

# Generation and purification of rabbit or goat polyclonal antibodies

## *Melchior's lab protocols 2002*

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### 1. GENERATION OF ANTIGEN

#### a) recombinant protein

##### General considerations

1. The purity of the recombinant antigen should be as high as possible. Bacterial contaminations are often more antigenic than the intended antigen, consequently, with dirty antigens the resulting antibodies may be directed predominantly against the impurities. Moreover, if the antibodies are subsequently used in experiments with several different recombinant proteins they may recognize too many contaminating proteins. For GST- or His-tagged proteins, we generally include at least one gel filtration step in physiological buffer after the initial enrichment by GST- or nickel sepharose.
2. With GST-tagged proteins, we usually use the fusion protein for the primary injection. This is because GST is very immunogenic, and serves well to stimulate the immune response. However, to subsequently enrich for antibodies that are specific for the antigen, we use the untagged protein (e.g. upon cleavage of the tag by thrombin- or FactorXa) for all subsequent booster injections.
3. See also chapter 5.

#### b) peptide - ovalbumin conjugates

##### General procedure

General procedure: peptides (10-15 amino acids in length) with an N-terminal Cysteine residue are coupled to amine groups in a carrier protein (ovalbumin, BSA, or KHL, we prefer ovalbumin), using a bifunctional crosslinker (Sulfo-SMCC, Pierce).

##### Material

1. Ovalbumin: Chicken egg Ovalbumin (Sigma)

2. PBS
3. SMCC: 20 mM Sulfo-SMCC (Pierce) in DMSO, prepare directly before use
4. PD10 column (prepacked G25 column, 10 mL bed volume, GE Healthcare)
5. peptide (must be reduced !, see comment / protocol below)

#### Procedure

1. Dissolve 2.5 mg Ovalbumin in 1 ml PBS, add 100  $\mu$ L of freshly prepared SMCC, and incubate for 30 min at RT (take gel sample).
2. In the mean time, wash a PD-10 column with 25 mL PBS.
3. To remove unbound crosslinker, apply the mixture to the PD10 column, let the sample run into the column, wash with 1.5 ml PBS, and elute with 3.5 mL PBS (take gel sample).
4. Dissolve 1 mg peptide with the column eluate and incubate over night at 4°C (take gel sample).
5. Freeze 500  $\mu$ L aliquots (for goats) in liquid N<sub>2</sub> and store at -70°C.
6. Analyze the 3 samples in SDS-PAGE (15%), a significant size shift of ovalbumin should be observed.

## 2. GENERATION OF EMULSION FOR INJECTION

### General procedure

A stable emulsion between antigen and adjuvant needs to be prepared prior to injection. This serves to increase antigenicity, and to increase stability of the antigen in the animal. We use **Titermax Gold** (available from Sigma) for the first injection, and **Freund's Adjuvant incomplete** (Sigma) for all subsequent injections. Titermax Gold forms a very stable emulsion, and in our hands has always induced a very good immune response. In principle, it could also be used for boosts. However, Freund's Adjuvant incomplete is much cheaper, and serves the purpose.

For detailed informations on Titermax and Titermax gold see: <http://www.titermax.com/>

There are many ways how to make the emulsion. We follow the procedure below (copied from the Titermax website, similar instructions come with the product from Sigma):

### Materials

1. TiterMax® Research Adjuvant
2. Two all-plastic syringes (1-3 ml, depending on the volume used)
3. One 18 gauge needle for withdrawing TiterMax® from the vial, or use syringe without needle or positive displacement precision pipette if you open entire vial
4. One 18 gauge double hub emulsifying needle
5. Antigen in saline or other suitable fluid

### Procedure

**NOTE:** Prior to preparation of a TiterMax® water-in-oil emulsion, warm the TiterMax® to room temperature and vortex for 30 seconds. Make sure the TiterMax® is a homogeneous suspension of copolymer-coated microparticles before proceeding to emulsify by any method.

For 1 ml of a 50:50 water-in-oil emulsion you will need 0.5 ml of the aqueous antigen and 0.5 ml of TiterMax® .

1. After TiterMax® has been vortexed, load a syringe with 0.5 ml TiterMax® . Load the second syringe with 0.25 ml of antigen in aqueous medium. Set aside the other 0.25 ml of antigen.

**NOTE:** It is important to add the aqueous antigen phase to the TiterMax® in at least 2 small volumes.

2. Connect the two syringes via a 18 gauge double hub emulsifying needle. Mix the TiterMax® with the antigen by forcing the materials back and forth through the needle for approximately 1 minute.

