

Chapter 2

Antibody Production with Synthetic Peptides

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Abstract

Peptides (usually 10–20 amino acid residues in length) can be used as effectively as proteins in raising antibodies producing both polyclonal and monoclonal antibodies routinely with titers higher than 20,000. Peptide antigens do not function as immunogens unless they are conjugated to proteins. Production of high quality anti-peptide antibodies is dependent upon peptide sequence selection, the success of peptide synthesis, peptide-carrier protein conjugation, the humoral immune response in the host animal, the adjuvant used, the peptide dose administered, the injection method, and the purification of the antibody. Peptide sequence selection is probably the most critical step in the production of anti-peptide antibodies. Although the process for designing peptide antigens is not exact, several guidelines and computational B-cell epitope prediction methods can help maximize the likelihood of producing anti-peptide antibodies that recognize the protein. Antibodies raised by peptides have become essential tools in life science research. Virtually all phospho-specific antibodies are now produced using phosphopeptides as antigens. Typically, 5–20 mg of peptide is enough for anti-peptide antibody production. It takes 3 months to produce a polyclonal anti-peptide antibody in rabbits that yields ~100 mL of serum which corresponds to ~8–10 mg of the specific antibody after affinity purification using a peptide column.

Key words Anti-peptide antibody, Immunogenicity, Antigenicity, Peptide synthesis, Peptide-carrier protein conjugation, Keyhole limpet hemocyanin (KLH), Polyclonal and monoclonal antibodies, Phospho-specific antibodies, Antibody titer

1 Introduction

Antibodies are routinely used in a variety of biomedical fields including biotechnology, medicine, immunotherapy, and diagnosis, and antibodies are one of the most useful biomolecules for life science research. With their high specificity and binding ability (the typical equilibrium dissociation constant of an antibody-antigen complex is $\sim 10^{-6}$ – 10^{-12} M), antibodies are mainly used for protein recognition [1–4]. Proteins, either prepared from biological specimens or made by recombinant methods, are traditionally used as immunogens to produce antibodies. Generating antibodies against a protein yields antibodies against numerous epitopes in the protein, which

maximizes the chance of an antibody recognizing the protein. However, this pool of antibodies does increase the possibility of cross-reactions with other proteins (*see* **Note 1**). Generating antibodies against a carefully selected synthetic peptide [1, 2, 5–7] (*see* **Note 2**), by contrast, will produce antibodies that are specific to the target protein. There are instances where using a peptide as antigen is advantageous over a protein, such as raising antibodies for a specific protein isoform, phosphorylated/glycosylated proteins [8–10] (*see* **Note 3**), and proteins that are not easily purified such as large membrane proteins. Peptide antigens are often used to generate polyclonal antibodies (mainly of IgG subclass) in either goat or rabbit that target unique epitopes, especially for protein families of high homology. The applications of antipeptide antibodies include gene product detection and identification, protein processing studies, diagnostic tests, protein localization, and determination of a protein active site.

1.1 Overall Strategy for Antipeptide Antibody Production

Low-cost, high-purity peptides can be obtained commercially (a list of companies which offer customer peptide synthesis can be found on the Peptide Resource Page [www.peptideresource.com]). There are many companies that also supply antipeptide antibodies (a list of companies which provide antibody services can be found on the Antibody Resource Page [www.antibodyresource.com]). However, not all antipeptide antibodies have a high titer and some cannot recognize native proteins. The potential drawback of choosing a peptide sequence that does not elicit a strong immune response and won't correspond to an exposed region of the endogenous protein can be reduced substantially by carefully analyzing the protein sequence and structure using protein structure/antigenicity prediction websites such as IEDB (www.iedb.org). Additionally, co-immunization of several peptide antigens or immunization of a larger peptide antigen from a protein will statistically increase the chances of obtaining antibodies that will recognize the target protein (*see* **Note 4**). Peptides containing phosphorylated amino acids can also be used to produce phospho-specific antibodies [8, 9] (*see* **Note 3**). Although short peptides have been reported to generate good antibodies by themselves, a peptide is usually too small to induce an immune response and produce high titer antibodies. The minimum molecular weight needed to induce a sufficient immune response is ~5 kDa. A carrier protein which contains multiple epitopes helps elicit T helper- and B-cell responses and is therefore conjugated to the peptide. The most commonly used carrier protein is keyhole limpet hemocyanin (KLH, 4.5×10^5 – 1.3×10^7 Da), which 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 a large numbers of peptide molecules. With the advancements in peptide synthesis, peptide selection, and peptide-carrier protein conjugation, peptides are becoming the method of choice for antibody production .

1.2 Analysis of Protein Sequence and Structure

Before the commencement of a new antipeptide antibody project, it is essential to know some basic features of the protein. The first step is ensuring that the correct species and protein sequence have been identified. Databases of protein structure (e.g., PDB [www.rcsb.org/pdb]) can aid in choosing epitopes that are readily accessible to the antibodies. Any potential cross-reactivity with other closely related proteins, e.g., domain structures, should be avoided. Obtaining the protein homology information at NCBI (www.ncbi.nlm.nih.gov), UniProtKB (www.uniprot.org), PIR (pir.georgetown.edu), ExPASy (us.expasy.org/tools), or HomoloGene (www.ncbi.nlm.nih.gov/homologene), predicting extracellular domains of transmembrane proteins using TMpred program (www.ch.embnet.org/software/TMPRED_form.html), determining the secondary structure and solvent accessibility/protein disorder/flexibility (IEDB and others), and predicting the 3D structure of a protein using I-Tasser (zhanglab.ccmb.med.umich.edu/I-TASSER) [11] is helpful. A list of commercially available antibodies can be obtained at www.antibodyresource.com/findantibody.html.

1.3 Selection of Peptide for Antibody Production

Although the exact peptide sequence to achieve the strongest immune response has to be determined empirically, many B-cell epitope databases/prediction servers (*see Note 6*) and selection guidelines/tips can help maximize the likelihood of success in producing antibodies of high quality. Other factors to be considered are the ease of peptide synthesis and peptide-carrier protein conjugation, peptide stability and solubility in buffers, and the specificity for the target protein. Since antibodies bind to epitopes on the surface of proteins, when examining a protein sequence for potential antigenic epitopes it is important to choose sequences that are found on the surface of the native protein, are flexible, contain both hydrophilic and hydrophobic amino acid residues, and preferably have antigenic amino acids (lysine, arginine, glutamic acid, aspartic acid, glutamine, asparagine, etc.). In addition, sequence regions with a β -turn or amphipathic helix character have been found to be antigenic. Peptide length, hydrophobicity, and a series of specific amino acids, which can render the peptide useless, should also be considered while designing the peptide antigen.

