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Instruction Manual

Streptavidin-AP Conjugate
Streptavidin-HRP Conjugate
Streptavidin-FITC Conjugate

For immunodetection of biotinylated recombinant proteins

Catalog nos. SA100-01, SA100-02, SA100-03

Version A
July 15, 2002
25-0523

Table of Contents

Important Information

Types of Products

This manual is supplied with the streptavidin conjugates listed below.

Product	Catalog no.
Streptavidin-Horseradish Peroxidase (HRP) Conjugate	SA100-01
Streptavidin-Fluorescein Isothiocyanate (FITC) Conjugate	SA100-02
Streptavidin-Alkaline Phosphatase (AP) Conjugate	SA100-03

Shipping and Storage

Each streptavidin conjugate is shipped and should be stored at +4°C. Each product is guaranteed for six months from the date of receipt if stored properly.

Note: Avoid freezing as this may result in loss of activity.

Contents

Each streptavidin conjugate is supplied as described below. The amount of streptavidin conjugate provided is sufficient for 25 western blots using 10 ml of working solution per reaction or 25 immunostaining reactions using 1 ml of working solution per reaction as appropriate.

Conjugate	Concentration	Buffer	Amount
Streptavidin-AP	Refer to label on tube	3.0 M NaCl 1.0 mM $MgCl_2$ 0.1 mM $ZnCl_2$ 30 mM triethanolamine, pH 7.6	125 µl
Streptavidin-HRP	Refer to label on tube	MOPS Buffer	50 µl
Streptavidin-FITC	Refer to label on tube	0.01 M sodium phosphate, pH 7.2 0.15 M NaCl 0.05% sodium azide	50 µl

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Important Information, continued

Conjugation

Each streptavidin conjugate is produced using the method described below. Streptavidin biotin-binding activity and enzyme activity (for AP and HRP conjugates only) are retained after conjugation.

Conjugate	Method of Production
Streptavidin-AP	Chemical coupling of the enzyme, calf intestinal alkaline phosphatase (CIAP), to streptavidin (Fernley, 1971)
Streptavidin-HRP	Chemical coupling of the enzyme, horseradish peroxidase, to streptavidin
Streptavidin-FITC	Coupling of the FITC fluorophore to streptavidin (3-4 FITC molecules/streptavidin molecule) under high pH conditions

Applications

Each streptavidin conjugate is suitable for use in the following applications.

Conjugate	Application
Streptavidin-AP	• Immunoblotting • ELISA • Immunocytochemistry where penetration of the conjugate is not limited • *In situ* hybridization with biotinylated DNA/RNA probes
Streptavidin-HRP	• Immunoblotting • ELISA • Immunomicroscopy
Streptavidin-FITC	• Immunomicroscopy • Fluorescence-activated cell sorting and analysis (single label experiments or in conjunction with other streptavidin conjugates in double label experiments)

Methods

Overview

Introduction

The streptavidin-AP, streptavidin-HRP, and streptavidin-FITC conjugates use the sensitive streptavidin-biotin interaction to allow flexible, rapid, and sensitive immunodetection (Bayer and Wilchek, 1980; Guesdon *et al.*, 1979).

General Guidelines

- Establish optimal concentrations of each streptavidin conjugate to use for your experiment depending upon your application. If you are performing western blot analysis using the streptavidin-AP or streptavidin-HRP conjugate, see page 3 for a sample protocol and recommended conditions for use. If you are performing immunofluorescence using the streptavidin-FITC conjugate, see page 6 for a sample protocol and recommended conditions for use.
- If you need to dilute the streptavidin conjugate, we recommend using a buffer containing an exogenous protein source (*e.g.* bovine serum albumin; BSA) to act as a carrier and to help reduce potential background.

Using the Streptavidin-AP Conjugate

Consider the following when using the streptavidin-AP conjugate:

- Calf intestinal alkaline phosphatase (CIAP) is a zinc metalloenzyme and can be temporarily inhibited in the presence of cation chelators. The enzyme is inactivated at a pH of less than 5.0.
- The streptavidin-AP conjugate may not be an appropriate reagent to use for all immunocytochemical procedures because of its large size, which may prevent the conjugate from reaching some intracellular antigens.

