Calculate dye:protein (F/P) molar ratios

TR0031.7

Introduction

Quantitation of protein:dye conjugation (dye:protein or F/P molar ratio) is essential for predicting the amount of probe necessary for an experiment and for controlling fluorescence intensity between experiments. The degree of labeling may be calculated by separately determining the protein and fluorophore molar concentrations of the conjugate based on absorbance measurements and then expressing these concentrations as a ratio. The ratio represents the average number of dye molecules conjugated to each protein molecule; some individual protein molecules in the solution will have greater than the average number of dye molecules and others will have less, presumably in frequencies described by a statistical distribution about the mean. This Tech Tip describes how to calculate dye:protein molar ratios for proteins labeled with various fluorescent dyes.

The degree to which a probe is labeled is often dependent on the conjugation process. Labeling reactions are influenced by the molar ratio of the reactants, contaminants, and the activity of labeling reagent. In general, a high level of labeling is desirable in fluorescence-based assays because it allows high sensitivity. However, over-labeling can cause quenching as a result of fluorescent emissions from one dye molecule being absorbed by neighboring dye molecules. In addition, over-labeling can result in loss of biological activity of a molecule or decreased solubility. In contrast, too few labels will yield weak fluorescence and possibly an ineffective probe. Therefore, when labeling an antibody or other molecule with a fluorescent dye, test different dye:protein molar ratios in the conjugation reaction to determine which conditions allow for optimal labeling levels and result in signal-to-noise ratios compatible with the intended assays.

Important Information

- Fluorescent dyes are hydrophobic and may bind noncovalently to proteins. For accurate determination of dye:protein molar ratios, extensive dialysis must be performed to remove any nonspecifically bound dye.
- To determine dye-to-protein molar ratio, the extinction molar coefficient (ε) of the unlabeled protein must be known. For more information about protein extinction coefficient (ε), including conversion between percent absorbances for a 1% solution and molar extinction coefficient, please see the related Tech Tip #6: Extinction coefficients.
- Each kind of fluorescent dye molecule absorbs maximally at a particular wavelength to an extent described by its extinction coefficient (ε´). A_{max} is the absorbance (A) of a dye solution measured at the wavelength maximum (λ_{max}). Together, the A_{max} and ε´ may be used to calculate the molar concentration of dye in a sample. Measurement of A_{max} is also necessary for correctly determining the protein concentration (see next bullet point). Values for λ_{max} and ε´ for several Thermo Scientific Pierce Fluorescent Dyes are listed in Table 1.
- Absorbance at 280 nm (A_{280}) is used to determine the protein concentration in a sample. However, because fluorescent dyes also absorb at 280 nm, a correction factor must be used to adjust for amount of A_{280} contributed by the dye. The correction factor (CF) equals the A_{280} of the dye divided by the A_{max} of the dye. Correction factors for several Thermo Scientific Pierce Fluorescent Dyes are listed in Table 1.

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Table 1. Critical values for various Thermo Scientific Fluorescent Dyes.

Fluorophore	Wavelength Maximum (λ_{max})	Extinction Coefficient (ε´)	Correction Factor (CF)
DyLight® 350	353 nm	15,000 M^{-1} cm^{-1}	0.1440
DyLight 405	405 nm	30,000 M^{-1} cm^{-1}	0.5640
DyLight 488	493 nm	70,000 M^{-1} cm^{-1}	0.1470
DyLight 550	562 nm	150,000 M^{-1} cm^{-1}	0.0806
DyLight 594	595 nm	80,000 M^{-1} cm^{-1}	0.5850
DyLight 633	627 nm	170,000 M^{-1} cm^{-1}	0.1100
DyLight 650	652 nm	250,000 M^{-1} cm^{-1}	0.0371
DyLight 680	684 nm	140,000 M^{-1} cm^{-1}	0.1280
DyLight 755	754 nm	220,000 M^{-1} cm^{-1}	0.0300
DyLight 800	777 nm	270,000 M^{-1} cm^{-1}	0.0452
Fluorescein Isothiocyanate (FITC), NHS-Fluorescein, 5-IAF	494 nm	68,000 M^{-1} cm^{-1}	0.3000
Tetramethyl-rhodamine-5-(and 6) -isothiocyanate (TRITC)	555 nm	65,000 M^{-1} cm^{-1}	0.3400
NHS-Rhodamine	570 nm	60,000 M^{-1} cm^{-1}	0.3400
Texas Red® Sulfonyl Chloride	595 nm	80,000 M^{-1} cm^{-1}	0.1800
R-Phycoerythrin	566 nm	1,863,000 M^{-1} cm^{-1}	0.1700
AMCA-NHS, AMCA-Sulfo-NHS or AMCA-Hydrazide	346 nm	19,000 M^{-1} cm^{-1}	0.1900

