Antipeptide Antibodies: Immunological Methods and Protocols

Antipeptide antibodies have a number of uses in biological research and bioassay, for example they can be very useful for the identification and quantification of proteins containing the peptide sequence chosen.[12] They are used routinely in such techniques as immunoprecipitation, as probes in Western blot analysis and in immunohistological identification and localization of proteins.[22] As suppliers of custom-made antipeptide antibodies, Mimotopes aims to supply you with antibodies that have the best prospects of working in your particular application.

Topics in this article:

1	**Introduction**	**2**
2	**Selection of Peptides for Raising Antipeptide Sera**	**2**
2.1	Simple Approaches	2
2.2	Predictive Algorithms for the Selection of Antigenic Peptides	3
3	**Peptide Feasibility**	**3**
4	**Selection of Peptide Length, Purity and Quantity**	**4**
4.1	Peptide Length	4
4.2	Peptide Purity	4
4.3	Peptide Quantity	4
4.4	Number of Peptides	4
5	**Peptide Conjugation**	**6**
5.1	Coupling Chemistry	6
5.1.1	Coupling using a Heterobifunctional Agent	6
5.1.2	Coupling using the Homobifunctional Agent Glutaraldehyde	7
5.1.3	MAPs (Multiple Antigenic Peptides)	7
6	**Animal Requirements**	**8**
6.1	Choice of Species to Raise Antipeptide Antibodies.	8
6.2	Number of Animals to be Used?	8
6.3	Immunization Protocol	8
7	**Assessment of the Antipeptide Antibody Response**	**8**
7.1	Design of Peptides for Assessment of Antipeptide Titers	9
7.1.1	N-Terminal Peptides of Native Proteins	9
7.1.2	C-Terminal Peptides of Native Proteins	9
7.1.3	Internal Peptide Sequences	9
8	**Affinity Purification**	**10**
9	**Epitope Mapping of Antipeptide Antibodies**	**10**
10	**Conclusions**	**11**
11	**References**	**11**

1. Definitions

So that we will have a good grasp of the issues relating to antipeptide sera, it is important to define some of the terms used:

Antigenic

A peptide can be antigenic, i.e., capable of stimulating antibody generation, resulting in antibodies that will bind to the peptide. The term antigen is also often used to describe a substance which can bind to specific antibodies, but which of itself is not capable of stimulating the formation of those antibodies. "Hapten" is a term used for this same purpose, and for clarity we have decided to distinguish between these two terms by confining our use of the term antigenic to describe materials which can of themselves generate antibody formation. Thus, many small peptides are nonantigenic but are good haptens. It is usually feasible to immunize animals with peptides, when rendered antigenic by coupling to a protein, and to demonstrate the presence in the animal sera of antibodies to the peptide using a solid phase immunoassay (however, see section 7 on the avoidance of artifacts).

Protein-reactive Antigenicity (PRA)

An antigenic peptide can also have "protein-reactive antigenicity" (called PRA for convenience in this article) if the antibodies that were generated to the peptide are also able to bind to the protein, such as protein in a denatured form, presumably binding to the portion which is homologous with the antigenic peptide's sequence.[17,20]

Native Protein-reactive Antigenicity (Native PRA)

A peptide which has PRA may still not be capable of stimulating the formation of antibodies which will bind to the native protein from which its sequence was derived. This may be because in the native protein, the target peptide sequence is either not readily accessible to antibodies (i.e. it is "buried" in the protein structure), or the structure of the peptide in the native protein is too rigid to allow the antipeptide antibodies to "mold" to it. Conversely, antibodies to a peptide displaying Native PRA may, in some instances, not bind readily to denatured forms of the protein (presumably due to inaccessibility of the required peptide sequence in the denatured forms of the protein).

The difference between simple peptide antigenicity and protein-reactive Antigenicity (PRA) is critical, because in most applications the aim is to generate antibodies that, although generated to a peptide, are also able to bind to the "target" protein. Most small peptides are generally not antigenic by themselves, and to be made antigenic must be coupled to an antigenic carrier protein. The reason is, although small peptides almost always can be recognized by the receptor (i.e. antibody) on some preexisting clones of B cells, and are thus potentially antigenic, they are generally monomeric and poor at direct stimulation of B cells. Another critical factor is that they are generally not large enough to contain an effective T helper epitope, which is required to stimulate the production of a lasting antipeptide antibody response, and to promote switching from IgM to IgG-type antibodies.

Coupling of the peptide to an antigenic carrier protein makes the peptide multimeric and also overcomes the T helper epitope requirement. It is the general experience that most peptides are rendered antigenic by coupling them to an antigenic carrier protein.

MAPs peptides, made on a branching lysine polymer structure to create a peptide multimer,[21] are said to be antigenic, however in a significant number of cases they have failed to stimulate the formation of antibodies, and thus, in these constructs, the peptides used should always incorporate a suitable T helper epitope within the peptide sequence.[1]

2. Selection of Peptides for Raising Antipeptide Sera

The real challenge in producing useful antipeptide antibodies is to choose peptides that are not only antigenic but which also have PRA. The selection of antigenic peptides has been the subject of a great deal of investigation[13,22] and although there are a number of predictive algorithms that can help in the selection,[14] the choice of an antigenic peptide is still an art, with many factors that can affect the peptide antigenicity needing to be taken into account. In order to maximize your chances of success we have put together guidelines that will assist with your selection of antigenic peptides.

2.1 Simple Approaches

The following guidelines offer a relatively simple approach to choosing a peptide (or peptides) for raising sera.

2.1.1 If a peptide is hydrophilic, it is likely to be at the surface of the protein in the native structure, which is favourable for binding of antipeptide antibodies to the protein itself. On the other hand, if it is too highly charged, it may be difficult for the immune system to create the highly charged complementary antibody combining site (paratope) necessary to allow strong specific binding. A plot of average hydrophilicity (or its inverse, hydropathicity) along the protein with a moving "window" of 6 residues allows hydrophilic regions to be readily located.[9]

2.1.2 The very N- and C-terminal peptide sequences of proteins (say, the first and last 5 residues) are usually at the surface of the protein and have the added advantage

that, because they are only tethered at one end, they can be more flexible than a continuous peptide (such as a loop, helix, sheet etc.) which is tethered at both ends. This is a factor in favour of Native PRA. The natural charged amino and carboxy terminal groups help the hydrophilicity of those peptides. N- and C-terminal peptides are thus often a good first empirical choice for raising antisera,[13] and it is possible to simulate both N- and C-terminal structures of a protein using just one peptide (see section 4.4 below). However it should be remembered that many proteins are produced as a pre- or pro-protein which is modified so that residues are clipped from the N-terminus of the pro-protein to produce the "mature " protein, so it is important to ensure that if the terminal sequences as deduced from a gene are used, that they are indeed the true termini of the mature protein.

