

TechNote #TNPJ200

PHYCOLINK[®] CONJUGATE EVALUATIONS

INTRODUCTION

PhycoLink Conjugation Kits provide a quick and simple method to attach fluorescent pigments to antibodies or other proteins of interest. When necessary, these conjugates can be further refined by purification with the PhycoLink Purification Kit.

Lot-to-lot comparisons and predictions of suitability for a specific application can be facilitated through the measurement of various conjugate properties; characteristics that affect performance include the molar ratios of the components, the average molecular weight of the conjugate (*i.e.* the numbers of molecules of each component joined together) and the amount of remaining unincorporated reactants.

In addition, where a purification is particularly exacting, the characterization techniques described here can be applied to individual chromatographic fractions, aiding in the selection of only the most desirable fractions for inclusion in the purified conjugate pool.

Even after purification, it is expected that some amount of phycobiliprotein (PB) and/or ligand will remain in the preparation. Quantifying this residual contamination is an important parameter in the characterization of the conjugate batch or in selecting fractions for pooling.

Characterizations are important where multiple batches of conjugate are intended for use in the same application. Batches can differ significantly from one another due to differences in reaction conditions, length of reaction time, or the characteristics and purity of the reactants. In particular, characteristics can change as the conjugation is scaled up; characterization is often essential to successful scale-up.

Techniques for characterization include absorbance measurements, affinity chromatography and HPLC. Absorbance measurements can be used to estimate:

- Protein concentration and molarity of individual components and total conjugate
- Molecular weight (minimum)
- Correction factors for determinations in the presence of blue dextran

Affinity chromatography can be used to provide more precise estimates of the incorporation of reactants into a conjugate, and HPLC can provide more detailed information on conjugate mixture composition and molecular weight.

ABSORBANCE

Spectrophotometric absorbance measurements are routinely used for concentration determinations in solution for strongly and uniformly colored substances such as PBs and other fluorescent dyes. Such determinations are very precise when performed properly due to the highly reliable internal calibration of most modern spectrophotometers.

For each PB, a known extinction coefficient can be applied to absorbance measurements to calculate concentration (see Table 1). However, the proportionality between absorbance and concentration (Beer's Law) only applies at low absorbances. Absorbance is due to the "shading" of specific wavelengths of light by molecules in the light path. As concentration increases, so does the probability that one molecule will shade another, preventing it from making its own absorbance contribution, and leading to underestimates of concentration.

Table 1 - Protein Characteristics

	λ_{Max} (nm)	$E_{\lambda_{\text{max}}}^{1\%}$ *	Blue Dextran Factor	A_{280} Factor	MW (kDa)
Streptavidin	280	32	$A_{280}/A_{690} = 5.4$		55
IgG	280	14	$A_{280}/A_{690} = 5.4$		150
Protein A	280	1.4	$A_{280}/A_{690} = 5.4$		42
Peridinin-chlorophyll- protein complex (PerCP)	482	116	$A_{482}/A_{690} = 0.2$	4.6	35.5
B-Phycoerythrin (BPE)	545	100	$A_{545}/A_{690} = 0.88$	5.4	240
R-Phycoerythrin (RPE)	566	82	$A_{566}/A_{690} = 1.24$	5.2	240
Allophycocyanin (APC)	650	70	$A_{650}/A_{690} = 1.8$	5.0	104

*The absorbance of a 1% (10 mg/ml) solution in a cuvette with a 1 cm pathlength.

Practical Considerations

Cuvette Selection - Quartz glass cuvettes or microcuvettes are necessary for estimating the relative abundance of conjugate and unincorporated PB in a sample, since accurate measurements of A_{280} are required. Cuvettes made of plastic or common glass are not highly transparent to 280 nm light and are thus not suitable for this application.

Measuring Range - For quantitative purposes, absorbance measurements in the range 0.3 – 0.8 are recommended. This avoids both measurements that are too low, and thus excessively sensitive to various sources of noise, and ones that are too high, leading to deviations from Beer's Law. More concentrated samples can be used in applications such as column profile determinations, where exact determinations of relative concentrations are not required.

Highly Dilute Samples - Dilution of sample may be necessary because the total volume of sample is simply not large enough to fill a cuvette. In such cases, lower than optimum absorbances may be obtained, and special care

in making determinations is required. Specifically, make sure that the cuvette is optically clean and free from scratches. In addition, the spectrophotometer must be blanked frequently and carefully in order to obtain precise measurements at low absorbances. A suitable dilution buffer is PBS Buffer - 25 mM sodium phosphate pH 7 and 150 mM sodium chloride.

