

## Introduction

This is intended as a brief guide to making rabbit polyclonal antibodies within the lab, not as a definitive work on the matter. For further reading, see the references given at the end of these sheets.

What will not be covered here is the raising of monoclonal antibodies.

## How Much Protein or Coupled Peptide is Required?

For the initial immunization about 200ug of protein or coupled peptide is needed and for each of the (normally six) subsequent boosts about 70-100ug is required.

To make an antibody affinity purification column, minimally 3mg of protein or coupled peptide is required, but more is definitely desirable. Try and aim for about 10mg, since the yield of purified antibody will be much greater. Affinity purification of antibodies (see later) takes several hours.

So the bottom line is that 10mg of protein or peptide should cover both the amount necessary to cover the rabbit immunizations and enable the affinity column to be made as well.

## Anti-Peptide Antibodies

### *Choosing a Peptide to Raise antibodies (see Section on Antigen Design and Administration)*

Try to choose a region on the protein where it is predicted to have good antigenicity (where known). Peptides derived from C- or N-terminal regions are often quite successful in this respect. For a review on this see C.M. Field et al 1998 Also for coupling the peptide to the affinity column matrix it would be advantageous to include a cysteine at either the N or C-terminal of the peptide to enable crosslinking.

### *Notes on handling of Peptides*

Peptide Reconstitution and Storage. Source: Eurogentec Bel s.a.

To avoid contamination with condensation, never open a vial containing peptide, until it has equilibrated with room temperature. Try to dissolve the peptide in pure water. If it does not dissolve easily, try sonication. If necessary add a small amount of aqueous acetic acid for a basic peptide, or aqueous ammonia for an acidic peptide. Once the peptide is dissolved, the buffer for the experiment can be added.

For some neutral or very hydrophobic peptides, polar organic solvents such as DMF or DMSO can be used. These should be added little by little while stirring until the peptide is dissolved. Then water or the buffer can be added to reach the final desired concentration. Note that organic solvents may have a harmful effect on biological systems.

Lyophilized peptides should be stored in a dessicator at 2-6C. Once reconstituted, peptides should be used as soon as possible to avoid degradation in solution. If this is not possible, it is recommended they are dissolved in a sterile buffer solution and then aliquotted and stored at -20C.(for a limited amount of time only). Peptide solutions where the peptide contains C, M, N, Q and W are particularly limited in their shelf life. Repeated freezing and thawing should be avoided.

## Coupling of Peptides to Carrier Proteins such as KLH or BSA

We use the Pierce Inject Activated Immunogen Conjugation Kit (Product Number 77611) so for more details the Pierce instruction sheet should also be referred to. Coupling occurs via an activated Maleimide group. An abridged version is as follows:

1. Dissolve up to 2mg of the sulfhydryl containing peptide or hapten in 200-500ul Conjugation Buffer. Save 20ul of this peptide solution for quantitation of the conjugation.

2. Dissolve 2mg (one vial) of pre-activated carrier protein with 200ul deionized or distilled water. Alternatively, the peptide may be added as a solid to the activated carrier solution if it is freely soluble. Use up to 2mg peptide per 2mg activated carrier protein.
3. Mix the peptide and activated carrier protein together and allow them to react for 2hrs at room temperature.
4. Save 20ul of this mix for the assay for conjugation efficiency.

The conjugate now has to be purified on the gel filtration column provided as now described:

5. Dissolve the contents of one bottle of purification salts in 60ml degassed, deionized water. This can be done in the bottle. Allow the column storage solution to drain. Wash the column with at least 4 volumes of Purification Buffer.
6. Apply the Hapten-Carrier mix directly to the top of the disc (not to the column sides).
7. Add 0.5ml aliquots of Purification Buffer and collect each fraction in a separate tube.
8. Test for the presence of conjugate by measuring the absorbance at 280nm.
9. If the immunogen is to be stored for more than a few days, sterile filter the conjugate fractions and store them at 4C, or freeze the aliquots at -20C.

