Non Standard Chapter

Production of Antipetide Antibodies

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Summary

Peptides (8-20 residues) are as effective as proteins in raising antibodies, both polyconal and monoclonal with a titer above 20,000 easily achievable. A successful antipeptide antibody production depends on several factors such as peptide sequence selection, peptide synthesis, peptide-carrier protein conjugation, the choice of the host animal, and antibody purification. Peptide sequence selection is likely the most difficult and critical step in the development of antipeptide antibodies. Although the format for designing peptide antigens is not precise, several guidelines can help maximize the likelihood of producing high quality antipeptide antibodies. Typically, 5-20 mgs of peptide is enough for raising an antibody, for preparing a peptide affinity column, and for antibody titer determination using an Enzyme-Linked ImmunoSorbent Assay (ELISA). Usually, it takes three months to raise a polyclonal antipeptide antibody from a rabbit that yields ~90 mL of serum which translates into approximately 8-10 mgs of the specific antibody after peptide affinity purification.

Key Words: Antipeptide antibody; peptide-carier protein conjugation; keyhole limpet hemocyanin (KLH); polyconal and monoclonal antibodies; phosphospecific antibodies; antibody titer.

1. Introduction

With their high specificity and binding ability (dissociation constant of 10^{-6} - 10^{-12}), antibodies are the most widely used reagents for protein recognition in many biochemical applications ***(1-4)***. Native or recombinant proteins are used traditionally to produce antibodies. Generating polyclonal antibodies against a protein yields antibodies against multiple epitopes, which maximizes the chance of recognizing the protein. However, this pool of antibodies does increase the cross-reactivity with other proteins (*see* **Note 1**). Generating polyclonal antibodies against a synthetic peptide **(*1-2,5-6*)** (*see* **Note 2**), by contrast, will produce antibodies that are specific to the target protein. There are instances in which a peptide serves as a better choice than a protein, e.g., raising antibodies for a specific protein isoform or for a phosphorylated protein and in cases where the protein is not available.

1.1. Overview of Producing Antipeptide Antibodies

Peptides at low cost with high purity can be obtained form numerous companies [a list of companies can be found on the Peptide Resource Page (www.peptideresource.com)]. Also, there are many companies that will produce antipeptide antibodies for a fee [a list of companies can be found on the Antibody Resource Page (www.antibodyresource.com)]. Nevertheless, not all antipeptide antibodies recognize native proteins and have a high titer. The potential drawback of choosing a peptide sequence that does not posses a high antigenicity and won't correspond to an exposed region of the native protein can be reduced substantially by carefully analyzing the protein sequence and structure using the large number of protein structure and antigenicity prediction software. Additionally, co-immunization of multiple peptide antigens from a protein will statistically increase the chances of obtaining antibodies that will recognize the target protein (*see* **Note 3**). Peptides containing phosphorylated amino acids can also be used to produce phosphospecific antibodies (*see* **Note 4**). However, a peptide by itself is typically too small to induce an immune response producing high titer antibodies. The minimum molecular weight needed to induce sufficient immune response is 5 - 15 kDa. A carrier protein is therefore conjugated to the peptide to induce an immune response in the animal.

The most commonly used carrier protein is keyhole limpet hemocyanin (KLH, MW 4.5 x 10^5 – 1.3 x 10^7) that has been shown to aid in the production of high titer antipeptide antibodies (*see* **Note 5**). KLH contains numerous exposed lysine residues, which allows for the covalent attachment of large numbers of peptide molecules. With the advancement in peptide synthesis, peptide selection, and peptide-carrier protein conjugation, peptides rather than proteins are becoming the method of choice for antibody production.

1.2. Analysis of Protein Sequence and Structure

Before producing a new antipeptide antibody, it is essential to know some basic features of the protein. Ensuring that the correct species and protein sequence have been identified is the first step. Information of the protein's structure can aid in choosing epitopes that are readily accessible to the antibodies. Any potential crossreactivity with other closely related proteins, e.g., domain structures, should be avoided. Searching the protein NCBI database (www.ncbi.nlm.nih.gov), Uni-Prot (www.uniprot.org), PIR (pir.georgetown.edu), ExPASy (us.expasy.org/tools), etc. might be helpful. Searching a list of commercially available antibodies at www.antibodyresource.com/findantibody.html may help you find the antibody that you need.

