Protein quantification: BCA™, Modified Lowry and Bradford assays

Absorbance measurements on Infinite™ F200 and Infinite M200

Introduction

Protein quantification is often required before proceeding with protein samples for isolation, chromatographic or electrophoretic analysis, or immunohistochemical methods. Two different techniques are generally used for colorimetric detection and quantification of proteins: protein-dye binding and protein-copper chelation.

In this note we describe the use of Tecan's Infinite F200 and Infinite M200 instruments for easy and sensitive protein quantification using different absorbance-based assays (see Table 1).

BCA™ Protein Assay

The BCA Protein Assay uses bicinchoninic acid (BCA) for colorimetric quantification of total protein in a sample [1]. The method is based on the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium. Cu^{1+} complexes with BCA, forming a colored water-soluble chelate that can be measured at its absorption maximum at 562 nm (see Fig 1). The linearity of protein concentrations can be detected within a 20-2,000 µg/ml range using a 2 ml test tube. If protein quantification is needed in smaller volumes, the detection range is about 125-2,000 µg/ml.

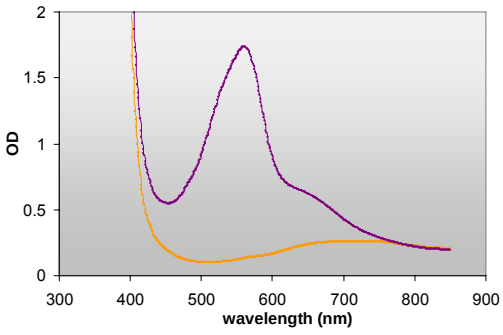

Figure 1: Absorbance scan (300-900 nm) of BCA with (—) and without (—) BSA on Infinite M200 reader.

The BCA Protein Assay may be the best choice if protein samples contain one or more detergents.

Modified Lowry Protein Assay

The Modified Lowry Protein Assay is based on the original protocol of Oliver Lowry's method [2] but with a modified, more stable formulated product. The protein reacts with cupric sulfate and tartate in an alkaline solution, which results in formation of a tetradentate copper-protein complex, reducing

the Folin-Ciocalteu Reagent. The blue colored, water-soluble product can be measured at 750 nm (see Fig 2). The linearity range for protein detection is 1-1,500 µg/ml.

The Modified Lowry Protein Assay can also be performed in small volumes if protein probes are measured in microplates.

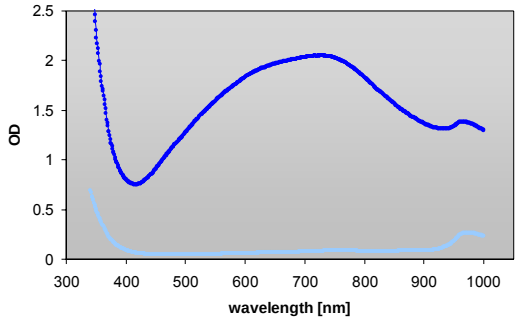

Figure 2: Absorbance scan (300-1,000 nm) of Modified Lowry Protein Assay reagent with (—) and without (—) BSA on Infinite M200 reader.

BioRad Protein (Bradford) Assay

The BioRad Protein Assay used in this application is a dye-binding assay based on the method developed by Bradford [3]. The Coomassie® Brilliant Blue G-250 dye binds to basic and aromatic amino acid residues, especially to arginine, which induces a shift of the absorbance maximum of the dye from 465 nm to 595 nm (see Fig 3). A typical standard curve, which is needed to calculate protein concentration, ranges between 20-140 µg/ml if prepared with BSA.

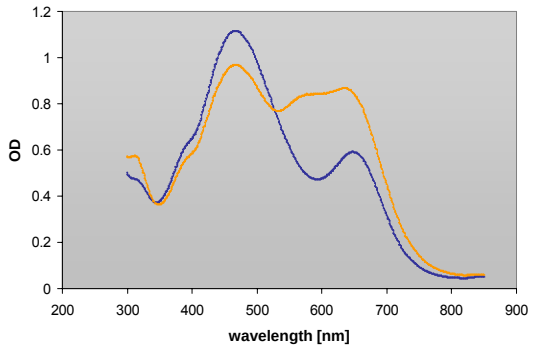

Figure 3: Absorbance scan (300-900 nm) of BioRad Protein assay reagent with (—) and without (—) BSA on Infinite M200 reader.

