ELISA SECONDARY ANTIBODY CONJUGATION

(Nichols and Wright, 2004; Rillig, 2004; Wright, 1994; & Wright *et al,* 1996)

Introduction

The ELISA is a procedure used to measure specific protein concentrations in with very little non-specific binding (or measuring of other co-extracted protein or other molecules) at low concentration levels for a more accurate concentration determination. (See Fig. 1 for a illustration of what is occurring during the assay procedures as described in the following sentences. The full details for the ELISA procedure are given below and should be followed. The following sentences give further information about what is occurring during the procedure and why it is so specific.)

In this assay, a sample of the extract solution, containing the target protein (glomalin) diluted in phosphate buffered saline (PBS) is placed in a 96-well plate. The sample is allowed to dry overnight – the water in the solution will evaporate, the salts [PBS and citrate, pyrophosphate, or borate (depending upon what was used for extraction)] will precipitate on the bottom of the well, and proteinaceous material, such as glomalin will bind to the surface of the well. Note the binding ability of the proteinaceous material to the well is dependent upon the shape of the well and type of material from which the well is made. Previous testing has found that Dynex u-shaped microtiter plates (see inventory for ordering info) provide the best binding characteristics for glomalin. After the proteinaceous material (i.e. glomalin) is bound to the well, the wells are filled with a milk solution. This solution is high in proteins which will bind to the well in spots where glomalin or other proteins are not located to cover up any open binding sites on the well. The plate is inverted to remove any unbound proteins in the milk solution as well as the precipitated salts. Next, a solution containing the anti-glomalin antibody (MAb32B11) is added to the well. This monoclonal antibody has a high specificity for an antigenic site on glomalin. Previous testing has demonstrated very little if any cross-reactivity between the anti-glomalin antibody (MAb32B11) and other soil proteins or molecules that may have been co-extracted during the extraction procedure. However, because the MAb32B11 antibody was generated against spores isolated from pot cultures, the antigenic site on extracted glomalin may have been lost during the prolonged heat or other extraction conditions (Nichols and Wright, 2004). This may reduce the binding of the antibody to extracted glomalin fractions and result in an underestimation of glomalin. Because of these issues it is advised to refer to the extracted material as glomalin-related soil protein (Rillig, 2004) and to observe trends between data rather than relying in concentration values. The cell line to produce this antibody was created in mice and therefore contains antigenic sites to bind to antibodies against mice. (The anti-glomalin antibody is currently not available commercially, but the B cell line for production of the antibody is available for purchase from ATCC (American Type Culture Collection). The catalog number is CRL-2559 and the cost is ca. $200. See the procedure for antibody production to learn how to grow the cell line or follow the procedures recommended by ATCC.) Unbound anti-glomalin antibodies are removed by inverting and washing the plate, and a solution containing a biotinylated anti-mouse IgM antibody with a long spacer arm is added. This antibody was produced in goats to bind to mice antigenic sites, such as those found on MAb32B11. This antibody is also biotinylated with a long spacer arm. The biotin addition will allow this antibody to bind to any proteins added to the solution and the long spacer arm increases the binding capability. Unbound anti-mouse IgM antibodies are removed by inverting the plate and washing. Next, an ExtAvidin, or Steptavidin, solution containing a protein that is covalently attached to a reporter enzyme (such as peroxidase or phosphatase) and exhibits low levels of non-specific binding is added to the wells. This molecule will bind to the biotin in the anti-mouse

IgM. Unbound ExtAvdin is removed and a solution containing a substrate molecule which reacts with the covalently attached reporter enzyme is added. When the enzyme reaction occurs, the chromogenic substrate molecule produces a soluble colored substance. The absorbance of this color is measured and compared to a standard curve to calculate glomalin concentration in the unknowns (see below for the calculations). The high specificity of this procedure allows the use a very tight curve (0.005 to 0.04 ug of protein). However, there is no standard glomalin solution to use for the standard curve; instead glomalin extracted from pot culture or soil samples may be used (see more information about the standard curve below).