## **Quantitation of the Conjugation**

See the Pierce Sheet for details of a microtiter plate method. This is easily expanded for cuvettes.

Please take into account the dilution of the peptide sample by the carrier protein solution!

10. Add 200ul of conjugation buffer to each appropriate well of a microtiter plate (or tube). For the blank add 210ul of this buffer.
11. Add 10ul of sample to the sample wells containing this buffer.
12. Add 10ul of conjugate solution to the sample wells containing this buffer
13. Add 20ul of Ellmans Reagent (1mg/ml in conjugation buffer) to all the test wells and blank.
14. Read at 412nm.

## **Proteins as Antigens**

Proteins do not need to be coupled to a carrier protein as is the case for peptides. As a consequence, proteins can be used for immunization purposes from a number of sources:

### **Expressed or purified proteins**

These should preferably be in an aqueous buffer. Where the protein is not soluble, detergents, urea, guanidine hydrochloride can be used as necessary, but used in minimal amounts. If the protein is denatured, the antibody generated may only recognize the denatured protein and not the native form.

### **Protein from bands in a gel slice**

Bands should be cut from the gel and briefly washed in water to reduce the concentration of methanol and acetic acid. For injection purposes, the gel slice should be taken up in a small amount of PBS and homogenized by passing through a large gauge needle. This can then be used for immunization directly. Protein is released from the gel slice slowly which is good for generating a good antibody response. On the down side, the protein is denatured and the antibodies may not later recognize the protein in the native state.

Proteins bound to nitrocellulose

This has been done but I have no experience of this. Companies are not too enamoured of this method either, but in theory this method offers the possibility of raising antibodies from a much smaller amount of antigen.

## **Affinity Purification of Antibodies (an overview)**

We have used:

For peptides we have added a C or a CG to the N-Terminal and then used the Pierce SulfoLink Coupling gel. Principle: Sulfhydryls react with an alkylating reagent that is on the support.

For Proteins or Fusion Proteins we have used the Pharmacia NHS-activated HiTrap columns

For both types of column, the elution procedure is the same (see later section). In both cases the procedure involves circulating the sera (diluted) repeatedly over the affinity column to bind the antibodies. After binding, the column is washed and the antibodies are eluted with Glycine at either (or both) pH 2.5 or pH 1.9\* and immediately neutralized to pH 7.0, and further eluted with Triethylamine pH 11.5 (again neutralized.) All eluted antibodies are dialyzed against PBS and concentrated to between 1-2mg/ml.

\* Elution with Glycine at pH 1.9 is recommended only when antibodies not eluted at pH 2.5 (most antibodies are eluted above this pH). If it really is necessary to elute at the lower pH, then care must be taken to elute as rapidly as possible, and to neutralize the eluates immediately, in order to minimize the degradation to the bound antigen or to the antibody.

## **Making Affinity Purification Columns**

### ***Coupling of Proteins to Pharmacia NHS-Activated HiTrap affinity columns***

See also the Product Sheet.

1. Dissolve the ligand (up to 10mg/ml) in Coupling buffer (0.2M NaHCO<sub>3</sub>, 0.5M NaCl, pH 8.3), dissolve in 1ml for the 1ml column and 5ml for the 5ml column. SDS can be added in the case of the ligand not being readily soluble. I have not tried this protocol using urea myself, but it should work.
2. Remove the top cap of the column and place a drop of ice cold 1mM HCl to the column to prevent air bubbles.
3. Connect adapter and remove the twist off end.
4. Using a syringe, wash out the isopropanol with 6 volumes of ice-cold 1mM HCl. Keep the flow rate low to avoid crushing the matrix.
5. Immediately inject the ligand solution onto the column. Seal the column.
6. Let it stand for 15-30 mins at room temperature (25C)
7. To deactivate any excess active groups, wash the column with the following (figures for 5ml column in parentheses).