Procedure for Determining Degree of Protein Labeling

A. Measure A_{280} and A_{max} of the Dye-labeled Protein

1. Remove excess dye from the sample by dialysis or gel filtration. The nonconjugated dye must be completely removed for optimal results and accurate determination of the dye:protein ratio.
2. Measure the absorbance of the protein:dye conjugate at 280 nm using a spectrophotometer cuvette that has a 1 cm path length.

   Note: If initial absorbance measurements exceed 2.0, dilute the sample, or an aliquot thereof, by a factor necessary to obtain absorbance values less than 2.0. Record the dilution factor, which will be required in the calculations.
3. Measure the absorbance of the protein:dye conjugate at the λ_{max} of the dye (see Table 1).

B. Calculate the Degree of Labeling

1. Calculate molarity of the protein:
   - ε = protein molar extinction coefficient (e.g., the molar extinction coefficient of IgG is ~210,000 M^{-1} cm^{-1})
   - A_{max} = Absorbance (A) of a dye solution measured at the wavelength maximum (λ_{max}) for the dye molecule
   - CF = Correction factor; adjusts for the amount of absorbance at 280 nm caused by the dye (see Table 1)
   - Dilution factor = the extent (if any) to which the protein:dye sample was diluted for absorbance measurement

$$\text{Protein concentration (M)} = \frac{A_{280} - (A_{max} \times CF)}{\varepsilon} \times \text{dilution factor}$$

2. Calculate the degree of labeling:
   - ε′ = molar extinction coefficient of the fluorescent dye

$$\text{Moles dye per mole protein} = \frac{A_{max} \text{ of the labeled protein}}{\varepsilon' \times \text{protein concentration (M)}} \times \text{dilution factor}$$

Example Calculations

Goat IgG (2mg/mL) was reacted with a 15-fold molar excess of DyLight 550 NHS Ester (Part No. 62262).

A. Measure A_{280} and A_{max} of the DyLight 550-conjugated Goat IgG

1. The final labeling reaction solution was dialyzed overnight to remove excess, nonconjugated dye.
2. The dialyzed conjugate was diluted 1/10 in phosphate buffered saline (PBS); the absorbance of the diluted conjugate at 280nm = 0.289
3. The absorbance of the conjugate at 562nm (λ_{max} of DyLight 550) = 0.906

B. Calculate the Degree of Labeling

1. Calculate molarity of the antibody:
   - ε = 210,000M^{-1} cm^{-1} for IgG
   - A_{max} = 0.906 for the conjugate (see A.3.)
   - CF = 0.0806 (see Table 1)
   - Dilution factor = 10

$$\text{Protein concentration (M)} = \frac{A_{280} - (A_{max} \times CF)}{\varepsilon} \times \text{dilution factor}$$

$$\frac{0.289 - (0.906 \times 0.0806)}{210{,}000\ M^{-1}\ cm^{-1}} \times 10 = 0.00001028\ M\ IgG$$

2. Calculate the degree of labeling:
   - ε' = molar extinction coefficient of the fluorescent dye

$$\text{Moles dye per mole protein} = \frac{A_{max}\ \text{of the labeled protein}}{\varepsilon' \times \text{protein concentration (M)}} \times \text{dilution factor}$$

$$\frac{0.906}{150{,}000\ M^{-1}\ cm^{-1} \times 0.00001028\ M} \times 10 = 5.87\ \text{moles dye per mole of IgG}$$

Additional Information

Please visit the web site for additional information relating to this method, including the following items:

- Our complete line of DyLight Fluors and other fluorescent dyes and labeling kits
- Tech Tip #43: Protein stability and storage
- Tech Tip #20: Dialysis overview
- Tech Tip #6: Extinction coefficients guide

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