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Overview, continued

Using the Streptavidin-HRP Conjugate

Consider the following when using the streptavidin-HRP conjugate:

- If you use azide in your dilution buffer, be sure to wash the western blot or microtiter wells thoroughly before adding the substrate for detection. Azide will inhibit horseradish peroxidase activity.
- Horseradish peroxidase is light sensitive. Avoid excessive exposure to light.
- In some cases, the presence of endogenous peroxidase activity in your sample may increase the background signal and must be eliminated or reduced before you can proceed with your experiment. See the **Appendix**, page 10 for procedures that may be used to lessen endogenous peroxidase activity.

Using the Streptavidin-FITC Conjugate

Use the appropriate fluorescence microscope and filters to detect your signal. For the best signal, view slides with epi-fluorescence. Use a high pressure mercury lamp and appropriate excitation and barrier filters.

- For FITC staining, we recommend using a standard FITC filter set (blue excitation) with an exciter filter at 450-490 nm, a chromatic beam splitter at 510 nm, and a barrier filter at 520 nm.
- For cell sorting, use an argon laser at 488 nm to excite cells labeled with fluorescein. Pass the emitted light through a 510-55 nm band pass filter before reaching the detector (Parks *et al.*, 1984).

Western Blotting (Immunoblotting)

Introduction

A general protocol is provided below for detection of biotinylated recombinant fusion proteins using the streptavidin-AP or streptavidin-HRP conjugate. Other protocols are suitable.

Recommended Dilutions

For western blots, we recommend diluting the streptavidin conjugates into Tris-Buffered Saline (TBS) containing 0.1% (v/v) Tween-20 and 1% (w/v) nonfat dry milk:

- Streptavidin-AP: dilute 1:2000
- Streptavidin-HRP: dilute 1:5000

Materials Needed

You should have the following materials and solutions on hand:

- Tris-Buffered Saline (TBS: 20 mM Tris-HCl, 140 mM NaCl, pH 7.5)
- Tris-Buffered Saline + Tween 20 (TBST: TBS plus 0.1% Tween-20, v/v)
- Blocking buffer (TBS + 5% nonfat dry milk, w/v)
- Dilution buffer (TBST + 1% nonfat dry milk, w/v)
- Streptavidin-AP or streptavidin-HRP conjugate
- Appropriate reagents for detection

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Western Blotting, continued

Polyacrylamide Gel Electrophoresis

To facilitate separation of your recombinant protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE® and Novex® Tris-Glycine polyacrylamide gels and electrophoresis apparatus are available from Invitrogen. In addition, Invitrogen carries a large selection of molecular weight protein standards and staining kits for visualization of proteins. For more information about the appropriate gels, standards, and stains to use to visualize your recombinant protein, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 11).

Western Blot Protocol

Prepare an SDS polyacrylamide gel (either Tris/Glycine or Tris/Tricine) designed to resolve your biotinylated protein or use Novex® pre-cast gels (see above). Prepare your samples for electrophoresis. For information about SDS-polyacrylamide gel electrophoresis, see Ausubel *et al.*, 1994. Load a sufficient amount of your recombinant protein onto the gel to get a strong signal.

1. Load your samples and electrophorese your SDS polyacrylamide gel.
2. Transfer proteins to nitrocellulose or any other suitable membrane electrophoretically. using 25 mM Tris, pH 8.3, 192 mM glycine, 20% (v/v) methanol as a transfer buffer. Other transfer buffers are suitable.

   Note: If you are using NuPAGE® gels or other types of pre-cast gels, other transfer buffers may be required. Refer to the manufacturer's instructions to transfer proteins to the membrane of choice.
3. Run at 100V, 150 mA (100V, 240 mA at the finish) for 1 hour. Be sure to have a cooling system in place. You may also transfer overnight at 30V, 40 mA (which will be 30V, 90 mA at the finish).
4. Remove membrane and incubate it in 10 ml blocking solution. Gently agitate using a rocker platform for 1 hour at room temperature. Proceed to Step 5.

   Note: You can store the blot overnight at this step if needed. Keep the blot in blocking solution and store at +4°C covered with plastic wrap.

