SAMPLE EXPERIMENTAL PROTOCOL

Prepare sample solution:

1. Use 50 to 100 µg maleimide sample (protein or other polymers).
2. Adjust the volume to 100 µL with Assay Buffer (Component B). Note: The maleimide-linked antibody or protein sample should be in pH = 6.0 buffer and without free maleimide.

Run Maleimide Assay:

1. Add the maleimide sample to one vial of Maleimide Blue™ (Component A).
2. Mix them well by repeatedly pipetting for a few times or vortex the vial for a few seconds.
3. Keep the reaction mixture at room temperature and rotate or shake for 30 - 60 minutes.

Prepare Spin Column for Sample Purification:

1. Invert the Spin Column (Component C) several times to resuspend the settled gel and remove any bubbles.
2. Snap off the tip and place the column in the Washing Tube (2 mL, Component D). Remove the cap to allow the excess packing buffer to drain by gravity to the top of the gel bed. If column does not begin to flow, push cap back into column and remove it again to start the flow. Discard the drained buffer, and then place the column back into the Washing Tube. However, centrifuge immediately if the column is placed into a 12 x 75 mm test tube (not provided).
3. Centrifuge for 1 min in a swinging bucket centrifuge at 1,000 x g (see Centrifugation Notes section) to remove the reaction buffer. Discard the buffer.
4. Apply 1 mL Assay Buffer (Component B) to the column, let the buffer drain out by gravity, or centrifuge the column for 1 min to remove the buffer. Discard the buffer from the collection tube. Repeat this process for 3 - 4 times.
5. Centrifuge for 2 minutes in a swinging bucket centrifuge at 1,000 x g (see Centrifugation Notes section) to remove the excess packing buffer. Discard the buffer.

Note: Spin Column (Component C) can fit into 2 mL microcentrifuge tubes or 12 x 75 mm test tubes for sample collection during centrifugation. Use the 2 mL microtubes provided with the columns for the initial column equilibration step.

Note: Swinging bucket centrifuges capable of generating a minimum force of 1,000 x g are suitable for Bio-Spin column use. The gravitational force created at a particular revolution speed is a function of the radius of the microcentrifuge rotor. Consult the swinging bucket centrifuge instruction manual for the information about conversion from revolutions per minute (RPM) to centrifugal or g-force. Alternatively, use the equation to calculate the speed in RPM required to reach the gravitational force of 1,000 x g.

\[
\text{RCF}(g) = 1.12 \times 10^{-5} \times (\text{RPM})^2 \times r
\]

\[
\text{RCF} = \text{the relative centrifugal force}
\]

\[
\text{RPM} = \text{the speed of the rotor}
\]

\[
r = \text{the radius in centimeters measured from the center of the rotor to the middle of the Bio-Spin column}
\]

Purify Maleimide Reaction Product:
1. Place the column in a clean Collecting Tube (1.5 mL, Component E). Carefully load the sample (100 µL) directly to the center of the column.

2. After loading the sample, add 10 µL Assay Buffer (Component B) to the top and centrifuge the column for 5 min at 1,000 x g, and collect the solution into the collecting tube.

Run Absorption Spectra with 0.2mL or 0.5 mL Quartz Cuvette:

1. Dilute the maleimide reaction product by 5 - 10 folds with Assay Buffer (Component B) depending on the cuvette size used and the absorbance reading. The dilution factor doesn’t affect the final maleimide quantitation result.

2. Measure the absorption spectrum from 900 nm to 250 nm range, or only read the absorbance number at 280 nm and 782 nm.

EXAMPLE DATA ANALYSIS AND FIGURES

For illustrating purpose we use a BSA-maleimide as an example to calculate the number of maleimide groups on maleimide-linked BSA sample with 0.5 mL cuvette measurement.

Sample: BSA-maleimide, 10.75 mg/mL in pH=6.0 Buffer

Procedures:

1. Use 4.7 µL (50 µg) of BSA-maleimide, and then add 95.3 µL Assay Buffer (Component B) to have total volume of 100µL.
2. Add above 100µL solution to Maleimide Blue™ vial (Component A), mix well.
3. Rotate for 60 min at room temperature.
4. Purify with Spin Column (Component C), and collect the product.
5. Take 50 µL of the product, and add 400 µL Assay Buffer (Component B) to the 0.5mL cuvette and measure the absorbance spectra.

Constants needed:

BSA extinction coefficient at 280 nm: 43824 M⁻¹ cm⁻¹
Maleimide Blue™ extinction coefficient at maximum absorption (780 ± 3nm): 275,000 M⁻¹ cm⁻¹
Correction Factor of Maleimide Blue™ at 280 nm (CF₂₈₀nm): 0.207

Results:

OD readings obtained with the above BSA-maleimide sample: 
A₂₈₀nm = 0.290, 
A₇₈₂nm = 1.214

Calculations:

Calculate BSA-maleimide amount with Equation:

\[
\frac{\text{(Moles of Maleimide)}}{\text{(Moles of protein or antibody)}} = \frac{[A_{782\text{nm}}]}{[A_{280\text{nm}}] \times \text{CF}_{280\text{nm}} \times [A_{280\text{nm}}] \times [A_{782\text{nm}}]} \times [\text{Moles of protein or antibody at 280 nm}]
\]

Maleimide Ratio = (1.214/275000)/(0.290 - 0.207 \times 1.214)/43824 = 5.00

Figure 1.