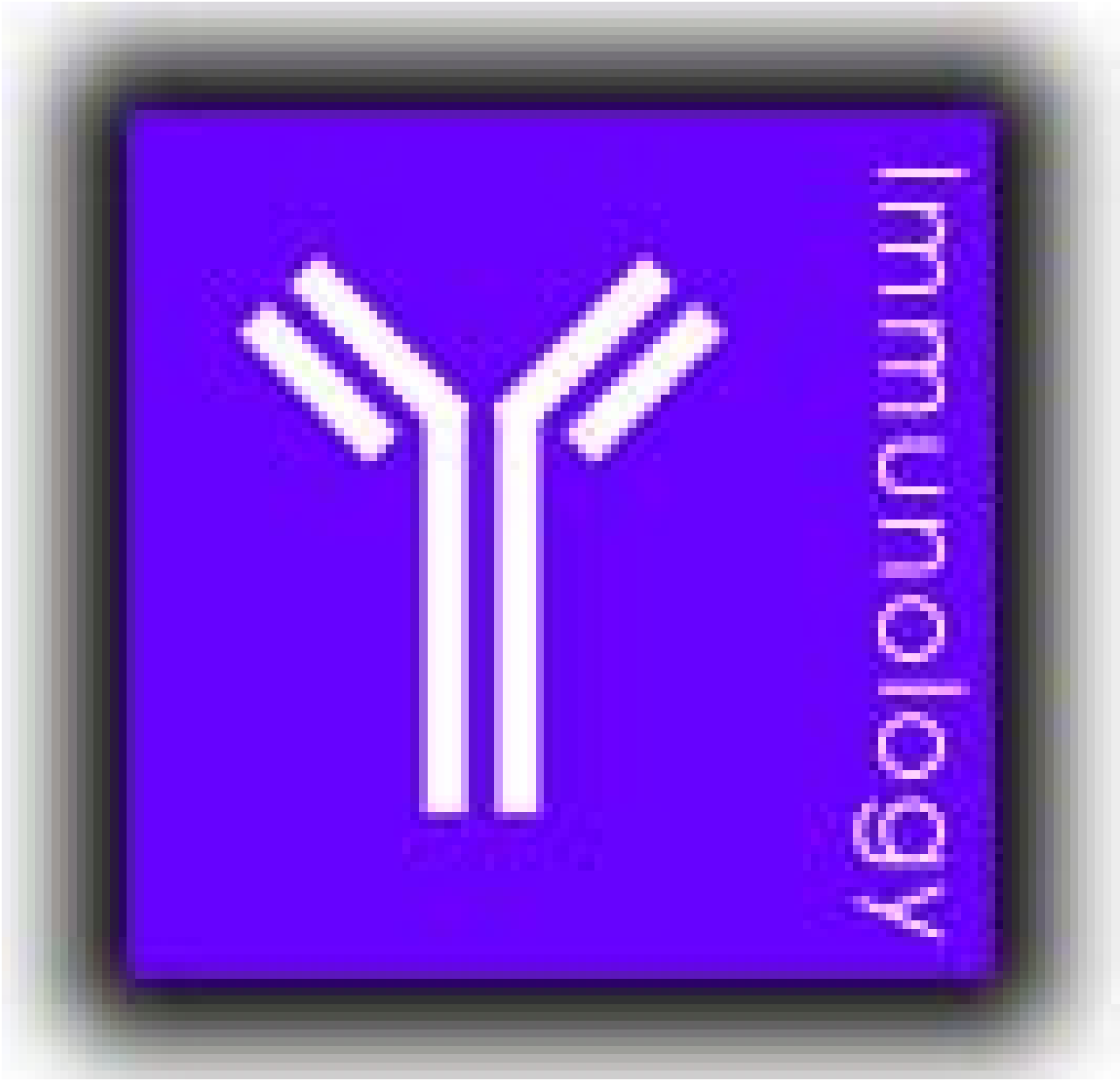
Polyclonal Antibody Production

Very useful for rapid and simple generation of antibodies for western blots, ELISA assays, and immunoprecipitation.



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I.Rabbit Immunization

Initial Preparation

Your antigen should at least have a molecular weight of 20 kDa (kilodaltons) for development of a strong immune response in animals. If it is a small protein, it can be linked to a larger protein by chemical methods or cloned and expressed as a fusion protein with a larger protein. If it is a peptide or small molecule, a carrier-hapten conjugate must be made. Purify your antigen well to obtain antibodies with good specificity.

