READ ME FIRST

- This is an electronic version, produced in Adobe Acrobat, of the instructions supplied with the product.
- On most monitors, use of the file is simplest at 115-150% magnification in Acrobat. This magnification should make the print size easy to read and permit orientation through the instructions.
- The page numbering of the Acrobat file does not match the page numbering of the instruction booklet, since two instruction booklet pages have been assembled onto one Acrobat page. Therefore, references to page numbers within the instructions are referring to the page numbers within the instructions and not to the numbers of the Acrobat pages.

A M E R S H A M B I O S C I E N C E S

Expression Module/ Recombinant Phage Antibody System

INSTRUCTIONS

The Recombinant Phage Antibody System (RPAS) is based on a phage-display technology where fragments of antibodies are expressed as a fusion with gene 3 protein and displayed on the tips of M13 phage. Once antigen-positive clones have been identified, they can then be used to infect a nonsuppressor strain of *E. coli* for the purpose of producing soluble antibodies for use as immunological reagents.

The RPAS is designed in a flexible modular format consisting of three parts: Mouse ScFv Module; Expression Module; and Detection Module. Anti-E Tag Antibody is provided separately for detection and purification of soluble antibodies. The module you are now working with – the Expression Module – is designed to clone the antibody ScFv gene into the pCANTAB 5 E phagemid and to express phage-displayed recombinant antibodies or soluble antibodies. A complete overview of the system is provided separately. Instructions for the other modules of the system and the Anti-E Tag Antibody are provided with those products.

The technology was developed in collaboration with Cambridge Antibody Technology Ltd., UK. See page 3 for important licensing information.

XY-040-00-08
Rev. 4

Amersham Biosciences

CONTENTS

PATENT AND LICENSE INFORMATION

Before using this product, carefully read the Patent and License Information below. By using this product, you indicate your acceptance of the conditions described below.

Considerable time and money have been spent developing technology which covers, among other things, the phagemid vector, antibody primers, and amplification methods, and patent applications have been filed in the U. S. and in other countries, including PCT applications numbers PCT/GB89/01344 and PCT/GB91/01134. Amersham Biosciences recognizes the importance of providing a kit using this technology for ongoing research in the area of recombinant antibody development and for studies in molecular and cellular biology, and we therefore want to make the kit available as widely as possible to the research market. Understandably, however, we feel the need to control its distribution. Therefore, a license for research use only of the technology and antibodies or molecules derived from them is granted by Cambridge Antibody Technology (the developer of the technology) to the end-user to use the technology, antibodies and molecules, provided this kit has been purchased from Amersham Biosciences.

If any antibodies or molecules derived from them (in either case isolated using the kit) are to be sold as a commercial product or incorporated into a commercial product or used in the manufacture of a commercial product, it will first be necessary to obtain a license for commercial use from Cambridge Antibody Technology at the Science Park, Melbourn, Cambridgeshire SG8 6EJ, UK, Telephone 44 0763 263233 or Fax 44 0763 263413. If you are in any doubt as to whether your use of antibodies or molecules derived from them would require a commercial license, please discuss the matter with Cambridge Antibody Technology.

COMPONENTS

pCANTAB 5 E:	Aqueous solution (50 ng/µl) in TE buffer (pH 7.6).
M13KO7 Helper Phage:	Titered phage in YT medium.
E. coli TG1 Cells:	Lyophilized; K12 Δ(*lac-pro*), *supE*, *thi*, *hsd*Δ5/F'[*traD*36, *proAB*, *lacI*^{q}, *lacZ*ΔM15].
E. coli HB2151 Cells:	Lyophilized; K12 Δ(*lac-pro*), *ara*, *nal*^{r}, *thi*/F'[*proAB*, *lacI*^{q}, *lacZ*ΔM15].
10X One-Phor-All Buffer PLUS: *(10X OPA+ Buffer)*	100 mM Tris-acetate (pH 7.5), 100 mM magnesium acetate, and 500 mM potassium acetate.
Control Insert:*	A10B, *Sfi* I/*Not* I digested.
ScFv Marker:	pUC18/A10B, *Sfi* I/*Not* I digested.

All components provided as part of the Expression Module of the Recombinant Phage Antibody System are in **boldface type**; additional materials and reagents are described in Appendix 2, page 34.

*Note: The **Control Insert** is meant to be used as a control for ligation only. It cannot be used to produce a functional soluble antibody.

☞ NOTE: Store **M13KO7** at 4°C. DO NOT FREEZE.

PROTOCOL

The Expression Module is designed to clone an antibody ScFv fragment, generated in the Mouse ScFv Module (27-9400-01), into the phagemid **pCANTAB 5 E**. Because of its unique construction, this single vector can be used in conjunction with different host *E. coli* strains to produce both phage-displayed recombinant antibodies for affinity purification on immobilized antigen and soluble antibodies for use as immunological reagents. The general protocol is summarized in the timetable on page 7.

To produce phage-displayed recombinant antibodies, competent *E. coli* **TG1** cells are first transformed with the phagemid containing the antibody ScFv gene. This host strain produces a suppressor tRNA which allows readthrough (suppression) of the amber stop codon located between the ScFv and gene 3 sequences of **pCANTAB 5 E**. Since suppression of the amber stop codon in TG1 cells is only about 20% efficient, soluble antibodies will be produced in addition to phage-displayed recombinant antibodies. Transformed cells are then infected with **M13KO7** helper phage (1) to rescue the phagemid with its ScFv gene insert. Recombinant phage which are produced contain a single-stranded DNA copy of the phagemid and antibody ScFv gene and display one or more copies of the recombinant antibody at their tips.

The switch to soluble recombinant antibody production is accomplished by infecting *E. coli* **HB2151** cells with phage from an antigen-positive clone. Since **HB2151** is a nonsuppressor strain, the amber stop codon will be recognized and only soluble antibodies will be produced. A detailed map of **pCANTAB 5 E** and a description of important control regions are presented in Appendix 1, page 29. For an in-depth description of the procedure, please refer to the Overview booklet.

Essential Preliminaries

Assembled ScFv product should be ligated into **pCANTAB 5 E** and used to transform competent *E. coli* **TG1** cells so that phage-displayed antibodies can be panned (affinity-purified) on immobilized antigen.

The preparation of competent cells requires several days (see Appendices 3 and 4, pages 38 and 39, for procedures). For best results, the cells should be transformed immediately after they are made competent. Preparation of the cells should begin three days prior to ligation (Procedure B, page 10) and transformation (Procedure C, page 12).

Panning (affinity enrichment of recombinant phage on the antigen of interest) is highly recommended regardless of whether hybridoma cells or spleen were used as the source of antibody mRNA. The incorporation of one or more panning steps will greatly reduce the number of clones that must be screened by ELISA.

Panning can be performed on a solid support or in solution (e.g. using biotinylated antigen). Although both procedures accomplish similar end results, solution-based selection may permit more controlled enrichment for antibodies with specific affinities. The procedure in this instruction booklet provides instructions for panning on a solid support.

Prior to selection (panning) and screening of colonies, an immunoassay should be performed to determine the optimal binding conditions for the antigen. This is especially important if solid-phase panning will be carried out since plastics vary in their binding characteristics.

Timetable

1. Quantitate ScFv product on an agarose gel (1 hour).
2. Ligate ScFv gene fragments into **pCANTAB 5 E** phagemid (1 hour).
3. Transform competent **TG1** cells and grow (4.5 hours).
4. Perform a rescue of phagemid by infecting with **M13KO7** helper phage (overnight). End of Day 1.
5. PEG-precipitate the resulting phage-displayed recombinant antibodies and select against the antigen of interest (5 hours).*
6. Reinfect log-phase **TG1** cells with bound phage (3.5 hours).
7. Plate reinfected cells and incubate (overnight). End of Day 2.
8. Rescue individual colonies (2 days). Infect **HB2151** cells with recombinant phage from each clone and grow (overnight). End of Day 4.
9. Using reagents from the RPAS Detection Module and Anti-E Tag Antibody, perform ELISAs to select for antigen-positive phage clones and soluble ScFvs (6 hours). Once antigen-positive clones have been identified by ELISA, streak individual plates with infected **HB2151** culture from each antigen-positive clone and incubate (overnight). End of Day 5.
10. Grow overnight cultures from colonies (overnight). End of Day 6.
11. Produce soluble antibodies in **HB2151** cells and assay cellular fractions (4.5 hours). End of Day 7.

Proceed to the instruction booklet included with the Anti-E Tag Antibody for Western blot protocols designed to detect soluble antibodies in the cellular fractions.