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Western Blotting, continued

Western Blot Protocol, continued

5. Wash membrane in 20 ml TBST for 5 minutes with gentle agitation. Repeat twice.
6. Transfer membrane to a tray containing the streptavidin-AP or streptavidin-HRP diluted 1:2000 or 1:5000, respectively in 10 ml dilution buffer. Incubate with gentle agitation for at least 1 hour at room temperature.

   Note: In most cases, a 1 hour incubation is sufficient for detection. Overnight incubation is possible, but may increase background.
7. Transfer membrane to a tray containing 20 ml TBST and wash for 5 minutes with gentle agitation. Repeat wash three times.
8. Transfer membrane to a tray containing 20 ml TBS and wash for 5 minutes with gentle agitation. Repeat wash three times.
9. Transfer membrane to a tray containing 20 ml of sterile water and wash for 5 minutes with gentle agitation.
10. Proceed to **Detection Reaction**, below.

Detection Reaction

We use a chemiluminescent substrate for detection using enhanced chemiluminescence. Other substrates (*e.g.* colorimetric) and methods for detection are suitable. Refer to the manufacturer's instructions to perform the detection reaction.

Note: The WesternBreeze® Chromogenic Kits and WesternBreeze® Chemiluminescent Kits are available from Invitrogen to facilitate detection of streptavidin conjugates by colorimetric or chemiluminescent methods. For more information, see our Web site (www.invitrogen.com) or call Technical Service (see page 11).

Immunofluorescence

Introduction

A general protocol is provided below for detection of biotinylated recombinant fusion proteins using the streptavidin-FITC conjugate. Other protocols are suitable.

Recommended Dilution

For immunofluorescence, we recommend diluting the streptavidin-FITC conjugate 1:500 into Phosphate-Buffered Saline (PBS) containing 10% FBS (v/v).

Materials Needed

You should have the following materials and solutions on hand:

- Phosphate-Buffered Saline (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \bullet 7H_2O$, 1.4 mM KH_2PO_4, pH 7.3)
- Fetal Bovine Serum (FBS)
- Methanol
- Blocking Buffer (PBS + 10% FBS, v/v)
- Streptavidin-FITC conjugate
- Fluorescence microscope
- FITC filter or other appropriate filter (see page 2)

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Immunofluorescence, continued

Immuno-fluorescence Protocol

A protocol is provided below to perform immunofluorescence on cells plated in a 35 mm tissue culture dish or a single well in a six-well tissue culture plate. Note that volumes may need to be adjusted if you are using larger or smaller plates.

1. Plate cells in a 35 mm dish or a single well in a six-well tissue culture plate. Incubate cells overnight at 37°C in serum-containing medium or until they reach 50% confluence.
2. Transfect cells with the expression construct of choice and allow an appropriate amount of time to elapse for expression of your biotinylated recombinant fusion protein to occur.
3. Remove the medium and wash cells twice with PBS.
4. Fix the cells by adding 2 ml of room temperature, 100% methanol. **Note:** Depending on the nature of the protein being detected, other fixatives may be used. Some empirical experimentation may be necessary.
5. Incubate for 5 minutes at room temperature. Do not exceed 5 minutes.
6. After incubation, wash cells 5 x 2 minutes with PBS (2 ml/wash).
7. Add 2 ml of Blocking Buffer (PBS containing 10% FBS) and incubate for 20 minutes at room temperature to reduce non-specific binding of the streptavidin-FITC conjugate.
8. Remove the Blocking Buffer and add 1 ml of Blocking Buffer containing the streptavidin-FITC conjugate (1:500 dilution). Incubate for 1 hour at room temperature in the dark.
9. Wash cells 2 x 5 minutes with PBS and observe cells with a fluorescence microscope equipped with a FITC filter (or appropriate filter).

Troubleshooting Western Blotting

Introduction

The table below lists some potential problems and possible solutions that you may use to help you troubleshoot your western blotting experiment.

