



GENERATION & PURIFICATION OF RABBIT POLYCLONAL ANTIBODIES

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September 2016

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BACKGROUND

This protocol describes how to generate polyclonal antibodies against a peptide of interest in rabbits. This process takes a minimum of 3 months and should be carefully planned at each step. Briefly, the protein of interest that you wish to generate antibodies against is analyzed via pBLAST to identify regions that are highly unique. It is advisable to consider what regions (epitopes) of the protein are intra/extra cellular and are accessible in their native form. This is particularly important when the antibody will be applied in immunoprecipitation, immunohistochemistry, flow cytometry and fluorescence microscopy. Western blotting is more robust as it is generally performed on denatured proteins.

Due to the fact that the peptide that will be generated is a hapten and therefore has low immunogenicity, it will be coupled to a carrier protein, Keyhole Limpet Hemocyanin (KLH) to increase immunogenicity. KLH is very large (390kDa) protein that has many immunogenic epitopes and many lysine residues that allow for coupling of a peptide. Coupling can be done using a variety of crosslinking mechanisms including glutaraldehyde to link the N-terminus of the peptide to the carrier, carbodiimide (EDC) to attach the C-terminus of the peptide to the carrier, succinimide esters (MBS, SMCC) to bind free amino groups and Cys residues and benzidine (BDB) to link Tyr residues to the carrier. Each method has its pros and cons and should be evaluated after a peptide is selected.

Once the peptide is coupled to the carrier, it is emulsified first in Freud's complete adjuvant (FCA); mineral oil with inactivated *Mycobacterium tuberculosis* (Mtb) This "prime" immunization is given to a rabbit followed by 3 "boosts" at 3 week intervals. The boost is composed of the conjugated peptide emulsified in Freud's incomplete adjuvant (which does not contain Mtb). After the third boost, a small sample of rabbit blood is collected to isolate the serum. Serum is then purified via peptide affinity purification columns and the purified antibody is tested via Western blot. When an adequate titer of antibodies is reached, the rabbit is sacrificed and exsanguinated and serum is stored for later purification.

NOTES

- Rabbits must be ordered and acclimated to their environment in the animal facility prior to beginning immunizations. A large amount of preparation and time is required before usable antibodies are available.

EQUIPMENT

- New Zealand White (NZW Rabbits) – usually 2 per antibody/peptide. ~5kg weight, male. We find 2 rabbits is safer, as sometimes we get weak responses in 1 rabbit. This protocol assumes 2 rabbits are being immunized.
- 3 mL syringes
- 20G needles
- Phosphate buffered saline (PBS)
- Bis-diazonium-benzidine (BDB, generated from Benzidine, Hydrochloric acid and Sodium Nitrate; see below). **HIGHLY CARCINOGENIC. BE CAREFUL.**
- Your peptide of interest. Usually we order it from GenScript on a 14mg scale.
- Standard pipettes, pipette tips and eppendorf tubes
- Sodium borate buffer (0.2M sodium borate, 0.15M NaCl, in water, pH=9)
- Keyhole Limpet Hemocyanin (KLH) – Sigma Cat #H7017-20MG
- Freud's Complete Adjuvant – Sigma Cat#F5881-10mL
- Freud's incomplete Adjuvant- Sigma Cat# F5506-10mL

- A sterile syringe emulsion adapter/connector. This is a metal or plastic connector to connect two 3 mL syringes together when preparing the injections.
- Affigel 10 (Bio Rad)
- 20 mM HEPES, pH =7
- 1M Ethanolamine pH = 8
- Plastic resin columns (various vendors)
- PBS with 0.01% Sodium Azide
- 70% ethanol
- 50mL Glycine, 100mM, pH = 2.7
- 50mL TRIS, 1M, pH= 9.0
- Dialysis tubing or cassettes
- Large (2L or 4L) beaker
- Stir bar
- Stir plate

