ALTERNATIVE CONJUGATION PROTOCOLS

For direct conjugation to antibodies (Abs) or other proteins of interest, ProZyme offers PhycoLink® Conjugation Kits, which includes Phycobiliproteins (PB) activated with SMCC (succinimidyl 4-[N-maleimidomethyl]-cyclohexane-1-carboxylate). The standard protocol is available on the webpage at:

http://www.prozyme.com/phycolink/pj25kbooklet.html#protocols

The target protein is treated with dithiothreitol (DTT) to expose free sulfhydryls. Excess DTT is then removed by running the reduced protein over a desalting gel-filtration column. The SMCC-PB is covalently coupled to the IgG through reaction of the maleimide groups with the free sulfhydryl on the IgG; typically only one of the IgG sulfhydryls will react with the SMCC-PB due to steric hindrances. Any remaining free sulfhydryl groups are covalently blocked by treatment with NEM. The conjugate is then exchanged into an appropriate storage buffer by gel filtration.

Many sulfhydryl-containing molecules besides immunoglobulins may be conjugated this way. Other molecules may also be conjugated if they are first treated with a reagent to introduce sulfhydryls, such as iminothiolane (Traut's Reagent) or SPDP (N-succinimidyl 3-[2-pyridyldithio]-propionate).

Some monoclonal Abs may precipitate upon DTT reduction, or fail to react with maleimide groups once they are reduced. In these cases, the SPDP protocol may be successful.

Protocols for these alternative methods are given here.

Molecular Weight of Reactants	
IgG	150 kda
R-Phycoerythrin (RPE)	240 kda
Allophycocyanin (APC)	104 kda
SMCC	334 da
SPDP	312 da
2-iminothiolane	138 da

IMINOTHIOLANE PROTOCOL

Sulfhydryls may be introduced into a protein by thiolation of the primary amines with 2-iminothiolane.

Reagents

Target Protein in PBS or other non-amine containing buffer between pH 7 and pH 9. A good buffer for this reaction is 50 *mM* Triethanolamine, 150 *mM* NaCl, 1 *mM* EDTA pH 8.0

NOTE: For best results, the starting concentration should be greater than 5 mg/ml, so that the conjugate concentration will be greater than 1 mg/ml after the desalting column (step 3).

Iminothiolane - **Immediately** prior to use, prepare a 1 mg/ml stock of 2-imino-thiolane (Pierce #2610ZZ) in dry DMSO.

PBS Buffer (25 *mM* sodium phosphate, 150 *mM* NaCl pH 7.0)

Exchange Buffer (50 *mM* MES pH 6.0, 2 *mM* EDTA and 1 µg/ml pentachlorophenol as an antimicrobial agent)

SMCC-PB

NEM Solution (*Optional*) 10 mg/ml N-ethyl-maleimide dissolved in dry DMSO

Storage Buffer: 10 *mM* Tris, 150 *mM* NaCl and 1 µg/ml pentachlorophenol pH 8.2

Procedure

1. Prepare 1 mg/ml stock of iminothiolane and **immediately** add enough to give a 5:1 molar ratio with your protein. For example, for 1 mg of an Ab, add 5 µl of iminothiolane stock.

2. Mix and incubate 45 minutes at room temperature.

NOTE: After the initial mixing, avoid further mixing to prevent oxidation of the thiol groups.

3. Desalt the thiolated protein into Exchange Buffer.

4. Immediately combine your thiolated protein with SMCC-PB.

NOTE: For SMCC-RPE start with a 1:1 molar ratio. For SMCC-APC, start with a 2:1 molar ratio.

5. Incubate the mixture for 1 - 2 hours at room temperature.

6. *Optional:* Block further reaction by adding 10 µl of NEM Solution per ml of reaction. Incubate the mixture for 20 minutes at room temperature.

7. Use a desalting column to exchange the conjugate into the desired storage buffer.

8. To optimize the yield and size distribution of the conjugate, alter the amount of iminothiolane in step 1 or the ratio of SMCC-PB to your protein used in step 4.

SPDP PROTOCOL

Some monoclonal Abs do not conjugate well after DTT reduction. This protocol may give better results.

Reagents

SPDP Reaction Buffer (10 *mM* Sodium Carbonate, 350 *mM* NaCl and 10% Glycerol pH 8.5

TCEP Stock (35 *mM* Tris[2-carboxyethyl]-phosphine hydrochloride {TCEP-HCl, Pierce #20490ZZ} in 50 *mM* Phosphate pH 7.2)

SPDP Stock (1 mg/ml SPDP [Pierce #21857ZZ] in absolute ethanol)

Procedure

1. Exchange 1.0 mg of Ab into SPDP Reaction Buffer and concentrate to 5 mg/ml (0.2 ml)

2. Add 10.4 µl of SPDP Stock and incubate at room temperature for 5 hours.

3. Add 24 µl of 0.5 M Sodium Phosphate pH 7.0.

4. Add 2.4 µl of TCEP Stock (final 350 µ*M*). Incubate at room temperature for 15 minutes, then incubate on ice for 15 minutes more.

5. Add the SMCC-PB and incubate overnight at 4°C.

NOTE: For SMCC-RPE start with a 1:1 molar ratio. For SMCc-APC, start with a 2:1 molar ratio.

6. To optimize the yield and size distribution of the conjugate, alter the amount of SPDP in step 2 above or the ratio of SMCC-PB your protein used in step 5.

CONJUGATE PURIFICATION

PhycoLink Conjugation Kits are designed for easy conjugation of Abs and other proteins to PBs and their tandems. Conjugates produced with these kits contain 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 unincorporated reactants (Product Code KPK13 or KPK80, available from ProZyme).

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 be considered before choosing to add this step, as losses of over 50% are possible when processing small quantities.

TECHNICAL SERVICE

This and other TechNotes are available on ProZyme's webpage located at:

<http://www.prozyme.com/technical/index.html#technotes>

ProZyme customers are an important source of information regarding advanced or specialized uses of our products. We encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

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