2.1.3 Synthetic peptide antigens which are very long may adopt a stable nonnative structure which may give rise to antibodies which do not bind to the protein (non-PRA). Long peptides are also more difficult to make, to an acceptable purity level. It is therefore a good idea in most cases to limit the length of peptide antigens to a maximum of about 25 residues.

2.1.4 Very short peptides may induce such a restricted set of antibodies that few or none will bind to the protein (non-PRA). For example, short peptides may stimulate the formation of antibodies to the end(s) of the peptide itself, which (except in the case of native N- and C-terminal ends of proteins) are unlikely to bind to the protein because of the lack of an homologous "end" structure in the protein. It is therefore a good idea to make peptide antigens (except for the very ends of proteins, see point 2.1.2 above) at least 6 residues in length, preferably longer.[13]

2.1.5 Peptides which may be post-translationally modified are not good choices as antigens unless the known modification is built into the peptide antigen. For example, cysteine-rich regions can be crosslinked in unknown ways through disulfide bonds; potential glycosylation sites (Asn-X-Ser and Asn-X-Thr; Ser-X-X-Pro and Thr-X-X-Pro) may be glycosylated in the mature protein, preventing antipeptide antibodies from binding; the ends of some proteins may be trimmed, amidated, acetylated or capped with a fatty acid; Tyr, Ser or Thr may be phosphorylated; or the protein may be cleaved into subunits. Unless the exact nature of the modification is known, it is better to avoid likely sites of post-translational modification when choosing a peptide antigen.

2.1.6 When a peptide is being chosen, the limitations of synthetic peptide chemistry and of the chemistry of peptide conjugation need to be taken into account. Peptides which are fine by all other criteria need to be assessed and the best one(s) for chemical synthesis selected as the ones to proceed with. The bases of this selection are dealt with in a later section ("Peptide Feasibility", see below).

2.2 Predictive Algorithms for the Selection of Antigenic Peptides

Beyond the relatively simple approaches mentioned above, a number of other predictive methods can be applied to a protein sequence. Many of these algorithms are based on the hydrophilicity approach mentioned above, and differ only in the derivation of the parameters applied to each amino acid.[9] Other algorithms predict that antigenic peptides are associated with surface flexibility, secondary structure (helices and sheets), and turns in proteins. A program developed by Pellequer and Westhof, called PREDITOP, is very useful because it offers a large number of such parameter sets.[14] Pellequer et al.'s preferred parameter scale is a propensity scale for each of the amino acids, used to predict turns in a protein. The authors claim that the success rate of finding antigenic peptides using the program is about 70%, higher than that achieved using other predictive parameters. It must be emphasized however that predicted antigenic peptides may still turn out not to have PRA, and that there will therefore be a significant "failure rate" for this approach (perhaps up to 30% failures).

3. Peptide Feasibility

While most peptides can be synthesized by standard methods, a significant number of peptides are difficult to make by chemical synthesis. These difficult peptides are best avoided. Many of the difficult peptides can be identified using an assessment of the peptide based on criteria established by experience. Peptide feasibility assessment is available as a free service by contacting Mimotopes, or there is appropriate software ("Pinsoft") available for downloading from Mimotopes' website. The following notes summarize some of the criteria:

3.1 Hydrophobic Peptides

Peptides that are hydrophobic, as well as being poorly soluble, are generally difficult to make and purify. Peptide hydrophilicity can be improved with the addition of charged residues such as lysine. The peptide, modified to make it more hydrophilic, may still not be successful as an immunogen because it may be found not to have the desired Native PRA, a consequence of the unavailability of highly hydrophobic regions of proteins to access by antibodies.

3.2 Peptides Containing Homopolymers

Homopolymers of some amino acids tend to form stable structures which may create solubility problems.

3.3 Peptides Containing Long Sequences of Sterically Hindered Residues

Beta-branched amino acids or amino acids with bulky sidechain protecting groups can be difficult to couple to the

growing peptide chain; if many of these amino acids are adjacent in a sequence, the purity of the peptide "as synthesized" can be low. This in turn creates difficulty in purification to a desired purity specification.

3.4 Peptides containing Cys or Met residues

These peptides will tend to oxidize on exposure of their aqueous solutions to air, resulting in altered structure. Oxidation can be minimized or avoided by taking certain precautions during handling.

4. Selection of Peptide Length, Purity and Quantity

In choosing a peptide to be used for immunization, the feasibility, cost and likelihood of success are influenced by the choice of peptide length, purity and quantity.

4.1 Peptide Length (see also sections 2.1.3 and 2.1.4).

To some extent the length of the peptide chosen is dependent on the eventual application and specificity of the antipeptide antibodies required. If the peptide represents the termini of the protein then quite short peptides can be chosen (e.g. up to 8 residues), the aim being to generate antibodies that are specific to the very last few residues of the protein chain (see section 2.1.2 above). A potential application for the use of antibodies to short N- or C-terminal peptides is to detect the protease cleavage products of a protein, for example, detecting a mature protein in the presence of a pro-protein.

As the length of the immunizing peptide increases then the number of possible epitopes within the peptide increases, so for peptides longer than 10 residues it is common to find that more than one epitope is present within the sequence (Fig. 1). A greater number of potential epitopes displayed by a peptide can be an advantage because it can increase the likelihood of the peptide displaying PRA. However, if the aim is to generate antibodies that can differentiate between protein variants, then using a long peptide which encompasses more than 3 or 4 continuous amino acids which are common to the variants could give rise to antibodies to these "common" sequences, and the antiserum will then fail to satisfy the specificity requirement.

As the length of peptides increases it is also possible that the peptide will assume a preferred stable structure, allowing antibodies to be generated to peptide structural features that are not present in the corresponding peptide within the protein. This effect would run counter to the aim of making a peptide with PRA.

As a general rule, where antibodies are required to a peptide from within a protein sequence, then peptides of between 15 and 20 residues are suitable.

