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CHAPTER 12

Epitope Mapping of a Protein Using the Geysen (*PEPSCAN*) Procedure

J. Mark Carter

1. Introduction

1.1. General

Immunity to many diseases is dependent on the ability of the host's antibodies to recognize foreign antigens, such as surface proteins or toxins, and bind them tightly and specifically. This binding is an important aspect of the immune response, and it is often required for subsequent immune processes that ultimately result in re-establishment of a disease-free state.

One of the toughest problems encountered in vaccine development is that of delineating the antibody response to a protein antigen. Whereas the overall response to an antigen may involve various molecular species of antibodies, each antibody molecule can bind specifically to one unique part of the antigen referred to as that antibody's epitope. Often only a subset of these epitopes is involved in blocking a protein's function, clearing of infectious organisms, or other steps in an effective immune response.

The *PEPSCAN* procedure, developed by Mario Geysen and marketed by Chiron Mimotopes (Victoria, Australia), is a variation of solid-phase peptide synthesis. It comprises the synthesis and immunochemical assay of hundreds of peptides covalently linked to plastic pins. This technology represents a major advance in the epitope mapping of protein antigens because of its ability to create the large numbers of overlapping peptides necessary for complete epitope mapping (1).

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Fmoc-NHCH₂CH₂CO-NHCH₂CH₂CH₂CH₂CH₂CH₂NH-COCH₂polymer-polyethylene

Fig. 1. Structure of the prederivatized polyethylene pins commercially available for peptide synthesis.

Currently, the plastic pins for attachment of peptides are commercially available from Chiron Mimetopes. They are now prepared according to a modification of the method originally published (1). First, the polyethylene matrix of the rods is grafted with an acrylic-like polymer. Then, the free carboxylic acid moiety on the polymer is amidated with one end of 1,6-diaminohexane. The other end of the amine is further derivitized with one of a number of linking moieties. Finally, the linker is acylated with Fmoc-β-alanine, yielding the structure shown in Fig. 1.

1.2. Issue of Reproducibility

One of the limitations plaguing early application of the *PEPSCAN* technique was poor reproducibility in the substitution level of these derivitized pins. In our tests of pins from the same lot at the Walter Reed Institute of Research (WRAIR), we found substitution levels ranging from 6–26 nmol NH₂/pin, with a mean of 12 nmol and an SD of 3 nmol. The issue here is not that this variability might prevent accumulation of worthwhile data. Rather, because of this limitation, all results from *PEPSCAN* must usually be considered qualitative. For confirmation, such results may be double-checked via synthesis and immunoassay of peptides via classical solid-phase methods.

Recent advances in pin design incorporate increased surface area as well as improved level and stability of pin derivitization. As the new pins become more widely available, the peptide pin methodology itself is expected to become more widely accepted and more generally utilized.

The pins for peptide synthesis are arranged in 8 × 12 arrays on 9-mm centers, like commercially available microtiter plates. This geometry allows for familiarity and simplification in subsequent enzyme-linked immunosorbent assay (ELISA) for the detection of antibody reactivity. Many laboratory technicians are already quite familiar with standard ELISA assays, and only minor modifications to this procedure are necessary to perform a *PEPSCAN* ELISA. Furthermore, automated microtiter plate readers are widely available for rapid determination of absorbance data in assays performed with these 96-well plates.

1.3. Computer-Automated Amino Acid Indexer

Other than variable substitution level in the pins, the most significant problem in *PEPSCAN* is the logistics of the simultaneous synthesis of several hundred peptides. Clearly computer support is required, but even a computer-generated synthesis schedule, such as the output from the software distributed by Chiron with the pins, leaves possible a large margin of error. The person performing the synthesis must manually transpose amino acid locations from the hard copy list to the microtiter wells. In our own laboratory, this procedure takes about 4 h to fill 10 microtiter plates, and it results in approx 3% error rate.

In order to address this problem, we have developed a computer-driven device that locates and identifies each of the different wells, and indicates their respective amino acid derivative requirements via illumination with LEDs. Using this computer-driven amino acid indexer, the time in filling 10 microtiter plates, for simultaneous synthesis of 960 peptides, is reduced sixfold to 40 min, and error becomes undetectable. The device is thoroughly described in VanAlbert et al. (2), and it is commercially distributed by CRACO (Vienna, VA).