1.3. Selection of Peptide for Antibody Production

The choice of peptide sequence for antipeptide antibody production is the single most important step in the process. Despite there being no infallible method for predicting antigenic peptides, there are several guidelines that can be followed to determine the peptide epitopes in a protein that are likely to be antigenic. These guidelines also increase the odds of an antibody recognizing the native protein. Other crucial parameters are the ease of peptide synthesis and conjugation, peptide stability and solubility in water, immunogenicity in the host animals, and specificity for the target protein.

1.3.1. Peptide Antigenicity/Accessibility Factors

It is unpredictable whether a designed peptide antibody will recognize the native protein due to conformation and other structural differences between synthetic peptides and peptide epitopes in native proteins. In general, ideal antigenic epitopes are hydrophilic, surface orientated and flexible. This is because in most natural environments, hydrophilic regions tend to reside on the surface, whereas hydrophobic regions are likely found hidden in the protein conformation. Antibodies can therefore only bind to epitopes found on the surface of proteins and tend to bind with higher affinity when those epitopes are flexible enough to move into accessible positions. The following are some guidelines to increase the likelihood of successful peptide selection.

1. All recommended peptides are compared for sequence homology with other proteins by Basic Local Alignment Search Tool (BLAST) database searches.
2. BLAST protein search with Protein Lounge (database containing numerous antigenic peptide targets, http://www.proteinlounge.com.)
3. Choosing peptides that are in the N- or C-terminal region of the protein is effective because these regions of proteins are usually solvent accessible and unstructured and antibodies developed against these peptides are also more likely to recognize the native protein. The N terminal capped with an acetyl group and the C terminal with an amide group will make the peptide appear more like a native protein and will reduce the degradation of the peptide in the animal.
4. Peptides lying in long loops connecting secondary structure motifs are preferable, avoiding those that are located in helical regions. This will increase the odds of the antibody recognizing the native protein. For proteins with known 3D coordinates, secondary structures can be obtained from the sequence link of the relevant entry at the Brookhaven data bank (www.rcsb.org/pdb/home/home.do). Solvent accessibility can be determined using a variety of programs such as DSSP (swift.cmbi.ru.nl/gv/dssp), NACESS

(www.bioinf.manchester.ac.uk/naccess) or WHATIF (swift.cmbi.kun.nl/whatif; swift.cmbi.ru.nl/servers/html/index.html).

5. When no structure information is available, secondary structure and accessibility predictions can be obtained from the following servers with 80% accuracy in predicting α-helix, β-strand, and loop **(*7*)**: PHD (www.predictprotein.org), JPRED (www.compbio.dundee.ac.uk/~www-jpred), PSI-PRED (bioinf.cs.ucl.ac.uk/psipred), PredAcc (bioserv.rpbs.jussieu.fr/RPBS/html/fr/T0_Home.html), SSPRED (coot.embl.de/~fmilpetz/SSPRED/sspred.html), PREDATOR (www-db.embl-heidelberg.de/jss/servlet/de.embl.bk.wwwTools.GroupLeftEMBL/argos/predator/down_predator.html), etc.
6. Websites for antiginicity prediction such as www.innovagen.se, immunax.dfci.harvard.edu/Tools/antigenic.pl **(*8*)**, www.immuneepitope.org/home.do, www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp, and ca.expasy.org/tools/protscale.html are useful as they check the peptide sequence against a number of criteria to estimate its suitability for antipeptide antibody production. Several software programs such as MacVector, DNAStar, and PC-Gene that incorporate one or more of several accepted algorithms for predicting peptide antigenicity are also very useful.
7. Not all areas of the protein such as the transmembrane regions are accessible to antibodies and should be avoided. Accessible (hydrophilic, surface-oriented, and flexible) regions of the native protein are exposed on the surface of the protein and in contact with the aqueous environment. Antigenic peptides are generally located in solvent accessible regions and may contain both hydrophobic and hydrophilic residues.

8. Generally, 8-20 amino acid long peptides should be used. Longer peptides increase the risk of losing specificity and shorter peptides may elicit antibodies that would not recognize the native protein with sufficient affinity.
9. Incorporation of proline and tyrosine residues confers some structural motif to the immunogen which is likely to be found in the native protein.

