GE Healthcare

Anti-E Tag Antibody

for the detection of the peptide tag ('E-tag') in the Recombinant Phage Antibody System (RPAS)

Product Booklet

Code: 27-9412-01

Page Finder

1. Legal

http//www.gehealthcare.com/lifesciences

GE Healthcare UK Limited.
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2. Handling

2.1 Safety warnings and precautions

Warning: *For research use only*. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.

2.2 Storage

Stored at 2–8°C.

2.3. Expiry

For expiry details see outer packaging.

3. Components

Anti-E Tag Antibody
27-9412-01, 1 mg

4. Other materials and equipment required

For immunoassays

- **Anti-mouse conjugated antibody**, whole molecule or Fc-specific (HRP- or AP- conjugated).
- **6 x Sample loading buffer**
  To 1 ml of distilled water, add the following: 0.625 ml of 0.5 M Tris-HCl (pH 8), 0.25 ml of β-Mercaptoethanol (bME), 0.3 g of Sodium Dodecyl Sulphate (SDS), 2 ml of Glycerol and 2.5 mg of Bromophenol Blue. Mix to dissolve. Adjust the volume to 5 ml with distilled water.
- **1 x PBS**
  To 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4, add distilled water to a total volume of 1 litre. Adjust the pH to 7.4 with NaOH and autoclave.
- **1 x PBST**
  PBS containing 0.05% Tween™20.
- **BB**
  PBS containing 10% nonfat dry milk.
- **BBT**
  Blocking buffer containing 0.05% Tween 20.
- **PBS/BBT**
  An equal volume mixture of PBS and BBT.
- **20% TCA**
  Prepare 100% Trichloroacetic Acid (TCA) by adding 227 ml of distilled water to a bottle of TCA containing 500 g. Allow to dissolve completely. Add 200 ml of 100% TCA to 800 ml of distilled water to make 1 litre of 20% TCA.

For anti-E tag affinity purification

- **5 ml Protein G column or slurry** (HiTrap Protein G, GE Healthcare code 17-0405-01 or Protein G Sepharose 4 Fast Flow, GE Healthcare code 17-0618-01).
- **20 mM Sodium Phosphate pH 8.2**
  Prepare a 100 mM solution of NaH_2PO_4 and a 100 mM solution of Na_2HPO_4. Mix the two solutions until a pH of 8.2 is achieved. Dilute to 20 mM and filter using a 0.22 µm filter.
- **0.2 M Triethanolamine pH 8.2**
  Add 2.7 ml of triethanolamine (98%, Aldrich catalogue number TS-830-0, F.W. =149.19, d=1.124) to approximately 90 ml of distilled water. Mix well. pH to 8.2 with HCl. Adjust to 100 ml final volume with distilled water.
- **0.1 M Ethanolamine, pH 8.2**
  Add 0.61 ml of Ethanolamine (99%, Aldrich catalogue number 11 016-7, F.W. 61.08, d=1.012) to approximately 90 ml of distilled water. Mix well. pH to 8.2 with HCl. Adjust to 100 ml final volume with distilled water.
- **0.1 M Glycine, pH 5.0**
  Prepare a 2 M stock of Glycine (free base, F.W. 75.07) by dissolving 15 g in a final volume of 100 ml of distilled water. For each 100 ml of 0.1 M Glycine, add 5 ml of 2 M stock to approximately 85 ml of distilled water. pH to 5.0 with HCl. Adjust to 100 ml final volume with distilled water.
- **0.1 M Glycine, pH 2.8**
  For each 100 ml of 0.1 M Glycine, add 5 ml of 2 M stock to approximately 85 ml of distilled water. pH to 2.8 with HCl. Adjust to 100 ml final volume with distilled water.
- **Dimethyl Pimelimidate Dihydrochloride** (Aldrich catalogue number 36 034-1).
- **2.0 M Tris-HCl, pH 8.0**

5. Description

Anti-E Tag Antibody is an integral part of the Recombinant Phage Antibody System (RPAS). The Anti-E Tag Antibody is used to detect the peptide tag ('E-tag') located at the carboxyl terminus of single-chain fragment variable (ScFv) recombinant antibodies expressed using the pCANTAB 5 E vector. A secondary conjugated antibody (not provided) is necessary to generate a signal in an immunoassay.