1.4. Linear vs Conformational Epitopes

PEPSCAN is particularly effective in the detection of linear (continuous) epitopes. Unfortunately, however, most antibodies are probably directed against discontinuous epitopes (3–5). This fact becomes especially important when *PEPSCAN* is used to study the specificity of monoclonal antibodies (MAbs). In many cases, the results of such experiments are weak and equivocal. Nonetheless, Geysen has suggested that binding of antibodies to discontinuous epitopes (such as are reported for most MAbs) may be detected on peptide pins, at least in some instances. Such binding typically involves two or three discontinuous regions of the protein sequence that fold into a discrete conformation on the solvent-accessible surface of the native structure of the antigen. Theoretically, the antibodies should bind, although much more weakly, to each of these regions when presented separately. In fact, we have observed many sets of data suggesting this conclusion, but the binding so detected is often not statistically significant above background (nonspecific) binding.

Excellent results are generally obtainable using immune serum as a source of antibody. It is probably true that serum raised against a native protein antigen will contain only a limited subset of antibodies reactive

to linear epitopes presented by the peptide pins (6). However, there is usually such a large variety of reactivities represented by a polyclonal serum that a fair number of linear epitopes can be readily demonstrated by binding to the pins. Antisera raised against peptide antigens and peptide conjugates tend to contain a greater proportion of antibodies that are reactive to linear epitopes because of their more limited conformational freedom. Consequently, this type of immune serum generally gives the highest level of detected binding on *PEPSCAN*.

2. Materials

2.1. Synthesis

1. Prederivitized polyethylene pins and polyethylene microtiter plates.
2. *N,N*-dimethylformamide (DMF).
3. Methanol.
4. PIP solution: 20% piperidine in DMF. (See Note 2).
5. Amino acid solution: 30 mM amino acid derivative and 30 mM 1-hydroxybenzotriazole (HOBt) in DMF. Derivatives used are 9-fluoreno-methoxycarbonyl (Fmoc) amino acids as *O*-pentafluorophenol or *O*-dihydrobenzotriazine esters.
6. Dichloromethane (DCM)—**Note: This solvent is a suspected carcinogen.**
7. Basified DCM: 5% diisopropylethylamine in DCM. Prepare fresh within 1 h of use.
8. Acetylation cocktail: 5% acetic anhydride and 1% diisopropylethylamine (DIEA) in DMF. Prepare fresh immediately before use.
9. Deblocking cocktail: 2.5% phenol and 2.5% 1,2-dithioethane in trifluoroacetic acid. Prepare fresh within 1 h of use. **Note:** This reagent is extremely corrosive, and it smells absolutely terrible. Wear appropriate protective devices, and use it in a fume hood.
10. Deionized water.
11. Silica gel desiccant.
12. Plastic baths and sealable bags.

2.2. Disruption

1. Sonicator: See Note 10 about choice of ultrasonic cleaner.
2. Disruption buffer: 1% reagent-grade sodium dodecylsulfate, 0.1% 2-mercaptoethanol, and 0.1M sodium phosphate, pH 7.2.
3. Explosion-proof heating bath, filled with boiling methanol.
4. Silica gel.
5. Sealable bags, tongs.

2.3. ELISA Analysis

1. Peptide pins.
2. Phosphate-buffered saline (PBS): 150 mM NaCl and 25 mM phosphate, pH 7.4. Prepare in 1-L batches, filter-sterilize, and store at 4°C. PBS keeps for about 2 or 3 wk. For indefinite storage, add 2 g/L sodium azide.
3. PBS/Tween 20 (PBST): Phosphate-buffered saline (PBS, as above) with 0.1% Tween 20. Prepare in 1-L batches (see Notes 13 and 18).
4. Blocking solution (see Note 14): Use a commercial ELISA blocker solution or one of the following two solutions. Prepare in 1-L batches, filter-sterilize, and store at 4°C. Solution keeps for about 2 or 3 wk. For extended storage (up to 8 wk), add 2 g/L sodium azide.
 - a. 1% Bovine serum albumin (BSA) and 1% chicken ovalbumin (OVA) dissolved in PBST.
 - b. 2% Casein in PBST: Boil 20 g casein in 100 mL 1N NaOH until completely dissolved. Adjust pH to 7.4 by addition of HCl. Add PBST to make final volume 1 L.
5. Test antibody solution. For serum or ascites fluid, the concentration used should be the same as that which gives a good, strong signal on a standard ELISA. If a standard ELISA titer is not available, then use a dilution of 1/500. For a purified antibody, use 1–10 µg/mL.
6. Second antibody solution (see Notes 19 and 20): The working concentration of the second antibody is usually specified by the manufacturer. Make the dilution in PBST.
7. Substrate solution: 0.1M diethanolamine, pH 9.8, with 0.01% MgCl₂ and 0.02% NaN₃. This buffer may be stored at 4°C for several months. However, it should be allowed to warm to room temperature before use. Immediately before use, dissolve *p*-nitrophenyl phosphate (the substrate) to a final concentration of 1 mg/mL.
8. Plastic boxes with tight-fitting lids (e.g., Tupperware).

