffer of the test sample.

4.4 Borosilicate glass culture tubes 12 x 75 mm BDP PN 20143, and 16 x 125 mm, BDP PN 20144 or BDP approved equivalent.

4.5 Glass Beaker

4.6 Disposable Pasteur Pipette, BDP PN 20843 or BDP approved equivalent.

4.7 Calibrated Pipettes

4.8 Timer, NIST Traceable

4.9 Thermometer, ± 2ºC tolerance or better

4.10 Pipette tips, BDP PN 25006 and 25007 or BDP approved equivalent.

4.11 Gloves, BDP PN 20766 or BDP approved equivalent.

4.12 Eppendorf Repeater Pipette and Combitips 25mL, BDP PN 21406, or BDP approved equivalent.

4.13 Beckman DU 800 Spectrophotometer for PA/QC assays or BDP approved spectrophotometer for manufacturing operations.

4.14 Polystyrene Disposable 1.5 mL Cuvettes, BDP PN 20070 or BDP approved equivalent.

4.15 Vortex Mixer

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted].

5.0 Procedure

5.1 Sample Protocol for Test Tube Procedure

NOTE: For determining protein concentrations in the range of 125 μg/mL to 1000 μg/mL, refer to the instruction manual on variations of the standard protocol. The range may be expanded as needed for manufacturing operations, or in process, and FIO samples.

5.1.1 Turn on the spectrophotometer and set to 595 nm.

5.1.2 Turn on the VIS lamp.

NOTE: Refer to ***SOP 22941 - Operation of the Beckman Coulter DU 800 Spectrophotometer*** or the appropriate SOP for the spectrophotometer to be used for the operation instructions of the spectrophotometer.

5.1.3 Mix the Coomassie® Plus reagent in bottle by gentle inversion. Decant enough reagent for the standards and samples into a glass beaker with overage as needed for the repeater pipet and for any potential additional dilutions (example: 25 mL reagent for standard curve + 5 mL per sample dilution x 1.5 = 45 mL). Allow the reagent to acclimate to room temperature prior to use.

5.1.4 Prepare a known protein concentration series by diluting a stock bovine serum albumin (BSA) standard (or other protein standard) in the same diluent as the protein sample whose concentration is to be determined. The protein standard series should cover the range of concentration between 125 and 1000 µg/mL. Convenient, standard concentration data points are 125, 250, 500, 750 and 1000 µg/mL. Prepare them as follows in triplicate in 12 x 75 mm Borosilicate glass tubes or per Batch Production Records (BPR's) directions.

NOTE: For PA/QC personnel to complete the assay:

One standard curve can be used continually for one working day as needed for testing associated with bulk product concentration, diafiltration steps, time course studies, etc. Once a standard curve has been generated it can be used throughout the course of 1 working day if the same buffer and same reagent lot are used throughout analysis.

A new standard curve should be generated with any subsequent analysis in the event of excessive laboratory temperature fluctuations (± 3ºC) as this can affect the rate of the colorimetric reaction. It is important to match the incubation time of the sample to that of the standard curve as closely as possible (Example: If the standard curve was measured after incubating at room temperature for 11 minutes then the sample incubation should be as close to 11 minutes as possible, ± 2 minutes with a minimum of 10 minutes or 10-13 minutes).

Samples and standards must be incubated 10-15 minutes prior to measuring the absorbance at 595 nm (See minimum incubation time in section 7.0) unless it has been determined that a specific sample, buffer or combination thereof require different incubation durations.

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted].

Volume of the BSA to Add	Volume of Diluent to Add	Final BSA Concentration
175 µL of (Stock)	175 µL	1,000 µg/mL (A)
75 µL of (Stock)	125 µL	750 µg/mL (B)
175 µL of (A)	175 µL	500 µg/mL (C)
175 µL of (C)	175 µL	250 µg/mL (D)
175 µL of (D)	175 µL	125 µg/mL (E)

5.1.5 Dilute test samples so they fall within the working range of the standard curve (125 µg/mL – 1000 µg/mL). Test samples are prepared in 12 x 75 mm glass tubes in triplicate or per BPR directions.

NOTE: Additional dilutions may be performed as needed by manufacturing personnel for use in BPR's with in-process and FIO samples. Document any additional dilution used in the BPR.

5.1.6 Pipet 0.05 mL dilute standard, unknown protein sample, and sample diluent, labeled by concentration for the standard and by dilution factor for the unknown, into large 16 x 125 mm Borosilicate glass tubes.

5.1.7 Add 1.5 mL Coomassie® Plus Reagent using the Eppendorf Repeater Pipette. Mix well by vortexing the samples briefly. Add the reagent in the same order that the standards and samples will be read.

5.1.8 Incubate samples for 10 – 15 minutes at ambient temperature. Record the laboratory temperature and incubation time on form 22164-01 for the standard curve and for the samples.

If samples are analyzed separately from the standard curve:

- Maintain a sample incubation within 3 minutes (≥ 10 minute minimum) of the standard curve.
- Confirm that the laboratory temperature is within 3°C from when the standard curve was run.

5.1.9 Blank the spectrophotometer using the sample diluent "blank," followed by triplicate readings (for PA/QC assays) of each standard and unknown at 595 nm.

5.1.10 Prepare a standard curve by plotting the average net absorbance at 595 nm for each dilute BSA or protein standard. Using the standard curve, determine the protein concentration for each unknown protein sample.

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted].

NOTE: The software of the Beckman spectrophotometer can be programmed to automatically plot the standard curve and unknown values as per ***SOP 22941 - Operation of the Beckman Coulter UV Spectrophotometer.*** Use a linear, zero-intercept curve fit. Excel (or another spreadsheet program) can also be used to create a standard curve if the Beckman spectrophotometer is not being used for the assay or if the software is unavailable.

5.1.11 Print the standard curve and the calculated values for the unknown samples. The printouts must be initialed and dated by the analyst and attached to the QC Request Form. Manufacturing personnel will attach the initialed and dated printout to the BPR.

5.1.12 A valid assay consists of a correlation coefficient of ≥ 0.98 for manufacturing operations, as well as coefficient of variation (CV) of ≤ 5.0% for all standards and unknowns. Report results that fall between the working range of the standards used.

5.1.13 For PA/QC personnel to complete the assay, the data should be saved to the appropriate network folder. To save the acquired data, click the Save File button. Once the Save File dialog box appears, select “Scidata (S:) from the drop-down box in order to save the data on the network. Click on the PA Public Folder. Data is saved in the Du 800 Data Folder using an appropriate filename. Typically, the data is saved using the QC Test Request number. Click on the OK button to save the data.

