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ProductInformation

Goat ExtrAvidin® Peroxidase Staining Kit

Product Number **EXTRA-1**
Storage Temperature 2-8 °C

TECHNICAL BULLETIN

Product Description

The unique avidin reagent, ExtrAvidin®, combines the high specific activity and sensitivity of avidin with the low background staining of streptavidin.
ExtrAvidin binds biotin with the affinity of egg white avidin ($K_a = 10^{15}\ M^{-1}$); however, it does not exhibit the unwanted nonspecific binding reported for egg white avidin at physiological pH, such as the staining of mast cells. The Goat ExtrAvidin Staining Kit consists of ExtrAvidin-Peroxidase and Biotinylated Mouse Monoclonal Antibody to Goat IgG. The biotinylated antibody shows minimal cross reaction with human tissue or cell preparations. It does cross-react strongly with sheep IgG and slightly with bovine IgG.

This kit contains reagents for use with goat primary antibodies in immunohistology and in solid-phase immunoassays such as ELISA, immunoblotting or dot immunobinding. The kit reagents for each lot were tested in immunohistology, ELISA and dot immunobinding (dot blot) using the procedures as described.

Components

Qty	Reagent	Amount
1 vial B 6523	Monoclonal Anti-Goat IgG Biotin Conjugate Purified mouse immunoglobulin Clone GT-34, Mouse IgG1 isotype in buffer containing preservative.	3 ml
1 vial E 8386	ExtrAvidin® Peroxidase 0.5 mg/ml in buffer containing preservative.	3 ml

The kit reagents have been tested in immunohistology, ELISA and dot immunobinding (dot blot) using the procedures described in this insert. The amount of reagents supplied is sufficient for 500 tests in immunohistological staining. The reagents may be used for at least 2,000 ELISA or dot immunobinding tests.

Precautions and Disclaimer

The components of this kit are for Research Use only and are not intended for diagnostic procedures.
See Material Safety Data Sheets.

Storage/Stability

Store at 2-8 °C. Do Not Freeze.

Procedure for Immunohistology

(Staining procedure for paraffin-embedded tissue sections)

Reagents required but not supplied
(Numbers in parentheses are Sigma Product or Stock Numbers)

1. 0.01M Phosphate Buffered Saline (PBS), pH 7.4 (P 4417)
2. Bovine Serum Albumin (BSA) (A 7030)
3. Diluent: 1% BSA in PBS
4. Xylene (X 2377)
5. Absoulute Ethyl Alcohol (E702-3 or 45,983-6)
6. 0.1% Trypsin in PBS or 0.1% Protease in PBS
7. Hydrogen Peroxide (H 6520) 3% solution
8. Peroxidase substrate: 3-Amino-9-ethylcarbazole (AEC) Tablets, (A 6926)
9. Dimethylformamide (D 4551)
10. 0.05M Acetate buffer, pH 5.0

Reagents recommended but not required

1. Normal Human Serum (S 2257)
2. Normal Mouse Serum (M 5905)

Method (for Immunohistology)

Removal of Paraffin and Rehydration

1. Place slides in a 56-60°C oven for 15 minutes (Caution: oven temperature must not exceed 60 °C).
2. Transfer slides directly into a xylene bath for 5 minutes.
3. Repeat step 2.
4. Shake off excess liquid and place slides in fresh absolute ethyl alcohol for 3 minutes.
5. Repeat step 4.
6. Shake off excess liquid and place slides in fresh 95% ethyl alcohol for 3 minutes.
7. Shake off excess liquid and place slides in fresh 80% ethyl alcohol for 3 minutes.
8. Rinse slides in gently running tap water for 30 seconds (avoid a direct jet which may wash off or loosen the section).

Staining (for Immunohistology)

For detection of antigens requiring "unmasking", digest formalin-fixed material with 0.1% trypsin or 0.1% protease before proceeding further.