1.3.1 Database of Experimental B-Cell Epitope and B-Cell Epitope Prediction (See Note 6)

The majority of published research has focused on peptidic B-cell epitopes, for which the amino acid residues are the structural units in computational analyses. B-cell epitopes, protein antigens that are recognized by the B cells, are classified as either continuous (linear) or discontinuous (conformational) epitopes. A continuous epitope is a single continuous stretch of amino acids within a protein sequence, while a discontinuous epitope has amino acid residues that are distantly separated in the sequence and are brought into physical proximity by protein folding. The majority of B-cell epitopes are conformational. The first step of designing peptides for raising

antipeptide antibodies is to rely on finding one suitable peptide in a database of experimental epitopes. If this is not successful, numerous algorithms are used to provide a collection of possible antigenic peptides. The common epitopes from all the predictions are considered. Many computational methods [12–15] (*see Note 6*) have been proposed for predicting either linear or conformational B-cell epitopes. Methods for predicting linear B-cell epitopes range from simple propensity scale profiling to state-of-the-art machine learning prediction servers whereas conformational B-cell epitopes are predicted utilizing structure and physicochemical features derived from antibody-antigen complexes that could be correlated with antigenicity. Despite the large number of B-cell epitope prediction methods proposed in the literature, it was a major challenge in immunoinformatics to locate B-cell epitopes in the protein. However, recent advances using multiple parameters (e.g., antigen preprocessing and mimotope analysis [16, 17]) has dramatically improved the accuracy of predictions for B-cell epitopes. A mimotope is a peptide mimic of an epitope. It elicits an antibody response similar to its corresponding epitope and binds to antibodies raised by its corresponding epitope. Mimotopes are commonly identified from phage display libraries. The following sections list the guidelines/tips for the selection of peptides for successful antibody production.

1.3.2 Peptide

Hydrophobicity/Surface

Accessibility/

Flexibility/Antigenicity

Factors

There is no foolproof way to determine whether a selected peptide can produce high quality antibodies recognizing the native protein other than injecting the peptide and analyzing the antibodies produced. From 0 to 75 % of antipeptide antibodies has been reported in the literature to recognize the native protein. Antibodies 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. 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 found hidden in the protein interior. Proline acts as a structural disruptor in the middle of regular secondary structural elements and is commonly found as the first amino acid residue in an alpha helix, in turns, and loops and in the edge strands of beta sheets. This may account for the fact that proline is usually solvent exposed and often forms part of a known epitope. Algorithms for predicting protein characteristics such as hydrophilicity/hydrophobicity and secondary structural regions aid in selection of a potentially exposed, immunogenic internal sequence for antibody generation. Some guidelines/tips to increase the likelihood of successful peptide selection are as follows:

1. Use a BLAST protein search with Protein Lounge (database containing numerous antigenic peptide targets, <http://www.proteinlounge.com>.) to compare all recommended peptides for sequence homology to other proteins.

2. Use the peptide antigen database (www.genscript.com/peptide-antigen-database.html) to predict linear epitopes.
3. Choose peptides that are in the N- or C-terminal region of the protein because these regions are usually solvent accessible and unstructured. 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 make the peptide appear more like a native protein and reduce the degradation of the peptide in the animal. In addition, the orientation of the peptide is extremely critical in certain cases.
4. Preferably choose peptides lying in long loops connecting secondary structure motifs. Avoid peptides that are located in inaccessible helical and beta sheet regions. Antigenic peptides should be located in solvent accessible regions and contain both hydrophobic and hydrophilic amino acid residues. This will increase the odds of the antibody recognizing the native protein. For proteins with known 3D coordinates, secondary structure and solvent accessibility can be obtained from the sequence link of the relevant entry at the Brookhaven data bank (www.rcsb.org/pdb) and can be calculated using a variety of programs such as DSSP (swift.cmbi.ru.nl/gv/dssp), NACCESS (www.bioinf.manchester.ac.uk/naccess), WHATIF (swift.cmbi.kun.nl/whatif; swift.cmbi.ru.nl/servers/html/index.html), or PDBsum (www.ebi.ac.uk/pdbsum). When no structural information is available, secondary structure and accessibility predictions can be obtained from the following servers with 80% accuracy in predicting α -helices, β -strands, and loops [11, 18]: I-Tasser (zhanglab.ccmb.med.umich.edu/I-TASSER), PHD (www.predictprotein.org), JPRED (www.compbio.dundee.ac.uk/~www-jpred), PSIPRED (bioinf.cs.ucl.ac.uk/psipred), PredAcc (bioserv.rpbs.jussieu.fr/RPBS/html/fr/T0_Home.html), RPBS (<http://bioserv.rpbs.univ-paris-diderot.fr/index.html>), ACCpro (scratch.proteomics.ics.uci.edu), SSpro (scratch.proteomics.ics.uci.edu), SSPRED (coot.embl.de/~fmilpetz/SSPRED/sspred.html), PREDATOR (<http://mobyly.pasteur.fr/cgi-bin/portal.py?#forms::predator>). Websites such as PROFsec (www.predictprotein.org) predict secondary structure elements and solvent accessibility using evolutionary information from multiple sequence alignments and a multi-level system. Meta-Disorder (MD) predicts intrinsically disordered proteins from protein sequences. The prediction is based on a system of neural networks that combines the outputs from several original prediction methods (NORSnet, DISOPRED2, PROFbval, and Ucon), with the evolutionary profiles and sequence features that correlate with the protein disorder, such as predicted solvent accessibility and protein flexibility.

5. Check the peptide sequence against a number of criteria to estimate its suitability for antipeptide antibody production using websites for antigenicity prediction such as www.innovagen.se, immunax.dfci.harvard.edu/Tools/antigenic.html, 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. 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 useful.
6. Avoid areas of the protein, such as the transmembrane regions, that are not accessible to antibodies.
7. Generally, use 10–20 amino acid long peptides although longer peptides give a good immune response and may provide relevant secondary structure (*see Note 7*). Peptide sequences of this length minimize synthesis problems and are reasonably soluble in aqueous solution. Longer peptides increase the risk of losing specificity and shorter peptides may elicit antibodies that would not recognize the native protein with sufficient affinity.
8. Design peptide antigens with a preference for hydrophilic or charged amino acid residues and regions containing at least 30% immunogenic amino acids such as lysine, arginine, glutamic acid, aspartic acid, glutamine, and asparagine.
9. Incorporate proline and tyrosine residues into the immunogen to confer some structural motifs that are likely to be found in the native protein.
10. For antipeptide antibody production, avoid: (1) internal cysteines (replace with serine), (2) long chains of hydrophobic amino acid residues, (3) any coupling method that binds to an internal amino acid residue, (4) numerous serine, glutamine, or proline residues in a peptide sequence, (5) N-terminal glutamine or asparagine and C-terminal proline or glycine in a peptide, (6) Arginine–glycine–aspartic acid motifs, (7) small molecule binding sites, (8) biologically active regions, and (9) posttranslational modification sites.

