pFliTrx™ Peptide Display Vector

A Cloning Vector for Studying Protein-Protein Interactions

Catalog no. V1126-01

Version D
010402
28-0144

Invitrogen™
life technologies

www.invitrogen.com
tech_service@invitrogen.com

Table of Contents

Important Information

Shipping and Storage

pFliTrx™ is shipped on dry ice.

- Store lyophilized vector and sequencing primers at -20°C
- Store the GI826 glycerol stock at -80°C

Kit Contents

20 µg pFliTrx™ vector, lyophilized

GI826 *E. coli* glycerol stock

Primer	Sequence	Amount	Total Moles
FliTrx™ Forward Sequencing, lyophilized	5´-ATTCACCTGACTGACGAC-3´	2.2 µg	4.05×10^{-10}
Rsr Reverse Sequencing, lyophilized	5´-CCCTGATATTCGTCAGCG-3´	2.2 µg	4.05×10^{-10}

Genotype of GI826

F$^-$, *lac*I^q, *amp*C:: P_{trp} *c*I, Δ*fli*C, Δ*mot*B, *eda*::Tn*10*.

Note: This strain is wild-type for recombination (*rec*A$^+$), endonuclease A (*end*A$^+$), and restriction. Transformation efficiencies and DNA yield may be lower than expected. Please see page 7 for more information.

Materials Supplied by the User

The following solutions and equipment may be needed to use this product.

- 60 mm tissue culture plates (We recommend Nunclon® Delta, Naperville, IL.)
- 100 to 200 ml culture flasks
- 100 mg/ml ampicillin
- Blocking Solution (see **Recipes**, page 20)
- Wash Solution (see **Recipes**, page 20)
- IMC medium (see **Recipes**, page 19)
- RM medium (see **Recipes**, page 19)
- RMG ampicillin plates (see **Recipes**, page 19)
- 10 mg/ml L-tryptophan
- Nonfat, dry milk (Blocking powder)
- 5 M NaCl (sterile)
- 20% α-methyl mannoside (sterile, methyl α-D-mannopyranoside; Sigma)
- 25°C incubator
- Orbital shaker
- Vortex

continued on next page

Important Information, continued

The FliTrx™ Panning Kit

The FliTrx™ Panning Kit (Catalog no. K1129-01) is available to make panning for epitopes that bind your FLITRX fusion peptide easier and less time-consuming. The kit contains all of the reagents necessary for growing cells, inducing expression, and preparing the solutions used for panning.

Item	Amount
Induction Base Medium (used to make IMC medium)	5 pouches*
10 mg/ml tryptophan	15 ml
5 M NaCl	15 ml
20% α-methyl mannoside	87.5 ml
Blocking powder	5 g
RM Base Medium	1 pouch*
RMG Agar Base Medium	1 pouch*

*each pouch contains reagents to prepare 1 liter of medium.

The FliTrx™ Random Peptide Display Library

The FliTrx™ Random Peptide Display Library (Catalog no. K1125-01) is a premade peptide library constructed in the pFliTrx™ vector. A diverse library of random dodecapetides was cloned into the active site of the thioredoxin protein (*trx*A) in place of the multiple cloning site. Each peptide is then displayed on the cell surface, readily accessible for ligand binding. The library contains 1.77×10^8 primary clones.

Purchaser Notification

This product is sold under patent license from Genetics Institute, Inc. **for research use only**. Licenses for commercial manufacture or use may be obtained directly from:

Genetics Institute, Inc.
87 Cambridge Park Drive
Cambridge, MA 02140
Tel: 617-876-1170

Overview

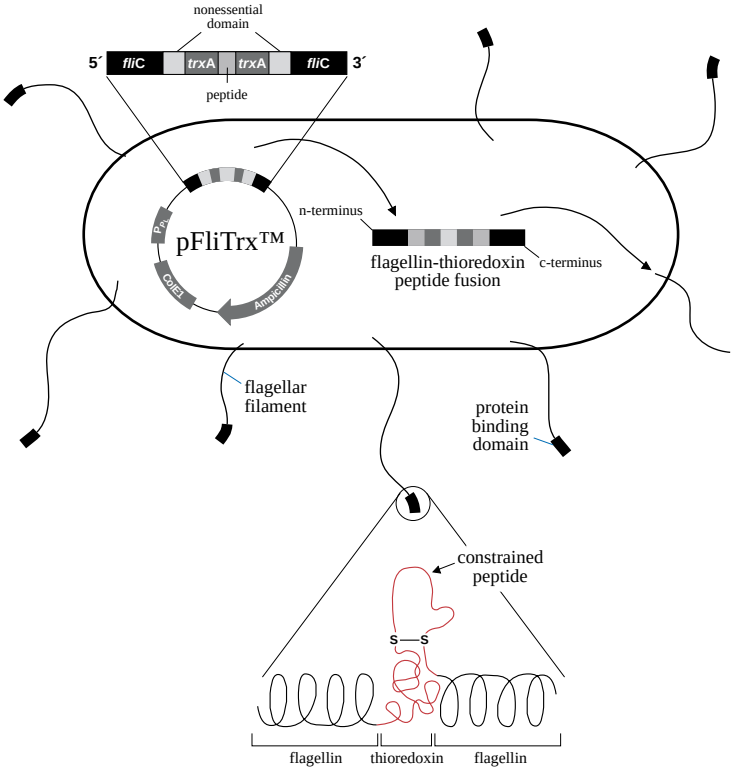
Introduction

The pFliTrx™ vector allow the display of peptides on the surface of *E. coli* by using the major bacterial flagellar protein (FliC) and thioredoxin (TrxA) (Lu *et al.*, 1995). Peptides are cloned into the active site loop of thioredoxin which is itself inserted into the dispensable region of the flagellin gene (*fli*C). This peptide fusion construct is expressed from the bacteriophage lambda major leftward promoter (P_L). When induced, the fusion protein (FLITRX) is exported and assembled into flagella on the bacterial cell surface, allowing display of the constrained peptide. The vector can be used for studying protein-protein interactions involved in receptor-ligand binding, enzyme-substrate specificity, and monoclonal antibody-antigen recognition.

Construction of the Peptide Library

The multiple cloning site of the pFliTrx™ vector allows peptides to be inserted in frame *within* the thioredoxin active site loop. This constrains the N- and C- terminal ends of the peptide resulting in a very well defined structural context (Lu *et al.*, 1995). The thioredoxin active site loop is known to protrude into the solvent, making the peptide accessible to binding (Katti *et al.*, 1990).

Thioredoxin is not normally displayed on the surface of *E. coli,* so DNA encoding the thioredoxin peptide fusion was cloned into the *fli*C gene replacing a large, solvent-exposed, nonessential domain. Expression of the FLITRX peptide fusion is controlled by the P_L promoter in the vector pFliTrx™. When induced, the construct is expressed and displayed on the surface of E. *coli*. The figure below shows schematically how the peptide is displayed.



continued on next page

Overview, continued

Expression from pFliTrx™

pFliTrx™ uses the P_L promoter from bacteriophage λ to drive expression. This promoter is one of the most efficient promoters for bacterial expression (Buell and Panayotos, 1986). It is also tightly regulated. The bacteriophage λ *c*I repressor binds to the operator region in front of the P_L promoter and controls the level of transcription from this promoter.