Use of Microcuvettes - Microcuvette readings are highly sensitive to cuvette positioning; spectrophotometers may vary in reproducibility of cell positioning. In many models, the cuvette can be moved small distances laterally, which can have a large impact on readings. Be sure to take multiple blank (buffer only *vs.* air blank) readings to gain an understanding of how the individual instrument performs. Remove and replace the cuvette several times, jiggling it back and forth in the holder to see the effects on buffer blank readings. The objective is to develop a technique for mounting the cuvette that gives a highly reproducible blank. In addition, always keep the same face of the cuvette forward, eliminating orientation as a variable.

Calculating Protein Concentrations

Protein concentrations are calculated by measuring absorbance at two wavelengths, the PB λ_{max} and 280 nm. Except in rare cases where the ligand is colored, $A_{\lambda_{\text{max}}}$ will be due entirely to PB absorbance. A_{280} , on the other hand, is characteristic for all proteins, due to the absorbance of aromatic amino acids, and thus reflects the concentrations of both the PB and the ligand.

Procedure

Dilute the conjugate in a suitable buffer and determine the absorbance at 280 nm and at the PB λ_{Max} (from Table 1) with a buffer blank. Choose a dilution factor that yields an absorbance at λ_{Max} of 0.3 - 0.8.

NOTE: These steps correspond to those in the Concentration Determination Worksheet (Table 2)

1. *Determine the concentration of PB.* Multiply the undiluted absorbance at λ_{Max} by 10 and divide by the extinction coefficient to yield the concentration of the PB component (mg/ml).
2. *Calculate A_{280} due to PB.* The A_{280} is calculated by dividing the PB absorbance at λ_{Max} by the A_{280} factor (shown in Table 1).
3. *Calculate ligand A_{280} .* Subtract the PB A_{280} from the total A_{280} . The remaining A_{280} is presumed to be due to the ligand component of the conjugate.
4. *Calculate the concentration of the ligand.* Multiply the remaining A_{280} by 10 and divide by the ligand extinction coefficient to yield the concentration of the ligand component (mg/ml).
5. *Calculate total conjugate.* Total conjugate concentration (in mg/ml) is the sum of the concentrations of the individual components: line 5 of the worksheet.

Calculating Molarity

Calculation of the actual number of conjugate molecules in solution is desirable, in addition to the mg/ml concentration, because it is always a single molecule of conjugate that reacts with a single antigen binding site. Unfortunately, this value can only be obtained when the molecular weight of the conjugate is known.

Procedure

The μmolar concentration of any given component of a conjugate is obtained by dividing the mg/ml concentration of that component by its molecular weight in kilodaltons (kDa) and multiplying by 1000. Be sure to use the total molecular weight of the protein molecule, not a subunit molecular weight (see Table 1 for some useful molecular weights).

This value is useful for determining the relative amounts of the components of a conjugate, and for calculating ligand-binding capacity.

NOTE: The concentration obtained by this calculation refers to the components as though they were free in solution (unconjugated), and is distinct from the molar concentration of the conjugate itself.

The same calculation as for a component protein (divide the mg/ml concentration by the molecular weight in kDa and multiply by 1000) can be used to estimate the concentration of conjugate in solution. For this calculation, however, the molecular weight of the conjugate will have to be estimated.

Estimating Conjugate Molecular Weight

When PB's are activated with SMCC, a few lysine residues are activated for conjugation. Similarly, only a few free sulfhydryl groups on the antibody are usually available, but since there are multiple conjugation sites on each participant, conjugates obtained are not limited to 1:1 (PB:Ab) constructs, but may also contain larger aggregates (2:1, 2:2, 3:1, etc.).

The minimum molecular weight for any conjugate can be calculated from the molecular weights of each of the components and their molar concentrations. For example, APC has a molecular weight of about 104,000 daltons; streptavidin has a molecular weight of about 50,000 daltons. Typically for ProZyme's PJ25S Streptavidin-Allophycocyanin Conjugate, the ratio of SA and APC molar concentrations in the purified preparation is about 1:1. Therefore, the minimum molecular weight of the conjugate would be 150-160,000 daltons.