*Each additional round of panning will add a day to the protocol.

Procedure A: Gel Quantitation of Assembled ScFv Product

The amount of assembled product added to a ligation mixture containing the cut vector can strongly influence the efficiency of ligation. Since ligation is most efficient with a molar ratio of insert to vector of approximately 3-5 to 1, it is important to determine the amount of assembled product to add to the ligation mixture. This is accomplished using a gel quantitation step. In this procedure, a portion of the assembled product is run in parallel on an agarose gel with a known amount of **ScFv Marker**, then visualized following ethidium bromide staining. The intensity of the assembled product is compared with the intensity of the band of the **ScFv Marker** that corresponds to the size of the assembled product (a second band of higher molecular weight will also be present in the marker), and an estimate is made of the relative amount of DNA in the portion of assembled product loaded on the gel. Adjustments to the amount of DNA added to the ligation mixture can then be made based on this visual estimate.

- Pour a 1% agarose gel that is 0.75 cm thick (approximately 60 ml in a GNA-100 gel apparatus). Use a comb with wells that are 3 mm wide. If desired, add ethidium bromide to the gel and running buffer to a final concentration of 0.5 µg/ml in each.
- To prepare the marker, mix 2.5 µl of **ScFv Marker**, 7.5 µl of distilled water and 2 µl of agarose gel loading dye (see the recipe given in Appendix 1 of the Mouse ScFv Module) in a microcentrifuge tube. Also prepare a second tube containing 5 µl of **ScFv Marker**, 5 µl of water and 2 µl of dye.

- For the assembled ScFv product, mix 2.5 µl of the assembled product, 7.5 µl of water and 2 µl of dye in a microcentrifuge tube. Also prepare a second tube containing 5 µl of assembled product, 5 µl of water and 2 µl of dye.
- Load each of the four samples into separate wells on the gel.
- Electrophorese until the bromophenol blue in the loading dye has migrated about two-thirds the length of the gel.
- Stain the gel with ethidium bromide (if ethidium bromide was not added to the gel and running buffer) and photograph under UV light. It may be necessary to overexpose the picture to visualize the assembled product.
- Compare the intensity of the assembled product with that of the ~750 bp band of the **ScFv Marker**. This band contains approximately 12.5 ng of the ScFv fragment in the 2.5 µl aliquot and 25 ng of the fragment in the 5 µl aliquot.
- Estimate the amount of DNA present in the 2.5 µl aliquot of assembled product by comparing its intensity to that of the **ScFv Marker** band. Divide this amount by 2.5 to obtain the number of ng per µl of assembled product. Determine the number of µl of assembled product that will give 150 ng of assembled product. Add this volume of assembled product to the ligation mixture (see Procedure B, page 10).

Procedure B: Ligation of ScFv Gene into pCANTAB 5 E

The antibody ScFv gene, generated in the Mouse ScFv Module, contains restriction-digested *Sfi* I and *Not* I sites located at the 5'-end of the heavy chain and the 3'-end of the light chain, respectively. The **pCANTAB 5 E** vector also contains restriction-digested *Sfi* I and *Not* I sites for ligation of the ScFv gene. When the vector is used for the first time, we recommend that a ligation of the vector without insert (vector background reaction) and a ligation of the vector to the **Control Insert** be performed in parallel. Sufficient vector is provided for 10 ligations and sufficient **Control Insert** is provided for at least one ligation.

Advance Preparation

- The assembled product should be gel-quantitated as described in Procedure A.
- Prepare a 10 mM solution of ATP in sterile distilled water.

Ligation

Set up three ligation reactions as described below:

- For ligation of the ScFv gene to **pCANTAB 5 E**, add the following to a 1.5 ml microcentrifuge tube:

Antibody ScFv gene fragment (150 ng)	x µl
10X OPA$^+$ Buffer	5 µl
pCANTAB 5 E (50 ng/µl; 250 ng)	5 µl
10 mM ATP	5 µl
T4 DNA ligase (5-7 U)	1 µl
Sterile distilled water	to 50 µl
Total Volume	50 µl

- For ligation of **pCANTAB 5 E** to itself, add the following to a 1.5 ml microcentrifuge tube:

10X OPA$^+$ Buffer	5 µl
pCANTAB 5 E (50 ng/µl)	5 µl
10 mM ATP	5 µl
T4 DNA ligase (5-7 U)	1 µl
Sterile distilled water	34 µl
Total Volume	50 µl

- For ligation of the **Control Insert** to **pCANTAB 5 E**, add the following to a 1.5 ml microcentrifuge tube:

Control Insert (10 ng/µl)	15 µl
10X OPA$^+$ Buffer	5 µl
pCANTAB 5 E (50 ng/µl)	5 µl
10 mM ATP	5 µl
T4 DNA ligase (5-7 U)	1 µl
Sterile distilled water	19 µl
Total Volume	50 µl

- Incubate all three reactions at 16°C for 1 hour.
- Heat inactivate the ligase by incubating the samples at 70°C for 10 minutes.
- Chill the reactions on ice for 5 minutes.

Procedure C: Transformation

In this procedure, competent *E. coli* **TG1** cells (prepared as described in Appendix 4, page 39) are transformed with **pCANTAB 5 E** containing the antibody ScFv gene insert. In addition, cells are also transformed with the vector background reaction and the reaction containing the **Control Insert**. (If electroporation is used to transform the cells, see Appendix 5, page 40. Otherwise, proceed as described below.) We recommend that 1 ng of an uncut (supercoiled) vector DNA (e.g. pUC18) be transformed in parallel with ScFv fragment/**pCANTAB 5 E** ligations to determine the efficiency of each competent cell preparation.

See Appendix 2, page 34, for buffer and media recipes.

- For each of the three ligation reactions and the uncut vector control, add 1 ml of freshly prepared competent *E. coli* **TG1** cells to separate pre-chilled 50 ml sterile disposable centrifuge tubes. Store on ice.
- Add 50 µl of each ligation reaction or 1 ng of uncut vector to the competent cells, swirl gently to mix, and place on ice for 45 minutes.
- Incubate the tubes in a 42°C water bath for 2 minutes, then chill briefly on ice.
  - Note: Transformed cells may be stored at 4°C overnight if the rescue is not performed on the same day.
- **If generating ScFvs from hybridoma cells**, transfer 900 µl of the transformed cells containing ScFv/**pCANTAB 5 E** to a sterile 50 ml disposable polypropylene centrifuge tube and rescue as outlined in Procedure D, page 14. Do not rescue transformed cells from the vector background, the control insert or the uncut vector samples. **If generating ScFvs from spleen**, see Appendix 6, page 42, for instructions on rescue of phage antibodies.

- For each of the four samples, immediately transfer 100 µl of the remaining transformed cells to separate 17 x 100 mm tubes (Falcon) containing 900 µl of LBG medium (prewarmed to 37°C) and incubate for 1 hour at 37°C with shaking at 250 rpm.
- Plate 100 µl of the diluted, transformed cells from the three ligated samples and 10 µl of the diluted, transformed cells from the uncut vector sample onto separate SOBAG plates. Also plate 100 µl of untransformed, competent **TG1** cells as a negative control. Incubate the plates overnight at 30°C.
  - Note: *E. coli* TG1 cells grow slowly at 30°C and require a relatively long period of incubation (20-24 hours) before growth is apparent.
- To prepare a frozen stock culture, add 900 µl of the diluted, transformed cells containing ScFv/**pCANTAB 5 E** to 250 µl of sterile 80% glycerol and mix with a pipet tip. Store at -70°C.
  - Note: To prepare a fresh culture, briefly thaw the frozen cells and transfer 500 µl to a sterile 50 ml centrifuge tube containing 9.5 ml of 2x YT-AG medium. Incubate at 37°C with shaking at 250 rpm until an A_{600} of 0.8 is reached. Add 4 x 10^{10} pfu of **M13KO7** to the cell suspension (volume of stock to add = 4 x 10^{10} pfu ÷ **M13KO7** pfu/ml). **DO NOT ADD AMPICILLIN.** Continue with Procedure D, page 14.

Procedure D: Rescue of Recombinant Phage Antibody Library

In this procedure, the helper phage **M13KO7** is used to rescue the phagemid with its antibody ScFv gene insert from the transformed *E. coli* **TG1** cells. **M13KO7** provides the necessary genetic information to package the recombinant **pCANTAB 5 E** phagemid which encodes the recombinant antibody expressed on the phage tip. The rescue is performed using 900 µl of the undiluted transformed cells from Procedure C (the remaining 100 µl is diluted and used to determine transformation efficiency and to prepare a glycerol stock) .

