

Determining the 4FB / Oligo 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/oligonucleotide is accomplished by a colorimetric assay presented in Figure 1 using a 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.

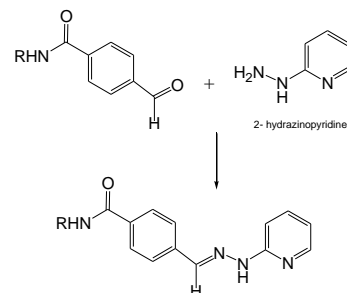


Figure 1: Scheme presenting the colorimetric assay used to quantify the number of 4FB groups on a biomolecule. The bis-arylhydrazone product absorbs at 350 nm has a molar extinction coefficient of 24,500 M⁻¹ cm⁻¹.

4FB Colorimetric MSR Assay Oligonucleotide / Spectrophotometer 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 (M.W. 182.1).
 - b. Create a 50 mg/mL solution of 2-hydrazinopyridine-2HCl 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 490 μ L of 0.5 mM 2-HP solution.
 - a. Add 10 μ L of 1x Conjugation Buffer, pH 6.0 to the first microcentrifuge tube, which will be the "Blank".
 - b. Add 10 μ L of 4FB modified oligonucleotide to the second and third microcentrifuge tubes to create duplicates.
 - c. Label all microcentrifuge tubes.
 - d. Reaction volumes may be proportionately decreased depending on cuvette size.
3. Incubate the microcentrifuge tubes at 37° C for 30 minutes.
4. Add the whole volume of the "Blank" microcentrifuge tube to a 1-cm cuvette and blank the spectrophotometer at 350nm.
5. Measure the A₃₅₀ of the duplicates.
6. Input the average A₃₅₀ value of the duplicates into the [4FB/Oligonucleotide MSR calculator](#).
7. If the value is between 0.8 and 1.2, then the oligonucleotide is modified and desalted.
 - a. If the value is below 0.8 the amino incorporation during oligonucleotide solid phase synthesis was inefficient or the modification reaction with S-4FB did not succeed. The oligonucleotide can be re-modified by desalting the oligonucleotide back into 1x Modification Buffer, pH 7.4 and repeating the modification steps.
 - b. If the value is above 1.2, then there is most likely excess 4FB in solution. Use a a Diafiltration spin column (Cat#: S-4004) to desalt the excess 4FB.