Note that it is recommended to use at least two duplicate samples of extract from each sample in the 96-well plate 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. The standard curve should have an $R^2 > 0.89$. If the protein values for the samples are above or below the standard curve values (0.005 to 0.04 ul), the variances between duplicate samples are too high (>25), or the R^2 value is too low (< 0.89), than the assay needs to be rerun on those samples (see below).

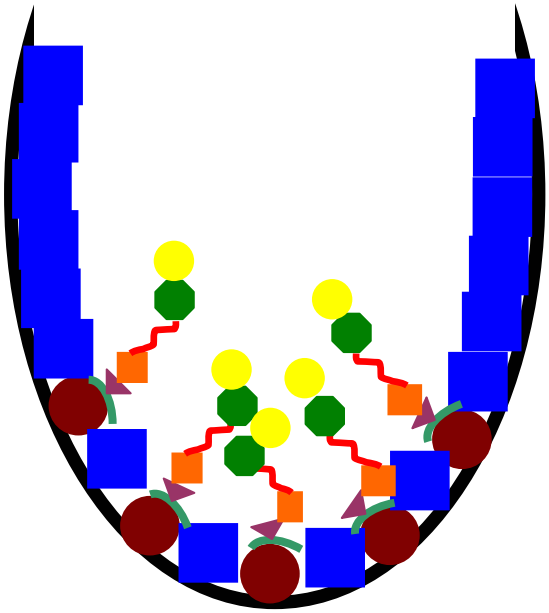

Figure 1. The ELISA procedure used here utilizes a secondary antibody conjugation which improves specificity. In this procedure, a layered sandwich is created in each well as pictured here where proteins and antibodies bind to each other. The glomalin molecule is designated as reddish-brown circles, while milk proteins are blue squares. The anti-glomalin antibody (MAb32B11) has a binding site for glomalin (green semi-circle) and an antigenic site (purple triangle) for the IgM antibody (orange diamonds with a tail) binding. Finally, the ExtrAvidin molecule (green hexagons) attaches to the biotin (tail) in the IgM and has an enzyme to which reacts with the substrate (yellow circles) molecule to produce the color.

Materials

1. Thermo Scientific 96-well, vinyl, u-bottom microtiter plate
2. tilt table or shaker
3. plate reader with a 405 or 410 filter
4. 2% non-fat milk (2g powdered milk/100ml of PBS)
5. PBS (Phosphate buffered saline), pH 7.4
6. PBST (PBS with 0.2 ml/L Tween 20), pH 7.4
7. 1% BSA (1 g BSA/100 ml PBS)*
8. MAb32B11 antibody (Dilute 1 ml antibody in 5 ml PBS)**
9. Biotinylated goat anti-mouse IgM (1:2500 in 1% BSA) (4.8µl/6ml)**
10. ExtrAvidin alkaline phosphatase (1:2000 in 1% BSA) (3µl/6ml)** (optional***)
11. TBST (Tris Buffered Saline) (250 mM NaCl in 10 mM Tris (hydroxymethyl)aminomethane)) plus 0.2 ml/L of Tween 20 at pH 7.4) (optional***)
12. DEA buffer (Mix 97 ml of diethanolamine with 1 L of 0.01% $MgCl_2$ solution, and adjust the pH to 9.8 with 1 N HCl. The solution must be kept sterile and stored covered at room temperature). (optional***)
13. Phosphatase substrate (optional***)
14. ExtrAvidin peroxidase (1:2000 in 1% BSA) (optional***)
15. 30% Hydrogen peroxide (optional***)
16. 1.05% Citric acid solution (optional***)
17. ABTS [2,2'-azino-di-(3-ethylbenzthiaoline sulfonic acid)] (optional***)

*Make about 500-1000 ml of stock and dispense in 6 ml aliquots that will be frozen until needed.
**These are recommended concentrations. Chemicals obtained from different companies or with different lot numbers may need to be optimized for your conditions. If you are not getting the expected results, concentrations may need to be adjusted (see below).
***Some of these chemicals are optional based on what enzyme and substrate are used for color development (see below for more details).

Methods

1) Prepare standard curve.