1.3.2. Peptide Synthesis and Solubility Factors

Investigating protein sequence to identify the best candidate peptides that optimize synthesis success and solubility is an essential step in antipeptide antibody production. Several observations are listed as follows:

1. Peptide solubility is strongly influenced by amino acid composition. Peptides with a high content of hydrophobic residues, such as Leu, Val, Ile, Met, Phe and Trp, have either limited solubility in aqueous solution or are completely insoluble. It is advisable to keep the hydrophobic amino acid content below 50% and to ensure that there is at least one charged residue for every five amino acids. A single conservative replacement, such as replacing Ala with Gly, or adding a set of polar residues to the N- or C-terminal, may also improve solubility.
2. During synthesis, β-sheet formation causes incomplete solvation of the growing peptide and results in a high degree of deletion sequences in the final product. This problem can be avoided by choosing sequences that do not contain multiple and adjacent residues comprising Val, Ile, Tyr, Phe, Trp, Leu, Gln, and Thr. If sequences cannot be chosen to avoid stretches of these residues, it often helps to break the pattern by making conservative replacements, for example, inserting a Gly or Pro at every third residue, replacing Gln with Asn, or replacing Thr with Ser.
3. Peptides containing multiple Cys, Met, or Trp residues are also difficult to obtain in high purity, partly because these residues are susceptible to oxidation and/or side reactions.

4. The following amino acids or sequences are best avoided:
   a. A sequence starting or ending with Pro.
   b. Ala, Val, Thr, Pro, or Ser doublets for synthetic purposes and ending sequences in Val, Ile, Trp, Tyr, and Phe.
   c. Extremely long repeats of the same amino acid (e.g., ArgArgArgArgArg) and Gln or Asn at the N-terminal.
5. A peptide having an overall charge close to neutral is desirable.
6. Ending sequences with hydrophilic residues are favorable as side groups will promote increased solubility along with free alpha reactive groups. Terminal ends are highly exposed to their environment.
7. Limiting the number of contiguous charged or hydrophobic residues is helpful as they can isolate a portion of the peptide leading to increased solubility problems
8. Due to the nature of glycine and its lack of a side group, it does not behave as a hydrophobic residue unless contiguous stretches exist**.**

1.4. Peptide Synthesis (9-*12*)

Usually peptides used for antipeptide antibody production contain 10-25 amino acid residues and are obtained either from a core facility or a commercial vendor. A state-of -the-art peptide synthesizer would have little problem producing 50 mgs of peptide with > 90% purity even with several phophorylated amino acids in the peptide. Peptides are routinely synthesized using stepwise Fmoc solid-phase synthesis chemistry starting from the c-terminus. The procedure for synthesizing a peptide is as follows (*see* **Note 6**): (I) The Fmoc group of the amino-acid-preloaded resin is removed by 20% piperidine, (II) Fmoc-amino acid (with or without modification) is coupled 60 min to the resin-bound peptide using 0.1 *M* 2-(1H-Benzotriazole-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate (HBTU) in dimethylformamide (DMF) containing 0.4 *M* 4-methylmorpholine for,

(III) Steps 1 and 2 are repeated untill the last amino acid is added, (IV) The Fmoc group of the resin-bound peptide is removed by 20% piperidine, (V) The peptide is then deprotected and cleaved from the resin using trifluoroacetic acid (TFA), (VI) Ethyl ether is added to precipitate the peptide from the TFA solution and the precipitated peptide is lyophilized, (VII) The crude peptide is purified on a reversed-phase C18 column using a preparative HPLC system. A flow rate of 20 mL/min with solvent A (0.1% TFA in deionized water) and solvent B (0.1% TFA in acetonitrile) is used. The column is equilibrated with 5% solvent B. After sample loading, the column is eluted with a linear gradient from 5% solvent B to 100% solvent B in 60 min, and (VIII) The pure peptide fraction is identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

