PROzyme®

GLYKO®

PhycoLink®

CONJUGATE PURIFICATION KIT

Rapid and convenient small-scale purification of phycobiliprotein conjugates by size exclusion chromatography.

- Everything needed to remove unincorporated reactants from conjugates; specifically formulated for use with PhycoLink Conjugation Kits
- Complete protocols, thoroughly tested for trouble-free purification
- Refills available for consumable components to allow reuse of "hardware"
- Two sizes to fit specific needs:

Product Code	Qty Conjugate	Column Vol
KPK13	0.25 mg	13 ml
KPK80	1 mg	80 ml

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KIT CONTENTS

NOTE: Please read this entire booklet before unpacking or using this kit.

Item	Qty
Column (80-ml or 13-ml)	1 ea
Top Fitting A (3-way valve and tubing)	1 ea
Bottom Fitting B (stopcock, luer adapter, union, retention nut, ferrule and tubing)	1 ea
Column Matrix	1 bottle
20x Storage Buffer	1 bottle
Ultrafilter C with Filtrate Collection Tube (0.5 ml, 30 kDa MWCO)	1 ea
Ultrafilter D with Filtrate Collection Tube (15 ml, 30 kDa MWCO)	1 ea
Microcuvette E1 (8.5-mm beam height)	1 ea
Microcuvette E2 (15-mm beam height)	1 ea
Syringe F (10-ml or 30-ml, with stopcock adapter)	1 ea
Bulb Pipettes G (disposable)	6 ea
CD-ROM (contains Tips & Hints, TechNotes and illustrations referenced in this booklet)	1 ea

Additional Required Supplies/Equipment

Miscellaneous laboratory glassware
Ring stand or similar mounting apparatus
Shelf or other support for buffer reservoir
Gloves for handling buffers and matrix containing preservative
Spectrophotometer
Vacuum pump or aspirator
Water (distilled or deionized for all steps, room temperature)
Refrigerated high-speed centrifuge (optional)
Rotors and/or holders for 2.2-ml or 50-ml tubes (optional)
Fraction collector (optional)

STORAGE CONDITIONS

Store Storage Buffer refrigerated long term. The rest of the Purification Kit should be stored at room temperature.

Phycobiliprotein conjugates should be protected from light and stored at 4°C; DO NOT FREEZE.

REFERENCES

Technical literature and additional information may be found on the enclosed CD-ROM and on ProZyme's website:

FAQ's:
http://www.prozyme.com/faqs/faqs.html#phycolink

TechNotes:
http://www.prozyme.com/technical/techindex.html#phycolink

INTRODUCTION

PhycoLink Conjugation Kits are designed for easy conjugation of antibodies (Abs) and other proteins to phycobiliproteins (PBs), such as R-Phycoerythrin (RPE), B-Phycoerythrin (BPE), Allophycocyanin (APC), Peridinin-chlorophyll-protein complex (PerCP) or their tandems. Conjugates produced with these kits contain residual unincorporated PB (since PB is supplied in excess), and may also contain small amounts of unincorporated Ab. The PhycoLink Purification Kit provides the means to remove these unincorporated reactants.

When to Purify

In many applications the presence of unincorporated reactants does not compromise conjugate performance. However, further purification may be necessary to increase sensitivity or precision, or to evaluate or compare different conjugate lots.

The disadvantages of purification should also be considered before choosing to add this step, as losses of over 50% are possible when processing small quantities. Choosing a smaller column permits more efficient recovery, but provides less complete purification.

Purification is achieved by size exclusion chromatography, which separates molecules according to size using porous gel beads. When a sample containing molecules of varied size travels through the matrix, molecules smaller than a certain size (the "exclusion limit") enter the pores of the beads and follow a longer path through their interior. Those too large to enter the pores travel only through the spaces between the

beads (the "void volume") and reach the bottom most rapidly. They are then followed by the molecules that are smaller than the exclusion limit in approximate order of size from largest to smallest.

Molecules are sorted based on the probability that they follow a shorter or longer path through the matrix, with diffusion causing additional spreading of the peak for molecules of a given size. Thus, in practice, only molecules of significantly different sizes separate cleanly. Fortunately, such large differences can be expected in PB-Ab conjugates, where complexes consist of relatively small numbers of large molecules linked together.

NOTE: The Column Matrix provided has been specially selected to minimize nonspecific binding by PBs. Substitution of other matrices may result in the loss of conjugate.