Table 2 - Concentration Determination Worksheet

Fill in the table with absorbance measurements:

- Dilute the conjugate in an appropriate buffer and determine the absorbance at 280 nm and at the λ_{Max} for the phycobiliprotein (PB) component of the conjugate (choose a dilution factor that yields an absorbance at λ_{Max} of 0.3 - 0.8). Enter results in Absorbance (A) column.
- Dilution = _____
- Read a buffer blank at 280 nm and the PB λ_{Max} and enter results in the Background column.
- Calculate: Net A = A - Background Undiluted A = Net A x Dilution

λ	Absorbance (A)	Background	Net A	Undiluted A
$\lambda_{\text{Max}} =$				
280				

Determine the concentrations of the PB and Ligand components of the conjugate, and from them, the conjugate concentration using values from Table 1:

- (Undiluted $A_{\lambda_{\text{Max}}}$ _____) $\times 10 \div$ PB $E_{\lambda_{\text{max}}}^{1\%}$ (_____) = _____ mg/ml PB
- Undiluted $A_{\lambda_{\text{Max}}}$ _____ \div PB A_{280} Factor (_____) = _____ PB A_{280} contribution
- Undiluted A_{280} _____ - PB A_{280} _____ = _____ Ligand A_{280}
- (Ligand A_{280} _____) $\times 10 \div$ Ligand $E_{280}^{1\%}$ (_____) = _____ mg/ml Ligand
- mg/ml PB + mg/ml Ligand = _____ mg/ml Conjugate

Sample Calculation - Concentration of an Antibody-RPE conjugate was determined:

Dilution = 20

λ	Absorbance (A)	Background	Net A	Undiluted A
$\lambda_{\text{Max}} = 566$	0.473	0.035	0.438	8.76
280	0.168	0.041	0.127	2.54

- (Undiluted $A_{\lambda_{\text{Max}}}$ [8.76]) $\times 10 \div$ PB $E_{\lambda_{\text{max}}}^{1\%}$ [82] = 1.07 mg/ml PB
- Undiluted $A_{\lambda_{\text{Max}}}$ [8.76] \div PB A_{280} Factor [5.2] = [1.68] PB A_{280} contribution
- Undiluted A_{280} [2.54] - PB A_{280} [1.68] = [0.86] Ligand A_{280}
- (Ligand A_{280} [0.86]) $\times 10 \div$ Ligand $E_{\lambda_{\text{max}}}^{1\%}$ [14] = [0.61] mg/ml Ligand
- mg/ml PB + mg/ml Ligand = 1.68 mg/ml Conjugate

It is not necessarily the case, though, that all conjugates present are 1:1. The preparation might also contain 2:2 or higher order conjugates, or possibly even offsetting quantities of 2:1 and 1:2 and their multiples. Thus, the minimum molecular weight should be viewed with caution, although in many cases it turns out to be a useful working estimate.

If it is important to determine an average molecular weight for the conjugate, a sample can be run over a calibrated gel filtration column (preferably with HPLC – see below).

Procedure

Begin with a reasonably pure conjugate; applying this technique with significant amounts of unincorporated reactants will produce spurious results. Estimate the molar concentrations of the components ([PB], [Ab]) as described above, then calculate the values of both [PB]/[Ab] and [Ab]/[PB]. With a little luck, one of these values will fall reasonably close to an integer. For example, a [PB]/[Ab] value of 2.90 would suggest a 3:1 conjugate, while an [Ab]/[PB] ratio of 2.07 would suggest a 1:2 conjugate. Multiply the number of each protein in the conjugate by its molecular weight to obtain the minimum molecular weight of the conjugate.

Again, this represents a very crude estimate. Even a conjugate that gives a seemingly “clean” molecular weight estimate in this fashion may well contain a wide variety of constructs. Still, these estimates are frequently useful in predicting conjugate behavior.

Blue Dextran Correction Factors

We recommend the use of blue dextran as a tracking dye when using the Phycolink Conjugation Protocol as only those fractions containing blue dye need to be collected from the antibody desalting column. This makes collection of reduced antibody easier as the need to monitor absorbance of the eluted fractions is avoided. However, blue dextran has a high molecular weight (so it will co-purify with conjugate) and a broad absorbance spectrum. Its presence should not affect conjugate performance, but will interfere with the spectrophotometric concentration determination described previously.

