



SIGMA-ALDRICH

**ExtrAvidin[®]
Alkaline Phosphatase
Staining Kit**

Rabbit

Stock No. EXTRA-3A

Table of Contents

Materials Supplied	3
Working Dilutions	4
Introduction	5
Procedure for Immunohistology	6
Procedure for ELISA	13
Procedure for Dot Immunobinding (Dot Blot)	16
Storage	21

Materials Supplied

<u>Qty</u>	<u>Reagent</u>	<u>Amount</u>
1 vial B 6648	Biotinylated Goat Anti-Rabbit IgG, Affinity Purified Antibody, in buffer containing preservative	3 ml
1 vial E 4889	ExtrAvidin® Alkaline Phosphatase, in buffer containing preservative	3 ml

These kit reagents have been tested for use 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 2000 ELISA or dot immunobinding tests.

Working Dilutions

Immunohistology:

Biotinylated Goat Anti-Rabbit Igs: 1:15-1:20

ExtrAvidin® Alkaline Phosphatase: 1:15-1:20

ELISA:

Biotinylated Goat Anti-Rabbit Igs: 1:1,000-1:1,500

ExtrAvidin® Alkaline Phosphatase: 1:1,000-1:1,500

Dot Immunobinding:

Biotinylated Goat Anti-Rabbit Igs: 1:1,000-1:1,500

ExtrAvidin® Alkaline Phosphatase: 1:20,000-1:30,000

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.

Introduction

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 ($K_a = 10^{15} \text{ M}^{-1}$); 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 Rabbit ExtrAvidin[®] Staining Kit consists of ExtrAvidin[®] Alkaline Phosphatase and Biotinylated Goat Anti-Rabbit IgG. The purified antibody has been adsorbed with human IgG and IgM to ensure minimal cross reaction with human tissue or cell preparations.

The Rabbit ExtrAvidin[®] Staining Kit contains reagents for use with rabbit 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.

Procedure for Immunohistology

(Staining procedure for paraffin embedded tissue sections)

Reagents not provided

1. 0.01 M Tris Buffered Saline, pH 8.2 (TBS)
2. 0.01 M Phosphate Buffered Saline, pH 7.4 (PBS) (P 4417)
3. Bovine Serum Albumin (BSA) (A 7030)
4. Diluent: 1% BSA in PBS
5. Xylenes (X 2377)
6. Absolute Ethyl Alcohol (E702-3 or 45,983-6)
7. 0.1% Trypsin in PBS or 0.1% Protease in PBS
8. Fast Red Substrate Solution (SIGMA *FAST™* Fast Red TR/Naphthol AS-MX, Product Code F 4523)

Method

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 90% 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 detection of antigens requiring “unmasking”, digest formalin-fixed material with 0.1% trypsin or 0.1% protease before proceeding further (see note 2).

1. Place slide in PBS wash bath for 2 minutes.
2. Allow slide to drain, shake off excess fluid and carefully wipe the slide around sections.
3. Dilute the respective rabbit 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.
4. Apply enough reagent (100 μ l) to cover the section.
5. Tilt the slide in two different directions.
6. Incubate for at least 60 minutes at room temperature.
7. Rinse gently in PBS wash bath for 5 minutes (with continuous slow stirring)

Staining (cont'd)

8. Allow slide to drain, shake off excess fluid and carefully wipe the slide as before.
9. Dilute the biotinylated antibody in PBS containing 1% BSA.
Optional: For elimination of residual background staining of human tissue, dilute the biotinylated second antibody in PBS containing 4% normal human serum.
10. Apply 100 μ l to all slides, cover the section.
11. Incubate for 30 minutes at room temperature.
12. Rinse gently with PBS from a wash bottle.
13. Place slide in PBS wash bath for 5 minutes (with continuous slow stirring).
14. Allow slide to drain. Shake off excess fluid and carefully wipe slide as before.
15. Dilute ExtrAvidin-Alkaline Phosphatase in diluent to its optimal concentration.
16. Apply 100 μ l to all slides, cover the section.
17. Tilt the slide in two different directions.