4.2 Peptide Purity

The choice of peptide purity is a very individual matter. From experience, we have found that a purity of >70% results in antipeptide sera which are satisfactory for many users. However, some users are more comfortable with high purity peptides (>95%) and this may be more appropriate when a serum is intended to distinguish between peptides or proteins differing by very few residues. It is possible for antibodies to be generated to deletion and truncation peptide sequences present in the less pure peptides, even though the quantity of any individual contaminant is usually an order of magnitude less that the quantity of the target peptide. It should be remembered that it is unlikely that these contaminant-specific antibodies would interfere with the antibodies specific for the "target" protein, and thus for practical purposes they may be regarded as part of the general background of unwanted antibodies present in any serum. Where it is intended to proceed to affinity purify the antipeptide antibodies from an antipeptide serum (see section 8, below), it would be appropriate to choose a higher purity peptide to minimize the copurification of contaminant antibodies. Even with the use of very high purity peptides, one cannot completely rule out the possibility of a "false positive" binding of antipeptide antibodies to a non-targeted protein which has a sequence related to the peptide antigen used.

4.3 Peptide Quantity

Antipeptide antibodies can be raised with very small amounts of peptide (<0.1mg). However, this may require specialized immunization techniques, and severely limits the number of animals which can be used, as well as the number of doses which can be given. For general purpose work, we have found that an amount of peptide in the 2-3 micromole range (about 5mg of a 15mer peptide) is adequate to immunize several animals with several doses, e.g. when an amount of 200-300 nanomole of peptide is injected per dose. In the case of peptide being in short supply, the dose can be decreased to 100 nanomole per injection without badly affecting the antibody response.

4.4 Number of Peptides

In many instances, it is worth using more than one peptide to raise antipeptide sera. For example, use of two or more peptides from a protein will increase the likelihood that a serum will be obtained which will bind to the whole protein, i.e. the more peptides that are used, the greater the prospect that one will have PRA. In many cases it is more important that a suitable serum is obtained the first time it is attempted, rather than trying a single peptide with the idea that if it doesn't work, a second one can be tried later. The peptides can be injected into the same animals, i.e. extra animals are not necessary. Two spinoffs from use of multiple peptides in the same animals are that 1. the animal holding and handling costs of the work are minimized, by comparison with making individual sera for each peptide, and 2. the prospect of obtaining a

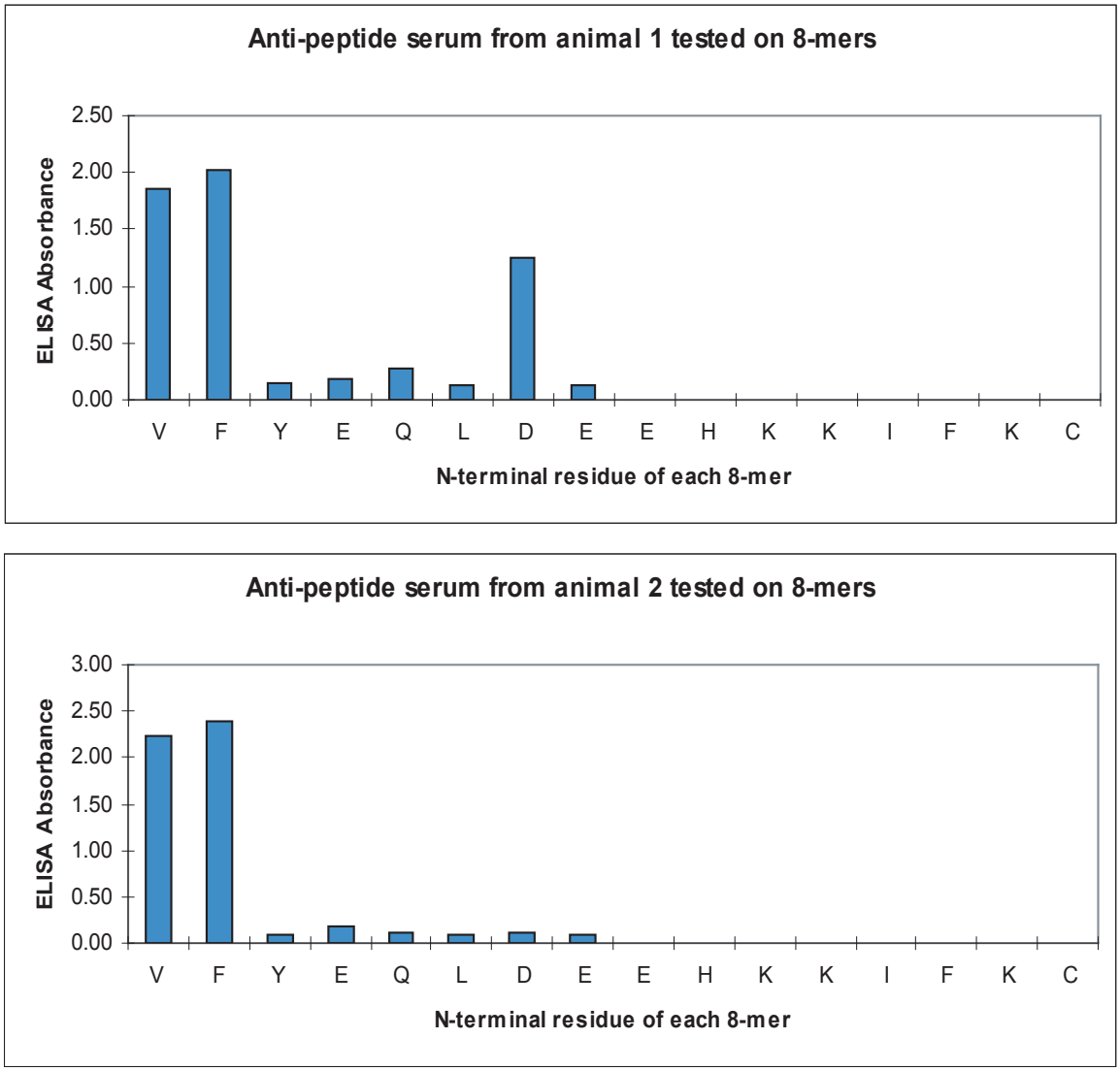

Fig.1 An illustration of the difference between two animals in the antibody response to a peptide

Both animals were hyperimmunized with a conjugate of the peptide VFYEQLDEEHKKIFKC. The peptide was conjugated to a carrier protein via the cysteine residue (see section 5.1.1). The first animal has responded by producing populations of specific antipeptide antibodies to two regions of the immunizing peptide: The 1st antibody-binding region consists of 2 sequential peptides overlapping by 7 residues, i.e. VFYEQLDE and FYEQLDEE. The implied minimal epitope (common to both peptides) is FYEQLDE. The second antibody-binding region is defined by peptide DEEHKKIF, i.e. the implied epitope is DEEHKKI

"precipitating" antipeptide serum is improved, since antibodies to two or more sites in the protein will give the improved precipitation effect as seen with a two-site monoclonal antibody combination.