Dissolving peptides in aqueous solutions is not trivial. In practice, the peptide sequence should contain at least 20% charged residues to facilitate solubilization. Hydrophilic peptides containing >25% charged residues (Glu, Asp, Lys, Arg, and His) and <25% hydrophobic residues also generally dissolve in aqueous media provided that the charged residues are fairly distributed throughout the sequence. Both acidic peptides (Glu+Asp residues> Lys+Arg+His residues) and basic peptides (Lys+Arg+His residues> Glu+Asp residues) are more soluble at neutral pH than at acidic pH. Hydrophobic peptides containing 50% to 75% hydrophobic residues may be insoluble or only partially soluble in aqueous solutions even if the sequence contain 25% charged residues. It is best to first dissolve these peptides in a minimal amount of stronger solvents such as DMF, acetonitrile, isopropyl alcohol, ethanol, acetic acid, 4-8 *M* guanidine HCl or urea, dimethyl sulfoxide (DMSO), and then slowly add (drop wise) the solution to a stirred aqueous buffer solution. Very hydrophobic peptides containing >75% hydrophobic residues will generally not dissolve in aqueous solutions. These peptides generally require initial solubilization in very strong solvents such as TFA and formic acid and may precipitate when added into an aqueous buffered solution. Peptide sequences containing a very high (>75%) proportion of Ser, Thr, Glu, Asp, Lys, Arg, His, Asn, Gln or Tyr are capable of forming extensive intermolecular hydrogen bond networks and have a tendency to form gels in concentrated aqueous solutions. Sonication may

increase solubility to an extant. 10% acetic acid in the solvent will help dissolve basic peptides whereas10% ammonium bicarbonate will help dissolve acidic peptides. For peptides with extremely low solubility in aqueous solutions, organic solvents (such as DMSO, isopropanol, methanol, and acetonitrile) should be used first. Once the peptides are completely dissolved, deionized water may be gradually added until the desired concentration is obtained. If peptide samples need to be frequently or periodically taken from a stock at -20 °C, it is recommended to make a series of aliquots from the stock. Peptide sequences containing Cys, Met, or Trp are prone to air oxidation. It is recommended to purge the air out of the vial and replace it with a blanket of nitrogen or argon. Lyophilized peptides can be stored with drierite long term at -20 °C.

1.5. Animal Immunization

A number of animals are suitable hosts for antipeptide antibody production, including mice, guinea pigs, rats, hamsters, rabbits, chickens, pigs, goats, sheep, bovines, donkeys, and horses (*see* **Note 7**). Selection of the appropriate animal species is dependent on several factors: the presence of a homologous protein in the species being immunized, the amount of antibody required, the amount of antigen available for immunization, the time required to obtain an antibody response, and the cost. Information on emulsion of adjuvant mixed with antigen, immunization routes, bleed, and antiserum preparation are widely available ***(1,2)***. The most common host animal for polyclonal antipeptide antibody production is the New Zealand White rabbit (female, 8 weeks of Age, used 95% of the time). It has the ability to respond to a broad classes of antigens and can produce good antibodies in 77 days. For rabbit projects, the titers remain relatively level after the 2nd booster and additional immunizations are used to maintain antibody titers rather than to increase them. Freund's adjuvant (*see* **Note 8**) is used in these rabbit projects. An adjuvant is a substance that serves to enhance the immune response against the antigen. Freund's adjuvant should be emulsified aseptically using syringes or sonication. An

insoluble antigen is as good as a soluble one. In the case of rabbits, 20 mL of serum is collected prior to the initial immunization by injection at four separate subcutaneous (s.c.) sites (two inguinal, two axillary) of 0.25 mgs peptide-KLH emulsified with Freund's complete adjuvant (FCA). A booster injection with 0.25 mgs peptide-KLH emulsified with Freund's incomplete adjuvant (FIA) is given 14 days after the initial immunization. Subsequent booster injection with 0.25 mgs peptide-KLH emulsified with Freund's incomplete adjuvant (FIA) are given every 4 weeks. 20 mL of serum is collected 10 days after each booster injection. At the conclusion of the animal immunization at day 77, a large-volume terminal bleed (90 mL) is collected by the rabbit exsanguination.