5.2 Sample Protocol for Low Concentration Test Tube Procedure

The low concentration test tube procedure is employed for determining the protein concentration of samples just below or at the lower end of the standard assay ≤ 250 ug/mL. Use Form 22164-03 for this procedure. See note in section 5.1.4 regarding standard curve guidelines.

5.2.1 A standard curve is prepared as follows:

Standard Dilutions:

Standard	Vol. of Diluent	Vol. of Bovine Std
(A) 500 μg/mL	225 μL	75 μL of (Stock)
(B) 250 μg/mL	150 μL	150 μL of (A)
(C) 125 μg/mL	150 μL	150 μL of (B)
(D) 62.5 μg/mL	150 μL	150 μL of (C)
(E) 31.3 μg/mL	150 μL	150 μL of (D)

5.2.2 The blank is the sample buffer diluent only. Blank the spectrophotometer using the sample diluent prior to measuring the standard curve.

5.2.3 Triplicate 50 uL aliquots of each prepared standard (as prepared in the table above) and sample are transferred to 12 x 75 mm Borosilicate glass culture tubes.

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted].

5.2.4 Using a repeater pipet, aliquot 1.0 mL of Pierce Coomassie Plus reagent to each standard tube.

5.2.5 Vortex each tube briefly and incubate at room temperature for 10-15 minutes. Record the laboratory temperature and incubation time on form 22164-03 for the standard curve and for the samples. If samples are to be analyzed separately from the standard curve, confirm that the laboratory temperature is within 3°C from when the standard curve was run.

5.2.6 Transfer standards and samples to Polystyrene Disposable 1.5 mL Cuvettes.

5.2.7 Measure the absorbance at 595 nm using a Non-linear (quadratic) curve fit.

5.2.8 A valid assay consists of coefficient of variation (CV) of ≤ 5.0% for all standards and unknowns ≥ 31.3 ug/mL. Report results that fall between the standards used for the assay.

5.2.9 Follow section 6.0, using Form 22164-03.

6.0 Documentation

6.1 BQC Documentation

6.1.1 Record the results from this procedure on the QC Test Request, Form 22001-01. Attach all raw data printouts from this procedure to the QC Test Request Form.

6.1.2 Record the sample preparation on Form 22164-01 Total Protein Sample Preparation (Attachment I) or Form 22164-03 LOW CONCENTRATION - Total Protein Sample Preparation (Attachment II). Attach the form to the QC Test Request and submit to PA/QC and BQA review.

6.1.3 Record all use of the UV/VIS in the Equipment Logbook, refer to ***SOP 21531 - Equipment/Facility Logbooks***.

6.2 Manufacturing Operations Documentation

6.2.1 Record results from this procedure in the BPR. Attach the spectrophotometer printout to appropriate location in the BPR.

6.2.2 Enter standard curve data into a spreadsheet program and create the standard curve if appropriate. Print this curve and attach to the appropriate location in the BPR.

6.2.3 If needed, record standard curve preparation on Form 22164-02 Standard Curve Determination (Attachment 3). Attach the form to the BPR if required.

6.2.4 Record all use of the spectrophotometer in the Equipment Logbook, refer to ***SOP 21531 - Equipment/Facility Logbooks***.

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted].

7.0 Assay and Sample Considerations

7.1 Protein-to-Protein Variation

All proteins are unique, and their varying structures can give variable responses. Each of the commonly used assay methods exhibits some degree of varying color response toward different proteins. These differences relate to dissimilarity among proteins due to amino acid sequence, pI, structure, and the presence of certain side chains or prosthetic groups that can dramatically alter the protein's color response.

Most protein assay methods utilize bovine serum albumin (BSA) or immunoglobulin (IgG) as the standard against which the concentration of protein in the sample is determined. Using either of these proteins as the standard works well in most assay methods. However, if great accuracy is required, the standard curve must be prepared from a pure sample of the target protein to be measured. If a pure sample of the target protein is not available, select the standard protein from those proteins that generate a color response that is close to the color response of the target protein.

7.2 Effect of Temperature on the 595 nm Readings

The absorbance readings at 595 nm obtained with the Coomassie® Plus Reagent are dependent on the temperature of the reagent to some extent. As the reagent temperature increases to room temperature, the 595 nm readings will increase. Therefore, it is important that the Coomassie® Plus Reagent be at room temperature during the assay.

7.3 Dye-dye and Dye-protein Aggregate Formation in the Coomassie® Plus Reagent

The Coomassie® Plus Protein Assay Reagent contains additives that help to slow down the formation of dye-dye and dye-protein aggregates that occur in all Coomassie® dye-based protein reagents. Over time, if left undisturbed, the aggregates will become large enough to be easily visible. When left overnight in a clear glass tube, the reagent forms a dye-dye aggregate that is visible as a dark precipitate of dye in the bottom of the tube with nearly colorless liquid over the dye aggregate. The dye-dye aggregates can form within hours in the stored reagent while the dye-protein-dye aggregates seem to form even more quickly. Fortunately, gentle mixing completely disperses the aggregates. Therefore, it is good practice, with all Coomassie® based protein assay reagents, to mix the Coomassie® Plus Reagent prior to pipetting and to again mix each tube or plate just before the readings are taken.