Obtain at least two 6 week old New Zealand White rabbits and have them delivered to the Animal Resources Center. Don't put all your eggs in one basket by using only one rabbit. Allow them to get accustomed to their new environment for two weeks before the first pre-bleed.

II. Preparation Of Peptide-KLH Conjugates For Immunization

These are basically Tim's procedures and have been used successfully by numerous members of our lab and by others around us. Of course you can get the service from ShineGene.

A. Checking Peptide Thiol Groups

Do this before thiol coupling, either to KLH or to resin. Peptide thiol groups have a tendency to get lost after synthesis.

1. Make up 5 mM Ellman's reagent (dithio-bis-2-nitrobenzoic acid) in 0.1M NaPi pH 7.2.
2. Weigh out about 1 mg of peptide into a tared tube.
3. Add 0.5 ml reagent. It should go bright yellow.
4. Dilute the mixture 1/50 in buffer. Read A412 against reagent at the same concentration.
5. Calculate the apparent molecular weight of the peptide based on thiol groups, using a molar extinction coefficient of 14,000. Compare this to the expected molecular weight of the peptide. They should agree within a factor of three, with the apparent molecular weight usually higher. If the thiol concentration is anomalously low, i. e., the apparent molecular weight is very high, there may be something wrong with the peptide- anyway it probably will not couple well. You may need to regenerate the thiol groups by reducing the peptide with excess DTT and running a P2 column.

B. Coupling of Peptide to KLH

This recipe is for two bunnies for about five injections per bunny

1. Weigh out 100mg of keyhole limpet hemocyanin (KLH). Dissolve in 2ml water. It generally takes about 4 hours to dissolve- you will need to sonicate and vortex. Be patient and put on a rotator at 4 deg C. Dialyze against 2l of 0.1M NaPhoshate pH 7.8 overnight. This is to remove any contaminating thiols or amino compounds.
2. Spin 10 minutes at full speed in microfuge to remove aggregates (don't be surprised to see a substantial pellet).
3. Split the KLH into 2 aliqouts for -SH and -NH2 coupling.
4. For -NH2 coupling, add 5mg peptide to one aliquot, followed by glutaraldehyde to 0.1% final. Add the peptide as a solid if it is soluble, otherwise from a 100 mg/ml stock in DMSO. Precipitation does not matter and often happens. After adding the glut, check the pH with pH paper, and adjust to 7.8 if necessary using NaOH. Incubate 8-12 hrs at 4 degrees, rotating gently.
5. Add a **tiny** pinch of NaBH4 to kill remaining glut. Make sure the sample is in a large tube since it tends to fizz up. Incubate 8-12 hrs at 4 degrees. This is the glut conjugate.
6. For the -SH coupling, warm the other aliquot of KLH to room temp. Add 1/9 th volume of Iodoacetic acid N-hydroxysuccinimide ester at 100mg/ml in DMSO. Make the DMSO stock fresh, and protect the iodoacetamide reagent from light. We make our own IAA-NHS ester, but it can be purchased from Sigma.
7. After 10 minutes at room temp the KLH will start to get a little cloudy. Load it onto a P-10 column equilibrated with 0.1M NaPhosphate pH 7.8. Make sure the column is at least 10 times the volume of the sample. Pool the KLH containing fractions by color (it will be sort of greyish green). Add 5mg of peptide to them, as in step 5 above. Incubate at least 8 hrs at 4 degrees, rotating gently.
8. Pool the coupled peptide from the two proceedures. Dilute to 5ml with 0.15M NaCl. If there is a precipitate, sonicate vigorously to break it up. Split the immunogen into 1 ml aliquots (each aliquot will immunize two rabbits) and freeze it.

III.Pre-bleed

1. Calmly and gently place rabbit in a restrainer. If they are relaxed they bleed much easier.
2. With a razor blade, shave the top middle of the ear you wish to bleed, completely visualizing the large vein running down the center of the ear.
3. If you find it necessary, dilate the ear vein by rubbing xylene on it with a Q-tip.
4. Using a perfusion kit with a butterfly needle at one end and a rubber enclosed needle at the other, insert the butterfly needle in the vein. Immediately after blood is seen in the tubing, quickly insert the needle at the other end into the rubber cork of a 20 mL evacuated blood collection tube. This step works best with two people.
5. When you have the required amount of blood (1-5 mL is sufficient for a pre-bleed--for later bleeds take 20 mL), remove the needle and apply pressure to the wound. When

the bleeding has stopped (~10 seconds), sterilize the ear with ethanol. If you used xylene, rinse two or three times with ethanol to remove it.