The Bradford Assay can be performed as a microassay procedure with a linearity range of 1.2-10 µg/ml BSA. The microassay procedure is ideally suited for measurements in microtiter plates using small volumes.

Assay	Abs-max (nm)	Colour	Linearity (µg/ml BSA)
BCA	562	purple	25-2000
Lowry	750	blue	1-1500
Bradford	595	blue	1.2-10
Bradford	595	blue	8-80

Table 1: Summary of the three protein assays. Shown are the different absorption maxima, the resulting color of the reagent and the linearity range. A standard curve has to be prepared for each assay to calculate unknown protein concentration.

Material and methods

Instruments

- Infinite F200, filter-based detection system (Tecan Austria, Austria)
- Infinite M200, quad4 monochromator™ detection system (Tecan Austria, Austria)

Microplates

- 96-well flat bottom transparent microplate (Greiner Bio-One, Germany)
- UV-cuvette micro (Brand, Germany)

Reagents and assay performance

Reagents

- BCA Protein Assay (Pierce, Illinois):
  the kit contains BCA Reagent A, BCA Reagent B and BSA protein standard (2 mg/ml).
- Modified Lowry Protein Assay (Pierce, Illinois):
  the kit contains Modified Lowry Protein Reagent, 2N Folin-Ciocalteu reagent and BSA protein standard (2 mg/ml).
- Bio-Rad Protein Assay (Bio-Rad, California):
  the kit contains protein assay dye reagent concentrate, lyophilized BSA protein standard.

Assay protocol

The working solutions, protein standards etc. for all assays were prepared as described in the manufacturers' recommendations.

BCA Protein Assay

Two different procedures are described for the BCA Protein Assay: one for cuvettes and the other for microplates, both within a working range of 20-2,000 µg/ml.

For the microplate protocol, 25 µl sample is mixed with 200 µl BCA working reagent; for the cuvette protocol, 100 µl sample

and 2 ml BCA working reagent are mixed. When using cuvettes, 50 µl sample mixed with 1 ml BCA working reagent is sufficient.

For both the cuvette and microplate procedures, samples are incubated for 30 minutes at 37 °C and cooled to room temperature before absorbance measurements (see Table 2).

Infinite F200 (microplate)	
Parameter	Setting
mode	absorbance
wavelength	562 nm
bandwidth	10 nm
number of readings	25
Infinite M200 (microplate, cuvette)	
mode	absorbance
wavelength	562 nm
bandwidth	9 nm
number of readings	25

Table 2: Measurement parameters and settings for the BCA Protein Assay on Infinite F200 and Infinite M200.

Modified Lowry Protein Assay

The Modified Lowry Protein Assay can be performed in two different ways: the cuvette protocol is ideal for high volumes of protein samples, and a microplate procedure is available for small volumes of unknown protein. Both procedures have a working range of about 1-1,500 µg protein/ml. This assay requires accurate pipetting to be performed exactly on time, therefore a multichannel pipettor is recommended.

For microplate assays, 40 µl sample is mixed with 200 µl Modified Lowry Reagent, the plate is shaken immediately for exactly 30 seconds and incubated at room temperature for 10 minutes. Folin-Ciocalteu Reagent (20 µl) is added and the plate is shaken for an additional 30 seconds then incubated for 30 minutes at room temperature, prior to the absorbance measurement at 750 nm.

For the cuvette procedure, 200 µl of each sample is pipetted into cuvettes and 1 ml Modified Lowry Reagent is added after a 15 second interval. Each cuvette well is mixed then incubated for 10 minutes before adding 100 µl Folin-Ciocalteu Reagent using exactly the same time regime as previously. Cuvettes are mixed then incubated for 30 minutes at room temperature before measuring absorbance at 750 nm (see Table 3).