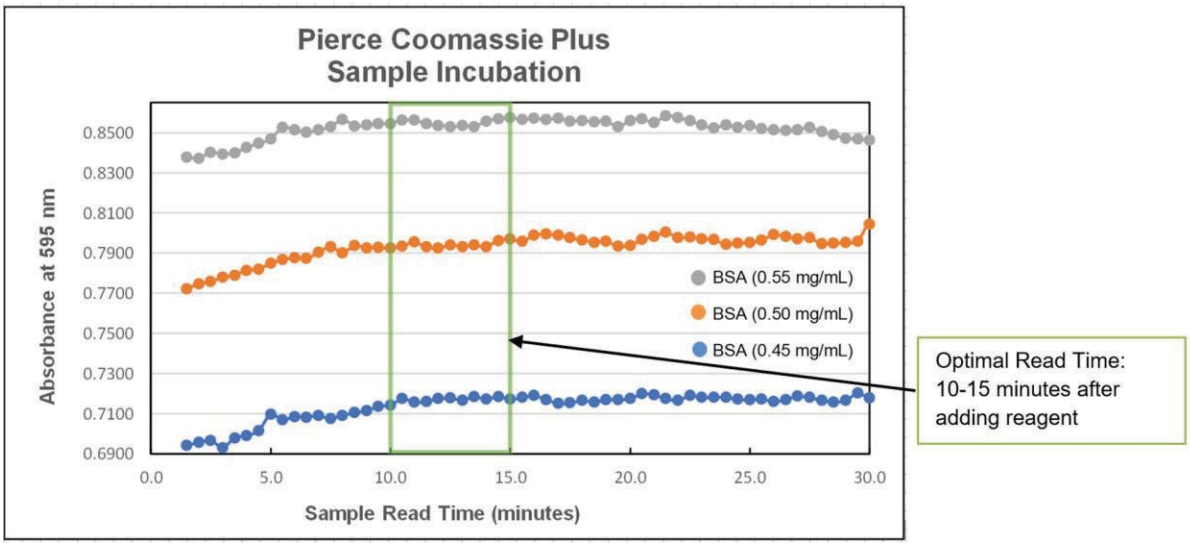
7.4 Substances Known to Interfere

Certain substances are known to interfere with Coomassie® based protein assays including most ionic and nonionic detergents, which reduce color development and can cause precipitation of the assay reagents. For strategies for eliminating or minimizing the effects of interfering substances and for a list of compatible substances concentrations in the Coomassie Plus Assay, see the Coomassie Plus Kit Instruction Manual.

7.5 Minimum Incubation Time

Standards and samples must be incubated at room temperature for 10-15 minutes prior to measuring the absorbance at 595 nm to allow complete color development.

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted].



8.0 References

8.1 Instruction Manual, Coomassie® Plus Protein Assay Reagent Kit.

8.2 Protein Assay, Principles of Operation/Standards Mode, Beckman Coulter DU 800 Manual

8.3 **SOP 11102** – *Operation of the Genesys 2 Spectrophotometer*

8.4 **SOP 14132 –** *Operation of the Beckman DU Series 600 Spectrophotometer at the* [redacted]

8.5 **SOP 22158 –** *Operation of the Beckman DU Series 600 Spectrophotometer*

8.6 **SOP 22941** – *Operation of the Beckman Coulter DU 800 Spectrophotometer*

8.7 **SOP 22946 –** *The Operation of the Nanodrop 1000 Spectrophotometer*

8.8 **SOP 21531 –** *Equipment/Facility Logbooks*

9.0 Attachments

9.1 **Attachment 1** Form 22164-01, Total Protein Sample Preparation

9.2 **Attachment 2** Form 22164-03, LOW CONCENTRATION – Total Protein Sample Preparation

9.3 **Attachment 3** Form 22164-02, Standard Curve Determination – Manufacturing Operations

9.4 **Attachment 4** Coomassie Plus (Bradford) Assay Reagent

9.5 **Attachment 5** Protein Assays, Standard Mode

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted].

Attachment 1 (Page 1 of 2)

Form 22164-01, Total Protein Sample Preparation

FNLCR, BDP
Form No.: 22164-01
SOP No.: 22164
Revision 05 APR 16 2020

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Total Protein Sample Preparation

QCTR Number: _____ Analyst: _____ Date: _____
Spectrophotometer MEF #: _____ Calibration Due Date: _____
Bovine Standard: _____ Lot# _____ Exp. Date: _____
Buffer/Diluent: _____ Lot# _____ Exp. Date: _____
Coomassie Lot#: _____ Exp. Date: _____
Standard Curve: Laboratory Temperature _____ °C Incubation time _____ minutes
Samples: Laboratory Temperature _____ °C Incubation time _____ minutes
Thermometer ID# _____ Cal Due Date: _____

Standard Dilutions:

Standard	Vol. of Diluent	Vol. of Bovine Std	Average %CV (≤ 5.0%)
(A) 1000ug/mL	175µL	175µL (Stock)	_____
(B) 750ug/mL	125µL	75µL (Stock)	_____
(C) 500ug/mL	175µL	175µL of (A)	_____
(D) 250ug/mL	175µL	175µL of (C)	_____
(E) 125ug/mL	175µL	175µL of (D)	_____

Valid Assay Acceptable Criteria:

Correlation Coefficient (R): ≥ 0.98 Pass / Fail
Coefficient of Variation (%CV): ≤ 5.0% for each Std Pass / Fail

Sample Dilutions:

Test Sample #1
Name: _____
Lot # _____
Protein Concentration: _____ mg/mL
Buffer: _____
Lot# _____ Exp. Date: _____

Dilution: _____
Volume of Diluent: _____ µL
Volume of Test Sample: _____ µL

Mean Result: _____ µg/mL
Protein Concentration = _____ mg/mL

Test Sample #2
Name: _____
Lot # _____
Protein Concentration: _____ mg/mL
Buffer: _____
Lot# _____ Exp. Date: _____

Dilution: _____
Volume of Diluent: _____ µL
Volume of Test Sample: _____ µL

Mean Result: _____ µg/mL
Protein Concentration = _____ mg/mL

Acceptable Criteria:

%CV (≤ 5.0%): Pass / Fail

%CV (≤ 5.0%): Pass / Fail

Performed by: _____ Date: _____
Reviewed by: _____ Date: _____

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted].

Attachment 1 (Continued Page 2 of 2)

Form 22164-01, Total Protein Sample Preparation

FNLCR, BDP
Form No.: 22164-01
SOP No.: 22164
Revision 05 APR 16 2020

Sample Dilutions:

Test Sample #3	Test Sample #4
Name: _____	Name: _____
Lot # _____	Lot # _____
Protein Concentration: _____mg/mL	Protein Concentration: _____mg/mL
Buffer:_____	Buffer:_____
Lot# _____ Exp. Date:_____	Lot# _____ Exp. Date:_____
Dilution: _____	Dilution: _____
Volume of Diluent: _____µL	Volume of Diluent: _____µL
Volume of Test Sample: _____µL	Volume of Test Sample: _____µL
Mean Result:_____µg/mL	Mean Result:_____µg/mL
Protein Concentration =_____mg/mL	Protein Concentration =_____mg/mL

Acceptable Criteria:

%CV (≤ 5.0%): Pass / Fail	%CV (≤ 5.0%): Pass / Fail

Comments:

Performed by: _____ Date: _____
Reviewed by: _____ Date: _____

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted].