A strategy which has worked well in our experience is to use two peptides: one corresponding to a hydrophilic "internal" sequence of the primary protein structure, the other peptide being the combined N-terminal+C-terminal peptide mentioned in section 2.1.2. The combined N-terminal+C-terminal peptide is made up of (for example) the 7 N-terminal amino acids plus the 7 C-terminal amino acids of the protein, with a Cys residue placed in the middle to act as a point for conjugation to the antigenic carrier protein (a "Y-shaped peptide", see section 7.1.3.5). This combined N+C peptide is effectively an antigen representing two sites in the protein, and in six out of six proteins tested was able to generate antibodies binding to the whole protein coated on plates, i.e. the combined N+C peptides displayed PRA in every case. When a combined N+C peptide is used together with the hydrophilic internal peptide, there are then, in just two peptides, three sequences which could potentially display PRA.

Another application for the N+C peptide concept is to make antibodies to both of the new N- and C-terminal epitopes which are created when a protein is cleaved by a protease. In this case, the N-terminus of the N+C peptide is the newly revealed N-terminus of the C-terminal portion of the cleaved protein; and the C-terminus of the N+C peptide is the C-terminus of the N-terminal portion of the cleaved protein.

In instances where more than one peptide has been used, it may be important to know which of the peptides used was effective at raising protein-reactive antibodies. The successful serum can be fractionated using the same immunizing peptides, attached to individual batches of gel to make an affinity matrix (see section 8 below).

We would not recommend any more than 4 peptides be used in this multipeptide immunization protocol because of the possibility of "antigenic competition" between the peptide antigens.

In summary, advantages associated with using multiple peptides in the same animals are:

- Improved chances of obtaining Native PRA active sera,
- Improved chances of obtaining "precipitating" antipeptide sera, since antibodies to two or more sites in the protein will give the enhancing effect as seen with a two-site monoclonal antibody combination,
- The animal holding and handling costs of the work are minimized, by comparison with making individual sera for each peptide.

5. Peptide Conjugation

To ensure the immunogenicity of peptides, most peptides must be coupled to an immunogenic carrier protein. The main requirement for the carrier protein is that it must be antigenic in the species being used to raise the antibodies. For example, Bovine Serum Albumin (BSA) would be a satisfactory carrier protein when generating antibodies in rabbits but a poor carrier protein when used in bovines. We routinely use and recommend Diphtheria Toxoid (DT) and Keyhole Limpet Hemocyanin (KLH) as being excellent carrier proteins. These are both from species phylogenically distant from those used to raise antibodies. Generally we find that KLH is a more difficult protein to work with, in that it tends to result in insoluble (though still antigenic) conjugates. It must be remembered that as well as raising antibodies to the peptide, antibodies will also be generated to the carrier protein. This is generally not a problem, because these anticarrier antibodies would not be expected to have any specificity for the "target" proteins. While BSA is a satisfactory carrier protein, because BSA is used widely as a blocking agent in subsequent serological tests, complications could arise as a result of the presence of anti-BSA antibodies. We therefore do not generally recommend BSA as a carrier protein for raising sera, although it is satisfactory as a peptide carrier protein for in vitro work.

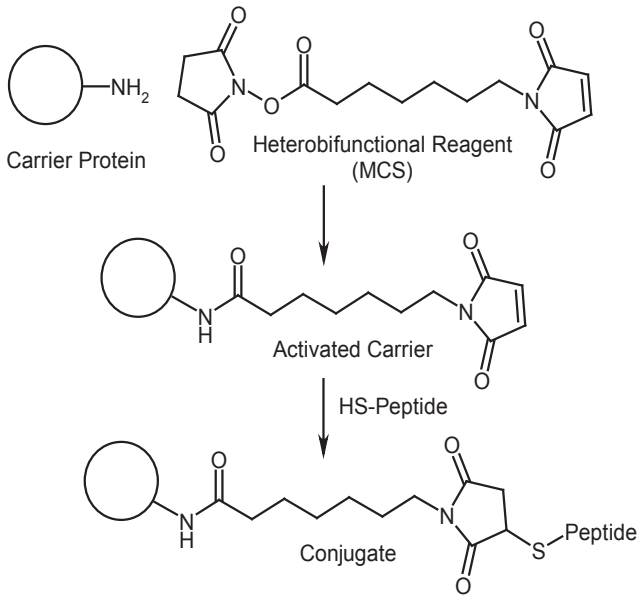

Fig. 2 The chemistry of coupling of Cys-containing peptides to maleimide-activated carrier protein

Amino groups (lysine side chains) on the surface of the carrier protein are activated by reaction with MCS to create available maleimide groups. Exposure of the activated carrier to peptide containing reduced sulfhydryl groups (such as Cys sidechains) results in spontaneous formation of a conjugate in which stable thioether linkages join the peptide to the carrier protein.

In the rare event when anti-carrier antibodies, or antibodies that were present in the animal before immunization, interfere in subsequent usage of the sera, then affinity purification of the antipeptide antibodies can be used to remove the crossreactive antibodies (see section 8).

5.1 Coupling Chemistry

Short peptides generally must be coupled covalently to a carrier protein in order to achieve the strongest antibody response (see section 1). There are many possible conjugation methods, but we find that one of the two following chemistries suits most cases.

5.1.1 Coupling using a Heterobifunctional Agent (MCS method). This is one of the best methods to couple a peptide to the carrier protein via a specific amino acid residue in the peptide, and to maintain peptide antigenicity.[11] It relies on the specificity of reaction between a maleimide group and a sulfhydryl (Fig. 2). For this procedure, the peptide to be conjugated must contain one or more fully reduced cysteines. The reagent MCS is first reacted with the carrier protein to add maleimido groups to

lysine side chains. When the MCS-activated protein is mixed with peptide (containing cysteines in the fully reduced form), the maleimido groups react with cysteine sulfhydryl groups to form stable thioether bonds, linking the peptide to the carrier protein. In the procedure we use, we generally aim to couple 1 to 1.5 peptide molecules per 10,000 Dalton of carrier protein.