3. Methods

3.1. Synthesis

Historically, Cambridge Research Biologicals (Cheshire, UK) distributed a recipe for synthesis of peptides via Fmoc chemistry. In it, the prederivitized polyethylene pins are deprotected, washed, neutralized, washed, and amino acylated repeatedly until peptides of the desired length are completed. These peptides are then N α -acetylated, side-chain deblocked, and washed once more. Finally, the peptide pins are subjected to ultrasonic disruption before ELISAs are performed. All reactions are

performed at room temperature in a fume hood. Details of the method, as well as a few modifications we and others have suggested, are described below.

1. Deprotection (removal of the N^{α} -Fmoc group): Pins are first pre-equilibrated in DMF baths for 5 min. Perform deprotection batchwise in polyethylene boxes (*see* Note 1) poured to a depth of about 2.5 cm with PIP solution for 1 h (*see* Note 2).
2. Wash: Deprotection is followed by washes in DMF (two washes, 5 min) and then methanol (three washes, 3 min each) (*see* Note 3). Then the blocks are allowed to air-dry in a fume hood for at least 1 h (*see* Note 4).
3. Coupling (amino acylation): After pre-equilibration in DMF bath 5 min, pins are amino acylated individually with 100 μ L/well in the polyethylene microtiter plates. The plates bearing the peptide pins are carefully oriented and lowered so that the pins are inserted into their respective wells. In order to reduce evaporation and contamination, the reaction is allowed to proceed overnight inside a sealed ziplock bag (*see* Note 5).
4. Wash: Following the amino acylation reaction, pins are again washed with DMF (two washes, 5 min each) and methanol (four washes, 3 min each), and then air-dried again for at least 1 h.
5. Elongation: Deprotection, washing, amino acylation, and washing are repeated until peptides of the desired length are produced. After the last amino acid is coupled, final deprotection, washing, and air-drying are performed as above (*see* Notes 6 and 7).
6. N^{α} -acylation: After pre-equilibration of the peptide pins in DMF bath for 5 min, α amino groups on the peptides are acetylated in polyethylene microtiter plates with 100 μ L/well solution of the acetylation cocktail for 90 min.
7. Wash: Pins are then washed with DMF (two washes, 5 min each), methanol (four washes, 3 min each), and air-dried again for 1 h.
8. Deblocking: Blocking groups are removed from the peptide amino acid side chains by incubation of the pins for 4 h in 2.5-cm deep baths of deblocking cocktail.
9. Wash: The pins are then washed in baths of DCM (two washes, 2 min each), basified DCM (two washes, 2 min each), DCM (5 min), allowed to air-dry (1 h), then washed in deionized water (2 min), methanol (overnight), and air-dried. Finally, the peptide pins are dried over silica gel in ziplock bags overnight. The finished blocks of peptide pins are stored at -20°C over silica gel in ziplock bags.
10. Disruption: Before they will perform properly in ELISA assays, the peptide pins must be disrupted, (*see* Section 3.2.).

3.2. Disruption

After peptide synthesis is complete, ELISAs are typically unsuccessful without prior ultrasonic "disruption." In order to make the peptides on the pins accessible to antibody binding, high-power ultrasonic treatment at elevated temperature is absolutely necessary.

1. The sonicator (*see* Notes 8 and 9) is filled with the disruption buffer and allowed to heat to $60\text{--}70^{\circ}\text{C}$. The polyethylene blocks bearing peptide pins are floated in the buffer, with the pins pointing downward. The sonicator is then operated for 20 min.
2. Pins are removed from the sonicator with tongs and rinsed briefly, but thoroughly in $60\text{--}70^{\circ}\text{C}$ water.
3. At this point, the pins may be used immediately for ELISA. If they are to be stored for more than a few minutes, they should be boiled in methanol for 2 min, air-dried for at least an hour in the fume hood, and finally stored in ziplock bags at -20°C over silica gel (*see* Notes 10 and 11).

