

# 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  $\mu\text{l}$  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  $\mu\text{l}$  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  $\mu\text{l}$  of 1X Conjugation Buffer, pH 6.0, to 25  $\mu\text{l}$  of 0.5 mM 2-HP in a microcentrifuge tube.
  - Prepare a 4FB MSR Reaction by adding 25  $\mu\text{l}$  of 4FB-modified protein to 25  $\mu\text{l}$  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  $\mu\text{l}$  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  $\mu\text{l}$  of 1X Conjugation Buffer, pH 6.0, to 10  $\mu\text{l}$  of 0.5 mM 2-HP in a microcentrifuge tube.
  - Prepare a 4FB MSR Reaction by adding 10  $\mu\text{l}$  of 4FB-modified protein to 10  $\mu\text{l}$  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  $\mu\text{l}$  of water if necessary (NanoDrop ND-1000 only).
- Blank the instrument with 2  $\mu\text{l}$  of the MSR Blank.
- Set the  $\lambda_1$  wavelength to 280 nm and the  $\lambda_2$  wavelength to 360 nm.
- Place 2  $\mu\text{l}$  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.