The peptide generally will not contain a "natural" cysteine (see section 3.4 above) and a cysteine normally has to be added to the peptide sequence. The choice of where to place the cysteine residue in the peptide is assisted by the following guidelines. Where the peptide is from an internal sequence of a protein then the cysteine can be positioned at either the N- or the C-terminus of the peptide.[18] Where the peptide is from the very N-terminus of the protein, the cysteine should be at the C-terminus of the peptide. Conversely, where the peptide represents the very C-terminus of the protein, the cysteine should be at the N-terminus of the peptide.

A novel application exists (see section 2.1.2 above), where the peptide represents a hybrid of the N- and C-termini of the protein sequence. In this case the cysteine should be in the "centre" of the peptide.

5.1.2 Coupling using the Homobifunctional Agent Glutaraldehyde. In its pure form, glutaraldehyde has two aldehyde groups on the one molecule, each of which can react with an amine (and with some other chemically reactive functional groups). Thus, glutaraldehyde has the potential to couple the amine of a peptide to the amine of a protein. This is a less controlled procedure than the MCS method, and thus is considered unsuitable for making conjugates of known structure. However, it is widely used, often results in immunogenic conjugates, and can be the method of choice under certain circumstances. The peptide is theoretically coupled via a lysine residue and/or the amino terminus of the peptide, to lysine sidechains on the protein surface. By controlling the pH of the coupling reaction it is possible to bias the coupling in favour of the amino terminus of the peptide, however some coupling via lysines present in the peptide sequence cannot be avoided. The method is more suitable where the native peptide does not have a lysine present, and coupling can then be effected by a free amine N-terminus or by adding a lysine to either the N- or C-terminus of the peptide. The method is also suitable where the native peptide is cyclised via a disulfide bridge, and the addition of a third cysteine to the peptide to facilitate coupling using the MCS chemistry would complicate peptide synthesis and purification.

The glutaraldehyde method may also be suitable where the aim is to generate antibodies specific for the reduced cysteine residue in a peptide. Here the strategy is to oxidise the peptide as a way of protecting the cysteine residues (glutaraldehyde coupling, although mainly via an amine group, can also couple via a free sulfhydryl group). After coupling with glutaraldehyde via amino groups, the peptide conjugate is reduced just prior to immunization, using an excess of dithiothreitol, to reveal the cysteine sulfhydryls.

In the few cases where one of the above coupling chemistries is unsuitable, there are others available.

Where the investigator's aim is to reproduce antipeptide antibodies reported by other workers, as described in a publication, it would generally be best to follow the published procedure closely. However, even this is not a guarantee of success, as it must always be remembered that the generation of useful antipeptide antibodies is empirical and that unknown or unreported factors can influence the results. Animal ethics considerations may also make it impractical to reproduce an immunization technique from the older literature.

The peptide-carrier conjugate can be injected with an adjuvant to increase the strength of the antibody response (see section 6.3).

5.1.3 MAPs (Multiple Antigenic Peptides). MAPs have been claimed to be a solution to the problem of poor immunogenicity of short peptides. A MAP consists of many "copies" of a peptide, all attached to a core structure such as a branching structure made from several lysine residues. One molecule of a MAP may thus contain four, eight or more "copies" of the particular peptide sequence in question.

While there is no doubt that MAPs, by presenting peptides in a polymeric form, can be very efficient antigens for recognition by pre-formed antibodies,[21] there is mixed data on their usefulness as immunizing antigens for antibody formation[1] or T cell stimulation.[19] This is expected, because unless the peptide sequence used in the MAP contains a T helper cell epitope, it will only be able to stimulate a short term B cell antibody response which will be primarily of the IgM isotype. The MAP will also not be able to stimulate the affinity maturation and isotype switching to IgG, which are characteristic of the strong, durable response to a good protein antigen. However, when a MAP is suitably antigenic (e.g. contains a good T helper epitope for the species and individual animal being immunized), it has the advantage that no conjugation to a carrier (antigenic protein) is needed, and there is thus no antibody response to the carrier. The same observation applies to immunization with single copy peptides containing a T helper cell epitope, i.e. single copy peptides can be quite antigenic without conjugation to a carrier protein if they contain the required T helper epitope.

Disadvantages associated with using MAP constructs:

1. Characterization of the product is difficult due to its high molecular mass and the high likelihood of a defect (deletion or truncation) in one or more of the multiple "copies" of the peptide sequence.

2. Purification to molecular homogeneity is very difficult. As a result, there is an increased potential for lack of specificity in the antibody response.

6 Animal Requirements

6.1 Choice of Species to Raise Antipeptide Antibodies

To a large extent this is based on the intended purpose for the antipeptide antibodies. Where the aim is to duplicate previous work then it is probably best to choose the species used previously. Because many antipeptide antibodies will be used in applications like ELISA, Western Blotting or histochemistry, where it is necessary to detect the antipeptide antibody using an enzyme labeled anti-species conjugate, then the choice may be influenced by the range of anti-species conjugates available in the user's laboratory.

If no preexisting limitations are present then the following factors should be considered.

Species available include rabbits, sheep, mice, rats and chickens. Rabbits and sheep are suitable for most applications, with sheep being especially suitable where large quantities of sera are required. Mice may be the species of choice where only a small amount of antipeptide antibody is needed, and where it is useful to obtain a measure of the diversity of individual animal responses. It is much more cost-effective to use many mice per peptide than for other species (see section 6.2). Chickens are a good source of large quantities of semi-purified antibodies (extracted from egg yolks, eggs being obtained on a daily basis).

When selecting the species, consideration should be given to selecting a species whose normal sera do not contain antibodies giving nonspecific/background reactions. This is less of a consideration where affinity purification of the hyperimmune serum is intended.