6. Remove the rabbit from the restrainer and place it in its cage.
7. Take the collected blood and place at 37°C for 30 minutes to inactivate complement.
8. Place the tube at 4°C overnight to clot.
9. Loosen the clot from the tube wall with a spatula and decant the blood into a plastic centrifuge tube. Centrifuge at 4°C 10,000g for 10 minutes.
10. Decant or pipet off the supernatant. This is your serum. Dispose of all blood waste in appropriate biohazardous containers.

IV.Antigen Injection

1. For injection of **two** rabbits, your antigen should be in 1 mL of a buffer such as phosphate buffered saline (PBS) or Tris that contains no chemicals harmful to the rabbit. 100 µg/rabbit of antigen is best, but you can use less and get good results.
2. Again for two rabbits, prepare RIBI (or Freund's) adjuvant by warming in a beaker of warm water, injecting 1 mL of your antigen solution into the adjuvant, and vortexing vigorously for 2 minutes to get a good suspension. **Do not get adjuvant in your eyes, or anywhere else.**
3. Calmly take the rabbit out of its cage, reassuringly petting it. There is no need for a restrainer. Place on a flat surface for the injection. With a 1 mL syringe, draw in 1 mL of your antigen/adjuvant solution. Four subcutaneous injections are done, two on the lower back and two on the thigh. To inject, rub the hair away from the injection site and sterilize with a squirt of ethanol. Pinch the skin and pull up slightly to pull the skin away from the muscle, insert the needle 1-2 cm at a 15 degree angle so as to not hit muscle, and inject 250 µL of your innoculum. After the required volume has been injected, let the needle remain in place for a few seconds, then pull out and gently rub the injection site so nothing leaks out. Repeat for all four sites and place the rabbit back in its cage. Repeat the process for the other rabbits.
4. Injections will be done every 4-6 weeks, with bleeds 7-10 days after each injection. Bleeds are performed identically to the pre-bleed, but obtain at least 20 mL per rabbit, with 40 mL being the upper limit for an adult rabbit to prevent anemia.

V． Monitoring of Titer

The quality of the antibodies in serum (humoral immune response) of the bleeds is monitored by indirect ELISA. Titer is defined as the dilution that gives you 1/2 the maximal absorbance of this assay.

Testing Rabbit Bleeds By Elisa

You can test whether or not you have gotten an immune response to the peptide and how strong that immune response is by doing ELISAs against peptide conjugated to BSA. By conjugating to BSA, you will eliminate any signal for antibodies generated to KLH during immunization.

A. Coupling Peptide to BSA

You need:

- 1 ml of 2 mg/ml BSA in 0.1 M NaHCO3.
- 1 ml of 0.2% glutaraldehyde in 0.1 M NaHCO3.
- 0.5 ml peptide (1 mg/ml in DMSO). Peptide can be added as a solid if soluble.

1. Mix, adding glutaraldehyde last. Peptide and BSA turn a little yellow even before adding glutaraldehyde.
2. Incubate 90' at 37 deg C.
3. Add 0.1 volumes of 0.1 M NaBH4 in 0.1 M NaHCO3. There will be some bubbling. Add same amount of NaBH4 after 15'. Do a quick microfuge spin if there are too many bubbles.

B. ELISA with Peptide conjugated to BSA

1. Coat 10 ug/ml antigen (peptide conjugated to BSA) diluted in TBS (50 ul/well) ON at 4 deg C.
2. Remove antigen and rinse wells 2Xs with TBST.
3. Block 2 hr with 200 ul of 5% NFDM in TBST.
4. Remove blocking reagent and rinse wells 2Xs with TBST.
5. Incubate in primary antibody diluted in Blocking Buffer for 2 hr at RT. I do tripling dilutions beginning at 1/10 (50 ul/well).
6. Remove primary antibody and rinse wells 4Xs with TBST.
7. Incubate in secondary antibody (1/5000 Goat anti-rabbit conjugated to AP) diluted in Blocking Buffer for 1 hr at RT (50 ul/well).
8. Remove secondary antibody and rinse wells 4Xs with TBST.
9. Rinse wells 2Xs with 50 mM HCO3; 0.5 mM MgCl2, pH 10.
10. Develop in 1 mg/ml p-Nitrophenyl phosphate in 50 mM HCO3; 0.5 mM MgCl2, pH 10 (50 ul/well).
11. Read A410 in ELISA reader.