A. Make up a stock solution of 0.08 ug of protein/100 ul of PBS and store at 4°C (Do not freeze the ELISA stock solution or use it if it appears to be contaminated or is several months old.)

Stock solution:

a) Use protein extracted from fresh hyphae or soil that is nearly 100% immunoreactive. To determine this, run a Bradford assay and measure protein concentration. Use this information to determine the volume of extract solution to use in the ELISA which should give a concentration in the middle of the ELISA standard curve (0.005 to 0.04 ug). Dilute the sample to 50 ul using PBS.
b) Run an ELISA assay as described below using another standard curve or comparing the values to the curve value ranges listed below and calculate the concentrations.

c) If Bradford and ELISA values are nearly the same, make an ELISA curve and test the values by comparing results to another standard curve or comparing the values to the curve value ranges listed below. If the sample values compared to known values generate a curve that has an $R^2 \geq 0.95$ then you can use this as your standard.
d) Make up 500 ul aliquots of the stock with a concentration of 0.08 ug of protein in 100 ul of PBS or 0.04 ug of protein in 50 ul of PBS.

B. Conduct a serial dilution of the standard by following the steps below:

Serial dilution:

a) Put 100 ul stock (concentration in stock = 0.08 ug protein/100 ul of PBS) in 2 of the wells and 50 ul PBS in the other 10 wells.
b) Transfer 50 ul of the 0.08 ug sample to a neighboring well that has 50 ul PBS (Fig. 1).
c) Mix 3-4 times with the micropipette by pulling the sample up and down.
d) Remove 50 ul from these 2 wells and transfer to 2 neighboring wells (Fig. 1). Mix 3-4x. Repeat for these 2 wells (Fig. 1).
e) After the 3rd dilution, remove 50 ul from the 2 wells that have 100 ul and dispose of it (Fig. 1). The row will then have a standard curve with the values shown in Table 1.

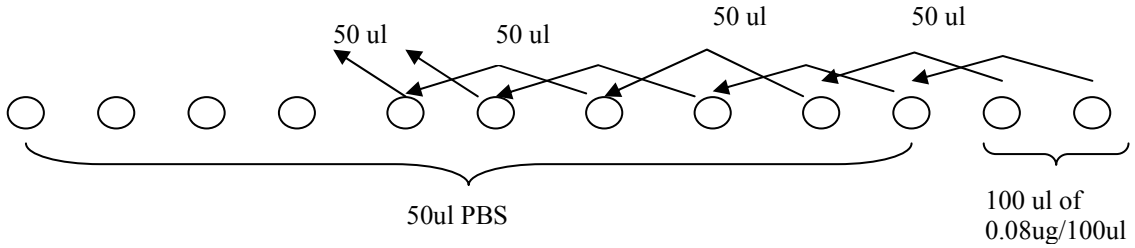

Figure 1. Serial dilution starting with 100 ul of standard solution containing 0.08 ug/100 ul.

Table 1. Glomalin concentrations in wells.

Well #	Protein concentration (ug/50 ul)
1	0
2	0
3	0
4	0
5	0.005
6	0.005
7	0.01
8	0.01
9	0.02
10	0.02
11	0.04
12	0.04

3) Calculate the volumes of sample needed to place in each well from the total protein assay values (as discussed below).

Calculating the volume of sample needed in each well:

a) Take the ug/ul value obtained in step 5 of the Bradford protein assay and divide it into 0.02 ug (which is a value in the middle of the ELISA curve). This gives you the number of ul needed to give a concentration of 0.02 ug/well. By targeting a concentration of 0.02 ug/well, you can dilute a little more or a little less per sample so you can standardize your dilutions. In other words, the number of ul calculated could be 2.16, 3.04, 2.78 for three different samples and you could just use 2.5 ul for each. See Example below.

Example:
Total protein assay ug/well concentration = 2.54 ug/well
Total protein assay ul of sample/well = 10 ul/well

0.02 ug ÷ (2.54 ug/well ÷ 10 ul/well) = 0.08 ul ≈ 0.1 ul

b) Place this amount of sample in a well and add enough PBS to equal a total volume of 50 ul or pre-dilute the sample. Pre-diluting consists of multiplying the amount needed per well from step a above by 20 (since there are twenty 50 ul amounts in 1 ml) and add this amount to a microfuge tube plus enough PBS to equal 1 ml (1000 ul).