Anti-E Tag Antibody may also be used to purify soluble recombinant antibodies from *E. coli* proteins through affinity chromatography. Purified antibodies may be used directly in a variety of immunological procedures.

This booklet provides protocols for the detection of soluble antibodies in different cellular preparations, guidelines for use of the Anti-E Tag Antibody in Westerns, ELISAs, and dot blots, and recommendations for purification of soluble antibodies using

Anti-E Tag Antibody affinity or hydrophobic interaction chromatography (HIC).

6. Overview

Detection of soluble antibodies in supernatant, periplasmic extract, and whole cell extract

Once the supernatant, periplasmic extract, and whole cell extract have been generated from a soluble antibody preparation (refer to Procedure 11 in the Expression Module of the Recombinant Phage Antibody System, 27-9401-01), the three fractions are analysed by Western blot to verify that soluble antibodies are being produced and to determine in which fraction(s) the soluble antibodies are concentrated. The location of soluble antibodies is expected to vary depending on the clone. The yield of soluble antibodies may also vary widely with different clones.

Note: Analysis of the whole cell extract is for diagnostic purposes only, i.e. to determine if soluble antibodies are being produced but not exported to the periplasm or supernatant. Antibodies present in the cytoplasm may not be folded properly for antigen binding.

Samples of each fraction are run on a denaturing polyacrylamide gel, proteins from the gel are transferred to nitrocellulose or other membrane, and the blot is incubated with Anti-E Tag Antibody. A secondary antibody that is conjugated to an enzyme [e.g. alkaline phosphatase (AP) or horseradish peroxidase (HRP)] is then used to detect the bound anti-E tag antibody. Detection occurs following the addition of the appropriate colorimetric or chemiluminescent detection reagents.

Immunoassays using soluble antibodies to detect the antigen

Once the production of soluble antibodies is verified and the location(s) of the soluble antibodies has been determined, the supernatant and/or periplasmic extract may be used directly in an immunoassay with the desired antigen. Alternatively, the soluble antibodies may be purified from *E. coli* proteins (see overleaf).

Recommendations for use of soluble recombinant antibodies in enzyme-linked immunosorbent assays (ELISAs), Western blots, and dot blots are provided. Soluble antibodies may require concentration for use in immunoassays. See page 22.

ELISAs are quantitative assays performed to examine specific binding of soluble antibodies to native antigen. Dot blots are performed for qualitative determination of antibody binding to native antigen. Western blots are performed to examine specific binding of antibodies to denatured antigen.

Purification of soluble recombinant antibodies

Purification of soluble antibodies may be performed if desired. For some immunoassays, purification may not be necessary unless components of the *E. coli* extract interfere with the immunoassay.

Protocols are provided for the preparation and use of Anti-E Tag Antibody affinity medium and suggestions are given for the use of hydrophobic interaction chromatography (HIC) to purify soluble recombinant antibodies. Since HIC does not rely upon specific antigen-antibody interactions as affinity chromatography does, purification of soluble antibodies using HIC will require customization for each antibody preparation.

Affinity purification using the antigen is another option for purification of soluble recombinant antibodies. We recommend HiTrap™ NHS-activated affinity columns (GE Healthcare code 17-0716-01 or 17-0717-01) for covalent coupling of antigens to a solid support.

7. Quality control

The Anti-E Tag Antibody is tested using Western blotting for its ability to detect an ScFv containing the E-tag peptide.

8. Protocol

8.1. Detection of soluble antibodies in supernatant, peri-plasmic extract and whole cell extract by Western blot

Soluble recombinant antibodies may be located in any one of the cellular fractions or in all three. However, soluble antibodies located in the cytoplasm may not be biologically functional. Western blot analysis of all three fractions is recommended to verify that soluble antibodies are being produced and in which cellular fraction(s) the soluble antibodies are concentrated. Below are some general protocols to follow. We recommend loading a range of volumes of each cellular fraction on to the gel. If a signal is not visible, TCA precipitation may be necessary to concentrate the proteins prior to loading onto the gel (see page 25).

Note: Antibody preparations that have been TCA-precipitated will not possess functional activity and cannot be used for immunoassays.

