GE Healthcare

HRP/Anti-E Tag Conjugate

for the **direct** immunodetection of E-tagged recombinant ScFv generated with the Recombinant Phage Antibody System (RPAS)

Product Booklet

Code: 27-9413-01

Page finder

1. Legal

http//www.gehealthcare.com/lifesciences

GE Healthcare UK Limited.
Amersham Place, Little Chalfont,
Buckinghamshire, HP7 9NA UK

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

Store at -15°C to -30°C for long-term storage.

May be stored at -70°C for short periods. Avoid multiple freeze-thaw cycles.

2.3. Stability

The expiry date is printed on the outer packaging.

2.4. Quality control

HRP/Anti-E Tag Conjugate is tested in an ELISA and by Western blot analysis for its ability to detect the E-tag on a recombinant ScFv.

3. Other materials required

All reagents and media should be prepared using distilled, deionized water.

Equipment

Centrifuge equipped with adaptors for microplates

Multi-channel pipette and tips

Dispensing tray to accommodate multi-channel pipette

Polyvinyl, polystyrene and V-bottom polypropylene microplates

Buffers and solutions

10x PBS

While stirring, add 700 ml of water, 80 g of NaCl, 2.0 g of KCl, 14.4 g of Na_2PO_4 and 2.4 g of KH_2PO_4 to a 1 litre graduated cylinder. Add water to a total volume of 1 litre and mix to dissolve. 10x PBS can be sterilized by autoclaving; however, for most of the HRP/Anti-E Tag Conjugate applications, this will not be necessary.

1x PBS

To 100 ml of 10 x PBS, add water to a total volume of 1 litre and mix well. The pH should be ~7.2-7.4. If it is not within this range, adjust with either HCl or NaOH.

PBS + 1 mM EDTA

Prepare 50 ml of 1 x PBS and add 100 µl of 0.5 M EDTA. Mix well.

0.5 M EDTA

Add 14.6 g of EDTA (MW 292.2) to 60 ml of water and mix well. Adjust the pH to 8.0. Add water to a total volume of 100 ml.

20% Glycerol

Mix 20 ml of Glycerol with 80 ml of water. Filter sterilize and store at room temperature.

0.05 M Carbonate buffer
(pH 9.6)
Buffer A: Add 5.3 g of Na_2CO_3 to 900 ml of water and stir until dissolved. Add water to a total volume of 1 litre.

Buffer B: Add 4.2 g of $NaHCO_3$ to 900 ml of water and stir until dissolved. Add water to a total volume of 1 litre.

Titrate Buffer A with Buffer B until a pH of 9.6 is reached. Store at room temperature in a sealed container. Prepare weekly.

Blocking buffer (1x PBS containing 3% non-fat dry milk)
Add 30 g of non-fat dry milk powder to 900 ml of 1x PBS and stir until dissolved. Adjust the final volume to 1 litre with 1x PBS.

Wash buffer (PBS containing 0.05% Tween 20)
Mix 400 ml of 10x PBS and 3 600 ml of water. Add 2 ml of Tween™ 20 and mix well.

Triethanolamine buffered saline
Add 7.5 g of NaCl and 2.8 ml of Triethanolamine to 800 ml of water. Adjust the pH to 7.5 with concentrated HCl (~1.1 ml), then add water to a total volume of 1 litre.

Note: Tris buffered saline can be used in place of Triethanolamine buffered saline. To prepare Tris buffered saline, dissolve 8.77 g of NaCl in 800 ml of 10 mM Tris-HCl buffer (pH 7.5). Add buffer to 1 litre and mix well.

4-Chloro-1-Naphthol substrate
To prepare a stock solution of 4-CN, dissolve one 4-CN tablet (30 mg of 4-CN/tablet; Sigma catalogue number C-6788) in 10 ml of Methanol.

To prepare 12 ml of substrate for use, add 2 ml of the 4-CN stock solution and 5 µl of 30% H_2O_2 to 10 ml of Triethanolamine buffered saline and mix well. Use within 5–10 minutes.

Note: Prepare 12 ml of 4-CN substrate per 50 cm^2 of nitrocellulose.

0.05 M Citric Acid (pH 4.0)
Dissolve 10.507 g of Citric Acid monohydrate (MW 210.14) in 1 litre of water. Adjust to pH 4.0 with 10 N NaOH (~6 ml).

ABTS
Dissolve 10 mg of ABTS™ in 45 ml of 0.05 M Citric Acid (pH 4.0). Filter sterilize, then store at 2–8°C. Immediately prior to use, add 36 µl of 30% H_2O_2 to 21 ml of ABTS.

6x Loading buffer
To 9 ml of 1 M Tris-HCl (pH 6.8), add 4.625 g of Dithiothreitol, 3.6 g of SDS and 1.8 g of Bromophenol Blue. Mix well, then add 18 ml of Glycerol. Adjust the volume to 50 ml with water.

Media
20% Glucose
Dissolve 100 g of D-(+)-Glucose (anhydrous) in 300 ml of distilled water. Add water to a final volume of 500 ml. Filter sterilize through a 0.2 µm filter.

50 mg/ml Ampicillin
Dissolve 0.5 g of Ampicillin (e. g. Sigma catalogue number A-9393) in 10 ml of distilled water. Filter sterilize through a 0.2 µm filter. Store at -15°C to -30°C.

1 M IPTG
Dissolve 2.38 g of IPTG in 7 ml of water. Adjust the volume to 10 ml with distilled water, then filter sterilize through a 0.2 µm filter. Store at -15°C to -30°C.

SB medium
Dissolve 35 g of Bacto-tryptone, 20 g of Bacto-yeast extract and 5 g of NaCl in 900 ml of distilled water. Adjust to pH 7.5 with NaOH. Add water to a final volume of 1 litre. Autoclave to sterilize.

SB-AG medium
SB medium containing
100 µg/ml Ampicillin and 2% Glucose. For plates, prepare SB medium, add 15 g of Bacto-agar, then autoclave. Allow the medium to cool to 50–60°C, then add Ampicillin and Glucose, mix well, and pour.

SB-AI medium
SB medium containing
100 µg/ml Ampicillin and 1 mM IPTG. For plates, prepare SB medium, add 15 g of Bacto-agar, then autoclave. Allow the medium to cool to 50–60°C, then add Ampicillin and IPTG, mix well, and pour.

2 x YT medium
Dissolve 17 g of Bacto-tryptone, 10 g of Bacto-yeast extract and 5 g of NaCl in 900 ml of distilled water. Add water to a final volume of 1 litre. Autoclave to sterilize.

2 x YT-AG medium
2 x YT medium containing 100 µg/ml Ampicillin and 2% Glucose. For plates, prepare 2 x YT medium, add 15 g of Bacto-agar, then autoclave. Allow the medium to cool to 50–60°C, then add Ampicillin and Glucose, mix well, and pour.

2 x YT-AI medium
2 x YT medium containing 100 µg/ml Ampicillin and 1 mM IPTG. For plates, prepare 2 x YT medium, add 15 g of bacto-agar, then autoclave. Allow the medium to cool to 50–60°C, then add Ampicillin and IPTG, mix well and pour.

4. Component

HRP/Anti-E Tag Conjugate 0.5 mg of antibody protein conjugated to HRP (sufficient for 50 ELISAs or 10 Western blots).

5. Critical parameters

- It is critical that an optimized ELISA protocol is developed prior to commencement of detection procedures.
- Always wear gloves and use forceps when handling nitrocellulose filters.
- It is important to use polypropylene microtitre plates for storage of periplasmic extracts as unpurified ScFv may adsorb to polyvinyl plastics.

