

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



Materials Required

Reagents	Catalog #	Equipment
1X MES Buffer (100 mM MES, pH 5.0)	S-4026	1.5 mL microcentrifuge tubes
2-Hydrazinopyridine-2HCl	S-2002	Spectrophotometer or NanoDrop
Conjugation Buffer	S-4002	Nuclease-free H ₂ O

The determination of the number of 4FB groups/protein is accomplished by a colorimetric assay presented in Figure 1 using a Nanodrop spectrophotometer. In the assay, 2-hydrazinopyridine-2HCl (2-HP) forms a chromophoric bis-arylhydrazone product with incorporated 4FB groups that absorbs at 350 nm and has a molar extinction coefficient of 24,500 M⁻¹ cm⁻¹.

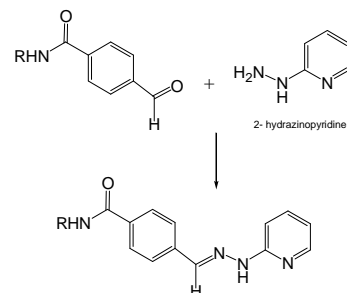


Figure 1: Scheme presenting the colorimetric assay used to quantify the number of 4FB groups on a biomolecule. The bis-arylhydrazone product absorbs at 350 nm and has a molar extinction coefficient of 24,500 M⁻¹ cm⁻¹.

4FB Colorimetric MSR Assay Protein / NanoDrop Protocol

1. Prepare a 0.5 mM working solution of 2-hydrazinopyridine-2HCl (2-HP) solution in 0.1 M MES buffer, pH 5.0 as follows:
 - a. Weigh 5 mg of 2-hydrazinopyridine-2HCl.
 - b. Create a 50 mg/mL solution of 2-hydrazinopyridine-2HCl (M.W. 182.1) in 100 μ L nuclease-free H₂O.
 - c. Add 91 μ L of this solution to a 50 mL conical tube containing 50 mL of 100 mM MES Buffer, pH 5.0.
 - d. Label the solution "0.5 mM 2-HP solution."
 - e. Protect the solution from light and keep refrigerated. This solution remains stable for up to 30 days at 4° C.
2. Fill three microcentrifuge tubes with 18 μ L of 0.5 mM 2-HP solution.
 - a. Add 2 μ L of 1x Conjugation Buffer, pH 6.0 to the first microcentrifuge tube, which will be the "Blank".
 - b. Add 2 μ L of desalted 4FB-modified protein solution (~2-5 mg/ml in 1x Conjugation Buffer, pH 6.0) to the second and third microcentrifuge tubes to create duplicates.
 - c. Label all microcentrifuge tubes.
3. Incubate all reaction tubes at 37° C for 30 minutes or at room temperature for 2 hours.
4. Open the NanoDrop and choose the UV-Vis option.
5. Uncheck the "Normalization" box.
6. Add a 2 μ L sample of the "Blank" microcentrifuge tube. Click the "Blank" button to blank.
7. Read the duplicates by adding 2 μ L to the NanoDrop. Measure the A₃₅₀ of the duplicates.
 - a. If the BLACK line is used to determine the A₃₅₀, adjust for the 1-mm path length by multiplying the given number from the NanoDrop by 10 to obtain the A₃₅₀ for a 1-cm path length.
 - b. If the RED line is used to determine the A₃₅₀, adjust for the 0.1-mm path length by multiplying the given number from the NanoDrop by 100 to obtain the A₃₅₀ for a 1-cm path length.
8. Using the averaged values obtained, calculate the 4FB/protein MSR with the aid of our [4FB/Protein MSR Calculator](#) or calculate the MSR by determining the hydrazone concentration using the known molar extinction coefficient (i.e. 24,500 M⁻¹ cm⁻¹ at 350nm) and dividing by the known molar protein concentration.