- For a standard-sized polyacrylamide gel, we recommend a range of sample volumes from 3 µl to 20 µl. If PhastGel™ is used, scale volumes down as appropriate. Add 1/6 volume of sample loading buffer (see page 6) to each sample from the supernatant, periplasmic extract, and whole cell extract. Loading of a molecular weight marker (e.g. LMW Molecular Weight Markers, GE Healthcare code 17-0446-01) is recommended. Heat samples for 10 minutes at 95–100°C prior to loading.

Electrophoresis and protein transfer may be accomplished using a variety of equipment and reagents (1–3). Using PhastSystem™ Separation-Control and Development Unit 120 VAC (18-1018-23) and PhastTransfer™ Kit(18-1001-23) from GE Healthcare, protein blots may be prepared in less than an hour (3).

- Load each sample into a separate well of an SDS-polyacrylamide

gel (e.g. 10–12.5% homogeneous gel) and electrophorese in buffer containing SDS (1) until the Bromophenol Blue in the sample loading buffer reaches the lower edge of the gel.

- Following electrophoresis, transfer the proteins from the gel to nitrocellulose or other membrane (4).
- When the transfer is complete, block the membrane with blocking buffer (BB – see page 6) for 1–2 hours at room temperature. Blocking can also be done overnight at 2–8°C.
- Dilute only the amount of Anti-E Tag Antibody that is required for the assay. Dilute the appropriate amount of Anti-E Tag Antibody (supplied at 1 mg/ml) to 8 µg/ml (i.e. 80 µl in 10 ml final volume) using blocking buffer with Tween™ (BBT – see page 6). Use at least 10 ml of diluted antibody for a 50 cm^2 blot. Incubate at room temperature on a shaker table for 1 hour.
- Wash the membrane once in PBST (see page 6) and four times with PBS, followed by a 5-minute soak in PBS. Drain the blot.
- Dilute an HRP- or AP-conjugated antibody (Anti-Mouse IgG, whole molecule or Fc-specific, not supplied) according to the manufacturer's instructions in 10 ml of BBT. Add to the blot and incubate at room temperature on a shaker table for 45–60 minutes.
- Wash the membrane once with PBST and four times with PBS, followed by a 5-minute soak in PBS. Drain the blot.
- Add an appropriate substrate for HRP or AP and develop the blot.

Note: Either colorimetric or chemiluminescent substrates can be used. The reagents in the Detection Module of the Recombinant Phage Antibody System (27-9402-01) cannot be used as a substrate for HRP in a Western blot since it will not form a precipitate on the membrane.

- If soluble antibodies are present, a band at ~30 kDa will be apparent.

Note: Some breakdown products may also be visible.

Once the production of soluble antibodies has been verified and the location(s) of the soluble antibodies has been determined, scale up the production of soluble antibodies to generate sufficient material for further evaluation/purification. The protocol provided in the Expression Module (Procedure 11) can be scaled up directly.

Immunoassays may be performed to determine the ability of the soluble antibodies produced to recognize the antigen of interest. Options include ELISAs, Westerns, and dot blots. Any or all of these assays may be performed in any order.

Note: Results of assays in which the antigen is presented in a native form (ELISAs and dot blots) versus those in which the antigen is presented in a denatured form (Western blots) may differ and may depend upon the nature of the epitope recognized by the antibody.

8.2. ELISAs

The amount of coating antigen for an ELISA should be optimized. The amount of antigen must be determined empirically and will depend upon the nature of the antigen, the purity of the antigen, and the nature of the specific antibody used. The optimal amount of antigen per well should be between 1 µg and 100 µg in a volume of equal to or greater than 100 µl when diluted (see below – the exact volume used will depend upon the capacity of the wells). Antigens that are similar biochemically to the antigen of interest should also be tested with the soluble antibody preparation to determine specificity.

- Dilute the antigen in a buffer that is appropriate for that antigen (PBS, for example). Dilute to a concentration that will yield the optimal amount of antigen/well. Coat wells of a 96-well microplate with the diluted antigen for 1–2 hours at room temperature or 37°C, or at 2–8°C overnight in a humidified container or incubator to prevent evaporation.

Note: We recommend polyvinyl microplates.

The volumes used in all following steps except for blocking should equal the volume of antigen (e.g. 50–100 µl) used to coat the wells.

