

Texas Red-BSA conjugation of Antibodies

Overview:

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

You can also use the short, less-detailed protocol for reference.

Contents:

Conjugation protocol

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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. Reduction of IgG

The hinge disulfide bonds are reduced to yield free sulfhydryls.

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

IgG solutions should be at 4 mg/ml or higher for best results. The reduction can be carried out in almost any buffer; MES, phosphate, and TRIS buffers (pH range 6 to 8) have been used successfully. The antibody should be concentrated if less than 2 mg/ml. Include an extra 10% for losses on the buffer exchange column.

Make each IgG solution 20 mM in DTT: add 20 μ l of DTT stock per ml of IgG solution while mixing. Let stand at room temp for 30 minutes without additional mixing (to minimize reoxidation of cysteines to cystines).

Pass the reduced IgG 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 IgG. This can be done either spectrophotometrically or colorimetrically (see hints on using columns for separation of nonfluorescent proteins).

Carry out the conjugation as soon as possible after this step.

Note: for conjugations which are poor or fail, it may help to reduce the DTT concentration.

III. Covalent conjugation

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

Add 3 mg of SMCC-TR-BSA per mg of IgG. Wrap the reaction tube in aluminum foil and rotate for 60 minutes at room temp. Note: These molar ratios (~6 TR-BSA per IgG) have worked very well; however, more BSA per IgG can only help ensure that all of the MAb is conjugated. Unconjugated BSA will be washed away during cell staining (or during protein A or G purification of the conjugate, if desired). The cost of the TR-BSA (~\$3.50/mg) is inconsequential compared to other factors.

After 60 minutes, unreacted free sulfhydryls on the IgG must be blocked.

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

Add 34 μ g (3.4 μ l) per mg of IgG. 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. It may be useful to spin TR-BSA conjugates prior to use in staining, especially if background seems to be a problem (e.g., at 10,000g in a microcentrifuge, at 4C). See also general hints on storing conjugates.

Materials, Chemicals, and Buffers

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:

TR-BSA - Texas Red-conjugated Bovine Serum Albumin. (3.2 mole TR per mole BSA)

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

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 is based on an original protocol devised by Alan Stall, based on the phycobiliprotein conjugation protocol by Randy Hardy.

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.