TECH TIP # 5

Attach an antibody onto glass, silica or quartz surface

TR0005.4

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Introduction

Researchers are increasingly engaged in assay development and affinity purification methods for specialized applications and instrumentation. A versatile platform for affinity assays or purification involves immobilizing antibodies or other proteins onto glass surfaces. This Tech Tip describes a simple and flexible four-step method for covalently attaching an antibody to glass through native or added sulfhydryl (-SH) groups (Figure 1). A glass surface is derivatized with primary amines ($-NH_2$) using an aminosilane reagent. These amines are subsequently reacted to the heterobifunctional crosslinker Sulfo-SMCC, resulting in a maleimide-activated surface able to react with sulfhydryl groups on antibodies and other proteins.

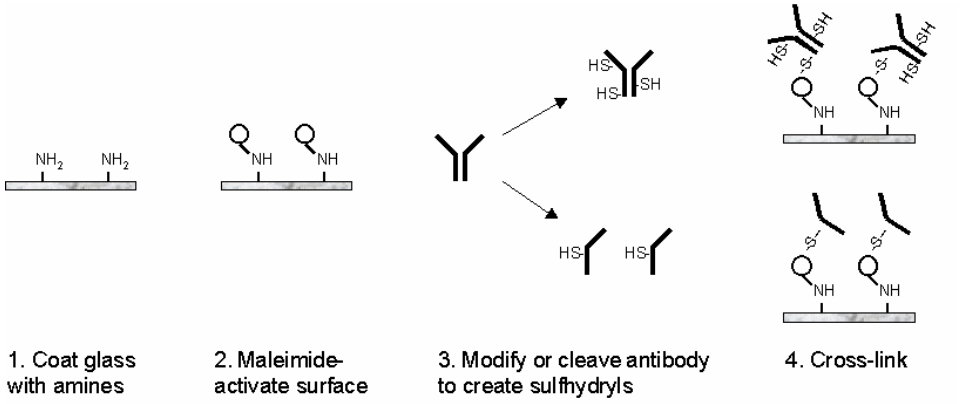

Figure 1. Basic steps involved in attaching an antibody onto a glass surface. See text and protocol for details. Functional groups and molecules are not drawn to scale.

One of two alternative strategies may be used to ensure that sulfhydryl groups are made available on an antibody for this immobilization method. Most proteins contain cysteines, whose sulfur atoms exist as sulfhydryls (-SH) or paired in disulfide bonds (-S-S-). Sulfur atoms must be in their reduced (-SH) state for covalent coupling to the maleimide-activated surface.

One strategy is to reduce native disulfide bonds in the antibody molecule. These disulfide bonds join heavy and light polypeptide chains together in a manner that ensures proper antibody structure and antigen-binding function. Therefore, complete reduction of antibody disulfides by treatment with reducing agents will usually inactivate the antibody. However, with the proper conditions, it is possible to selectively reduce only the more labile disulfides between heavy chains in the hinge region of IgG molecules; the result is functional half-antibodies with sulfhydryls available for reaction to the activated glass surface. Such partial reduction of antibody disulfides usually results in sulfhydryl group attachment points that will not sterically hinder antigen binding.

A second strategy for creating the necessary sulfhydryl groups is to add them to the antibody with specific reagents. Traut's Reagent and SATA are sulfhydryl-containing modification reagents that react with primary amines ($-NH_2$), which are present in the side-chain of lysine residues in antibodies and other proteins. Traut's Reagent creates sulfhydryl groups that are available for immediate reaction to the maleimide-activated surface. SATA creates protected sulfhydryl groups that are then exposed upon treated with hydroxylamine to yield sulfhydryl groups for coupling to the activated surface. With sulfhydryl addition methods, there is no risk of completely reducing and fragmenting an antibody; however, disruption of antigen-binding capability remains possible as a result of modification of the antigen-binding sites.

The following protocol is divided into four discrete sections. Section 3 presents the two alternative options for creating sulfhydryl groups on the antibody; choose one or the other of these two options. Read through all four sections before starting, and ensure that the activated surface (Section 2) and the sulfhydryl modified antibody (Section 3) are ready at the same time for the final conjugation reaction (Section 4).