Infinite F200 (microplate)	
Parameter	Setting
mode	absorbance
wavelength	750 nm
bandwidth	10 nm
number of readings	25
Infinite M200 (microplate, cuvette)	
mode	absorbance
wavelength	750 nm
bandwidth	9 nm
number of readings	25

Table 3: Measurement parameters and settings for the Modified Lowry Protein Assay on Infinite F200 and Infinite M200.

BioRad Protein (Bradford) Assay

The dye reagent and sample volumes are variable depending on the protocol, and these should be determined using the manufacturer's manual. In general, the standard samples and the unknown protein samples are pipetted into wells, resp. cuvettes, and the corresponding volume of dye reagent is added before incubating for 5 minutes at room temperature. Absorbance of the samples is measured at ~595 nm (see Table 4).

Infinite F200 (microplate)	
Parameter	Setting
mode	absorbance
wavelength	590 nm
bandwidth	10 nm
number of readings	25
Infinite M200 (microplate, cuvette)	
mode	absorbance
wavelength	595 nm
bandwidth	9 nm
number of readings	25

Table 4: Measurement parameters and settings for BioRad Protein (Bradford) Assay on Infinite F200 and Infinite M200.

Results

BCA Protein Assay

The BCA Protein Assay can be performed in 96-well microplates using the standard assay procedure when measured on the Infinite M200 or Infinite F200 (see Fig 4). The calculated sensitivity for protein detection is about 10 µg/ml with the Infinite 200 series of instruments. This assay was also performed with its original protocol in cuvettes measured on the Infinite M200.

Figure 4 shows the results of the microplate and cuvette measurements. Comparable and reliable results are achieved using either cuvettes or microplates.

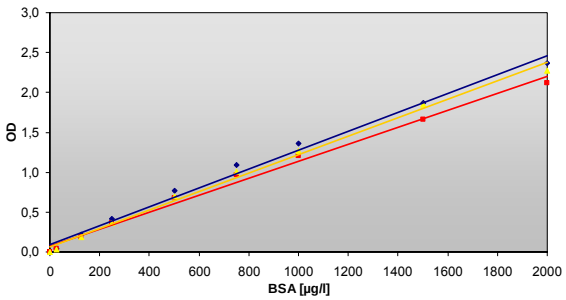

Figure 4: BCA Protein Assay measurements in cuvette using an Infinite M200 (—) and in 96-well microplate measured on Infinite F200 (—) and Infinite M200 (—). The linearity range is 25-2,000 µg/ml.

Modified Lowry Protein Assay

The Modified Lowry Protein Assay was performed in 96-well microplates and in cuvettes. Using microplates on both the Infinite M200 and Infinite F200, nearly identical results could be obtained.
In contrast to microplate-based measurements, the cuvettte measurements display few higher absorbance values, which can be explained by the fact that handling cuvettes takes longer than measuring a 96-well plate, and the color development increases during this time (see Fig 5).

The sensitivity of the Modified Lowry Protein Assay using an Infinite F200 or Infinite M200 instrument is about 2 µg/ml (see Table 5).

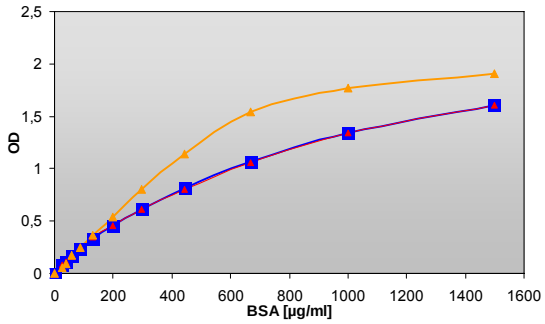

Figure 5: Modified Lowry Protein Assay measurements in cuvette using an Infinite M200 (—) and in 96-well microplate measured with Infinite F200 (—) and Infinite M200 (—).

