

Texas Red conjugation of Annexin V

Overview:

This protocol serves not only to describe the conjugation of Texas Red (in the form of Texas Red BSA, TR-BSA) to Annexin V, but to serve as a model for TR-BSA conjugation of any protein. The molecular weight of Annexin V is about 40 kDaltons. When conjugating other proteins, take into account the relative molecular weight.

This protocol is similar to that of conjugation of TR-BSA to immunoglobulins. One difference arises from the much smaller size of Annexin V compared to IgG (one-fourth the molecular weight). The primary difference is that there is no available sulfhydryl on Annexin V. To introduce one, a heterobifunctional linking reagent is used which reacts with Annexin V lysines with one end of the molecule, and has a sulfhydryl on its other end. This sulfhydryl is reacted with the SMCC-derivatized APC through reductive cross-linking. Annexin V can be purchased from some commercial sources or obtained in collaborative agreements with researchers who make recombinant protein.

Texas Red (TR) is most commonly used on FACS machines with a dye laser (excitation 595-605 nm). It emits at about 620 nm, much shorter than the APC emission. It makes for a relatively bright reagent, allowing for discrimination of weakly-expressed antigens. Direct TR conjugates of antibodies, however, are often sticky and are quite unstable, lasting only a few months at 4C. Therefore, for many years TR was used only as an avidin conjugate, i.e., for use as a second step for biotin-labeled antibodies. However, when TR is conjugated to BSA as a carrier, it is no longer sticky nor unstable. A single TR-BSA (typically carrying 3 TR molecules) is conjugated to an antibody in a site-specific manner, resulting in a bright reagent that is stable at 4C for extended periods of time and shows very little background binding.

Refer to notes about the following procedures used by this protocol:

Reductive cross-linking of antibodies

Column chromatography

Reagent storage

Contents:

Conjugation protocol

I. Derivatization of TR-BSA

II. Preparation of Annexin V

III. Covalent conjugation

Materials, chemicals, and buffers

References

Conjugation protocol.

The entire conjugation can be performed easily in a single day. In addition to the materials listed below, you will need to have a solution of your antibody at a concentration of at least 2 mg/ml. You should be familiar with how to use desalting columns and how to measure protein concentration spectrophotometrically.

The protocol is divided into three phases: derivitization of the TR-BSA, reduction of the IgG, and coupling of the two. SMCC-derivitized TR-BSA can be stored in the refrigerator for extended periods (probably many months).

Thus, this step can be undertaken once for several antibody conjugations subsequently performed on separate days. However, considering the low cost of the TR-BSA, this is probably not necessary.

I. Derivatization of TR-BSA

The amino groups on the BSA react with the succinamide of SMCC to yield a maleimide-labeled TR-BSA.

Dissolve the TR-BSA into "Dialysis Buffer", at a concentration of 10 mg/ml. Prepare 3 mg TR-BSA per mg of antibody.

Prepare a 10 mg/ml stock solution of SMCC in dry DMSO immediately prior to use.

Add 45 μ l of SMCC per mg of TR-BSA while vortexing. Wrap the reaction tube in aluminum foil and rotate at room temperature for 60 minutes.

Note: for conjugations which are poor or fail, it may help to increase or decrease the molar ratio of SMCC to TR-BSA, or use an alternative heterobifunctional crosslinking reagent.

Pass the SMCC-TR-BSA over a filtration column pre-equilibrated with "Exchange Buffer". (See hints on using columns with fluorescently-conjugated proteins). Calculate the concentration of the SMCC-TR-BSA assuming a 90% recovery of starting material.

II. Preparation of Annexin V

A heterobifunctional linking reagent is reacted with Annexin V (via lysines) to provide free sulfhydryl groups.

Dissolve (or dialyze or exchange) the Annexin V into any standard PBS solution (azide-free!); the Annexin V should be at a concentration of greater than 5 mg/ml if possible.

Dissolve 2-IT in the same PBS solution at a concentration of 10 mg/ml. Use immediately: for a molar ratio of 4, add 15.2 μ g of 2-IT per mg of Annexin. See notes above about using different molar ratios of 2-IT to Annexin V.