Finally, plan reaction volumes and procedures in advance so as not to waste valuable antibody. Most importantly, notice in Section 4 that the final reaction requires antibody solution at a concentration greater than 10 µg/ml in sufficient volume to cover the glass surface, while Section 3 describes default conditions for preparing exactly 4 mg of antibody. Depending on the amount of antibody needed for the final coupling reaction, one may prepare more or less total antibody than assumed in Section 3. However, avoid altering the absolute concentrations of reagents (including antibody); otherwise molar ratios and incubation times will require optimization. Also, ensure that appropriate desalting columns are available for efficiently processing the reaction volumes used.

Materials Required

- Aminosilane Reagent: 3-Aminopropyltriethoxysilane (Product No. 80370)
- Acetone: solvent/diluent for Aminosilane Reagent
- Coupling Buffer: PBS-EDTA (50 mM Phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2). Use BupH™ Phosphate Buffered Saline Packs (Product No. 28372) and add EDTA to a concentration of 10 mM. Alternatively, other neutral or slightly alkaline amine-free buffers such as borate, HEPES, and bicarbonate also may be used. Whatever buffer is chosen, include 5-10 mM EDTA to prevent metal-catalyzed oxidation of sulfhydryls during the final cross-linking step. Also, avoid sulfhydryl-containing components in the buffer, as these will react with the maleimide portion of the crosslinker and inhibit conjugation of the antibody.
- Crosslinker: Sulfo-SMCC (Product No. 22322)
- Reagent to expose or add sulfhydryl groups - choose one of the following methods (see Introduction):
  - Reducing Agent: 2-Mercaptoethylamine (2-MEA) (Product No. 20408)
  - Sulfhydryl Addition Reagent - choose one of the following sets of reagents:
    - Traut's Reagent (2-Iminothiolane•HCl, Product No. 26101)
    - SATA (Product No. 26102) and Hydroxylamine•HCl (Product No. 26103) and DMSO (Product No. 20688)
- Desalting Column: D-Salt™ Polyacrylamide Desalting Columns (Product No. 43240) or D-Salt™ Dextran Desalting Columns (Product No. 43230)

Section 1: Aminosilylate the Glass Surface

1. Thoroughly wash and dry the glass, silica or quartz surface to be coated.

   Note: Perform Steps 2 and 3 in a fume hood.

2. Prepare a 2% solution of the Aminosilane Reagent (3-Aminopropyltriethoxysilane) in acetone. For example, mix 1 part Aminosilane Reagent with 49 parts dry (i.e., water-free) acetone. Prepare a volume sufficient to immerse or cover the surface material.
3. Immerse surface in the diluted reagent for 30 seconds.
4. Rinse surface with dry acetone.
5. Allow surface to air-dry.

   Note: The dried silylated surface may be stored for later use.

Section 2: Maleimide-Activate the Amino-Modified Surface

1. Add 2 mg Crosslinker (Sulfo-SMCC) to 1 ml Coupling Buffer. This solution may be scaled as needed, and must be used immediately to avoid hydrolysis.
2. Cover silylated surface with the Crosslinker solution.
3. Incubate for 1 hour at room temperature (RT).
4. Rinse the modified surface with Coupling Buffer.

   Note: The maleimide-activated surface may be dried and stored desiccated at 4°C for later use.

Section 3, Option 1: Partially Reduce Antibody to Produce Sulfhydryls for Coupling

Note: Perform either Option 1 or Option 2 of this section (see Introduction)

1. Dissolve 4 mg antibody (IgG) in 450 µl Coupling Buffer.
2. Dissolve 6 mg Reducing Agent (2-MEA) in 100 µl Coupling Buffer (results in 0.5 M stock solution).
3. Add 50 µl of Reducing Agent solution to the 450 µl antibody solution, and mix.
4. Incubate for 90 min at 37°C.
5. Purify the reduced antibody from the Reducing Agent using a Desalting Column equilibrated with Coupling Buffer. Collect 500 µl fractions.
6. Identify the fraction(s) that contain antibody by measuring for those having peak absorbance at 280 nm.
7. Pool the fractions that contain antibody (now with sulfhydryls). Proceed immediately to Section 4 to minimize disulfide formation, which will require having to repeat this section of the procedure.

Section 3, Option 2: Add Sulfhydryl Groups to Antibody for Coupling

Note: Choose one of the following two methods for adding sulfhydryl groups to the antibody (see Introduction).