– Note: Panning should be performed as soon as possible following rescue since some phage-displayed recombinant antibody preparations may be unstable.

- Add 9.1 ml of 2x YT-G medium (***NO AMPICILLIN***) to the 900 µl of transformed cells from Procedure C.

  – Note: Polypropylene tubes are recommended since phage may adsorb nonspecifically to other plastics.

- Incubate the culture for 1 hour at 37°C with shaking at 250 rpm.

  – Note: Do not allow the culture to overgrow since the seed culture will be rather dense.

- Add 50 µl of 20 mg/ml ampicillin and 4 x 10^{10} pfu of **M13KO7** to the cell suspension (volume of stock to add = 4 x 10^{10} pfu ÷ **M13KO7** pfu/ml).
- Incubate the culture for 1 hour at 37°C with shaking at 250 rpm.
- Spin at 1000 x g* in a clinical centrifuge for 10 minutes to sediment the cells. Carefully remove and discard the supernatant.

*For a force of 1000 x g, the appropriate speed can be calculated from the following formula: RCF = 1000 = (1.12)(r)(rpm/1000)^2, where RCF = relative centrifugal force; r = radius in mm measured from center of spindle to bottom of rotor bucket; and rpm = revolutions per minute. The above equation resolves to:

$$\text{rpm} = (1000)(\sqrt{893/r}).$$

For example, with a rotor having a radius of 170 mm, the appropriate speed would be 2,291 rpm.

- Gently resuspend the entire sample in 10 ml of 2x YT-AK medium (***NO GLUCOSE***) in a sterile 50 ml disposable polypropylene centrifuge tube. Incubate overnight at 37°C with shaking at 250 rpm.
- Spin at 1000 x g for 20 minutes in a clinical centrifuge to sediment the cells.
- Transfer the supernatant which contains the recombinant phage to a sterile 50 ml disposable polypropylene centrifuge tube and store at 4°C or proceed with the panning reaction described in Procedure E, page 16. We recommend that the supernatant be filtered through a 0.45 µm filter if it will be stored.

  – Note: The typical phage yield is 10^{10} to 10^{11} ampicillin-transducing units per ml.

  – Note: PEG precipitation and panning of phage should be performed as soon as possible following rescue since some phage-displayed recombinant antibody preparations may be unstable.

Procedure E: Panning to Select for Antigen-Positive Recombinant Phage Antibodies

The purpose of panning (selection) is to selectively capture antigen-positive recombinant phage antibodies with an antigen bound to a solid support or with antigen in solution. Prior to panning, recombinant phage are precipitated from solution with PEG to separate them from soluble antibodies which may also react with the antigen. The recombinant phage are then panned against the antigen. Those which bind to the antigen are retained while those which fail to react with the antigen are removed during subsequent wash steps. When log phase *E. coli* **TG1** cells are added to the panning vessel, antigen-reactive phage infect the cells, which can then be rescued and selected again or plated onto SOBAG plates for screening of individual colonies.

Panning is highly recommended regardless of whether hybridoma cells or spleen were used as the source of antibody mRNA. The incorporation of one or more panning steps will greatly reduce the number of clones that must be screened by ELISA.

Panning can be performed on a solid support or in solution (e.g. using biotinylated antigen). Although both procedures accomplish similar end results, solution-based selection may permit more controlled enrichment for antibodies with specific affinities. The following procedure provides instructions for panning on a solid support.

Solid-phase panning can be conducted in a variety of containers including microtiter plates, tissue culture flasks or dishes. In the following protocol, a plastic 25 cm^2 tissue culture flask is used. Regardless of the solid support chosen, its binding characteristics must be evaluated since plastics differ in their binding capacities depending upon the antigen.

Advance Preparation

- An immunoassay should be performed to determine the optimal binding conditions for the antigen of interest prior to selection of antigen-positive phage and screening of individual clones.
- Prepare log phase **TG1** cells as follows. Transfer a colony from a minimal medium plate to 5 ml of 2x YT medium and incubate overnight at 37°C with shaking at 250 rpm.
- Inoculate 10 ml of fresh 2x YT medium with 100 µl of the overnight culture and incubate at 37°C with shaking at 250 rpm until the culture reaches an A_{600} of 0.3.

PEG Precipitation of Recombinant Phage

E. coli **TG1** cells produce an amber suppressor tRNA which allows read-through (suppression) of the amber stop codon located between the cloned ScFv and gene 3 sequences of **pCANTAB 5 E**. Since suppression of the amber stop codon in TG1 cells is only about 20% efficient, soluble antibodies will be produced in addition to phage-displayed recombinant antibodies. By PEG precipitating the phage-displayed antibodies, they are purified away from the soluble antibodies which may also compete for antigen during selection.

- Add 2 ml of PEG/NaCl (see Appendix 2, page 34) to the 10 ml of recombinant phage antibody supernatant (from Procedure D, page 15). Mix well and place on ice for 30-60 minutes.
- Spin at 10,000 x g in a Beckman JA-20 rotor for 20 minutes at 4°C. Discard the supernatant. Invert the tube over a clean paper towel to remove all the buffer. Resuspend the pellet (which may not be easily visible) in 16 ml of 2x YT medium. We recommend that the supernatant be filtered through a 0.45 µm filter if it will be stored (at 4°C).

– Note: PEG precipitation and panning of phage should be performed as soon as possible following rescue since some phage-displayed recombinant antibody preparations may be unstable.

Panning

- Coat a 25 cm^2 tissue culture flask with 5 ml of antigen diluted to 10 µg/ml in an appropriate buffer, e.g. PBS or 0.05M Na_2CO_3 (pH 9.6). Coating with antigen may be performed for 1-2 hours at room temperature or overnight at 4°C.
  – Note: The conditions for coating the plate, i.e. buffer and incubation temperature and time, depend on the antigen and should be similar to the immunoassay conditions used for the original polyclonal or monoclonal antibody from which the new recombinant was derived. The coating concentration of the antigen can be varied depending on the affinity (antigen-binding capability) of the recombinant phage antibody desired. Less antigen is required for high affinity antibodies than for those with low affinity. However, solution-based selection may be preferable to solid-phase selection for isolating antibodies with specific affinities since the amount of antigen used in the selection can be more accurately controlled.
- Wash the flask three times with PBS, emptying it completely after each wash.
- Fill the flask completely with blocking buffer (see Appendix 2, page 34) to block any remaining sites on the flask surface. Incubate at room temperature for 1 hour. Wash the flask three times with PBS, emptying it completely after each wash.

- Prepare 14 ml of blocking buffer containing 0.01% thimerosal or 0.01% sodium azide as a preservative.
- Dilute the 16 ml of PEG-precipitated recombinant phage with 14 ml of blocking buffer (which contains a preservative) and incubate at room temperature for 10-15 minutes.
  – Note: Non-specific, hydrophobic protein-protein interactions may occur between native M13 phage proteins and some antigens during the panning step. This interaction can be reduced if Triton X-100 is added to the diluted phage supernatant to a final concentration of 0.1%.
- Add 20 ml of the diluted recombinant phage to the flask and incubate for 2 hours at 37°C. Empty the flask.
- Wash the flask 20 times with 30-50 ml of PBS and 20 times with PBS containing 0.1% Tween 20 (a wash bottle works well for dispensing the wash solutions). Empty the flask completely each time.
- To isolate colonies for small-scale rescue, reinfect *E. coli* **TG1** cells with bound phage directly in the panning vessel and plate the reinfected cells (Procedure F, page 20). If subsequent rounds of panning are to be performed, proceed directly to Appendix 7, page 43.

Procedure F: Reinfection of *E. coli* with Enriched Phage Clones

- Add the entire 10 ml of log-phase **TG1** cells (see Advance Preparation, page 17) to the flask or panning vessel. Incubate at 37°C for 1 hour.
- After 1 hour, remove 100 µl of the 10 ml cell suspension. Prepare ten-fold dilutions of the cell suspension in 2x YT medium (1:10, 1:100, 1:1000).
- Plate 100 µl of undiluted cells and 100 µl of each dilution onto separate SOBAG plates using a sterile glass spreader. When dry, invert the plates and incubate overnight at 30°C. If the colonies are too small to pick after incubation, leave at 30°C for an additional 4-8 hours.
  - Note: To prepare a frozen stock culture, add 800 µl of reinfected cells from the flask to 200 µl of sterile 80% glycerol and mix with a pipet tip. Store at -70°C. Refer to the note on the bottom of page 13 for instructions concerning the preparation of a fresh culture from a glycerol stock. The remainder of the reinfected cells can be stored for several days at 4°C in a polypropylene centrifuge tube for later use if the plating was unsuccessful.
  - Note: The SOBAG plates can be handled as follows:

    a) Scrape the cells from the plate to generate stock cultures. Flood the plate with 5 ml of 2x YT medium and scrape the cells into the medium with a sterile glass spreader. Add glycerol to a final concentration of 15-30% and store at -70°C.

    b) Seal the plates and store for up to 2 weeks at 4°C for rescue at a later time.