If the chosen sequence does not contain cysteine, it is common to place a cysteine residue at the N- or C-terminus to obtain controlled linking of the peptide to the carrier protein. Undesirable amino acid residues should be replaced with conservative amino acid residues to improve peptide properties, e.g., alanine or valine can be used to replace internal cysteine. If possible, the use of more than one peptide, a carboxyl-terminal hydrophilic sequence, an amino-terminal hydrophilic sequence, internal hydrophilic regions, and peptides lying in long loops connecting secondary structure motifs are desirable. The antigen, injection conditions, or host can be modified to increase the immune response.

1.3.3 Peptide Solubility and Stability Factors

Investigating a protein sequence to identify the best peptides that can be successfully synthesized with high solubility is an essential step in antipeptide antibody production. Several things to consider are as follows:

1. Peptide solubility is strongly influenced by amino acid composition. Peptides with a high content of hydrophobic amino acid residues, such as leucine, valine, isoleucine, methionine, phenylalanine, and tryptophan, have either limited solubility in aqueous solution or are completely insoluble. It is advisable to keep the hydrophobic amino acid content below 50% and ensure that there is at least one charged amino acid residue for every five amino acids. A single conservative replacement, such as replacing alanine with glycine or adding a set of polar amino acid residues to the N- or C-terminus, may also improve solubility.
2. During synthesis, β -sheet formation causes incomplete solvation of the growing peptide and results in many deletion sequences in the final product. This problem can be avoided by choosing sequences that do not contain multiple and adjacent valine, isoleucine, tyrosine, phenylalanine, tryptophan, leucine, glutamine, and threonine amino acid residues. If sequences cannot be chosen to avoid stretches of these amino acid residues, it often helps to break the pattern by making conservative replacements such as inserting a glycine or proline at every third amino acid residue, replacing glutamine with asparagine, or replacing threonine with serine.
3. Peptides containing multiple cysteine, methionine, or tryptophan residues are also difficult to obtain in high purity because these amino acid 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 proline.
 - (b) A sequence containing an acid labile aspartic acid–glycine bond.
 - (c) Alanine, valine, threonine, proline, or serine doublets and sequences ending in valine, isoleucine, tryptophan, tyrosine, and phenylalanine.
 - (d) Extremely long repeats of the same amino acid (e.g., arginine–arginine–...) and glutamine or asparagine at the N-terminus.
5. A peptide having an overall charge close to neutral is desirable.
6. Sequences ending with hydrophilic amino acid residues or free alpha reactive groups are preferred as these side groups will promote solubility.

7. Limiting the number of contiguous charged or hydrophobic amino acid residues is helpful as they can create solubility problems.
8. Due to the nature of glycine and its lack of a side group, it does not behave as a hydrophobic amino acid residue unless continuous stretches exist.

1.4 Peptide Synthesis

Usually peptides used for anti-peptide antibody production contain 10–20 amino acid residues and are readily obtainable either from a core facility or commercial vendors [19–22]. A state-of-the-art peptide synthesizer would have little problem producing 50 mg of peptide with >90% purity even with several phosphorylated amino acids economically. Peptides are routinely synthesized in our laboratory using stepwise Fmoc solid-phase synthesis chemistry starting from the C-terminus as follows (*see Note 8*): (1) the Fmoc group of the amino-acid-preloaded resin is removed by 20% piperidine, (2) the Fmoc-amino acid (with or without modification) is coupled 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 30–60 min, (3) **steps 1 and 2** are repeated until the last amino acid is added, (4) the Fmoc group of the resin-bound peptide is removed by 20% piperidine, (5) the peptide is then deprotected and cleaved from the resin using trifluoroacetic acid (TFA), (6) ethyl ether is added to precipitate the peptide from the TFA solution and the precipitated peptide is lyophilized, (7) the crude peptide is purified on a reversed-phase C18 column using a preparative high-performance liquid chromatography 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 (8) the pure peptide fraction is identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) or electrospray ionization mass spectrometry (ESI MS).

Dissolving peptides in aqueous solutions is not trivial. In practice, the peptide sequence should contain at least 20% charged amino acid residues to facilitate solubilization. Hydrophilic peptides containing >25% charged amino acid residues (glutamic acid, aspartic acid, lysine, arginine, and histidine) and peptides containing <25% hydrophobic amino acid residues also generally dissolve in aqueous media, provided that the charged amino acid residues are properly distributed throughout the sequence. Both acidic peptides (glutamic acid + aspartic acid residues > lysine + arginine + histidine residues) and basic peptides (lysine + arginine + histidine residues > glutamic acid + aspartic acid residues) are more soluble at neutral pH. Hydrophobic peptides containing 50–75% hydrophobic amino acid residues may be insoluble or only partially soluble in aqueous solutions even if the sequence

contains 25% charged amino acid residues. It is best to first dissolve these peptides in a minimal amount of stronger solvents (such as DMF, acetonitrile, isopropyl alcohol, ethanol, 4–8 M guanidine HCl, 2–8 M urea, DMSO, ammonium bicarbonate, or acetic acid) and then slowly add the solution to a stirred aqueous buffer solution until the desired concentration is obtained. Very hydrophobic peptides containing >75% hydrophobic amino acid residues will generally not dissolve in aqueous solutions and 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 serine, threonine, glutamic acid, aspartic acid, lysine, arginine, histidine, asparagine, glutamine or tyrosine are capable of forming extensive intermolecular hydrogen bond networks and have a tendency to form gels in concentrated aqueous solutions. Sonication may help dissolve peptides to a small degree. Acetic acid (10%) in the solvent will help dissolve basic peptides, whereas 10% ammonium bicarbonate will help dissolve acidic peptides. Most of the peptides used for generating custom antibodies are reasonably hydrophilic and will dissolve readily in PBS, water, or saline solutions. If a small test batch of peptide solubilizes easily in PBS, then dissolving at a 1 mg/mL concentration to create a working stock of the peptide is advisable. Peptide sequences containing cysteine, methionine, or tryptophan 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 and the peptide should be dissolved in the absence of oxidant. Lyophilized peptides are extremely stable and can be stored with Drierite at –20 °C for years. Storing all peptides in their lyophilized state is recommended.