6. Description

HRP/Anti-E Tag Conjugate is used for *direct* immunodetection of E-tagged recombinant ScFv generated with the Recombinant Phage Antibody System (RPAS). The reagent was developed by conjugating horseradish peroxidase to mouse monoclonal Anti-E Tag Antibody. This monoclonal has previously served as the 'secondary' antibody in immunoassays designed to detect ScFv displaying the 'E-tag' peptide. In this scenario, since the resulting antigen/ScFv/Anti-E Tag Antibody complex is unlabelled, it can only be detected with an anti-mouse antibody conjugate. The need for this additional reagent, however, is eliminated if Anti-E Tag Antibody itself is labelled. The use of a direct detection format of this type not only streamlines the assay, but also avoids background problems which may occur with anti-mouse antibody conjugates.

7. Overview

In the Recombinant Phage Antibody System (RPAS), antibody ScFv (single chain fragment variable) genes (obtained using the Mouse ScFv Module) are cloned into the pCANTAB 5 E phagemid vector and expressed as soluble recombinant ScFv antibodies in *E. coli* (using the Expression Module). Although the location and concentration of ScFv will vary depending upon the specific antibody cloned and the prevailing culture conditions, in most cases the bacterial periplasmic extract will yield the highest concentration of functional ScFv antibodies.

HRP/Anti-E Tag Conjugate can be used as a reagent in enzyme-linked immunosorbent assays (ELISAs) to quickly identify E. *coli* colonies expressing soluble antigen-positive E-tagged ScFv. Because they can be quantitative, assays such as ELISAs provide efficient methods to assess ScFv levels and determine growth conditions for optimal expression. In other assays, such as Western blot analyses, the conjugate can be used to detect expressed ScFv in cell fractions or alternatively, to detect ScFv bound to antigen. Depending upon the antigen, HRP/Anti-E Tag Conjugate can also be used as a preliminary rapid screening reagent to detect positive clones within a large plate population of *E. coli* colonies using a colony lift adaptation.

8. Procedures to detect E-tagged ScFvs using HRP/Anti-E Tag Conjugate

8.1. ELISA

In the following ELISA procedure, HRP/Anti-E Tag Conjugate is used to detect E-tagged ScFvs (from periplasmic extract) bound to an antigen-coated microtitre well (Figure 1). Because ELISAs are quantitative in nature, the signal will vary with the expression level and affinity of the ScFvs. It is thus possible to correlate expression with culture conditions and ultimately define those conditions that lead to optimal ScFv expression. For example, expression levels can vary depending upon the formulation of the growth medium, concentration of IPTG inducer, incubation time and temperature, and even the type of container (glass or plastic) in which cultures are grown. Additionally, in some cases, cloned ScFvs are simply not stable in *E. coli*. The cumulative effect of all of these factors can be easily monitored in an ELISA using HRP/Anti-E Tag Conjugate.

Note: Prior to beginning this procedure, it is CRITICAL that an optimized ELISA protocol be developed. This is mandatory for any type of immunological work where samples must be screened for the presence of antibodies to a specific antigen. Failure to optimize the assay may lead to reduced sensitivity or even false positive results. A general protocol for optimizing an ELISA is given on page 33.

- Dilute the antigen in coating buffer (e. g. PBS or carbonate) to an appropriate concentration. In general, concentrations between 1 and 100 µg/ml in a volume of 50-200 µl should be evaluated for coating the wells. Prepare a diluted negative control antigen in similar fashion.

Note: See page 33, for information concerning assay optimization.

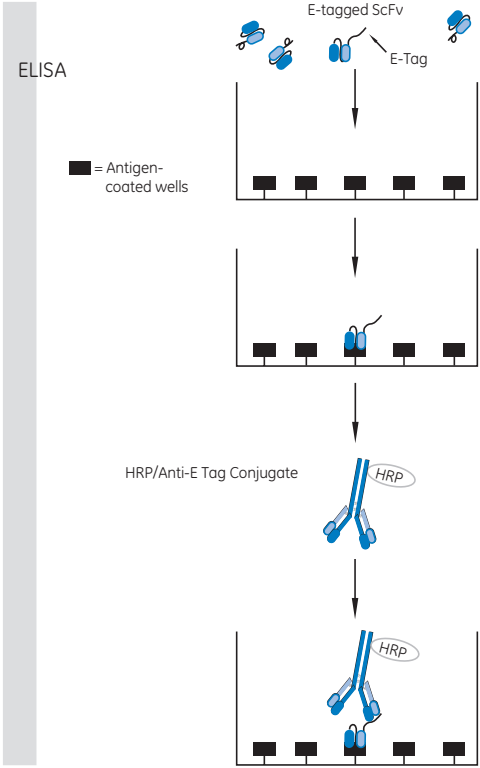

Figure 1. Schematic showing use of HRP/Anti-E Tag Conjugate to detect antigen-binding ScFvs in an ELISA.

- To coat the wells, add 50–200 µl of the diluted antigen or negative antigen control to each well of a polyvinyl microplate.

Note: Excluding the blocking step, the volume of each reagent added to wells in the microplate should be kept constant throughout the assay. For example, if 50 µl is chosen initially as the well volume, then this volume should be used for each additional reagent.

- Using a moist paper towel, wipe off the bottom of the microplate to remove any static charge on the surface.
- Incubate the plate in a humidified container (e. g. a plastic box containing wet paper towels) for at least 1 hour at room temperature.

Note: Plates may also be incubated for 1–2 hours at 37°C or overnight at 2–8°C in a humidified container. All of the following incubation steps should be performed at the same temperature to prevent spurious results due to well-to-well temperature fluctuations.

- Prepare blocking buffer and wash buffer as described on page 6.
- Empty the microplate by inverting and flicking the well contents into a suitable container. Blot the inverted plate onto a dry paper towel to remove any residual liquid in the wells.
- Fill each well with blocking buffer and incubate for 1 hour at room temperature.

Note: If plates are blocked for extended periods of time, the antigen may be displaced from the well surface by components in the blocking buffer.

- Empty the contents of the microplate and blot as before.

Note: For some stable antigens, plates can be washed once with wash buffer, tapped dry, and then stored dry at 2–8°C for several weeks. Prior to use, the plates should be washed once with wash buffer since subsequent reagents may nonspecifically adhere to dry wells.

- Dilute the periplasmic extract containing soluble recombinant E-tagged ScFvs with an equal volume of blocking buffer.

Note: The total volume of sample required will depend upon the well volume initially selected for the assay and the number of wells to which the diluted ScFv will be added.

- Add 50–200 µl of the diluted ScFv solution to a set of antigen-coated and negative antigen control wells.
- Incubate the plate for 1–3 hours at room temperature in a humidified container.

Note: The plate can be incubated at 37°C. However, if this temperature is chosen, all subsequent incubations should also be carried out at 37°C.

- Empty the contents of the microplate and blot as before.
- Wash the microplate by completely filling the wells with wash buffer and emptying. Repeat this step twice for a total of three washes. Blot any residual liquid as before.
- Dilute the HRP/Anti-E Tag Conjugate 1:8 000 in blocking buffer.

Note: It may be necessary to optimize the amount of HRP/Anti-E Tag Conjugate used to detect various recombinant ScFv antibodies. See page 33, for assay optimization.

- Add 50–200 µl of the diluted conjugate to each well.
- Incubate the plate for 1 hour at room temperature in a humidified container.
- Empty the contents of the microplate and blot as before.
- Wash the wells three times with wash buffer.
- Prepare the ABTS substrate as described on page 6. Be sure to add H_2O_2 to the solution. Add 50–200 µl of the substrate to each well.
- Incubate the plate for 20 minutes at room temperature.
- Read the absorbancy for each well at 410 nm.