Expression of the *c*I repressor is also regulated. pFliTrx™ is propagated in *E. coli* cells (GI826) where the *c*I repressor gene is under control of the *trp* promoter. The *trp* repressor regulates expression of the *c*I repressor. When cells are grown in tryptophan-free medium (IMC medium), the *c*I repressor gene is transcribed, and the *c*I repressor protein binds to the P_L promoter preventing transcription. Expression is induced by adding tryptophan to the medium which shuts down *c*I repressor synthesis. This allows transcription from the P_L promoter (Mieschendahl *et al.*, 1986). The figure below shows how expression from pFliTrx™ is induced.

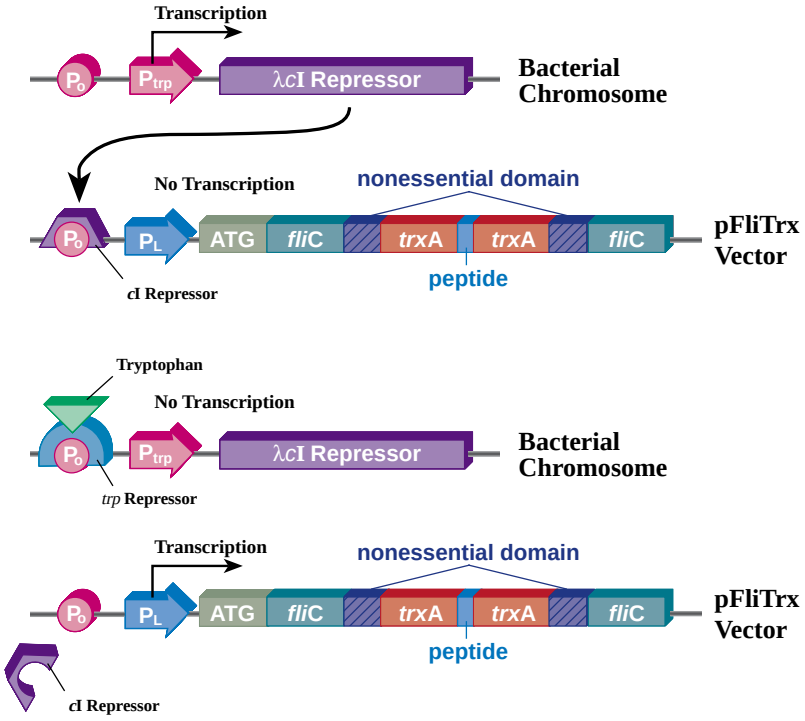
It is estimated that greater than 90% of individual peptide fusions assemble into functional flagella, and that they generally can be produced to a level of between 10-20% of the total cellular protein (Lu *et al.*, 1995).

1. In the absence of tryptophan, expression of the *c*I repressor is driven by the *trp* promoter.

2. The *c*I repressor protein binds to the operator region upstream of the P_L promoter and prevents transcription of the FLITRX peptide fusion.

3. Tryptophan is added to the medium and a tryptophan-*trp* repressor complex is formed. This complex binds tightly to the *trp* operator blocking expression of the *c*I repressor.

4. The *c*I repressor dissociates from the P_L operator, allowing transcription of the flagellin/ thioredoxin peptide fusion.



pFliTrx™

Description

The pFliTrx™ plasmid (5032 bp) was designed to express peptide fusions inserted into the active site of thioredoxin. Thioredoxin-peptide fusions are displayed on the surface of *E. coli* by inserting the fusions into the nonessential domain of flagellin.

Features of pFliTrx™

pFliTrx™ contains the following elements. All features have been functionally tested.

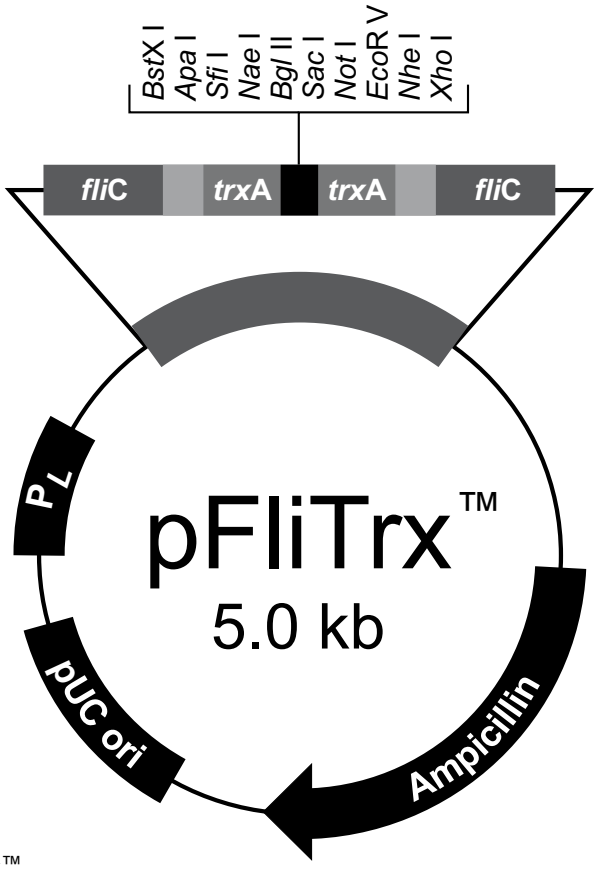
Feature	Benefit
P_L promoter	Permits efficient, tightly regulated, high-level expression of FLITRX peptide fusions (Buell and Panayotos, 1986)
*fli*C gene	Encodes the major bacterial flagellar protein Allows the thioredoxin fusion peptides to be displayed on the cell surface
*trx*A gene	Encodes the thioredoxin protein
Multiple cloning site	Allows insertion of your peptide into the active site of thioredoxin
pUC origin	High-copy number replication and growth in *E. coli*
Ampicillin resistance gene (β-lactamase)	Selection of vector in *E. coli*

continued on next page

pFliTrx™, continued

Map of pFliTrx™

The map below shows the elements of pFliTrx™. **The complete sequence of this vector is available for downloading from our Web site (www.invitrogen.com) or from Technical Services (see page 23).**



Comments for pFliTrx™
5032 nucleotides

Ampicillin Resistance Gene: bases 200-1061
pUC origin: bases 1206 - 1879
P_L Promoter: bases 2061-2510
fliC 5´ ORF: bases 2706-3434
trxA 5´ ORF: bases 3435-3536
Multiple Cloning Site: bases 3537-3597
trxA 3´ ORF: bases 3598-3821
fliC 3´ ORF: bases 3825-4271

Cloning into pFliTrx™

Introduction

The pFliTrx™ vector is designed to express and display a peptide on the cell surface. To accomplish this, the peptide insert must be cloned in frame with both the *trx*A 5′ ORF and the *trx*A 3′ ORF. The pFliTrx™ vector works best with peptides that are smaller than 36 base pairs in length.