Problem	Reason	Solution
No signal	Poor or no transfer	Stain membrane with Ponceau S to check degree of transfer, then re-run the gel and repeat transfer
	Streptavidin conjugate too dilute	Use more streptavidin conjugate
	Protein too dilute	Load more protein
	Old detection reagents	Prepare fresh detection reagents immediately before use
High background	Streptavidin conjugate too concentrated	Titrate the streptavidin conjugate and use the maximal dilution that gives a detectable signal in a reasonable amount of time
	Insufficient blocking	Increase incubation time in blocking solution Include Tween-20, BSA, or other blocking agents in the blocking and washing solutions
Multiple protein bands	Proteolysis of the protein	Use protease inhibitors when preparing cell lysates
	Inefficient reduction of the protein	Resuspend samples in SDS-PAGE sample buffer containing fresh reducing agent and boil the samples for 5 minutes prior to electrophoresis

Troubleshooting Immunofluorescence

Introduction

The table below lists some potential problems and possible solutions that you may use to help you troubleshoot your immunofluorescence experiment.

Problem	Reason	Solution
No signal	Little or no biotinylated protein expressed	Repeat transfection and expression. Harvest cells and use western blot analysis to check for protein expression
	Streptavidin conjugate too dilute	Use more streptavidin conjugate
	Poor fixation	Try alternative fixation methods
High background	Streptavidin conjugate too concentrated	Titrate the streptavidin conjugate and use the maximal dilution that gives a detectable signal in a reasonable amount of time
	Insufficient blocking	Increase incubation time in blocking solution

Appendix

Endogenous Peroxidase Activity

Introduction

In certain situations, endogenous peroxidase (or pseudo-peroxidase) activity may be found in samples being assayed. If you are using the streptavidin-HRP conjugate, this endogenous peroxidase activity can increase background and must be eliminated or reduced to allow for interpretable results. A number of procedures are available to help reduce endogenous peroxidase activity (see below). Use the procedure which best suits your needs.

Procedures

The procedures below may be used to diminish peroxidase activity. Treatment is generally performed prior to primary antibody incubation, but may be changed if the antigen is susceptible to the treatment.

- Treat rehydrated sections with H_2O_2 diluted to 0.1-3.0% in methanol for 15 to 30 minutes (DeLellis, 1981; Straus, 1979). Wash.
- For antigens sensitive to organic extraction, treat rehydrated sections with dilute H_2O_2 for 15 to 60 minutes (DeLellis, 1981). Wash.
- Treat rehydrated sections with dilute acid (0.74% HCl in ethanol for 15 to 30 minutes (Weir *et al.*, 1974). Wash.
- Treat rehydrated sections with 7.5% H_2O_2. Wash. Incubate with 0.1 M periodic acid. Wash. Block aldehyde groups with 0.02% potassium borohydride (Heyderman and Neville, 1977). Wash.
- Treat rehydrated sections with 0.1% phenylhydrazine, pH 7.0, for 1 hour at 37°C.

Technical Service

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Technical Service, continued

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2. Follow instructions on the page and fill out all the required fields.
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Product Qualification

Introduction

Each streptavidin conjugate is qualified as described below.

Streptavidin-AP

1. The conjugate has 1600-2600 units per ml of alkaline phosphatase activity and a concentration ranging from 0.75-1.2 mg/ml. Refer to the label on the tube for the activity and concentration of your lot of conjugate.
2. When tested in an ELISA assay
   - Maximum signal is obtained at dilutions ≥ 1:2000.
   - The background must be ≤ 0.1 at 405 nm.
   - 5 ng of biotinylated BSA is detected.
3. When tested in a Western blot with 25 ng of biotinylated BSA, a signal is obtained using a 1:2000 dilution of streptavidin-AP and detection with a chemiluminescent substrate after a 5 minute exposure to film.

Streptavidin-HRP

1. The concentration of the conjugate ranges from 0.75-1.2 mg/ml. Refer to the label on the tube for the concentration of your lot of conjugate.
2. When tested in a Western blot with 5 ng of biotinylated BSA, a signal is obtained using a 1:5000 dilution of streptavidin-HRP and detection with a chemiluminescent substrate after a 5 minute exposure to film.

Streptavidin-FITC

1. The conjugate must contain a ratio of 2.5-4.0 molecules of FITC per molecule of streptavidin as determined by UV absorption.
2. The concentration of the conjugate ranges from 0.5-1.2 mg/ml. Refer to the label on the tube for the concentration of your lot of conjugate.

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Notes

Notes

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