BIO-RAD BRADFORD TOTAL PROTEIN ASSAY

(Bradford, 1976 & Wright *et al.,* 1996)

Introduction

This procedure is used to measure protein concentration in samples extracted for glomalin. This assay does not give the most accurate glomalin concentration, because it is not specific for glomalin and will measure any protein (>3000 daltons) in the extract solution. The dye also reacts with polyphenolics which may be co-extracted with glomalin. Also, if sodium pyrophosphate is used to extract glomalin, a modification to this procedure must be used (see alternate procedure below). Sodium pyrophosphate cross-reacts with the Bradford dye reagent and must be added to the standard to remove any cross-reaction as background. Therefore, these values should be listed as Bradford-reactive soil protein (BRSP) as described in Rillig, 2004 and should not be relied upon as exact glomalin values. Also, Bradford total protein values are usually higher than the values calculated from the ELISA procedure, but running this assay will give an estimate of glomalin concentration and help determine the volume of extract needed for the ELISA. The Bradford assay is based upon a shift in the absorption spectra of Coomassie Brillinat Blue G-250 when the dye binds to protein in an acidic solution. Glomalin precipitates in acidic solutions, so this assay must be conducted rapidly (use 5 minutes as the time between adding the dye and reading the samples).

Note: It is recommended to use at least two duplicate samples in the 96-well plate of extract from each sample and to calculate the percentage of the coefficient of variance (which should be <25) between these two duplicate samples to identify any variances that may have resulted because of variances within the plate or operator error. In addition the standard curve should have an $R^2 > 0.94$. If the protein values for the samples are above or below the standard curve values (1.25 to 5.0 ul), the variances between duplicate samples are too high (>25), or the R^2 value is too low (< 0.95), than the assay needs to be rerun on those samples (see below).

Materials

PBS (phosphate buffered saline), pH 7.4
BSA (bovine serum albumin)
1-ml microcentrifuge tubes
Bio-Rad protein dye
96-well microtiter plates or ELISA strips
Micro-pipetter and tips
Dissecting needle or syringe attached to a 12-channel manifold (Fig. 1)
Plate reader (Fig. 2), with 590 or 595nm filter
Microtiter tubes

Figure 1. Recently my laboratory has been using a 12-channel manifold attached to a syringe with the plunger all the way down to prevent capillary action from drawing any sample out of the well to mix the dye with the protein sample. This reduces the mixing time and does not cause bubbles to form as does occur when mixing with a multi-channel pipette. Caution must be used to make sure the bottom of the well is not scratched. To prevent scratching, stir in a circular motion with the manifold sitting just above the bottom to mix horizontally and move straight up and down touching the bottom to mix vertically.

Figure 2. Typical computer and plate reader set-up for the Bradford total protein and ELISA immunoreactive protein assays. Software that either comes with the plate reader or may be purchased separately may be used to assist in the analysis of the data. We use Softmax® software. If software is not available or a computer interface is not available, data may be collected directly from the plate reader and inputted into a spreadsheet program, like Excel, for analysis.

Methods

1) Prepare standard curve, using BSA. A new curve must be used on each new plate or each time the assay is run.

BSA standard curve preparation

A. Make a 200 ml stock solution of 10 ug BSA/200ul PBS (10 mg/200 ml), aliquot into 1-ml microcentrifuge tubes, and freeze, until needed. (Larger amounts of the stock solution may be prepared. Part of this larger stock solution or a portion of the 200 ml solution may be placed in bottles and frozen. The frozen stock may then be thawed and with 1 ml aliquots made at a later time to reduce the amount of 1-ml tubes stored at any one time.)

B. Thaw and dilute with PBS* into one row of 12 wells in the 96-well plate (either the top or bottom row works the best) as outlined in Table 1:

Table 1. BSA standards for Bradford protein assay

Well designation	ug/well	BSA stock solution(ul)	PBS (ul)
Blank (wells 1 and 2)	0	0	200
Standard 1 (wells 3 and 4)	0	0	200
Standard 2 (wells 5 and 6)	1.25	25	125
Standard 3 (wells 7 and 8)	2.5	50	150
Standard 4 (wells 9 and 10)	3.75	75	125
Standard 5 (wells 11 and 12)	5	100	100

2) Add 200 ul of PBS* minus the volume of extract to each well. (For example, if there is 5 ul of sample in the well, 195 ul of PBS is needed.) Extract volume will typically be anywhere from 1 to 50 ul, depending on color. (Follow color chart and examples (Table 2 and Fig. 3) to determine amount of sample to add). Although there may be variations in color between samples placed in wells of a particular plate, use a standard concentration that represents the average color for all samples. This will prevent you from having to keep track of different volumes across the 96-well plate and will allow you to use the multi-channel pipettes. The sample volume may differ in different 96-well plates or with different assays.