1.5 Animal Immunization

Once the antigen has been selected, the production of the antibody is dependent on the animal's immune system. Despite decades of trying to create a comparable protein detection method, no other system (phage display, aptamers, etc.) has ever come close to mimicking the specificity of antibodies raised from an animal's immune system. A number of species of animal are suitable hosts for antipeptide antibody production, including mice, guinea pigs, rats, hamsters, rabbits, chickens, pigs, goats, sheep, bovines, donkeys, and horses (*see Note 9*). Selection of the appropriate animal species is dependent on several factors (NIH animal care guidelines are generally followed): the presence of a homologous protein in the species being immunized, the amount of antibody required, the amount of antigen available for immunization, the necessity of monoclonal antibodies, the time required to obtain an antibody response, and the cost. In order to achieve maximum immune response, choosing an animal that is genetically very different from the source of the immunogen is important to avoid self-recognition of the immunogen by the host animal. As an example, it is more suitable to use a rabbit or mouse than a monkey for human proteins. For highly conserved mammalian

proteins, raising antibodies in chickens is often a preferred alternative. Although the egg yolks can produce high levels of antibody, IgY purification is required before the antibody can be used in assays. Information on emulsion of adjuvant mixed with antigen, immunization routes, bleed, and antiserum preparation are widely available [1, 2]. Typically, a single sample bleed will yield 25 mL of serum from a rabbit, 200 μ L from a mouse, and 2 mL from a rat, hamster, or guinea pig. Beside mice/rats, 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 broad classes of antigens, is easy to maintain and handle, can be safely bled repeatedly, and produces well characterized and easily purified antibodies in 77 days. In rabbit, the titers remain relatively level after the second booster and additional immunizations are used to maintain antibody titers rather than to increase them. For rabbit immunization, Freund's adjuvant (*see* **Note 10**) is used, which should be emulsified aseptically using syringes or sonication. Solubility of antigen does not play an important role here. Twenty milliliter of serum (prebleed) is collected prior to the initial immunizations. The rabbits are then immunized by injection at four separate subcutaneous sites (two inguinal, two axillary) with 0.25 mg peptide-KLH emulsified together with Freund's complete adjuvant (FCA). Injected material will drain quickly into the local lymphatic system and will become concentrated in the lymph nodes closest to the injection sites. A booster injection with 0.25 mg peptide-KLH emulsified with Freund's incomplete adjuvant (FIA) is given 14 days after the initial immunization. Subsequent booster injections with 0.25 mg peptide-KLH emulsified with Freund's incomplete adjuvant (FIA) are given every 4 weeks. Twenty milliliter 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 (~100 mL) is collected from the rabbit by exsanguination. Animals from various species reach immune maturation at different times. Generally, mice at 6 week of age and rabbits at 8 week of age are used. Even in genetically identical animals, the same antigen will elicit different immune responses. To combat this, in the case of the rabbit, at least two animals should be used, with three to four being preferable. For mice or other rodents, six animals are often used.

2 Materials

All solutions should be filtered through a 0.2 μ m filter.

2.1 Peptide–Carrier Protein Conjugation

1. 0.01 M phosphate buffer pH 7.0: Use 0.01 M Na_2HPO_4 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: Use 0.05 M Na_2HPO_4 to adjust the pH of a 0.05 M solution of NaH_2PO_4 to pH 6.0.

3. 0.5 N HCl.
4. 2 N NaOH.
5. 0.1 M ammonium bicarbonate.
6. Dimethylformamide (DMF).
7. *M*-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS).
8. Keyhole limpet hemocyanin (KLH).
9. Bovine serum albumin (BSA).
10. pH paper (wide range: 1–14).
11. PD-10 column (Pharmacia Bioscience, Piscataway, NJ).
12. Lyophilizer.

2.2 Antipeptide Antibody Titer Determination by ELISA

1. Carbonate Buffer: 15 mM Na₂CO₃, 35 mM NaHCO₃, 0.02 % NaN₃. Adjust pH to 9.6 with 1 N NaOH.
2. Synthetic peptide (0.2–2.5 μM) in carbonate buffer.
3. Phosphate-buffered saline (PBS): 140 mM NaCl, 27 mM KCl, 7.2 mM Na₂HPO₄, 14.7 mM K₂HPO₄, pH 7.2.
4. PBS containing 0.05 % Tween 20 (PBST).
5. Blocking solution: 10 mg/mL BSA in PBST.
6. Secondary antibody: Goat anti-rabbit globulin conjugated to alkaline phosphatase.
7. Enzyme substrate: 1 mg/mL *p*-nitrophenyl phosphate, 0.2 M Tris buffer, 5 mM MgCl₂, pH 8.0.
8. Stopping solution: 0.01 M ethylenediaminetetraacetic acid or 3 N NaOH.
9. 96-well flat bottom microtiter plate.
10. Microtiter plate reader with 405 nm filter.

2.3 Peptide Affinity Purification

1. CNBr-activated Sepharose 4 B (Pharmacia Bioscience, Piscataway, NJ).
2. Centrifuge.
3. 1 mM HCl.
4. 0.1 M Tris-HCl.
5. 1 M Tris-HCl buffer, pH 9.0.
6. 0.1 M acetate buffer containing 0.5 M NaCl, pH 4.0.
7. 0.1 M Tris-HCl buffer containing 0.5 M NaCl, pH 8.0.
8. Synthetic lyophilized peptide.
9. Coupling buffer: 0.1 M NaHCO₃, pH 8.3, 0.5 M NaCl.
10. Washing buffer 1: 50 mM Tris-HCl, pH 8.0, 0.1 % Triton X-100, 0.5 M NaCl.
11. Washing buffer 2: 50 mM Tris-HCl, pH 9.0, 0.1 % Triton X-100, 0.5 M NaCl.

12. Washing buffer 3: 50 mM sodium phosphate, pH 6.3, 0.1 % Triton X-100, 0.5 M NaCl. Use 50 mM Na_2HPO_4 containing 0.1 % Triton X-100 and 0.5 M NaCl to adjust the pH of a 50 mM solution of NaH_2PO_4 containing 0.1 % Triton X-100 and 0.5 M NaCl to pH 6.3.
13. Elution buffer: 50 mM glycine-HCl, pH 2.5, 0.1 % Triton X-100, 0.15 M NaCl.
14. Phosphate-buffered saline (PBS): 140 mM NaCl, 27 mM KCl, 7.2 mM Na_2HPO_4 , 14.7 mM K_2HPO_4 , pH 7.2.
15. 50 mM Tris-glycine, 0.15 M NaCl, 40 % glycerol, 0.02 % sodium azide, 0.05 % BSA, pH 7.4.
16. PD-10 column (Pharmacia Bioscience, Piscataway, NJ).
17. 1×10 cm chromatography column.
18. 0.2 μm Syringe filter.
19. UV spectrophotometer.