General Molecular Biology Techniques

For help with DNA ligations, *E. coli* transformations, restriction enzyme analysis, DNA sequencing, and DNA biochemistry, please see *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989) or *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

Preparing Competent Cells

You may wish to prepare competent GI826 cells for transformation prior to setting up ligations. Please see the protocols for preparing chemically competent or electrocompetent GI826 cells on pages 8 and 9, respectively. Please note that only cells **without plasmid** may be grown in LB medium at 37°C. Growing cells containing plasmid at temperatures above 30°C or in LB medium may result in plasmid deletions or rearrangements.

Maintenance of pFliTrx™

In order to propagate and maintain the pFliTrx™ vector, we recommend that you re-suspend the lyophilized vector in 20 µl sterile water to make a 1 µg/µl stock solution. Store at -20°C.

Use this stock solution to transform the GI826 *E. coli* strain. Transformants are selected on RM plates containing 100 µg/ml ampicillin (see **Recipes**, page 19) and grown at 30°C. **Other *E. coli* strains are not suitable for use with pFliTrx™. DO NOT use LB medium to grow GI826 cells containing plasmid.**

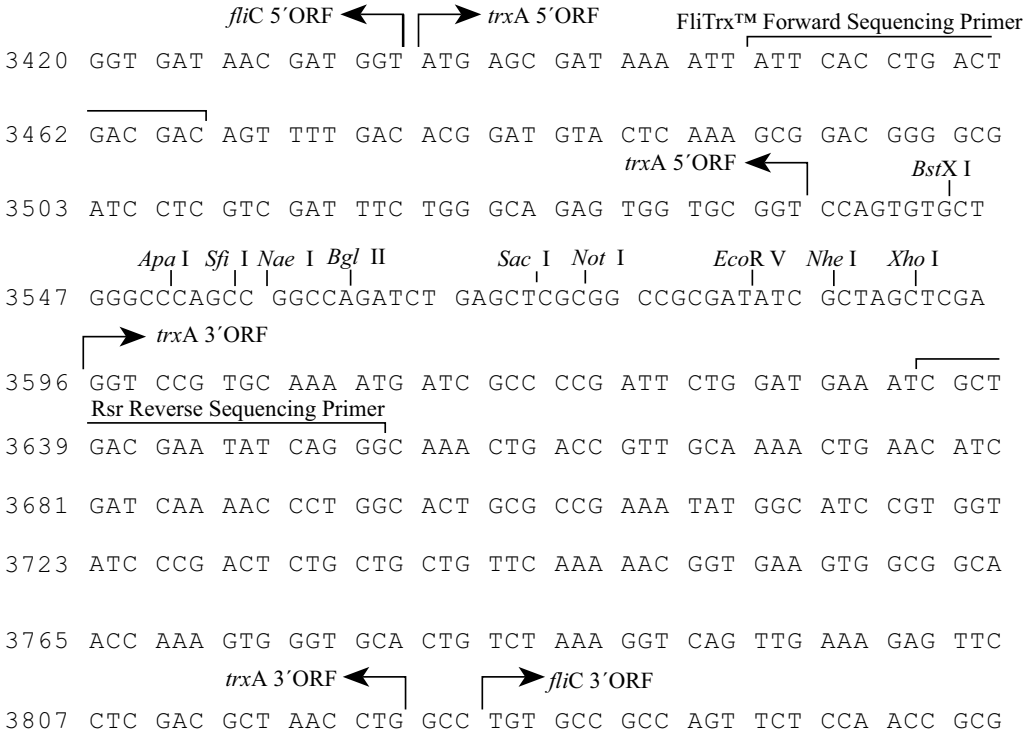
Note

The pFliTrx™ vector is a fusion vector. To ensure proper expression of your recombinant peptide, you must clone your gene in frame with both the 5′ open reading frame (ORF) and the 3′ ORF of the thioredoxin protein. This will create a fusion peptide with thioredoxin. It may be necessary to PCR amplify your gene of interest with ends that are appropriate for cloning into pFliTrx™. See page 6 to develop a cloning strategy.

Cloning into pFliTrx™, continued

Multiple Cloning Site of pFliTrx™

Below is the multiple cloning site for the pFliTrx™ vector. Restriction sites are labeled to indicate the cleavage site. The multiple cloning site has been confirmed by sequencing and functional testing. **The complete sequence of this vector is available for downloading from our Web site (www.invitrogen.com) or from Technical Services (see page 23).**



Transformation

Introduction

At this point you should have ligation reactions which are ready to be transformed into competent GI826. For procedures to prepare chemically competent cells, see page 8 and for preparing electrocompetent cells, see page 9. Transformed cells will be plated onto RMG-Amp plates (see **Recipes**, page 19) to select ampicillin resistant colonies.

Genotype and Characteristics of the *E. coli* Host

F$^-$, *lac*I^q, *amp*C:: P_{trp} *c*I, Δ*fli*C, Δ*mot*B, *eda*::Tn*10*.

Characteristics of the host strain, GI826

- Contains the *c*I repressor gene under control of the *trp* promoter (see page 2 for details of FLITRX expression).
- Deleted for the wild-type *fli*C gene that codes for flagellin to ensure that only the FLITRX peptide fusion proteins are displayed on the surface of the cell.
- Deleted for the gene *mot*B. Loss of MotB prevents flagellar rotation and improves binding of the cells to the target molecule.
- Tn*10* transposon confers tetracycline resistance. It is inserted into the *eda* locus which is near *mot*B.
- The *eda* locus encodes the enzyme 2-keto-3-deoxygluconate 6-phosphate aldolase.

Transformation Efficiency of GI826

GI826 is wild-type for restriction and modification of DNA; therefore your transformation efficiencies may be much lower than for strains like DH5α, JM109, or HB101. Typical transformation efficiencies are 5 x 10^5 cfu/µg DNA for chemically competent cells and 1 x 10^6 cfu/µg DNA for electrocompetent cells. You may have to transform with more DNA (1-10 ng), plate a larger volume of cells (100-300 µl), or increase the number of plates (2-5 plates) to obtain a reasonable number of transformants.

Before Starting

Before you begin your transformation procedure, please do the following:

- If transforming by electroporation, be sure to have on hand sterile, glass transfer pipettes, electroporation cuvettes, and sterile, 15 ml, polypropylene snap-cap tubes
- Determine the total number of transformations. Since you will be plating two different volumes from these reactions, you will need two RMG-Amp plates per transformation
- Make sure the SOC medium is at room temperature

continued on next page

Transformation, continued

Preparation of Chemically Competent Cells

Sterile technique is absolutely essential to avoid contamination of the competent cells. Remember to use **sterile** solutions, medium, and supplies. For **Recipes** see pages 20-22.

1. Streak GI826 on an LB plate, invert the plate, and incubate at 37°C overnight. **LB plates and 37°C incubators are only used for cells without plasmid.**
2. Inoculate 5 ml of SOB medium in a sterile culture tube with one colony from the LB plate.
3. Grow overnight (12-16 hours) in a shaking incubator (200-225 rpm) at 37°C.
4. Inoculate 100 ml of fresh SOB medium in a 500 ml or 1 liter culture flask with 2.5 ml of the overnight culture.
5. Grow the culture at 18°C at 200-225 rpm in a shaking incubator until the OD_{600} reaches between 0.5-0.6.
6. Place on ice for 15 minutes.
7. Centrifuge at 3000 x g for 10 minutes at 0-4°C.
8. Decant the medium and resuspend the pellet in 8 ml cold (0-4°C) TB buffer. Incubate on ice for 10 minutes.
9. Centrifuge the tube at 3000 x g for 10 minutes at 0-4°C.
10. Decant the buffer and resuspend each pellet in 2.5 ml cold TB buffer.
11. While gently swirling the tube, slowly add 938 µl of DMSO to the tube. Incubate on ice for 15 minutes.
12. While gently swirling the tube, slowly add an additional 938 µl of DMSO to the tube. Incubate on ice for 10 minutes.
13. For each cell preparation, aliquot 100 µl into screw-topped microcentrifuge tubes on ice. Keep cell suspension on ice.
14. Quick-freeze aliquots in liquid nitrogen. Store at -80°C.