4) Add the sample plus enough PBS to the well for a total volume of 50 ul in each well.

5) Let the samples and standard protein bind to the wells overnight. (As the water slowly evaporates, protein with bind to the plastic of the wells. **Note: Do not run assay until the wells are completely dry.**

6) Add 250 ul/well of freshly prepared 2% non-fat milk* to wells and incubate on shaker for 15 min. Invert the plate over a sink followed by blotting with vigorously tapping of the inverted plate on an absorbent paper towel.

7) Add 50 ul/well of diluted MAb 32B11antibody* and incubate on shaker for 1 hr. Flip plate to remove followed by blotting with vigorously tapping of the inverted plate on an absorbent paper towel. Wash with PBST 3x, blotting between washes.

8) Add 50 ul/well of biotinylated IgM antibody*, diluted in 1% BSA, and incubate on shaker for 1 hour. Flip plate to remove followed by blotting with vigorously tapping of the inverted plate on an absorbent paper towel. Wash with PBST 3x, blotting between washes.

9) Add 50 ul of ExtrAvidin Alkaline Phosphatase* diluted in 1% BSA, and incubate on shaker for 1 hr. After incubation, flip plate to remove followed by blotting with vigorously tapping of the inverted plate on an absorbent paper towel. Wash with PBST 3x, blotting between washes.

10) Conduct a fourth wash of the plate using TBST not PBST, because PBST will react with the phosphatase enzyme.

11) Make the color developer solution* by dissolving one tablet of Phosphatase Substrate (5 mg tablets) in 5 ml of DEA buffer (ca. 6 ml color developer is needed per plate). Add 50 ul to each well and incubate for 10 to 30 min. A blue-green color will develop in the wells according to glomalin protein concentration (Fig. 2).

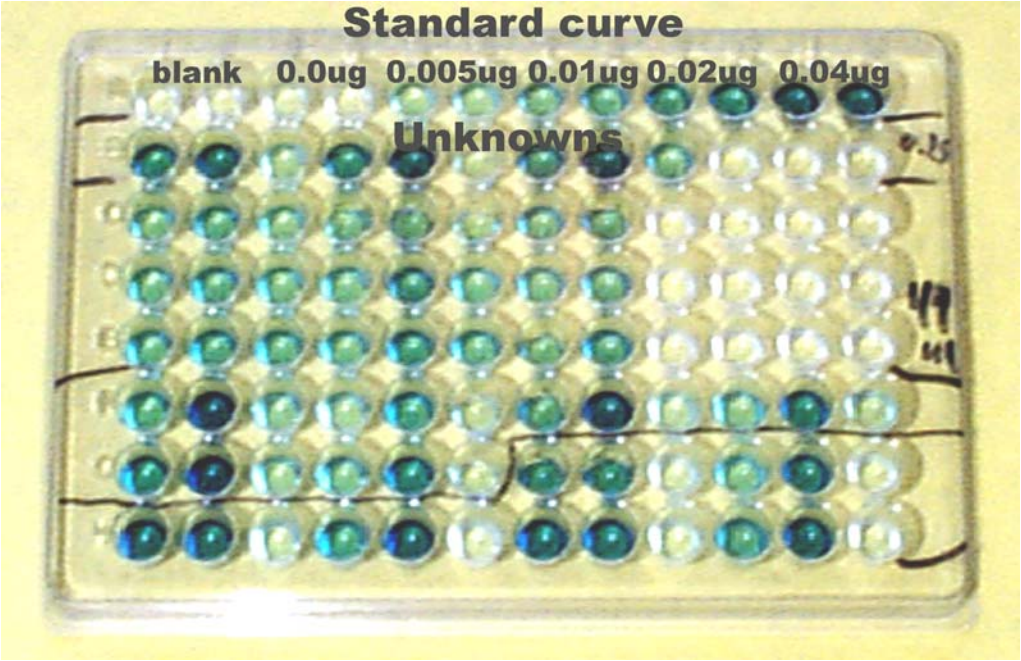

Figure 2. The intensity of color corresponds to concentration. Optical Density values from row A (standard curve) are used to determine an equation for the regression line for the standard curve values. This equation is then used to calculate concentration values for samples.