VI. Purification of Antibodies

Although for many purposes it is not necessary to purify the antibodies away from other serum proteins, if desired this is best accomplished by Protein A Affinity Chromatography.

A。Coupling Peptides To Resin For Affinity Purification

We use Affigel-10, converting its functional group first to amino and then to iodoacetyl. You can also buy amino resin (it's more expensive) and start at step 6. All washes are performed on a glass-fritted filter funnel sucking until a wet cake is formed. Do not dry the resin completely or you will introduce lots of air bubbles. All reactions up to peptide addition are performed in the funnel by covering the spout with parafilm. This minimizes loss. Assume the affigel is a 50% slurry.

1. Wash with 5 volumes of 100% cold EtOH.
2. Wash with 5 volumes of 50% cold EtOH.
3. Wash with 5 volumes of cold water.
4. Add 5% ethylene diamine in water. Incubate 15' at RT.
5. Wash with 10 volumes water. At this point you have amino-affigel.
6. Wash with 3 volumes of 0.1 M NaPi pH 7.8.
7. Resuspend resin in 0.1 M NaPi pH 7.8. Add iodoacetic acid-NHS ester, and incubate for 10 min at RT. The resin has about 10 5mol groups/ml, so add about 20 5mol reagent per ml (7 mg/ml resin). Dissolve the IAA-NHS ester in dry DMSO and add it while stirring the resin. This step and subsequent steps up to the blocking of residual iodoacetate groups should be done in dim light since the iodo group is light sensitive.
8. Wash with 10 volumes of 0.1 M NaPi pH 7.8= buffer.
9. Resuspend the resin as a 50% slurry in buffer. Add the peptide. If the peptide is soluble directly in buffer, add it as a solid. Many peptides go in better added as a 100 mg/ml stock in DMSO. Generally, if your peptide was readily soluble when you checked for thiol groups, it will be soluble in the 50% slurry because the concentration is the same. Some hydrophobic peptides will crash out. It is possible to couple such peptides in 20% buffer, 80% DMSO. In an extreme case you can use 100% DMSO containing triethylamine. The amount of peptide to add is a question. We usually add 1-2 mg peptide/ml resin, hoping to make a resin that will bind 10-100 mgs/ml of specific antibody.
10. Mix gently on a rotating wheel ON at 4 !C. Add betamercaptoethanol to 0.2% to block residual iodoacetate groups. Incubate 1 hr at RT.
11. Wash resin sequentially with 5 volumes 0.1 M NaHCO3, 5 volumes of 1 M Na2CO3, 5 volumes of water, 5 volumes of 0.2 M glycine, pH 2.0; 150 mM NaCl, 5 volumes of TBS and 5 volumes of 6 M Guan-HCl in TBS. Reequillibrate into TBS + 0.1% NaN3.

Before adding valuable peptide, we recommend checking the resin chemistry using a quick eyeball test. The amino resin will react with an NHS ester, whereas the original affigel and the iodoacetate will not. Take an aliquot (50ul) of resin at each step. Resuspend in 100 ul of buffer. Add 1 ul of 0.1 M NHS fluoroscein or NHS-rhodamine in DMSO. Incubate 5' RT. Wash the resin

twice in buffer by centrifugation. The original resin, and the resin after step 8 should be only lightly labeled, wherease the resin after step five should be heavily labeled.

B．Purification Of Anti Peptide Antibody

All steps up to the dialysis at room temperature.