Preparation for Screening

The ELISA to be used for screening should be optimized before preparing phage-displayed and soluble recombinant antibodies. The amount of

antigen to be used for screening must be determined empirically and will depend on the nature of both the antigen and the specific antibody used. In general, concentrations between 1 and 100 µg/ml in a volume of 50 to 200 µl should be evaluated for coating the wells.

Biochemically similar proteins should also be tested with the phage-displayed and soluble antibody preparations to determine specificity. We recommend that a microtiter plate be coated with the antigen of interest and a separate plate coated with a negative control antigen.

Soluble antibody clones require only one period of overnight growth and will be ready for screening by ELISA the following day. Phage-displayed clones require two periods of overnight growth before screening can be performed. We recommend that both phage-displayed and soluble antibodies be prepared concurrently and screened for binding to the antigen of interest.

- Add 400 µl of 2x YT-AG medium to each cluster tube (96 tubes in a microtiter format, Costar #4411).
  - Note: Alternatively, single colonies can be grown up in sterile 17 x 100 mm polypropylene tubes.
- Transfer individual well-isolated colonies to separate tubes using sterile toothpicks or pipet tips. This is the ***Master Plate.*** It will serve as the source from which phage recombinant antibodies and soluble antibodies will be prepared for screening. See Procedures G and H.
- Incubate overnight at 30°C with shaking at 250 rpm.
  - Note: The 30°C incubator should be humidified to prevent evaporation of medium from the cluster tubes. Alternatively, the cluster tubes can be placed in a sealable plastic box containing moist paper towels.

Procedure G: Screening of Phage Antibodies from Enriched Clones

In Procedure F, *E. coli* **TG1** cells were infected with recombinant phage antibodies and plated onto SOBAG medium. The recombinant phage-infected cells contain the **pCANTAB 5 E** phagemid with the antibody ScFv gene insert. In the following procedure, each clone or colony is transferred to a separate tube for rescue with **M13KO7**. Initiation of the M13 phage life cycle ultimately leads to the production of phage which display recombinant antibodies. The recombinant phage antibodies which arise from each clone are then assayed for antigen-binding activity in an immunoassay (e.g. an ELISA).

Essential Preliminaries

In Procedure I, log phase *E. coli* **HB2151** cells are infected with recombinant phage produced in Procedure G. Since an overnight incubation is required for growth of the cells, it is important to inoculate the culture when Procedure G is begun. See Procedure I, page 26, for instructions concerning preparation of log phase **HB2151**.

Phage Rescue

- For each set of 96 cluster tubes comprising the ***Master Plate*** (see Procedure F, page 20), prepare 50 ml of 2x YT-AG medium containing 2.5×10^{10} pfu of **M13KO7**.
- Pipette 400 µl of the **M13KO7** phage suspension into each tube of a new set of 96 cluster tubes. Label this set of tubes as plate P1.
  - Note: Alternatively, use polypropylene tubes or plates, or treat polystyrene plates with blocking buffer to prevent phage from sticking nonspecifically to the plastic.
- Transfer 40 µl of saturated culture from each tube of the ***Master Plate*** to a corresponding tube in plate P1.

- Incubate plate P1 for 2 hours at 37°C with shaking at ~150 rpm. The cultures should appear turbid after incubation.
  - Note: The 37°C incubator should be humidified to prevent evaporation of medium from the cluster tubes. Alternatively, the cluster tubes can be placed in a sealable plastic box containing moist paper towels.
- Centrifuge the P1 plate at 1500 x g for 20 minutes at room temperature in an IEC Refrigerated Centrifuge, or equivalent (see Appendix 2, page 34) fitted with microtiter plate adaptors. Alternatively, the contents of each tube can be transferred to individual microcentrifuge tubes and sedimented at 1500 x g for 20 minutes in a microcentrifuge.
- Carefully remove the supernatant from each pellet and discard the supernatants in an appropriate waste container.
- Prepare 50 ml of 2x YT-AK medium (***NO GLUCOSE***). Transfer 400 µl of medium to each tube in the P1 plate.
- Incubate the plate overnight at 37°C (in a humidified incubator) with shaking at 250 rpm.
- Centrifuge the plate as described above. Carefully transfer 322 µl of each supernatant (which contains recombinant phage antibodies) to a corresponding tube in a new set of 96 cluster tubes. Label this plate as P2.
- Prior to adding blocking buffer (see below), transfer 2 µl of recombinant phage supernatant from each clone to tubes containing log phase *E. coli* **HB2151** cells prepared according to instructions in Procedure I, page 26.
  - Note: If the ELISA is not continued at this point, the supernatants may be stored at 4°C in the cluster tubes, microcentrifuge tubes or polypropylene tubes. However, some phage antibodies may be unstable during storage.
- Add 80 µl of blocking buffer (see Appendix 2, page 34) to each tube in plate P2 containing 320 µl of supernatant and incubate for 10 minutes at room temperature.
- Perform an ELISA as described in the RPAS Detection Module.

Procedure H: Screening of Soluble Antibodies from Enriched Clones

E. coli **TG1** produces a suppressor tRNA which will allow readthrough (suppression) of the amber stop codon located between the ScFv and gene 3 sequences of **pCANTAB 5 E**. Since suppression of the amber stop codon in TG1 cells is only about 20% efficient, soluble antibodies will be produced, as well as phage-displayed recombinant antibodies. Small-scale soluble antibody preparations may therefore be produced and screened in **TG1** cells as an alternative to immediately infecting **HB2151** cells. However, since the yield of soluble antibodies will be greater in the **HB2151** nonsuppressor strain, we recommend that following identification of antigen-positive clones, the corresponding phage be used to infect **HB2151** cells (see Procedure I, page 26) for the purpose of large-scale production of soluble antibodies.

- Pipette 400 µl of 2x YT-AG into each tube in a new set of 96 cluster tubes. Label this plate as S1.
- Transfer 40 µl of saturated culture from each tube of the ***Master Plate*** (see Procedure F, page 20) to a corresponding tube in plate S1.
  - Note: At this point, the ***Master Plate*** can be sealed with Parafilm® or plate sealer (see Appendix 2, page 34) and stored at 4°C for up to 2 weeks. Alternatively, add glycerol to a final concentration of 15-30% and store at -70°C.
  - Note: After antigen-positive clones have been identified, an additional set of individual freezer stocks should be prepared in cryovials.
- Incubate plate S1 for 2 hours at 30°C with shaking at 250 rpm. The cultures should appear turbid after incubation.

  - Note: The 30°C incubator should be humidified to prevent evaporation of medium from the cluster tubes. Alternatively, the cluster tubes can be placed in a sealable plastic box containing moist paper towels.
- Centrifuge the plate at 1500 x g for 20 minutes at room temperature in an IEC Refrigerated Centrifuge, or equivalent, fitted with microtiter plate adaptors. Alternatively, the contents of each tube can be transferred to individual microcentrifuge tubes and sedimented at 1500 x g for 20 minutes in a microcentrifuge.
- Carefully remove the supernatant from each pellet and discard the supernatants in an appropriate waste container.
- Prepare 50 ml of 2x YT-AI medium (***NO GLUCOSE***). Transfer 400 µl of medium to each tube in plate S1.
- Incubate the plate for at least 3 hours at 30°C (in a humidified incubator) with shaking at 250 rpm.
- Centrifuge the plate as described above. Carefully transfer 320 µl of each supernatant (which contains soluble recombinant antibodies) to a corresponding tube in a new set of 96 cluster tubes. Label this plate as S2.
  - Note: If the ELISA is not continued at this point, the supernatants may be stored at 4ºC in the cluster tubes, microcentrifuge tubes or polypropylene tubes.
- Add 80 µl of blocking buffer (see Appendix 2, page 34) to each tube containing 320 µl of supernatant and incubate for 10 minutes at room temperature.
- Perform an ELISA as described in the instruction booklet which accompanies the Anti-E Tag Antibody (27-9412-01 and -02).

Procedure I: Infection of *E. coli* HB2151 Cells

In this procedure, recombinant phage from individual clones are used to infect *E. coli* **HB2151** cells. Once antigen-positive clones have been identified by ELISA, infected cells will be used to produce a soluble antibody preparation for each clone.