Incubate and rotate at room temperature for 60 minutes.

Prepare a fresh solution of 1 M DTT (15.4 mg/100 μ l) in distilled water.

Make each Annexin V reaction 20 mM in DTT: add 20 μ l of DTT stock per ml of reaction while mixing. Let stand at room temp for 15 minutes without additional mixing (to minimize reoxidation of cysteines to cystines).

Pass the reduced 2-IT-Annexin V over a filtration column pre-equilibrated with "Exchange Buffer". Collect 0.25 ml fractions off the column; determine the protein concentrations and pool the fractions with the majority of the Annexin V. This can be done either spectrophotometrically or colorimetrically (see hints on using columns for separation of nonfluorescent proteins).

Carry out the TR-BSA conjugation as soon as possible after this step.

III. Covalent conjugation

The TR-BSA is covalently coupled to 2-IT-Annexin V through reaction of the maleimide groups with the free sulfhydryl on the of the 2-IT. Do not delay this step since the 2-IT sulfhydryls will reoxidize.

Add 12 mg of SMCC-APC per mg of 2-IT-Annexin V. Wrap the reaction tube in aluminum foil and rotate for 60

minutes at room temp.

After 60 minutes, unreacted free sulfhydryls on the 2-IT-Annexin V must be blocked.

Prepare a fresh solution of 10 mg NEM in 1.0 ml dry DMSO.

Add 20 µg (2.0 µl) per mg of 2-IT-Annexin V. Wrap and rotate for 20 minutes at room temperature.

The product can be either dialyzed or exchanged over a column into an appropriate buffer (e.g. "Storage Buffer"). It is best to keep the product at high concentration (> 1 mg/ml) for optimal stability. Never freeze the conjugates. See also general hints on storing conjugates.

Materials:

For column separations, we often use one of two types of pre-poured columns:

For 1.25ml to 2.5ml sample volumes: PD-10 (Sephadex G-25M).

For <0.5 ml sample volumes: NAP5 columns (Sephadex G-25 DNA grade).

Chemicals:

2-IT - 2-iminothiolane * HCl ("Traut's reagent"), mw 137.6

SMCC - succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate, mw 334.42

NEM – N-Ethylmaleimide, mw 125.1

DMSO - anhydrous dimethyl sulfoxide.

Note: keep the DMSO absolutely dry at all times. We keep the bottle in a dessicator. Pour out an amount of DMSO sufficient for your need and then pipette that; don't pipetter directly into the bottle.

NaHCO₃ - sodium bicarbonate, mw 84.01

NaCO₃ - sodium carbonate, mw 106

NaCl - Sodium Chloride, mw 58.44

TRIZMA pre-Set crystals 8.0 - Combination of Tris base and TrisHCl, average mw 141.8

NaN₃ – Sodium Azide, mw 65

Standard PBS (free of sodium azide)

Buffers:

"Dialysis Buffer"

50 mM Sodium phosphate, 1 mM EDTA, pH 7.0

To make 1 Liter:

13.41g Sodium phosphate dibasic (7*H₂O)

0.37 g EDTA

"Exchange Buffer"

50 mM MES, 2 mM EDTA, pH. 6.0

To make 1 Liter:

9.76 g MES

0.74 gm EDTA

pH to 6.0

"Storage Buffer"

10 mM Tris, 150 mM NaCl, 0.1% NaN₃, pH 8.2

To make 1 Liter:

1.42g TRIZMA 8.0

8.77g NaCl

1g NaN₃

pH to 8.2

See hints on storing buffers.

References and credits:

This protocol was devised and tested by Mario Roederer, and is based on the reductive cross-linking protocol used for phycobiliprotein conjugates.

Hardy, RR: Purification and coupling of fluorescent proteins for use in flow cytometry. In: Handbook of Experimental Immunology, 4th ed. DM Weir, LA Herzenberg, C Blackwell, and LA Herzenberg, editors. Blackwell Scientific Publications, Boston, 1986, pp. 31.1-31.12.