2. Materials

2.1. Peptide-Carrier Protein Conjugation

1. 0.01 *M* phosphate buffer, pH 7.0. Prepare by using 0.01 *M* Na_2HPO4 to adjust the pH of a 0.01 *M* solution of NaH_2PO_4 to pH 7.0.
2. 0.05 *M* phosphate buffer, pH 6.0. Prepare by using 0.05 *M* Na_2HPO4 to adjust the pH of a 0.05 *M* solution of NaH_2PO_4 to pH 6.0.
3. Dimethylformamide (DMF, Sigma, St. Louis, MO).
4. M-maleimidobenzoic acid N-hydroxysuccinimide ester (MBS, Sigma, St. Louis, MO).
5. Keyhole limpet hemocyanin (KLH, Pierce, Rockford, IL).
6. Bovine Serum Albumin (BSA, Sigma, St. Louis, MO).
7. PD-10 column (Pharmacia Bioscience, Piscataway, NJ).

2.2. Antipeptide Antibody Titer Determination by ELISA

1. Carbonate Buffer: 15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.02% NaN_3 , adjust pH to 9.6 with 1 *N* NaOH.
2. 0.2 to 2.5 μ*M* synthetic peptide in carbonate buffer.

3. Phosphate-buffered saline (PBS): 140 m*M* NaCl, 27 m*M* KCl, 7.2 m*M* Na_2HPO_4, 14.7 m*M* K_2HPO_4, pH 7.2.
4. PBS containing 0.05% Tween-20 (PBST,).
5. Blocking solution: 10 mgs/mL BSA in PBST.
6. Secondary antibody: Goat anti-rabbit globulin conjugated to akaline phosphatase (Sigma, St. Louis, MO).
7. Enzyme substrate: 1 mg/mL p-nitrophenyl phosphate (Sigma, St. Louis, MO), 0.2 *M* Tris buffer, 5 m*M* $MgCl_2$.
8. Stopping solution: 0.01 *M* ethylenediaminetetraacetic acid or 3 *N* NaOH.
9. Immulon 2 or equivalent 96-well flat bottom microtiter plate (Dynetech Laboratory, Chantilly, VA; BD, Franklin Lakes, NJ).
10. Microtiter plate reader (Dynetech Laboratory, Chantilly, VA): spectrophotometer with 405 nm filter.

2.3. Peptide Affinity Purification

1. CNBr-activated Sepharose 4 B (Pharmacia Bioscience, Piscataway, NJ).
2. Eppendorf Centrifuge 5804R (Brinkmann Instrument, Westbury, NY).
3. 1 m*M* HCl.
4. Synthetic peptide powder.

3. Coupling buffer: 0.1 *M* $NaHCO_3$, pH 8.3, 0.5 *M* NaCl.

4. Washing buffer 1: 50 m*M* Tris-HCl, pH 8.0, 0.1% Triton X-100, 0.5 *M* NaCl.

5. Washing buffer 2: 50 m*M* Tris-HCl, pH 9.0, 0.1% Triton X-100, 0.5 *M* NaCl.

6. Washing buffer 3: 50 m*M* Sodium Phosphate, pH 6.3, 0.1% Triton X-100, 0.5 *M* NaCl. Prepare by using 50 m*M* Na_2HPO4 containing 0.1% Triton X-100 and 0.5 *M* NaCl to adjust the pH of a 50 m*M* solution of NaH_2PO_4 containing 0.1% Triton X-100 and 0.5 *M* NaCl to pH 6.3.

7. Elution buffer: 50 m*M* glycine-HCl, pH 2.5, 0.1% Triton X-100, 0.15 *M* NaCl.

8. Phosphate-buffer saline (PBS): 140 m*M* NaCl, 27 m*M* KCl, 7.2 m*M* Na_2HPO_4, 14.7 m*M* K_2HPO_4, pH 7.2.

9. 1 x 10 cm chromatography column.

10. 0.2 um syringe filter (Whatman, Piscateway, NJ).