Method 1: Sulfhydryl Addition with Traut's Reagent

1. Adjust about 10 ml of prepared Coupling Buffer (PBS-EDTA, pH 7.2) to pH 8.0 with concentrated NaOH.
2. Dissolve 4 mg antibody (IgG) in 475 µl the pH-adjusted Coupling Buffer.
3. Dissolve 2 mg Traut's Reagent in 1 ml of the pH-adjusted Coupling Buffer (results in 14.5 mM stock solution).
4. Immediately add 25 µl Traut's Reagent solution to the antibody solution (results in a 12-fold molar excess of reagent).
5. Incubate for 45 minutes at room temperature.
6. Purify the modified antibody from excess Traut's Reagent using a Desalting Column equilibrated with Coupling Buffer (pH 7.2). Collect 500 µl fractions.
7. Identify the fraction(s) that contain antibody by measuring for those having peak absorbance at 280 nm.
8. Pool the fractions that contain antibody (now with sulfhydryls).
9. Proceed immediately to Section 4.

Method 2: Sulfhydryl Addition with SATA

1. Dissolve 4 mg antibody (IgG) in 475 µl of Coupling Buffer.
2. Dissolve 3 mg SATA in 1 ml of DMSO (results in 13 mM stock solution).
3. Immediately add 25 µl SATA solution to the antibody solution (results in a 25-fold molar excess of reagent).
4. Incubate for 30 minutes at room temperature.
5. Purify the modified antibody from excess SATA and other reaction by-products using a Desalting Column equilibrated with Coupling Buffer (pH 7.2). Collect 500 µl fractions.

6. Identify the fraction(s) that contain antibody by measuring for those having peak absorbance at 280 nm.
7. Pool the fractions that contain antibody.

   Note: At this point, the modified antibody may be stored indefinitely; however, once the following steps for deacylating (deprotecting) the sulfhydryl groups are performed, the antibody must be desalted and used immediately for coupling.
8. Dissolve 348 mg of Hydroxylamine•HCl in 9 ml Coupling Buffer and then adjust to pH 7.2 with NaOH. Finally adjust the volume to 10 ml with additional Coupling Buffer (results in 0.5 M Hydroxylamine•HCl).
9. Add 100 µl Hydroxylamine solution to each 1 ml of SATA-modified antibody solution.
10. Incubate for 2 hours at room temperature.
11. Purify the antibody from the Hydroxylamine using a Desalting Column equilibrated with Coupling Buffer (pH 7.2). Collect 500 µl fractions.
12. Identify the fraction(s) that contain antibody by measuring for those having peak absorbance at 280 nm.
13. Pool the fractions that contain antibody.
14. Proceed immediately to Section 4.

Section 4: Cross-Link Sulfhydryl-Containing Antibody to Activated Surface

1. Cover the maleimide-activated surface material with the antibody solution. The antibody solution may be diluted in Coupling Buffer to a volume sufficient to cover the surface material. For optimal results, ensure that the final protein concentration is greater than 10 µg/ml.
2. Incubate for 2-4 hours at room temperature.
3. Remove the reaction solution, which contains any antibody that did not attach to the surface.
4. Thoroughly rinse the surface with Coupling Buffer to ensure that only covalently attached antibody molecules remain.
5. The surface is now ready to use for detection assays and other applications. Depending on stability of the particular antibody, the surface material may be dried for storage or kept covered in buffer containing 0.02% sodium azide.

Related Thermo Scientific Pierce Products

22582	**Ellman's Reagent**, 5 mg
77712	**Immobilized TCEP Disulfide Reducing Gel**, 5 ml
23225	**BCA Protein Assay Reagent Kit**
20036	**Bioconjugate Techniques**, 2nd Edition, Greg T. Hermanson, Academic Press, Inc., 2008.

Additional Information

This procedure was adapted from methods presented in Bioconjugate Techniques (Product No. 20036) and individual product instructions. Literature references and more detailed information are available in the individual product instructions, which may be obtained from our web site.

A related Tech Tip (#1) for attaching a protein to a glass, silica or quartz surface by a cleavable crosslinker is also available.

Current versions of product instructions are available at *www.thermo.com/pierce*. For a faxed copy, call 800-874-3723 or contact your local distributor.