ProductInformation

BIOTINYLATION KIT
BK-200

TECHNICAL BULLETIN

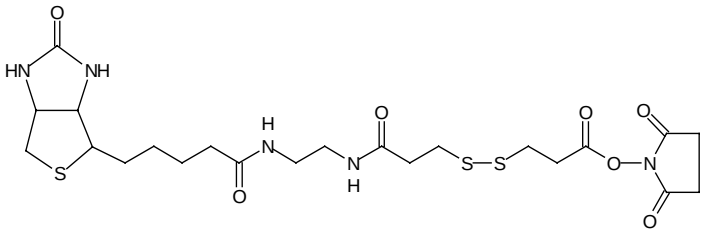
Product Description

The Avidin/biotin system is a versatile method which has been used extensively to detect and isolate a large variety of molecules. It is based on the high affinity of the cofactor Biotin for the protein Avidin (K_{diss} 10^{-15}) and the ease of spectrophotometric measurement. One disadvantage to this system is the irreversibility of the Avidin/Biotin interaction. Harsh conditions are required to effect dissociation. Various biotin derivatives have been used to label molecules. This biotinylation kit utilizes Biotin disulfide N-hydroxysuccinimide ester, a labelling reagent containing an active ester to react with primary amines and a cleavable disulfide linkage to facilitate removal of the biotin moiety at a later time. This allows for recovery of the target molecule essentially unmodified. After cleavage of the disulfide linkage, a 4 atom chain containing a free sulfhydryl group remains on the target molecule. Applications of this system include biotinylation of enzymes, proteins, nucleotides, or other compounds which contain or have been modified to contain a primary amine group. Other uses include receptor purification and cell surface labelling.

Also included in this kit is a reagent to determine Biotinylation levels. This assay is based on the binding of the dye HABA to Avidin and the ability of Biotin to displace the dye in stoichiometric proportions. This displacement of dye is accompanied by a change in absorbance at 500 nm which has a known extinction coefficient. See reference 1 for further details

Components

1. Biotin disulfide N-Hydroxysuccinimide ester
   B4531 3 X 10mg MW= 575.7
   Biotinylation reagent which is used to label primary amines of proteins or other compounds. This reagent is supplied in an amount sufficient to biotinylate at least 200mg of protein of MW approx. 100,000 when using a 15 molar excess of labeling reagent. This reagent contains an extended spacer with a cleavable disulfide linkage to allow for easy recovery of labeled compounds.



2. N,N'-Dimethylformamide (DMF) D 8654 5ml
   Solvent to dissolve biotinylation reagent.
   B4531 is soluble up to 50 mg/ml in DMF.
3. Phosphate buffer, pH 7.6 P 7834 1 vial
   Reaction buffer for biotinylating proteins or other compounds.
   When reconstituted with 10ml deionized water, this vial yields 0.1M sodium phosphate pH 7.6.
4. Phosphate buffered saline (PBS) packet
   P 3813 1 packet
   For equilibration of Sephadex G-25 column and avidin-agarose. When reconstituted with 1L of deionized water, this packet yields 0.01M sodium phosphate, 0.138M NaCl, 2.7mM KCl, pH 7.4.
5. Sephadex G-25 Gel filtration Column
   5-4805 1 column
   For separation of excess biotinylation reagent from labeled proteins or other compounds with a molecular weight of 5000 or greater. This column has a bed volume of 9.1ml and can be used to desalt samples of 1.1ml or less. This column may be regenerated by washing with at least 3 column volumes 0.5M NaCl, then re-equilibrating with PBS.
6. HABA/Avidin Reagent H 2153 1 vial
   For spectrophotometric determination of Biotin incorporation levels.
   When reconstituted with 10ml deionized water, this vial yields a ready-to-use solution containing HABA dye and Avidin in buffered saline with preservative, pH approx. 7.3. One vial is sufficient for 10 assays.

7. Dithiothreitol (DTT) D 0632 100mg
   For cleavage of disulfide linkage of the Biotinylation reagent (to separate the labeled compound from the affinity matrix). One hundred milligrams will prepare 11ml of 50mM solution.
8. The Avidin-agarose A 9207 1 ml
   For affinity separation of biotinylated compounds. Enough resin is supplied for one separation of approx. 2.5-4.5 mg protein. This resin is not capable of being regenerated and should be discarded after use. Additional affinity matrix is required for chromatography of greater amounts of protein. Some choices of other matrices include:
   A 4808 Avidin-acrylic beads
   S 1638 Streptavidin-agarose
   S 2415 Streptavidin-superparamagnetic iron oxide particles

Procedure

A. Biotinylation

1. Dissolve contents of Phosphate buffer vial (P 7834) with 10ml deionized water.

2. Dissolve protein or compound to be labeled at 5-15mg/ml in phosphate buffer from step one. Please note: Samples which contain buffers with primary amine groups (Tris, glycine) must be dialyzed or desalted before use because these compounds will react with the biotinylation reagent.

3. Dissolve biotinylation reagent at 25mg/ml in DMF. This stock solution is stable for up to 24 hours at 2 – 8 °C. For a 15 molar excess, the amount of reagent required is:

$$\text{\_\_\_\_ mg B4531} = \frac{\text{mg sample X 15 X 575.7 mg/mmole}}{\text{MW sample}}$$

____ ml DMF = mg B4531 ÷ 25 mg/ml

Molar excesses of 10-30 may be used depending on reactivity of the sample and level of biotinylation required.