PROCEDURE

Design, Preparation and Conjugation of Peptide

1. Design your peptide using approximately the last 15-20 C-terminal residues. Preferably your peptide will start with a glycine residue.
Note: you may prefer to choose N-terminal residues depending on your antibody applications. Avoid selecting peptides from regions of the protein that may be buried in a cell membrane or within the protein itself.
2. It is imperative to ensure the peptide is specific for your protein of interest by performing a protein BLAST.
3. Add adventitious Lys (K) and Tyr (Y) residues to the N-terminus of the chosen peptide. Your peptide will read **KY(G)XXXXXXXX**. The purpose of the Lys-residue is for coupling to the Affigel 10 beads during purification and the Y residue is used to couple to protein to the KLH carrier.
4. Order the peptide from a commercial vendor. We prefer GenScript and order a 14mg-scale peptide. It should take 2-3 weeks. When the peptide arrives, store the lyophilized powder at -80C before use. Keep the spec paper.
5. Synthesize Bis-diazonium-benzidine (BDB) by dissolving 0.115 g of Benzidine HCL in 22.5 mL 0.2N HCL. Dissolve 87.5 mg Sodium Nitrate in 2.5 mL H₂O. Combine the two liquids and incubate with gentle mixing at 4C for 1 hour. **BE CAREFUL. BDB IS HIGHLY CARCINOGENIC. ALL LAB & MSDS SAFETY PROTOCOLS SHOULD BE FOLLOWED!** Excess BDB aliquots can be frozen and stored at -80C.
6. Prepare sodium borate buffer if it has not already been prepared. See above for recipe.
7. Read the certificate of analysis of the peptide and find the total quantity of the peptide (10-14 mg). Add sterile water to the peptide to make a 100 mg/mL stock. For example if you have 10.3 mg, you will add 103 uL sterile water. NOTE: your peptide may require a different solvent depending on the polarity and size. Consult GenScript's solubilization procedure included with your peptide.
8. Add 5mg of the peptide (50 uL) to 450 uL sodium borate.
9. Dissolve KLH in borate buffer to a final concentration of 8 mg/mL. Try to avoid frothing and oxidization of the protein.
10. Add 4 mg of KLH (500 uL) to the peptide solution for a total volume of 1mL.
11. Test the pH using pH paper. It should be between 8 and 9. If not, adjust accordingly with acid or base.
12. Add 100 uL of BDB to the 1 mL peptide-KLH solution to initiate coupling. Invert the tube a couple times. There should be a quick change in color to either a darker yellow or a maroon-brown color.
13. Mix for 2 hours at 4C with gentle rocking. The color may become darker.
14. Aliquot the solution in 400 ug aliquots and store at -80 C. The volume will be around 50 uL.
Note: Rabbits are immunized with 400 ug. Mice with 100 ug. You may want to adjust accordingly if using mice.

Preparation of Immunizations

- 15.** The rabbits are immunized by the Vaitukaitis protocol with 4 intradermal immunizations at once. First, an initial prime is given using Freud's complete adjuvant. After 3 weeks, a boost is performed using incomplete adjuvant, followed by two more additional boosts in 3 week intervals. It is important to know which rabbits will receive which antigens when creating different antibodies simultaneously to avoid mixing up immunizations and ruining the production.
- 16.** If the immunization is being performed by a technician, submit a request for service (RFS) form **48 hours** before the immunization is to be performed. AUP: 10-08-56. Species: NZW/Rabbit. Room: 1U87.
The RFS should state "Please Inject Rabbit X (the ID of the rabbit) with syringe labeled X subcutaneously into four sites per animal with 0.25mL of antigen/adjuvant for a total of 1 mL per animal.
If required, a sedative such as ACEpromazine can be used for easier injection.
For 1 week post-injection, please inspect injection sites daily for signs of infection or abscess.
- 17.** 1-2 hours before the immunization is performed, prepare the immunization by thawing one 400 ug stock of peptide/KLH per rabbit.
- 18.** Add 450 uL PBS to each tube.
- 19.** Sterilize the rubber cap of Freud's complete adjuvant (if performing a prime) or incomplete adjuvant (if performing a boost). Using a 20G needle, draw 1 mL adjuvant into a 3 mL syringe. Now you will have 1 syringe with 1 mL peptide solution in PBS and 1 syringe with 1 mL adjuvant.
- 20.** Carefully remove the needle from each syringe and connect the ends to the emulsifying adapter.
- 21.** Firmly push the plungers back and forth to emulsify the adjuvant and peptide solutions. The solution should turn white/cream colored and become increasingly hard to push back and forth.
- 22.** After 15-20 pushes, let the syringe sit for 5 minutes. If the emulsion has not separated into oily and aqueous phases, your immunization is ready. Adjust the volumes so 1mL is in each syringe.
- 23.** Disconnect the adapter and connect 20G needles to each syringe. Clean out the adapter with distilled water and ethanol.
- 24.** Take the preparations to the animal facility. Be sure the syringes are well labeled with rabbit ID #s.
- 25.** Repeat this procedure for at least 3 boost immunizations (with incomplete adjuvant) at 3-week intervals.

