HyNic Protein MSR Instructions

Determining the HyNic/Protein Molar Substitution Ratio (MSR)

Determination of the number of HyNic groups per protein is accomplished by a colorimetric assay as presented in Figure 1. In this assay 2-sulfobenzaldehyde (2-SB) forms a chromophoric bis-arylhydrazone product with HyNic groups on proteins which absorbs at 347 nm with a molar extinction coefficient of 28,500 M^{-1} cm^{-1}. The HyNic MSR may be measured using either a conventional UV-Vis spectrophotometer or a NanoDrop™ spectrophotometer. After preparing the 2-sulfobenzaldehyde solution, follow the MSR procedure below for the type of instrument available.

Materials Required

Reagents	Equipment
S-Sulfobenzaldehyde	1.5 mL microcentrifuge tubes
Conjugation Buffer (10X)	Spectrophotometer or NanoDrop Spectrophotometer
1X MES Buffer (100 mM MES, pH 5.0)	
Nuclease-free H_2O	

Prepare 2-Sulfobenzaldehyde (2-SB) Solution (For Protocols Below)

1. Prepare a 0.5 mM working solution of 2-SB in 0.1 M MES buffer, pH 5.0, as follows:
   a. Weigh approximately 10 mg of 2-sulfobenzaldehyde into a microcentrifuge tube while noting the exact mass.
   b. Dissolve the 2-sulfobenzaldehyde in water to create a 50 mg/mL solution.
   c. Add 104 µL of the 2-SB solution to a 50 mL conical tube containing 50 mL of 100 mM MES Buffer, pH 5.0.
   d. Mix well.
   e. Protect the solution from light and keep refrigerated. This solution remains stable for up to 60 days at 4°C.

Protocols:

Conventional UV-Vis Spectrophotometer MSR Protocol

1. MSR reaction setup.

   Note: *The following procedure is designed for a 500 µL cuvette. Volumes of the blank and MSR reactions can be lowered proportionally for smaller volume micro-cuvettes to preserve HyNic-modified protein.*

   a. Prepare an MSR blank by adding 25 µL of 1X Conjugation Buffer, pH 6.0, to 25 µL of 0.5 mM 2-sulfobenzaldehyde in a microcentrifuge tube.
   b. Prepare a HyNic MSR reaction by adding 25 µL of HyNic-modified protein to 25 µL of 0.5 mM 2-sulfobenzaldehyde in a separate microcentrifuge tube.
   c. Vortex both reactions to mix
2. Incubate the reactions at 37°C for 60 minutes or at room temperature for 90 minutes.
3. Briefly centrifuge the tubes at 10,000 x g to collect condensation from the cap.
4. Add 450 µL of water to each tube and vortex to mix.
5. Program the spectrophotometer to scan from 220 nm to 420 nm.

   Note: *If wavelength scanning is not available, the absorbance may be measured at 280 nm and 347 nm individually.*
6. Using a UV-transparent plastic or quartz cuvette, blank the spectrophotometer from 220 – 420 nm with the diluted MSR blank sample.
7. Scan the diluted HyNic MSR reaction from 220 – 420 nm, recording the absorbance at 280 nm and 347 nm.
8. Enter these values into the HyNic-Protein MSR Calculator, along with the required protein information.
9. The calculator will display the HyNic MSR.

NanoDrop Spectrophotometer MSR Protocol

1. MSR reaction setup.
   a. Prepare an MSR Blank by adding 10 µL of 1X Conjugation Buffer, pH 6.0, to 10 µL of 0.5 mM 2-sulfobenzaldehyde in a microcentrifuge tube.
   b. Prepare a HyNic MSR reaction by adding 10 µL of HyNic-modified protein to 10 µL of 0.5 mM 2-sulfobenzaldehyde in a separate microcentrifuge tube.
   c. Vortex both reactions to mix.
2. Incubate the reactions at 37°C for 60 minutes or at room temperature for 90 minutes.
3. Briefly centrifuge the tubes at 10,000 x g to collect condensate from the cap.
4. Vortex both reactions to mix.
5. Launch the NanoDrop software and select the UV-Vis menu option.
6. Initialize the instrument with 2 µL of water if necessary (NanoDrop ND-1000 only).
7. Blank the instrument with 2 µL of the MSR Blank.
8. Set the λ1 wavelength to 280 nm and the λ2 wavelength to 347 nm.
9. Place 2 µL of the MSR reaction on the pedestal and click the “Measure” icon. The 1-mm pathlength A280 and A347 will be displayed.

   Note: *Ensure the absorbance values displayed correspond to a 1-mm pathlength rather than a 10-mm (1-cm) pathlength. If the values are given for a 10-mm pathlength divide them by 10 before entering them into the MSR calculator.*
10. Enter the calculated values into the HyNic-Protein MSR Calculator, along with the required protein information.
11. The calculator will display the HyNic MSR.