

4FB Oligonucleotide MSR Instructions

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

Determination of the number of 4FB groups per oligonucleotide is accomplished by a colorimetric assay. In this assay, 2-hydrazinopyridine (2-HP) forms a chromophoric bis-arylhydrazone product with 4FB groups that absorbs at 360 nm and has a molar extinction coefficient of $24,500 \text{ M}^{-1} \text{ cm}^{-1}$. Either a conventional UV-Vis spectrophotometer with 1-cm pathlength cuvette or a NanoDrop™ spectrophotometer can be used for MSR determination. Follow the procedure below to prepare a 2-hydrazinopyridine working solution, then prepare the MSR samples and read the absorbance value based on the type of instrument available.

If the 4FB-oligonucleotide concentration is unknown, first measure the concentration using a spectrophotometer as described in the [Oligo Concentration Determination protocol](#). The 4FB-oligo concentration should be between approximately 0.25 – 0.50 OD₂₆₀/μl.

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

- Set up MSR and blank reactions:
 - Prepare a 2-HP blank by adding 5.0 μl of 1X Conjugation Buffer to 45.0 μl of 2-HP reagent in a microcentrifuge tube. Label this tube "2-HP Blank."
 - Prepare a 4FB-oligo MSR sample by adding 5.0 μl of 4FB-modified oligo to 45.0 μl of 2-HP reagent in a separate microcentrifuge tube. Label this tube "4FB-Oligo MSR."
 - Vortex both solutions to mix, then briefly spin the tubes to collect all liquid at the bottom.

- Incubate both tubes at 37°C for 60 minutes or at room temperature for 90 minutes.
 - After the incubation period, centrifuge both tubes at 15,000 x g for 15 seconds to collect condensation from the lids.
- Prepare a 1:10 dilution of the 2-HP Blank and 4FB-Oligo MSR reaction by adding 450 μl of water to each tube.
 - Using a 1-cm pathlength cuvette, blank the spectrophotometer at 360 nm with the diluted 2-HP Blank.
 - Measure the 360 nm absorbance of the diluted 4FB-Oligo MSR reaction.
 - Enter this value directly into the [4FB-Oligonucleotide MSR Calculator](#).
 - Ensure the required 4FB-oligonucleotide information has been entered into the calculator. The calculator will display the oligo 4FB MSR.

NanoDrop Spectrophotometer MSR Protocol

- Set up MSR and blank reactions.
 - Prepare a 2-HP blank by adding 2.0 μl of 1X Conjugation Buffer to 18.0 μl of 2-HP reagent in a microcentrifuge tube. Label this tube "2-HP Blank."
 - Prepare a 4FB-oligo MSR sample by adding 2.0 μl of 4FB-modified oligo to 18.0 μl of 2-HP reagent in a separate microcentrifuge tube. Label this tube "4FB-Oligo MSR."
 - Vortex both solutions to mix, then briefly spin the tubes to collect all liquid at the bottom.
 - Incubate both tubes at 37°C for 60 minutes or at room temperature for 90 minutes.
 - After the incubation period, centrifuge both tubes at 15,000 x g for 15 seconds to collect condensation from the lids.
 - Vortex both tubes to mix.
- Launch the NanoDrop software and select the UV-Vis option.
- Initialize the instrument with 2 μl of water, if necessary (NanoDrop ND-1000 only).
- Blank the instrument with 2 μl of 2-HP Blank.
- Set the λ1 absorbance wavelength to 360 nm.
- Load 2 μl of the 4FB-Oligo MSR reaction and read the 360 nm absorbance.

Note: This value should be displayed as a 1-mm pathlength absorbance rather than a 10-mm (1-cm) pathlength. If the instrument displays the 10-mm pathlength absorbance, divide this value by 10 before entering it into the calculator.
- Enter the 1-mm absorbance value into the [4FB-Oligonucleotide MSR Calculator](#).
- Ensure the required 4FB-oligo information has been entered into the calculator. The calculator will display the oligo 4FB MSR.