12) Read plate at 405 or 410 nm every five minutes. Compare OD values to the expected values in Table 2.

Table 2. OD value range for the ELISA standard curve after 15 min of incubation using ExtrAvidin alkaline phosphatase with a phosphate substrate.

Concentration (ug/well)	OD value
0	0 ± 0.050
0.005	0.250-0.500
0.01	0.600-0.950
0.02	1.200-1.700
0.04	2.000-2.800
Slope value	50.000-75.000

Note: Because this curve has a slight quadratic shape, it is advised to dilute samples at the top of the curve in half and recheck them. It is also advised to rerun the sample at the bottom of the curve at 2x the concentration. This will give you ideal concentrations right in the middle of the curve.

13) Use OD values for the standards to calculate the concentrations (mg glomalin g^{-1} soil) for the samples from their OD values (see calculations below).

***** The milk, MAb32B11, IgM, Alkaline phosphatase, and color developer solutions may be placed in solution basins to have working solutions in a container that will allow easy pipetting with the multi-channel pipettes and/or prevent possible contamination of the stock solutions.

Color development in ELISA

Other enzymes and substrate molecules in a number of different solutions may be used for color development in ELISA. Initially, a procedure using a peroxidase enzyme with a hydrogen peroxide-ABTS [2,2'-azino-di-(3-ethylbenzthiaoline sulfonic acid)] color developer solution was recommended for use with glomalin assays. (This procedure is outlined below.)

Because this procedure required the use of hydrogen peroxide which is readily decomposes into water and oxygen, it was difficult to maintain a fresh 30% hydrogen peroxide solution. Also, ABTS readily reacts with light and may be already developed before using it as a color developer in the peroxidase reaction.

Color development with peroxidase:

1) Follow steps 1-5 as outlined in the methods above.
2) Dilute the ExtrAvidin Peroxidase in 1% BSA (3ul/6ml)* and add 50 ul of the diluted solution to each well.
3) Incubate for 1 hr. After incubation, flip plate to remove followed by blotting with vigorously tapping of the inverted plate on an absorbent paper towel.
4) Wash with PBST 4x, blotting between washes.
5) Make the color developer solution fresh as outlined below.

Color developer*

Just before use mix the solutions in the amounts outlined in the table below:

Citrate buffer	ABTS soln.	30% H2O2
10 ml	200 ul	10 ul
7.5 ml	150 ul	7.5 ul
5 ml	100 ul	5 ul
2.5 ml	50 ul	2.5 ul

Citrate buffer: For 100 ml use 1.05 g of citric acid (not sodium citrate) and adjust the pH to 4.0 using 6 N and 2 N NaOH. This may be made up ahead of time and stored until needed. **Note: Check pH before each use.**

ABTS solution: 0.015 g 2,2'-azino-di-(3-ethylbenzthiaoline sulfonic acid)/1 ml ddH2O (Milli-Q) **Note: Must be made fresh and not turn bluish-green prior to adding to wells.**

Hydrogen peroxide: Add to solution right before using in the amounts listed above. **Note: Test freshness by diluting 30% hydrogen peroxide in PBS at a 1:1000 concentration and measure the optical density (OD). The OD value should be ~0.7 at 230 nm (see p. 150 of Goding, 1986)**

6) Add 50 ul of color developer to each well. A blue-green color will develop in the wells according to glomalin protein concentration (Fig. 2).
7) Incubate 15 minutes and read at 405 nm with plate reader. Compare OD values to the expected values in Table 3.
8) Use OD values for the standards to calculate the concentrations (mg glomalin g^{-1} soil) for the samples from their OD values (see calculations below).

***** These are recommended concentrations. Chemicals obtained from different companies or with different lot numbers may need to be optimized for your conditions. If you are not getting the expected results, concentrations may need to be adjusted (see below).