3.3. ELISA Analysis

A typical ELISA has five main steps (plus washes): First, the antigen is allowed to bind to the microtiter plate wells overnight in a dilute solution with PBS (*see* Note 13). Next, the excess antigen solution may be removed, or even a brief rinse performed, before a "blocking" solution is added. After an hour or two, the blocking solution is removed, and the test or "first" antibody is added (*see* Note 14). The first antibody is usually allowed 1 or 2 h to bind the antigen on the plates. After a series of thorough washes, an enzyme-conjugated "second" antibody is added and allowed to bind to the first antibody. After an hour or two, another thorough wash is made, and then a chromogenic substrate solution for the enzyme is added and allowed to develop for a few minutes to 2 h. Finally, the results are read on an automated microtiter plate reader that generally stores the values for absorbance for each well in a computer file. (A general recipe for this type of basic ELISA appears in Chapter 10, Section 8).

The peptide pin ELISA is performed very much like a typical ELISA. Persons experienced in the latter generally have little trouble with the technical aspects of pin ELISAs. There is, indeed, but one major difference between the two. In a typical ELISA, the first step comprises the adsorption of the antigen onto the bottom of a microtiter plate. This molecule acts as a solid-phase "capture" antigen for the subsequent binding of antibodies. Contrarily, with peptide pins, the peptide antigen remains covalently linked to the solid-phase support pin at all times.

This means that a peptide pin ELISA has only four steps. Briefly, the pins are "blocked" with a suitable buffer, they are subjected to binding of a first antibody, they are probed with an enzyme-labeled second antibody, and they are developed with a substrate. Each of these steps, as well as washing between them, is detailed below.

1. Blocking: Into each well of a microtiter plate, pipet 200 μL of blocking solution. Insert the pins and incubate 1 h at room temperature.
2. Test antibody: Pipet 175- μL test antibody solution into each well of a microtiter plate. Insert the pins and leave to incubate overnight at 4°C, rocking gently on a platform (*see* Notes 15 and 16).
3. Wash: Pour PBST (*see* Note 17) into a clean plastic box so that the level of liquid comes at least halfway up the pins when the blocks of pins are inserted with their pins downward. Put the box with wash buffer and pins on a rotating platform for 10 min. Discard the used wash buffer down the sink. Repeat for a total of three washes.
4. Second antibody: Pipet 150 μL second antibody solution (*see* Notes 17, 19 and 20) into each well of a microtiter plate, insert pins, and allow 1–2 h for binding.
5. Second wash: After the second antibody, make another thorough wash to remove excess enzyme conjugate reagent. Again, three washes of 10 min each are sufficient.
6. Substrate: Pipet 125 μL substrate solution into each well of a good-quality ELISA plate. Before inserting them into the plate, carefully orient the pins so that the numbered edges of the plates correspond with the numbered edges of the block containing the peptide pins. This will prevent confusion when the plates are being read after development.
7. Development: Allow development to proceed until the positive reactions are well colored, usually 30–60 min (*see* Note 21). Stop the development by removing the pins. Do not allow development to proceed until the negative peptides give a strong color reaction (*see* Note 22). After development is complete, remove the pins and rinse them immediately in water.
8. Plate reader: Read the plates on an automated microtiter plate reader within an hour.
9. Disruption: Disrupt as soon as possible. If this cannot be done within a couple of hours, store the pins overnight in a methanol bath. Do not let any of the ELISA reagents dry onto the pins.

3.4. Data Interpretation

3.4.1. Epitope Analysis

For each plate, individually, subtract the mean of the lowest 10–25% of absorbance readings. This is background. This is facilitated by means of a spreadsheet program. In lieu of any officially established criterion

for differentiating between positive and negative reactions, positive responses are identified through the judgment of the experimenter. In general, the highest responses will be scored as positive reactions, whereas most sequences will be unreactive. Peptides with intermediate reactivity are often borne out as positive or negative after a repeat of the ELISA experiment.

There are several different combinations of antibody and peptides commonly used in the peptide pin system. Each combination may be expected to give different results, although they will all generally allow the same conclusions to be drawn.

3.4.2. Polyclonal Antibody Epitopes

One of the most common applications of this system is epitope mapping of a full protein sequence of overlapping octamers, where the immunogen was the intact native protein (or even an entire organism). ELISA reactivity of such a polyclonal immune serum typically gives several peaks, each corresponding to an epitope. Frequently there is one relatively strong immunodominant epitope that stands out among the others (*see* Fig. 2).