#### ***Buffer A***

0.5 M Ethanolamine, 0.5M NaCl, pH 8.3.

#### ***Buffer B***

0.1M Acetate, 0.5M Na Cl., pH 4.0.

8. Inject 3X2ml (3X10ml) of Buffer A.
9. Inject 3X2ml (3X10ml) of Buffer B

10. Inject 3X2ml (3X10ml) of Buffer A
11. Let it stand for 30mins at Room Temperature
12. Inject 3X2ml (3X10ml) of Buffer B
13. Inject 3X2ml (3X10ml) of Buffer A
14. Inject 3X2ml (3X10ml) of Buffer B
15. Finally inject 2ml (10ml) of a buffer with a neutral pH.

For long-term storage inject a storage solution e.g. 50mM Na<sub>2</sub>HPO<sub>4</sub> containing 0.1% azide.

## **Making the buffers**

### ***Coupling buffer***

0.2M NaHCO<sub>3</sub>, 0.5M NaCl, pH8.3

For 2L weigh out 33.6g NaHCO<sub>3</sub>, and add 58.44g NaCl

**1mM HCl is 91ul Conc. HCl to 1L with dist. H<sub>2</sub>O**

### ***Wash Buffer A***

0.5M Ethanolamine, 0.5 M NaCl pH 8.3

15ml Ethanolamine and 14.61g NaCl. Make to 500ml with H<sub>2</sub>O

### ***Wash Buffer B***

0.1M acetate, 0.5M NaCl

=2.86ml Acetic acid (100%) and 14.61g NaCl. PH4.0 Make to 500ml with dist. H<sub>2</sub>O.

## **Coupling of Peptides with Cysteine to SulfoLink Coupling Gel**

See Product Information Sheet.

Special Note: This coupling protocol works even in the presence of 5M Urea making it useful when the peptide/protein is insoluble in the normal coupling buffer. To date, SDS has not been tried with this protocol. In this case only the coupling reaction step was performed in the presence of urea.

1. Bring the Coupling gel to Room Temperature.
2. Pack a polypropylene disposable column with an appropriate amount of SulfoLink Gel. Up to 1mg of peptide/ reduced protein can be bound per ml gel.
3. Equilibrate the column with 6 volumes 50mM Tris, 5mM Na-EDTA, pH 8.5.
4. Replace the bottom cap on the column.
5. Dissolve the peptide in 50mM Tris, 5mM Na-EDTA, pH 8.5 (up to 10mg/ml).
6. Add the peptide solution to the column, cap and mix the column at room temperature for 15 mins.
7. Incubate the column for a further 30 mins at room temperature without mixing.
8. Drain the buffer.
9. Wash the column with 3 volumes of 50mM Tris, 5mM Na-EDTA, pH 8.5

10. Block the non specific binding sites on the gel in the following way:
  - Make a 50mM Cysteine solution in 50mM Tris, 5mM Na-EDTA, pH 8.5.
  - Apply 1ml of 50mM Cysteine solution per ml of Gel to the column.
  - Mix the column at room temperature for 15 mins.
  - Incubate the column for a further 30 mins at room temperature without mixing.
11. Wash the column with 16 vols 1M NaCl and 16 volumes of degassed 0.05% Na Azide respectively.

## **Antibody Elution from Antigen-Affinity Columns**

Use approximately 15ml serum per 5-10mg bound antigen. Serum needs to be diluted (see below)

If it is uncertain who used the column last or how well it was washed, it is advisable to wash the column extensively. This should be done in the following way:

1. Wash column with 10 column volumes of 20mM Tris pH 7.5
2. Wash column with 10 column volumes of 20mM Tris pH 7.5, 0.5M NaCl
3. Wash column with 10 column volumes of 100mM Glycine pH 2.5\*\*
4. Wash column with 10 column volumes of 20mM Tris pH 8.8
5. Wash column with 10 column volumes of 100mM Triethylamine pH11.5
6. Finally, wash the column back into 20mM Tris pH 7.5 (20 column volumes)

\*\*or Glycine pH 1.9, if the antibody is to be eluted at this pH.