1. Place slides on a flat level surface. Do not allow slides to touch each other. Do not allow the sections to dry out at any time.
2. Add enough drops of 3% hydrogen peroxide to cover the whole section.
3. Incubate 5 minutes at room temperature.
4. Rinse with PBS from a wash bottle.
5. Place slide in PBS wash bath for 2 minutes.
6. Allow slide to drain, shake off excess fluid and carefully wipe the slide around sections. Dilute the respective goat primary antibody or negative control to its optimal concentration in PBS containing 1% BSA. The diluent alone can serve as a negative control. A positive control slide (tissue known to contain the antigen under study) should also be run.
7. Apply enough reagent (100 µl) to cover the section.
8. Tilt the slide in two different directions.
9. Incubate for at least 60 minutes in a humidified chamber at room temperature.
10. Rinse gently in PBS wash bath for 5 minutes (with continous slow stirring).
11. Allow slide to drain, shake off excess fluid and carefully wipe the slide as before.
12. Dilute the Monoclonal Anti-Goat IgG-Biotin Conjugate in PBS containing 1% BSA.
    Optional: For elimination of residual background staining of tissue, dilute the Monoclonal Anti-Goat IgG-Biotin Conjugate in PBS containing 5% normal mouse serum or 0.1% BSA. If the tissue is of human origin, 4% normal human serum maybe substituted.
13. Apply 100 µl to all slides, cover the section.
14. Incubate for 30 minutes in a humidified chamber at room temperature.
15. Rinse gently with PBS from a wash bottle.
16. Place slide in PBS wash bath for 5 minutes (with continuous slow stirring).
17. Allow slide to drain. Shake off excess fluid and carefully wipe slide as before.
18. Dilute ExtrAvidin-Peroxidase in diluent to its optimal concentration.
19. Apply 100 µl to all slides, cover the section.
20. Tilt the slide in two different directions.
21. Incubate 30 minutes in a humidified chamber at room temperature.
22. Rinse gently with PBS from a wash bottle.
23. Place slide in PBS wash bath for 5 minutes (with continuous slow stirring).
24. It is convenient to prepare the substrate mixture during the final wash bath as follows:
    a) Prepare an AEC stock solution by dissolving 1 AEC Tablet in 2.5 ml of dimethylformamide. This solution is stable at room temperature. For long term storage, store at 2-8 °C.
    b) Mix 0.2 ml of AEC stock solution with 3.8 ml of 0.05 M acetate buffer, pH 5.0. Immediately before use add 20 µl of 3% H_2O_2. The resultant yellowish solution is stable for 2-3 hours at room temperature. Properly discard any solution not used after this period of time. The formation of a fine precipitate does not interfere with the performance of the substrate mixture. If desired, the mixture may be filtered onto the tissue section. Each 4 ml of substrate mixture is sufficient for 20 slides.
25. Allow slide to drain. Shake off excess fluid and carefully wipe slide as before.
26. Apply enough substrate solution (100 µl) to cover the tissue section.
27. Incubate 5-10 minutes or until the red dye is adequate when monitored with a microscope. Terminate the reaction before generalized background staining appears in the negative controls.

28. To terminate the reaction, rinse gently with distilled water from a wash bottle.

Counterstaining Procedure

1. Apply Mayer's hematoxylin to cover the section or place slides in a bath of Mayer's hematoxylin. **Note**: Do not use alcohol containing solutions for counter-staining (e.g. Harris' hematoxylin, acid alcohol) since the AEC stain used with this kit is soluble in organic solvents. The slide must NOT be dehydrated, brought back to toluene or xylene, or mounted in toluene containing mounting media.
2. Incubate for 1-5 minutes, depending upon the strength of the hematoxylin used.
3. Rinse slide gently with distilled water from a wash bottle.
4. Rinse slide under gently running tap water for 5 minutes (avoid a direct jet which may wash off or loosen the section).
5. Mount sections using aqueous mounting medium such as glycerol gelatin.

Notes (for immunohistology):

1. This Goat ExtrAvidin Peroxidase Staining Kit has been optimized primarily for the adequate staining of formalin-fixed, paraffin-embedded human tissue sections. It may also be used for staining cryostat sections, cell smears, imprints, cytospins and cultured cells.
2. Pre-incubation with 5% BSA for 20 minutes prior to Step 9 of the *Staining* section may also decrease background staining. For best results with other animal tissues use similarly diluted normal mouse serum. It is recommended that reagents and buffers be prepared immediately prior to use for optimal staining.
3. Alternative substrates such as DAB may also be used.
4. In cases where weak or no staining at all occurs, or for antigens requiring "unmasking" add an enzyme digestion step for 15-30 minutes at 37 °C, prior to the removal of endogenous peroxidase (Step 1 of the *Staining* section). For the enzyme digestion use either 0.1% Trypsin in PBS or 0.1% Protease in PBS. Increased incubation times may also enhance specific staining.