**NOTE:** It is important to push the antigen into the TiterMax® syringe first, so that the aqueous phase enters the oil phase rather than vice versa. Hold the syringes carefully so that they do not come apart from the double hub needle during emulsification. The formation of a water-in-oil emulsion is signaled by a sudden increase in viscosity \*, i.e., more force is required to move the material through the needle.

\* Make sure, needle sits very tightly on syringes - at this step one can easily splash the antigen all over the bench!!!

**NOTE:** After ~ 1 minute a whipped-cream-like water-in-oil emulsion forms. Push all of the emulsion into one syringe and disconnect the empty syringe.

3. Load the empty syringe with the remaining 0.25 ml aqueous antigen solution. Reconnect the syringes and emulsify for another 30 to 60 seconds.

**NOTE:** Again, first push the antigen into the water-in-oil emulsion. Care must be taken in holding the syringes together since the oil may lubricate and loosen the connection. That is why it is preferable to use a lock tip syringe. Push all of the emulsion into one syringe and disconnect the empty syringe. If necessary, load the syringe you have chosen for injecting your species of animals.

4. To test for stability, place a drop of emulsion on water.

#### **Amount and volume of antigen per injection**

We use approx. 200 µg antigen in 200-500 µl buffer per rabbit injection, and approx. 500 µg antigen in 500-1000 µl buffer per goat injection. Don't use much more than that - there seem to be cases where too much antigen inhibits response.

### **3. INJECTION / BLEEDING SCHEME**

Injections (subcutaneous) into rabbits or goats are carried out by the personnel of the animal facility. Please keep in mind that appointments need to be made ahead of time. Most importantly, sacrifices can only be carried out at specific days, so plan ahead for the terminal bleed. For blood, provide the animal facility with labeled 50 ml falcon tubes (labeled with the log number of the animal).

**Procedure days indicated below are approximate. However, for maximal titer, blood should always be taken between days 10 and 14 after injection!**

**Day 1:** obtain 50-200 ml blood (pre-serum) **and** have

1. Injection with **Titermax Gold**

**Day 40:** 2. Injection (with Freund's Adjuvant incomplete)

**Day 50:** obtain 200 ml blood for goat, 40 ml for rabbit (1st serum)

**Day 70:** 3. Injection (with Freund's Adjuvant incomplete)

**Day 80:** obtain 200 ml blood for goat, 40 ml for rabbit (2nd serum)

*Test immediately by western, whether titer in 2nd serum is good!*

**Outcome A --- titer good:**

**Day 84:** Terminal blood (approx 1 liter for goat, 200 ml for rabbit)

**Outcome B --- titer not yet satisfactory:**

**Day 100:** 4. Injection (with Freund's Adjuvant incomplete)

**Day 110:** obtain 200 ml blood for goat, 40 ml for rabbit (3rd serum)

*Test immediately by western, whether titer in 3rd serum is good!*

**Outcome C --- titer good:**

**Day 114:** Terminal blood

**Outcome D --- titer not yet satisfactory:**

Continue with injections...

#### 4. FROM BLOOD TO SERUM

Once blood is received from the animal facility, stir it with a glass rod (for approx. 1 min), let it sit at room temperature for 1 hour, stir it again, and store it at 4°C over night. On the next day, stir again, then centrifuge in a swing out rotor for 30 min at 5000 g (e.g. in Beckmann J6B centrifuge at 4200 rpm). Collect supernatant, aliquot, and store at -20°C.

#### 5. TEST OF SERUM / PURIFIED ANTIBODIES

##### General procedure

We use two parameters to evaluate our sera and purified antibodies, the titer (how much specific antibodies are in the serum?), and the sensitivity (how little antigen can be recognized by the antibody?)

1. First, we test the titer by carrying out immunoblots with a fixed amount of antigen (10 ng) in each strip, and serial dilutions (1:100 - 1:5000) of the antibody. To control for nonspecific background, we always apply the antigen as a mix with total HeLa cell extract, and for each antibody dilution load two samples, one with (A) and one without (B) antigen in the HeLa extract. When serum is tested, you should also include a pre-serum control.

**A decent titer in the serum should allow serum dilutions of at least 1:1000 (with 10 ng antigen). Ideally, the antibody gives a clean signal without background, but in most cases affinity purification is advisable (see below).**

2. Second, we carry out immunoblots with a suitable dilution of the antibody (one that gave a decent signal in 1), and serial dilutions of antigen from 100 ng to 0.1 ng per gel lane. Like above, the antigen is always mixed with total HeLa cell extract, and one lane contains cell extract only.