Note: Weak ELISA signals may occur if the expression level or affinity of antigen-positive ScFvs is low, if the ScFvs fail to recognize the antigen, if the ScFvs have degraded, or if the ELISA is not optimized. In some cases, signals can be enhanced by incubating the plate overnight at 2–8°C in a humidified container. If a suitable colour reaction does not develop after this time, the ScFv concentration is below the detection limits of the assay.

8.2. Immune complex ELISA (ICELISA)

HRP/Anti-E Tag Conjugate has two paratopes (antigen-binding domains), each of which recognizes a single E-tag peptide displayed at the carboxy-terminus of an ScFv recombinant antibody. Each E-tagged ScFv also displays its own unique paratope which reacts with a specific antigen. Since the conjugate and ScFv are separate and distinct antibodies, their interactions with their respective antigens are independent of each other. When mixed with E-tagged ScFvs, the paratopes of the conjugate react with the ScFvs via the E-tag peptides to form an immune complex (IC) consisting of the conjugate and the ScFv (Figure 2). This IC can develop while the ScFv is reacting with its specific antigen in the ELISA – hence the acronym ICELISA. This approach eliminates the extra steps required if these reagents are used sequentially in the assay.

In the following protocol, antigen-reactive E-tagged ScFvs are detected using an ICELISA.

- Prepare blocking buffer and wash buffer as described on page 6.
- Prepare antigen-coated and blocked microplate wells according to the procedure in 8.1., page 13. Prepare a set of negative antigen control wells in a similar manner.
- Dilute HRP/Anti-E Tag Conjugate 1:4 000 in blocking buffer.
- Add 25, 50 or 100 µl of the diluted HRP/Anti-E Tag Conjugate to each well of the microplate.

Note: The volume of conjugate added to each well is one-half the volume of antigen used to coat the wells. The combined volume of conjugate and ScFv (see below) should not exceed the volume of antigen initially used to coat the wells.

- Immediately add an equal volume of E-tagged ScFv recombinant antibodies to a set of antigen-coated and negative antigen control wells containing the diluted conjugate.

Note: As an option, HRP/Anti-E Tag Conjugate and E-tagged ScFvs can be pre-mixed, then added to the microtitre wells.

- Incubate the plate in a humidified container for 1–3 hours at room temperature.
- Empty the microplate by inverting and flicking the well contents into a suitable container. Blot the inverted plate onto a dry paper towel to remove any residual liquid in the wells.
- Wash the microplate by completely filling the wells with wash buffer and emptying. Repeat this step twice for a total of three washes. Blot any residual liquid as before.
- Prepare the ABTS substrate as described on page 6. Be sure to add H_2O_2 to the solution. Add 50-200 µl of the substrate to the appropriate wells.
- Incubate for 20 minutes at room temperature.
- Read the absorbancy for each well at 410 nm.

Note: Weak ELISA signals may occur if the expression level or affinity of antigen-positive ScFvs is low, if the ScFvs fail to recognize the antigen, if the ScFvs have degraded, or if the ELISA is not optimized. In some cases, signals can be enhanced by incubating the plate overnight at 2–8°C in a humidified container. If a suitable colour reaction does not develop after this time, the ScFv concentration is below the detection limits of the assay.

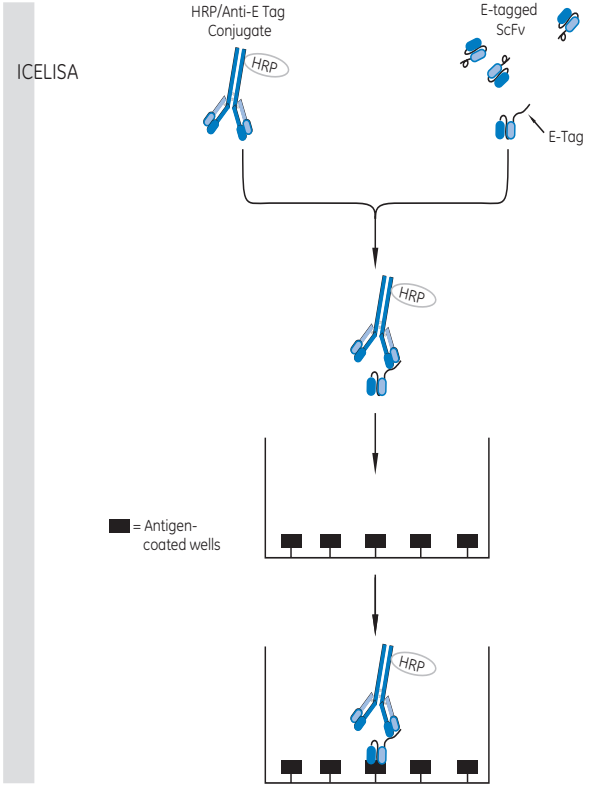

Figure 2. Schematic showing an ICELISA protocol

8.3. Western blot analysis

When electrophoresed through acrylamide gels, ScFvs typically appear as 30–32 kDa bands. After transfer to nitrocellulose membranes, ScFvs displaying the E-tag peptide are conveniently detected using HRP/Anti-E Tag Conjugate and a precipitable substrate.

In the following protocol, E-tagged ScFvs are detected by Western blot analysis (1–3) using HRP/Anti-E Tag Conjugate.

- For standard-size polyacrylamide gels, mix 3–20 µl of periplasmic extract containing E-tagged ScFv antibodies with 1/6 volume of 6x loading buffer (see page 7). A prestained molecular weight marker should be run in parallel with the samples.
- Heat all samples at 95–100°C for 2–5 minutes, cool to room temperature, and spin briefly.
- Load each sample onto an SDS-polyacrylamide gel and electrophorese until the Bromophenol Blue dye in the sample loading buffer reaches the lower edge of the gel.
- Following electrophoresis, transfer the proteins from the gel to a nitrocellulose membrane.
- Prepare blocking buffer and wash buffer as described on page 6.
- When the transfer is complete, incubate the membrane in a pre-measured volume of blocking buffer (use 10 ml of blocking buffer per 50 cm^2 of nitrocellulose) for 1 hour at room temperature.

Note: If the blot is blocked for more than 10 minutes, it should be incubated in a humidified container to prevent evaporation.

- While the blot is incubating, prepare a 1:1 000 dilution of HRP/Anti-E Tag Conjugate in blocking buffer. Use 10 ml of blocking buffer per 50 cm^2 of nitrocellulose and add 1 µl of conjugate per ml of buffer. Mix well.

- Remove the blocking buffer from the blot and replace with the diluted HRP/Anti-E Tag Conjugate.
- Incubate the blot for at least 1 hour at room temperature in a humidified container with gentle shaking.
- Prepare the 4-Chloro-1-Naphthol (4-CN) substrate immediately prior to use. Be sure to add H_2O_2 to the solution. See page 6.

Note: Substrates other than 4-CN may be used. LumiGLO (Kirkegaard and Perry Laboratories, catalogue number 54-61-00), a chemiluminescent HRP substrate, is more sensitive than 4-CN, while diaminobenzidine (Sigma catalogue number D-4418) is less sensitive. For optimal results, it may be necessary to vary the concentration of HRP/Anti-E Tag Conjugate if a substrate other than 4-CN is used.

- Remove the diluted conjugate solution from the blot.
- Wash the blot six times (30 seconds to 2 minutes per wash) with an excess of wash buffer, and once with distilled, deionized water. Drain any excess water from the blot.
- Place the blot in the 4-CN substrate until a suitable colour (blue) reaction develops.
- Rinse the blot with distilled, deionized water to stop the reaction.