Preparation of Affinity Purification Column

- 26.** 10-11 days after the third boost immunization has been given, prepare an affigel affinity purification column by creating a 50% slurry of AffiGel 10 in 70% ethanol.
- 27.** Take 8 mL slurry into a 15 mL tube and centrifuge at 4000 rpm for 2 minutes at 4 degrees. This will yield 3-4 mL packed AffiGel 10
- 28.** Wash the AffiGel in 20 mM HEPES pH = 7.0 2-3 times.
- 29.** Resuspend the AffiGel in 8 mL 20 mM HEPES pH = 7 (total volume should be 12 mL) and add 5mg (50 uL) of the purified peptide (**NOT THE KLH CONJUGATED PEPTIDE**) to the AffiGel.
- 30.** Incubate at 4C with gentle mixing for 2 hours.
- 31.** Wash the AffiGel with 20 mM HEPES pH = 7 three times.
- 32.** Resuspend the resin in 1M ethanolamine pH = 8 and incubate with gentle mixing at 4C for 1 hour.
- 33.** Wash the AffiGel with 20 mM HEPES pH = 7 three times. You now have peptide-conjugated AffiGel.
- 34.** Clean the resin column with 70% ethanol and wash with sterile water.
- 35.** Resuspend the peptide-conjugated AffiGel in PBS with 0.01% sodium azide.
- 36.** Transfer the suspension to the column (bottom must remain capped) and store at 4C until use.

Purification of Polyclonal IgG from Serum (Test Bleed)

37. 10-11 days after the third boost immunization, send a RFS form for a test bleed. The information will remain the same as above, however it will read "Please bleed each rabbit for 10 mL and label each tube with the ID of the rabbit. As before, if ACEpromazine is required, it may be used".
38. Put the blood in a 37C incubator and allow clotting for 30 minutes.
39. Transfer the tube to a 4C fridge for 30 minutes. Break the clotted blood using a pipette.
40. Transfer the liquid fraction to a new 15 mL tube.
41. Centrifuge at 4000 rpm for 10 minutes at 4 C.
42. Repeat steps 40 and 41 once more.
43. The serum should be diluted 1:1 in PBS and can now be purified or stored at -80C for later use.
44. Wash the column with PBS twice.
45. Load the diluted serum into the column, collect the flow through and run it through the column a second time.
46. Collect the twice-run serum and re-freeze. There may still be antibodies remaining, depending on how well the immunization worked.
47. Wash the column with PBS 3 times.
48. Determine the ratio of 1M TRIS pH = 9.0 to 100mM Glycine pH = 2.7 in order to get a neutral 1mL solution. This is typically approximately 50-55uL TRIS and 945-950uL Glycine. This is so when the antibodies are eluted using acidic Glycine, the fractions can be placed directly into pre-aliquotted TRIS in order to neutralize the solution rapidly. Use pH paper to determine the correct ratio.
49. Label 12 eppendorf tubes #1-12 and aliquot the determined amount of 1M TRIS pH = 9.0 into each.
50. With a second person in charge of vortexing and closing the tubes ready, load 20mL 100mM Glycine pH= 2.7 and unscrew the stopper. Fill the first tube to the 1mL mark and quickly switch to the second tube. At the same time, pass the first tube to the helper who will quickly close the tube and vortex it 3-4 times. Repeat until 1-2mL Glycine is left in the column and screw in the stopper.
51. Test the protein fractions either by nanodrop or BCA/Bradford assay, using a neutral TRIS/Glycine mix as the blank. Keep the best 4-8 protein concentrations, with a minimum of around 0.3mg/mL. Record the concentrations and pool the 3-4 fractions with the best concentrations (usually in the 150-300ug/mL range).
52. Load the solution into a dialysis membrane cassette using a 21 gauge syringe. Be extra careful not to puncture the membrane. Dialysis tubing can be used alternatively. If using a cassette, attach it to a piece of Styrofoam.
53. Fill a 2L beaker with around 1.5L PBS and add a stir bar. Carefully place the cassette/Styrofoam piece into the solution. Let this gently stir at 4°C overnight.
54. The next morning, change the PBS and let stir for an additional 2 hours.
55. Remove the solution from the cassette using a 21 gauge syringe and aliquot the antibody. If storing at 4°C, add 0.01% Sodium Azide to prevent contamination. This is not required for aliquots stored at -20°C.
56. The antibody is now ready for testing. You may want to quantify the final concentration by standard methods such as a Bradford or BCA assay. We recommend testing the antibody by western blot. **ENSURE YOU HAVE BOTH A POSITIVE AND NEGATIVE CONTROL LYSATE.**
57. If the antibody has reached a desired titer after the third boost, you can submit an RFS to sacrifice the rabbit by exsanguination (removing as much blood as possible). Serum is collected and stored as above and can be purified as needed.

REFERENCES

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