Staining (cont'd)

18. Incubate 20 minutes at room temperature.
19. Rinse gently with TBS from a wash bottle.
20. Place slide in TBS wash bath for 5 minutes (with continuous slow stirring).
21. Prepare the substrate mixture during the final wash.
Levamisole may be added to the substrate solution at a concentration of 1 mM to block endogenous alkaline phosphatase.
22. Allow slide to drain. Shake off excess fluid and carefully wipe slide as before.
23. Apply enough substrate solution (100 μ l) to cover the tissue section.
24. 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.
25. 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 substrate 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

1. The Rabbit ExtrAvidin Staining Kit has been optimized primarily for the staining of formalin-fixed, paraffin-embedded human tissue sections. It may also be used for staining cryostat sections, cell smears, imprints, cytopspins and cultured cells.
2. In cases where weak or no staining occurs, or for antigens requiring "unmasking", add an enzyme digestion step for 15-30 minutes at 37 °C, prior to 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.
3. Preincubation with 5% BSA for 20 minutes prior to step 2 of the Staining section may also decrease background staining. For best results with other animal tissues, use similarly diluted normal goat serum. We recommend preparation of reagents and buffers immediately prior to use for optimal staining.

Notes (cont'd)

4. Optimal dilutions and incubation times should be determined for each primary antibody prior to the use of this kit.

Procedure for ELISA

Reagents not provided

1. 0.01M Tris Buffered Saline, pH 8.2 (TBS).
2. Phosphate Buffered Saline, pH 7.4 (PBS).
3. Dilution buffer/Washing buffer: PBS + 0.05% TWEEN™ 20 (PBS-T).
4. Blocking Solution:
 - a. 1% Bovine Serum Albumin (BSA)
 - b. 1% Normal Goat Serum
5. *p*-Nitrophenyl Phosphate (pNPP) Substrate Solution (SIGMA FAST™ *p*-Nitrophenyl Phosphate, Product Code N 2770)
6. Stop Solution: 3 N NaOH.

Method

1. Dissolve the antigen to the appropriate concentration in PBS.
For immunoglobulins, the recommended concentration is 2.5 $\mu\text{g/ml}$.
2. Pipette 200 μl of the antigen solution to each well of a multiwell plate.
3. Incubate overnight at 4 $^{\circ}\text{C}$ (the same results can be obtained by 2 hour incubation at 37 $^{\circ}\text{C}$).
(All subsequent steps are carried out at room temperature.)
4. Remove the coating solution, wash three times with PBS-T and twice with distilled water.
5. Block nonspecific binding sites by filling wells with blocking solution for 10 minutes. Wash as in step 4.
6. Dilute primary antibody produced in rabbit, to its optimal concentration in PBS-T or prepare serial dilutions in order to find optimal activity range.

Method (cont'd)

7. Add 200 μ l of the diluted primary antibody to each well.
8. Incubate at room temperature for 2 hours.
9. Wash as in step 4.
10. Dilute the biotinylated antibody to its optimal concentration in PBS-T.
11. Add 200 μ l of the diluted second antibody to each well.
12. Incubate at room temperature for 2 hours.
13. Wash as in step 4.
14. Dilute ExtrAvidin[®] Alkaline Phosphatase to its optimal concentration in TBS-T.
15. Add 200 μ l of the diluted ExtrAvidin[®] Alkaline Phosphatase to each well.
16. Incubate at room temperature for 60 minutes.
17. Wash 4 times with TBS-T and 3 times with distilled water.
18. Add 200 μ l of freshly prepared substrate solution.

Method (cont'd)

19. Incubate in the dark at room temperature for 30 minutes.
20. Stop the reaction with 50 μ l of stop solution.
21. Read results at 405 nm with an ELISA reader.
Positive reaction is indicated by a yellow color.