8.4. Colony lift assay

Depending upon the antigen, individual *E. coli* colonies expressing antigen-positive E-tagged ScFvs can be identified among a large population of colonies on agar medium using HRP/Anti-E Tag Conjugate in conjunction with a colony lift protocol. In this assay, *E. coli* (e. g. TG1 or HB2151) colonies expressing soluble ScFv to an antigen are initially grown on a Glucose-containing agar medium (e. g. 2 x YT-AG) in a standard Petri dish (master plate). Nitrocellulose membrane filters coated with the antigen are then placed onto the surface of a Glucose-deficient agar medium (e. g. 2 x YT-AI) in a

second Petri dish. Colonies are lifted from the surface of the master plate using a fresh piece of nitrocellulose (the master filter), and the master filter is carefully layered (colony-side up) onto the surface of the antigen-coated nitrocellulose filter in the second Petri dish (see Figure 3).

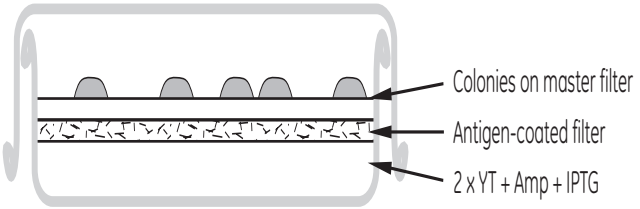

Figure 3. Schematic showing arrangement of filters in colony lift assay.

The cells on the master filter are incubated overnight in contact with the antigen-coated filter to allow for ScFv expression. As ScFv antibodies are produced, they diffuse through the master filter and bind to the antigen-coated filter beneath. The master filter is then removed and stored (colony-side up) at 2–8°C on sterile agar medium. The antigen-coated filter is probed with HRP/Anti-E Tag Conjugate and developed with an HRP-precipitable substrate to detect E-tagged ScFvs bound to antigen on the filter surface. Finally, the master filter is aligned with the antigen-coated filter to identify and recover those colonies expressing E-tagged ScFvs reactive with the antigen.

The following protocol describes a colony lift assay whereby ScFv-expressing clones of *E. coli* can be rapidly identified. In some cases, due to the large number of colonies that can be simultaneously

screened, it may be possible to completely circumvent phage rescue and antigen-affinity selection procedures normally used in the identification of positive clones (especially those originating from a hybridoma source).

Note: Some antibodies produced by hybridoma cells cannot recognize their respective antigens if they are immobilized onto nitrocellulose. Consequently, the colony lift assay cannot be used to detect recombinant antibodies generated from these hybridoma cells since, in general, the recombinant antibodies are not likely to recognize the nitrocellulose-bound antigens either.

- Prepare a master plate by inoculating *E. coli* transformed with pCANTAB 5 E/ScFv onto 2 x YT-AG or SB-AG agar medium. Incubate overnight at 30°C or until the colonies are 1–2 mm in diameter.

Note: It is important that colonies be well-isolated for this procedure. To achieve this, it may be necessary to plate several dilutions of the transformed *E. coli* cells.

Note: The plates containing the colonies can be stored at 2–8°C for several days at this point; however, some ScFv genes or expressed proteins may be unstable upon storage. The stability of the ScFv gene in *E. coli* or the ScFv protein expressed by *E. coli* cannot be predicted. As the cells age, there is a greater risk that the ScFv gene or protein product will be lost.

- Place a piece of Parafilm™ (approximately 110 mm x 110 mm) onto the lab bench.
- Place two pieces of nitrocellulose (pre-cut to fit a standard Petri plate) onto the Parafilm.

Note: Generally, there is no need to autoclave the nitrocellulose if it has been stored in a closed, clean container.

Note: We have found nitrocellulose membranes from Schleicher and Schuell (BA85, 82 mm diameter) to be suitable for this protocol. Background problems may occur if other filters are used.

CAUTION: ALWAYS wear gloves and use a forceps when handling the nitrocellulose filters.

- Completely wet one nitrocellulose filter with 0.75–1.5 ml of antigen diluted to an appropriate concentration (e. g. 10 µg/ml) in a suitable buffer (e. g. PBS or carbonate). Incubate for 10–20 minutes at room temperature.

Note: It may be necessary to gently rock the filter by hand to ensure that it is completely wetted with the antigen solution.

- Prepare blocking buffer and wash buffer as described on page 6.
- Holding the filter by the edge with a forceps, drain any excess fluid by gently touching the opposite edge of the filter onto a paper towel.
- Place the filter (antigen-side up) onto a paper towel and air-dry for 5–10 minutes at room temperature.

Note: It may be advantageous to mark the antigen side of the filter for easy identification.

- Completely immerse the filter in blocking buffer for 20–60 minutes at room temperature.
- Drain any excess fluid from the filter as described before.
- Wash the filter briefly with 100–200 ml of PBS to remove excess blocking buffer.
- Drain excess fluid from the filter as before.
- Place the filter (antigen-side up) onto a paper towel and air-dry for 5–10 minutes at room temperature.
- Transfer the blocked antigen-coated filter (antigen-side up) onto the surface of 2 x YT-AI or SB-AI agar medium in a Petri dish.

- Carefully place the second nitrocellulose filter (which is uncoated and unblocked) onto the master plate containing the ScFv-expressing colonies of *E. coli.*

Note: It is important to apply the filter gently and evenly onto the plate surface without smearing the colonies. To do this, gently curl the filter up into a curved semi-circle. Bring the base of the semi-circle into contact with an imaginary center line across the agar medium. Then slowly lower the filter onto the surface of the medium.

- Incubate at room temperature until the filter appears moist (approximately 1–5 minutes).
- Using a forceps, carefully lift the nitrocellulose (master filter) from the surface of the master plate.
- Place the master filter (colony-side up) onto the surface of the antigen-coated filter in the Petri dish. Invert the plate and incubate overnight at 30°C.
- Mark both filters simultaneously by poking a pattern of holes through the filters with a 16-gauge needle. This will be used at a later step to align the two filters for the purpose of identifying colonies expressing antigen-positive ScFvs.
- Remove the master filter and transfer it (colony-side up) onto the surface of a fresh agar medium plate. Store at 2–8°C until needed. This will protect the cells from desiccation and loss of viability.
- Transfer the antigen-coated filter to a suitable container to which 50–100 ml of blocking buffer has been added. Incubate for 15 minutes at room temperature with gentle shaking.

Note: To avoid background problems associated with *E. coli* cellular material, the antigen-coated filter must be adequately washed with blocking and/or wash buffer.

CAUTION: Do **NOT** blot the antigen-coated filter with a paper towel during any of the following steps. If the filter is blotted, the reaction products will smear.

- Transfer the antigen-coated filter from the blocking buffer to 100–200 ml of wash buffer. Incubate for 15–30 minutes at room temperature with gentle shaking. Repeat this wash step using fresh wash buffer.
- Prepare a 1:1 000 dilution of HRP/Anti-E Tag Conjugate in blocking buffer.

Note: Approximately 7 ml of diluted conjugate is sufficient to completely cover the surface of the antigen-coated filter placed into a standard sized Petri plate.

- Remove the filter from the wash buffer. While holding the filter with forceps, examine it for the presence of yellow, shiny *E. coli* cellular debris. If debris is present, wash the filter in wash buffer as described before until no debris is visible.
- Transfer the filter into the diluted HRP/Anti-E Tag Conjugate. Incubate for 1 hour at room temperature with gentle shaking.
- Transfer the filter from the conjugate solution to a suitable container to which 100–200 ml of wash buffer has been added. Rinse briefly, then pour off the wash buffer. Repeat for a total of six washes.
- Wash the filter briefly in distilled water and drain any excess water from the filter as described earlier.