Procedure for ELISA

Reagents not supplied (numbers in parentheses are Sigma product or stock numbers)

1. Dilution Buffer: 0.01 M Phosphate Buffered Saline (PBS), pH 7.4 (P 4417)
2. TWEEN® 20 (P 1379)
3. Blocking Solution:
   a. 1% Bovine Serum Albumin (BSA) (A 7030) **or**
   b. 1% Normal Mouse Serum (M 5905)
4. Substrate: 0.2 mg/ml 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphuric acid) (A 9941) in 0.05 M citrate-phosphate buffer with urea hydrogen peroxide (P 9305), pH 5.3. Prepare immediately before use.
5. Stop Solution: 0.4% (w/v) NaF. (S 1504)

Method (for ELISA)

1. Dissolve the antigen to the appropriate concentration in PBS. Example: For immunoglobulins the recommended concentration is 2.5 µg/ml.
2. Pipet 100 µl of the antigen solution to each well of a microtiter plate.
3. Incubate overnight at 2-8 °C.

All subsequent steps are carried out at room temperature.

4. Remove the coating solution, wash three times with PBS and twice with distilled water.
5. Block non-specific binding sites by filling wells with blocking serum for 10 minutes. Wash as in Step 4.
6. Dilute primary antibody produced in goat, to its optimal concentration in dilution buffer or prepare serial dilutions in order to find the optimal activity range.
7. Add 100 µl of the diluted primary antibody to each well.
8. Incubate at room temperature for 1 hour.
9. Wash as in Step 4.
10. Dilute the Monoclonal Anti-Goat IgG-Biotin Conjugate to its optimal concentration in dilution buffer.
11. Add 100 µl of the diluted biotin conjugate to each well.
12. Incubate at room temperature for 1 hour.

13. Wash as in Step 4.
14. Dilute ExtrAvidin-Peroxidase to its optimal concentration in dilution buffer containing 0.05% TWEEN 20.
15. Add 100 µl of the diluted ExtrAvidin-Peroxidase to each well.
16. Incubate at room temperature for 30 minutes.
17. Wash 5 times with washing buffer.
18. Add 100 µl of freshly prepared substrate.
19. Incubate, in the dark, at room temperature for 30 minutes.
20. Stop the reaction with 50 µl of stop solution.
21. Read results at 405 nm with an ELISA reader. Positive reaction is indicated by a green color.

Note: For best results with animal biological material use normal mouse serum as a blocking agent. We recommend preparation of reagents and buffers immediately prior to use.

Procedure for Dot Immunobinding (Dot Blot)

Reagents not supplied (numbers in parantheses are Sigma product or stock numbers)

1. 0.05 M Tris Buffered Saline (TBS), pH 7.4 (T 5030)
2. 0.01 M Phosphate Buffered Saline (PBS), pH 7.4, (P 4417)
3. Bovine Serum Albumin (BSA) (A 7030)
4. Nitrocellulose (NC) Blot, 0.45 µm pore size
5. Normal Horse Serum (H 0146)
6. Dilution buffer: TBS or PBS + 1% Normal Horse Serum
7. TBS or PBS + 0.05% TWEEN® 20 (P 1379) (TBS-T or PBS-T)
8. Wash buffer: TBS-T or PBS-T + 1% Normal Horse Serum
9. Peroxidase substrate: 3-Amino-9-ethylcarbazole (AEC) Tablets (A 6926)
10. Dimethylformamide (D 4451)
11. 0.05 M Acetate buffer, pH 5.0
12. Whatman Filter Paper No. 1
13. Hydrogen Peroxide 30% (H 1009)

Main Steps in Dot Immunobinding Assay

1. Application of antigen onto a nitrocellulose sheet.
2. Blocking/Quenching of nonspecific adsorption.
3. Immunodetection by a primary antibody.
4. Immunodetection by a biotinylated secondary antibody.
5. Avidin-Biotin linkage of the ExtrAvidin- Peroxidase conjugate.
6. Peroxidase substrate reaction.