4. Add the biotinylation reagent slowly to a solution of the compound to be labelled while stirring or agitating.

5. Gently mix for one hour at room temperature.

6. While the biotinylation is in progress, prepare the Sephadex G-25 column as follows:
   a. Dissolve the contents of PBS packet with 1L deionized water.
   b. Remove the top cap of the column then clip open the bottom with a scissors or razor blade.
   c. Equilibrate the column with at least 3 column volumes (approx. 30 ml) PBS.

7. When labelling is complete, add the reaction mix to the top of the column. The reaction vessel may be washed with a few drops of PBS and added to the top of the column.

8. Elute biotinylated sample with 1.5 column volumes (approx. 13 ml) PBS, collecting 1/20th of a column volume fractions. For the 9.1ml column supplied, this is approximately 0.5ml.

9. Check for fractions containing protein by A_{280} or a qualitative detection method such as a dye binding assay.

10. Protein containing fractions up to 66% of a column volume may be combined. For the column supplied, collecting 0.5ml fractions, this would be fraction number 12. If fractions past this point are combined, they may contain free biotinylation reagent.

11. Determine sample concentration as appropriate for the compound that was biotinylated (*i.e.* A_{280nm}, Biuret, etc.).

B. Determination of degree of biotinylation by HABA/Avidin assay.
HABA/Avidin Reagent, H 2153, is suitable for the spectrophotometric determination of biotin levels. This is a modified version of a literature preparation which eliminates the need for supplements to avoid non-specific or ionic interactions with most proteins.*

When reconstituted with 10 ml deionized water, each vial will yield the following solution:
0.3mM HABA (4-Hydroxyazobenzene-2-carboxylic acid)
0.45 mg/ml Avidin
0.3M NaCl
0.01M HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid], (a buffer with pKa=7.5)
0.01M $MgCl_2$
0.02% Sodium azide (a preservative)
pH approx. 7.3

Reconstituted vials should be stored at 0-5 °C. Freezing and thawing of solutions is not recommended. When stored at 0-5 °C, this reagent as reconstituted is stable at least one month. Some minor insolubles may develop over time. These may be removed by filtration or centrifugation without affecting the integrity of the reagent or assay results.

*Except in special cases such as lectins which require additon of an appropriate sugar (0.1 M to 0.5M).

1. Samples

Approx. 1 mg protein/ml for biotinylated proteins of MW approx. 100,000 and with approx. 5 moles biotin/mole protein. Otherwise, samples should be approx. 0.08µmole biotin/ml. This should result in a change in A_{500} of 0.1-0.4. If a change greater than 0.4 is observed, the sample should be diluted because more biotin is present than Avidin, and the dye displacement is no longer being measured. Also, samples containing glycerol must be dialyzed or desalted before use because glycerol interferes with this assay.

2. Protocol

In a 1 ml cuvette, pipet 900µl HABA/Avidin reagent (reconstituted as above). Read A_{500}. Add 100µl sample, mix by inversion, then read A_{500}. In some cases, as with a biotinylated protein, the absorbance may slowly decrease with time. If this occurs, a wait of 2 min. before reading is recommended.

Also, for colored samples, the following blank must be the following used:

900µl water or diluent + 100µl sample.

Calculations

a) $A_{500} = 0.9(A^{\text{HABA/Avidin}}_{500}) - A^{\text{HABA/Avidin + sample}}_{500}$

0.9 = Dilution factor of HABA/Avidin upon addition of sample

Note: For colored samples, the absorbance of the blank must be used:

$A_{500} = 0.9(A^{\text{HABA/Avidin}}_{500}) + A^{\text{sample blank}}_{500} - A^{\text{HABA/Avidin + sample}}_{500}$

b) µmole biotin/ml = $(A_{500}/34)(10)$
34 = mM extinction coefficient at 500nm
10 = dilution factor of sample into cuvette

c) mole biotin/mole protein = $\dfrac{\text{µmole biotin/ml sample}}{\text{µmole protein/ml sample}}$

At this point, the biotinylated compound may be used directly in detection systems. See reference 2 for possible applications. The following procedure for affinity chromatography and recovery is optional. It is advantageouly used after the biotinylated compound was used to scavenge another molecule of interest from a mixture (*i.e.* receptor purification or antigen/antibody interactions).

C. Affinity separation of biotinylated compound

1. Prepare affinity matrix by washing with 3 volumes PBS in a small column or microcentrifuge tube.

2. Add resin to solution containing biotinylated compound in PBS. Approx. 2.5-4.5 mg biotinylated protein or 25-50 µg biotin will bind per ml of Avidin-agarose.

3. Gently agitate resin mixture for 30 minutes at room temperature.

4. Wash resin with 5 - 10 volumes PBS as above.

5. Recover compound of interest by incubating resin with 3 volumes PBS supplemented to 50mM with DTT.

6. Gently agitate resin mixture for 1 hour at room temperature to ensure complete cleavage of disulfide linkage.

7. Remove supernatant from resin then wash gel with 1-2 column volumes PBS containing 50mM DTT. Combine wash with supernatant. This should contain the compound of interest which is no longer biotinylated. The resulting sample may be dialyzed or desalted to remove DTT.

8. The previously biotinylated compound now contains a free sulfhydryl group which may be used for later fractionation on a thiol- or disulfide-containing affinity chromatography matrix.

References

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