CAUTION: Do **NOT** blot the filter with a paper towel.

- Place the filter (antigen-side up) onto a paper towel and air-dry briefly for 1–5 minutes.
- Prepare the 4-CN substrate immediately prior to use. Be sure to add H_2O_2 to the solution. See page 6.

Note: Substrates other than 4-CN can also be used. However, since their sensitivity varies, it may be necessary to optimize the concentration of HRP/Anti-E Tag Conjugate used in the colony lift assay.

- Transfer the filter (antigen-side up) into the substrate and incubate at room temperature until a suitable purple/blue colour reaction develops (approximately 5–20 minutes).

Note: If a visible colour reaction does not develop within 20 minutes, continue incubation overnight at 2–8°C to enhance the sensitivity of the colour reaction. In some cases, the *E. coli* ScFv expression level may be too low for detection.

- Transfer the filter from the substrate to distilled water to stop the colour reaction. Incubate for 1–10 minutes at room temperature.
- Place the antigen-coated filter (antigen-side up) onto a paper towel and air-dry.
- Align the antigen-coated filter with the master filter. Colonies which produce a purple/blue colour reaction on the antigen-coated filter are expressing ScFvs reactive with the antigen.

Note: The shapes of the coloured areas on the antigen-coated filter should correlate with the shapes of their respective colonies on the master filter. If the shapes are not similar, the result may represent an artifact. These artifacts can occur if the filter is accidentally blotted or if the filter is not washed sufficiently.

- Using sterile pipette tips, transfer colonies producing ScFv from the master filter to sterile tubes containing 2–3 ml of fresh 2 x YT-AG or SB-AG medium. Incubate 3–4 hours at 30°C with shaking at 150–250 rpm.

Note: Some ScFvs are unstable in *E. coli*. As a precaution, transfer colonies from the master filter to plates containing 2 x YT-AG or SB-AG. Streak for isolation and incubate overnight at 30°C. If the colony

picked from the master filter fails to produce antigen-positive ScFvs reactive in subsequent immunoassays, assay individual colonies on the streak plate for ScFv production.

- Prepare frozen Glycerol stocks using aliquots from the broth cultures. The remainder of the cultures can be used as starter cultures for the production of soluble ScFvs in *E. coli* HB2151 (see the Expression Module booklet).

8.5. Screening for E-tagged ScFvs expressed by microplate colonies

In the Expression Module booklet, individual clones of pCANTAB 5 E-transformed *E. coli* are isolated on SOBAG plates. These clones can be grown in microplates for the purpose of small-scale screening for soluble ScFv production. Either the culture medium or periplasmic extract is assayed by ELISA for the presence of ScFvs reactive with the antigen. With the microplate format, a large number of colonies can be assayed and positive clones easily identified. However, since the expression level for some recombinant antibodies may be quite low, the concentration of ScFvs in the culture medium or periplasmic extract may fall below the limit of detection in an immunoassay. This limitation can be circumvented by growing cultures in larger microtitre wells or in cluster tubes (Costar). ScFvs can then be concentrated from the periplasmic extract obtained from the larger cell numbers.

Note: If ELISA plates have been pre-coated with antigen and blocked, they can be used to immediately screen for the presence of antigen-positive soluble antibodies obtained from the periplasmic extract of colonies grown in microplates. An ELISA to detect E-tagged soluble ScFv antibodies using HRP/Anti-E Tag Conjugate is described in 8.1., page 13.

Unless otherwise noted, all of the following steps should be carried out aseptically. The use of a multi-channel pipette equipped with sterile pipette tips will greatly facilitate the dispensing and transfer of liquids between microplates in this procedure.

- Add 200 µl of either 2 x YT-AG or SB-AG medium to each well of a sterile 96-well polypropylene microplate.

Note: V-bottom polypropylene microplates are especially effective for pelleting the cells.

Note: For 24-well plates, aseptically add 2–2.25 ml of medium to each well.

Note: Cover the plates with sterile lids to prevent contamination.

- Using sterile pipette tips or toothpicks, transfer well-isolated colonies from the agar medium to microtitre wells containing either 2 x YT-AG or SB-AG medium. Label this plate as S1.

Note: For some *E. coli* expressing ScFvs, the following three steps can be omitted, but this must be determined experimentally. In these cases, transfer colonies directly into microtitre wells containing 200 µl of 2 x YT-AI or SB-AI medium. Continue with the 3–4 hour incubation at 30°C as described below.

- Incubate the microplate for 3–4 hours (96-well plate) at 30°C with gentle shaking (100–150 rpm) in a humidified incubator. If a humidified incubator is unavailable, place the plates in a sealable plastic box containing moist paper towels prior to incubation.

Note: For 24-well plates, incubate the cultures until they become turbid.

Note: It is advisable to prepare Gycerol stocks of the clones at this point. To do this, transfer 25 µl of the log phase cultures to polypropylene microplates. Add sterile 20% Glycerol to a final concentration of 15% and mix well. Cover the plate and store at -15°C to -30°C or -70°C.

- Using a clinical centrifuge equipped with microplate holders, centrifuge plate S1 at 1 500 x g for 20 minutes.
- Remove and discard the supernatants from the cell pellets.
- Add 200 µl of 2 x YT-AI or SB-AI medium to the cell pellets in each well of plate S1. Incubate the plate for 3–4 hours at 30°C in a humidified incubator.

Note: For 24-well plates, aseptically add 2–2.25 ml of medium to each well. Incubate the cultures until they become turbid.

- Centrifuge plate S1 as described above.
- Aseptically transfer the supernatants from the cell pellets to a second sterile polypropylene microtitre plate. Label this plate as S2 supernatants.

Note: The culture supernatants can be assayed for the presence of soluble ScFv antibodies by ELISA. However, for some recombinant antibodies, the concentration of soluble ScFv in the supernatant may fall below the detection limit of the assay.

- To prepare periplasmic extracts, resuspend the cell pellets in the wells of plate S1 with 10–20 µl of PBS containing 1 mM EDTA (see page 4). Incubate plate S1 on ice for 20–30 minutes.

Note: For 24-well plates, resuspend the cell pellets in 100–200 µl of PBS containing 1 mM EDTA.

- Centrifuge the microplates as described above.
- Transfer the supernatants (periplasmic extracts) to wells of a polypropylene microplate and either store at -15°C to -30°C or assay by ELISA (see 8.1., page 13) or ICELISA (8.2., page 17).

Note: It is important to use polypropylene microplates for storage of periplasmic extracts since unpurified ScFvs may adsorb to polyvinyl plastics.

9. Procedures to detect antigen using E-tagged ScFvs and HRP/Anti-E Tag Conjugate

9.1. Western blot analysis

Recombinant E-tagged ScFv antibodies can be used as immunological reagents in Western blot analysis to detect antigens transferred to nitrocellulose membranes. In this capacity, the E-tagged ScFv serves as the primary antibody which binds to the antigen on the membrane. Because it bears the E-tag, the bound ScFv can be detected using HRP/Anti-E Tag Conjugate and a suitable HRP substrate.

Note: Some antibodies produced by hybridoma cells cannot recognize their respective antigens if they are immobilized onto nitrocellulose, as in a Western blot assay. Consequently, recombinant antibodies generated from these hybridoma cells are not likely to recognize the nitrocellulose-bound antigens either.