Attachment 2 (Page 1 of 2)
Form 22164-03, LOW CONCENTRATIO N – Total Protein Sample Preparation

FNLCR, BDP
Form No.: 22164-03
SOP No.: 22164
Revision 05: APR 16 2020

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LOW CONCENTRATION - Total Protein Sample Preparation

QCTR Number: _____ Analyst: _____ Date: _____
Spectrophotometer MEF #: _____ Calibration Due Date: _____
Bovine Standard: _____ Lot# _____ Exp. Date: _____
Buffer/Diluent: _____ Lot# _____ Exp. Date: _____
Coomassie Lot#: _____ Exp. Date: _____
Standard Curve: Laboratory Temperature _____ °C Incubation time _____ minutes
Samples: Laboratory Temperature _____ °C Incubation time _____ minutes
Thermometer ID# _____ Cal Due Date: _____

Standard Dilutions:

Standard	Vol. of Diluent	Vol. of Bovine Std	Average %CV (≤ 5.0%)
(A) 500 µg/mL	225 µL	75 µL of (Stock)	_____
(B) 250 µg/mL	150 µL	150 µL of (A)	_____
(C) 125 µg/mL	150 µL	150 µL of (B)	_____
(D) 62.5 µg/mL	150 µL	150 µL of (C)	_____
(E) 31.3 µg/mL	150 µL	150 µL of (D)	_____

50uL Sample + 1000uL Pierce Coomassie Plus Reagent
Blank on sample diluent without Pierce Coomassie Plus Reagent
Non-Linear Curve Fit

Valid Assay Acceptance Criteria:

Coefficient of Variation (%CV): ≤ 5.0% for each Standard Pass / Fail

Sample Dilutions:

Test Sample #1

Name: _____
Lot # _____
Protein Concentration: _____ mg/mL
Buffer: _____
Lot# _____ Exp.Date: _____
Dilution: _____
Volume of Diluent: _____ µL
Volume of Test Sample: _____ µL

Mean Result: _____ µg/mL
Protein Concentration = _____ mg/mL

Test Sample #2

Name: _____
Lot # _____
Protein Concentration: _____ mg/mL
Buffer: _____
Lot# _____ Exp.Date: _____
Dilution: _____
Volume of Diluent: _____ µL
Volume of Test Sample: _____ µL

Mean Result: _____ µg/mL
Protein Concentration = _____ mg/mL

Acceptance Criteria:

%CV (≤ 5.0%): Pass / Fail

%CV (≤ 5.0%): Pass / Fail

Performed by: _____ Date: _____
Reviewed by: _____ Date: _____

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted].

Attachment 2 (Continued Page 2 of 2))

Form 22164-03, LOW CONCENTRATION – Total Protein Sample Preparation

FNLCR, BDP
Form No.: 22164-03
SOP No.: 22164
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LOW CONCENTRATION - Total Protein Sample Preparation

Sample Dilutions:

Test Sample #3

Name: _____
Lot # _____
Protein Concentration: _____mg/mL
Buffer: _____
Lot# _____ Exp.Date: _____

Dilution: _____
Volume of Diluent: _____µL
Volume of Test Sample: _____µL

Mean Result: _____µg/mL
Protein Concentration = _____mg/mL

Test Sample #4

Name: _____
Lot # _____
Protein Concentration: _____mg/mL
Buffer: _____
Lot# _____ Exp.Date: _____

Dilution: _____
Volume of Diluent: _____µL
Volume of Test Sample: _____µL

Mean Result: _____µg/mL
Protein Concentration = _____mg/mL

Acceptance Criteria:

%CV (≤ 5.0%): Pass / Fail

%CV (≤ 5.0%): Pass / Fail

Comments:

Performed by: _____ Date: _____
Reviewed by: _____ Date: _____

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted].

Attachment 3
Form 22164-02, Standard Curve Determination – Manufacturing Operations

FNLCR, BDP
Form No.: 22164-02
SOP No.: 22164
Revision 05: APR 16 2020

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Standard Curve Determination – Manufacturing Operations

Spectrophotometer MEF:		Cal. Exp. Date:	

	Part Number	BDP Lot Number	Expiration Date
BSA Standard	30060		
Buffer/Diluent			
Coomassie Reagent	30055		

Standard Dilutions:	Standard	Concentration	Recommended Vol. (µL)		Actual Vol. (µL)		Performed by / Date:
			Diluent	BSA Standard	Diluent	BSA Standard	
	A	1000 µg/mL	200	200 (Stock)			
	B	750 µg/mL	125	75 (Stock)			
	C	500 µg/mL	200	200 of (A)			
	D	250 µg/mL	200	200 of (B)			
	E	125 µg/mL	200	200 of (C)			
	F	62.5 µg/mL	200	200 of (D)			

Acceptance Criteria:

Correlation Coefficient (R) ≥ 0.98	Yes / No NA	%CV ≤ 5.0	Yes / No NA

Performed By/Date:		Verified By/Date:	

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted].

Attachment 4 (Page 1 of 8)

INSTRUCTIONS

Coomassie Plus (Bradford) Assay Kit

Thermo SCIENTIFIC

Pub. No. MAN0011203
Rev B.0
Pub. Part No. 2160229.11

23236

Number	Description
23236	**Coomassie Plus (Bradford) Assay Kit**, sufficient reagents for 630 test tube or 3160 microplate assays

Kit Contents:

Coomassie Plus (Bradford) Assay Reagent, 950mL, containing coomassie G-250 dye, methanol, phosphoric acid and solubilizing agents in water; store at 4°C

Caution: Phosphoric acid is a corrosive liquid.

Albumin Standard Ampules, 2mg/mL, 10 × 1mL ampules, containing bovine serum albumin (BSA) at 2.0mg/mL in a solution of 0.9% saline and 0.05% sodium azide; store unopened ampules at room temperature (Available separately as Product No. 23209)

Storage: Upon receipt store each component as indicated. Product shipped at ambient temperature.

Note: Discard any reagent that shows discoloration or evidence of microbial contamination.

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Introduction

The Thermo Scientific™ Coomassie Plus™ Kit is a quick and ready-to-use coomassie-binding, colorimetric method for total protein quantitation. This modification of the well-known Bradford method greatly reduces the tendency of coomassie reagents to give nonlinear response curves by a formulation that substantially improves linearity for a defined range of protein concentration. In addition, the Coomassie Plus Reagent results in significantly less protein-to-protein variation than is observed with other Bradford-type coomassie formulations.

When coomassie dye binds protein in an acidic medium, an immediate shift in absorption maximum occurs from 465nm to 595nm with a concomitant color change from brown to blue. Performing the assay in either test tube or microplate format is simple: Combine a small amount of protein sample with the assay reagent, mix well, incubate briefly and measure the absorbance at 595nm. Protein concentrations are estimated by reference to absorbances obtained for a series of standard protein dilutions, which are assayed alongside the unknown samples.