BioRad Protein (Bradford) Assay

Two different microassay protocols are available for low protein concentrations, varying in dye/sample ratio:

- a cuvette protocol for protein concentrations from about 1-12 µg/ml
- a microplate protocol for protein concentrations ranging from 8-80 µg/ml.

When the Bradford microassays are performed in microplates and cuvettes, the sensitivity is calculated to be about 1 µg/ml protein.

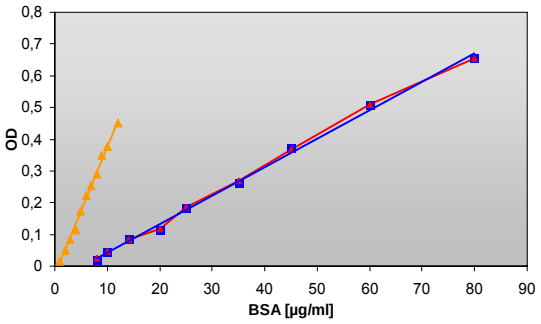

Figure 6: BioRad Protein (Bradford) microassays measured on Infinite F200 (—) and on Infinite M200 (—) in 96-well microplates, and on Infinite M200 (—) using cuvettes.

Sensivitiy

Assay	BCA	Lowry	Bradford
Protocol [µg/ml]	25-2,000	26-1,500	8-80
Sensitivity [µg/ml]	10	2	1

Table 5: Sensitivity [µg protein/ml] of the BCA, Modified Lowry and BioRad (Bradford) Protein Assays measured on Tecan's Infinite 200 instruments. Linearity ranges of the different protocols are given in µg/ml.

Discussion

Three different protein quantification assays were compared using Tecan's filter-based Infinite F200 and the quad4 monochromator-based Infinite M200.

For the BCA assay, the standard procedure was used for cuvetttes and microplates, although this protocol is not thought to be suitable for microplates. Both instruments could detect proteins at concentrations of about 10 µg/ ml.

The Modified Lowry Protein Assay is useful for lower protein concentrations, using one protocol for each dilution with estimated sensitivity of 2 µg/ml. This assay is the most laborious, and sample handling and pipetting become more complicated with increasing sample numbers. Therefore, we used the Infinite 200 injector system to perform the Modified Lowry Protein Assay in 96-well plates. Details and instrument settings are shown in the application note 'Protein quantification on Infinite 200 with injectors' (#395179).

For sensitive protein detection, the BioRad Protein (Bradford) microassay protocols can be used. Measurements can be performed in 96-well plates and cuvettes and, using the standard assay protocol, protein concentrations of up to 500 µg/ml can be measured.

Conclusion

The assay chosen for protein quantification depends on the quantity of protein available, the composition of the sample (buffer, salts, reducing agents and detergents) and finally on the nature of the protein itself. On the instrument side, limitations occur if larger numbers of samples are to be measured when the reader can only read cuvettes, this often requires larger amounts of protein in the sample.
These disadvantages can be overcome using Tecan's Infinite 200 series of microplate readers, which offer high flexibility and high sensitivity. As we have shown, small amounts of protein in small volumes can easily be measured in microplates (see Table 5). Additionally, the Infinite M200 monochromator reader can measure protein samples in cuvettes as well as in microplates. The Infinite M200 can also perform absorbance scans to detect absorbance maxima of unknown substances. This was demonstrated for the protein assays described here, by performing an absorbance scan of the colored complexes with and without BSA.

List of abbreviations

BCA	bicinchoninic acid
BSA	bovine serum albumin
Cu	copper

Literature

[1] Smith, P.K., et al.: Measurement of protein using bicinchoic acid. *Anal Biochem*., 150, 76-85, 1985
[2] Lowry, O.H. et al.: Protein measurement with Folin Phenol Reagent. *J. Biol. Chem*., 193, 265-275, 1951
[3] Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quatities of protein utilizing the principle of protein dye binding. *Anal. Biochem*., 72, 248-254, 1976

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395 245 V1.0, 09-2007