BY JOHN MARK CARTER, STEVE VANALBERT, JAIME LEE, JEFF LYON, & CAROLYN DEAL

Shedding Light on Peptide Synthesis

One of the toughest problems encountered in vaccine development is delineating the antibody response to a protein antigen. Whereas the overall response to an antigen may involve various antibody species, each antibody molecule can bind specifically to only one unique epitope. Often, antibodies to a small subset of these epitopes will block a protein's function, clearing infectious organisms or provoking other processes in an effective immune response.

Protein epitopes can be reproduced by synthetic processes that duplicate the protein's amino acid sequence. Antibodies often bind to these synthetic peptides much as they would bind to the intact protein antigen. One effective way to analyze and map the binding specificity of an antibody is to make a number of synthetic peptides and test them for antibody reactivity in a standard capture ELISA. A simple method for making and testing large numbers of peptides has been developed by Mario Geysen (Geysen et al., 1984). In this technique, numerous peptides are synthesized simultaneously on individual chemically derivatized plastic pins, and hundreds of these pins are later assayed for antibody binding via a modified ELISA. This technique has enjoyed great success in mapping medically and scientifically significant epitopes on numerous proteins (e.g., Geysen et al., 1984; Stover et al., 1990).

The peptides made using the Geysen method are typically eight amino acids long and overlap each other throughout the entire protein sequence. For example, if a certain protein antigen is composed of a string of one hundred amino acids, the first peptide would comprise amino acids 1-8. The second one would comprise amino acids 2-9, and so on, up to the 94th peptide, which would be 94-100. The peptides are prepared on an 8 x 12 rectangular matrix of plastic pins (Figure 1). Each peptide chain is assembled onto one pin throughout the synthesis. The peptides are synthesized from their C-termini according to well-defined peptide synthesis chemistry. Each day for eight days, one amino acid is added to each growing peptide chain. This is done by pipetting a solution containing an activated derivative of the correct individual amino acid into the appropriate corresponding wells of a solvent-resistant, 96-well microtiter plate. When all the wells are filled with the correct amino acids, the pins are placed into the plates to react in the solutions overnight.

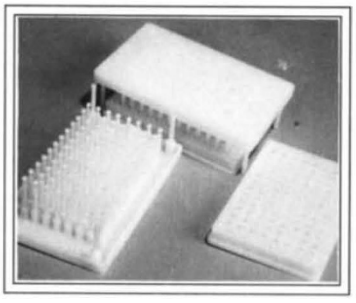


Figure 1. Prederivatized peptide pins. The polyethylene pins are arranged in an 8 x 12 matrix on 9-mm centers (like standard microtiter plates) and are commercially available from Cambridge Research Biochemicals and ICI Bio Products & Fine Chemicals.

Figure 2. Amino acid indexer. The prototype is capable of simultaneous synthesis of 960 peptides on 10 microtiter plates.

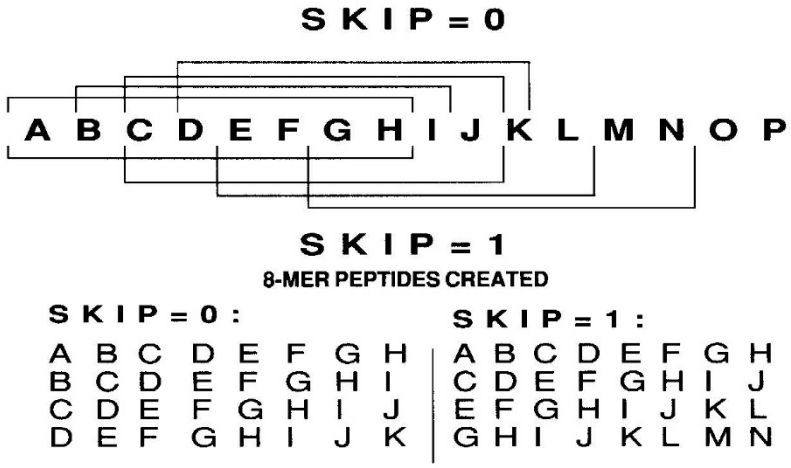
The major weakness of the peptide pin procedure is the complexity of simultaneously synthesizing hundreds or even thousands of peptides. The "amino acid indexer" presented here was designed to simplify the technical problems of matching the different amino acids to the hundreds of microtiter plate wells for each day's work in a Geysen peptide synthesis. We have developed computer software and hardware that allows the relatively straightforward creation of up to 960 separate peptides (10 different microtiter plates) simultaneously (VanAlbert et al., 1991).