1. Pour column in TBS (=0.15M NaCl, 20mM TrisCl pH 7.4). We use a 5 ml column for 25 mls serum. Wash extensively in TBS after prewashing as indicated in the protocol for coupling peptide to the resin.
2. Thaw serum- dilute 1:1 with TBS and filter through a 0.2 um filter
3. Load the serum over the column, taking at least 20 minute total.
4. Run the breakthrough over the column five times. Alternatively you can use a parastaltic pump and recirculate the serum ON or just batch bind the serum ON.
5. Wash with 5 col vols TBS.
6. Wash with 10 col vols 0.5 M NaCl, 20mM TrisCl pH 7.4, 0.2% Triton- X-100.
7. Wash 5 col vols TBS
8. Elute with 0.15 M NaCl, 0.2 M Glycine-HCl pH 2.0. Collect 1 ml fraction, with each tube containing 0.1ml of 2 M TrisCl pH 8.5
9. Wash with TBS until pH is reequillibrated.
10. Elute with 6 M GuanidineHCl in TBS, collecting 1ml fractions.
11. Wash with TBS + 0.1% NaN3, and store at 4 deg C.
12. To determine where to pool fractions, spot 1 ul of each fraction onto nitrocellulose paper and stain with ponceau S. Pool all fractions that show pink color.
13. Dialyze ON into TBS or your favorite buffer.
14. If necessary the antibody can be concentrated by sweating the dialysis bags, or by spin-concentrating.
15. Bring the azide concentration up to 0.1% and store at 4 deg C for up to three months. For longer storage freeze in aliquots and store at -80 degC or add glycerol to 50% and store at -20 deg C.

Note: Do not pool the low pH and GuHCl eluates as they may have significantly different properties. We have found that the GuHCl may have higher affinities, but also may contain a higher fraction of partially denatured antibody that could contribute to staining background. The proportion in each pool varies with the peptide immunogen.

The quality of the anti-peptide serum seems to increase with multiple boosts - the first bleed may be feeble.

C.Affinity purification of antibodies from crude serum

A. **Binding Antigen to Column perform all steps at 4°C.**

1. Depending on application, affigel-10 or affigel-15 are appropriate as supports for binding of antigen. Affigel-10 is best for binding proteins at near to or below their isoelectric point, while affigel-15 is better for binding proteins near or above their isoelectric point. Thus, when coupling at or close to neutral pH, affigel-10 is better for proteins with pI of 6.5 to 11.0 while affigel-15 is better for proteins with pI less than 6.5. The following conditions refer to affigel-10 coupling at pH 7.5.
2. Wash 1ml of affigel beads two times in 10ml cold dH20, spin down between washes at 3500rpm for ca. 30". Resuspend beads in 1ml binding buffer (100mM MOPS, pH 7.5)
3. Dialyse 4mg antigen protein (at about 1mg/ml) against 1 litre 100mM MOPS pH 7.5 overnight at 4°C. Retain 10ul of protein for assaying binding.
4. Add to beads and agitate gently for 4 hours at 4°C.
5. Take 10ul supernatant and run on SDS PAGE gel with sample from 3 above to assess how much protein has bound column. Samples can also be taken during binding to assess rate of binding. Expect to see at least 90% of the protein bound after 2 hours and complete binding after 4 hours. If binding has not gone to completion, adding CaCl2 to 80mM can improve binding.
6. Add 100ul 1M ethanolamine HCl pH8, agitate gently for another hour at 4°C. This will block any free binding sites on the resin.
7. Transfer gel to econocolumn (Biorad).
8. Wash column with the following solutions:

   - 10ml 100mM MOPS pH 7.5
   - 10ml PBS
   - 10ml 100mM glycine HCl pH 2.4 / 150mM NaCl
   - 10ml PBS

9. Store column in PBS/0.2% sodium azide at 4°C.

Binding antibodies to column

10. Wash column with the following solutions:

    - 10ml PBS,
    - 10ml 100mM glycine HCl pH 2.4 / 150mM NaCl
    - 10ml PBS

11. Spin serum (ca 1.5ml) 13000 rpm 10 minutes.
12. Apply supernatant to the column to which a 27 gauge needle has been fitted to slow rate at which column flows. Collect run-through and apply to column at least twice more. remove the needle.
13. Wash column with 10ml PBS.
14. Elute antibody with 100mM glycine HCl pH 2.4 / 150mM NaCl. Collect at least ten 1ml fractions into 200ul 1M Tris HCl pH 8, to immediately neutralise.
15. Run 20 ul samples of the fractions on SDS PAGE to assay elution of specific antibody. Antibody should be in fractions one to four.
16. Wash column with the following solutions:

    - 10ml PBS,
    - 10ml 100mM glycine HCl pH 2.4 / 150mM NaCl
    - 10ml PBS

17. Store column in PBS/0.2% sodium azide at 4°C.

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