Transformation

1. Equilibrate a water bath or heat block to 42°C. Remove the appropriate number of tubes of frozen GI826 chemically competent cells (100 µl/tube) and thaw on ice.
2. Add 3-5 µl of each ligation reaction to a separate tube of competent cells. Mix gently with the pipette tip. DO NOT PIPETTE UP AND DOWN. Repeat for all ligations.
3. Transfer all tubes to 42°C heat block or water bath and incubate for exactly 90 seconds, then place on ice for 1-2 minutes.
4. Add 800 µl of room temperature SOC medium to each tube and shake at 225 rpm for **45 minutes at 37°C**. Incubate the tubes horizontally and secure with tape to maximize aeration. Incubation at 37°C does not cause rearrangements or deletions in the plasmid for this short amount of time.
5. Plate 100 µl and 300 µl of each transformation mix on the RMG-Amp transformation plates. Let all the liquid absorb, invert, and **incubate at 30°C** overnight. Store the remaining transformation mix at +4°C. If you need to screen more transformants, plate out the remaining transformation mix the following day.

continued on next page

Transformation, continued

Preparation of Electrocompetent Cells

These cells are only to be used for electroporation. Do not use them for any other transformation protocol. **Sterile technique is absolutely essential to avoid contamination of the electrocompetent cells.** For **Recipes** see pages 20-22.

1. Streak GI826 on an LB plate, invert the plate, and incubate at 37°C overnight. **LB plates and 37°C incubators are only used for cells without plasmid.**
2. Inoculate 50 ml of LB medium in a 250 ml culture flask with a single colony from the LB plate and incubate at 37°C with shaking (200-225 rpm) for 12-16 hours (overnight).
3. Inoculate 1 liter of LB medium in a 2 liter or 4 liter flask with the 50 ml overnight culture. Grow the 1 liter culture in shaking incubator (200-225 rpm) at 37°C until the OD_{550} is between 0.5 and 0.6 (approximately 2-3 hours).
4. Transfer the 1 liter culture to two chilled, sterile, 500 ml centrifuge bottles and incubate on ice for 30 minutes.
5. Centrifuge the cultures at 2000 x g for 15 minutes at 0-4°C. Keep the cell pellet and decant the broth. Place bottles back on ice.
6. Resuspend the cell pellet in each bottle in approximately 500 ml of cold (0-4°C), sterile water.
7. Centrifuge cells at 2000 x g for 15 minutes at 0-4°C. Keep the pellet and decant the water. Place bottles back on ice.
8. Resuspend the cells in each bottle in approximately 250 ml of cold (0-4°C), sterile water.
9. Centrifuge cells at 2000 x g for 15 minutes at 0-4°C. Decant the water and place bottles back on ice.
10. Using a prechilled, sterile 25 ml pipette, resuspend cells in each bottle in 20 ml cold (0-4°C), sterile, 10% glycerol and transfer each cell suspension to a chilled, sterile, 50 ml centrifuge tube.
11. Centrifuge cells at 4000 x g for 15 minutes at 0-4°C. Decant the 10% glycerol and place tubes on ice.
12. Resuspend each cell pellet in 1 ml cold (0-4°C), sterile, 10% glycerol. Using a prechilled 5 ml pipette, pool the cells into one of the 50 ml tubes. Keep on ice.
13. For each cell preparation, place thirty-five 1.5 ml microcentrifuge tubes on ice and pipette 55 µl of the cell suspension into each tube. Keep cell suspension and tubes on ice until all of the cell solution is aliquoted.
14. After all of the cell suspension is aliquoted, quick-freeze tubes in a dry ice/ethanol bath and store at -80°C until ready for use.

continued on next page

Transformation, continued

Electroporation Transformation

1. Remove the appropriate number of microcentrifuge tubes of GI826 electrocompetent cells from the -80°C freezer and thaw on ice. Chill electroporation cuvettes on ice.
2. Set up your electroporation device for electroporation of bacteria using the manufacturer's instructions.
3. Add 1-2 µl of a ligation reaction to a tube containing 50 µl competent cells. Repeat for all ligation reactions.
4. Take one sample at a time and transfer the cell/DNA mix to an electroporation cuvette. Place the cuvette in the chamber and discharge the electrical pulse.
5. Remove cuvette and **immediately** add 800 µl room temperature SOC medium and transfer using a sterile glass pipette to a 15 ml snap-cap polypropylene tube (Falcon 2059 or similar). Place on ice.
6. Repeat steps 3-5 until all samples have been transferred to 15 ml tubes.
7. Incubate all tubes with shaking (200-225 rpm) at **37°C for 45 minutes**. Incubation at 37°C does not cause rearrangements or deletions in the plasmid for this short amount of time.
8. Plate 25 µl and 100 µl of the transformation mix on the RMG-Amp transformation plates. After the liquid is absorbed, invert and **incubate at 30°C** overnight.

Analysis

Once your peptide is cloned into pFliTrx™, we recommend that you sequence your construct to ensure that the peptide is in frame with the *trx*A gene. For sequencing, the FliTrx™ Forward and Rsr Reverse sequencing primers are provided in the kit.

Important

Constructs based on pFliTrx™ must always be propagated in GI826 in RM medium plus 100 µg/ml ampicillin and grown at 30°C. Other *E. coli* strains are not suitable for use with pFliTrx™. **DO NOT use LB medium with plasmid-containing GI826**. Growing cells containing plasmid at temperatures above 30°C or in LB medium may result in plasmid deletions or rearrangements.

Preparing a Glycerol Stock

Once you have identified the correct clone, be sure to purify the colony and make a glycerol stock for long-term storage. It is also a good idea to keep a DNA stock of your plasmid at -20°C.

- Steak the original colony out on an RM plate containing 100 µg/ml ampicillin. Incubate overnight at 30°C.
- Select a single colony and inoculate into 1-2 ml RM medium containing 100 µg/ml ampicillin.
- Grow the culture to mid-log phase (OD_{600} = 0.5-0.7).
- Mix 0.85 ml of the culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
- Store at -80°C.

Troubleshooting

If plasmid yield is low, try growing the cells longer to increase cell density.

If you get plasmid deletions or rearrangements, make sure you are transforming into GI826. Remember to always grow at 30°C and use RM medium with ampicillin.

If isolating plasmid by alkaline lysis, be sure to extract with phenol/chloroform to remove nucleases. **(Remember that GI826 is *end*A$^+$).** This will increase the stability of the plasmid preparation.