6.2 Number of Animals to be Used?

The simple answer is: the more the better. The more animals used, the greater the chance that at least one will produce antibodies of high titer and with the desired specificity. As a minimum, for most work two individual animals should be used, however where possible more animals should be used. If the initial assessment shows that the two animals used did not give antibodies of sufficiently high titer or specificity it may be necessary to immunize additional animals. Outbred animals have the greatest variability but their use is recommended because they maximize the prospect of getting at least one strong serum; inbred animals may be uniformly poorly responsive to a particular antigen. Inbred animals sometimes have the advantage that a known T helper epitope can be used in an unconjugated peptide antigen to ensure a good antipeptide response while avoiding the formation of large amounts of anti-carrier antibody.[1]

6.3 Immunization Protocol

Many immunization protocols have been published and there is no clear information as to which is the "best" protocol. Important factors include the amount of antigen, the route of injection, the type of adjuvant used, the way the adjuvant and antigen are combined, the number of doses and the interval between them etc. There are many variations on how the conjugate is adjuvanted.[10] We have found the following protocol to be suitable, as it generally results in the generation of high titres of antibodies, with the minimum number of immunizations, in a short time (approx. 6 weeks from the start of the immunizations).

Day 0 Take bleed for preimmune serum sample. Immunize each rabbit subcutaneously at multiple sites with a total of 1mL of emulsion consisting of 200nmol peptide (in the form of a peptide-protein conjugate) in the aqueous phase, emulsified with 2 volumes of Freund's complete adjuvant (an oily adjuvant containing mycobacteria).

Day 14 Immunize each rabbit subcutaneously at multiple sites with a further 1mL of emulsion, similar to the first injection except that the adjuvant is Freund's incomplete adjuvant (without mycobacteria) rather than the complete adjuvant.

Day 35 Take a large bleed for preparation of immune serum.

Day 42 Take a large bleed for preparation of immune serum.

The protocol for sheep is similar except that a single large bleed is taken on day 35, rather than two bleeds a week apart.

Note: These are general purpose protocols and that if a special protocol is preferred then we may be able to apply it. If required, it is also feasible to hold animals for prolonged periods with regular boosting to maintain antibody levels or to allow "affinity maturation".

7. Assessment of the Antipeptide Antibody Response

It is always useful to know if an immunization with a peptide has been successful at raising antipeptide antibodies, even though the eventual practical usefulness of such antibodies cannot be predicted. Antipeptide antibody titrations serve a number of functions:

1. They establish the success (or otherwise) of the whole process of making the peptide, conjugation, and immunization.[8]

2. They provide a relative measure of the antipeptide response of different animals, and allow comparison of different peptides (provided the testing is carried out in a consistent way).

3. They permit sequential monitoring of the progress of the antipeptide response, such as whether the antipeptide titer

has declined when animals are being held without booster immunizations, or whether later booster immunizations have improved the antipeptide antibody level.

The simplicity of solid phase immunoassays (e.g. ELISA) makes it easy to monitor the progress of the antipeptide response. Using a constant level of peptide attached to the solid phase (e.g. a microtiter plate), a measure of the antipeptide antibody level can be obtained by testing serial dilutions of the serum across the plate. The less straightforward aspects of this process include: the method of immobilization of the peptide on the solid phase; and the design of the peptide to avoid artifacts.[2,4,6] For peptide immobilization, it is common to:

1. Passively coat the immunizing peptide onto polystyrene or PVC plates. This can lead to poor coating[7] or steric hindrance of antibody binding to epitopes in the peptide, problems which diminish as longer peptides are used.

2. Use a biotinylated form of the peptide, captured onto avidin or streptavidin plates.[15,23] For relatively short peptides (up to 20 residues), we recommend this method. Provided a spacer arm is included between the biotin and the target peptide sequence, the peptide is present at a consistent antigen density on the plate surface and is free to interact with antipeptide antibodies.

3. Form a conjugate of the immunizing peptide with a different carrier protein and to coat that conjugate onto plates. This method suffers from the disadvantage that even though anticarrier antibodies will not be measured, antibodies to the linker system will be detected.[2,4]

7.1 Design of Peptides for Assessment of Antipeptide Titers

In measuring antipeptide titers, it is often important to know if the particular antibody population being measured is relevant to the purpose for which the antibodies are being raised. For example, if an "internal" peptide of a protein is chosen for raising antibodies, then antibodies directed against a free end of the peptide will be irrelevant for reactions involving the whole protein, because such a free end does not exist at that point in the continuous peptide chain of the whole protein. Especially for short peptides, antibodies to a free end can dominate the antipeptide response.[3,6] Of course, where the peptide is the same as a natural free end of the protein, it can be highly desirable to generate antibodies to that free end, since these may be the most effective at recognizing the whole protein. The peptide used in assessing the antipeptide response should therefore be designed to detect such anti-free end antibodies, e.g. by making a detector peptide which is captured (via biotinylation) to the solid phase through the end opposite that representing the natural free end.

When assessing antipeptide sera, our aim then is to design, for assessment of the serum, a biotinylated peptide that will give a measure of the "useful" antipeptide antibodies generated. The following guidelines summarize the most common situations. The sequence "PEPTIDE" is used to indicate the sequence taken from the protein of interest. It is assumed in most cases that the peptides for immunization are coupled to carrier protein through the sulfhydryl group of a Cys residue. Spacer residues of Ser and Gly are placed between biotin and the peptide sequence of interest, to prevent steric hindrance from occurring between the avidin and the antipeptide antibodies which attach to the peptide. With additional information about a particular application, other designs of the biotinylated peptide may be recommended, which would better suit the particular application.

7.1.1 N-Terminal Peptides of Native Proteins. The peptide used in the conjugation/immunization would be Amino-PEPTIDE-Cys.

The biotinylated peptide should be Amino-PEPTIDE-GSG-Bc (Bc = biocytin amide, a lysine residue with a biotin group attached to its side chain).

Note: Generally the N-terminus of the protein will have a free amino group. However, it may be post-translationally modified, so it is important that the N-terminus of the peptide antigen and the biotinylated peptide match that which is actually present in the protein.

7.1.2 C-Terminal Peptides of Native Proteins. The peptide used in the conjugation/immunization would be Cys-PEPTIDE-carboxyl (Acid). The biotinylated peptide should be Biotin-SGSG-PEPTIDE- carboxyl (Acid).

Note: The C-terminus of the protein will usually be the free acid. However, it may be post-translationally modified, so it is important that the C-terminus of the immunizing peptide and the biotinylated peptide match that which is actually present in the protein.

7.1.3 "Internal" Peptide Sequences

7.1.3.1 If the peptide used for the conjugation was Cys-PEPTIDE-Amide, the biotinylated peptide should be Acetyl-PEPTIDE-GSG-Bc.