- Empty the contents of the microtitre wells by inverting the plate and flicking the well contents into a suitable container. Tap the inverted plate on a paper towel to remove any liquid remaining in the wells.
- Prepare BB. Fill each well with BB. Block for 1 hour at room temperature or overnight at 2–8°C.
- Empty the contents of the microplate and blot it on a paper towel as before.
- In a separate container, dilute at least 50 µl/well of soluble antibodies with an equal volume of BBT for a total volume of at least 100 µl per well. Mix and incubate for 10 minutes at room temperature. Add diluted soluble antibodies to the wells.

Note: The appropriate amount of soluble antibodies to use in an ELISA must be determined empirically. If the volume of soluble antibodies used is not sufficient to generate an acceptable signal, a larger volume may need to be used or the preparation may require concentration via filtration prior to use. We recommend using a filter with a 10 kDa cut-off. Do not concentrate using TCA since antibodies will not retain functionality.

- Incubate at room temperature, or at 37°C in a humidified incubator, for 1–2 hours.
- Empty the contents of the microplate and blot it on a paper towel as before.
- To wash the wells, fill each well with PBST. Empty the wells. Repeat twice for a total of three washes.
- Empty the contents of the microplate and blot it as before.
- Dilute only the amount of Anti-E Tag Antibody that is required for

the assay. Dilute the Anti-E Tag Antibody to 1 µg/ml (1 µl for every 1 ml of buffer) in an equal volume of PBS/BBT (see page 6). Add the appropriate volume to each well, i.e. the same volume as the volume of antigen originally used to coat each well. Incubate at room temperature or at 37°C in a humidified incubator for 1–2 hours.

- Empty the contents of the microplate and blot it on a paper towel as before.
- To wash the wells, fill each well with PBST. Empty the wells. Repeat twice for a total of three washes.
- Empty the contents of the microplate and blot it on a paper towel as before.
- Dilute the conjugated antibody (not supplied) in BBT, according to the manufacturer's instructions. Add the appropriate volume to each well. Incubate for 1 hour at room temperature, or at 37°C in a humidified incubator.
- Empty the contents of the microplate and blot it on a paper towel as before.
- To wash the wells, fill each well with PBST. Empty the wells. Repeat twice for a total of three washes.
- Empty the contents of the microplate and blot it on a paper towel as before.
- Detect using the appropriate substrate for HRP or AP.

8.3. Dot blots/Western blots

Dot blots

Use 0.1–1.0 µg of antigen in a volume of 1–2 µl, diluted in an appropriate buffer such as PBS, per spot in a dot blot. Spot on to nitrocellulose or other membrane. We recommend also spotting related antigen(s) to help determine the specificity of binding of the

soluble antibodies. In addition, prepare a second dot blot to verify that incubation using the original hybridoma supernatant, ascites fluid or serum will give a positive signal against the antigen. Allow the dots to dry completely after the antigen has been spotted on to the nitrocellulose.

- Block the membranes in separate containers with BB for 1–2 hours at room temperature or overnight at 2–8°C.

Note: Do not block for more than 24 hours or antigen may be displaced.

- In a separate container, dilute 5–100 ml of supernatant or at least 5 ml of periplasmic extract with an equal volume of BBT. Mix and incubate at room temperature for 10 minutes.
- Add the entire volume of the diluted soluble antibodies to the first blot and incubate on a shaker table for 1–2 hours at room temperature.
- For the positive control, incubate the second blot with hybridoma supernatant, ascites fluid or serum.
- Wash the membranes once with PBST and four times with PBS, followed by a 5-minute soak in PBS. Drain the blots.
- For the first blot, dilute only the amount of Anti-E Tag Antibody that is required for the assay. Dilute the Anti-E Tag Antibody to 8 µg/ml (80 µl into 10 ml final volume) in 10 ml of PBS/BBT. Add to the blot and incubate on a shaker table for 1 hour at room temperature.
- Do not add Anti-E Tag Antibody to the second (positive control) blot.
- Wash the first blot once with PBST and four times with PBS, followed by a 5-minute soak in PBS. Drain the blot.
- For the first blot, dilute the conjugated antibody (not supplied) according to the manufacturer's instructions (dilute only the amount required for the assay) in 10 ml of PBS/BBT. Add to the first blot. Incubate on a shaker table for 1 hour at room temperature.