The *E. coli* strain **HB2151** infects quite poorly, and selection of transductants on medium containing ampicillin, glucose, and nalidixic acid (SOBAG-N, see below) ensures that resulting colonies are true nal^r **HB2151** transductants and not due to carryover of infected **TG1** cells.

See Appendix 3, page 38 for instructions on preparing and maintaining *E. coli* **HB2151** cells.

- Prepare log-phase **HB2151** cells as follows. Transfer a colony from a minimal medium plate to 5 ml of 2x YT medium and incubate overnight at 37°C with shaking at 250 rpm. Inoculate 50 ml of fresh 2x YT medium with 500 µl of the overnight culture and incubate at 37°C with shaking at 250 rpm until the culture reaches an A_{600} of 0.3-0.5.
- Transfer 400 µl of the log phase culture into each tube in a new set of 96 cluster tubes.
- For each clone, add 2 µl of recombinant phage antibodies (see Procedure G, page 22) to 400 µl of log phase **HB2151** cells. Incubate with intermittent gentle shaking for 30 minutes at 37°C.
- Once antigen-positive clones have been identified by ELISA, streak individual SOBAG-N plates (using a sterilized inoculating loop) with a loopful of the infected culture from each antigen-positive clone and incubate overnight at 30°C.
- Transfer individual colonies for production of soluble antibodies as outlined in Procedure J, page 27.

Procedure J: Production of Soluble Antibodies

After infecting *E. coli* **HB2151** cells with antigen-positive phage clones, soluble antibodies can be produced. It is important to prepare and assay the supernatant, periplasmic extract, and whole cell extract to verify that soluble antibodies are produced and to determine the site(s) in which they are concentrated.

Supernatant

- Prepare an overnight culture as follows: Transfer a colony from a SOBAG-N plate to 5 ml of freshly prepared SB-AG or 2x YT-AG medium and incubate overnight at 30°C with shaking at 250 rpm.
  - Note: More than one clone may require evaluation. It is possible that a clone may contain a stop codon which may not interfere with the production of phage-displayed antibodies in the *supE* strain **TG1** but may interfere with the production of soluble antibodies in the nonsuppressor strain **HB2151**.
- Add the 5 ml overnight culture to 50 ml of freshly prepared SB-AG or 2x YT-AG medium. Incubate for 1 hour at 30°C with shaking at 250 rpm.
- Centrifuge the culture at 1500 x g for 20 minutes at room temperature in an IEC Refrigerated Centrifuge (or equivalent).
- Carefully remove the supernatant from the sedimented cells and discard the supernatant in an appropriate waste container.
- Resuspend the sedimented cells in 50 ml of freshly prepared SB-AI or 2x YT-AI medium (***NO GLUCOSE***) and incubate for at least 3 hours at 30°C with shaking at 250 rpm.
  - Note: 250 ml baffled flasks should be used to ensure adequate aeration. Longer incubation times (up to 24 hours) may increase the yield of soluble antibodies. The optimal time of incubation for each clone must be determined empirically.

- Divide the culture into two separate centrifuge tubes and centrifuge at 1500 x g for 20 minutes at room temperature in an IEC centrifuge.
- Carefully remove the supernatants (which contain the extracellular soluble antibodies) from both pellets and transfer to a single clean container. Filter through a 0.8 µm filter or through a 0.45 µm filter with a pre-filter and store at -20°C. Reserve one pellet for preparation of the periplasmic extract and one pellet for preparation of the whole cell extract.

Periplasmic Extract

This procedure is modified from reference 3. See reference 4 for an alternate procedure for preparation of periplasmic extracts.

- To prepare the periplasmic extract, resuspend one of the two cell pellets in 0.5 ml of ice-cold 1X TES (see Appendix 2, page 34). Add 0.75 ml of ice-cold 1/5X TES and vortex to resuspend (this induces a mild osmotic shock). Incubate on ice for 30 minutes. Transfer the contents to a 1.5 ml microcentrifuge tube and centrifuge at full speed for 10 minutes. Carefully transfer the supernatant, which contains the soluble antibodies from the periplasm, to a clean tube. Store at -20°C until needed. Discard the pellet.

Whole Cell Extract

- To prepare the whole cell extract, resuspend the second pellet from the supernatant preparation in 0.5 ml of PBS and boil for 5 minutes. Pellet the cell debris as above and carefully transfer the supernatant, which contains the intracellular soluble antibodies, to a clean tube. Store at -20°C until needed. Discard the pellet.
  - Note: For procedures to detect soluble antibodies in the cellular fractions, recommendations for purification of soluble antibodies, and procedures for immunoassays including ELISAs, Western blots and dot blots, refer to the booklet included with the Anti-E Tag Antibody (27-9412-01 or -02).

Appendix 1: pCANTAB 5 E

The map of **pCANTAB 5 E**, presented on pages 30 and 31, shows the control regions and genealogy of the vector. The section above the vector map (V_H-Linker-V_L) depicts the orientation of a hypothetical ScFv fragment cloned into **pCANTAB 5 E**. Detailed sequence analysis of the vector follows. The sequence of **pCANTAB 5 E** was assembled from the sequences of its constituents. The restriction analysis was compiled using DNASIS™ software. The enzymes chosen for the analysis are those which we believe to have been commercially available in June, 1992. **pCANTAB 5 E** has not been tested with all of these enzymes, and therefore the accuracy of the tables cannot be guaranteed. Please contact your local Amersham Biosciences representative if a discrepancy is identified.

Control Regions

Expression control region: *lac* promoter: -35: 2144-2149; -10: 2168-2174; Operator: 2180-2200

Gene 3 signal sequence: 2269-2313; protein synthesis begins at the start of the gene 3 signal sequence (with GTG at position 2269)

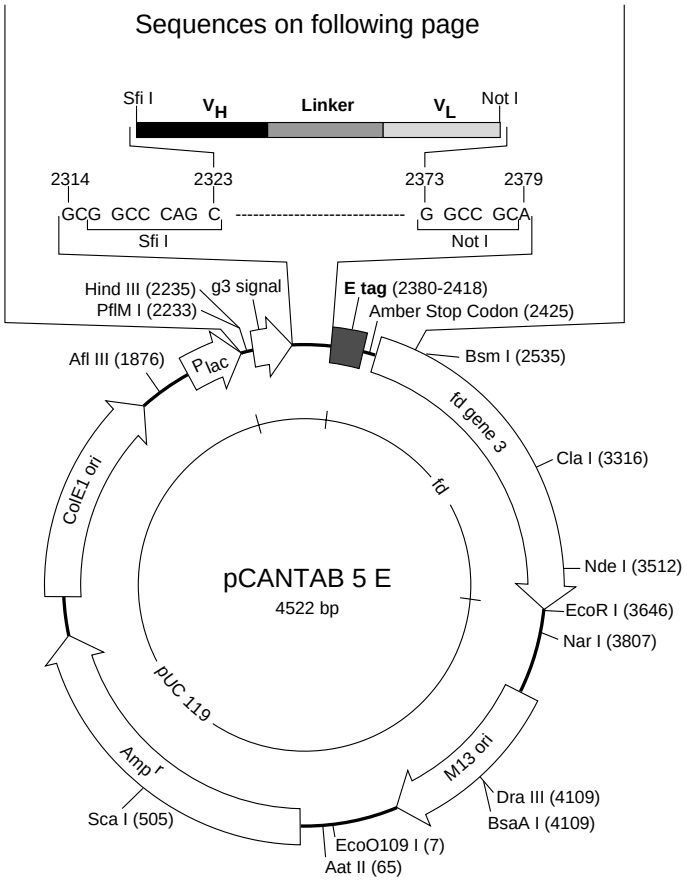
Gene 3 protein: 2428-3639 (two stop codons follow at bases 3640-3645)

M13 region: 3863-4336

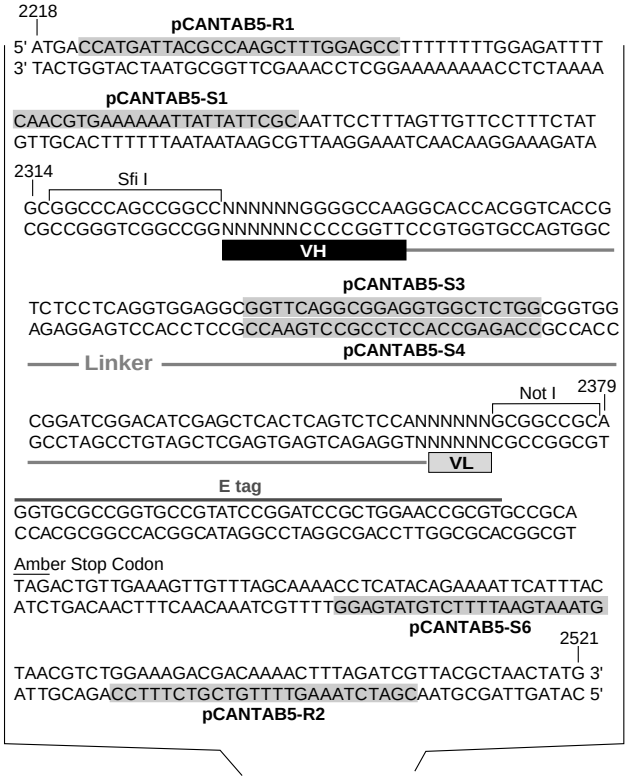
M13 origin of replication: 4173 (base 4174 is the first base of the newly synthesized strand)

- Note: Earlier instructions (anything prior to print code XY-038-00-05, Rev. 3) indicated the M13 ori in the opposite direction. This was incorrect. The map on page 30 shows the correct orientation.