7.1.3.2 If the peptide used for the conjugation was Amino-PEPTIDE-Amide coupled to the carrier via glutaraldehyde, which tends to couple via the N-terminal amino group of the peptide, the biotinylated peptide should be Acetyl-PEPTIDE-GSG-Bc.

7.1.3.3 If the peptide used for the conjugation was Acetyl-PEPTIDE-Cys, the biotinylated peptide should be Biotin-SGSG-PEPTIDE-Amide.

7.1.3.4 If a cysteine residue occurring naturally in the internal peptide sequence was used for conjugation, e.g. the peptide format Acetyl-PEPTIDE1-Cys-PEPTIDE2-Amide, the biotinylated form of the peptide should be BIOTIN-

SGSG-PEPTIDE1-Cys-PEPTIDE2-Gly-Amide. Insertion of the C-terminal Gly prevents recognition of the original C-terminal amide residue.

7.1.3.5 If a combined N- and C-terminal peptide, with a Cys in the middle (Amino-PEPTIDE1-Cys-PEPTIDE2-acid, a "Y-shaped peptide", see section 4.4), was used for immunization, then it should not be necessary to make a separate peptide for assessment; the unconjugated immunizing peptide can be air oxidized to dimerize it prior to coating plates for ELISA, thereby doubling its size and enabling it to both coat the plate and bind to antipeptide antibodies.

8. Affinity Purification

Specific affinity purified antipeptide antibodies can be prepared from the immune sera.[16] Applications for such purified antibodies include: cases where the sera have high background or crossreactivity, due to unwanted preexisting antibodies; where antibodies to the carrier protein are undesirable; where fractionation of multiple antipeptide antibody populations is desirable (e.g. where two or more peptides have been used in immunization); or where the antipeptide antibodies are to be directly labeled, e.g. with radioactive iodine or with an enzyme.

Where the peptide used to generate the antipeptide antibodies had been coupled to the carrier protein using a cysteine residue, the same peptide can be used for affinity purification by coupling the peptide to Thiopropyl Sepharose 6B™ gel. The sulfhydryl group of the cysteine residue reacts with the pre-activated gel to form a disulfide linkage (Fig. 3). This gel-peptide link is stable in the absence of thiols, and the peptide-gel can be used to affinity purify the antipeptide antibodies from serum.

The advantage of using this procedure is that only antipeptide antibodies will bind to the gel and will be purified. Antibodies generated to the carrier protein or to the linker used in coupling to the antigenic carrier protein will not bind specifically to the gel, nor will the other serum proteins. The bound antibodies are then eluted from the gel using high or low pH elution conditions. Recovery of the antipeptide antibodies is measured by ELISA titration and purity is checked by silver stained PAGE analysis in comparison with unpurified serum. The purified antipeptide antibodies show greatly reduced ELISA reactivity with the carrier protein used in immunization.

An alternative gel coupling chemistry is the attachment via an oxime link, achieved by making peptide with an N-terminal aminooxy acetyl group, and reacting with a gel containing aldehyde functionality (e.g. Pierce Aminolink™).

Where the peptide used to raise the antisera was coupled to the carrier protein using glutaraldehyde, then we generally prepare a suitable affinity purification gel by coupling the peptide, via an amine group present in the peptide, to an

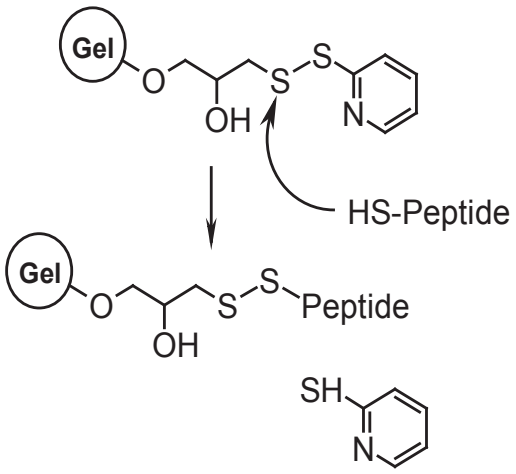

Fig. 3 Attachment of Cys-containing peptide to Thiopropyl Sepharose gel

Displacement of the thiopyridine group from the activated gel by reduced sulfhydryl of a Cys residue in the peptide results in covalent attachment of the peptide to the gel through a disulphide bridge and release of the chromophore pyridinethione (shown as the enthiol form)

activated gel such as Bio-Rad Affigel-10™. Many of the comments and limitations of this method of coupling are similar to those discussed for the coupling of the peptide to the carrier protein using the homobifunctional glutaraldehyde.

The peptide-gel can be re-used many times (10 or more) to prepare further batches of affinity-purified antibody or to process larger amounts of serum at one time.

A further application of peptide-gel affinity media is the removal of specific unwanted crossreactivity from antipeptide sera, by use of a peptide which is related to the immunizing peptide but is from another protein known to (or likely to) crossreact.

9. Epitope Mapping of Antipeptide Antibodies

When the peptide antigen is of 10 or more residues, it is possible that the antipeptide serum will contain antipeptide antibodies to distinctly different parts of the peptide (see Fig. 1). Thus, it can be said that the peptide antigen itself contains multiple epitopes, as defined by an antipeptide serum. This information can be important in understanding the biological properties, or practical utility, of particular antipeptide antisera, e.g. whether a serum is able to neutralize infectivity, or whether a serum is useful diagnostically. One of the simplest and easiest ways to map the binding sites of antipeptide sera is with the use of

multiple overlapping peptides homologous with the immunizing peptide (e.g. a PepSet™, made by Mimotopes or made using a kit supplied by Mimotopes).[5] A further application of the peptide-based affinity purification technique is to affinity purify just one specific portion of the antipeptide antibodies, using as the adsorbent the peptide sequence, found, in an epitope mapping experiment, to be correlated with a desired property of the antipeptide serum.

10. Conclusions

As a supplier of high quality custom antipeptide antibodies, our aim is to supply the most suitable product that will have the greatest chance of performing well in your applications. Please see separate literature for details of the services offered. Our scientists have extensive experience gained by raising antibodies to many hundreds of peptides. However, it must be understood that even high titered antipeptide antibodies may fail in your particular application. Our technical staff are always available to offer you advice in the choice of peptides, species, conjugation chemistry, and immunization protocol that will maximize the probability of producing antisera that will be effective for your purposes. Options include use of predictive methods, multiple peptide immunogens, the unique "Y-shaped peptides", and affinity purification. Quality is assured through a combination of quality assessment of the immunizing peptides, appropriate choice of peptide for assessment of the antipeptide titer, and our titer guarantee.

