

Anti-Xpress[™] Antibody **Anti-Xpress[™] -HRP Antibody**

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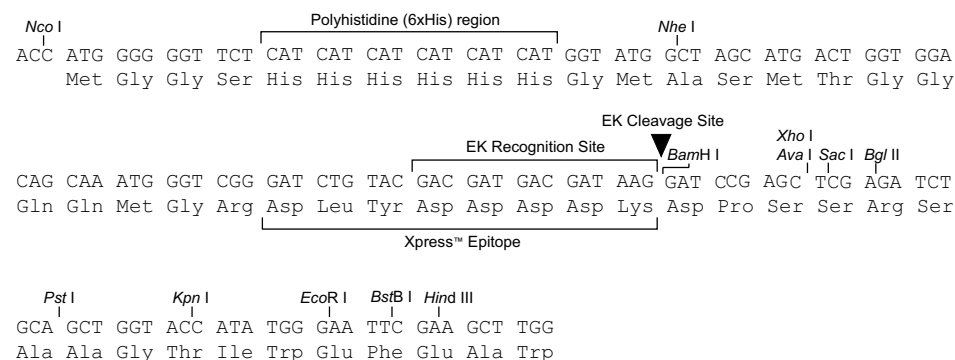
Overview

Introduction

The Anti-Xpress™ and Anti-Xpress™-HRP (horseradish peroxidase-conjugated) Antibody allows detection of recombinant proteins containing the N-terminal leader peptide (Xpress™ epitope). This N-terminal peptide contains a polyhistidine sequence, the Xpress™ epitope (part of the bacteriophage T7 gene 10 protein), and an enterokinase cleavage site. The Anti-Xpress™ antibodies recognize the Xpress™ epitope sequence **-Asp-Leu-Tyr-Asp-Asp-Asp-Lys-** found in this leader peptide. The antibodies can be used to detect expression of N-terminal Xpress™ fusion proteins from bacterial, insect, and mammalian cells.

Location of the Anti-Xpress™ Epitope

The diagram below shows the location of the epitope recognized by the Anti-Xpress™ antibodies. The example shown is the leader peptide from the pTrcHis B vector. The leader peptide is the same in all of the Xpress™ vectors up to and including the aspartic acid following the lysine in the enterokinase recognition site. The multiple cloning site which follows the *Bam*H I site varies between vectors.



Contents

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

The Anti-Xpress™ Antibody is a mouse monoclonal IgG₁ antibody. Anti-Xpress™-HRP Antibody was prepared by crosslinking the Anti-Xpress™ Antibody with horseradish peroxidase using glutaraldehyde.

Antibody	Concentration	Buffer	Amount
Anti-Xpress™	refer to label on tube	PBS, 0.01% azide	50 µl (25 westerns) [†]
Anti-Xpress™ - HRP	refer to label on tube	PBS	50 µl (25 westerns) [†]

[†]Assumes 10 ml buffer per western blot.

Shipping/Storage

The Anti-Xpress™ 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.

Continued on next page

Overview, Continued

Antibody Specificity

Both antibodies have been tested in immunoblotting and ELISA procedures. No crossreactivity was detected in any of the host cell lines.

In western blot experiments with purified protein, 50 ng (for Anti-Xpress[™]-HRP Antibody) or 100 ng (for Anti-Xpress[™] Antibody) of recombinant Positope[™] protein gave a detectable signal using the protocol on page 3.

Note: The Anti-Xpress[™] Antibodies have also been used successfully to immunoprecipitate fusion proteins that contain the Xpress[™] tag.

Crossreactivity

Using chemiluminescence as the detection method, no crossreactivity has been observed in bacterial lysates or mammalian lysates.

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 PBST and test dilutions to determine the best dilution of antibody for your application.
 - You may use other buffers of your choice for dilution or blocking agents such as bovine serum albumin (BSA) or gelatin.
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Using Secondary Antibodies

If you are using the unconjugated Anti-Xpress Antibody, review the information below:

- 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 the microtiter wells thoroughly before adding the secondary antibody. azide will inhibit horseradish peroxidase activity.
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Product Qualification

Both antibodies are functionally tested by western blot using the protocols on page 3.

Anti-Xpress[™] Antibody

The antibody must react specifically with 100 ng of an *E. coli* expressed fusion protein containing the Xpress[™] epitope. Western blots must reveal a strong signal, with no non-specific background, after 10 minutes of color development.

Anti-Xpress[™]-HRP Antibody

The antibody must react specifically with 50 ng of an *E. coli* expressed fusion protein containing the Xpress[™] 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 proteins containing the Xpress™ epitope by western blot. We have included a general protocol for your convenience. 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 PVDF or nitrocellulose membrane.
3	Probe the blot with Anti-Xpress™ or Anti-Xpress™-HRP Antibody.
4	If you probe with the Anti-Xpress™ 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 detection method of choice.

Suggested Solutions

We use chemiluminescence to detect binding of the Anti-Xpress™ 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 (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄)
- 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

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Western and Dot Blot, Continued

Immunoblotting Protocol

Prepare an SDS polyacrylamide gel (either Tris/Glycine or Tris/Tricine) designed to resolve your recombinant protein. Prepare your samples for electrophoresis. Remember that you need to load about 100 to 500 ng of your recombinant protein into each lane of the gel for the best results. (For information about SDS-polyacrylamide gel electrophoresis, please see Ausubel, *et al.*, 1990.)