- For standard size polyacrylamide gels, mix 3–20 µl of antigen (2–10 µg of total protein) with 1/6 volume of 6 x loading buffer (see page 7). A pre-stained molecular weight marker should be run in parallel with the samples.
- Heat all samples at 95–100°C for 2 to 5 minutes, cool to room temperature, and spin briefly.
- Load each sample onto an SDS-polyacrylamide gel and electrophorese until the Bromophenol Blue dye in the sample loading buffer reaches the lower edge of the gel.
- Following electrophoresis, transfer the proteins from the gel to a nitrocellulose membrane.

- Prepare blocking buffer and wash buffer as described on page 6.
- When the transfer is complete, place the membrane into a measured volume of blocking buffer (use 10 ml of blocking buffer per 50 cm^2 of nitrocellulose). Incubate for 1 hour at room temperature in a humidified container.
- Dilute the E-tagged ScFv sample with an equal volume of fresh blocking buffer (use 10 ml of blocking buffer per 50 cm^2 of nitrocellulose). Transfer the blot into the solution and incubate for at least 1 hour (but no more than 3 hours) at room temperature in a humidified container with gentle shaking.
- Pour off the diluted ScFv solution.
- Wash the blot six times (30 seconds/wash) with an excess of wash buffer.
- Prepare a 1:1 000 dilution of HRP/Anti-E Tag Conjugate in blocking buffer (use 10 ml of blocking buffer per 50 cm^2 of nitrocellulose).
- Add the diluted conjugate to the blot. Incubate for at least 1 hour at room temperature in a humidified container with gentle shaking.
- Prepare the 4-Chloro-1-Naphthol (4-CN) substrate immediately prior to use. Be sure to add H_2O_2 to the solution. See page 6.
- Remove the diluted conjugate solution from the blot.
- Wash the blot six times with an excess of wash buffer, and once with water. Drain any excess water from the blot.
- Place the blot in the 4-CN substrate until a suitable colour (blue) reaction develops.
- Rinse the blot with deionized, distilled water to stop the reaction.

10. Additional information

10.1. Optimizing an ELISA

A number of variables may influence the results of an ELISA (4). Failure to optimize these variables may result in poor sensitivity or even false positive results. Variables which should be optimized include:

- antigen-coating concentration
- concentration of primary (first) antibody used to detect the bound or captured antigen
- concentration of secondary antibody (labelled with a reporter molecule such as horseradish peroxidase or alkaline phosphatase) used to detect the primary antibody
- the plastic from which the microplates are constructed
- incubation times and temperatures
- blocking reagents
- buffer pH, salt and detergent concentrations

In some cases, even the choice of the reporter molecule or substrate used in the ELISA can influence the assay results. For example, if the antigen contains peroxidase, the ELISA should be developed using an enzyme/substrate system that does not involve peroxidase. Ideally, all of these variables should be optimized prior to even beginning any recombinant antibody work since immunoassays are constantly used for screening clones for ScFv expression.

For E-tagged ScFv-based ELISAs using HRP/Anti-E Tag Conjugate, the E-tagged ScFv is the primary antibody and the conjugate is the secondary antibody. The optimum antigen-coating concentration should have been determined previously using hybridoma medium, mouse serum or ascites as the primary antibody source and an

appropriate labelled secondary antibody [such as goat anti-mouse IgG (Fc-specific) antibody conjugated to HRP].

Generally, optimum concentrations of the various reagents are determined by performing a series of dilution experiments using the 96-well microplate format. The following protocol (5), known as a 'checkerboard titration', can be used to optimize an ELISA.

Note: In a 96-well microplate (Figure 4), there are 12 columns (designated numerically from 1 to 12) and 8 rows (designated alphabetically from A to H).

- Dilute the antigen to 100 µg/ml in coating buffer [e. g. PBS or 0.05 M carbonate buffer (pH 9.6)].
- Add 100 µl of coating buffer to each well of the microplate.

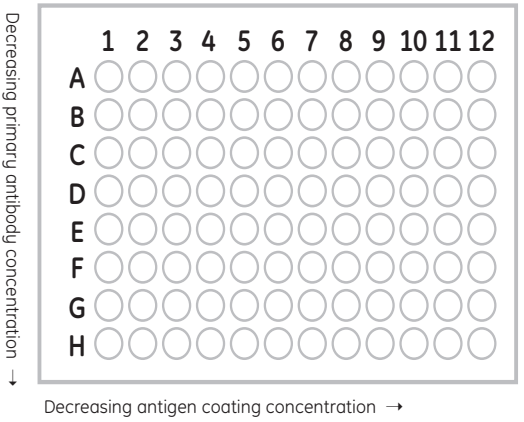

Figure 4. Typical 96-well microplate used for ELISAs

- Add 100 µl of the diluted antigen to each well in column 1 and mix. Change the pipette tips after each step to prevent well-to-well carryover of undiluted reagents.
- Transfer 100 µl of the diluted antigen from each well in column 1 to corresponding wells in column 2 and mix.
- Continue this two-fold dilution process through column 11. Wells in column 11 will contain 200 µl of diluted antigen.
- Remove 100 µl of the diluted antigen from each well in column 11 and discard. Do ***NOT*** add diluted antigen to the wells in column 12. Wells in column 12 will serve as negative (N) controls to determine if nonspecific interactions are occurring between the primary antibody and the blocking buffer or plastic.
- Using a moist paper towel, wipe off the bottom of the microplate to remove any static charge on the surface.
- Incubate the microplate for 1 hour at either room temperature or 37°C or overnight at 2–8°C in a humidified container (e. g. a plastic box containing wet paper towels).

Note: Spurious results may occur when plates are subjected to temperature changes during the course of the assay. Uniform results are more easily obtained if all steps in the ELISA are performed at room temperature.

- Empty the microplate by inverting and flicking the well contents into a suitable container. Blot the inverted plate onto a dry paper towel to remove any residual liquid in the wells.
- Fill all of the wells of the plate with blocking buffer (e. g. 10% normal goat serum, 1% BSA, 3% nonfat dry milk, 1% casein or 1% Gelatin diluted in PBS). The proteins in the buffer will coat any unoccupied sites on the plastic. This prevents adsorption of the primary antibody to the plastic.
- Incubate the microplate for 1 hour at room temperature.

- Empty the contents of the microplate and blot as before.

Note: For some stable antigens, plates can be washed once with wash buffer, tapped dry, and then stored dry at 2–8°C for several weeks. Prior to use, the plates should be washed once with wash buffer since subsequent reagents may nonspecifically adhere to dry wells.

- Add 100 µl of blocking buffer to each well of the plate.

Note: If the ELISA plate was stored dry, wash the plate several times with wash buffer before use.

- Dilute the primary antibody in blocking buffer.

Note: Dilute immune serum or ascites 1:50. If the antibody is purified, dilute it to 100 µg/ml. Hybridoma supernatant or ScFvs should not be diluted at this point.

- Add 100 µl of the primary antibody to each well in row A and mix.
- Transfer 100 µl of the primary antibody from each well in row A to the corresponding well in row B and mix.
- Repeat this two-fold dilution series through wells in row G. Wells in row G will contain 200 µl of the diluted primary antibody.
- Remove 100 µl of diluted antibody from each well in row G and discard. Do ***NOT*** add primary antibody to the control wells in row H. Wells in row H will serve as negative controls to determine if nonspecific interactions are occurring between the secondary antibody and various concentrations of the antigen.
- Incubate the plate for 1 hour at room temperature or 37°C in a humidified container.
- Empty the contents of the microplate and blot as before.
- Rinse the plate by completely filling and emptying the wells three times with wash buffer. Blot to remove any residual liquid in the wells.