11. References

1. Ahlborg, N., Paulie, S. and Braesch-Andersen, S. (1997) Generation of antibodies to human IL-12 and amphiregulin by immunization of Balb/c with diepitope multiple antigen peptides. J. Immunol. Meth. 204; 23-32.

2. Briand, J.P., Muller, S. and Van Regenmortel, M.H.V. (1985) Synthetic peptides as antigens: pitfalls of conjugation methods. J. Immunol. Meth. 78; 59-69.

3. Dyrberg, T. and Oldstone, M.B.A. (1986) Peptides as antigens. Importance of orientation. J. Exp. Med. 164; 1344-1349.

4. Edwards, R.J., Singleton, A.M., Boobis, A.R. and Davies, D.S. (1989) Cross-reaction of antibodies to coupling groups used in the production of anti-peptide antibodies. J. Immunol. Meth. 117; 215-220.

5. Geysen, H.M., Rodda, S.J., Mason, T.J., Tribbick, G. and Schoofs, P.G. (1987) Strategies for epitope analysis using peptide synthesis. J. Immunol. Meth. 102; 259-274.

6. Gras-Masse, H.S., Jolivet, M.E., Audibert, F.M., Beachey, E.H., Chedid, L.A. and Tartar, A.L. (1986) Influence of CONH2 or COOH as C-terminus groups on the antigenic characters of immunogenic peptides. Mol. Immunol. 23; 1391-1395.

7. Geerligs, H.J., Weijer, W.J., Bloemhoff, W., Welling, G.W. and Welling-Wester, S. (1988) The influence of pH and ionic strength on the coating of peptides of herpes simplex type I in an enzyme-linked immunosorbent assay. J. Immunol. Meth. 106; 239-244.

8. Groome, N.P. (1994) Immunoassays of proteins and anti-peptide antibodies. In Peptide antigens: a practical approach, pp139-179, Ed. Wisdom, G.B. (Oxford).

9. Hopp, T.P. (1993) Retrospective: 12 years of antigenic determinant predictions, and more. Peptide Research 6; 183-190.

10. Houen, G., Jakobsen, M.H., Svaerke, C., Koch, C. and Barkholt, V. (1997) Conjugation to preadsorbed preactivated proteins and efficient generation of anti peptide antibodies. J. Immunol. Meth. 206; 125-134.

11. Lee, A.C.J., Powell, J.E., Tregear, G.W., Niall, H.D. and Stevens, V.C. (1985) A method for preparing beta-hCG COOH peptide-carrier conjugates of predictable composition. Mol. Immunol. 17; 749-756.

12. Lerner, R.A. (1982) Tapping the immunological repertoire to produce antibodies of predetermined specificity. Nature 299; 592-596.

13. Palfreyman, J.W., Aitcheson, T.C. and Taylor, P. (1984) Guidelines for the production of polypeptide specific antisera using small synthetic oligopeptides as immunogens. J. Immunol. Meth. 75; 383-393.

14. Pellequer, J.-L., Westhof, E. and van Regenmortel, M.H.V. (1994) Epitope predictions from the primary structure of proteins. In Peptide antigens: a practical approach, pp7-25, Ed. Wisdom, G.B. (Oxford).

15. Picard, M.D., Litwak, G., Waite, B.C.D., Pettye, C.L., Foster, K.A. and Thomas, I.J. (1996) Sensitive ELISA method for quantitating antibodies to specific protein epitopes. BioTechniques 21; 628-630.

16. Price, M.R. and Beyzavi, K. (1994) Preparative immunoaffinity techniques. In Peptide antigens: a practical approach, pp117-137, Ed. Wisdom, G.B. (Oxford).

17. Rodda, S.J., Geysen, H.M. and Tribbick, G. (1996) Epitope delineation using multiple peptide synthesis. In Structure of Antigens Vol 3, pp37-60, Ed. Van Regenmortel, M.H.V. (CRC, Boca Raton).

18. Schaaper, W.M.M., Lankhof, H., Puijk, W. and Meloen, R.H. (1989) Manipulation of antipeptide immune

response by varying the coupling of the peptide with the carrier protein. Mol. Immunol. 26; 81-85.

19. Schott, M.E., Wells, D.T., Schlom, J. and Abrams, S.I. (1996) Comparison of linear and branched peptide forms (MAPs) in the induction of T helper responses to point-mutated ras immunogens. Cellular Immunol. 174; 199-209.

20. Tainer, J.A., Deal, C.D., Geysen, H.M., Roberts, V.A. and Getzoff, E.D. (1991) Defining antibody-antigen recognition: towards engineered antibodies and epitopes. Intern. Rev. Immunol. 7; 165-188.

21. Tam J.P., Clavijo, P., Lu, Y.-A., Nussenzweig, V., Nussenzweig, R. and Zavala, F. (1990) Incorporation of T and B epitopes of the circumsporozoite protein in a chemically defined synthetic vaccine against malaria. J. Exp. Med. 171; 299-306.

22. Walter, G. (1986) Production and use of antibodies against synthetic peptides. J. Immunol. Meth. 88; 149-161.

23. Weiner, A.J., Geysen, H.M., Christopherson, C., Hall, J.E., Mason, T.J., Saracco, G., Bonino, F., Crawford, K., Marion, C.D., Crawford, K.A., Brunetto, M., Barr, P.J., Miyamura, T., McHutchinson, J. and Houghton, M. (1992) Evidence for immune selection of hepatitis C virus (HCV) putative envelope glycoprotein variants: potential role in chronic HCV infections. Proc. Natl. Acad. Sci. USA 89; 3468-3472.

Mimotopes International
11 Duerdin St, Clayton
VIC, Australia 3168
mimotopes@mimotopes.com
www.mimotopes.com

Mimotopes Asia Pacific
Tel: +61 3 9565 1111
Fax: +61 3 9565 1199
australia@mimotopes.com

Mimotopes Europe
Tel: +44 870 460 1500
Fax: +44 870 460 1501
europe@mimotopes.com

Mimotopes US East
Tel: +1800 633 8161
Fax: +1800 424 3970
useast@mimotopes.com

Mimotopes US West
Tel: +1800 644 1866
Fax: +1800 655 1866
uswest@mimotopes.com