**Very good antibodies recognize 1 ng antigen (or less) in immunoblots. If you need more than 10 ng antigen the antibody may still be acceptable, but if you detect only a signal with 100 ng or more antigen your antibody is pretty useless for most purposes.**

#### 6. AFFINITY PURIFICATION

Specific antibodies can be purified from serum by binding to - and elution from - immobilized antigen. Two methods for generating columns with immobilized antigen are described below: method a) involves coupling of a protein to cyanogen bromide activated sepharose; method b) involves coupling of peptides to EAH-sepharose (a bifunctional crosslinker couples a cysteine in the peptide to primary amines presented on the sepharose). The subsequent procedure of antibody purification is the same for either column material, and is described in detail under c). Briefly, upon incubation of the column material with serum, specific antibodies bind to the immobilized antigen. Elution with acetic acid (our preferred choice), MgCl<sub>2</sub>, or guanidinium chloride, dialysis and concentration follow.

##### a) Generation of antigen columns

###### General consideration

Recombinant protein, either the same that was used for injection, or a larger one that contains the domain to which antibodies were generated, is coupled to cyanogen activated sepharose. Ideally, the protein that is used for purification is not identical with the one that was injected (e.g. injection of a GST-fusion protein, purification against a His-tagged protein). This is to avoid both co - enrichment of antibodies against contaminating bacterial proteins, and antibodies to the fusion domain.

**Protocol:**

1. Dialyze protein extensively against carbonate buffer (0.2 M, pH 8.9), with several buffer changes, over night. All traces of free amines (such as in Tris, some protease inhibitors, etc) have to be removed !
2. Take 0.8 g CNBr beads (Sigma, catalog number # C-9142), swell for 10 min in 1 mM HCl, wash 1 x in carbonate buffer. Use immediately for step 3, it inactivates rapidly!
3. Add protein (0.5 mg protein / ml beads is pretty good for affinity purification of antibodies). Concentration of protein can be anything between 0.2-2 mg/ml prior to coupling, but don't forget to check OD280 prior and after coupling to determine coupling rate. Incubate 1 h at RT, than at 4°C overnight (or 3 h RT).
4. Wash beads 2 x with carbonate buffer.
5. Incubate with 100 mM Ethanolamine for 1 h at RT to block all remaining coupling sites.
6. Wash 3 x with carbonate buffer.
7. Equilibrate with 500 mM NaCl in PBS.
8. Beads can be stored at 4°C (for prolonged storage add 1 mM sodium azide).

**b) Generation of peptide columns**

**Please consider the following:** if peptide coupled to a carrier was injected, antibodies against the crosslinker may also be present in the serum, and will be co-purified. More importantly, it may be desirable to enrich for antibodies recognizing the protein (not just the flexible peptide). Consequently, if at all possible, it may be better to purify the antibodies against the protein rather than against the peptide (see procedure a).

**Protocol:**

1. 0.3 g EAH-Sepharose are swollen in 50 ml 0.5 M NaCl, harvest beads by centrifugation.
2. Wash beads 2x in PBS.
3. Resuspend in 1 bead volume PBS.
4. Add 0.2 ml of 50 mg / ml Sulfo-SMCC in water.
5. Incubate at RT for 1 h (rotate slowly).
6. Wash 3x with 50 ml PBS.
7. Dissolve 10 mg peptide in 5 ml PBS (peptide must be reduced, see comment / protocol below). Measure OD at 215 and 225 nm (peptide bond).
8. Add to beads and rotate slowly at RT over night. Harvest beads by centrifugation. Measure OD of supernatant at 215 and 225 nm (should be much lower than in 7.).
9. Wash beads 1x in PBS, 1x in PBS + 1 M NaCl, 1x in 0.1 M NaHCO<sub>3</sub> + 0.5 M NaCl, 1x in 0.2 M acetic acid + 0.5 M NaCl, and finally 1x in PBS + 0.5 M NaCl.
10. Beads can be stored at 4°C, for prolonged storage, add 1 mM sodium azide.

**c) Affinity purification of serum**

1. Incubate 1 - 2 ml affinity matrix (prepared as in a or b) with 20 ml serum and 30 ml PBS in a 50 ml Falcon tube at 4°C over night (slow rotation).
2. Harvest beads by centrifugation (keep supernatant at 4°C, in case that antibodies did not bind efficiently to the beads).
3. Wash 2x with 50 ml PBS + 500 mM NaCl.
4. Transfer into column, continue to wash until no more protein in flow through (can be rapidly tested by spotting 1 µl of the wash onto nitrocellulose and staining with Ponceau red).
5. Run column, until no more liquid is above the column material.