Color chart for sample volume determination

Table 2. The color of the extract can help determine the right sample volume to use.

Sample color	ul sample/well
Golden	50+
Golden-brown	25-50
Brown	10-25
Reddish brown	5-10
Reddish black	1-5

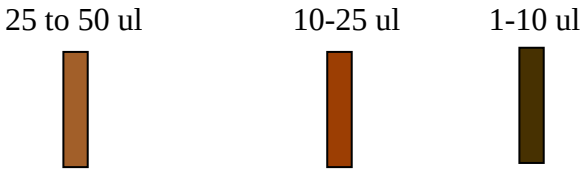

Figure 3. Examples of extract solution colors and sample volume needed.

3) Shake the bottle of dye solution and carefully add 50 ul of Bio-Rad protein dye* to each well being careful to keep the pipette tips from touching the solution (Fig. 4). (If the solution is touched, dispose of the tips and any remaining dye before moving to a new well or row.) Start 5 min on timer. Mix thoroughly with pipette.** **Note: Mixing frequently produces bubbles that must be popped prior to reading the plate. Since it takes time to pop the bubbles, it is advised when starting out to only add dye to 5 (i.e. four sample rows and one standard row) rows or strips at a time using use a different curve for each set of samples.**

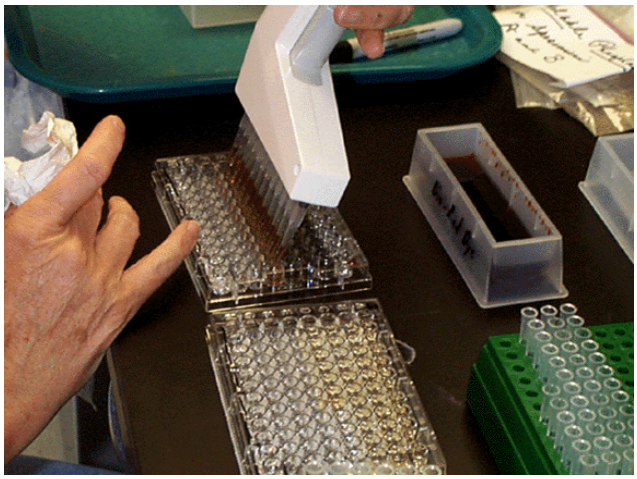

Figure 4. Bio-Rad protein dye is added to each well.

4) Pop bubbles with dissecting needle (cleaning needle between samples) and read after the 5 min has expired (Fig. 5).