Basic Panning Protocol

Introduction

The panning procedure described below was developed to screen the pFliTrx™ fusion peptide for a monoclonal antibody epitope (Blond-Elguindi *et al.*, ; Lu *et al.*, 1995; Scott and Smith, 1990). In theory, other proteins can be used as a target as long as they can be immobilized on a solid support. Elution of selected bacteria is accomplished by simple mechanical shearing of the flagella.

If you wish to study an interaction between your particular peptide and a specific target molecule without panning, the following induction protocol (page 13) is still applicable for displaying the FLITRX fusion peptide on the cell surface.

Time Line of Panning Process

Once the FLITRX peptide fusion protein is expressed and displayed, cells may be screened by panning. Panning involves immobilizing the target protein and incubating cells displaying the peptide with the target. Cells expressing a peptide fusion that recognizes the target bind to the target. Unbound cells are washed away, and bound cells are eluted by mechanical shearing. Selected cells are further screened by repeat pannings. The table below outlines the panning process.

Day	Step	Action
1	1	Inoculate and grow the recombinant pFliTrx™ vector
2	2	First Panning: Dilute culture and induce expression of FLITRX peptide fusion protein
	3	Immobilize target protein to culture plate
	4	Block the plate to prevent non-specific binding
	5	Incubate induced recombinant pFliTrx™ vector with the target protein
	6	Wash off unbound cells
	7	Elute bound cells by mechanical shearing
	8	Inoculate new culture with eluted cells and grow overnight
3	9	Second Panning: Repeat steps 2 through 8
4	10	Third Panning: Repeat steps 2 through 8, and so forth
5	11	Fourth Panning
6	12	Fifth Panning
7	13	After growing the cells overnight from the fifth panning, streak or plate out cells to isolate single colonies
8	14	Select individual colonies and isolate plasmid DNA for sequencing

Important

- It is very important to use the IMC medium described on page 19. It contains low amounts of tryptophan which represses transcription from the P_L promoter and keeps the peptide fusion from expressing. Uncontrolled expression from the P_L promoter leads to plasmid deletions and rearrangements.
- Ampicillin should be fresh. **Degradation of ampicillin or failure to include ampicillin will lead to loss of the pFliTrx™ plasmid from the cells.**

continued on next page

Basic Panning Protocol, continued

pFliTrx™ Media Requirements

It is very important to use the correct medium to obtain optimal results. The table below describes the media used with pFliTrx™ and the function of each medium.

Medium	Purpose	Comments
IMC Medium (no tryptophan)	Preinduction growth of the pFliTrx™ vector	Do not use to grow selected clones for DNA isolation. The yield of plasmid is very low.
IMC Medium (100 µg/ml tryptophan)	Induction and expression of displayed peptides	Use **only** when inducing the vector for panning. Do not maintain cells on this medium as loss or rearrangement of individual plasmids may occur.
RM Medium	Used to increase the yield of plasmid for sequencing positive clones	Do not use as preinduction growth medium. Induction and expression of the peptides is better with IMC.
RMG plates	Used for selection of positive clones in pFliTrx™	--

Before Starting

Be sure to have the following reagents and equipment on hand before starting the panning procedure. See pages 18-19 for recipes. Most of the reagents can be found in the FliTrx™ Panning Kit (Catalog no. K1129-01). For more information on this kit, please see page vi.

- A culture flask containing 50 ml IMC Medium, 100 µg/ml ampicillin (**no tryptophan**)
- A culture flask containing 50 ml IMC Medium, 100 µg/ml ampicillin, **100 µg/ml tryptophan**
- RMG plates containing 100 µg/ml ampicillin (for plating of the panned library)
- 25°C shaking incubator and 30°C incubator
- 20 µg monoclonal antibody in 1 ml sterile water (DO NOT use a polyclonal antibody)
- 60 mm Nunclon® Delta plates
- Vortex
- 10 mg/ml L-tryptophan
- IMC Medium containing 100 µg/ml ampicillin
- Nonfat, dry milk
- 5 M NaCl (sterile)
- 20% α-methyl mannoside (sterile; methyl α-D-mannopyranoside, Sigma)
- Blocking Solution
- Wash Solution

continued on next page

Basic Panning Protocol, continued

Growth of Peptide Library (Day 1)

1. Inoculate 50 ml IMC Medium containing 100 µg/ml ampicillin **(no tryptophan)** with the cells transformed with pFliTrx™ and **grow with shaking (225-250 rpm) to saturation (OD_{600} = ~3) for 15 hours (overnight) at 25°C.**

Induction of Fusion Peptide (Day 2)

1. Take a sample from the culture and read the absorbance at 600 nm. You may have to dilute the cells 10-fold in medium to obtain an accurate reading. Calculate the volume needed to give 1 x 10^{10} cells (1 OD_{600} = ~1 x 10^9 cells for a 1 ml sample).
2. Induce expression of the pFliTrx™ fusion peptide by adding 1 x 10^{10} cells (~3 ml) of the overnight culture to 50 ml IMC Medium containing 100 µg/ml ampicillin and **100 µg/ml tryptophan.**
3. **Grow culture at 25°C with shaking for 6 hours.**

Immobilization of Antibody (Day 2)

1. Meanwhile, take a 60 mm tissue culture plate (Nunclon® Delta) and coat with 20 µg of antibody in 1 ml sterile water. Add antibody solution to the plate and gently agitate at 50 rpm on a orbital shaker for 1 hour.
2. Rinse the plate with 10 ml of sterile water.
3. Decant the water, add 10 ml of Blocking Solution, and gently agitate at 50 rpm for 1 hour.

Selection of Induced Cells (Day 2)

1. Just before the end of the 6 hour incubation, take a 50 ml sterile, conical centrifuge tube and add the following reagents:

   0.1 g dry milk
   300 µl 5 M NaCl
   500 µl 20% α-methyl mannoside
2. After the 6 hour incubation, remove 10 ml from the induced culture and add it to the centrifuge tube prepared in Step 1. Swirl to mix. Final concentration: 1% nonfat, dry milk; 150 mM NaCl; 1% α-methyl mannoside.

   Note: α-methyl mannoside is required to prevent selection of fimbriated *E. coli*. Fimbriated *E. coli* adhere to antibodies via lectin-like interactions. Inclusion of α-methyl mannoside to the cultured library and in the washing and blocking solutions prevents the isolation of false positives.
3. Decant the Blocking Solution from the plate and add the 10 ml induced cell solution from Step 2. Gently agitate at 50 rpm for 1 minute, then incubate at room temperature for 1 hour. It is very important to handle the plates gently as the flagella will shear, resulting in the loss of positive clones.
4. Decant bacterial culture (unbound cells).
5. Gently wash the plate with 10 ml Wash Solution at 50 rpm for **only** 5 minutes and decant. Repeat the wash 4 more times. **Do not wash longer than 5 minutes. Selection efficiency will decrease.**
6. After the fifth wash is decanted, elute the cells into the small volume of residual wash solution by vortexing the plate for 30 seconds. To vortex plate, place the plate flat against the top of the vortex cup and hold for 30 seconds.

continued on next page

Basic Panning Protocol, continued

Growth of Selected Cells (Days 2, 3, 4, 5, and 6)

1. Add 10 ml of IMC Medium containing 100 µg/ml ampicillin **(no tryptophan)** to the plate and pipet into a 50 or 125 ml culture flask.
2. Grow the 10 ml culture with shaking (225-250 rpm) at **25°C** for 15 hours.
3. Return to **Induction of Fusion Peptide**, Step 1 and repeat all steps to this point four more times.
4. After the fifth panning **and overnight growth** (Step 2, above), proceed to **Isolation of Positive Clones,** below.

