Anti-His(C-term) Antibody
Anti-His(C-term)-HRP Antibody

Catalog nos. R930-25, R931-25

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Table of Contents

Overview

Introduction

The Anti-His(C-term) and Anti-His(C-term)-HRP Antibodies allow detection of recombinant proteins containing a C-terminal polyhistidine tag with a free carboxyl group (-His-His-His-His-His-His-COOH). They are derived from the clone #3D5 (Lindner, et al., 1997). The antibodies can be used to detect expression of C-terminal His fusion proteins from bacterial, insect, and mammalian cells.

Contents

The table below provides information on the concentration of antibody, buffer, and amount supplied.

The Anti-His(C-term) Antibody is a mouse monoclonal IgG_{2b} antibody. Anti-His(C-term)-HRP Antibody was prepared by crosslinking the Anti-His(C-term) Antibody with horseradish peroxidase using glutaraldehyde.

Antibody	Concentration	Buffer	Amount
Anti-His(C-term)	refer to label on tube	PBS, 0.01% azide	50 µl (25 westerns)†
Anti-His(C-term)-HRP	refer to label on tube	PBS	50 µl (25 westerns)†

†Assumes 10 ml buffer per western blot.

Shipping/Storage

The Anti-His(C-term) Antibodies are shipped and stored at +4°C. Each product is guaranteed for six months from the date of receipt.

For long-term storage, aliquot the antibody and store at -20°C or -80°C. Repeated freezing and thawing is not recommended as it may result in loss of antibody activity.

Antibody Specificity

Both antibodies have been tested in immunoblotting and ELISA procedures. Low background was observed using chemiluminescent or alkaline phosphatase reagents for detection.

In western blot experiments with purified protein, 50 ng (for Anti-His(C-term)-HRP Antibody) or 100 ng (for Anti-His(C-term) Antibody) of recombinant Positope™ protein gave a detectable signal using the protocol on page 4.

Note: The Anti-His(C-term) Antibody has also been used successfully to immuno-precipitate fusion proteins that contain the 6xHis tag.

Crossreactivity

Using chemiluminescence as the detection method, no crossreactivity has been observed in bacterial lysates. In mammalian lysates, a few crossreactive proteins have been observed upon overexposure of blots.

continued on next page

Overview, continued

Recommended Dilutions

We recommend the following dilutions of the supplied antibody for these applications:

- For western blots, dilute 1:5000 into Phosphate-Buffered Saline (PBS) containing 0.05% Tween-20 and 5% nonfat, dry milk (PBSTM).
- For ELISA assays, serially dilute into PBSTM and test dilutions to determine the best dilution of antibody for your application.

If you use a different buffer for washing and blocking your blots, then dilute as described above with that buffer. You may use other blocking agents such as bovine serum albumin (BSA) or gelatin.

Using Secondary Antibodies

If you are using the unconjugated Anti-His(C-term) Antibody, please review the following information.

- If you use alkaline phosphatase-conjugated secondary antibody, do not use PBS. Phosphate inhibits alkaline phosphatase. Use Tris-Buffered Saline (TBS) instead.
- If you use horseradish peroxidase-conjugated secondary antibody, be sure to wash the western blot or microtiter wells thoroughly before adding the secondary antibody. Azide will inhibit horseradish peroxidase activity.

Product Qualification

The Anti-His(C-term) Antibody and the Anti-His(C-term)-HRP Antibody are functionally tested by western blot using the protocols described in the manual.

Anti-His(C-term) Antibody

The antibody must react specifically with 500 ng of an *E. coli* expressed fusion protein containing a C-terminal polyhistidine (6xHis) epitope. Western blots must reveal a strong signal, with no non-specific background, after development with a chemiluminescent substrate followed by a 10 minute exposure to x-ray film.

Anti-His(C-term)-HRP Antibody

The antibody must react specifically with 100 ng of an *E. coli* expressed fusion protein containing a C-terminal polyhistidine (6xHis) epitope. Western blots must reveal a strong signal, with no non-specific background, after development with a chemiluminescent substrate followed by a 1 minute exposure to x-ray film.

Western and Dot Blot

Introduction

This procedure can be used for detection of fusion protein expression particularly when levels of expression are low. The table below outlines the basic steps of a western blot.