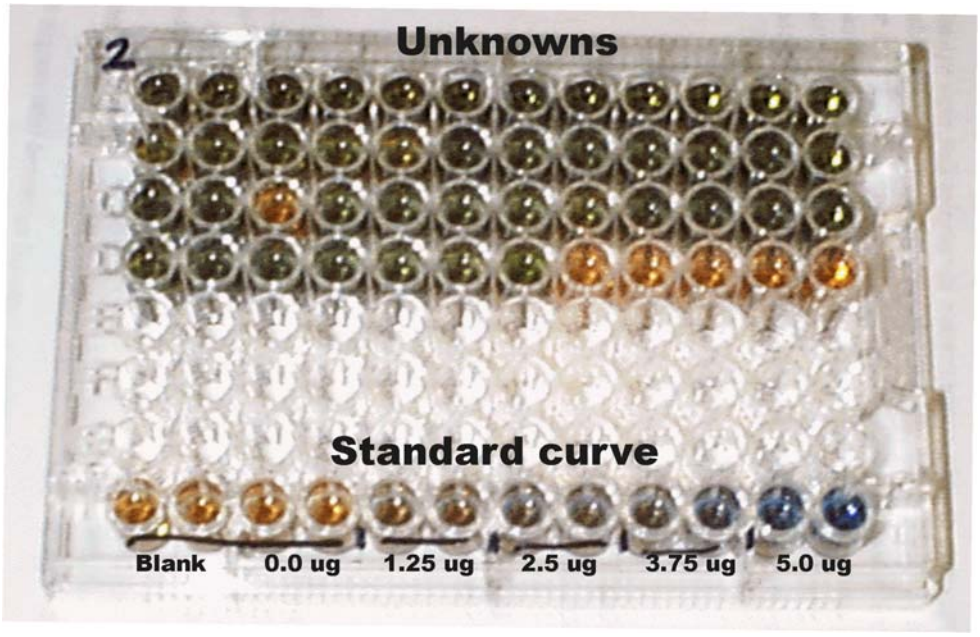

Figure 5. Example of plate after dye has been added and mixed – red indicates no protein while the intensity of blue [measured as optical density (OD)] is related to concentration of protein based off known concentrations of protein and the OD values for the standard.

5) The OD values should fall within the expected ranges as shown in Table 3 with slight increases possible with the addition of sodium pyrophosphate to the standard curve and slight variations possible depending upon the age of the dye and if you are using a new bottle or at the bottom of an old bottle of dye. **Note: If your equipment is not capable of blanking the OD values based on the blanks in the standard row in wells A and B, then you must subtract the average OD values from these two wells from all your samples before comparing the standard curve values to those in Table 3. Alternatively, you can add the average blank value to the ranges of OD values in Table 3 and then compare the values from your standard to these new ranges.**

Table 3. Typical, OD value ranges for the total protein standard curve. As noted in the table, the age of the dye solution may impact the OD values.

Older Dye		Newer Dye	
Concentration (ug/well)	OD value	Concentration (ug/well)	OD value
0	0	0	0
1.25	0.100-0.200	1.25	0.175-0.225
2.5	0.200-0.300	2.5	0.325-0.400
3.75	0.300-0.400	3.75	0.450-0.550
5.0	0.400-0.550	5.0	0.600-0.700

6) Use BSA standard curve to calculate mg protein/g material extracted (see below).

***** The PBS solution and Bio-Rad total protein dye solution may be poured into solution basins to have working solutions in a container that will allow easy pipetting with the multi-channel pipettes. Having working solution separate from stock solutions will prevent possible contamination.

** We have recently purchased a manifold system for mixing which does not produce the bubbles (Fig. 1).

BIO-RAD BRADFORD TOTAL PROTEIN ASSAY WITH SODIUM PYROPHOSPHATE MODIFICATION

(Bradford, 1976; Nichols and Wright, 2004 & Wright et al., 2006)

Introduction

If sodium pyrophosphate is used to extract glomalin, a modification to this procedure must be used (see below). Sodium pyrophosphate cross-reacts with the Bradford dye reagent and must be added to the standard to remove any cross-reaction as background. Pyrophosphate will be added to the standard wells at the same volume as used for the unknowns which will decrease the amount of PBS added to the standard wells by the amount of pyrophosphate added. As the concentration of pyrophosphate in the standard curve increases, the slope will increase. Unfortunately, this relationship is not linear so an equation for calculating the slope at each new concentration of pyrophosphate cannot be determined.

Materials

PBS (phosphate buffered saline), pH 7.4
BSA (bovine serum albumin)
Sodium pyrophosphate (100 mM, pH 9.0)
Bio-Rad protein dye
1-ml microcentrifuge tubes
96-well microtiter plates or ELISA strips
Micro-pipetter and tips
Dissecting needle or syringe attached to a 12-channel manifold (Fig. 1)
Plate reader (Fig. 2), with 590 or 595nm filter
Microtiter tubes

Figure 1. Recently my laboratory has been using a 12-channel manifold attached to a syringe with the plunger all the way down to prevent capillary action from drawing any sample out of the well to mix the dye with the protein sample. This reduces the mixing time and does not cause bubbles to form as does occur when mixing with a multi-channel pipette. Caution must be used to make sure the bottom of the well is not scratched. To prevent scratching, stir in a circular motion with the manifold sitting just above the bottom to mix horizontally and move straight up and down touching the bottom to mix vertically.