3 Methods

3.1 *Peptide–Carrier Protein Conjugation*

To stimulate antibody responses for smaller peptides, the 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 for antibody production failure. It is critical that the peptide to carrier protein molar ratio is high (one mole of peptide per 50 amino acids of carrier is a reasonable coupling ratio), and that all epitopes on the peptides are properly oriented in order to induce a high titer specific immune response (*see Note 11*). The method of coupling the peptide to the carrier protein is an often overlooked factor while designing the synthetic peptide. It is important to ensure that the peptide is presented to the immune system in a manner similar to that of the native protein. For example, N-terminal sequences should be coupled through the C-terminal amino acid and vice versa for C-terminal sequences. Internal sequences can be coupled at either end. Another consideration for internal sequences is to acetylate or amidate the unconjugated end, because the sequence in the native protein molecule would not contain a charged terminus. The sequence chosen should not have multiple amino acid residues that can react with the chosen chemistry, otherwise, shortening the peptide or choosing the sequence such that they are all localized at either the amino or the carboxyl terminus of the peptide is helpful. For internal sequences, peptides which end furthest from the predicted epitope are normally favored as this circumvents potential masking problems by the carrier. Several popular coupling methods which couple through sulfhydryl (cysteine), amino (α -amino or lysine), carboxylic acid groups (aspartic

acid, glutamic acid or σ -carboxyl), or hydroxyl groups are available using coupling reagents such as glutaraldehyde, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), and *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) [5]. However, most peptides contain several amino, carboxyl, or hydroxyl groups in side chains of amino acid residues which result in multipoint attachment. It is preferable to attach a carrier protein through a sulfhydryl group in a cysteine residue present at either the N- or C-terminus of a peptide. The peptide-carrier protein ratio can be determined by MALDI-TOF MS or ESI MS. The EDC method is used for coupling the peptide and the carrier protein via the amino or carboxyl group of the peptide sequence, depending on the activation strategy. Carbodiimides can activate the side chain carboxyl groups of aspartic and glutamic acid as well as the carboxyl terminal group to make them reactive sites for coupling with primary amines. The activated peptides are mixed with the carrier protein to produce the final conjugate. If the carrier protein is activated first, the EDC method will couple the carrier protein through the N-terminal α -amine and possibly through the amine in the side-chain of lysine. The MBS is a heterobifunctional reagent that can be used to link peptides to carrier proteins via cysteine. The coupling takes place with the sulfhydryl group of the cysteine residue. A terminal cysteine may be added to the peptide sequence away from the epitope location to allow peptide conjugation to carrier proteins. If the peptide is derived from the N-terminus of the protein, the cysteine should be added to the C-terminus of the peptide and vice-versa. Our procedure, which is a popular protocol [23, 24], for coupling peptides to KLH or BSA through a cysteine is as follows [25]:

1. Dissolve 5 mg of KLH or BSA in 0.5 mL of 0.01 M phosphate buffer, pH 7.
2. Dissolve 3 mg of MBS in 200 μ L DMF.
3. Add 70 μ L of MBS solution to 0.5 mL of KLH or BSA solution and stir 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 25 mL 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 mg of peptide in 100 μ L of DMF. Rapidly add 1 mL of purified KLH/MBS or BSA/MBS. Shake rapidly and immediately add 11 μ L of 2 N NaOH.
6. Check the pH with pH paper. It should be 7.0–7.2. Too high a pH or too low a 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 h 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 will yield a positive reactivity of a particular epitope in an assay system such as ELISA. This value gives an indication of the quality 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 microtiter plate with 300 μ L of 0.2–2.5 μ M synthetic peptide, leaving wells at the end as blanks. Incubate overnight at 4 °C (*see Note 12*).
2. Discard the unbound synthetic peptide.
3. Wash the wells three times with PBST.
4. Block the unoccupied sites with 300 μ L/well of blocking solution.
5. Wash the wells three times with PBST.
6. Prepare serial dilutions of antiserum with PBST ranging from 1:300 to 1:300,000.
7. Add 300 μ L/well of the antiserum serial dilutions to the wells and incubate for 2 h at 37 °C.
8. Wash the wells three times with PBST.
9. Dilute the secondary antibody 1:7000 with PBST.
10. Add 300 μ L/well of the secondary antibody to the wells and incubate at 37 °C for 2 h.
11. Wash the wells three times with PBST.
12. Add 50 μ L enzyme substrate. Incubate for 10–30 min at 37 °C.
13. Terminate color development by addition of 100 μ L of stopping solution.
14. Measure absorbance at 405 nm with a microtiter plate reader.
15. In our laboratory, each animal showed exceptional titers of greater than 1:500,000 against the peptide sequence and confirmed that a strong and specific immune response had occurred.

3.3 Peptide Affinity Purification of Antipeptide Antibody

Among many antibody purification methods [1–6], peptide affinity purification is the most effective technique for purification of the antipeptide antibody. The peptide affinity purification is used for isolating antibodies that recognize a specific epitope with the same specificity as that of monoclonal antibodies.

1. Swell 1 g dried CNBr-activated Sepharose 4 B in 50 mL of 1 mM HCl for 30 min.
2. Centrifuge for 5 min at 1000 $\times g$ and discard the supernatant.

3. Wash the CNBr-activated Sepharose 4 B by swelling the resin in 50 mL of 1 mM HCl and after 15 min centrifuge at $1000\times g$ discarding the supernatant. Repeat this process twice.
4. Dissolve 10 mg of synthetic peptide in 5 mL of coupling buffer.
5. Mix the synthetic peptide solution with the swollen gel. Stir gently for 1 h.
6. Centrifuge for 5 min at $1000\times g$ and discard the supernatant.
7. Remove excess uncoupled synthetic peptide by washing the resin with 20 mL coupling buffer. Centrifuge for 5 min at $1000\times g$ 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 h.
9. Wash the resin with 0.1 M acetate buffer containing 0.5 M NaCl, pH 4.0. Centrifuge for 5 min at $1000\times g$ and discard the supernatant.
10. Wash the resin with 0.1 M Tris-HCl buffer containing 0.5 M NaCl, pH 8.0. Centrifuge for 5 min at $1000\times g$ 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 μm 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 buffer, pH 9.0.
22. Wash the column with 20 mL PBS (*see Note 13*).
23. Use PD-10 column to desalt the antibody by loading 2.5 mL antibody solution onto the column and eluting with 3.5 mL PBS as the desalting buffer. Aliquot and store purified antibody at -20°C in 50 mM Tris-Glycine, 0.15 M NaCl, 40% glycerol, 0.02% sodium azide, 0.05% BSA, pH 7.4 (*see Note 14*).
24. Determine the antibody concentration by measuring the absorbance at 280 nm of a 1 mL solution and multiply by 0.7 to determine the antibody concentration in mg/mL.