- For the second blot, dilute a conjugated antibody specific for the antibody used in the positive control according to the manufacturer's instructions. Dilute in 10 ml of PBS/BBT. Add to the second blot. Incubate on a shaker table for 1 hour at room temperature.
- Wash both membranes once with PBST and four times with PBS, followed by a 5-minute soak in PBS. Drain the blots.
- Detect using an appropriate colorimetric or chemiluminescent substrate for HRP or AP.

Western blots

- Load 1–2 µg of antigen into each well of a standard gel (1 x 3 mm wells) and electrophorese according to the manufacturer's instructions (see page 12).

Note: For a positive control, prepare duplicate gels and transfers or cut strips from the membrane and incubate with the hybridoma supernatant, ascites fluid, or serum.

- Following electrophoresis, transfer the proteins from the gel to nitrocellulose.
- Block and develop the blot according to the instructions provided for dot blots.

8.4. Preparation of anti-E tag antibody affinity medium

Soluble recombinant antibodies can be purified in a single step using Anti-E Tag Antibody affinity medium, eliminating the need for processes such as precipitation, dialysis and gel filtration chromatography. In this procedure, a soluble antibody preparation is allowed to interact with Anti-E Tag Antibody that has been covalently coupled to a solid support (e.g. Protein G Sepharose™, GE Healthcare code 17-0405-01 or 17-0618-01). After the solid support has been

washed, bound soluble antibodies are eluted with a drop in pH.

Protein G is the matrix of choice for binding the Anti-E Tag Antibody. Protein G has been shown to react more strongly with antibodies in the IgG1 subclass (to which the Anti-E Tag Antibody belongs) than Protein A.

In this protocol, the Anti-E Tag Antibody will be irreversibly bound to immobilized Protein G using dimethyl pimelimidate dihydrochloride. See pages 6–7 for materials and reagents required for the preparation and use of Anti-E Tag Antibody affinity medium.

Preparation of Anti-E Tag Affinity Column

Allow all reagents to equilibrate to room temperature.

We recommend that 5 mg of Anti-E Tag Antibody (supplied at 1 mg/ml) be used for coupling to 3–5 ml of swollen gel.

Note: Anti-E Tag Antibody, which belongs to the IgG1 subclass, will not bind as efficiently to the Protein G as antibodies from other subclasses (5). Do not alter the ratio of Anti-E Tag Antibody to be coupled to Protein G resin.

- Prepare the Protein G matrix for coupling with Anti-E Tag Antibody. We recommend HiTrap Protein G (17-0405-01) or Protein G Sepharose 4 FF (17-0618-01). The HiTrap Protein G column requires the use of a pump or syringe and cannot be used in a batch format. The Protein G Sepharose 4 FF is supplied as a bulk resin. If using the bulk Protein G Sepharose 4 FF, a column with approximately a 10 ml capacity must be purchased separately.

Note: Dried gel or pre-swollen slurries can also be purchased from other suppliers. Be sure to purchase resin with at least 2 mg of protein G coupled to each 1 ml of swollen gel. Follow the manufacturer's instructions for preparing the swollen gel.

- Equilibrate the gel matrix using three bed volumes of 20 mM sodium phosphate, pH 8.2.

- Pour the equilibrated gel matrix into a disposable column if using bulk resin or use a HiTrap. Allow the gel to settle while the remainder of the buffer passes through the column.
- Cap the bottom of the column so that the matrix will not dry out.

Note: Do not allow the matrix to dry out at any time during the procedure.

- Dilute 5 mg (5 ml) of Anti-E Tag Antibody with 5 ml of 20 mM Sodium Phosphate, pH 8.2.
- Add the diluted antibody to the gel matrix in the column. Cover the top of the column with Parafilm™ if the column does not have its own cap (closure) and mix by inversion.
- Incubate for 1–2 hours at room temperature with continuous gentle mixing by inversion.
- Remove the top and bottom closures and allow the gel to settle while the diluted antibody passes through the column using a syringe, a pump, or gravity.
- Wash the column with six bed volumes of 20 mM Sodium Phosphate, pH 8.2.
- Cap the bottom of the column before the gel dries out.

Covalently crosslink the Anti-E Tag Antibody to the column as follows:

- Carefully weigh out 40 mg of Dimethyl Pimelimidate Dihydrochloride (DMP) and dissolve in 10 ml of 0.2 M Triethanolamine, pH 8.2.