3. Methods

3.1. Peptide-Carrier Protein Conjugation (*1-2,5-6,13*)

Molecules smaller than 5 - 15 kDa may not elicit any significant immune responses. To stimulate antibody responses to smaller peptides, peptides need to be covalently conjugated to a larger immunogenic carrier protein (KLH, BSA, etc) prior to immunization. Poor conjugation of the peptide to the carrier protein is one of the reasons why peptides fail to induce antibody production. It is critical that the peptide to carrier protein molar ratio be very high, and that all epitopes on the peptides be properly oriented in order to induce a high titer specific immune response. Several popular coupling methods which couple through sulfhydryl, amino, carboxyl, or hydroxyl groups are available. However, most peptides contain several side chains such as amino, carboxyl, and hydroxyl groups resulting in a multipoint attachment. It is preferable to attach a carrier protein through a sulfhydryl group present in a cysteine residue at either the N- or C- terminus of a peptide. The procedure for coupling peptides to KLH or BSA through a cysteine is as follows **(*13*)**:

1. Dissolve 5 mgs of KLH or BSA in 0.5 mL of 0.01 *M* phosphate buffer, pH 7.
2. Dissolve 3 mgs of MBS in 200 µl DMF.
3. Add 70 µl of MBS solution to 0.5 mL of KLH or BSA solution and stir or rotate for 30 min at room temperature. Add 2 mL of 0.05 *M* phosphate buffer, pH 6.
4. Equilibrate a PD-10 column using approximately 25mL of 0.05 *M* phosphate buffer, pH 6. Add the 2.5 mL of the MBS/KLH or BSA/MBS solution to the column and elute with 3.5 mL of 0.05 *M* phosphate buffer, pH 6. Add 0.5 mL of deionized water to the 3.5 mL of purified KLH/MBS or BSA/MBS.
5. Dissolve 5 mgs of peptide in 100 µl of DMF. Rapidly add 1 mL of purified KLH/MBS or BSA/MBS. Shake rapidly and immediately add 11 µl of 2 N NaOH.
6. Check the pH with pH paper. It should be 7.0 – 7.2. Too high a pH or too a low pH will stop the reaction between KLH/MBS or BSA/MBS and peptide. If needed, immediately add an appropriate amount of 0.5 N HCl or 2 N NaOH to change the pH.
7. Stir or rotate the solution for 3 hrs or overnight at 4 °C. Finally, add 3 mL of ammonium bicarbonate (0.1 *M*) before lyophilizing the coupled peptide.

3.2. Antipeptide Antibody Titer Determination by ELISA

An antibody titer is defined as the highest antibody dilution that still yields a positive reactivity of a particular epitope in an assay system such as ELISA. This value gives an indication of the concentration of an antibody preparation. This section describes an assay used to determine the titer of antipeptide antibodies in serum raised against a peptide or a protein containing the peptide sequence.

1. Coat the wells of a mictrotiter plate with 300 µl of 0.2 to 2.5 µ*M* synthetic peptide, leaving wells at the end as blanks. Incubate overnight at 4 ^{o}C (*see* **Note 9**).
2. Discard the unbound synthetic peptide.

3. Wash the wells 3 times with PBST.

4. Block the unoccupied sites with 300 µl/well of blocking solution.

3. Wash the wells 3 times with PBST.

4. Prepare serial dilutions of antiserim with PBST ranging from 1: 300 to 1:300,000 .

5. Add the serial dilutions of the antiserium to the wells and incubate for 2 hrs at 37 ^{o}C.

6. Wash the wells 3 times with PBST.

7. Dilute the secondary antibody 1:7000 with PBST.

8. Add the secondary antibody to wells and incubate at 37 ^{o}C for 2 hrs.

9. Wash the wells 3 times with PBST.

10. Add 50 µl enzyme subtractsubtrate. Incubate 10 - 30 mins at 37 ^{o}C.

11. Terminate color development by addition of 100 ul of stopping solution.

12. Measure absorbance at 405 nm with a microtiter plate reader. The titer corresponds to the highest dilution that still yields a positive reading.

3.3. Peptide Affinity Purification

Among many antibody purification methods (1-6), peptide affinity purification is the most effective technique to purify the antipeptide antibody. The peptide affinity purification is used for isolating those antibodies that recognize a specific epitope with about the same specificity as that of monoclonal antibodies.

1. Resuspend 1g dried CNBr-activated Sepharose 4 B in 50 mL of 1m*M* HCl for 30 mins.

2. Centrifuge for 5 mins at 1000 x *g* and discard the supernatant.

3. Wash the CNBr-activated Sepharose 4 B by resuspending the resin in 50 mL of 1m*M* HCl and after 15 mins spin at 1000 x g discarding the supernatant. Repeat this process twice.

