

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 ₂ O	

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

- Prepare a 0.5 mM working solution of 2-SB in 0.1 M MES buffer, pH 5.0, as follows:
 - Weigh approximately 10 mg of 2-sulfobenzaldehyde into a microcentrifuge tube while noting the exact mass.
 - Dissolve the 2-sulfobenzaldehyde in water to create a 50 mg/ml solution.
 - 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.
 - Mix well.
 - 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

- 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.

- 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.
 - 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.
 - Vortex both reactions to mix
- Incubate the reactions at 37°C for 60 minutes or at room temperature for 90 minutes.

- Briefly centrifuge the tubes at 10,000 x g to collect condensation from the cap.
- Add 450 µl of water to each tube and vortex to mix.
- 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.
- Using a UV-transparent plastic or quartz cuvette, blank the spectrophotometer from 220 - 420 nm with the diluted MSR blank sample.
- Scan the diluted HyNic MSR reaction from 220 - 420 nm, recording the absorbance at 280 nm and 347 nm.
- Enter these values into the [HyNic-Protein MSR Calculator](#), along with the required protein information.
- The calculator will display the HyNic MSR.

NanoDrop Spectrophotometer MSR Protocol

- MSR reaction setup.
 - 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.
 - 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.
 - Vortex both reactions to mix.
- Incubate the reactions at 37°C for 60 minutes or at room temperature for 90 minutes.
- Briefly centrifuge the tubes at 10,000 x g to collect condensate from the cap.
- Vortex both reactions to mix.
- Launch the NanoDrop software and select the UV-Vis menu option.
- Initialize the instrument with 2 µl of water if necessary (NanoDrop ND-1000 only).
- Blank the instrument with 2 µl of the MSR Blank.
- Set the λ₁ wavelength to 280 nm and the λ₂ wavelength to 347 nm.
- 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.
- Enter the calculated values into the [HyNic-Protein MSR Calculator](#), along with the required protein information.
- The calculator will display the HyNic MSR.