Pierce Biotechnology 3747 N. Meridian Road | PO Box 117 Rockford, IL 61105 USA | (815) 968-0747 (815) 968-7316 fax | thermofisher.com

This procedure is made available through federal funds from the National Cancer Institute, NIH, under contract [redacted].

Attachment 4 (Continued Page 2 of 8)

Thermo SCIENTIFIC

Preparation of Standards and Assay Reagent

A. Preparation of Diluted Albumin (BSA) Standards

Use Table 1 as a guide to prepare a set of protein standards. Dilute the contents of one Albumin Standard (BSA) ampule into several clean vials, preferably in the same diluent as the sample(s). Each 1mL ampule of Albumin Standard is sufficient to prepare a set of diluted standards for either working range suggested in Table 1. There will be sufficient volume for three replications of each diluted standard.

Table 1. Preparation of Diluted Albumin (BSA) Standards

Dilution Scheme for Standard Test Tube and Microplate Protocols (Working Range = 125–1500µg/mL)

Vial	Volume of Diluent	Volume and Source of BSA	Final BSA Concentration
A	0	300µL of Stock	2000µg/mL
B	125µL	375µL of Stock	1500µg/mL
C	325µL	325µL of Stock	1000µg/mL
D	175µL	175µL of vial B dilution	750µg/mL
E	325µL	325µL of vial C dilution	500µg/mL
F	325µL	325µL of vial E dilution	250µg/mL
G	325µL	325µL of vial F dilution	125µg/mL
H	400µL	100µL of vial G dilution	25µg/mL
I	400µL	0	0µg/mL = Blank

Dilution Scheme for Micro Test Tube or Microplate Protocols (Working Range = 1–25µg/mL)

Vial	Volume of Diluent	Volume and Source of BSA	Final BSA Concentration
A	3555µL	45µL of Stock	25µg/mL
B	6435µL	65µL of Stock	20µg/mL
C	3970µL	30µL of Stock	15µg/mL
D	3000µL	3000µL of vial B dilution	10µg/mL
E	2500µL	2500µL of vial D dilution	5µg/mL
F	1700µL	1700µL of vial E dilution	2.5µg/mL
G	4000µL	0	0µg/mL = Blank

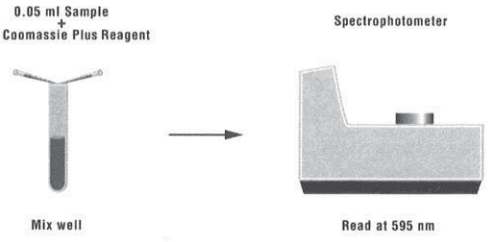
B. Mixing and Equilibrating the Coomassie Plus Reagent

Mix the Coomassie Plus Reagent solution immediately before use by gently inverting the bottle several times (Do not shake the bottle to mix the solution). Remove the amount of reagent needed and equilibrate it to room temperature (RT) before use.

Note: Coomassie Plus Reagent contains additives that retard formation of dye-dye and dye-protein aggregates that tend to form in all coomassie-based protein assay reagents. If left undisturbed, the aggregates will become large enough over time to be visible. For example, when left overnight in a clear glass tube, the reagent forms dye-dye aggregates that are visible as a dark precipitate in the bottom of the tube with nearly colorless liquid above. Dye-dye aggregates can form over several hours in stored reagent while dye-protein-dye aggregates form within seconds. Fortunately, gentle mixing completely disperses the aggregates. Therefore, it is good practice to mix the Coomassie Plus Reagent before dispensing and to mix each tube or plate immediately before measuring absorbances.

Procedure Summary

(Standard Test Tube Protocol):



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Test Tube Procedures

A. Standard Test Tube Protocol (Working Range = 125-1500µg/mL)

Note: The linear working range with BSA = 125-1000µg/mL; the linear working range with IgG = 125-1500µg/mL.

1. Pipette 0.05mL of each standard or unknown sample into appropriately labeled test tubes.
2. Add 1.5mL of the Coomassie Plus Reagent to each tube and mix well.
3. Optional: For the most consistent results, incubate samples for 10 minutes at room temperature (RT).
4. With the spectrophotometer set to 595nm, zero the instrument on a cuvette filled only with water. Subsequently, measure the absorbance of all the samples.
5. Subtract the average 595nm measurement for the Blank replicates from the 595nm measurements of all other individual standard and unknown sample replicates.
6. Prepare a standard curve by plotting the average Blank-corrected 595nm measurement for each BSA standard vs. its concentration in µg/ml. Use the standard curve to determine the protein concentration of each unknown sample.

B. Micro Test Tube Protocol (Working Range = 1-25µg/mL)

1. Pipette 1.0mL of each standard or unknown sample into appropriately labeled test tubes.
2. Add 1.0mL of the Coomassie Plus Reagent to each tube and mix well.
3. Optional: For the most consistent results, incubate samples for 10 minutes at room temperature (RT).
4. With the spectrophotometer set to 595nm, zero the instrument on a cuvette filled only with water. Subsequently, measure the absorbance of all the samples.
5. Subtract the average 595nm measurement for the Blank replicates from the 595nm measurements of all other individual standard and unknown sample replicates.
6. Prepare a standard curve by plotting the average Blank-corrected 595nm 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 Procedures

A. Standard Microplate Protocol (Working Range = 100-1500µg/mL)

1. Pipette 10µL of each standard or unknown sample into the appropriate microplate wells (e.g., Thermo Scientific™ Pierce™ 96-Well Plates, Product No. 15041).
2. Add 300µL of the Coomassie Plus Reagent to each well and mix with plate shaker for 30 seconds.
3. Remove plate from shaker. For the most consistent results, incubate plate for 10 minutes at room temperature (RT).
4. Measure the absorbance at or near 595nm with a plate reader.
5. Subtract the average 595nm measurement for the Blank replicates from the 595nm measurements of all other individual standard and unknown sample replicates.
6. Prepare a standard curve by plotting the average Blank-corrected 595nm 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: When compared to the Standard Test Tube Protocol, 595nm measurements obtained with the Microplate Protocols are lower because the light path is shorter. Consequently, this may increase the minimum detection level of the assay. If higher 595nm measurements are required, use 15µL of standard or sample and 300µL of Coomassie Plus Reagent per well.