Method (for Dot Blot)

1. Prepare nitrocellulose according to manufacturer's directions.
2. Apply antigen to a nitrocellulose sheet in a small volume (0.5 or 1.0 microliters). Small tight dots containing antigen at a high concentration will result in better color contrast against the background.
3. Dry the sheet under a cold air stream for 5 minutes.

Note: All incubation and washing steps are carried out at room temperature on an orbital shaker platform.

4. Incubate the sheet in 5% BSA (w/v) or 5% Normal Horse Serum in TBS or PBS for 16-20 hours at room temperature. The blocking ("quenching") step greatly minimizes nonspecific adsorption of antibody and detection reagents onto the blot. The quenching reagent should be chosen after considering the type of probe that will be subsequently used in the overlay procedure.
5. Wash the sheet once in PBS or TBS for 5 minutes.

For the following steps the nitrocellulose sheet may be cut in strips if more than one primary antiserum is used in immunodetection.

6. Dilute the goat primary antibody in dilution buffer. Other non-interfering proteins (e.g. BSA, Ovalbumin) may be substituted for Normal Horse Serum. The dilution buffer should be used as the negative control.
7. Place the strips with the dot side facing up in troughs or petri dishes and overlay with goat primary antibody at an appropriate dilution such that the strip is covered completely with liquid. Incubate for 2 hours.
8. Wash the strips four times for 5 minutes each wash, use 15 ml wash buffer per wash.
9. Dilute the Monoclonal anti-Goat IgG-Biotin Conjugate in dilution buffer to its optimal concentration.
10. Incubate the strips for 1 hour in diluted biotinylated antibody.
11. Wash the strips as in Step 8.
12. Dilute ExtrAvidin-Peroxidase in dilution buffer to its optimal concentration.
13. Incubate the strips for 1 hour in the diluted ExtrAvidin-Peroxidase.
14. Wash the strips as in Step 8.

15. It is convenient to prepare the substrate mixture during the final wash bath as follows:
    a) Prepare an AEC stock solution by dissolving 1 AEC Tablet in 2.5 ml of dimethylformamide. This solution is stable at room temperature. For long term storage, store at 2-8 °C.
    b) Mix 0.2 ml of AEC stock solution with 3.8 ml of 0.05 M acetate buffer, pH 5.0. Immediately before use add 20 µl of 3% H_2O_2. The resultant yellowish solution is stable for 2-3 hours at room temperature. Properly discard any solution not used after this period of time. The formation of a fine precipitate does not interfere with the performance of the substrate mixture. If desired, the mixture may be filtered prior to use.
16. Iincubate the strips in the substrate mixture for 5-10 minutes. A red insoluble precipitate characterizes the antigen-antibody complex in the dot. The nitrocellulose strip will normally have a slight reddish background.
14. Wash the strips in several changes of distilled water.
15. Dry the strips between sheets of filter paper under a cold air stream.
17. The peroxidase-labeled nitrocellulose strips may be stored in the dark, in a plastic sleeve.

Notes (for Dot Immunobinding Assay):

1. If background staining occurs when the sample is of human origin dilute the biotinylated purified antibody in PBS containing 2% normal human serum or 2% normal mouse serum. For best results with animal biological material use normal serum of the second antibody host as a blocking reagent. Preparation of reagents and buffers immediately prior to use is recommended.
2. This procedure can be stopped after each washing step. Strips should be kept in PBS at 2-8 °C or room temperature and the reaction can be continued some hours later or the following day.
3. Times of incubations and washings may be shortened, but only after the assay system has been optimized.

Results

Working Dilutions

Immunohistology:

Biotinylated Anti-Goat IgG:	1:15 - 1:20
ExtrAvidin-Peroxidase:	1:15 - 1:20

ELISA:

Biotinylated Anti-Goat IgG:	1:1,000 - 1:1,500
ExtrAvidin-Peroxidase:	1:250 - 1:500

Dot Immunobinding:

Biotinylated Anti-Goat IgG:	1:1,000 - 1:1,500
ExtrAvidin-Peroxidase:	1:500 - 1:1,000

In order to obtain best results in other assays or with different procedures, it is recommended that optimal working dilutions first be determined by a titration assay.

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