Figure 2. Typical computer and plate reader set-up for the Bradford total protein and ELISA immunoreactive protein assays. Software that either comes with the plate reader or may be purchased separately may be used to assist in the analysis of the data. We use Softmax® software. If software is not available or a computer interface is not available, data may be collected directly from the plate reader and inputted into a spreadsheet program, like Excel, for analysis.

Methods

1) Prepare standard curve, using BSA. A new curve must be used on each new plate or each time the assay is run.

BSA standard curve preparation

A. Make a 200 ml stock solution of 10 ug BSA/200ul PBS (10 mg/200 ml), aliquot into 1-ml microcentrifuge tubes, and freeze, until needed. (Larger amounts of the stock solution may be prepared. Part of this larger stock solution or a portion of the 200 ml solution may be placed in bottles and frozen. The frozen stock may then be thawed and with 1-ml aliquots made at a later time to reduce the amount of 1-ml tubes stored at any one time.)

B. Thaw and dilute with PBS* into one row of 12 wells in the 96-well plate (either the top or bottom row works the best) as outlined in Table 1:

Table 1. BSA standards for Bradford protein assay

Well designation	ug/well	BSA stock solution(ul)	PBS – sodium pyrophosphate* (ul)
Blank (wells 1 and 2)	0	0	200 - ?
Standard 1 (wells 3 and 4)	0	0	200 - ?
Standard 2 (wells 5 and 6)	1.25	25	175 - ?
Standard 3 (wells 7 and 8)	2.5	50	150 - ?
Standard 4 (wells 9 and 10)	3.75	75	125 - ?
Standard 5 (wells 11 and 12)	5	100	100 - ?

* The amount of PBS add to each well in the standard row is reduced by the volume of sodium pyrophosphate added to each well in the standard row. The volume of sodium pyrophosphate added to each well in the standard row is equal to the volume of extract solution added to the sample wells (as determined in step 2). For example, if 25 ul of sample is added to the sample wells, then 25 ul of sodium pyrophosphate is added to all the wells in the standard row, and the volume of PBS added to each well in the standard row (as stated in the table above) is reduced by 25 ul. The addition of sodium pyrophosphate to the standard row removes any background cross-reactivity due to sodium pyrophosphate in the extract solution of the sample.

2) Add 200 ul of PBS* minus the volume of extract to each well of the remaining sample wells (wells not used for the standard row). (For example, if there is 5 ul of sample in the well, 195 ul of PBS is needed.) Extract volume will typically be anywhere from 1 to 50 ul, depending on color. (Follow color chart and examples (Table 2 and Fig. 3) to determine amount of sample to add). Although there may be variations in color between samples

placed in wells of a particular plate, use a standard concentration that represents the average color for all samples. This will prevent you from having to keep track of different volumes across the 96-well plate and will allow you to use the multi-channel pipettes. The sample volume may differ in different 96-well plates or with different assays.

Table 2. The color of the extract can help determine the right sample volume to use.

Sample color	ul sample/well
Golden	50+
Golden-brown	25-50
Brown	10-25
Reddish brown	5-10
Reddish black	1-5

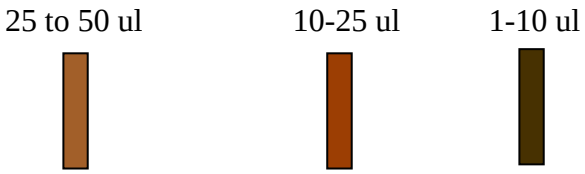

Figure 3. Examples of extract solution colors and sample volume needed.

3) Shake the bottle of dye solution and carefully add 50 ul of Bio-Rad protein dye* to each well being careful to keep the pipette tips from touching the solution (Fig. 4). (If the solution is touched, dispose of the tips and any remaining dye before moving to a new well or row.) Start 5 min on timer. Mix thoroughly with pipette.** **Note: Mixing frequently produces bubbles that must be popped prior to reading the plate. Since it takes time to pop the bubbles, it is advised when starting out to only add dye to 5 (i.e. four sample rows and one standard row) rows or strips at a time using use a different curve for each set of samples.**