   Note: If using curve-fitting algorithms associated with a microplate reader, a four-parameter (quadratic) or best-fit curve will provide more accurate results than a purely linear fit. If plotting results by hand, a point-to-point curve is preferable to a linear fit to the standard points.

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B. Micro Microplate Protocol (Working Range = 1-25µg/mL)

1. Pipette 150µL of each standard or unknown sample into the appropriate microplate wells.
2. Add 150µL of the Coomassie Plus Reagent to each well and mix with plate shaker for 30 seconds.
3. Remove plate from shaker. For the most consistent results, incubate plate for 10 minutes at room temperature (RT).
4. Measure the absorbance at or near 595nm on a plate reader.
5. Subtract the average 595nm measurement for the Blank replicates from the 595nm measurements of all other individual standard and unknown sample replicates.
6. Prepare a standard curve by plotting the average Blank-corrected 595nm measurement for each BSA standard vs. its concentration (µg/ml). Using the standard curve, determine the protein concentration estimate for each unknown sample.

   Note: If using curve-fitting algorithms associated with a microplate reader, a four-parameter (quadratic) or best-fit curve will provide more accurate results than a purely linear fit. If plotting results by hand, a point-to-point curve is preferable to a linear fit to the standard points.

Troubleshooting

Problem	Possible Cause	Solution
Absorbance of Blank is OK, but remaining standards and samples yield lower values than expected	Improper reagent storage	Store reagent refrigerated
	Reagent still cold	Allow reagent to warm to RT
	Absorbance measured at incorrect wavelength	Measure absorbance near 595nm
Absorbances of Blank and standards are OK, but samples yield lower values than expected	Sample protein (peptide) has a low molecular weight (e.g., less than 3000)	Use the Pierce BCA Assay Kit
A precipitate forms in all tubes	Sample contains a surfactant (detergent)	Dialyze or dilute sample or remove interfering substances from sample using Product No. 23215
		Use the Thermo Scientific™ Pierce™ Detergent Compatible Bradford Assay Kit (Product No. 23246)
	Samples not mixed well or left to stand for extended time, allowing aggregates to form with the dye	Mix samples immediately before measuring absorbances
All tubes (including Blanks) are dark blue	Strong alkaline buffer raises pH of formulation, or sample volume too large, thereby raising reagent pH	Dialyze or dilute sample
		Remove interfering substances from sample using Product No. 23215
Need to read absorbances at a different wavelength	Spectrophotometer or plate reader does not have 595nm filter	Color may be read at any wavelength between 575nm and 615nm, although the slope of standard curve and overall assay sensitivity will be reduced

A. Interfering substances

Certain substances are known to interfere with coomassie-based protein assays including most ionic and nonionic detergents, which reduce color development and can cause precipitation of the assay reagent. Other substances interfere to a lesser extent. These have only minor (tolerable) effects below a certain concentration in the original sample. Maximum compatible concentrations for many substances in the Standard Test Tube Protocol are listed in Table 2 (see last page). Substances were compatible in the Standard Test Tube Protocol if the error in protein concentration estimation (of BSA at 1000µg/mL) caused by the presence of the substance in the sample was less than or equal to 10%. The Blank-corrected 595nm absorbance measurements (for the 1000µg/mL BSA standard + substance) were compared to the net 595nm absorbances of the 1000µg/mL BSA standard prepared in 0.9% saline.

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B. Strategies for eliminating or minimizing the effects of interfering substances

The effects of interfering substances in the Coomassie Plus Assay may be overcome by one of several methods.

- Remove the interfering substance by dialysis or desalting.
- Dilute the sample until the substance no longer interferes.
- Precipitate proteins with acetone or trichloroacetic acid (TCA). Upon precipitation the liquid containing the substance that interfered is discarded and the protein pellet is solubilized in a small amount of ultrapure water or directly in the Coomassie Plus Reagent. Alternatively, use Product No. 23215 (see Related Thermo Scientific Products).

Note: For greatest accuracy, the protein standards must be treated identically to the sample(s).

Note: The Pierce Detergent Compatible Bradford Assay Kit (Product No. 23246) is an alternative related product compatible with a wide range of detergents.

Related Thermo Scientific Products

15041	**Pierce 96-Well Plates – Corner Notch,** 100/pkg
15075	**Reagent Reservoirs,** 200/pkg
15036	**Sealing Tape for 96-Well Plates,** 100/pkg
23208	**Pre-Diluted Bovine Serum Albumin (BSA) Set**, 7 × 3.5mL
23209	**Bovine Serum Albumin Standard Ampules,** 2mg/mL, 10 ×1mL
23212	**Bovine Gamma Globulin Standard Ampules**, 2mg/mL, 10 × 1mL
23213	**Pre-Diluted Bovine Gamma Globulin Fraction II (BGG) Set**, 7 × 3.5mL
23246	**Pierce Detergent Compatible Bradford Assay Kit**
23227	**Pierce BCA Protein Assay Kit**
23235	**Micro BCA™ Protein Assay Kit**
23215	**Compat-Able™ Protein Assay Preparation Reagent Set**

Additional Information

A. Please visit the web site for additional information on this product including:

- Tech Tip #9: Quantitate immobilized protein
- Application notes and more complete reference list

B. Response characteristics for different proteins

Each of the commonly used total protein assay methods 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. Most protein assay methods utilize BSA or immunoglobulin (IgG) as the standard against which the concentration of protein in the sample is determined. Thermo Scientific™ Bovine Serum Albumin Standard (BSA) (Product No. 23209) provides a consistent standard for protein estimations. Nevertheless, individual proteins, including BSA and IgG, differ slightly in their color responses in the Coomassie Plus Assay (Figure 1). For greatest accuracy, the standard curve should be prepared from a pure sample of the target protein to be measured.

Table 3 shows typical protein-to-protein variation in color response. All proteins were tested at 1000µg/mL using the Standard 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. The protein-to-protein variation observed with the Coomassie Plus Reagent is significantly less than that seen with other Bradford-type coomassie formulations.

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C. Measuring Absorbances at Wavelengths other than 595nm

If a photometer or plate reader is not available with a 595nm filter, the blue color may be measured at any wavelength between 570nm and 610nm. The maximum sensitivity of the assay occurs when the absorbance of the dye-protein complex is measured at 595nm. Measuring the absorbance at any wavelength other than 595nm will result in a lower slope for the standard curve and may increase the minimum detection level for the protocol.