Machine Characteristics

The outside of the amino acid indexer is constructed of a tough acrylic polymer. This material is fairly inexpensive, easily machined, and resistant to attack by the solvents used in the synthesis procedure. Ten polyethylene microtiter plates are mounted on the top face of the instrument, and a high-brightness red LED is mounted in

The authors are at the Walter Reed Army Institute of Research, Washington, DC. The views expressed here do not necessarily reflect those of the U.S. government.

Figure 3. Overlapping peptides. The sequences of the first four peptides that would be prepared from a hypothetical parent protein sequence are shown.



each of the wells. To use it, a researcher snaps an empty polyethylene microtiter plate into place over the top of each LED-containing plate. This second plate serves as a reaction vessel for the peptide syntheses. When the LEDs in the bottom plates are lit, they shine through the bottom of the upper (reaction vessel) plate, easily visible in typical ambient laboratory lighting conditions (Figure 2).

On the inside, the indexer has three major components: the main control circuit board, the latch circuit boards, and the LED circuit board. Each of the 10 LED plate modules is composed of an LED circuit board that accepts the individual LEDs, and a latch circuit board drives the LEDs. These circuit boards mate through two 50-pin connectors. The 10 latch boards are chained together and connected to the main control board using a bus cable. Data is communicated from the computer to the indexer using RS-232 asynchronous communication protocol. Because the microtiter plate wells are arranged in an 8 by 12 matrix, standard 8-bit ASCII code is used. A single 8-bit character represents the light pattern of each row, each bit corresponding to a single well. If the bit has a value of 1, the corresponding LED will be off; if the bit has a 0 value, the LED will be illuminated. For each amino acid, 120 ASCII characters are transmitted to the amino acid indexer: one character for each of 12 rows for each of 10 plates.

The indexer is designed to increase the accuracy and throughput of peptide pin synthesis. When a synthesis of a few hundred samples is performed with current techniques, even an experienced technician can suffer from mental fatigue and confusion while trying to make certain that all the microtiter plate wells are correctly identified and filled. The indexer simplifies the synthesis of

Figure 4. Typical ELISA Results. Shown are results of MAb 6-526-12 assayed on pins synthesized with overlapping octamers representing the gonococcal pilin protein sequence (Hagblom et al., 1985).

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

longer, more complicated peptide sequences, and it allows facile duplication of sequences for double-checking results.

Lighting Sequences

The software, running on an IBM PC/XT/AT compatible or IBM PS-2 series computer, reads a protein sequence from an existing ASCII file and creates separate pattern files. This software allows the creation of peptides from 8 to 12 amino acids in length. In the example shown in Figure 3, eight separate pattern files would be created since eight days are needed to create an octapeptide sequence. These pattern files contain information as to which amino acid should be placed in which plate well on each particular day. Each pattern file consists of 2400 8-bit ASCII characters. Each character in the pattern file is used to illuminate appropriate LED's on the indexer. The pattern information is sent to the indexer one amino acid at a time, lighting all the LEDs that correspond to that amino acid, thereby indicating which wells should be filled with amino acid derivative solution. The software allows total control over which amino acid is displayed and when it is displayed.