25. Measure the titer of the purified antibody with the synthetic peptide using ELISA.
26. Evaluate the purified antibody with a purified protein/cellular protein extract using ELISA/western blotting/flow cytometry (*see* **Note 15**).

4 Notes

1. Although large quantities of monoclonal antibodies (typically rat or mouse) with their high specificity and lower background noise can be produced with batch to batch homogeneity, they generally have a lower affinity, less utility, a longer response time for production, are less effective in immunoprecipitation/chromatin immunoprecipitation, are less tolerant of minor changes in the antigen, and are more expensive than polyclonal antibodies. Polyclonal antibodies, though prone to batch-to-batch variability, are the preferred choice for detection of denatured proteins on western blots and in ELISA (often, antigens are partially denatured) and provide complete affinity maturation in a short time period.
2. All synthetic peptides may contain low amounts of contaminants such as peptides (missing amino acid residues) other than the target peptide and peptides with modified or still protected side-chains. Nowadays peptide synthesis is extremely successful for short peptides (<~20 amino acid residues). It is common for long peptides to have failed sequences with missing amino acids, but this is not critical for antibody production. It is always a good idea to purify the peptides by reversed-phase high-performance liquid chromatography. Although pure peptides are always better, it is not crucial as even ~70% pure peptides can be used to generate antibodies successfully. Antibodies raised by highly immunogenic impurities are seldom a concern. However, if affinity purification using a peptide column is required, the purer the peptide, the higher the specificity of the purified antibodies.
3. For raising antibodies against phosphopeptides, there are three critical factors for success: (1) the stability of the phosphorylation, (2) the purity of the peptide, and (3) purification of the phospho-specific antibody. The generation of a phospho-specific antibody includes the synthesis of phosphorylated and non-phosphorylated peptides, immunization with the phosphorylated peptide, and construction of phosphorylated and non-phosphorylated peptide affinity columns for successive purification. The antigen design choices are constrained by the sequence directly surrounding the phosphorylated amino acid residue of interest. Antigen sequences used for generating these

phospho-specific antibodies are generally short. This forces the phosphorylated amino acid residue into the epitope recognized by the antibody. Co-immunization with either different lengths of peptides (in the case of a known phosphorylation site) or peptides containing predicted phosphorylation sites (in the case of unknown phosphorylation sites) may be used to increase the chance of success. The serum or purified IgG is passed through the non-phosphopeptide column first. The flow-through from the non-phosphopeptide column is applied to the phosphopeptide column to extract the phospho-specific antibody. This cycle can be repeated if resulting antibodies are still recognizing the nonphospho-peptide. Milk often contains high concentration of phospho-tyrosine and thus can give an abnormally high background if used as a blocking agent with phospho-tyrosine antibodies, so BSA is used instead.

4. A good antigen has three chemical features: (1) an epitope capable of being bound to a B cell antibody, (2) a site recognized simultaneously by a MHC class II molecule and the T cell receptor, and (3) it must be degradable by T-cells. In order to improve the chances of producing high titer antibodies, it is beneficial to use a few peptide sequences from a protein as antigens in a co-immunization protocol. However, a peptide that contains several known immunogenic epitopes may produce a lesser immune response than the individual epitopes. If mixed populations of antigens (individual KLH conjugates mixed in equimolar ratios) are used, an antibody response to several components is expected with antibodies to some of the compounds dominating the response. Peptide affinity columns can be used to isolate an antibody against each peptide.
5. Most proteins with an adequate number of side chains for coupling and minimal cross-reactivity with other proteins can serve as carriers. The resultant peptide-carrier protein complex is able to stimulate the immune system to produce antibodies against both the peptide and the carrier proteins. Thus, it is important that ELISA analysis be performed using either peptides or peptides conjugated to a different carrier protein. In addition, there is some evidence that antibodies that react to the cross-linker used to couple the peptide to carrier proteins can be produced. Invertebrate keyhole limpet hemocyanin (KLH, 4.5×10^5 – 1.3×10^7 Da), deactivated bacterial toxin tetanus toxoid (TT, 150,000 Da), bovine gamma globulin (BGG, 150,000 Da), bovine serum albumin (BSA, 69,000 Da), ovalbumin (OVA, 44,000 Da), flagellin (FLA, monomer, 40,000 Da), horse heart myoglobin (hhMb, 16,951 Da), and hen egg-white lysozyme (HEL, 15,000 Da) are the commonly used protein carriers. KLH is preferred since its large size and numerous [epitopes](#) generate a substantial immune response in

the majority of animals, but it does not cause an adverse immune response in humans. KLH has an abundance of [lysine](#) residues for coupling peptides allowing for a high peptide–carrier protein ratio and increasing the likelihood of generating peptide-specific antibodies. In addition, because KLH is derived from the limpet, a gastropod, it is [phylogenetically](#) distant from mammalian proteins, thus reducing false positives in immunologically based research techniques in mammalian model organisms. However, a high-quality KLH should be used because it is susceptible to aggregation and precipitation, which limits its ability to conjugate peptides. Since BSA is often a component in many tissue culture media and assays, an anti-peptide antibody raised against a BSA conjugated peptide should be avoided. Rabbits immunized with rabbit serum albumin (RSA) conjugate are less likely to raise antibodies to the carrier, as the RSA is recognized as self. Multiple Antigenic Peptide (MAP, juxtaposition of four or eight peptide molecules on a cross-linked lysine core) [26, 27] is simply an alternative peptide antigen. Its advantage is that the carrier protein conjugation step is not necessary and the core of lysines has superior adsorption characteristics for the detection of antibodies in solid-phase ELISA procedures. It is recommended for short (10–15 amino acid residues) or insoluble peptides. Difficulty in coupling amino acids occurs with longer peptides and the juxtaposition of more than 16 peptide molecules. Peptides located near the C-terminus of a protein require indirect MAP synthesis to have a preferable orientation. However, the major disadvantages of MAP is that it can bypass the immune response system in some hosts and MAP antibodies do not always react with the cognate protein.