(continued on page 32)



30



pCANTAB 5 E map on previous page

βlactamase gene region: Promoter: -35: 131-136; -10: 154-159; Start codon (ATG): 201; Stop codon (TAA): 1059*(continued on page 32)*

Plasmid origin of replication: 1819

Amber Stop Codon: 2425

E Tag: 2380-2418; **E Tag Sequence:** GGT GCG CCG GTG CCG TAT CCG GAT CCG CTG GAA CCG CGT

Restriction Enzyme Analysis

No sites: *Acc*65 I, *Afl* II, *Age* I, *Apa* I, *Apa*L I, *Asc* I, *Asu* II, *Ava* III, *Avr* II, *Bal* I, *Bbr*P I, *Bbs* I, *Bcl* I, *Bfr* I, *Bgl* II, *Bpu*1102 I, *Bsa*B I, *Bsg* I, *Bsi*W I, *Bss*H II, *Bst*1107 I, *Bst*B I, *Bst*E II, *Bst*X I, *Bsu*36 I, *Ecl*136 II, *Eco*47 III, *Eco*N I, *Eco*R V, *Esp* I, *Hpa* I, *Kpn* I, *Mam* I, *Mlu* I, *Msc* I, *Mun* I, *Nhe* I, *Nru* I, *Nsi* I, *Pac* I, *Pme* I, *Pml* I, *Ppu*M I, *Rsr* II, *Sac* I, *Sac* II, *Sau* I, *Sce* I, *Sma* I, *Sna*B I, *Spe* I, *Sph* I, *Spl* I, *Spo* I, *Srf* I, *Sse*8387 I, *Stu* I, *Swa* I, *Tth*111 I, *Xba* I, *Xcm* I, *Xma* I

One site: *Aat* II (65), *Acc* I (2350), *Afl* III (1876), *Ban* II (4036), *Bcg* I (461), *Bpm* I (898), *Bsa* I (916), *Bsa*A I (4109), *Bse*A I (2397), *Bsm* I (2535), *Bsp*D I (3316), *Bsp*E I (2397), *Bsp*M II (2397), *Cla* I (3316), *Dra* II (7), *Dra* III (4109), *Eag* I (2372), *Eam*1105 I (983), *Eco*O109 I (7), *Eco*R I (3646), *Hin*c II (2350), *Hin*d II (2350), *Hin*d III (2235), *Kas* I (3807), *Nar* I (3807), *Nco* I (2327), *Nde* I (3512), *Not* I (2371), *Pae*R7 I (2356), *Pfl*M I (2233), *Pst* I (2344), *Sal* I (2350), *Sca* I (505), *Sfi* I (2316), *Sgr*A I (2384), *Sty* I (2327), *Xho* I (2356)

Two sites: *Alw*N I (1462, 2976), *Ava* I (2356, 4218), *Bam*H I (2400, 3009), *Dsa* I (2327, 3552), *Eco*57 I (301, 1349), *Esp*3 I (4467, 4516), *Fsp* I (763, 3786), *Nae* I (2322, 4006), *Ngo*M I (2322, 4006), *Nsp* I (1876, 4481), *Pvu* I (616, 3766), *Pvu* II (2054, 3736), *Xmn* I (384, 3435)

pCANTAB 5 E, as supplied, has a 49-bp region removed by *Sfi* I/*Not* I digestion. The following restriction sites are affected. The numbers in parentheses indicate the location of the site(s) remaining.

One-site enzymes eliminated: *Acc* I, *Eag* I, *Hin*c II, *Hin*d II, *Nco* I, *Pae*R7 I, *Pst* I, *Sal* I, *Sty* I, *Xho* I

Two-site enzymes become one-site: *Ava* I (4218), *Dsa* I (3552), *Nae* I (4006), *Ngo*M I (4006)

Three-site enzymes become two-site: *Ava* II (624, 846), *Bgl* I (864, 3792), *Bsp*M I (2369, 3047), *Sin* I (624, 846)

Additional Restriction Sites added by Linker Primers and 3' V_H Primer

The linker primers consist of two 93-base oligonucleotides which are complementary to each other and have homology with the 3'-end of the V_H gene and the 5'-end of the V_L gene. Twenty-four bases on either end of the linkers are complementary to the ends of the V_H and the V_L. The central 45 bases of the linkers encode the flexible $(Gly_4Ser)_3$ linker which joins the V_H and the V_L to form an ScFv fragment. In addition to restriction sites encoded by the sequence of **pCANTAB 5 E** itself, numerous restriction sites are encoded by the sequence of the 93-base linkers and an additional 8 bases upstream of the linker which are encoded by the 3' V_H primer.

Sites present (numbers in parentheses indicate number of recognition sites present): *Aci* I (4), *Alu* I (1), *Alw* I (1), *Asu* I (1), *Ban* I (1), *Ban* II (1), *Bsa*J I (2), *Bsm*A I (2), *Bsp*1286 I (1), *Bsp*W I (2), *Bst*E II (1), *Bsu*36 I (1), *Dde* I (2), *Dpn* I (1), *Dpn* II (1), *Dsa* I (1), *Ecl*136 II (1), *Esp*3 I (1), *Hae* III (1), *Hgi*A I (1), *Hph* I (1), *Mae* III (1), *Mbo* I (1), *Mnl* I (3), *Nla* IV (2), *Sac* I (1), *Sau* I (1), *Sau*3A I (1), *Sau*96 I (1), *Sdu* I (1), *Sec* I (2), *Sty* I (1), *Taq* I (1)

Appendix 2: Additional Materials Required

Equipment

Clinical (tabletop) centrifuge with swinging-bucket rotor.

IEC Refrigerated Centrifuge (or equivalent) with adaptors for microtiter plates.

Microcentrifuge with variable-speed control.

Reagents

Cluster tubes (96 tubes in a microtiter format, Costar #4411).

Plate sealer (Costar #6524).

100 mM ATP (27-2056-01).

T4 DNA Ligase (27-0870-03).

IPTG (27-3054-01).

Media

Store media at room temperature and all plates (and LBG medium) at 4°C. Use media and plates within 2 weeks if they contain antibiotics or within 4 weeks if they do not contain antibiotics. Unless otherwise stated, additives should be added to medium after autoclaving and after medium has cooled to 50-60°C.

LBG Medium (LB + 20 mM glucose):
To 90 ml of sterile distilled water, add 1.0 g of Bacto-tryptone, 0.5 g of Bacto-yeast extract, and 0.5 g of NaCl. Stir until dissolved. Sterilize by autoclaving. After the medium has cooled to 50-60°C, add 1 ml of 2 M glucose. Adjust to 100 ml with sterile distilled water.

Minimal Medium Plates:
Prepare stocks of the following:

1 M $MgCl_2 \cdot 6H_2O$: Dissolve 20.33 g in distilled water to a final volume of 100 ml and autoclave.

1 M $CaCl_2 \cdot 2H_2O$: Dissolve 14.7 g in distilled water to a final volume of 100 ml and autoclave.

1 M thiamine hydrochloride: Dissolve 33.73 g in distilled water to a final volume of 100 ml and sterilize using a 0.22 µm filter.

20% glucose: Dissolve 20 g of D-(+)-glucose (anhydrous) in distilled water to a final volume of 100 ml and sterilize by filtration through a 0.2 µm filter. Do not autoclave.