Note:

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

Procedure for Dot Immunobinding (Dot Blot)

Reagents not provided

1. 0.05 M Tris-HCl Buffered Saline, pH 7.4, (TBS) (T 5030)
2. 0.01 M Phosphate Buffered Saline, pH 7.4, (PBS) (P 4417)
3. 5% Bovine Serum Albumin (BSA) (A 7030) in PBS containing 0.1% Sodiumazide

Reagents not provided

4. Nitrocellulose (NC) Blot, 0.45 μm pore size
5. TBS or PBS + 0.05% TWEEN™ 20 (TBS-T or PBS-T)
6. BCIP/NBT (SIGMA FAST™ BCIP/NBT, Product Code B 5655)

Method

1. Prepare nitrocellulose according to manufacturer's directions.
2. Sample Application: Apply antigen to a nitrocellulose sheet in a small volume (0.5 or 1.0 μl). 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. All incubation and washing steps are carried out at room temperature on an orbital shaker platform.

Method

4. Incubate the sheet in 5% BSA (w/v) in TBS or PBS for 16-20 hours at room temperature. The blocking (“quenching”) step greatly minimizes non-specific adsorption onto the blot. The choice of quenching reagent depends on the type of probe that will be subsequently used in the overlay procedure and should be chosen accordingly.
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.

6. Dilute rabbit primary antibody in PBS-T or TBS-T. The dilution buffer should be used as the negative control. Place the strips with the dot side facing up in troughs or petri dishes and overlay with rabbit primary antibody at an appropriate dilution such that the strip is covered completely with liquid. Incubate for 2 hours.

Method (cont'd)

7. Wash the strips four times, 5 minutes each wash, with 15 ml of PBS-T or TBS-T.
8. Dilute the biotinylated antibody in TBS buffer to its optimal concentration.
9. Incubate the strips for 1 hour in diluted biotinylated antibody.
10. Wash the strips four times, 5 minutes each wash, with 15ml of TBS.
11. Dilute ExtrAvidin® Alkaline Phosphatase in TBS to its optimal concentration.
12. Incubate the strips for 1 hour in the diluted ExtrAvidin® Alkaline Phosphatase.
13. Wash the strips as in step 10.
14. Incubate the strips in freshly prepared substrate mixture for 10-30 minutes.

A purple insoluble precipitate characterizes the antigen-antibody complex in the dot. The nitrocellulose strip will normally have a slight background color.

Method (cont'd)

15. Wash the strips in several changes of distilled water.
16. Dry the strips between sheets of filter paper under a cold air stream.
17. Store the alkaline phosphatase labeled nitrocellulose strips in the dark in a plastic sleeve.

Notes

1. If background staining with human biological material occurs, dilute the biotinylated purified antibody in PBS containing 2% normal human serum. For best results with animal biological material, use normal serum of the second antibody host as a blocking reagent. We recommend the preparation of reagents and buffers immediately prior to use.

Notes (cont'd)

2. This procedure can be stopped after each washing step and continued hours or days later. Strips should be kept in PBS at 4 °C or room temperature.
3. Incubation and washing times may be shortened, but only after optimization of the assay system according to the recommended method.

Storage

Store at 2-8 °C. Do Not Freeze.

LPG 7/03

TWEEN is a trademark of the ICI Group.

ExtrAvidin is a registered trademark of Sigma-Aldrich Biotechnology.

Notes:

Notes:



SIGMA-ALDRICH

3050 Spruce Street
Saint Louis, Missouri 63103 USA
Telephone (800) 325-5832 • (314) 771-5765
Fax (314) 286-7828

Sigma brand products are sold through Sigma-Aldrich, Inc.
Sigma-Aldrich, Inc. warrants that its products conform to the information contained
in this and other Sigma-Aldrich publications.

Purchaser must determine the suitability of the product(s) for their particular use.

Additional terms and conditions may apply.

Please see reverse side of the invoice or packing slip.