### ***Preparation of Serum***

Whilst the column is on the final washing step, prepare the serum for passing over the column.

Dilute the serum with an equal volume of 20 mM Tris pH7.5 and filter the diluted serum through a Millex-GV 0.22µm filter (For a column with 10mg bound antigen I normally use about 30 ml of diluted serum.). Alternatively, spin the diluted serum 3000 RPM for 20min to remove any particulate matter.

### ***Loading the Column***

Load all the diluted serum and let it pass over the column at least six times (this can be done at room temperature), or preferably let it circulate over the column overnight at 4C.

After binding, collect the flow through and store it at -20C in case the binding was unsuccessful.

### ***Washing and Elution of the Antibody***

Always perform this at 4C!

Wash the column with 20 column volumes 20mM Tris pH7.5 and then a further 20 volumes 20 mM Tris pH 7.5, 500mM NaCl.

Elution can be performed with 100mM Glycine at either pH 2.5 or (for more tightly bound antibodies) pH1.9. Care must be taken to rapidly neutralize (see below) the eluate since the antibody can be inactivated by prolonged exposure to low pH (particularly when Glycine pH 1.9 is used for elution).

To neutralize the antibody eluates, prepare 15X1.5ml Eppendorf tubes (into which the eluate will be collected) by adding 70ul (Glycine pH 2.5 elution) or 120ul (Glycine pH1.9 elution) 1M Tris pH8.8. Elute the antibody at 4C and collect 1ml of the eluate in each tube. Check the pH of the collected eluate on a tube-by-tube basis with pH paper

and adjust to pH 7.5. Using BioRad Protein Assay Reagent (10ul neutralized eluate +50ul diluted reagent), identify and pool those fractions containing antibody. These antibodies will be dialyzed and concentrated as described later.

After elution of antibodies at low pH the column should be washed with 20 volumes of 20mM Tris pH8.8 and check the pH of the flow through. An additional elution of antibody can now be made using 100mM Triethylamine pH11.5. The eluted antibody can be collected in 1ml amounts into tubes containing 9ul of concentrated HCl and 30ul 1M Tris pH 7.5. Like the low pH elution, the pH of the neutralized solution should be checked and adjusted to neutrality with 1M Tris pH 6.8 if necessary.

Pool the peak antibody fractions and dialyze against PBS (2X2L). Concentrate the antibody if necessary using a concentrator (Vivaspin) to around 2mg/ml.

Aliquot the antibody into 100ul amounts and flash freeze under Liquid N<sub>2</sub>.

### **Reagents**

1. PBS
2. Millex GV 0.2u filters (Cat. No. SLGV 025 BS)
3. Glycine (from Merck)
4. Triethylamine (from Sigma T-8521)
5. Vivaspin concentrators 15ml with a 30,000 MWCO (Cat. No. VS 1521)

## **Rabbit Injection Protocol**

Starting Samples:

1. Peptides coupled to Carriers such as Keyhole Limpet Hemocyanin (KLH), BSA etc.
2. Whole proteins or portions of proteins, Fusion Proteins etc.

When raising antibodies in rabbits, it is needed to give an initial injection of approximately 100-200ug protein/coupled peptide along with the adjuvant. For subsequent boosts, only 70-150ug protein/coupled peptide is necessary.

A total volume of 1ml of antigen/adjuvant mix should be injected per rabbit and is made up as follows:

Adjuvant-we use Ribi MPL+TDM+CWS Emulsion. These come as a vial containing a desiccated emulsion. The contents of the vial should be resuspended in a total volume of 1ml.

Per rabbit, take 0.5ml resuspended adjuvant and add the desired amount of protein/coupled peptide as indicated above. Make up the volume to 1ml with PBS. Take up this sample in a 2ml syringe taking care to remove any air bubbles. Provide the animal house with a 25-gauge (orange) needle on the syringe.