- Dilute the enzyme-conjugated secondary antibody in blocking buffer according to the manufacturere's instructions and transfer 100 µl of the diluted conjugate to each well of the plate.

Note: If HRP/Anti-E Tag Conjugate is the secondary antibody, the optimum dilution in an ELISA should be 1:8 000. However, the concentration may be varied slightly to improve the signal in the assay.

- Incubate the plate for 1 hour at room temperature or 37°C in a humidified container.
- Empty the contents of the microplate and blot as before.
- Rinse the plate three times with wash buffer.
- Add 100 µl of an appropriate substrate to all wells.
- Incubate the plate for 20 to 30 minutes at room temperature.
- Determine the absorbancy values for each well at a wavelength appropriate for the substrate.

Once the absorbancy values are obtained, the optimum antigen coating and primary antibody concentrations can be determined by calculating the P:N (+:–) ratio for the ELISA. To determine the optimum antigen coating concentration, use the following protocol.

- Divide the absorbancy values of the test (P) (positive antigen) wells in each **column** by the absorbancy value of the respective negative (N) control (no antigen) well in row H.
- Using linear graph paper, plot antigen concentration on the X-axis and P:N ratio on the Y-axis. If the assay is working properly, a curve should be obtained when all of the values are plotted. The antigen coating concentration located directly beneath the peak of the curve will be the optimum antigen coating concentration that should be used in an ELISA.

The optimum antibody concentration can be determined using a similar protocol.

- Divide the absorbancy values for the test (P) (primary antibody) wells in each **row** by the absorbancy value for the respective negative (N) control (no primary antibody) well in column 12.
- Using linear graph paper, plot primary antibody concentration on the X-axis and P:N ratio on the Y-axis. If the assay is working properly, a curve should be obtained when all of the values are plotted. The primary antibody concentration located directly beneath the peak of the curve will be the optimum primary antibody concentration that should be used in an ELISA.

In some cases, nonspecific interactions between the primary antibody or secondary antibody and the blocking buffer or negative control antigens are so extensive that the optimum antigen coating concentration and primary antibody concentration cannot be determined. In these cases, the blocking buffer, the type of plastic microtitre plate or the concentration of the secondary antibody can be changed. If these changes do not solve the problem, the primary antibody can be diluted in an equal volume of blocking buffer containing 0.2% Triton X-100 and/or 1–2 mM EDTA.

Note: The negative control well designated 12H in the above assay contains only blocking buffer and the conjugated secondary antibody. If a positive signal is obtained in well 12H, then the wells for the microplate were not blocked or washed sufficiently or the concentration of the conjugated secondary antibody was too high. If the concentration of the conjugate is not optimal, you will need to determine the optimal concentration by performing an ELISA. Vary the concentration of the conjugated antibody applied to test (P) wells which contain constant concentrations of the coating antigen and primary antibody. Also apply the conjugate to negative (N) control wells which contain blocking buffer only. Determine the

optimal conjugate concentration using the protocol described above for optimal antibody concentration. In some cases, an antigen may contain components or contaminants which react nonspecifically with and bind to the constant regions of specific classes of antibodies from certain animal species. If this occurs with the secondary antibody conjugate (e.g. HRP/Anti-E Tag Conjugate which is a mouse IgG monoclonal antibody), the nonspecific reaction can be blocked using blocking buffer comprising normal animal serum (e.g. mouse serum) diluted in PBS. Unlabelled antibodies in the normal animal serum will bind to the antigen-associated nonspecific components or contaminants, thereby leaving the secondary antibody conjugate free to interact with the primary antibody bound to the antigen.

11. Troubleshooting guide

Problem: No signal in the ELISA using antigen-coated wells

Possible causes	Remedies
1. The antigen did not adsorb to the plastic.	**1.** This should have been determined during optimization of the ELISA. Not all plastics are suitable for antigen adsorption and not all antigens adsorb equally well to plastics.
2. ScFvs are not reactive with antigen adsorbed to a plastic surface.	**2.** If antibodies produced by the hybridoma from which the ScFvs were generated do not react with antigen bound to microtitre wells (due to steric hindrance or epitope modification), it is unlikely that the ScFvs will recognize the antigen either.
3. ScFvs are not reactive with the antigen.	**3.** Verify that the hybridoma supernatant, ascites or serum is reactive with antigen adsorbed to plastic. If these antibodies can recognize the antigen, but the ScFvs cannot, it may be necessary to reisolate clones for screening.
4. The ABTS substrate was prepared incorrectly.	**4.** Add 1 µl of HRP/Anti-E Tag Conjugate to 1 ml of the substrate. A colour reaction should occur within 30 seconds. If the substrate does not produce a visible colour reaction, prepare fresh substrate, making sure to add H_2O_2.

Possible causes	Remedies
5. The concentration of E-tagged ScFvs in the sample was too low to detect by ELISA.	**5.** Check the sample in a dot blot immunoassay. Spot 1–2 µl of the ScFv sample onto a piece of nitrocellulose and allow the spot to air-dry. Block the membrane and detect the ScFvs using HRP/Anti-E Tag Conjugate according to 8.3., page 20. If 4-CN substrate is used and the dot turns blue in colour, then an E-tagged product is present in the sample. Perform a Western blot on the sample to verify if intact ScFvs (30–32 kDa) are present (see 8.3.). If ScFvs are detected in the sample, then either they are not reactive with the antigen or the ELISA was not optimized. Optimize the ELISA using serum or a polyclonal or monoclonal antibody to determine optimum antigen-coating and incubation conditions.

Problem: The signal was weak in the ELISA using antigen-coated wells

Possible causes	Remedies
1. Recombinant ScFv antibodies were expressed at very low levels.	**1.** In some cases, the level of antibody expression can be improved by first growing the cells overnight in medium such as 2 x YT-AG and then transferring to medium such as 2 x YT-AI for 20–24 hours to induce expression. Concentrate the ScFvs by purification or by ultrafiltration (e. g. using an Amicon

Possible causes	Remedies
	1. *Continued.* P10 disc membrane in conjunction with a stirred cell filtration unit).
2. Components in the blocking buffer may interfere with binding of the conjugate.	**2.** The activity of HRP/Anti-E Tag Conjugate can sometimes be improved by using normal goat serum (e. g. Sigma catalogue number S-6898) diluted 1:10 in PBS (10% NGS in PBS) as a blocking reagent throughout the assay instead of non-fat dry milk. In some cases, however, the background in the assay may also increase when 10% NGS is used as a blocking reagent.

Problem: The background was high in the ELISA using antigen-coated wells.

Possible causes	Remedies
1. The microplate was not washed sufficiently.	**1.** Wash the plate more thoroughly, either for longer periods, with more wash buffer or by increasing the number of washes. Alternatively, it may be necessary to use a detergent other than Tween™ 20.
2. The microplate was not blocked sufficiently.	**2.** Block the plate for a longer period of time or use 10% non-fat dry milk in PBS as a blocking reagent.
3. Too much HRP/Anti-E Tag Conjugate was used in the assay.	**3.** Dilute the conjugate as necessary to give the optimum signal to noise ratio. It may be necessary to optimize the concentration of conjugate used for detection. In any case, it is important that the ELISA be optimized.

Possible causes	Remedies
4. The sample contains endogenous peroxidases which react with the HRP substrate.	**4.** Add substrate only (no conjugate) to the antigen-coated and blocked wells. If a colour reaction occurs, use the unconjugated Anti-E Tag Antibody (27-9412-01, -02) and a secondary antibody (e.g. goat anti-mouse IgG) coupled to a different enzyme (e. g alkaline phosphatase).
5. HRP/Anti-E Tag Conjugate interacts non-specifically with the antigen used to coat the microtitre wells.	**5.** Some antigens react non-specifically with mouse IgG. The non-specific interaction between the conjugate and the antigen can therefore be inhibited by diluting the conjugate in blocking buffer containing 10% normal mouse serum (e. g. Sigma catalogue number M-5905) rather than non-fat dry milk.