D. Effect of Temperature on 595nm Absorbance

Absorbance measurements at 595nm obtained with the Coomassie Plus Reagent are dependent on the temperature of the reagent to some extent. As the reagent temperature increases to RT, the 595nm measurements will increase. Therefore, it is important that the Coomassie Plus Reagent remain at a constant temperature (i.e., RT) during the assay.

E. Cleaning and Re-using Glassware

Care must be exercised when cleaning glassware that will be used again for protein assays. Thorough cleaning often requires the use of a detergent such as Thermo Scientific PCC-54 Detergent (Product No. 72288), which must be completely removed in the final rinse. Coomassie dye will stain glass or quartz cuvettes. Disposable polystyrene cuvettes are a convenient alternative.

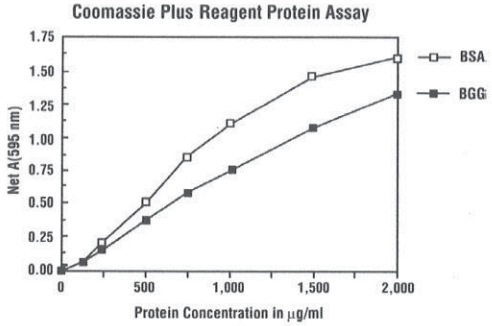

Figure 1. Typical color response curves for BSA and BGG using the Standard Test Tube Protocol.

Table 3. Protein-to-Protein Variation. Absorbance ratios (595nm) for proteins relative to BSA using the Standard Test Tube Protocol in the Coomassie (Bradford) Assay.

Ratio = (Avg "test" net Abs.) / (avg. BSA net Abs.)

Protein Tested	Ratio
Albumin, bovine serum	1.00
Aldolase, rabbit muscle	0.74
α-Chymotrypsinogen, bovine	0.52
Cytochrome C, horse heart	1.03
Gamma globulin, bovine	0.58
IgG, bovine	0.63
IgG, human	0.66
IgG, mouse	0.62
IgG, rabbit	0.43
IgG, sheep	0.57
Insulin, bovine pancreas	0.67
Myoglobin, horse heart	1.15
Ovalbumin	0.68
Transferrin, human	0.90
Average ratio	**0.73**
Standard Deviation	**0.21**
Coefficient of Variation	**28.8%**

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General References

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**:248-54.

Compton, S.J. and Jones, C.J. (1985) Mechanism of dye response and interference in the Bradford protein assay. *Anal Biochem* **151**:369-74.

Davies, E.M.(1988). Protein assays: A review of common techniques. *Amer Biotech Lab* **July** 28-37.

Sedmak, J.J. and Grossberg, S.E. (1977) A rapid, sensitive and versatile assay for protein using Coomassie brilliant blue G-250. *Anal Biochem* **79**:544-52.

Sherwood, J.K., *et al.* (1996) Controlled release of antibodies for long-term topical passive immunoprecipitation of female mice against genital herpes. *Nature Biotech* **14**:468-71.

Sorensen, K. (1994) Coomassie protein assay used for quantitative determination of sodium cyanoborohydride ($NaCNBH_3$). *Anal Biochem.***218**:231-33.

Tal, M., Silberstein, A. and Nusser, E. (1980) Why does Coomassie brilliant blue R interact differently with different proteins? *J Biol Chem* **260**:9976-80.

Product References

Kagan, A., *et al.* (2000) The dominant negative LQT2 mutation A561V reduces wild-type HERG expression. *J Biol Chem* **275**:11241-8.

Glover, B.P. and McHenry, C.S. (2001) The DNA polymerase III holoenzyme: an asymmetic dimerc replicative complex with leading and lagging strand polymerases. *Cell* **105**:925-34.

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Table 2. Compatible substance concentrations in the Coomassie Plus Protein Assay.

Substance	Compatible Concentration
Detergents	
Brij™-35	0.062%
Brij-56 (Brij-58)	0.031%(0.016%)
CHAPS, CHAPSO	5.0%
Deoxycholic acid	0.4%
Lubrol™ PX	0.031%
Octyl β-glucoside	0.5%
Nonidet P-40 (NP-40)	0.5%
Octyl β-thioglucopyranoside	3.0%
SDS	0.016%
Span™ 20	0.5%
Triton™ X-100, X-114	0.062%
Triton X-305, X-405	0.125%(0.025%)
Tween™-20	0.031%
Tween-60	0.025%
Tween-80	0.016%
Zwittergent™ 3-14	0.025%
Chelating agents	
EDTA	100mM
EGTA	2mM
Sodium citrate	200mM
Reducing & Thiol-Containing Agents	
N-acetylglucosamine in PBS, pH 7.2	100mM
Ascorbic acid	50mM
Cysteine	10mM
Dithioerythritol (DTE)	1mM
Dithiothreitol (DTT)	5mM
Glucose	1.0M
Melibiose	100mM
2-Mercaptoethanol	1.0M
Potassium thiocyanate	3.0M
Thimerosal	0.01%
Misc. Reagents & Solvents	
Acetone	10%
Acetonitrile	10%
Aprotinin	10 mg/L
DMF, DMSO	10%
Ethanol	10%
Glycerol (Fresh)	10%
Hydrochloric Acid	100mM
Leupeptin	10mg/L
Methanol	10%
Phenol Red	0.5mg/mL
PMSF	1mM
Sodium Hydroxide	100mM
Sucrose	10%
TLCK	0.1mg/L
TPCK	0.1mg/L
Urea	3.0M
o-Vanadate (sodium salt), in PBS, pH 7.2	1mM