Note: DMP is moisture sensitive. This solution must be prepared fresh immediately before each use. Once opened, store the container of DMP with desiccant.

- Immediately after preparation of the DMP solution, add 10 ml of this crosslinking solution to the column and resuspend the gel matrix. Replace the top closure. Mix by gently inverting the column

several times.

- Incubate for 1 hour at room temperature with continuous gentle mixing by inversion.
- Remove the top and bottom closures and allow the gel to settle while the antibody solution passes through the column using a syringe, a pump, or gravity.
- Wash the column with six bed volumes of 0.2 M Triethanolamine, pH 8.2.
- Cap the bottom of the column before the gel dries out.
- Add two bed volumes of 0.1 M Ethanolamine, pH 8.2 to block any remaining reactive sites.
- Replace the top closure. Mix continuously by gentle inversion for 15 minutes.
- Wash the column with three bed volumes of 0.1 M Glycine, pH 2.8 to remove any Anti-E Tag Antibody that is not covalently bound.
- Wash the column with six bed volumes of 20 mM Sodium Phosphate, pH 8.2 to return the column to a neutral pH.
- Replace the bottom cap and add buffer to cover the gel and ensure that it will not dry out during storage. Replace the top cap and store at 2–8°C until use.
- If the column will not be used for > 1 month, 0.01% Thimerosal can be added as a preservative.

Note: The column can be used at least 5–10 times. To avoid cross-contamination, a new column should be prepared for purification of different antibodies.

8.5. Use of anti-E tag antibody affinity medium

Before loading the soluble antibody preparation onto the affinity column, the preparation should be filtered. This will prevent the column from becoming clogged with cell debris or other particulates.

The periplasmic extract can be filtered through a 0.8 µm or 0.45 µm filter. If the extract is extremely viscous, dilute it with PBS. A 0.22 µm filter with a pre-filter is suitable for clarifying the supernatant fraction. To purify a large volume of supernatant it may be advantageous to concentrate the antibody by ultrafiltration using a filter with a 10 kDa cut-off (e.g. Filtron Brand Minisette™). Do not concentrate using TCA.

Purify the soluble antibodies at room temperature. Equilibrate all solutions and supernatant or periplasmic extract to room temperature.

- Equilibrate the column with approximately 10 bed volumes of PBS. The column may be equilibrated by gravity or by pump or syringe at a flow rate of 1 ml/minute. When the column has stopped dripping, replace the bottom closure to prevent the gel matrix from drying out.

Soluble antibodies may be purified using either a column or a batch method. If a HiTrap column is used, however, the column method must be performed.

Column method

- Pass the supernatant or periplasmic extract over the Anti-E Tag Antibody affinity column with a flow rate of approximately 1 ml/minute.
- When all of the antibody solution has passed through the column, wash with 5–10 bed volumes of PBS. Do not allow the gel matrix to dry out.
- Wash the column with 3–5 bed volumes of 0.1 M Glycine, pH 5.0.
- Proceed with elution as described on page 23.

Batch method

- Resuspend the gel matrix containing the coupled Anti-E Tag Antibody and transfer from the column to the sample containing the soluble antibodies.

- Allow binding to occur for 30–60 minutes at room temperature with continuous gentle mixing.
- Pass the entire antibody-gel solution through the column and allow the gel to settle. Rinse the container 2–3 times to ensure that all of the gel is recovered.
- Wash the column with 5–10 bed volumes of PBS.
- Wash the column with 3–5 bed volumes of 0.1 M Glycine, pH 5.0.
- Proceed with elution as described below.

Elution of soluble ScFv antibodies

- Elute the bound antibodies with 5–10 bed volumes of 0.1 M Glycine, pH 2.8. Collect 1.5 ml fractions in 2.0 ml microcentrifuge tubes containing 175 µl of 2.0 M Tris-HCl, pH 8.0 and mix well. Using litmus paper, verify that the pH of the fractions is approximately 7.0–8.0.

The purified soluble antibodies can be used directly in most applications.

A dot blot or Western blot can be used to determine which fractions contain purified antibodies.

- Regenerate the column by adding two bed volumes of 0.1 M Glycine, pH 2.8, then neutralize immediately with two bed volumes of 2.0 M Tris-HCl, pH 8.0. Wash the column extensively with PBS and store at 2–8°C. (If the column will not be used for >1 month, 0.01% Thimerosal can be added as a preservative.)

