

4FB Protein MSR Instructions

Determining the 4FB/Protein Molar Substitution Ratio (MSR)

Determination of the number of 4FB groups per protein is accomplished by a colorimetric assay. In this assay 2-hydrazinopyridine (2-HP) forms a chromophoric bis-arylhydrazone product with 4FB groups on proteins which absorbs at 360 nm with a molar extinction coefficient of $24,500 \text{ M}^{-1} \text{ cm}^{-1}$. The 4FB MSR may be measured using either a conventional UV-Vis spectrophotometer or a NanoDrop spectrophotometer. After preparing the 2-hydrazinopyridine solution, follow the MSR procedure below for the type of instrument available.

Materials Required

Reagents	Equipment
2-Hydrazinopyridine dihydrochloride	1.5 mL microcentrifuge tubes
Conjugation Buffer (10X)	Spectrophotometer or NanoDrop Spectrophotometer
1X MES Buffer (100 mM MES, pH 5.0)	
Ultrapure water	

Prepare 2-Hydrazinopyridine (2-HP) Solution (For Protocols Below)

- Prepare a 0.5 mM working solution of 2-HP in 0.1 M MES buffer, pH 5.0, as follows:
 - Weigh approximately 5 – 10 mg of 2-hydrazinopyridine dihydrochloride into a microcentrifuge tube while recording the exact mass weighed.
 - Dissolve the 2-HP solution in water at a concentration of 50 mg/mL. Vortex to completely dissolve.
 - Add 91 μL of this 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 4FB-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-HP in a microcentrifuge tube.
 - Prepare a 4FB MSR Reaction by adding 25 μL of 4FB-modified protein to 25 μL of 0.5 mM 2-HP 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 can be measured at 280 nm and 360 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 4FB MSR Reaction from 220 – 420 nm, recording the absorbance at 280 nm and 360 nm.
- Enter these values into the [4FB-Protein MSR Calculator](#), along with the required protein information.
- The calculator will display the 4FB 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-HP in a microcentrifuge tube.
 - Prepare a 4FB MSR Reaction by adding 10 μL of 4FB-modified protein to 10 μL of 0.5 mM 2-HP in a separate microcentrifuge tube.
 - Vortex both solutions to mix.
- Incubate the tubes 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.
- 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 λ_1 wavelength to 280 nm and the λ_2 wavelength to 360 nm.
- Place 2 μL of the MSR Reaction on the pedestal and click the “Measure” icon. The 1-mm pathlength A_{280} and A_{360} 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 these values into the [4FB-Protein MSR Calculator](#), along with the required protein information.
- The calculator will display the 4FB MSR.