3.4.3. Epitope Overlaps

Each of the peaks of epitope recognition will typically span several pins and, therefore, several overlapping peptides. The minimal region of recognition is the sequence contained in all the recognized peptides of a given epitope (*see* Fig. 3). This vital information is only accessible through synthesis of many overlapping peptides.

3.4.4. Antipeptide Antibodies

You may wish to map the fine specificity of a serum raised against a synthetic peptide immunogen. These experiments have generated the strongest ELISA signals we have seen. However, the results are often complicated by strong reactivity to two (or more!) closely neighboring epitopes. This gives a broad peak, so that it is difficult to tell where one epitope ends and another one begins. Of course, this is more of a problem with sera raised against larger peptides (30+ amino acids) as immunogens.

3.4.5. Monoclonal Antibody Epitopes

Most MAbs are raised with an intact protein as immunogen. The limited reactivity of these MAbs to peptide pins emphasizes the paradigm of underrepresentation of linear epitopes among the general population of

PEPTIDE PIN ELISA RESULTS

Monoclonal Antibody (MAb 6-526-12)

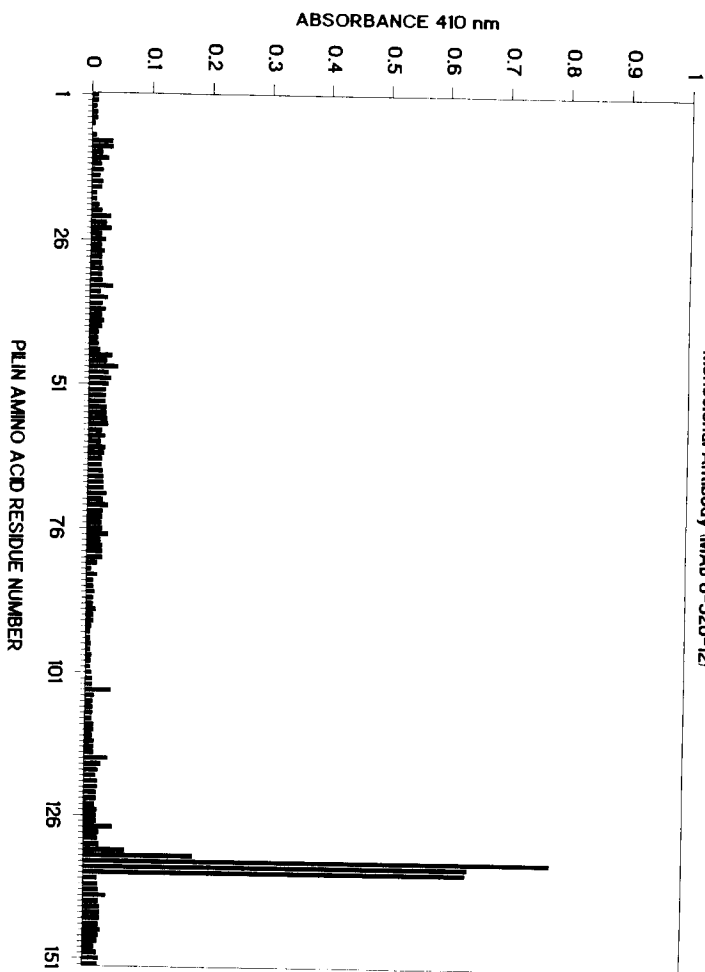


Fig. 2. Typical peptide pin ELISA. ELISA was performed according to standard methods presented in the text. Immune rabbit serum was used to probe pins bearing overlapping 8-mer peptides comprising a bacterial protein sequence. The X-axis of the figure represents the position in the protein sequence, and the Y-axis indicates absorbance (i.e., ELISA reactivity). Obviously, one epitope reacts much more strongly than the remainder of the protein. This phenomenon is referred to as immunodominance.

antibodies. Only about one out of every eight of the MAbs reacts strongly to any of the peptides on pins. Frequently, MAbs give somewhat ambiguous results with two or three peaks detected. This may seem like an artifact, since an MAb should only have one target sequence, but Geysen has suggested that these multiple regions of recognition suggest the location of the noncontiguous regions that would fold together in the native protein to give the conformational epitope for these MAbs. We have also seen gross crossreactivity of MAbs to several peptides with related sequences on pins. In our limited experiences with MAbs raised against peptide immunogens, we have seen only linear epitopes represented.