Outline of the Protocol

1. Column Assembly: The column is assembled, checked for leaks and equilibrated.

2. Sample Loading: The concentration of the conjugate solution is adjusted for optimal resolution, then introduced onto the column and washed into the matrix.

3. Sample Collection: Purified conjugate is collected by hand or fractions are pooled based on absorbance measurements.

4. Finishing: The conjugate is formulated and the column stored or prepared for reuse.

Measurements and Calculations

Absorbance measurements are used to select the optimum fractions of column eluate for purification. The procedures described employ measurements of absorbance at the absorbance peak of the PBs, which are more sensitively detected at low concentrations due to their intense color (see Table 1).

The column profile, which plots absorbance (at PB λ_{Max}) *vs.* volume of eluate, will usually have one or more peaks of conjugate at or near the void volume with a trailing peak of free PB. For free APC, this trailing peak falls about midway between the excluded and included volumes of the column. The trailing peaks for free RPE or BPE (~2x the molecular weight of APC), run closer to the void volume and may not separate as completely from the conjugate peak. For PerCP, the trailing peak runs almost fully included.

Free Ab, if present, will normally run just in front of free APC or PerCP or just behind free RPE or BPE, and appear as a peak of A_{280} without a corresponding peak at λ_{Max}. Because PBs are added in excess in the PhycoLink conjugation procedure, relatively little free Ab is present in the final conjugate. In the case of a failed conjugation however, the conjugate will exhibit relatively little absorbance at the PB λ_{Max}; the initial peak will be reduced relative to that for a successful conjugation.

Table 1 - PB Absorbance Maxima

Protein	λ_{Max}	A_{Max}/A_{280}
RPE	566 nm	5.8
BPE	545 nm	5.6
APC	650 nm	5.2
PerCP	482 nm	4.6

Column Profile (Sample Data) - Elution profiles for IgG, RPE and an IgG-RPE conjugate run on 13- and 80-ml columns are shown in Figure 1. Elution profiles for IgG, APC and an IgG-APC conjugate run on 13- and 80-ml columns are shown in Figure 2. The solid line represents the column profile of the conjugate. Standard curves for Ab (80-ml profile only) and the PB alone are superimposed for reference; they will not appear in the conjugate column profile.

The 80-ml column gives significantly better performance than the 13-ml column due to its greater running length, which is recommended except for very small conjugate quantities. For either column significantly purified conjugate can be obtained by selecting only early fractions. Selection of a cutoff point to combine fractions is a choice based on a tradeoff between purity and yield; the farther to the left the cutoff is set, the more highly purified the conjugate and the lower the yield.

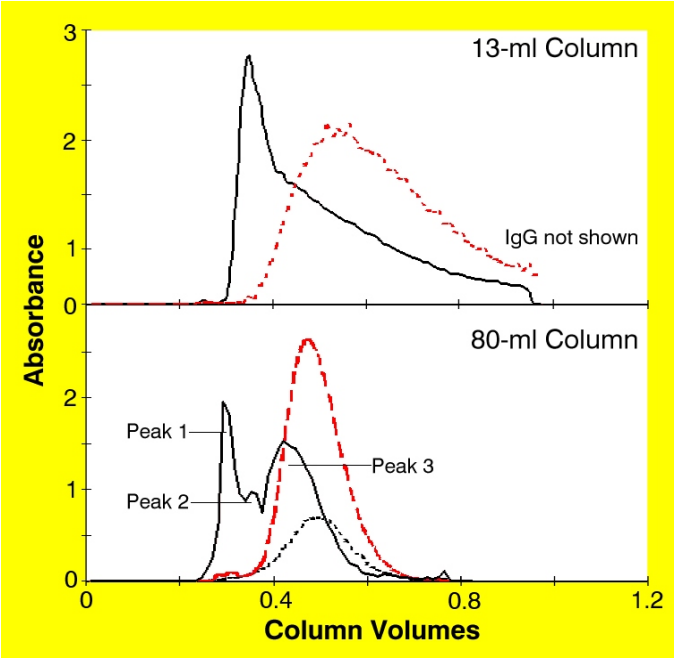

Figure 1 - RPE Conjugate Column Profiles
RPE (240 kD; – – –) runs slightly ahead of IgG (150 kD; – – –). Once conjugated (——), a significant quantity, consisting of RPE-IgG complexes, exceeds the exclusion limit of the column and runs as a leading peak (peak 1). Smaller complexes run as a smear (peak 2) between peak 1 and the IgG and RPE (peak 3). Note that the profile is not as well resolved on the 13-ml column owing to its smaller overall volume relative to the bead size of the matrix.