Problem: ScFv bands either disappear or appear as smears or as multimers on stained denaturing SDS-polyacrylamide gels.

Possible causes	Remedies
1. The concentration of ScFvs in the sample was too low to detect by Western blot analysis.	**1.** Concentrate the sample. Add 250 µl of 20% trichloroacetic acid in water to a microcentrifuge tube containing 250 µl of sample and mix well. Incubate on ice for at least 15 minutes, then centrifuge the sample for 10 minutes at 12 000-14 000 rpm. Remove the supernatant and resuspend the pellet in 10–25 µl of water. Add 1/6 volume of 6x loading buffer and prepare the sample for electrophoresis as described previously. If the loading buffer

Possible causes	Remedies
	1. *Continued.* becomes acidic (yellow), add a small crystal of Tris-base to the sample to return the sample to a pH of 7 or greater. **Note:** TCA precipitation destroys the functional activity of ScFvs.

Problem: The background on the Western blot was high

Possible causes	Remedies
1. Too much ScFv samples was loaded onto the gel.	**1.** Reduce the amount of sample loaded.
2. Too much HRP/Anti-E Tag Conjugate was used in the assay.	**2.** Dilute the conjugate as necessary to minimize background.
3. The blot was blocked insufficiently.	**3.** Block for a longer period of time or use 10% non-fat dry milk in PBS as a blocking reagent.

Problem: The reaction of ScFvs with antigen in the Western blot was weak or absent

Possible causes	Remedies
1. The amount of ScFvs in the sample was too low.	**1.** If the intensity of bands on the Western blot is weak, but the background is acceptable, incubate the sample with HRP/Anti-E Tag Conjugate for a longer period of time (overnight, if necessary). Alternatively, use a different blocking buffer or substrate.
2. The ScFvs do not react strongly with antigen on the membrane.	**2.** If HRP/Anti-E Tag Conjugate is used to detect an E-tagged recombinant ScFv antibody bound to an antigen on nitrocellulose and the signal is too weak, either use more antigen in the assay or incubate the blot with the ScFv sample for a longer period. In some cases, the antigenic site recognized by the ScFvs is disrupted prior to or during electrophoresis or cannot be recognized when immobilized onto nitrocellulose. Consequently, some recombinant ScFv antibodies, like some monoclonal antibodies, cannot be used for Western blots. Check for reactivity using the original hybridoma monoclonal antibody, serum or ascites.
3. Too little antigen was loaded onto the gel.	**3.** Increase the amount of sample loaded.

Possible causes	Remedies
4. Components in the blocking buffer may interfere with binding of the conjugate.	**4.** The activity of HRP/Anti-E Tag Conjugate can sometimes be improved by using normal goat serum (e. g. Sigma catalogue number S-6898) diluted 1:10 in PBS (10% NGS in PBS) as a blocking reagent throughout the assay instead of non-fat dry milk. In some cases, however, the background in the assay may also increase when 10% NGS is used as a blocking reagent.
5. Recombinant ScFv antibodies were expressed at very low levels.	**5.** In some cases, the level of antibody expression can be improved by first growing the cells overnight in medium such as 2 x YT-AG and then transferring to medium such as 2 x YT-AI for 20–24 hours to induce expression. Concentrate the ScFvs by purification or by ultrafiltration (e. g. using an Amicon P10 disc membrane in conjunction with a stirred cell filtration unit).

Problem: Antigen-reactive recombinant ScFv antibodies produce a signal in an ELISA but cannot be detected on a Western blot

Possible causes	Remedies
1. In some cases, the affinity of the ScFvs is high but the expression level is low. Consequently, the ScFvs can be easily detected in an ELISA but may not be detected in a Western blot.	**1.** In some cases, the level of antibody expression can be improved by first growing the cells overnight in medium such as 2 x YT-AG and then transferring to medium such as 2 x YT-AI for 20–24 hours to induce expression. Concentrate the ScFv by purification or by ultrafiltration (e. g. using an Amicon P10 disc membrane in conjunction with a stirred cell filtration unit).
2. The sample may contain insufficient ScFv for detection on a Western blot.	**2.** Concentrate the sample prior to analysis. As an alternative, concentrate an aliquot of the sample by TCA precipitation (see page 43–44 in the Troubleshooting guide for details.) **Note:** TCA precipitation destroys the functional activity of ScFvs.

12. References

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13. Related products

Mouse ScFv Module 5 reactions	27-9400-01
Expression Module 1 kit	27-9401-01
Detection Module 1 kit	27-9402-01
Selection Module 1 kit	27-9403-01
Purification Module 1 kit	17-1362-01
Anti-E Tag Antibody 1 mg†	27-9412-01

GE Healthcare offices:

GE Healthcare Bio-Sciences AB
Björkgatan 30 751 84
Uppsala
Sweden

GE Healthcare Europe GmbH
Munzinger Strasse 5 D-79111
Freiburg
Germany

GE Healthcare UK Limited
Amersham Place
Little Chalfont
Buckinghamshire
HP7 9NA
UK

GE Healthcare Bio-Sciences Corp
800 Centennial Avenue
P.O. Box 1327
Piscataway
NJ 08855-1327
USA

GE Healthcare Bio-Sciences KK
Sanken Bldg. 3-25-1
Hyakunincho Shinjuku-ku
Tokyo 169-0073
Japan

GE Healthcare regional office contact numbers:

Asia Pacific
Tel: +85 65 62751830
Fax: +85 65 62751829

Australasia
Tel: + 61 2 8820 8299
Fax: +61 2 8820 8200

Austria
Tel: 01/57606-1613
Fax: 01/57606-1614

Belgium
Tel: 0800 73 890
Fax: 02 416 8206

Canada
Tel: 1 800 463 5800
Fax: 1 800 567 1008

Central, East, & South East Europe
Tel: +43 1 972 720
Fax: +43 1 972 722 750

Denmark
Tel: 45 70 25 24 50
Fax: 45 45 16 2424

Eire
Tel: 1 800 709992
Fax: +44 1494 542010

Finland & Baltics
Tel: +358 9 512 3940
Fax: +358 9 512 39439

France
Tel: 01 69 35 67 00
Fax: 01 69 41 98 77

Germany
Tel: 0800 9080 711
Fax: 0800 9080 712

Greater China
Tel: +852 2100 6300
Fax: +852 2100 6338

Italy
Tel: 02 26001 320
Fax: 02 26001 399

Japan
Tel: +81 3 5331 9336
Fax: +81 3 5331 9370

Korea
Tel: 82 2 6201 3700
Fax: 82 2 6201 3803

Latin America
Tel: +55 11 3933 7300
Fax: + 55 11 3933 7304

Middle East & Africa
Tel: +30 210 96 00 687
Fax: +30 210 96 00 693

Netherlands
Tel: 0800-82 82 82 1
Fax: 0800-82 82 82 4

Norway
Tel: +47 815 65 777
Fax: +47 815 65 666

Portugal
Tel: 21 417 7035
Fax: 21 417 3184

Russia, C.I.S. & N.I.S
Tel: +7 495 956 5177
Fax: +7 495 956 5176

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Fax: 935 94 49 65

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Fax: 018 612 1910

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Fax: 0800 616 927

USA
Tel: +1 800 526 3593
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Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA
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