4. Dissolve 10 mgs of synthetic peptide in 5 mL of coupling buffer.

5. Mix the synthetic peptide solution with the swollen gel. Stir gently for 1 hr.

6. Centrifuge for 5 mins at 1000 x *g* and discard the supernatant.

7. Wash excess synthetic peptide with 20 mL coupling buffer. Centrifuge for 5 mins at 1000 x *g* and discard the supernatant. .

8. Block remaining active groups by transferring the resin to 0.1 *M* Tris-HCl, pH 8.0, stand for 2 hrs.

9. Wash the resin with 0.1 *M* acetate buffer containing 0.5 *M* NaCl, pH 4.0. Centrifuge for 5 mins at 1000 x *g* and discard the supernatant. .

10. Wash the resin with 0.1 *M* Tris-HCl pH 8.0 containing 0.5 *M* NaCl. Centrifuge for 5 mins at 1000 x *g* and discard the supernatant.

11. Transfer the resin into PBS.

12. Pack the peptide affinity column by pouring the resin into a vertically held column.

13. Wash the column with 100 bed volumes of PBS.

14. Filter 15 mL rabbit serum through a 0.2 um filter.

15. Dilute the serum with PBS to 50 mL.

16. Load the filtered serum onto the peptide affinity column.

17. Wash the column with 20 mL PBS.

18. Wash the column with 20 mL washing buffer 1.

19. Wash the column with 20 mL washing buffer 2.

20. Wash the column with 20 mL washing buffer 3.

21. Elute the antibodies from the column with 20 mL elution buffer and collect in a tube containing 4 mL of 1 *M* Tris-HCl, pH 9.0 .

22. Wash the column with 20 mL PBS (*see* **Note 10**).

23. Use multiple PD-10 columns to desalt the antibodies by loading in 2.5 mL antibody per column and eluting with 3.5 mL PBS as the desalting buffer (*see* **Note 11**).

24. Calculate the antibody concentration (mg/mL) by A_{280} (the absorbance reading at 280 nm for a 1

mL solution) x 0.7.

25. Measure the titer of the purified antibody with the synthetic peptide using ELISA (*see* **Note12**).

4. Notes

1. Although monoclonal antibodies with their high specificity can be the antibody type of choice, they generally have a lower affinity, narrower utility, longer response time, and are more expensive than polyclonal antibodies.
2. Although pure peptides are always better, it is not a requirement to have > 90% pure peptides for antibody production. Even ~ 70% pure peptides can be used to generate antibodies successfully. However, if affinity purification is required, the more pure the antigen that is coupled to the peptide affinity column the higher the specificity of the purified antibodies.
3. In order to improve the chances of producing high titer antibodies, using several peptide sequences from a protein as antigens in a co-immunization protocol is beneficial. Individual KLH conjugates are mixed in equimolar ratios and used as immunogen. Having several peptides derived from a single protein will mathematically increase the chances of obtaining antibodies that will recognize the target protein. Peptide affinity columns can be used to isolate an antibody against each peptide.
4. The generation of a phophospecific antibody includes the synthesis of phosphorylated and non-phosphorylated peptides, immunization with the phosphorylated peptide, and fabrication of phosphorylated and non-phosphorylated peptide affinity columns for successive purification. The serum undergoes two rounds of purification. The first is the purification of antibodies that recognize the phosphopeptide, followed by a depletion step whereby the antibodies that bind to the non-phosphorylated peptide are removed. The flow-through of the non-phosphopeptide column is repurified by the phosphopeptide column. To get the anti-nonphosphopeptide antibody, the serum

should be applied to the non-phosphopeptide column first, and then remove the non-specific antibodies by the phosphopeptide column. Co-immunization with either different length of peptides in the case of a known phosphorylation site or peptides containing predicted phosphorylation sites in cases of unknown phosphorylation sites may be used to increase the chance of success.