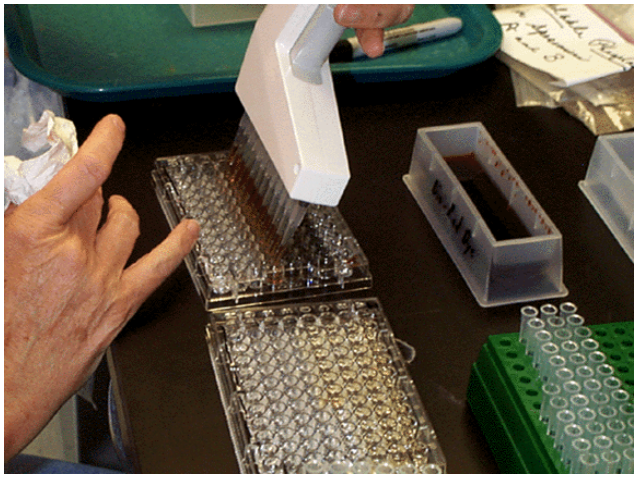

Figure 4. Bio-Rad protein dye is added to each well.

4) Pop bubbles with dissecting needle (cleaning needle between samples) and read using a plate reader after the 5 min has expired (Fig. 5).

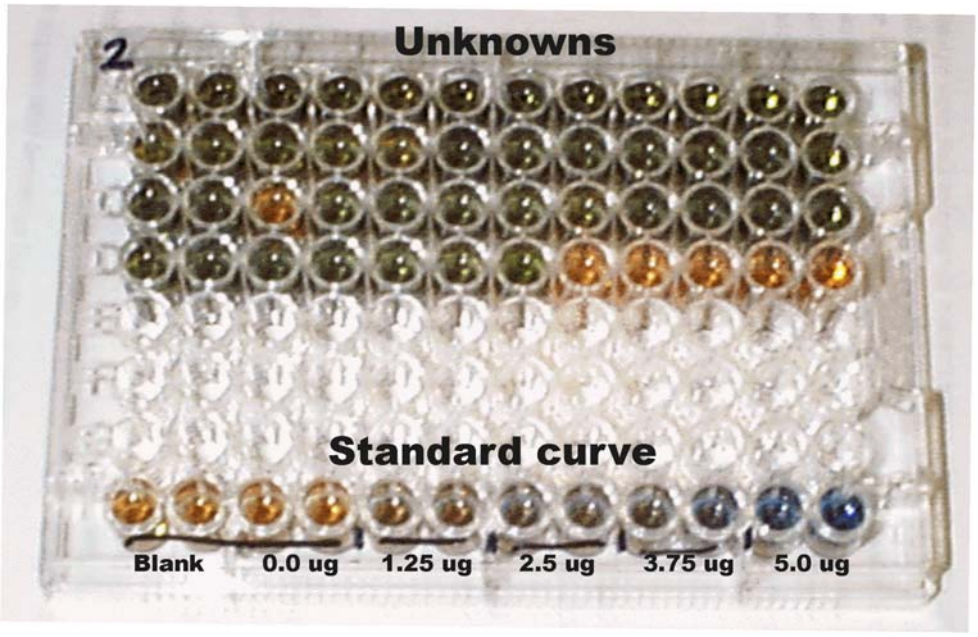

Figure 5. Example of plate after dye has been added and mixed and bubbles have been popped – red indicates no protein while the intensity of blue [measured as optical density (OD)] is related to concentration of protein based from regression analysis of known concentrations and the OD values for the BSA protein standard.

5) The OD values should fall within the expected ranges as shown in Table 3 with slight increases possible with the addition of sodium pyrophosphate to the standard curve and slight variations possible depending upon the age of the dye and if you are using a new bottle or at the bottom of an old bottle of dye. **Note: If your equipment is not capable of blanking the OD values based on the blanks in the standard row in wells A and B, then you must subtract the average OD values from these two wells from all your samples before comparing the standard curve values to those in Table 3. Alternatively, you can add the average blank value to the ranges of OD values in Table 3 and then compare the values from your standard to these new ranges.**

Table 3. Typical OD value ranges for the different concentrations of BSA used in calculating the total protein standard curve (slope values for the regression line are also given). The addition of sodium pyrophosphate to the standard curve row increases the OD values and the slope as indicated in the table.