1. Load your samples and electrophorese the SDS polyacrylamide gel.
2. Transfer proteins to nitrocellulose electrophoretically using the settings recommended by the manufacturer of your transfer apparatus. Transfer Buffer: 25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3.
3. Remove nitrocellulose and incubate it in 10 ml blocking buffer. Gently agitate using a rocker platform for 15-30 minutes at room temperature.
4. Wash nitrocellulose in 20 ml TBST for 5 minutes with gentle agitation. Repeat wash twice more.
5. Transfer membrane to a tray containing Anti-Xpress™ or Anti-Xpress™-HRP antibody diluted 1:5000 in 10 ml blocking buffer (2 µl of antibody diluted into 10 ml of blocking buffer). Incubate with gentle agitation for 1 hour at room temperature.
Overnight incubation may be preferred, since longer incubations may increase the sensitivity of detection. However, it may also increase background. In most cases, a 1 hour incubation is sufficient for detection.
6. Transfer membrane to a tray containing 20 ml TBST and wash for 5 minutes with gentle agitation. Repeat wash twice more. If you are using the Anti-Xpress™-HRP Antibody, proceed to detection.
7. If you are using the Anti-Xpress™ Antibody, transfer membrane to a tray containing the secondary antibody. Dilute the secondary antibody according to the manufacturer's recommendation into blocking buffer. Incubate for 1 hour with gentle agitation.
8. Wash the membrane 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

This protocol is used to quickly detect the presence of recombinant protein. This method can be used to screen a variety of baculovirus or mammalian clones in order 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 cm².
2. Spot 1 µl of each sample onto nitrocellulose paper, or alternatively, 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**.

Continued on next page

Western and Dot Blot, Continued

Troubleshooting

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

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
	Antibody too dilute	Use more antibody
	Protein too dilute	Load more protein
	Old detection reagents	Prepare fresh detection reagents immediately before use
High background	Antibody too concentrated	Titrate the antibody 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 electrophoresing your gel

Enzyme-Linked Immunosorbent Assay (ELISA)

Introduction

The Anti-Xpress™ antibodies can be used in immunoassays. A sample protocol for performing an ELISA is provided below 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 the optimal dilution of Anti-Xpress™ or Anti-Xpress™-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.
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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 this antibody.

- Phosphate-Buffered Saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM NaHPO₄, 1.8 mM KH₂PO₄)
 - Phosphate-Buffered Saline + Tween-20 (PBST: PBS plus 0.05% Tween-20, v/v)
 - PBST + Nonfat dry milk (PBSTM: PBST plus 5% nonfat dry milk, w/v)
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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*. Please note that other protocols are suitable.

1. Prepare a 10 µg/ml solution of antigen in PBS. This will be used for your standard curve so it should be as pure as possible.
2. Take a polystyrene 96-well plate and 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:** We recommend that you 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 to allow antigen to bind to the plate.
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 the Anti-Xpress™ or Anti-Xpress™-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-Xpress™-HRP Antibody, proceed to **ELISA Detection Reaction**, below.
10. For unconjugated Anti-Xpress™ 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 at room temperature for 30 to 60 minutes.
12. Remove the antibody solution and wash the 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. For other detection methods, please 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 separately from Invitrogen (Catalog no. 15980-014).

1. Dissolve 0.1 mg of TMB (3, 3', 5, 5'-tetramethylbenzidine, Invitrogen Catalog no. 15980-014) 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 the plates for 10-30 minutes at room temperature. Positives appear pale blue.
7. Add 50 µl of 1 M H₂SO₄ to each well. Positives now appear bright yellow.
8. Read the results at 450 nm using a spectrophotometer.

Continued on next page

ELISA, Continued

Analysis of 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-Xpress[™] Antibodies, you are ready to analyze your lysates.

1. Prepare an ~ 10 $\mu\text{g/ml}$ 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-Xpress[™] or Anti-Xpress[™]-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-Xpress[™]-HRP Antibody, proceed to **ELISA Detection Reaction**, previous page.
 10. For Anti-Xpress[™] Antibody, add 50 μl of anti-mouse HRP-conjugated secondary antibody diluted in PBSTM to each well. 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.
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Immunoprecipitation

Immuno-precipitation Protocol

The Anti-Xpress™ and Anti-Xpress™-HRP Antibodies can be used to immunoprecipitate fusion proteins that contain the -Asp-Leu-Tyr-Asp-Asp-Asp-Lys- epitope. The procedure below describes a general immunoprecipitation protocol. For more details, please refer to *Antibodies* (Harlow and Lane, 1988).

1. Prepare a lysis buffer of your choice. 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 μ M leupeptin, 1-10 mM EDTA, 1 μ M pepstatin, and 0.2-1 mM PMSF (from 100 mM stock in 2-propanol).
2. Prepare cell lysate using the appropriate lysis buffer. For mammalian and insect cells, use approximately 5×10^6 to 1×10^7 cells/ml of lysis buffer. For *E. coli* and yeast cells, use approximately 1×10^9 cells/ml of lysis buffer.
3. Centrifuge the lysate for 20 minutes at 10,000 x g at +4°C.
4. Carefully transfer the supernatant to a sterile microcentrifuge tube and place on ice.
5. Add 50 μ l 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 μ g (typically, 1-2 μ l) of the Anti-Xpress™ or Anti-Xpress™-HRP Antibodies and 50 μ l of the Protein-G Sepharose® resin slurry to the supernatant. Incubate the tube for 2-24 hours at +4°C with gentle rocking on a rocking platform.
10. Centrifuge for 1 minute at 10,000 x g at +4°C.
11. Remove the supernatant.
12. Wash the resin twice with 500 μ l of lysis buffer.
13. The fusion protein immune complexes may now be used in the appropriate assay.
14. For SDS polyacrylamide gels, add 50 μ l 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 the supernatant onto an appropriate gel.

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

Technical Service

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5. When you are finished entering information, click the 'Submit' button. Your MSDS will be sent within 24 hours.

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Technical Service, Continued

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- 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.
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