Isolation of Positive Clones (Day 7)

1. Streak the overnight culture from the fifth panning onto RMG plates containing 100 µg/ml ampicillin to select for single colonies. **Incubate overnight at 30°C.**
2. Add glycerol to 20% to the selected cells and store it at -80°C until needed.

   (If you need to return to these cells for more clones, streak a small portion of the frozen stock on an RMG plate containing 100 µg/ml ampicillin to select for single colonies. Incubate overnight at 30°C.)
3. Proceed to **DNA Isolation and Western Blot Analysis**, next page.

Western Blot Analysis and DNA Isolation

Introduction

If you are attempting to identify the epitope recognized by a particular antibody, it is recommended that you test your selected clones using Western blot analysis. In this section, selected clones are grown under noninducing conditions for DNA isolation. A small amount of this culture is used to inoculate a second culture that will be induced to express the fusion peptides. The induced cells can be tested in a Western blot using the target antibody to check for positive clones before proceeding with DNA sequencing. You will need to be familiar with SDS-PAGE and Western blot procedures.

Before Starting

You will need the following reagents and equipment:

- RM medium containing 100 µg/ml ampicillin **(for DNA isolation)**
- IMC medium containing 100 µg/ml ampicillin and 100 µg/ml L-tryptophan
- 30°C shaking incubator and 37°C incubator
- Microcentrifuge
- SDS-PAGE sample buffer
- Boiling water bath
- SDS-PAGE buffers and apparatus for an SDS-PAGE gel
- Reagents for Western blot
- Plasmid DNA isolation method:
  - S.N.A.P.™ MiniPrep Kit (Catalog no. K1900-01) for isolation of very pure DNA for automated and manual sequencing
  - Alkaline Lysis

Preparation of Positive Clones for Analysis

1. Select 20 colonies from the RM/ampicillin plate (**Isolation of Positive Clones**, page 14, Step 1) and inoculate each colony into **2 ml RM medium containing 100 µg/ml ampicillin.** It is very important to use RM medium for this step to obtain high yields of plasmid DNA.
2. **Grow at 30°C to saturation (16-24 hours) with shaking.** Cells grown for 16 to 18 hours should have an OD_{600} of between 2.0 and 3.0.
3. Inoculate 2 ml IMC containing 100 µg/ml ampicillin and 100 µg/ml tryptophan with 40 µl of the culture from Step 2. **Grow at 37°C with shaking until the cells reach mid-log phase (0.3 to 0.6 OD_{600}, 6 to 16 hours).** Save the remaining uninduced cultures at +4°C for DNA isolations (next page). **Do not use induced cells for DNA isolation.**
4. While the cells are growing, prepare an SDS-PAGE gel for a Western blot.
5. Transfer 1.5 ml of the induced cells to microcentrifuge tubes and harvest the cells by centrifuging at maximum speed for 10-20 seconds.
6. Decant the supernatant and resuspend the cells in 100 µl SDS-PAGE sample buffer.
7. Boil the samples 5 minutes, load 5 µl of each sample onto the SDS-PAGE gel, and process.
8. Perform a Western blot using the gel from Step 7. Positive clones can be analyzed by DNA isolation and sequencing (next page).

continued on next page

Western Blot Analysis and DNA Isolation, continued

DNA Isolation and Sequencing

1. Return to the cultures you stored at +4°C in Step 3, previous page, and select all the clones that yielded positives in the Western blot.
2. Isolate plasmid DNA using one of the following methods:
   - S.N.A.P.™ MiniPrep Kit (Catalog no. K1900-01) is a rapid method that provides ultra-pure DNA for both automated and manual sequencing.
   - Alkaline Lysis miniprep, or any other method that provides pure DNA for sequence analysis, can also be used.
3. If you wish to analyze your clones by agarose gel electrophoresis, digest with *Eco*R I to linearize the plasmid DNA. *Eco*R I is unique to pFliTrx™.
4. Sequence all of the plasmids using the FliTrx™ Forward Sequencing primer and/or the Rsr Reverse Sequencing primer. Please see the diagram of the multiple cloning site on page 6 for sequence information.
5. After acquiring sequence information, proceed to **Analysis of Data**, below.

Analysis of Data

If you have cloned several inserts into the pFliTrx™ or have constructed a peptide library, we recommend that you sequence DNA from several colonies to generate enough data to identify the peptide recognized by the target protein. In many cases, the target molecule will recognize a contiguous peptide sequence that can be identified by consensus.

In other cases, the target molecule may recognize a structural motif or a discontinuous epitope that could be formed by a variety of peptide sequences. In these cases, a consensus sequence may not be obvious. It is recommended that a few of your positive peptides be chemically synthesized with cysteines at the N- and C-termini (Koivunen *et al.*, 1994; Luzzago *et al.*, 1993; Pasqualini *et al.*, 1995). The peptide is gently oxidized to enable formation of a disulfide bond between the cysteines. This conformationally constrained peptide can be tested for binding to the target molecule.

For More Information

For more information on peptide display, please see *Gene*, 1993, Volume 128, Number 1. This whole volume contains articles on peptide display and applications.

Technical Assistance™

Troubleshooting

Please use the following table to troubleshoot any problems you might have.

Problem	Reason	Solution
Difficulty isolating single colonies after panning	Plates are too dense	Serially dilute the overnight cell culture obtained after the fifth panning and plate 50 µl of each dilution on an RMG plate containing 100 µg/ml ampicillin. Use RM medium as the diluent. Grow overnight at 30°C and select single colonies.
No signal from Western	No protein transferred to support. Antibody does not recognize epitope when protein is denatured	Use the Anti-Thio™ antibody (Catalog no. R920-25) to detect peptide fusions (positive control). **Note**: this will detect **all** peptide fusions whether they are true panning positives or not.
No induction of peptide fusion	Incorrect medium used	Use only IMC medium to induce GI826. Remember to add tryptophan to induce the peptide library.
Low amount of plasmid DNA isolated	Loss of plasmid from the cell	Be sure to use RM medium and plates containing 100 µg/ml fresh ampicillin (< 2 weeks old)
No DNA sequence	Poor yield or quality of DNA template	Try isolating DNA using the S.N.A.P.™ MiniPrep Kit (Catalog no. K1900-01).
		Use the FliTrx™ Forward and Rsr Reverse Sequencing primers and PCR to amplify the peptide containing fragment. Clone into pCR®2.1 (Catalog no. K2000-01) or any other vector with priming sites outside the multiple cloning site and sequence.
Peptide is not displayed on cell surface	Insert is not in frame with the *trx*A and *fli*C genes	Consult the map on page 6 to design a strategy for cloning in frame into the pFliTrx™ vector.
	Insert is too large	Limit the size of your peptide to 36 base pairs
DNA sequence does not yield a consensus peptide, but Western is positive.	Epitope or recognition site is not contiguous.	Synthesize peptides and test for binding.