Step	Description
1	Run an SDS polyacrylamide gel of the purified or partially purified protein or cell lysate with appropriate controls.
2	Transfer the proteins electrophoretically to a nylon or nitrocellulose membrane.
3	Probe the blot with Anti-His(C-term) Antibody or the Anti-His(C-term)-HRP Antibody.
4	If you probe with the Anti-His(C-term) Antibody, incubate the blot with anti-mouse IgG secondary antibody conjugated to an enzyme such as alkaline phosphatase or horseradish peroxidase (HRP).
5	Detect fusion protein using the appropriate method of choice.

Suggested Solutions

We use chemiluminescence to detect binding of the Anti-His(C-term) Antibodies to the recombinant protein. Other detection methods can be used. The following materials and solutions are needed for immunoblotting and chemiluminescent detection:

- Phosphate-Buffered Saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4
- Phosphate-Buffered Saline + Tween 20 (PBST: PBS plus 0.05% Tween-20, v/v)
- Blocking buffer (PBST + 5% nonfat, dry milk, w/v)
- Secondary Antibody: Anti-Mouse IgG (whole molecule) HRP

continued on next page

Western and Dot Blot, continued

Immunoblotting Protocol

Prepare an SDS polyacrylamide gel designed to resolve your recombinant protein or purchase Novex® pre-cast gels (call Technical Service for more information). For information about SDS-polyacrylamide gel electrophoresis, see Ausubel *et al.*, 1994.

1. Prepare and load your samples for electrophoresis. Load at least 5 to 50 ng of your recombinant protein onto the gel in order to get a good signal.
2. Transfer proteins to nitrocellulose membrane electrophoretically. We use 25 mM Tris (pH 8.3), 192 mM glycine, and 20% v/v methanol as transfer buffer.
3. Run at 100 V, 150 mA (100 V, 240 mA at the finish) for 1 hour. Be sure to have a cooling system in place and operational with these electrophoretic settings. You may also transfer overnight at 30 V, 40 mA (30 V, 90 mA at the finish).
4. Remove the nitrocellulose membrane and incubate it in 10 ml blocking buffer. Gently agitate using a rocker platform for 1 hour at room temperature.
5. Wash the nitrocellulose membrane in 20 ml PBST 2X for 5 minutes each with gentle agitation.
6. Transfer membrane to a tray containing either the Anti-His(C-term) or the Anti-His(C-term)-HRP Antibody diluted 1:5000 in blocking buffer (2 µl of antibody diluted into 10 ml blocking buffer). Incubate with gentle agitation for 1-2 hours.

   Overnight incubation may be preferred as longer incubations may increase the sensitivity of detection. Generally, a 1 hour incubation is sufficient.
7. Transfer membrane to a tray containing 20 ml PBST and wash for 2 x 5 minutes with gentle agitation. If you are using the Anti-His(C-term)-HRP Antibody, proceed to detection.
8. If you are using the Anti-His(C-term) Antibody, transfer membrane to a tray containing the secondary antibody. Dilute the secondary antibody according to the manufacturer's recommendation into blocking buffer. Incubate with gentle agitation for 1 hour.
9. Wash for 2 x 5 minutes in PBST as described in Step 7.

Detection Reaction

We use enhanced chemiluminescence to detect the fusion proteins. Please follow the manufacturer's instructions. Other detection methods are suitable.

Dot Blot Protocol

Use this protocol to quickly detect the presence of recombinant protein (e.g. to screen a variety of baculovirus or mammalian clones to find the highest expressing clone. Be sure to spot equivalent amounts of protein for each sample.

1. Make serial dilutions of samples (purified or partially purified protein or cell lysates) in 10 mM Tris-HCl, 25 mM EDTA, pH 8.0. The lowest dilution should have at least 30 ng of protein present. Nitrocellulose membrane can bind approximately 100 µg protein per cm^2.
2. Spot 1 µl of each sample onto nitrocellulose paper or use a slot blot apparatus.
3. Allow membrane to air-dry.
4. Proceed to the **Immunoblotting Protocol**, steps 4-9, above, then to the **Detection Reaction**.

Enzyme-Linked Immunosorbent Assay (ELISA)

Introduction

In addition to western blots, the Anti-His(C-term) Antibodies can also be used in immunoassays. A sample protocol is provided for your convenience. Other protocols are suitable. For more information, please refer to *Antibodies* (Harlow and Lane, 1988) and *Current Protocols in Molecular Biology* (Ausubel *et al*., 1994), unit 11.2.

Guidelines

Please consider the items below when setting up your ELISA.