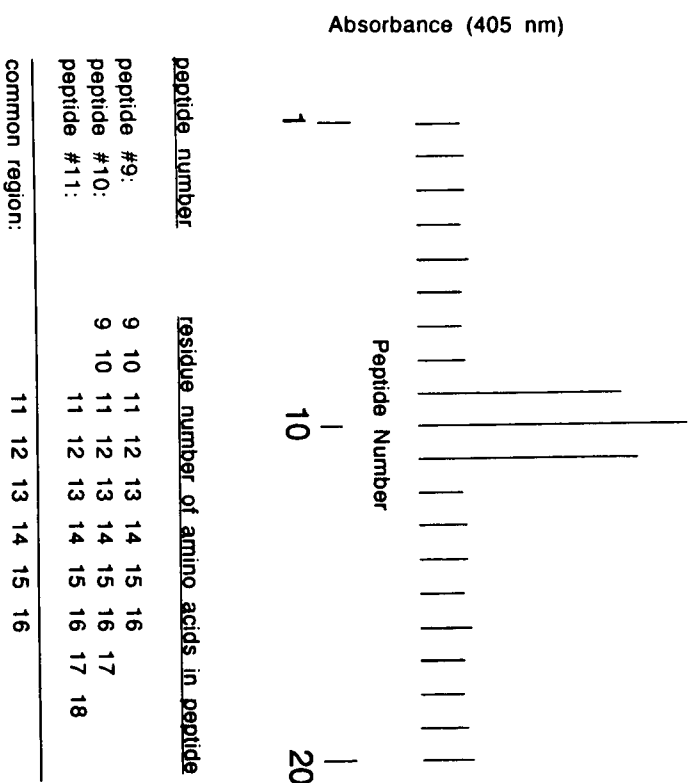


Fig. 3. Schematic of overlapping peptides representing a linear epitope. Peptides 1-20 are overlapping 8-mer peptides. The figure shows their reactivity with an antibody preparation. Peptides 9, 10, and 11 are reactive. The amino acid sequence in common to these three peptides is 11-16. This sequence represents the epitope of the test antibody.

3.4.6. Human Serum Epitopes

Immunologists who regularly work with human sera are generally familiar with its idiosyncracies. Chief among these is a remarkably high background reaction in ELISAs. This is presumably the result of the broad sensitization of humans, owing to diversity of exposure experiences, as well as a large amount of low-specificity antibody in the naive state. Monkey sera exhibit these same problems, although to a lesser degree.

A brief treatment at 56°C will kill most disease organisms and viruses, as well as neutralizing complement and many other serum proteases. However, heat treatment increases nonspecific "stickiness" of the serum. We have found that an ELISA blocking solution based on 2% casein is very effective in reducing this background reactivity.

3.4.7. Differential Responses

Different species of immune animals will often react to different sets of epitopes in a given immunogen. Different individuals within a species often react differently, especially if they are "outbred" (not genetically homogenous). Even with inbred strains, differences will arise because of heterogeneity in the animals' immune history and in injection technique.

3.5. Other Applications

3.5.1. Mimetopes

One application now widely touted by Geysen is the construction of mimetopes. These are artificial epitopes comprising peptides containing natural and nonnatural amino acids in nonnative sequences (7,8). Mimetopes can attain conformations in assays that have the same binding characteristics of naturally occurring conformational epitopes. It seems probable that mimetopes may also be able to elicit antibodies with affinity for naturally occurring conformational and even nonprotein (e.g., carbohydrate) epitopes (9). For this reason, they are promising candidates for future vaccines.

3.5.2. Cleavable Pins

Another approach utilizes the chemical spacer built onto the peptides. This is the nonpeptide moiety that attaches the peptides to the plastic support pins. Incorporation of an acid-labile amino acid sequence (Asp-Pro) at this position in the peptide facilitates acidolytic cleavage from the pin after synthesis is completed. This results in generation of a large number of soluble peptides although in limited quantities. This technique has proven useful in studies demonstrating T-lymphocyte epitope specificity through mitogenesis assays (10). More recently, pins bearing a water-cleavable chemical link have become commercially available from Chiron (11,12). Depending on the linker and respective cleavage chemistry, the new pins can be used to generate peptides with C-terminal free carboxy acids, carboxamides, or diketopiperazines.

One fairly simple variation is the use of proteins other than antibodies to probe the peptide pin arrays. This approach is promising for structure-function studies on biological receptor molecules. Another example takes advantage of the reversibility of binding of antibodies to peptides in the typical ELISA application of the pins. By eluting the bound antibodies from the individual peptides, it is possible to affinity purify small quan-

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tities of antibody. The amounts of antibody protein isolated from each pin by this technique are vanishingly small, but sufficient to be detected by means of binding to Western blots.