Thus, concentration calculations for conjugates containing blue dextran must include correction factors for the absorbance contribution of blue dextran at 280 nm and at the PB λ_{max} wavelength. These correction factors are obtained from the absorbance spectrum of blue dextran normalized to its absorbance at 690 nm (where essentially all absorbance is due to blue dextran). Table 1 includes a list of empirically-derived correction factors for blue dextran.

Procedure

1. Determine the absorbance of the conjugate at 280 nm, 690 nm and at the λ_{max} for the PB component of the conjugate.
2. Multiply the A_{690} by the appropriate “Blue Dextran Factor” for the λ_{max} of the PB to obtain the absorbance contribution of blue dextran at λ_{max} . Subtract this from the measured absorbance at λ_{max} to get the corrected value.
3. Multiply the A_{690} by the A_{280}/A_{690} correction factor of 5.4 to obtain the absorbance contribution of blue dextran at 280 nm. Subtract this from the measured A_{280} to get the corrected value.
4. Perform the protein concentration calculations as described previously, using the corrected absorbance values.

NOTE: This correction seems to be an imperfect one; at low conjugate concentrations it often results in deriving an improbably low antibody (or other ligand) concentration. Perhaps it overcorrects the A_{280} . If more accurate concentration measurements are needed, the blue dextran should be omitted.

AFFINITY CHROMATOGRAPHY

For some conjugates, it is important to determine the relative amounts of conjugated antibody and unincorporated phycobiliprotein in the final conjugate or in individual fractions during purification. For example, before conjugate concentration or molarity can be determined it is necessary to be certain that there is no significant unincorporated protein present.

An affinity column that will bind the antibody portion of the conjugate can be used to separate the PB in the final preparation into bound and unbound fractions by measurement of absorbance at the characteristic maximum for the PB before and after passing the sample over the column. A typical test: load a small amount of conjugate, wait a few minutes for binding to maximize, elute the unbound material, measure its $A_{\lambda_{max}}$ and determine the bound material by subtraction.

Column Selection - The procedure requires a 1-ml column of an affinity matrix, such as Protein A-Agarose, Protein G-Agarose, Protein A/G-Agarose, Secondary Antibody-Agarose or Immobilized Antigen. The selected column must be known to bind the antibody used in the conjugation. While immobilized antigen might seem the most obvious choice, the other options on the list are often more readily available and may offer higher binding affinity. On the other hand, the use of immobilized antigen in this determination will also provide an indication of whether the conjugation procedure has compromised the binding properties of the antibody. When immobilized Fc-binding protein (*i.e.* protein A or protein G) is used in the determination, be aware that some IgG species show significant differences in binding affinity for these two proteins and the proper matrix should be selected with this in mind.

NOTE: Affinity purification is not suitable for PB conjugates as the harsh elution conditions diminish fluorescent properties.

Calculations

AU Calculations - Absorbance units themselves may be used as surrogate measures of concentration since concentration is directly proportional to absorbance. For processes such as the determination of conjugate contamination with PBs, it is necessary to calculate the "total quantity" of PB absorbance in a sample; this is a surrogate measure for mg of PBs. Terms are defined as:

Q_{AU} - "quantity" of absorbance units in unknown sample

$A_{\lambda_{Max}}$ - absorbance at the PB's wavelength of maximum absorbance in diluted sample (blank-corrected by spectrophotometer)

D - dilution (total volume in diluted sample ÷ volume of evaluation sample in diluted sample)

V = volume of evaluation sample

The quantity of absorbance units in the unknown sample is then:

$$Q_{AU} = A_{\lambda_{Max}} \times D \times V$$

Example - A ten-ml aliquot of purified conjugate is to be evaluated for contamination with unincorporated PB. For determination of the PB concentration in the aliquot, 0.1 ml of aliquot is diluted with 0.4 ml of column buffer (D = 5). Absorbance of the diluted sample is 0.42.

In the aliquot:

$$\begin{aligned} Q_{AU} &= A_{\lambda_{Max}} \times D \times V \\ &= 0.42 \times 5 \times 10 \\ &= 21 \text{ AU} \end{aligned}$$

To load an affinity column, we wish to begin with 0.75 AU of purified conjugate. Thus, a sample of $0.75/21 \times 10$ ml, or about 0.36 ml, is required (0.75/21 is the fraction of the total AU in the aliquot that is to be used for affinity evaluation). This 0.36 ml sample is withdrawn from the original aliquot and loaded on the column.