Recipes

Stock Solutions for Media

The following list describes the stock solutions needed to make IMC medium and IMC plates. **Please note that all the reagents needed to make the solutions in this section (except ampicillin and glucose) are provided in the FliTrx™ Panning Kit (see page vi).**

10X M9 Salts (for 1 liter)

60 g Na_2HPO_4
30 g KH_2PO_4
5 g NaCl
10 g NH_4Cl

1. Dissolve chemicals in 900 ml deionized water and pH to 7.4 with 10 M NaOH.
2. Add water to 1 liter and autoclave for 20 minutes on liquid cycle.
3. Store at room temperature.

1 M $MgCl_2$

1. Dissolve 20.33 g $MgCl_2$ in 100 ml deionized water.
2. Autoclave for 20 minutes on liquid cycle.
3. Store at room temperature.

100 mg/ml ampicillin

1. Dissolve 1 g ampicillin in 10 ml deionized water.
2. Filter-sterilize and store at -20°C.

20% glucose

1. Dissolve 20 g glucose (dextrose) in 100 ml deionized water.
2. Filter sterilize and store at room temperature.

10 mg/ml L-tryptophan

1. Dissolve 100 mg of L-tryptophan in 10 ml deionized water.
2. Filter sterilize and store at +4°C in an amber tube or bottle.

Important

You will need casamino acids to make IMC medium. The casamino acids must contain low amounts of tryptophan to avoid constitutive expression of the pFliTrx™ vector. We recommend purchasing casamino acids from the vendors listed below. DO NOT USE TECHNICAL GRADE CASAMINO ACIDS.

Vendors

If you need to purchase casamino acids, you may order from the following vendors:

Vendor	Chemical	Amount	Catalog number
Difco (1-800-521-0851)	Casamino Acids	100 g	0230-15-5
		500 g	0230-17-3
Sigma (1-800-325-3010)	Casamino Acids (Amicase)	250 g to 5 kg	A 2427

continued on next page

Recipes, continued

IMC Medium

1X M9 Salts
0.2% casamino acids
0.5% glucose
1 mM $MgCl_2$
100 µg/ml ampicillin

1. For 1 liter of induction medium mix 2 g casamino acids with 875 ml water and autoclave for 20 minutes on liquid cycle.
2. After the autoclaved solution has cooled, add aseptically:

10X M9 Salts	100 ml
1 M $MgCl_2$	1 ml
20% glucose	25 ml
100 mg/ml ampicillin	1 ml

3. Mix well and store medium containing ampicillin at +4°C. Medium is good for 1 month if stored at +4°C.

RM Medium

1X M9 Salts
2% Casamino Acids
1% glycerol
1 mM $MgCl_2$
100 µg/ml ampicillin

1. For 1 liter of RM medium mix 20 g Casamino Acids, 10 ml 100% glycerol, and 890 ml water and autoclave 20 minutes on liquid cycle.
2. After the autoclaved solution has cooled, add aseptically:

100 ml	10X M9 Salts
1 ml	1 M $MgCl_2$
1 ml	100 mg/ml ampicillin

3. Mix well and store medium containing ampicillin at +4°C. Medium is good for 1-2 weeks at +4°C.

RMG-Amp Plates

1X M9 Salts
2% Casamino Acids
0.5% glucose
1 mM $MgCl_2$
100 µg/ml ampicillin
1.5% agar

1. For 1 liter of plating medium, mix 20 g Casamino Acids, 15 g agar, and 875 ml water and autoclave for 20 minutes on liquid cycle.
2. Cool autoclaved solution to ~55°C and add aseptically:

100 ml	10X M9 Salts
1 ml	1 M $MgCl_2$
25 ml	20% glucose
1 ml	100 mg/ml ampicillin

3. Mix well and pour into 10 cm petri plates. Yield: ~40 plates.
4. Let agar harden, invert, and store plates in the dark at +4°C. Plates are good for 1-2 weeks at +4°C.

continued on next page

Recipes, continued

TB Buffer

Composition:

10 mM HEPES
55 mM $MnCl_2$
15 mM $CaCl_2$
250 mM KCl
pH 6.7

1. For 1 liter, dissolve 2.38 g HEPES, 2.21 g $CaCl_2$, and 18.64 g KCl in 950 ml deionized water.
2. Adjust the pH of the solution to 6.7 with KOH and bring the volume up to 1 liter.
3. Add 10.88 g $MnCl_2$.
4. Autoclave on liquid cycle for 20 minutes at 15 lb/sq. inch.
5. Store at room temperature or at +4°C.

Blocking Solution

IMC medium containing 100 µg/ml ampicillin
1% nonfat dry milk
150 mM NaCl
1% α-methyl mannoside

1. Prepare this solution right before use. To prepare 10 ml Blocking Solution, add:

   0.1 g nonfat, dry milk
   300 µl 5 M NaCl (sterilize by autoclaving at 121°C, 15 psi)
   500 µl 20% α-methyl mannoside (sterilize by filter sterilization)
   to 9.2 ml IMC medium containing 100 µg/ml ampicillin.
2. Vortex to dissolve and use immediately. **Do not store this solution.**

Wash Solution

IMC medium containing 100 µg/ml ampicillin
1% α-methyl mannoside

1. Prepare this solution right before use. To prepare 50 ml of Wash Solution, add 2.5 ml 20% α-methyl mannoside (sterilize by filter sterilization) to 47.5 ml IMC medium containing 100 µg/ml ampicillin.
2. Vortex to mix and use immediately. **Do not store this solution.**

continued on next page

Recipes, continued

LB (Luria-Bertani) Medium and Plates

Composition:

1.0% Tryptone
0.5% Yeast Extract
1.0% NaCl
pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 ml deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes at 15 lb/sq. inch. Allow solution to cool to 55°C and add antibiotic if needed.
4. Store at room temperature or at +4°C.

LB agar plates

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes at 15 lb/sq. in.
3. After autoclaving, cool to ~55°C, add antibiotic (50 µg/ml of ampicillin), and pour into 10 cm plates.
4. Let harden, then invert and store at +4°C.

SOB Medium

SOB (per liter)

2%	Tryptone
0.5%	Yeast Extract
0.05%	NaCl
2.5 mM	KCl
10 mM	$MgCl_2$

1. Dissolve 20 g tryptone, 5 g yeast extract, and 0.5 g NaCl in 950 ml deionized water.
2. Make a 250 mM KCl solution by dissolving 1.86 g of KCl in 100 ml of deionized water. Add 10 ml of this stock KCl solution to the solution in Step 1.
3. Adjust pH to 7.0 with 5 M NaOH and add deionized water to 1 liter.
4. Autoclave this solution, cool to ~55°C, and add 10 ml of sterile 1 M $MgCl_2$.
5. Store at room temperature or +4°C.