4. Notes

1. Before deprotection, pins are first pre-equilibrated in DMF baths for 5 min. This step probably reduces nonspecific attachment of the piperidine molecule to the polyethylene pin matrix. Other chemists have increased the piperidine concentration in order to shorten the time for deprotection (12).
2. At WRAIR, the original piperidine solution is reused every day (each deprotection cycle) for the entire synthesis. This is possible because the reaction of piperidine with the Fmoc groups on the peptide pins is stoichiometric: each mole of Fmoc removed requires only 1 mol of piperidine. This means that very little of the piperidine is actually consumed in one use of the reagent solution. With this in mind, and partly because of the difficulty and expense of obtaining and storing large quantities of piperidine (which is a controlled substance), we investigated the possibility of reusing the 20% piperidine solution. We found that, after 13 daily uses for deprotection of the peptide pins, the solution was as effective as it was when freshly prepared. This was in spite of the observation that, after 4 d, it began to develop a white crystalline material. We presumed that this material was a piperidine formate salt resulting from hydrolysis of the DMF solvent (by atmospheric water in the presence of the piperidine base as a catalyst). When we repeated the experiment, storing the piperidine/DMF reagent over molecular sieves to keep it anhydrous, the crystalline material did not form, and the reagent maintained its clarity as well as its efficacy.
3. When washing, we found that it was necessary to rinse both sides of the pin blocks in order to remove contamination resulting from splashes and condensation of solvents and reagent, which otherwise accumulate on their undersides.
4. From the residual odor remaining on the pins, it is apparent that the piperidine is not completely removed by the organic solvent washes alone, as described above. Because piperidine is a base, its presence is easily confirmed by testing the pH of an aqueous solution of the final wash solvent. Worried that residual piperidine will affect the peptide syntheses, we now typically add a 5-min wash in 1% acetic acid (freshly prepared) in DMF. This is intended to neutralize the piperidine base and reduce its affinity for the polyethylene. After washing with this modified protocol, we prepared a 50% solution of the final methanol wash in water, and found that its pH was neutral, indicating essentially complete removal of piperidine. Note that this acid-wash step is not appropriate for chemistry modification incorporating *in situ* activation of the amino acid derivatives.

5. If you elevate the concentration of amino acid derivative and HOBt in the amino acylation solution to 100 mM, you may reduce the time for acylation to 4 h. This allows two or even three amino acids to be added to the growing peptides in a 24-h period. Some have suggested the use of BOP or HBTU equimolar with the amino acid derivative, to accelerate the coupling reaction (*see also* Chapter 1, *PSP*). Still others have substituted dimethylacetamide for the DMF solvent in the coupling step, claiming that this improves solvation efficiency of the pin matrix (13). Finally, Geyssen himself now uses Fmoc amino acid derivatives as free carboxy acids, activating *in situ* with dicyclohexylcarbodiimide.
6. In order to expedite and improve the accuracy of the placement of amino acid esters in the appropriate microtiter plate wells, an automated indexer is used in our laboratory (2). Driven by menu software on a PC-type computer, this device indicates the appropriate wells for each of the amino acids for the synthesis (14). Chiron is expected to market a similar device.
7. The laboratory at WRAIR typically makes peptides 6–12 amino acids in length. Shorter molecules may not have a measurable affinity for the test antibody, whereas longer molecules will probably contain little of the full-length peptide because of the limited efficiency of the non-sequence-optimized coupling chemistry.
8. We used an ultrasonic cleaner instrument manufactured by Blackstone and rated for 500 W at 25 kHz. This sonicator has an electrical heater and a thermostat that we operate at 70°C. Our several attempts using less-powerful sonicators were ineffective, resulting in high-background signals in the ELISA and residual protein on the pins, as detected via amino acid analysis. Similarly, poor results were obtained when the bath temperature was allowed to drop below 60°C.
9. Our sonicator has a vol of 20 L, so it can fit eight blocks of peptide pins at once, floating in a single layer on the top. Although we add fresh 2-mercaptoethanol every day, we routinely reuse the disruption buffer 10 or 12 times, until it begins to darken.
10. To keep the silica gel from intimate contact with the pins, pouches may be made from paper towels, filled with a generous handful of indicator-grade silica, and then stapled shut. These silica pouches may be regenerated when necessary by baking overnight at 120°C.
11. To keep the silica gel from intimate contact with the pins during storage, pouches may be made from paper towels, filled with a generous handful of indicator-grade silica, and then stapled shut. These silica pouches may be regenerated when necessary by baking overnight in an oven at 120°C.
12. It is critical to avoid microbial contamination of the peptide pins. Amino acid analysis indicates that the peptides are rapidly destroyed by microbial