Note: The column can be used at least 5–10 times. To avoid cross-contamination, a new column should be prepared for purification of different antibodies.

9. Additional information

9.1. TCA precipitation

If soluble antibody fractions are too dilute to visualize on a Western blot, it may be necessary to concentrate a portion of the sample prior to dot blotting or gel analysis.

Note: Antibodies precipitated with Trichloroacetic Acid (TCA) will not be functional for antigen binding.

- To precipitate, add an equal volume of ice-cold 20% TCA (see page 7) to the sample and mix well. Incubate on ice for 20 minutes.
- Centrifuge for 10 minutes in a microcentrifuge at full speed.
- Remove the supernatant and discard. Resuspend the pellet, which may be difficult to visualize, in sample loading dye. The sample will be acidic. Neutralize by adding 1 grain of Trizma™ base.

9.2. Hydrophobic interaction chromatography

Hydrophobic Interaction Chromatography (HIC) is an alternative to affinity purification for purifying soluble recombinant antibodies. However, it is less straight-forward than affinity chromatography since it is not based on specific antigen-antibody interactions, and may require some optimization. HIC is based on the interaction of hydrophobic residues of proteins with a hydrophobic matrix.
To purify soluble recombinant antibodies using HIC, Ammonium Sulphate is added to the crude periplasmic extract to a final concentration of 1.7 M. The sample is centrifuged to remove insoluble material and the supernatant is loaded on to a column, consisting of a hydrophobic matrix, which is equilibrated in buffer containing 1.7 M Ammonium Sulphate. The Ammonium Sulphate helps to maximize the hydrophobic interaction of the proteins to the resin. As the amount of Ammonium Sulphate in the buffer is gradually decreased in a linear gradient, the least hydrophobic proteins begin to elute from

the matrix. Proteins with a higher hydrophobicity will elute from the matrix as the concentration of Ammonium Sulphate is lowered. As an example of HIC purification of a soluble recombinant antibody preparation, a periplasmic extract of TEL9 (a recombinant antibody specific for turkey egg white lysozyme) was purified using FPLC™ on a Phenyl Superose™ HR10/10 column with a bed volume of approximately 8 ml (GE Healthcare code 17-0530-01).

Five ml of periplasmic extract was prepared from 250 ml of sedimented cells.

- The column was equilibrated with buffer A (20 mM Potassium Phosphate, pH 7.4 containing 1.7 M Ammonium Phosphate).
- Ammonium Sulphate (660 mg) was added to the 5 ml of crude periplasmic extract, mixed to dissolve, and incubated for 10 minutes on ice.
- The precipitate was removed by centrifugation at 10 000 x g and the supernatant was loaded on to the column.
- The column was washed with buffer A and the sample was eluted with a linear gradient (0-100%) of buffer B (20 mM Potassium Phosphate buffer, pH 7.4).
- Fractions of 1.5 ml each were collected.
- Aliquots of each fraction were spotted on to nitrocellulose.
- A dot blot assay was performed according to protocol 3, page 16.
- Fractions that gave a positive reaction in the dot blot were run on SDS-PAGE and analysed by Western blot to determine antibody purity.
- Antibody-containing fractions were then dialyzed against PBS to remove Ammonium Sulphate and analysed in an ELISA against the antigen.

Buffer recipes

- Buffer A: Add 224.6 g of Ammonium Sulphate to buffer B to a final

volume of 1 litre.

- Buffer B: Prepare a 100 mM solution of KH_2PO_4 and a 100 mM solution of K_2HPO_4. Mix the two solutions until a pH of 8.2 is achieved. Dilute to 20 mM and filter using a 0.22 µm filter.

10. References

1. Towbin, H. H. *et al., Proc. Natl. Acad. Sci.* USA **76**, 4350 (1979).

2. Tsang, V.C. W. *et al., Methods Enzymol.* **92**, 377 (1983).

3. PhastSystem Manual, Amersham Biosciences.

4. Sambrook, J., Fritsch, E. F., and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, second edition (1989).

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

11. Related products

Mouse ScFv Module	
5 reactions	27-9400-01
Expression Module	
1 kit	27-9401-01
HiTrap NHS-activated	
1 ml	17-0716-01
5 ml	17-0717-01

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