

Protein labeling by use of biotin derivatives

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The highly specific interaction and extremely high affinity of avidin for biotin is a useful tool to target and detect biomolecules (BAYER EA and WILCHEK M 1980; BAYER EA and WILCHEK M 1990; WILCHEK M and BAYER EA 1990). The avidin-biotin interaction is the strongest known non-covalent interaction between protein and ligand. The bond formed between biotin and avidin is rapid and once formed is virtually unaffected by extreme situations (pH, solvents or denaturing agents).

Biotin is a small molecule that can be easily conjugated to most proteins and other biomolecules without altering their biological activity. For example, an antibody molecule can be coupled with several biotin molecules which, in turn, can each bind a molecule of avidin. This greatly increases the sensitivity of assays.

There exist a number of biotinylation reagents for labeling a variety of functional groups such as primary amines, sulfhydryls, carboxyls or carbohydrates. The most frequently used biotinylation reagents that react with primary amines are *N-hydroxysuccinimide esters* (NHS) and *N-hydroxysulfosuccinimide esters* (sulfo-NHS). NHS-activated biotins react readily with primary amine groups at a pH range from 7 to 9 and form stable amide bonds. Proteins such as antibodies possess abundant primary amines (lysine residues) that are able as targets for labeling with NHS-biotin. Several NHS esters of biotin are available with varying properties and spacer arms. The non-sulfo-NHS-biotins require organic solvents (e.g. DMF) to become dissolved, while the sulfo-NHS-biotins are water soluble. Thus, NHS-esters are first dissolved in an organic solvent and subsequently diluted into the aqueous reaction mixture.

The target for the NHS-biotin is the deprotonated form of the primary amine, and significant reactions occur above neutral pH when the amine is able to react with the ester. The reactive biotin is quite unstable. Once the biotin is solubilized, it must be used immediately. The degree of biotin incorporation into proteins depends on a set of conditions that result in conjugates with optimum properties for a special application. Especially the number of amines available for conjugation will vary among proteins, thus, reaction conditions being optimal for one protein may not be optimal for another protein. The extent of labeling can be controlled in experiments in order to find out the appropriate molar ratio of biotin to protein.

When conjugating an antibody for the first time, a range of different NHS-biotin concentrations should be compared. For example, one can try a range of 10 to 350 μg biotin per mg of antibody, and to compare each conjugate in a set of experiments in order to select the conjugate with the best staining results and the lowest background. Usually, more dilute protein solutions need greater molar fold excess of biotin, f.e. >12-fold molar excess of biotin for a 10 mg/mL protein solution and >20-fold molar excess of biotin for a 2 mg/mL protein solution.

Usually, primary amines of antibodies can be biotinylated without loss immunological reactivity. However, if the antibody contains a lysine rich antigen-binding site, then amine-

reactive reagents may inhibit the antigen-binding site. In this case, one can try NHS-biotins with “spacer arms” of different lengths. Alternatively, another biotin derivative should be selected, f.e. a derivative that reacts with aldehydes such as biotin-hydrazide: aldehyde groups can be readily generated on antibody molecules by oxidation of carbohydrate moieties with periodate. Then, biotin-hydrazide molecules bind readily to oxidized carbohydrates through the hydrazide group (-NH-NH₂) by formation of stable hydrazone bonds.

The biotinylation protocol of antibodies by use of biotin-N-hydroxysuccinimide (BNHS) *via* free amino groups is described. *

Reagents and chemical solutions

Biotin-N-hydroxy-succinimide ester (BNHS)
Dimethylformamide (DMF)
BNHS solution in DMF: 0.1 mol/L BNHS in DMF
Affinity chromatography purified antibodies in saline
0.9% NaCl
0.2 mol/L NaHCO₃ buffer pH 8.5
0.9% NaCl in 0.01 mol/L phosphate buffer pH 7.2 (PBS)
Sodium azide; glycerol

Biotinylation of antibodies

1. Antibodies are dialysed against 0.9% NaCl and used at a concentration of 3 mg/mL (concentration determined by the *Lowry* method).
2. Biotinylation:
 - Molar ratio BNHS/amino group = 3.
 - Example: 10 mg antibodies plus 171 μL of 0.1 mol/L BNHS in DMF.
3. Antibodies are diluted with 0.2 mol/L carbonate buffer (v/v), e.g. 1 mL antibody solution plus 1 mL of 0.2 mol/L carbonate buffer; final antibody concentration 1.5 mg/1 mL.
4. Biotinylation procedure:
 - 2 mL antibody solution (= 3 mg antibodies) plus 51.30 μL of 0.1 mol/L BNHS in DMF are mixed at room temperature for 60 minutes.
 - Dialyse against large quantities of PBS (with at least two changes) at 4°C for 24 h.
5. Aliquots of biotinylated antibodies are stored refrigerated or at -20°C at a final concentration of 1.5 mg antibodies/mL in PBS, glycerol (50% final concentration) and sodium azide (0.05% final concentration).

References for further readings

Bayer EA and Wilchek M (1980)

* Reagents and chemicals used for protein labeling can be toxic. They must be handled with care

Bayer EA and Wilchek M (1990a, 1990b)
Wilchek M and Bayer EA (1990a, 1990b, 1990c, 1990d)

Full citation of publications is given in chapter *References*

link: <https://www.kuhlmann-biomed.de/wp-content/uploads/2020/12/References.pdf>

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