- Include controls for cross-reactivity and nonspecific binding to host cell proteins
- Be sure that all experimental conditions are kept constant to ensure reproducibility
- Determine optimal dilution of Anti-His(C-term) or Anti-His(C-term)-HRP Antibody for use with your antigen
- Always include a standard curve with each plate
- Analyze samples in duplicate
- Be sure that the concentration of antigen falls within the dynamic range of the standard curve.

Detection of Antigen

ELISAs can be used to detect 1 ng/ml to 1 µg/ml antigen in a bacterial cell lysate. Sandwich ELISAs are more sensitive than direct ELISAs.

Buffers

We have used the following buffers with these antibodies.

- PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM $NaHPO_4$, 1.8 mM KH_2PO_4
- PBST: PBS, 0.05% Tween-20
- PBSTM: PBST, 5% nonfat, dry milk

continued on next page

ELISA, continued

General Procedure

The procedure below is an example of a direct ELISA to detect the amount of antigen in a bacterial cell lysate. For details, please refer to Unit 11.2 of *Current Protocols in Molecular Biology* (Ausubel *et al*., 1994). Other protocols are suitable.

1. Prepare a 10 µg/ml solution of purified antigen in PBS.
2. In a polystyrene 96-well plate, serially dilute the antigen solution across the columns so that each row has a different dilution of antigen. Use PBSTM as the dilution buffer and 50 µl as the final volume in each well. Leave column 12 blank as a background control. Repeat with a second plate. **Note**: Include controls such as a lysate that does not contain the antigen of interest.
3. Cover the plates and incubate overnight at +4°C or 2 hours at room temperature.
4. Remove antigen solution and wash with wells with PBS three times.
5. Add 0.2 ml PBSTM to each well and incubate for 1 hour at room temperature.
6. Remove PBSTM and wash with PBST three times.
7. Serially dilute Anti-His(C-term) or Anti-His(C-term)-HRP Antibody across the rows. Use PBSTM as the diluting buffer and 50 µl as the final volume in each well. Start with a 1:500 or 1:1000 dilution in row A.
8. Cover and incubate plates at room temperature for 2 hours.
9. Remove antibody and wash wells three times with PBST. If you used Anti-His(C-term)-HRP Antibody, proceed to detection reaction, below.
10. For Anti-His(C-term) Antibody, add 50 µl of diluted anti-mouse HRP-conjugated secondary antibody to each well. Use PBSTM as the dilution buffer. To dilute the secondary antibody, see the manufacturer's instructions.
11. Incubate at room temperature for 30 to 60 minutes.
12. Remove antibody and wash wells four times with PBS.
13. Proceed to **ELISA Detection Reaction**, below.

ELISA Detection Reaction

The protocol uses a sensitive chromogenic substrate for detection of HRP-labeled reagents. Other detection reagents are suitable. For other detection methods, refer to *Antibodies* (Harlow and Lane, 1988). This protocol makes enough substrate solution for up to two 96-well microtiter plates. Pre-made substrate solution is available from other vendors (i.e. Sigma, Catalog no. T8865).

1. Dissolve 0.1 mg of TMB (3, 3', 5, 5'-tetramethylbenzidine, Sigma Catalog no. T2885) in 0.1 ml of dimethylsulfoxide (DMSO).
2. Add 9.9 ml of 0.1 M sodium acetate, pH 6.0.
3. Filter through Whatman No. 1 paper or equivalent.
4. Add hydrogen peroxide to a final concentration of 0.01%.
5. Add 50 µl of the substrate solution to each well.
6. Incubate 10-30 minutes at room temperature. Positives appear pale blue.
7. Add 50 µl of 1 M H_2SO_4, to each well. Positives now appear bright yellow.
8. Read the results at 450 nm using a spectrophotometer.

continued on next page

ELISA, continued

Analyzing Your Experiment

Plot absorbance versus known antigen concentration on semilog paper to analyze each antibody dilution. For a working dilution of antibody, choose the dilution that provides the maximum sensitivity over a linear range of antigen concentrations and a minimum binding (< 0.05 absorbance units) for background.

Analyzing Lysates

Once you have identified the optimal working dilution of Anti-His(C-term) or Anti-His(C-term)-HRP, you are ready to analyze your lysates.