6. B-cell epitope databases/prediction servers:

UniProt knowledgebase; www.uniprot.org, Protein data bank (PDB); www.rcsb.org/pdb, Immune epitope database (IEDB); www.iedb.org, Bcipep B-cell epitope prediction server (Bcipep); www.imtech.res.in/raghava/bcipep, Conformational epitope database (CED); immunet.cn/ced, Epitome database of structurally inferred antigenic epitopes in proteins (Epitome); www.rostlab.org/services/epitome, AntiJen database of published experimentally determined conformational B-cell epitopes (AntiJen); www.ddg-pharmfac.net/antijen/AntiJen/aj_bcell.htm, HIV molecular immunology database; www.hiv.lanl.gov/content/immunology/index, Peptide antigen database predicted by OptimumAntigen design tool; www.gen-script.com/peptide-antigen-database.html, Epitopia bound dataset of 66 nonredundant complex structures (Epitopia); epitopia.tau.ac.il/trainData, Liang's unbound dataset including 48 complexes and the unbound structures of antigens; sysbio.

unl.edu/services, Benchmark sequence datasets; www.imtech.res.in/raghava/cbtope, MimoDB mimotope database (MimoDB); immunet.cn/mimodb. Finding common results from the above databases is desirable. Linear B-cell epitope databases/prediction servers: Immune epitope database (IEDB); www.iedb.org, ABCpred B-cell epitope prediction server (ABCpred); www.imtech.res.in/raghava/abcpred, BepiPred B-cell epitope prediction server (BepiPred); www.cbs.dtu.dk/services/BepiPred, LEP-LP B-cell epitope prediction server (LEP-LP); biotools.cs.ntou.edu.tw/lep_d_antigenicity.php, BCPred B-cell epitope prediction server (BCPred); ailab.cs.iastate.edu/bcpreds, FBCPred B-cell epitope prediction server (FBCPred); ailab.cs.iastate.edu/bcpreds, Epitopia B-cell epitope prediction server (Epitopia); epitopia.tau.ac.il, BayesB B-cell epitope prediction server (BayesB); www.immunopred.org/bayesb, BROracle B-cell epitope prediction server (BROracle); sites.google.com/site/oracleclassifiers, LEPS B-cell epitope prediction server (LEPS); leps.cs.ntou.edu.tw, SVMTriP B-cell epitope prediction server (SVMTriP); sysbio.unl.edu/SVMTriP, LBtope B-cell epitope prediction server (LBtope); crdd.osdd.net/raghava/lbtope, JalView B-cell epitope prediction server (JalView); www.jalview.org, ScanProsite B-cell epitope prediction server (ScanProsite); prosite.expasy.org/scanprosite, PRATT B-cell epitope prediction server (PRATT); www.ebi.ac.uk/Tools/pfa/pratt, MimoScan B-cell epitope prediction server (MimoScan); immunet.cn/sarotup/cgi-bin/MimoScan.pl. Finding common results from the above predictions is advantageous. Conformational B-cell epitope databases/prediction servers: COBEpro B-cell epitope prediction server (COBEpro); scratch.proteomics.ics.uci.edu, CBTOPE B-cell epitope prediction server (CBTOPE); www.imtech.res.in/raghava/cbtope, BEST B-cell epitope prediction server (BEST); biomine.ece.ualberta.ca/BEST, Bprediction B-cell epitope prediction server (Bprediction); http://bcell.whu.edu.cn, DiscoTope B-cell epitope prediction server (DiscoTope); www.cbs.dtu.dk/services/DiscoTope, Epitopia B-cell epitope prediction server (Epitopia); epitopia.tau.ac.il, EPCES B-cell epitope prediction server (EPCES); sysbio.unl.edu/services/EPCES, EPSVR B-cell epitope prediction server (EPSVR); sysbio.unl.edu/EPSVR, EPMeta B-cell epitope prediction server (EPMeta); sysbio.unl.edu/EPMeta, SEPPA B-cell epitope prediction server (SEPPA); badd.tongji.edu.cn/seppa, EpiSearch B-cell epitope prediction server (EpiSearch); curie.utmb.edu/episearch.html, Pepitope B-cell epitope prediction server (Pepitope); pepitope.tau.ac.il, PepMapper B-cell epitope prediction server (PepMapper); informatics.nenu.edu.cn/PepMapper. Common results from the above predictions are located if possible.

7. Many short peptides are able to elicit a strong immune response. Antibodies against human angiotensin II Asp-Arg-Val-Tyr-Ile-His-Pro-Phe and bovine angiotensin II Asp-Arg-Val-Tyr-Val-His-Pro-Phe with a titer of 100,000 and 800,000, respectively, have been successfully produced. Other examples of shorter epitopes are the Flag epitope (DYKDDDDK) and His₆ tag epitope (HHHHHH), which are widely used in the purification, identification, and functional analysis of proteins.
8. 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.
9. There is little need to prescreen animals prior to immunization for antibodies reacting with the peptide in most instances, except 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.
10. Nonspecific stimulators of the immune response are known as adjuvants. Freund's Complete Adjuvant (FCA) should be used for the primary injection (first immunization) only. Freund's Incomplete Adjuvant (FIA) should be used for subsequent 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 heat-killed *Mycobacterium tuberculosis*, paraffin oil, and non-metabolizable mannide monooleate and elicits a delayed hypersensitivity reaction. The water-in-oil emulsion used in FCA is stable, 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: FCA should be avoided for studies of *Mycobacterium*. Though FCA has been a mainstay and has shown consistently superior results 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 used in this case.
11. Immunogenicity is influenced by multiple characteristics of an antigen including stability, foreignness, molecular size, chemical composition and heterogeneity, susceptibility to antigen processing and presentation, and modifications. Due to slow release and

rapid phagocytosis, particulate antigens are usually much better immunogens than soluble molecules. Polymeric antigens like Multiple antigen peptide (MAP) can also have a strong effect on immunogenicity. Peptide orientation is extremely important in certain cases. An antigen's immunogenicity can be improved in the following ways: (1) antigen modification with dinitrophenol or arsanilic acid, (2) denaturation of the antigen, (3) selection of a different carrier, (4) including t-cell receptor class II protein binding sites, (5) coupling antigen to sheep red blood cells, and (6) coupling antigen to beads. Alternate animal species should be tried and the dose of antigen increased. The optimum dose to achieve the strongest immune response has to be determined empirically. In general, 0.5–1 mg for rabbit and 50–100 µg for mouse are recommended.