The animal house will then inject the following:

- 0.5ml of the injection mix into six sites intradermally on the back.
- 0.3ml into two sites intramuscularly (thigh).
- 0.2ml into two sites subcutaneously in the neck region.

In the past we have used either Freund's or TiterMax adjuvant and in these cases they would have been injected subcutaneously. Subsequent injections (boosts) were of 100ug protein given at intervals of 28 days. About 50ml blood is removed from the rabbit ten days after each boost (when the antibody titer is at its peak).

In our experience, if we have not produced the desired antibody after two injections, then the protocol is unlikely to be successful.

**Materials**

Ribi Adjuvant - MPL+TDM+CWS Emulsion in 2% Oil-Tween-80-H2O  
Ribi Immunochem Research. Inc.,  
553 Old Corvallis Rd,  
Hamilton,  
MT 59840.  
USA.  
Tel. 406/363-6214

**Testing the Antibodies**

The Primary Screen is usually done by Western Blot using the sera at a dilution of anywhere between 1:50-1:500 against either whole cell extract or enriched antigen purification

Positive sera can then be enriched for antibody by affinity chromatography.

**Western Blotting Method**

Transfer of proteins onto membranes:

***Wet Transfer Method***

Suitable for overnight or rapid transfer.

**Buffer (10X) - For 1 Liter**

25g Tris Base 206.4mM

120g Glycine 1.6M

To make the transfer solution, dilute the 10X buffer to 1X and use. If problems are encountered in transferring larger proteins, this buffer can be made up to 20% with methanol. SDS can also be added to a final concentration of 0.037%

For rapid transfer, run at 50-55V for 2-5 hours depending on the size of the protein of interest (longer running time for high molecular weight proteins)

For overnight transfer, run at 10-15V

***Semi-dry Blotting Method***

**Dry Blot Buffer (1X)**

Tris Base 11.64 g

Glycine 5.86 g

SDS 3.75 mls of a 20% Stock Solution

**Methanol 200ml**

Make up to 2 Liters with Distilled water.

Transfer mini gels at 10-15 V for about 15-30 mins. Larger gels can be transferred at 15-25 V for 30-60 mins. Do not transfer at higher than the recommended voltages otherwise overheating may occur.

To visualize the transfer of the proteins to the membrane flood the membrane with Ponceau S solution

***Blotting Developing Protocol***

**Blocking Buffer**

3% Milk Powder (Fat free)

0.05% Tween 20

20mM Tris HCl pH 8.0

150mM Na Cl

### **Wash Buffer**

0.05% Tween 20

20mM Tris HCl pH 8.0

150mM Na Cl

1. Block the membrane in blocking buffer for 30 mins with rocking.
2. Dilute \* the antibody in the wash buffer and incubate for one hour with rocking.
3. Wash the membrane with wash buffer three times (10 mins per wash)
4. Dilute the secondary antibody in wash buffer and incubate (with rocking) for one hour
5. Wash the membrane with wash buffer three times (10 mins per wash).
6. Transfer to another container and develop according to protocol

\*Suggested dilutions:

- Serum - Try between 1:50-1:500
- Affinity Purified Antibody - Try between 1:1000-1:10,000

### **Storage of Antibodies**

Here are the common ones:

1. Made to 0.1% with Sodium Azide and stored at 4C. Caution! The presence of azide may interfere in assays (for example those involving cell extracts) so beware when using the antibodies for immunodepletion experiments.
2. Made to 50% with glycerol and stored at -20C
3. Aliquoted and stored at -80C

### **References**

C.M. Field, K. Oegema, Y. Zheng, T.J. Mitchison and C.E. Walczak. (1998): Purification of Cytoskeletal Proteins using Peptide Antibodies.

Methods in Enzymology 298 pp. 525-538.