Amount of sodium pyrophosphate in standard curve row (ul)	0-10	20-50	60-150
Concentration (ug well^{-1})	OD values		
0	0.000±0.025	0.000±0.070	0.000±0.070
1.25	0.100-0.300	0.100-0.400	0.200-0.500
2.5	0.300-0.500	0.150-0.750	0.250-0.800
3.75	0.450-0.700	0.250-1.000	0.500-1.000
5.0	0.550-0.850	0.300-1.100	0.650-1.400
Slope value	0.100-0.170	0.110-0.240	0.130-0.270

6) Use BSA standard curve to calculate mg protein/g material extracted (see below).

***** The PBS solution and Bio-Rad total protein dye solution may be poured into solution basins to have working solutions in a container that will allow easy pipetting with the multi-channel pipettes. Having working solution separate from stock solutions will prevent possible contamination.

** We have recently purchased a manifold system for mixing which does not produce the bubbles (Fig. 1).

Calculating protein concentration from OD values

A. After running the assay, plot the OD values for the standard curve against the known concentrations (0, 1.25, 2.5, 3.75, and 5.0 ug) of BSA from the standard curve and draw a regression line. Calculate the R^2 value and the equation for the line. Use the equation for the line to calculate the concentration in ug protein $well^{-1}$ for the unknowns.*
B. Each sample should have a protein concentration value calculated using the equation from the standard curve (Step A) between 0 to 5.0 ug. If the sample values are close to or above 5.0 ug or are close to or below 1.25 ug, then the samples need to be rerun at a different concentration (see below for more information about conducting reruns).
C. Take unknown concentration value and divide it by the number of ul of sample in the well, giving a ug/ul value. (See example below for more details.)
D. Multiply this number by the number of ul extracted (the volume of the extract solution) for an ug extracted value.
E. Divide this number by g weight extracted giving an ug/g value that can be converted in a mg/g value by dividing by 1000.

Example:

OD reading = 0.306
ug/well concentration (from OD value and standard curve) = 2.54 ug/well
ul of sample/well = 10 ul/well
amount of extract = 7650 ul (7.65 ml)
weight extracted = 1.0 g

2.54 ug/well ÷ 10 ul/well x 7650 ul ÷ 1.0 g = 1943.1 ug/g = 1.9431 mg/g

* The plate reader may come with software that can be set to plot the OD and concentration values from the standard curve, draw a regression line, and determine the R^2 value and the equation for the line. It may also be able to calculate the concentrations for the unknowns. **Note: These concentration values are not mg protein g^{-1} soil, but rather are ug protein $well^{-1}$ and must be feed into the equations described in steps B-E.** We use a program called Softmax®. If there is no software program, the data may be put into a spreadsheet program, like Excel®, where it may be plotted and analyzed as described in step A.

Advice for choosing to when rerun samples:

Samples should be rerun if any of the following are true:

A. if you had any trouble pipetting the PBS, sample, or dye, any trouble with bubbles that would not pop, or precipitation of the sample;
B. if the OD values are way outside of the ranges listed Table 3;
C. if one or more of the values calculated for the points on of the standards are not close to the known values. In other words, the value for standard 3 calculated using the equation for the regression line should be close to 2.5 ug;
D. if the CV (coefficient of variation) percentage is >25% for any of the duplicate standard values (Two wells are used for each standard and the CV is calculated from the values calculated for each well. For standard 1, the zero concentration, a percentage CV >25% is acceptable since the CV is calculated as the variation divided by the mean and if the values for this standard are close to zero than CV may be very large.);
E. if the R^2 for the standard curve is < 0.95, the intercept is not very close to zero (±0.07), and the slopes do not match slopes the expected values in Table 3 and/or are not very similar across plates run the same day (In other words, although a different standard curve is used on each plate to calculate concentrations, there should be strong similarity between the intercept and slope for plates run on the same day with reagents from the same source. If new dye or PBS is used, there may be some difference, but intercept and slope values should retain a fairly high similarity.);
F. if the calculated concentrations for the unknowns have an average above 3.75 or below 1.25 or a percentage CV >25%. (When rerunning samples, if the average value is below 1.25, you should increase the volume of extract added to the well by 2 to 4 times, or if the mean result is above 3.75, decrease the volume of extract added to the well by half or a third.);
G. if all three replicate or duplicate samples per sample bag do meet the above criteria.