5. Proteins such as thyroglobulin, Rabbit Serum Albumin, Bovine Serum Albumin, Ovalbumin, and Human Gamma Globulin are all very effective as carrier proteins. The resultant peptide-carrier protein complex is able to stimulate the immune system to produce antibodies against both the peptide and carrier proteins. Thus it is important that ELISA analysis be performed using peptide or peptide conjugated to a different carrier protein. Finally, there is some evidence that antibodies can also be produced that react to the crosslinker used to couple the peptide to carrier proteins. There is an alternative Multiple Antigenic Peptide (MAP peptide, juxtaposition of 4 or 8 peptide molecules on a crosslinked lysine core) method **(14-*15*)** for preparing peptide antigens, its advantage being that the conjugation step is not necessary. However, MAP is not a better method than KLH and it can bypass the immune response system in some hosts.
6. All reagents are of highest purity to ensure superb quality in synthesis. Water content is minimized in solvents and all containers are purged with nitrogen and sealed to ensure the longest possible shelf life.
7. There is little need to prescreen animals prior to immunization in most instances, but prescreening can be extremely important in studies of certain organisms such as yeast. All animals used for antibody production are certified specific pathogen-free, which provides a cleaner basis for antibody production. However, animals may already have generated closely related antibodies to something in their environment or their feed which may be similar to the antigen of choice. It is therefore a good idea to immunize more than one animal using a standard protocol. At the conclusion, the animal(s) with the best antibody response are continued on extended protocols.

8. Freund's Complete Adjuvant (FCA) should be used for the priming (first immunization) only. Freund's Incomplete Adjuvant (FIA) should be used for subsequence immunizations to prevent lesions at the sites of injection. Data shows positive and negative aspects about using FCA which was developed in the 1930s. It contains killed mycobacteria tuberculosis, paraffin oil, and mannide monoosleate and elicits a delayed hypersensitivity reaction. The water in oil emulsion using FCA is stable and provides a slow release of antigen and protects the antigen from degradation. The drawback with FCA is that it may cause granulomas and inflammation at the injection site with an intradermal injection. Note that one should avoid using FCA for studies of mycobacterium. Though FCA has been a mainstay and shown consistently superior result than alternative adjuvants in antibody production, some animal care and use committees reject the use of FCA due to its toxicity to the host animal. Therefore, other adjuvants such as Ribi, TiterMax, and Adjuvax ***(1)*** should be use in this case.

9. Most peptides can be coated on ELISA plates using carbonate buffer at pH 9.6. If the peptide does not adsorb completely, try other buffers in the pH 4 to 8 range. The coupling of peptides to BSA to facilitate coating is usually not necessary.

10. A typical peptide affinity purification of 20 mL of serum yields approximately 2 mgs of specific antibody. Although the capacity does decrease slightly with each use, the column can often be used for many additional purifications if stored at 4 ^{o}C with sodium azide.

11. For short term storage of antibodies, 4 °C is recommended. For longer term storage, we recommend storing at -20 °C or -80 °C. It is important, however, to avoid repeated cycles of freezing and thawing, as this will lead to partial denaturation of the antibodies. Sodium azide is an antimicrobial agent that prevents the growth of bacteria in the serum or purified antibody.

Antibody should be stored in ready-to-use aliquots thawing them only as they are needed. The addition of 50 % glycerol to the PBS is preferred, but not necessary.

12. In cases where an antibody doesn't work in a particular assay, likely explanations are: (I) The peptide sequence corresponds to a nonexposed region of the native protein, (II) the protein's conformation in the peptide region differs enough that the antibody has trouble recognizing the native protein, and (III) the target protein is not present in the sample. It is not uncommon to see multiple bands in protein western blots even when affinity-purified antibody is used. This is not indicative of a problem with the antibody's specificity. Rather, this typically occurs for one of the following reasons: (I) The antipeptide antibody recognizes a homologous protein in the sample that shares one or more epitopes with the peptide sequence, (II) the protein is a different molecular weight than previously predicted, (III) the antibody recognizes either cleaved fragments of the protein at lower molecular weights or aggregated multimers of the native protein at higher molecular weights. The advantage of using a western blot (native or sodium dodecyl sulfate polyacrylamide gel electrophoresis; one or two dimensional) ***(2-3,16-17)*** to evaluate the antibody over the ELISA method is that proteins are separated and probed by antibody individually. The downside is that western blotting is a labor intensive technique.

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