Substance	Compatible Concentration
Salts/Buffers	
ACES, pH 7.8	100mM
Ammonium sulfate	1.0M
Asparagine	10mM
Bicine, pH 8.4	100mM
Bis-Tris, pH 6.5	100mM
Borate (50mM), pH 8.5 (# 28384)	undiluted
B-PER™ Reagent (#78248)	1/2 dilution*
Calcium chloride in TBS, pH 7.2	10mM
Na-Carbonate/Na-Bicarbonate (0.2M), pH 9.4 (#28382)	undiluted
Cesium bicarbonate	100mM
CHES, pH 9.0	100mM
Na-Citrate (0.6M), Na-Carbonate (0.1M), pH 9.0 (#28388)	undiluted
Cobalt chloride in TBS, pH 7.2	10mM
EPPS, pH 8.0	100mM
Ferric chloride in TBS, pH 7.2	10mM
Glycine	100mM
Guanidine•HCl	3.5M
HEPES, pH 7.5	100mM
Imidazole, pH 7.0	200mM
MES, pH 6.1	100mM
MES (0.1M), NaCl (0.9%), pH 4.7 (#28390)	undiluted
MOPS, pH 7.2	100mM
Modified Dulbecco's PBS, pH 7.4 (#28374)	undiluted
Nickel chloride in TBS, pH 7.2	10mM
PBS; Phosphate (0.1M), NaCl (0.15M), pH 7.2 (#28372)	undiluted
PIPES, pH 6.8	100mM
RIPA lysis buffer; 50mM Tris, 150mM NaCl, 0.5% DOC, 1% NP-40, 0.1% SDS, pH 8.0	1/40 dilution*
Sodium acetate, pH 4.8	180mM
Sodium azide	0.5%
Sodium bicarbonate	100mM
Sodium chloride	5.0M
Sodium citrate, pH 4.8 or pH 6.4	200mM
Sodium phosphate	100mM
Tricine, pH 8.0	100mM
Triethanolamine, pH 7.8	100 mM
Tris	2.0M
TBS; Tris (25mM), NaCl (0.15M), pH 7.6 (#28376)	undiluted
Tris (25mM), Glycine (192mM), pH 8.0 (#28380)	undiluted
Tris (25mM), Glycine (192mM), SDS (0.1%), pH 8.3 (#28378)	1/4 dilution*
Zinc chloride in TBS, pH 7.2	10mM

*Diluted with ultrapure water.

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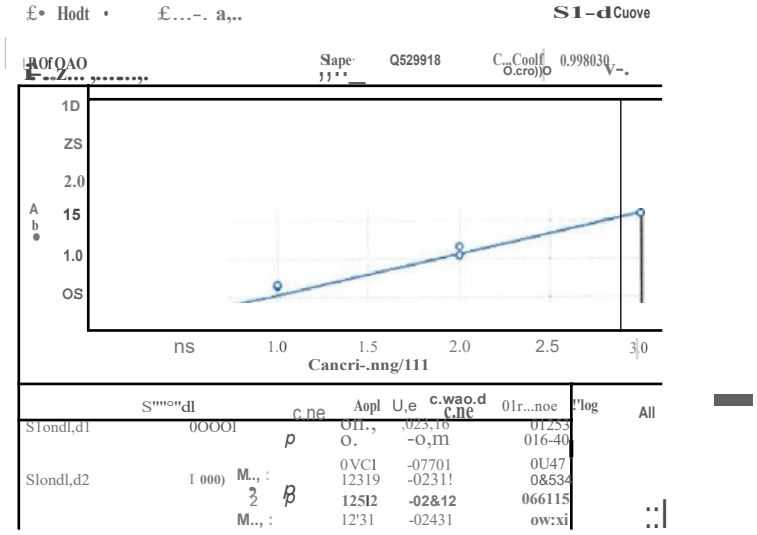
Protein Assay, Standards Mode

DU800 Applications Software - Protein Assay Analysis

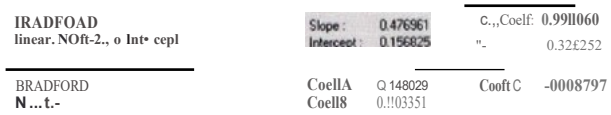
Standards Mode

[Introduction] [Menus/Functioos] (Application Modes] [ ] [ )

The *Standards Mode* is used to measure standards and calculate a standard curve for the quantitative analysis of samples containing one component. The standard curve Is made from standards of known concentratio ns. The standards data are fit to either a linear or non-linear (quadratic) curve.



By de fault and without a standard curve, only the selected *Curve Fit* is shown in the top panel. When the require d number of standards have been acquired and the curve has been calculated, the coefficients and quality parameters are displayed.



When opening the application, the default standard identification "Standard x" and the concentrati ons from the method are filled into the grid automatically, basedon the number of replicates. The *Use* box Is unchecked at this time and the cells to the right are empty.

The *Standard ID* items are shown in blue, indicating that the text can be edited. The entries In these cells will be changed to black and locked when the first replicate of the respective standard is acquired. After the acquisition of each standard, the *Use* box will be checked and the *Analytical Abs* and the *Net Abs* values will be displayed.

One standard always has the focus. This is indicated by the bold display of the *Replicate* entry . The latest acquired standard automatically gets the focus. The focus can be changedmanually by

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Protein Assay, Standards Mode

clicking on the respective row. In this case, a specific standard – the one that has the focus – can be re-read.

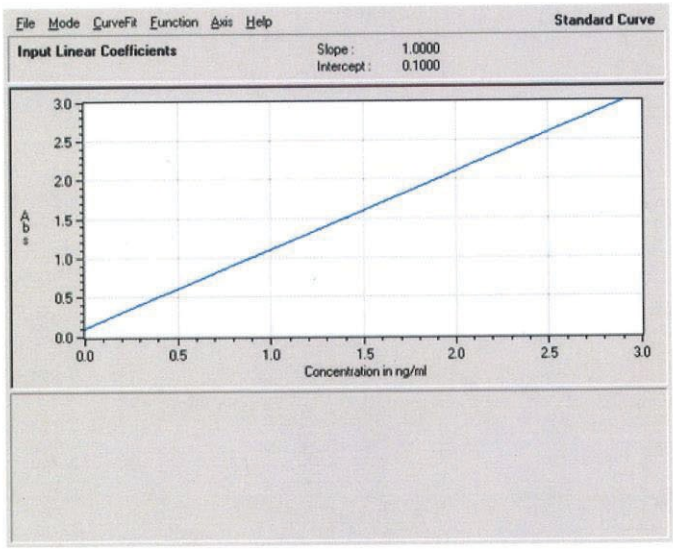
After all standards have been acquired, the curve is calculated and displayed automatically. Any change, such as a change in the *Use* of a standard replicate, will automatically cause a re-calculation and display of the curve and its standard points. Non-used points will not be displayed.

With replicates (2 or 3), the *Mean* values are calculated and displayed. A *Flag* is displayed when the %CV is greater than the value set in the method.

When the first sample is acquired, anything related to the standard curve becomes locked. In this case, the standard curve can only by viewed and no changes can be made anymore.

When saving a file in this mode, it contains all standard data.

Up to 30 standards and 3 replicates can be configured in the method.



With coefficients input by the user, the coefficients are shown in the top panel and the resulting curve is displayed. In this case, there are no quality parameters. Also, the grid with the standards is meaningless and will be invisible.

mk:@MSITStore:C:\Program%20Files\DU800%20System\Help\Du800Help.chm::/Applic... 2/15/2019

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