1. Prepare an ~10 µg/ml solution of your lysate in PBS.
2. Take a polystyrene 96-well plate and serially dilute the lysates across the columns so that each row has a different dilution of antigen. Use PBSTM as the diluting buffer and 50 µl as the final volume in each well. Leave column 12 blank as a background control. Repeat with a second plate.
3. Cover the plates and incubate overnight at 4°C or 2 hours at room temperature.
4. Remove antigen solution and wash with wells with PBS three times.
5. Add 0.2 ml PBSTM to each well and incubate for 1 hour at room temperature.
6. Remove PBSTM and wash with PBST three times
7. Add 50 µl of the appropriate dilution of Anti-His(C-term) or Anti-His(C-term)-HRP Antibody in PBSTM.
8. Cover and incubate plates at room temperature for 2 hours.
9. Remove antibody and wash wells three times with PBST. If you used Anti-His(C-term)-HRP Antibody, proceed to detection reaction.
10. For Anti-His(C-term) Antibody, add 50 µl of diluted anti-mouse HRP-conjugated secondary antibody to each well. Use PBSTM as the dilution buffer. For the appropriate dilution of secondary antibody, please see the manufacturer's instructions.
11. Incubate the plates for 30-60 minutes at room temperature.
12. Remove antibody and wash wells four times with PBS.
13. Proceed to **ELISA Detection Reaction**, previous page.

Immunoprecipitation

Immuno-precipitation Protocol

The Anti-His(C-term) and Anti-His(C-term)-HRP Antibodies can be used to immunoprecipitate fusion proteins that contain the -His-His-His-His-His-His-COOH epitope. The procedure below describes a general immunoprecipitation protocol; other protocols are suitable. For more details, please refer to *Antibodies* (Harlow and Lane, 1988).

1. Before beginning, you will need to prepare the appropriate lysis buffer. Many lysis buffers are suitable. We recommend using either:
   - RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris, pH 7.5) or
   - NP-40 lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris, pH 8.0)

   When preparing the lysis buffer, you may want to include a cocktail of protease inhibitors such as 10-100 µM leupeptin, 1-10 mM EDTA, 1 µM pepstatin, and 0.2-1 mM PMSF (from 100 mM stock in 2-propanol).
2. Lyse cells using one of the lysis buffers above or one of your choice. For mammalian and insect cells, use approximately 5 x 10^6 to 1 x 10^7 cells/ml of lysis buffer. For *E. coli* and yeast cells, use approximately 1 x 10^9 cells/ml of lysis buffer.
3. Centrifuge the lysate for 20 minutes at 10,000 x g at +4°C.
4. Carefully transfer supernatant to a sterile microcentrifuge tube and place on ice.
5. Add 50 µl of Protein-G Sepharose® resin slurry (50% slurry in lysis buffer) per 1 ml of supernatant to pre-clear the lysate.
6. Rock at +4°C for 1 hour.
7. Centrifuge for 1 minute at 10,000 x g at +4°C.
8. Transfer supernatant to a sterile microcentrifuge tube and place on ice.
9. Add 1-2 µg (typically, 1-2 µl) of the Anti-His(C-term) or Anti-His(C-term)-HRP Antibodies and 50 µl of the Protein-G Sepharose® resin slurry to the supernatant. Rock for 2-24 hours at +4°C.
10. Centrifuge for 1 minute at 10,000 x g at +4°C.
11. Remove supernatant.
12. Wash the resin 2X with 500 µl lysis buffer.
13. The fusion protein immune complexes may now be used in the appropriate assay.
14. For SDS polyacrylamide gels, add 50 µl of SDS-PAGE sample buffer (2% SDS, 10% glycerol, 100 mM DTT, 60 mM Tris pH 6.8, and 0.001% bromophenol blue) to the resin. Heat the sample at 85°C for 2 minutes. Centrifuge for 1 minute at 10,000 x g and load supernatant onto the gel.

Sepharose® is a registered trademark of Amersham Pharmacia Biotech, Ltd.

References

References

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1994) *Current Protocols in Molecular Biology*. Greene Publishing Associates and Wiley-Interscience, New York.

Harlow, E. and Lane, D. (1988) *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory. Cold Spring Harbor, NY.

For Immunoprecipitation, see Chapter 11, pp. 421-470.

For Immunoblotting (westerns), see Chapter 12, pp. 471-510.

For Immunoassays (ELISA), see Chapter 14, pp. 553-612.

Lindner, P., Bauer, K. Krebber, A., Nieba, L., Kremmer, E., Krebber, C., Honegger, A., Klinger, B., Mocikat, R., and Plueckthun, A. (1997) Specific Detection of His-tagged Proteins With Recombinant Anti-His Tag scFv-phosphatase or scFv-phage Fusions. *Biotechniques* **22**:140-149.

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continued on next page

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