- Carefully apply elution buffer (0.2 M acetic acid, pH 2.7, 500 mM NaCl) onto column (without disturbing gel bed) and elute antibodies with 10-20 ml (approximately 10 column volumes). Collect 0.5 ml fractions.
- Immediately add 100  $\mu$ l 1M Tris / Base to each 0.5 ml fraction to neutralize the pH (mix gently).
- Check for presence of antibodies by spotting 1  $\mu$ l onto nitrocellulose and staining with Ponceau red.
- Combine protein-containing fractions, concentrate to approx. 1 ml (we use centricon units, 30 kD cut off), change buffer to PBS by dialysis or repeated dilution / concentration.
- Mix antibodies with 1 vol of 87% glycerol and store in aliquots at -20 °C.

**Considerations:**

- acetic acid elution is just one of many alternative protocols (e.g. elution with 0.2 M glycine, 4 M MgCl<sub>2</sub>, 4 M guanidinium chloride etc). In our experience this works very well for most antibodies.
- In some cases, a second elution (after washing of the column in PBS + 0.5 M NaCl) with 4M guanidinium chloride pH 7 gives rise to more antibodies (possibly differing in their properties).
- Our protocol with PBS + 500 mM NaCl is quite stringent. Alternative protocols use PBS only.
- The optimal ratio between affinity matrix and serum needs to be determined empirically. Compare serum, unbound serum and eluted antibodies by immunoblotting.
- In principle, both types of affinity matrices can be used more than once. However, after some frustrating experience we prefer to use matrices with recombinant proteins only once. Antibodies with very high affinity may elute only partially. Consequently, the best sites on the antigen will be more and more blocked. In addition, acid elution may partially denature the antigen, and antibody selection (native versus unfolded antigen) may vary between a fresh and a reused column.

**7. SUPPLEMENTARY PROTOCOL: REDUCTION OF PEPTIDE**

Newly synthesized peptides come in reduced form. But the sulfhydryl group tends to get oxidized over time, and it might be necessary to pretreat the peptide with DTT prior to coupling. (The molar amount of reduced peptide can be determined using Ellman's reagent and a glutathione standard curve).

**Material:**

- Peptide
- 1% acetic acid
- Sephadex G10 column (10 mL bed volume, 1 cm diameter)
- 50 mM Hepes, pH 7.0
- DTT
- Ellman buffer: 0.1 M sodium phosphate buffer, pH 7.4, 5 mM EDTA
- Ellman's reagent: 1 mM dithiobisnitrobenzoic acid in methanol.

**Protocol: Reduction of synthetic peptide with DTT**

- Equilibrate a Sephadex G10 column (10 mL bed volume, 1 cm diameter) with 25 mL 1% acetic acid.
- In the meantime, dissolve 10 mg peptide in 1 mL 50 mM Hepes, pH 7.0, add 10 mg DTT and incubate for 1 h at RT.
- Separate peptide from free DTT by gel filtration in 1% acetic acid on the equilibrated G10 column. Collect 0.5 mL fractions.
- Analyze the fractions using Ellman's reagents: for that, add 10  $\mu$ l fraction to a test tube containing 900  $\mu$ l Ellman buffer and 100  $\mu$ l Ellman's reagent - fractions containing free sulfhydryl residues yield a bright yellow color. The first yellow peak contains the peptide, the second peak contains DTT.

5. Combine peptide containing fractions, lyophilize 8 aliquots in pre-weighed eppendorf tubes to complete dryness, weigh again (should be approximately 1 mg peptide per aliquot), and store at  $-70^{\circ}\text{C}$ .

The exact concentration of peptide can be calculated from the absorption below 230 nm, which is due to the peptide bond. Several different formulas can be found in the respective textbooks, one of which is:  $[\text{peptide}]\mu\text{g}/\text{mL}=144\times[\text{A}215-\text{A}225]$ . Alternatively, the exact concentration of free sulfhydryl groups, equivalent to the concentration of reduced peptide, can be calculated using Ellman's reagent in combination with a standard curve of glutathione. However, for the purpose of antigen / affi-column preparation the approximate weight is sufficient.

## BIBLIOGRAPHY

As a general reference for procedures involving antibodies we recommend "Using Antibodies: A Laboratory Manual" (Ed Harlow and David Lane, 1988).

Details to specific reagents, eg crosslinkers, adjuvants, column materials, can be found on the home pages of the respective companies.