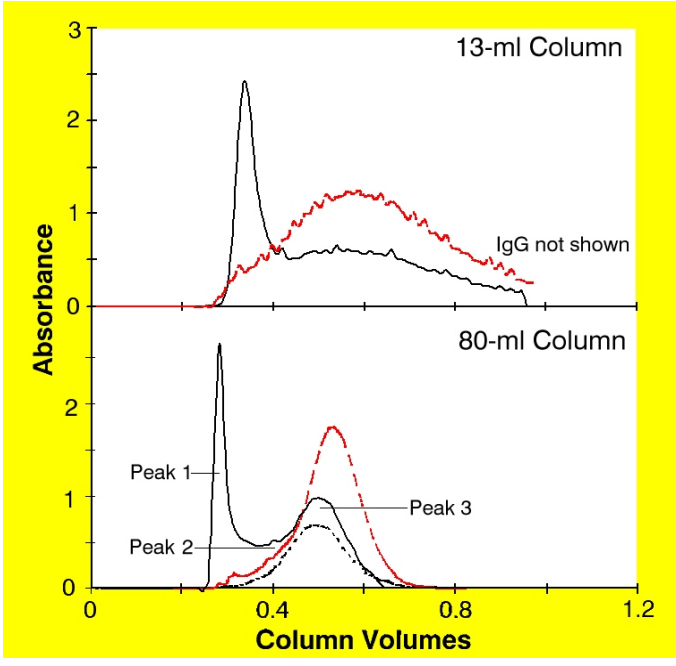

Figure 2 - APC Conjugate Column Profiles
APC (104 kD, – – –) runs just slightly behind IgG (150 kD, – – –). Once conjugated (——), APC-IgG complexes which exceed the exclusion limit of the column, run as a leading peak (peak 1). Smaller, included species of conjugate (peak 2) span the portion between the excluded material and the final broad peak of unincorporated APC and IgG (peak 3). Note that the profile is not as well resolved on the 13-ml column owing to its smaller overall volume relative to the bead size of the matrix.

Practical Considerations

The disposable microcuvettes supplied with the kit are suitable for measurement of absorbance at the PB λ_{Max} for selecting fractions to pool. They are unable to deliver the sensitivity required to characterize a finished conjugate.

Measuring Range - For quantitative purposes, absorbance measurements in the range 0.25 – 0.75 are recommended. This avoids both measurements that are too low (excessively sensitive to various sources of noise) or too high (where the relationship between absorbance and concentration may become nonlinear).

Highly Dilute Samples - Sample dilution may be necessary when the volume is 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; disposable cuvettes (supplied with this kit) may not be suitable. In addition, the spectrophotometer should be blanked frequently in order to obtain precise measurements, and longer reading times, where obtainable are suggested to improve precision.

Using the Microcuvette - Microcuvette readings are highly sensitive to cuvette positioning; spectrophotometers 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.

Preservatives - Storage Buffer contains pentachlorophenol as a preservative. See Finishing, page 25, for a discussion of alternatives.

Conjugate Characterization - The protein concentration, molar ratios of the components and average molecular weight of the conjugate can be estimated from absorbance measurements of the finished conjugate. These calculations and detailed methods for characterization are provided in TechNote TNPJ200 *PhycoLink Conjugate Evaluations*.

Kit Flexibility and Reuse - The column may be reused by washing or replacing the Column Matrix (Product Code KPK100, available from ProZyme). Since many prefer to reserve matrix for each Ab, PB or Ab-PB combination, instructions have been included in this booklet for reserving the matrix and repouring the column.

OTHER PROZYME PRODUCTS & KITS

ProZyme offers a variety of fluorescent labeling reagents and kits. A complete listing may be found on our website at:

http://www.prozyme.com/phycolink/

PRODUCT USE AND WARRANTY

Terms and conditions of sale may be found at:

http://www.prozyme.com/terms.html

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PLACING AN ORDER

For North American destinations: telephone orders may be placed between 8:00 am and 5:00 pm Pacific Time. Telefax or e-mail orders may be sent or messages recorded anytime.

TOLL FREE **(800) 457-9444** (US& CANADA)
PHONE **(510) 638-6900**
FAX **(510) 638-6919**
E-MAIL **info@prozyme.com**
WEB **www.prozyme.com**

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or place an international order directly if there is no distributor in your area:

http://www.prozyme.com/orders.html#exna

PROzyme®
1933 Davis Street, Ste 207
San Leandro, CA 94577-1258
USA

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

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

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