- action. Indeed, pins left overnight in PBS at room temperature are thereby completely ruined. It is also probably best to prevent any of the ELISA solutions from drying onto the pins.
13. In all buffers and reagent solutions used for the pin ELISA, 0.1% Tween 20 is typically added. Tween 20 is a very mild nonionic detergent. It serves as a wetting agent, thereby improving reproducibility and helping to reduce nonspecific binding. Because Tween 20 is surface active, all pipetting should be performed carefully so as to minimize aeration, since foaming will affect reproducibility.
 14. Two percent casein gives lower background for some antibody, such as human serum. Either of the blocking buffers described will keep for 2 or 3 wk if sterility is maintained. If desired, 0.2% NaN₃ may be added. This will increase the practical storage time for the reagent to several weeks, but 1 L is typically consumed in a few days of ELISA work. Blocking is generally performed for 1 h at room temperature, but if the solution contains 0.2% sodium azide, it may be left overnight in the refrigerator.
 15. As an alternative to a rocking platform, we have used a rotating (orbital) platform for incubations. It seems to work just as well.
 16. For the overnight incubation with test antibody, put the filled plates into a sealable plastic box lined with a moistened paper towel to maintain humidity and minimize evaporation. If this step is allowed longer than about 12 h, evaporation and condensation may nonetheless begin to affect reproducibility, especially for the pins closest to the edge of the plate. After the overnight incubation, the first antibody solution is usually discarded. However, we have occasionally pooled and reused this reagent up to four times without any discernible loss in signal-to-noise ratio. In these cases, we have added a single wash step between the blocking and first antibody to minimize dilution and contamination of the valuable test antibody solution. Of course, we also stored the antibody solution at 4°C.
 17. For PBST, we purchase 10X PBS in liter bottles, and then add Tween 20 and sodium azide. We store this 10X stock solution in a carboy at room temperature for up to 2 wk. From the stock, we prepare 1X PBST for each day's use by diluting 1/10 with deionized water.
 18. Remember that proper reactions for control peptides, if they are used, will probably require a different first antibody solution. They may also require a different second antibody solution.
 19. For second antibody (enzyme conjugates), we have successfully used commercially available reagents from various sources, as well as our own conjugates. Although we have also used conjugates with alkaline phosphatase (AP) and horseradish peroxidase (HRP), we prefer the AP conjugates for maximum reproducibility with good sensitivity since the HRP substrate

contains peroxides that damage the peptides. We perform incubations for second antibody binding on the laboratory benchtop, but it does not hurt to do it in the refrigerator or to use a rocking platform. This incubation may also be performed overnight.

20. In some cases, it may be necessary to prepare your own enzyme-conjugated second antibody. To make an AP-conjugated goat antibody to recognize *Aotus* monkey antibody in ELISAs, we used the following protocol: Isolate several milligrams of nonimmune antibody from the monkey serum by protein A affinity. Use most of this protein as an immunogen to raise antibody in a goat. Isolate several milligrams of goat immune antibody by protein A affinity. Couple a few milligrams of the *Aotus* antibody immunogen to Sepharose. Use the immobilized *Aotus* antibody to affinity purify the goat antibody vs *Aotus* antibody. Conjugate the purified antibody to commercial AP via glutaraldehyde. Dilute in PBS with 0.2% NaN₃. Test the second antibody conjugate at various concentrations to determine the appropriate working concentration for the reagent. Store frozen, avoiding refreezing. For details, refer to Lyon and Haynes (15).
21. To aid in visualization of color development, place the plate containing the substrate solution on a piece of white paper. If you are using more than one plate for the ELISA (which is likely), number them on their outer edges. During development, avoid thermal gradients. These may be caused, for example, by drafts or sunlight. We cover the pins to isolate them from environmental effects.
22. After development, removal of the pins stops the color generation catalyzed by the enzyme conjugate on the pins, but the substrate is thermolabile, and the chromophore is photolabile, so avoid unnecessary delays by setting up the reader while development is still taking place. Avoid touching the bottom of the ELISA plate before it is read. Do not discard the plates until you are certain that you have two legible copies (either "soft" or "hard" copies, according to your preference) of your data. If the signals are weak, you may return the pins to the plates for further development and read them again later.

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