Table 3. OD value range for the ELISA standard curve using ExtrAvidin peroxidase with a hydrogen peroxide substrate.

Concentration (ug/well)	OD value
0	0 ± 0.100
0.005	0.300-0.600
0.01	0.550-1.100
0.02	0.950-1.800
0.04	1.700-2.850
Slope value	40.000-70.000

Note: Because this curve has a slight quadratic shape, it is advised to dilute samples at the top of the curve in half and recheck them. It is also advised to rerun the sample at the bottom of the curve at 2x the concentration. This will give you ideal concentrations right in the middle of the curve.

Calculating mg/g concentration from OD values:

a) After running the assay, plot the standard curve OD values against the concentration values (0, 0.005, 0.01, 0.02, and 0.04 ug) for 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 between 0-0.04 ug. If the sample values are close to or above 0.04 ug or are close to or below 0.005 ug, then the samples need to be rerun at a different concentration (See below for more information about conducting reruns).
c) Take the calculated sample value and divide by the volume (ul) of sample that you placed in the well, giving you a ug/ul value. (See example below for more details.)
d) Multiply this number by the total volume (ul) of the extract solution for each sample (measured in a graduated cylinder following completion of extraction) for an ug extracted value.
e) Divide this number by g weight extracted giving a ug/g value that can be converted in a mg/g value by dividing by 1000

Example:
OD reading (with peroxidase) = 1.150
ug/well concentration = 0.022 ug/well
ul of sample/well = 1 ul/well
amount of extract = 7650 ul (7.65 ml)
weight extracted = 1.0 g

0.022 ug/well ÷ 10 ul/well x 7650 ul ÷ 1.0 g = 1683 ug/g = 1.683 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.

Calculating percentage of immunoreactive protein (%IR):

a) Take the ELISA value for the unknown (as calculated using the steps above) and divide by the total protein value obtained from the Bradford total protein assay (see Bradford total protein assay methodology). (See example below for more details.)
b) Multiply this number by 100 to get % IR (percentage immunoreactive protein).

Example:
Bradford total protein value = 1.9431 mg/g

ELISA glomalin protein value = 1.683 mg/g

% IR = (1.683 mg/g ÷ 1.9431 mg/g) x 100 = 86.61%

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 any of the solutions or there is no color in any of the wells;
b) if the OD values are way outside of the ranges listed in Tables 2 or 3;
c) if one or more of the values calculated for 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 0.01 ug;
d) if the CV (coefficient of variation) percentage is >25% for any of the duplicate standard values;
e) if the R^2 for the standard curve is < 0.90, the intercept is not very close to zero (±0.005), or the slopes do not match slopes the expected values in Tables 2 or 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 the MAb32B11, IgM, or Alkaline phosphatase come from a different bottle, tube, vial, source, etc., 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 0.02 or below 0.005 or a percentage CV >25%. (When rerunning samples, if the average value is below 0.005, you should increase the volume of extract added to the well by 2 to 4 times, or if the value is above 0.02, decrease the volume of extract added to the well by half or a third.);
g) if all three replicate or duplicate samples per field sample do meet the above criteria.

Adjusting concentrations of working solutions to optimize reaction:

a) Because chemicals obtained at different times or from different companies were made at different times and have different lot numbers, the concentrations needed for optimal results with this highly sensitive assay may not match those suggested.
b) If you are getting unexpected results, concentrations may need to be adjusted. Besides the sample, there are four solutions used in the ELISA procedure (32B11 antibody against glomalin, anti-mouse IgM, ExtrAvidin Alkaline Phosphatase, and the color developer). You may need to adjust one or more of these solutions or the incubation time of the color developer.
c) To check incubation time, read the plate every 2 to 5 min and optimize time for full development of standards and samples without overdeveloping the blanks.
d) To determine which solutions need to be optimized, you may use a checkerboard dilution procedure on a plate filled with one sample at one

concentration. This checkerboard serial dilution allows you to examine two of these solutions within a plate by keeping two solutions constant and serially diluting the other two solutions – one horizontally and the other(s) vertically across the plate (see Table 4 below for further details). You may also want to try the concentrations suggested by the manufacture or perform a serial dilution using a value that is 2 or more times the maximum to determine the optimum or minimal concentration.