SOC Medium

SOC (per liter)

SOB Medium
20 mM glucose

1. Prepare and autoclave the SOB medium as described above.
2. After autoclaving, cool the solution to ~55°C, and add 10 ml of sterile 1 M $MgCl_2$ and 7.2 ml of 50% glucose.
2. Store at room temperature or +4°C.

continued on next page

Recipes, continued

DMSO

It is very important to use fresh, analytical grade DMSO. If you routinely transform cells by chemical means using the method of Hanahan, 1983, you probably have frozen aliquots of DMSO in your laboratory. If you do not use this method, then follow this procedure:

1. Order the smallest amount of analytical grade DMSO you can.
2. When the DMSO arrives, take about 5-10 ml and aliquot 200-500 µl per microcentrifuge tube. You may use the rest of the DMSO for other applications or you may aliquot the remainder for competent cells. It depends on whether you plan to use the method described in this manual on a routine basis.
3. Freeze these tubes at -20°C and use one tube per preparation of competent cells. Discard any remaining DMSO in the tube. **Use a fresh tube for every preparation of competent cells.**

Preparing IMC Medium Using Induction Base Medium

To make IMC Medium (1 liter) using Induction Base Medium:

1. Dissolve 12.6 g of Induction Base Medium (1 pouch) in 900 ml deionized water.
2. Adjust final volume to 1 liter.
3. Autoclave 20 minutes at 15 psi, 121°C.
4. Cool to room temperature and aseptically add 25 ml of 20% glucose and 1 ml of 100 mg/ml ampicillin. (Final concentration: 0.5% glucose, 100 µg/ml ampicillin).
5. Store medium containing antibiotic at +4°C. Medium is good for 1-2 weeks.

Technical Service

World Wide Web

Visit the Invitrogen Web Resource using your World Wide Web browser. At the site, you can:

- Get the scoop on our hot new products and special product offers
- View and download vector maps and sequences
- Download manuals in Adobe® Acrobat® (PDF) format
- Explore our catalog with full color graphics
- Obtain citations for Invitrogen products
- Request catalog and product literature

Once connected to the Internet, launch your Web browser (Internet Explorer 5.0 or newer or Netscape 4.0 or newer), then enter the following location (or URL):

http://www.invitrogen.com

...and the program will connect directly. Click on underlined text or outlined graphics to explore. Don't forget to put a bookmark at our site for easy reference!

Contact Us

For more information or technical assistance, please call, write, fax, or email. Additional international offices are listed on our Web page (www.invitrogen.com).

United States Headquarters:
Invitrogen Corporation
1600 Faraday Avenue
Carlsbad, CA 92008 USA
Tel: 1 760 603 7200
Tel (Toll Free): 1 800 955 6288
Fax: 1 760 602 6500
E-mail:
tech_service@invitrogen.com

Japanese Headquarters
Invitrogen Japan K.K.
Nihonbashi Hama-Cho Park Bldg. 4F
2-35-4, Hama-Cho, Nihonbashi
Tel: 81 3 3663 7972
Fax: 81 3 3663 8242
E-mail: jpinfo@invitrogen.com

European Headquarters:
Invitrogen Ltd
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF, UK
Tel (Free Phone Orders): 0800 269 210
Tel (General Enquiries): 0800 5345 5345
Fax: +44 (0) 141 814 6287

E-mail: eurotech@invitrogen.com

MSDS Requests

To request an MSDS, please visit our Web site (www.invitrogen.com) and follow the instructions below.

1. On the home page, go to the left-hand column under 'Technical Resources' and select 'MSDS Requests'.
2. Follow instructions on the page and fill out all the required fields.
3. To request additional MSDSs, click the 'Add Another' button.
4. All requests will be faxed unless another method is selected.
5. When you are finished entering information, click the 'Submit' button. Your MSDS will be sent within 24 hours.

continued on next page

Technical Service, continued

Emergency Information

In the event of an emergency, customers of Invitrogen can call the 3E Company, 24 hours a day, 7 days a week for disposal or spill information. The 3E Company can also connect the customer with poison control or with the University of California at San Diego Medical Center doctors.

3E Company
Voice: 1-760-602-8700

Limited Warranty

Invitrogen is committed to providing our customers with high-quality goods and services. Our goal is to ensure that every customer is 100% satisfied with our products and our service. If you should have any questions or concerns about an Invitrogen product or service, please contact our Technical Service Representatives.

Invitrogen warrants that all of its products will perform according to the specifications stated on the certificate of analysis. The company will replace, free of charge, any product that does not meet those specifications. This warranty limits Invitrogen Corporation's liability only to the cost of the product. No warranty is granted for products beyond their listed expiration date. No warranty is applicable unless all product components are stored in accordance with instructions. Invitrogen reserves the right to select the method(s) used to analyze a product unless Invitrogen agrees to a specified method in writing prior to acceptance of the order.

Invitrogen makes every effort to ensure the accuracy of its publications, but realizes that the occasional typographical or other error is inevitable. Therefore Invitrogen makes no warranty of any kind regarding the contents of any publications or documentation. If you discover an error in any of our publications, please report it to our Technical Service Representatives.

Invitrogen assumes no responsibility or liability for any special, incidental, indirect or consequential loss or damage whatsoever. The above limited warranty is sole and exclusive. No other warranty is made, whether expressed or implied, including any warranty of merchantability or fitness for a particular purpose.

References

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

Blond-Elguindi, S., Cwirla, S. E., Dower, W. J., Lipshutz, R. J., Sprang, S. R., Sambrook, J. F., and Gething, M.-J. H. (1993). Affinity Panning of a Library of Peptides Displayed on Bacteriophages Reveals the Binding Specificity of BiP. Cell *75*, 717-728.

Buell, G., and Panayotos, N. (1986) Mechanism and Practice. In *Maximizing Gene Expression*, W. Reznikoff and L. Gold, eds. (Boston, MA.: Butterworth Publishers).

Katti, S. K., LeMaster, D. M., and Eklund, H. (1990). Crystal Structure of Thioredoxin from *E. coli* at 1.68 Angstroms Resolution. J. Mol. Biol. *212*, 167-184.

Koivunen, E., Wang, B., and Ruoslahti, E. (1994). Isolation of a Highly Specific Ligand for the Alpha 5 Beta 1 Integrin from a Phage Display Library. J. Cell Biol. *124*, 373-380.

Lu, Z., Murray, K. S., VanCleave, V., LaVallie, E. R., Stahl, M. L., and McCoy, J. M. (1995). Expression of Thioredoxin Random Peptide Libraries on the *Escherichia coli* Cell Surface as Functional Fusions to Flagellin: A System Designed for Exploring Protein-Protein Interactions. Bio/Technology *13*, 366-372.

Luzzago, A., Felici, F., Tramontano, A., Pessi, A., and Cortese, R. (1993). Mimicking of Discontinuous Epitopes by Phage-displayed Peptides, I. Epitope Mapping of Human H Ferritin Using a Phage Library of Constrained Peptides. Gene *128*, 51-57.

Mieschendahl, M., Petri, T., and Hänggi, U. (1986). A Novel Prophage Independent *trp* Regulated Lambda P_L Expression System. Bio/Technology *4*, 802-808.

Pasqualini, R., Koivunen, E., and Ruoslahti, E. (1995). A Peptide Isolated from Phage Display Libraries is a Structural and Functional Mimic of an RGD-binding Site on Integrins. J. Cell Biol. *130*, 1189-1196.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Second Edition (Plainview, New York: Cold Spring Harbor Laboratory Press).

Scott, J. K., and Smith, G. P. (1990). Searching for Peptide Ligands with an Epitope Library. Science *249*, 386-390.