In a 500 ml bottle, dissolve 6 g of Na_2HPO_4 (dibasic), 3 g of KH_2PO_4 (monobasic) and 1 g of NH_4Cl in distilled water to a final volume of 500 ml. Adjust the pH to 7.4 with NaOH. In a separate 1 liter bottle, add distilled water to 15 g of Bacto-agar to a final volume of 500 ml. Autoclave both bottles simultaneously to sterilize.

Cool both bottles to 50-60°C and combine. Add 1 ml of 1 M $MgCl_2 \cdot 6H_2O$, 1 ml of 1 M $CaCl_2 \cdot 2H_2O$, 1 ml of 1 M thiamine hydrochloride and 5 ml of 20% glucose.

Pour plates immediately.

SOBAG Medium:
To 20 g of Bacto-tryptone, 5 g of Bacto-yeast extract and 0.5 g of NaCl, add distilled water to ~900 ml and autoclave. After the medium has cooled to 50-60°C, add the following: 10 ml of sterile 1 M $MgCl_2$, 55.6 ml of sterile 2 M glucose and 5 ml of filter-sterilized 20 mg/ml ampicillin. For plates, add 15 g of Bacto-agar before autoclaving. Pour plates quickly.

SOBAG-N Medium:
SOBAG medium containing 100 µg/ml nalidixic acid.

2x YT Medium:
To 900 ml of distilled water add 17 g of Bacto-tryptone, 10 g of Bacto-yeast extract, and 5 g of NaCl. Stir until dissolved. Adjust to 1 liter with distilled water. Autoclave to sterilize.

2x YT-AG Medium:
2x YT medium containing 100 µg/ml ampicillin and 2% glucose.

2x YT-AK Medium:
2x YT medium containing 100 µg/ml ampicillin and 50 µg/ml kanamycin.

2x YT-AI Medium:
2x YT medium containing 100 µg/ml ampicillin and 1 mM IPTG.

2x YT-N Medium:
2x YT medium containing 100 µg/ml nalidixic acid.

2x YT-G Medium:
2x YT medium containing 2% glucose.

SB Medium:
To 900 ml of distilled water, add 35 g of Bacto-tryptone, 20 g of Bacto-yeast extract and 5 g of NaCl. Stir until dissolved. pH to 7.5 with NaOH. Adjust to 1 liter with distilled water. Autoclave to sterilize.

SB-AG Medium:
SB medium containing 100 µg/ml ampicillin and 2% glucose.

SB-AI Medium:
SB medium containing 100 µg/ml ampicillin and 1 mM IPTG.

Buffers and Solutions

TSS Buffer:
To 70 ml of sterile distilled water add 1.0 g of Bacto-tryptone, 0.5 g of Bacto-yeast extract, 0.5 g of NaCl, 10.0 g of polyethylene glycol (PEG, M.W. 3350), 5 ml of dimethylsulfoxide (DMSO), and 5 ml of 1 M $MgCl_2$. Stir until dissolved. Adjust the pH to 6.5 with HCl or NaOH. Adjust to 100 ml with sterile distilled water. Sterilize by filtering through a 0.22 µm filter. Store at 4°C. Stable up to 6 months.

1X 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 liter. Adjust the pH to 7.4 with NaOH and autoclave.

Blocking Buffer:
1X PBS containing 10% nonfat dry milk. Use immediately.

PEG/NaCl:
To 200 g of Polyethylene glycol 8000 and 146.1 g of NaCl, add distilled water to 1 liter. Heat to dissolve, and autoclave.

20 mg/ml Ampicillin:
Dissolve 100 mg in 5 ml of water, filter sterilize (using a 0.2 µm filter) and store at -20°C.

25 mg/ml Kanamycin:
Dissolve 125 mg in 5 ml of water, filter sterilize and store at -20°C.

2 M Glucose:
Dissolve 36 g in 70 ml of water. Add water to 100 ml and filter sterilize. DO NOT autoclave.

Nalidixic Acid (Sodium salt, Sigma catalog number N-4382):
Prepare a 100 mM stock solution (~25 mg/ml) in distilled water, filter sterilize and store at -20°C.

1X TES Buffer:
0.2 M Tris-HCl (pH 8.0), 0.5 mM EDTA, 0.5 M sucrose. Filter sterilize and store at 4°C. To prepare 1/5X TES Buffer, add 1 volume of 1X TES Buffer to 4 volumes of distilled water.

Appendix 3: Preparation/Maintenance of E. coli Stocks

IMPORTANT: The following steps should be carried out aseptically.

Growth

E. coli **TG1** and *E. coli* **HB2151** are supplied as lyophilized cultures.

Each lyophilized culture should be resuspended in 1 ml of LB or 2x YT medium. Incubate overnight at 37°C with shaking at 250 rpm. Using a sterilized inoculating loop, streak a minimal medium plate with a loopful of the overnight culture and incubate overnight at 37°C.

Short-Term Storage

Cells may be stored for up to two weeks (at 4°C) on minimal medium plates.

Long-Term Storage

Inoculate 5 ml of LB or 2x YT medium with a single colony from a minimal medium plate. Incubate overnight at 37°C with shaking at 250 rpm. To prepare glycerol stocks, add 200 µl of sterile 80% glycerol to 800 µl of stationary phase cells, mix well, and store at -70°C.

Appendix 4: Preparation of Competent Cells

The following protocol is derived from reference 2.

See Appendix 3, page 38 for instructions on preparing and maintaining *E. coli* **TG1** cells.

IMPORTANT: All steps in this procedure should be carried out aseptically.

- Using sterile technique, streak *E. coli* **TG1** cells from a glycerol stock onto a minimal medium plate. Grow overnight at 37°C.
- Transfer a colony from a minimal medium plate to 5 ml of 2x YT medium and incubate overnight at 37°C with shaking at 250 rpm. Inoculate 100 ml of fresh 2x YT medium with 1 ml of the overnight culture and incubate at 37°C with shaking at 250 rpm until the culture reaches an A_{600} of 0.4-0.5. (It is critical that the absorbance is not more than 0.5). This will take approximately 2.5-3 hours.
- Sediment the cells at approximately 2500 x g for 15 minutes at 4°C, then gently resuspend in 10 ml of ice-cold TSS and place on ice. Cells must be used for transformations within 2-3 hours.

Appendix 5: Electroporation

Preparation of Cells

- Inoculate 10 ml of 2x YT medium with an *E. coli* **TG1** colony from a minimal medium plate. Incubate at 37°C overnight with shaking.
- Inoculate 1 liter of 2x YT medium with the 10 ml overnight culture of **TG1** cells. Incubate at 37°C with shaking at 250 rpm until an A_{600} of 0.5-0.7 is achieved (approximately 2-2.5 hours).
- Place the flask on ice for 15-30 minutes.
- Spin at 4000 x g for 20 minutes at 4°C to sediment the cells.
- Decant the supernatant and resuspend the cells in 1 liter of ice-cold sterile 1 mM Hepes (pH 7.0).
- Spin as described above. Decant the supernatant and resuspend the cells in 500 ml of ice-cold sterile 1 mM Hepes (pH 7.0).
- Spin as described above. Wash the cells in 20 ml of sterile 1 mM Hepes (pH 7.0) containing 10% glycerol.
- Spin as described above. Resuspend the cells in a total volume of 2-3 ml of sterile 10% glycerol.
- Dispense in 50-100 µl aliquots and proceed to the Electroporation protocol on page 41 or freeze on Dry Ice and store at -70°C.

Preparation of DNA

- Extract the ligated ScFv/**pCANTAB 5 E** (as well as the two control reactions) once with an equal volume of phenol-chloroform and once with an equal volume of chloroform. Remove the aqueous phase and add 1/10 volume of 3 M sodium acetate and 2.5 volumes of ethanol. Place on Dry Ice for 15 minutes and then spin in a microcentrifuge at full speed for 5 minutes to pellet the DNA. Remove the supernatant and wash the pellet

with 1 ml of 70% ethanol. Spin for 5 minutes, discard the supernatant, and dry the pellet. Resuspend each DNA pellet in 20 µl of sterile distilled water. Alternatively, the DNA can be gel-purified. The DNA must be completely free of salt prior to electroporation.

Electroporation

– Note: The following protocol was developed using a Bio-Rad Gene Pulser.