Table 4. An example of the procedure to check for optimum concentration or to discover which chemical may not be performing correctly and needs to be used at a different concentration or to be reordered. On the grid representing a 96-well plate, 5 ul (or 0.02 ug) of a glomalin sample from a Baltimore soil was placed in each well. In the rows, the MAb32B11 antibody was serially diluted (horizontally) eight times starting with four times the recommended concentration or 4 ml in 2 ml PBS. In the columns, the ExtrAvidin Phosphatase was serially diluted (vertically) six times starting at three times the recommended concentration or 9 ul in 6 ml 1% BSA in PBS. In this example, the optimum concentration was found in row 3, column 4, (highlighted) or at 1 ml for the 32B11 antibody and ca. 1 ul for ExtrAvidin Phosphatase. At the higher concentrations, the molecules may be self-aggregating and causing a reduction in values.

0.432	0.500	0.555	0.578	0.526	0.317
0.679	0.978	1.356	1.659	1.153	0.330
1.049	1.468	1.502	1.975	1.009	0.157
1.429	1.519	1.624	1.725	1.036	0.149
0.658	0.552	0.468	0.538	0.487	0.189
0.468	0.579	0.456	0.339	0.285	0.090
0.135	0.182	0.199	0.134	0.145	0.079
0.075	0.129	0.157	0.055	0.039	0.008

e) After optimizing the assay for your conditions, if unexpected results occur, one or more of the chemicals may need to be replaced.
f) Use the checkerboard dilution procedure discussed above again to determine which chemical needs to be replaced.
g) Replacing solvents such as the PBS, PBST, TBST, or DEA buffer may also resolve some problems.

Note: It is a good idea to make several plates full (all 96 wells) of the standard curve dried to the wells when you have a good sample that you are using as your standard, this will help to (1) solve problems with the antibodies or when your standards are getting too old, and (2) can help you calibrate a new standard sample. Remember the concentrations used and the OD value range for the standard curve are just recommendations. Experimenting in your own lab using your best judgment will assist you in solving most of the problems that you may be having. Also, note that these procedures are continuing to evolve as we get further information and that

some of the changes in immunoreactivity (see below) may greatly impact your results.

Changes in immunoreactivity

(Nichols, 2003; Nichols and Wright, 2004)

a) Glomalin is a complex biomolecule which has only recently been examined on a molecular level.

b) It has been suggested that the three dimensional structure of a protein (or its conformation) may impact its function. In addition, conditions (such as temperature, pH level, chemical structure and reactivity) of the solute(s) used in analysis of the protein may impact the conformation [which might obscure the epitope for the antibody against glomalin (MAb32B11) and reduce immuno-reactivity]. Therefore, prior to extraction, during extraction, and while analyzing, purifying and storing glomalin, certain changes in glomalin conformation are likely due to the high temperature and aqueous salt solution used for glomalin extraction (i.e. solubilization).

c) Some of the actions that could induce conformational changes are:

   1. Binding of iron, other cations, or organic matter to glomalin in soil or pots
   2. Self-aggregation of the glomalin molecule with time in and conditions of the extract solution
   3. Prolonged exposure to heat during the extraction process
   4. Leaving samples in an aqueous citrate or pyrophosphate solution for more than a week or two
   5. Growth of other microbes in the extract solution
   6. Exposure to pH extremes (i.e. low pH HCl and high pH NaOH) during the purification procedures
   7. Freezing glomalin to obtain a freeze-dried sample

d) As more information is obtained about the structure of glomalin, ways to prevent reductions in immunoreactivity and improve the efficiency of extraction and quantification will be identified.

e) Typically, ELISA's used as positive or negative assays to show the presence or absence of a molecule. Trying to do quantification with ELISA is often difficult, especially without a good standard.

f) Over time the immunoreactivity of standards will change and you will have to extract a new standard from the reference soil.