After loading and rinsing, 2.1 ml are collected from the bottom of the column. This solution is only slightly colored, and is measured in the spectrophotometer with no dilution. Its absorbance at $A_{\lambda_{Max}}$ is 0.13. In the post-column pool, then:

$$\begin{aligned} Q_{AU} &= A_{\lambda_{Max}} \times D \times V \\ &= 0.13 \times 1 \times 2.1 \\ &= 0.27 \text{ AU} \end{aligned}$$

Since 0.75 AU of mixture was originally loaded, and 0.27 AU passed through the column, 0.48 AU are assumed to have been adsorbed by the column matrix. Thus, the aliquot is determined to contain about 64% (0.48/0.75) of its PB in the form of conjugate and about 36% as free PB.

Procedure

1. Equilibrate the column with PBS Buffer.
2. Determine the absorbance of the conjugate sample to be tested at the PB λ_{\max} (Table 1).
3. Calculate Total AU for the sample at $A_{\lambda_{\max}}$.
4. Load a volume of protein solution containing a total of 0.5–1 AU onto the column (note that this material will be lost, thus reducing the yield of conjugate).
5. Collect the flow-through buffer; discard if no color is evident.

NOTE: PB's are highly colored, which is, of course, part of the reason they make excellent tags. This also makes them extremely easy to work with, since concentrations more than a few tens of $\mu\text{g/ml}$ are readily visible.

6. Wash the column with 1.5 ml of PBS. (Pool the eluate from this wash with the eluate collected during the sample loading if the latter has any color.)
7. Determine the total volume of the pooled eluate and the absorbance of this material at the PB λ_{\max} .
8. Calculate the fraction of total absorbance that is not bound by the column. This approximates the contamination of your conjugate with unincorporated PB.

HPLC/GEL FILTRATION

Gel filtration provides molecular weight estimates that can be extremely useful in characterizing conjugates. Either conventional liquid chromatography (LC) or high-performance liquid chromatography (HPLC) may be employed. However, HPLC permits resolution not achievable with LC, due to its precise control of flow and pressure, combined with columns packed to rigorous specifications at a small scale, that minimizes spatial variability in flow characteristics. In addition, HPLC can be performed with much smaller samples than LC, reducing consumption of valuable conjugate.

Standards of known molecular weight are used to calibrate the columns; combined standards for this application – mixtures of several proteins of known molecular weight -- are available from a variety of vendors and should be chosen to fall within the exclusion limits of the column employed. The characteristic absorbance of protein at 280 nm is used to detect the various elution peaks. In these calibrations, the log of each standard's molecular weight is plotted as a function of elution volume for each associated peak. The result should be an approximately linear relationship with a negative slope (inverse proportion).

After calibration, conjugate samples can be run on the same column in the same manner, and the calibration line used to estimate conjugate molecular weight(s). Some caution is required in the interpretation of these results, however, since the relationship between log molecular weight and elution volume only strictly obtains for spherical globular proteins. PB conjugates often migrate on sizing columns with apparent molecular weights that are somewhat greater than their true molecular weights. However, this estimate should still be accurate enough to define a starting point for optimization experiments, and provides a way to compare the molecular weights of different batches of conjugate. Table 3 lists estimates for various conjugates made at ProZyme.

Most importantly, these estimated conjugate molecular weights, when taken together with the molecular weights of the component proteins (PB and antibody), permit the estimation of conjugate composition in terms of these components. The column elution profile will contain one or more peaks that will represent one or more of the possible species of antibody (A) - PB (D) conjugates (AD, ADD, AADD, etc.). In most cases, the estimated molecular weights for these peaks will be sufficiently accurate to distinguish between these possibilities, based on known component molecular weight.

NOTE: Bear in mind the exclusion limits of the column employed; a single leading peak running in the exclusion volume may contain multiple conjugate species.

Procedures for analysis using HPLC columns are outside the scope of this document and will not be included here.

Table 3 - Conjugate Molecular Weights

	kDa
Streptavidin-APC	≥400
Streptavidin-RPE	≥300
IgG-APC	≥400
IgG-RPE	≥400
IgG-BPE	≥400
IgG-PerCP	180 - 500
Protein A-APC	150 - 500

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1933 Davis Street, Suite 207
San Leandro, CA 94577-1258

TOLL FREE (800) 457-9444
PHONE (510) 638-6900
FAX (510) 638-6919

E-MAIL info@prozyme.com
WEB www.prozyme.com