- If electro-competent cells have been frozen, thaw vials on ice. Otherwise, proceed directly to the following step using freshly prepared cells.
- Transfer 50 µl of cells to a pre-chilled 0.2 cm cuvette. Add 2 µl of ***salt-free*** DNA containing ScFv/**pCANTAB 5 E** and shake to the bottom of the cuvette. Place on ice for 1 minute.
- Program the electroporator to give 25 µF, 2.5 kV at 200 ohms. Dry the cuvette with a tissue and place it in the electroporation chamber. Pulse once (should yield a pulse with a time constant of 4.5-5 msec).
- Immediately add 1 ml of fresh LB-G or 2x YT-G medium to the cuvette and cover and invert to resuspend the cells. Repeat nine times for a total of ten identical samples. Transfer the contents of nine cuvettes (~9 ml) to a 50 ml disposable culture tube containing 6 ml of 2x YT-G medium (***NO AMPICILLIN***). Incubate the culture for 1 hour at 37°C with shaking at 250 rpm.
- Add 75 µl of 20 mg/ml ampicillin and 6 x 10^{10} pfu of **M13KO7** to the cell suspension. Incubate the culture for 1 hour at 37°C with shaking at 250 rpm.
- Proceed with rescue according to Procedure D, page 14.
- Incubate the remaining 1 ml of electroporated cells for 1 hour at 37°C with shaking at 250 rpm. Use for plating and preparation of a glycerol stock.
- We also recommend that controls be electroporated (see Procedure C). Only one electroporation reaction is required for each control.

Appendix 6: Rescue of Library from Spleen

Although plating the transformed cells adds a day to the protocol, it does avoid the problem of clonal competition that can occur in broth cultures. By plating the cells, the chance for loss of recombinant antibody clones may be reduced.

- Incubate 900 µl of undiluted **TG1** cells transformed with the ScFv/**pCANTAB 5 E** for 1 hour at 37°C.
- Plate 100 µl of the transformed cells onto nine separate SOBAG plates using a sterile glass spreader. When dry, invert the plates and incubate overnight (20-24 hours) at 30°C.
- Scrape the cells from the plates by flooding each plate with 5 ml of 2x YT medium and scraping the cells into the medium with a sterile glass spreader.
- To perform the phage rescue, transfer 5 ml of the scraped cells into a sterile 50 ml disposable polypropylene centrifuge tube. Dilute the cells to an A_{600} of 0.3. Note the final volume of cells. Add ampicillin to 100 µg/ml and glucose to a final concentration of 2%.
- Incubate the culture for 1 hour at 37°C with shaking at 250 rpm.
- Add **M13KO7** to the culture at a multiplicity of infection (moi) of 5:1. Determine the number of pfu of **M13KO7** to add as follows: (5×10^8 cells/A_{600} Unit) x (moi of 5) x (A_{600} of ~0.5) x (final volume of cells).
- Incubate the culture for 1 hour at 37°C with shaking at 250 rpm.
- Sediment the cells by centrifugation and complete the rescue as described in Procedure D, page 14.
- To prepare frozen stocks from the remaining scraped cells, add glycerol to a final concentration of 15-30% and store at -70°C.

Appendix 7: Rescue of Phagemid Library Following Panning

- Add the entire 10 ml of log-phase **TG1** cells (see Procedure E, Advance Preparation, page 17) to the flask or panning vessel. Incubate with shaking (250 rpm) at 37°C for 1 hour.
- Transfer the entire 10 ml from the panning vessel into a sterile 50 ml disposable polypropylene centrifuge tube.
- To the cell suspension, add ampicillin to 100 µg/ml, and glucose to a final concentration of 2%. Also add 4×10^{10} pfu of **M13KO7** (volume of stock to add = 4×10^{10} pfu ÷ **M13KO7** pfu/ml).
- Incubate the culture for 1 hour at 37°C with shaking at 250 rpm.
- Sediment the cells by centrifugation and complete the rescue as described in Procedure D, page 14.

TROUBLESHOOTING GUIDE

After transformation, few or no colonies are visible on the plate.

- *The MicroSpin column purification was not performed according to the instructions provided in the Mouse ScFv Module.* The MicroSpin column should be spun as directed at 735 x g for 2 minutes. An increase of g-force or spin time may compromise the size selection properties of the gel matrix.
- *The PCR product was not fully cut with Sfi I and Not I (see Mouse ScFv Module)*. Restriction enzymes from Amersham Biosciences are highly recommended. The amount of restriction enzymes used and restriction digestion times should not be altered. Check efficiency of ligation of the Control Insert to pCANTAB 5 E.
- *The bacterial cells may not have been fully competent.* Determine the number of colonies per µg of uncut DNA. Efficiencies of $>10^7$ are achievable with chemically competent cells. Prepare fresh competent cells if necessary or use electroporation to obtain larger libraries.
- *An insufficient amount of assembled product was added to the ligation reaction.* Approximately 150 ng of assembled product is optimal in a ligation reaction with 250 ng of vector.

Colonies of untransformed, competent TG1 cells are visible after plating on SOBAG medium.

- *The SOBAG plates were not fresh*. Use SOBAG plates within 2 weeks of preparation to ensure full activity of the ampicillin.
- *The competent TG1 cells were contaminated with plasmid DNA*. Prepare competent cells using disposable plasticware.

Few or no colonies are visible on plates after reinfection of TG1 cells following panning.

- *Rescue of the recombinant phage library was not successful.*
  - Transformed cells stored at 4°C for longer than 2 weeks were used for rescue. Return to the stock of cells at -70°C.
  - The correct amount of helper phage was not used in the rescue. The number of pfu of **M13KO7** used for the rescue should not deviate from the amounts stated in the protocol – recheck calculations. Also, check the infectivity of the helper phage.
  - *E. coli* **TG1** cells were not stored on minimal medium; consequently, they lost the f' plasmid and the ability to produce f' pili. Without f' pili, reinfection of the cells by **M13KO7** is not possible. Also, **TG1** cells should be at log phase so that they are expressing the f' pili.
  - *E. coli* **TG1** cells were handled too vigorously during the rescue. Cells should not be spun at >1500 x g or be vortexed vigorously since this may compromise their capacity for phage infection.
  - The correct medium and temperature for incubation were not used at each step. Note that the final medium in the rescue contains ampicillin and kanamycin but NO glucose.
- *The panning step was not successful.*
  - Antigen coating of the plastic surface was unsuccessful. Conditions for coating the antigen onto the plastic surface should be tested with the hybridoma antibody or mouse polyclonal serum antibody to ensure that the antigen sticks to the plastic. The incubation time and temperature and the coating buffer must be compatible with the antigen. Alternatively, perform solution-phase panning.

– The blocking agent (2% nonfat dry milk) was not added with the recombinant phage supernatant during the binding step. When the blocking agent is omitted, recombinant phage antibodies may adsorb non-specifically to the plastic surface.

After panning and reinfection of TG1 cells with recombinant phage antibodies, all plates (even at 1000X dilution) are confluent with colonies.

- *During panning, the antigen-coated surface was not washed sufficiently prior to infection of TG1 cells with bound recombinant phage*. The washing steps should be followed according to the protocol.
- *The recombinant phage antibody solution was not diluted with blocking buffer before panning*; consequently, M13KO7 may have adhered nonspecifically to the antigen.

During small-scale rescue of enriched phage clones, tubes lose volume or dry out.

- *The incubators must be humidified to prevent evaporation.* Tubes or plates can also be incubated in sealed plastic boxes to minimize evaporation.

FUNCTION TESTING

pCANTAB 5 E is tested for its ability to ligate the **Control Inser**t and transform chemically competent TG1 cells at an efficiency greater than 1 x 10^6 transformants/µg. The **TG1** and **HB2151** cells are assayed for viability after lyophilization.

ORDERING INFORMATION

Expression Module	
27-9401-01	1 kit
Companion Products	
Mouse ScFv Module	
27-9400-01	5 reactions
Detection Module	
27-9402-01	20 96-well plates
Anti-E Tag Antibody	
27-9412-01	1 mg
27-9412-02	5 mg
pCANTAB 5 Sequencing Primer Set	
27-1585-01	250 pmol each
pCANTAB 5 Gene Rescue Primers	
27-1581-01	1 nmol each

Light Primer Mix 27-1583-01	10 µl
Heavy Primers 27-1586-01	10 µl each
Linker Primer Mix 27-1588-01	100 µl
RS Primer Mix 27-1589-01	100 µl
Anti-M13 Antibody 27-9410-01	1 mg
HRP/Anti-M13 Conjugate 27-9411-01	200 96-well plates

REFERENCES

1. Vieira, J. and Messing, J., *Methods Enzymol.* **153**, 3 (1987).
2. Chung, C. T., *Proc. Natl. Acad. Sci. USA* **86**, 2172 (1989).
3. Skerra, A. and Plückthun, A., Science **240**, 1038 (1988).
4. Neu, H. C., *J. Biol. Chem.* **240**, 3685-3692 (1965).