12. Most peptides can be coated on ELISA plates using carbonate buffer at pH 9.6. If the peptide does not adsorb completely, other buffers in the pH 4 to 8 range can be tried. The coupling of peptides to BSA to facilitate coating is usually not necessary.
13. A typical peptide affinity purification of 20 mL of serum yields approximately 2 mg of specific antibody. Although the capacity does decrease slightly with each use, the column can be reused many times depending on the stability of the immobilized peptide and should be stored at 4 °C in PBS with sodium azide (0.015–0.1 M). The columns should not be frozen.
14. Antibodies (purified, serum, plasma, ascetic fluid) are relatively stable proteins that retain their activity in a wide range of biological conditions due to their compact and stable protein domains. Although the storage of antibody solution is straightforward, precautions such as avoiding repeated freeze–thaw cycles, excessive protein concentration dilution (particularly of purified antibody), and bacterial or fungal growth should be taken into account. Serum, plasma, and ascites fluid that contain sodium azide (0.015–0.1 M) can be stored at 4 °C for a few months. Serum, plasma, and ascites fluid without sodium azide, however, should be stored in aliquots at –20 °C in order to avoid bacterial or fungal growth. Purified antibodies that contain sodium azide can also be stored at 4 °C for a few months to allow for use without repeated freeze–thaw cycles and at –20 °C for longer-term storage. Purified antibodies should be stored at relatively high concentrations (e.g., 10 mg/mL) at neutral pH. Sodium azide is poisonous and known to interfere with certain assays. Filter sterilization (0.2–0.45 µm pore size) is an alternative to sodium azide. The addition of 50% glycerol can help avoid freeze–thaw cycles while keeping antibody solutions at –20 °C. In addition, addition of BSA, if it does not interfere with the downstream applications, can increase protein concentration and improve stability of the

antibody. However, it is not required if the above guidelines and aliquot strategies are followed.

15. In cases where an antibody has a high titer as measured by ELISA against the peptide but a low titer against the native protein, likely explanations are as follows: (1) the peptide sequence corresponds to a nonexposed region of the native protein (using a different extraction buffer such as 85 % formic acid may help expose this region), (2) the protein's conformation in the peptide region differs from the peptide enough that the antibody has trouble recognizing the native protein, and (3) the target protein is not present in the sample. We have successfully raised an antibody that detects protein (vascular endothelial growth factor receptor) by using a larger peptide (9 kDa) without a carrier when an antibody raised by a short peptide with a carrier fails to detect the protein. Antipeptide antibodies raised with linear epitopes work well in western blots but may not work for flow cytometry because the majority of B-cell epitopes are conformational. The advantage of using western blotting (one or two dimensional native or sodium dodecyl sulfate polyacrylamide gel electrophoresis) [2, 3, 28, 29] over the ELISA method to evaluate the antibody is that in the western blot, proteins are separated and probed individually by antibody. 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 and typically occurs for one of the following reasons: (1) the antipeptide antibody recognizes a homologous protein in the sample that shares one or more epitopes with the peptide sequence, (2) the protein has a different molecular weight than previously predicted, and (3) the antibody recognizes either cleaved fragments of the protein at lower molecular weights or aggregated multimers of the native protein at higher molecular weights.

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References

1. Howard GC, Kaser MR (eds) (2007) Making and using antibodies: a practical handbook. CRC Press, Taylor & Francis, Boca Raton, FL
2. Harlow DL (1988) Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, New York
3. Rosenberg IM (1996) Protein analysis and purification benchtop techniques. Birkhauser, Boston
4. Walker JM (ed) (2009) The protein protocols handbook. Humana Press, Totowa, NJ

5. Coligan JE, Kruisbeek AM, Margulies DH et al (1996) Current protocols in immunology, vol 2. John Wiley & Sons, New York, pp 9.0.1–9.8.15
6. Wisdom GB (ed) (1993) Peptide antigen: a practical approach. Oxford University Press, Oxford
7. Delves PJ (1997) Antibody production essential techniques. John Wiley & Sons, New York
8. Archuleta AJ, Stutzke CA, Nixon KM et al (2011) Optimized protocol to make phospho-specific antibodies that work. *Methods Mol Biol* 717:69–88
9. Arur S, Schedl T (2014) Generation and purification of highly specific antibodies for detecting post-translationally modified proteins in vivo. *Nat Protoc* 9:375–395
10. Teo CF, Ingale S, Wolfert MA et al (2010) Glycopeptide-specific monoclonal antibodies suggest new roles for O-GlcNAc. *Nat Chem Biol* 6:338–343
11. Yang J, Yan R, Roy A et al (2015) The I-TASSER suite: protein structure and function prediction. *Nat Methods* 12:7–8
12. Blythe MJ, Flower DR (2005) Benchmarking B cell epitope prediction: underperformance of existing methods. *Protein Sci* 14:246–248
13. Gao J, Faraggi E, Zhou Y et al (2012) BEST: improved prediction of B-Cell epitopes from antigen sequences. *PLoS One*. doi:[10.1371/journal.pone.0040104](https://doi.org/10.1371/journal.pone.0040104)
14. Gao J, Kurgan L (2014) Computational prediction of B cell epitopes from antigen sequences. *Methods Mol Biol* 1184:197–215
15. Sanne MM, Hensen SMM, Derksen M et al (2014) Multiplex peptide-based B cell epitope mapping. *Methods Mol Biol* 1184:295–308
16. Huang J, He B, Zhou P (2014) Mimotope-based prediction of B-cell epitopes. *Methods Mol Biol* 1184:237–243
17. Sun P, Ju H, Zhang B et al (2015) Conformational B-cell epitope prediction method based on antigen preprocessing and mimotopes analysis. *Biomed Res Int*. doi:[10.1155/2015/257030](https://doi.org/10.1155/2015/257030)
18. Tsigelny IF (ed) (2002) Protein structure prediction: bioinformatic approach. International University Line, La Jolla, CA
19. Benoiton NL (ed) (2006) Chemistry of peptide synthesis. Taylor & Francis, New York
20. Howl J (ed) (2005) Peptide synthesis and applications. Humana, Totowa, NJ
21. Kates SA, Albericio F (2000) Solid phase synthesis: a practical guide. Marcel Dekker, New York
22. Fields GB (1997) Solid-phase peptide synthesis. Academic Press, New York
23. Hermanson GT (2008) Bioconjugate techniques. Academic Press, San Diego, CA
24. Sommer J, Garbers C, Wolf J et al (2014) Alternative intronic polyadenylation generates the Interleukin-6 trans-signaling inhibitor sgp130-E10. *J Biol Chem* 289:22140–22150
25. Lateef S, Gupta S, Jayathilaka G et al (2007) An improved protocol of coupling synthetic peptides to KLH for antibody production using MBS as bifunctional linker. *J Biomol Tech* 18:173–176
26. Posnett D, McGrath H, Tam JP (1988) A novel method for producing anti-peptide antibodies. *J Biol Chem* 263:285–288
27. Tam JP (1988) Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. *Proc Natl Acad Sci U S A* 85:5409–5413
28. Westermeier R (2001) Electrophoresis in practice. WILEY-VCH, Weinheim
29. Hames BD (1998) Electrophoresis of protein. Oxford University Press, Oxford