To select the string of amino acids to be synthesized, the user enters the residue number of the beginning and the ending amino acids. The program then highlights this section of the total sequence on the screen and prompts the researcher for acceptance. When accepted, the program creates the binary pattern files that will be used to light the LEDs on the indexer. It also creates a comprehensive report detailing all of the individual peptide strings that will be synthesized, and shows the actual peptide sequence to be synthesized on each pin. For each day of synthesis it gives a recipe for preparing the 20 different amino acid solutions in sufficient quantities for the day's work. At the end of the report is a summary showing the amount of each reagent required for the entire full-length synthesis.

This program also performs ELISA analysis and simplifies the processing of ELISA data once they have been recorded by the microtiter plate reader. It finds the lowest 25 absorbance values on each plate and subtracts either the average of the lowest 25 or the absorbance value for the negative control, whichever is lower, from each value on the plate. The resulting data matrix is then stored in a format suitable for import into a spreadsheet program for manipulation, labeling, and output. The final results of a typical peptide pin ELISA prepared with the use of the amino acid indexer are shown in Figure 4 on the facing page.

Pins and Indexers: Applications

As the peptide pin method becomes more widely used, indexer modifications will be needed in order to support novel and inventive applications of pin technology. One new application may involve the construction of mimetopes. These are artificial epitopes made of peptides containing natural and non-natural amino acids in non-native sequences (Geysen et al., 1986a,b). Mimetopes—promising candidates for future vaccines (McGregor et al., 1990)—can attain conformations in assays with the same binding characteristics as naturally occurring conformational epitopes. It seems likely that mimetopes may also be able to elicit antibodies with affinities for naturally occurring conformational and even non-protein (e.g., carbohydrate) epitopes.

A second approach utilizes the chemical spacer built onto the peptides. This is the non-peptide 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 the generation of a large number of soluble peptides, albeit in limited quantities. This technique has proven useful in studies demonstrating T-lymphocyte epitope specificity through mitogenesis assays (van der Zee et al., 1989; Jarboe et al., 1992), as well as peptide analog synthesis applications (DiPasquale et al., 1991).

Another fairly simple variation is the use of proteins other than antibodies to probe the peptide pin arrays. This method has proven useful in some structure-function studies on biological receptor molecules (Wang et al., 1991; Karabatsas et al., 1991). A fourth 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 quantities 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 (J.M. Carter, unpublished observations).

Finally, pin and indexer technology may also be adapted to other biological and biochemical investigations that utilize a microtiter plate and repeated, pseudo-random addressing, including lymphocyte proliferation, receptor binding, cytotoxicity, viral plaques, enzyme kinetics, and other procedures often performed in clinical chemistry and microbiology laboratories. It seems reasonable to expect that, as this methodology becomes more widely used and accepted, even more applications will present themselves. ///

References

1. DiPasquale, A., Bray, A.M., Geysen, H.M., Valerio, R.M., and Wang, J. 1991. Proceedings of the sixteenth annual conference on protein structure and conformation. Lorne, Australia.
2. Geysen, H.M., Meleon, R.H., and Barteling, S.J. 1984. *Proc. Natl. Acad. Sci. USA* **81**:3998.
3. Geysen, H.M., Rodda, S.J., and Mason, T.J. 1986a. Synthetic peptides as antigens. *Ciba Found. Symp.* **119**:130.
4. Geysen, H.M., Rodda, S.J., and Mason, T.J. 1986b. *Mol. Immunol.* **213**:709.
5. Hagblom, P., Segal, E., Billyard, E., and So, M. 1985. *Nature* **15**:156.
6. Jarboe, D.L., Cassels, F.J., Kodak, J.A., Nauss, J.L., Boedeker, E.C., Deal, C.D., Carter, J.M., and Reid, R.H. 1992. *J. Immunol.* (submitted).
7. Karabatsas, M., Seldon, M.A., Napier, M.A., and Geysen, H.M. 1991. Proceedings of the sixteenth annual conference on protein structure and conformation. Lorne, Australia.
8. McGregor, D., Triantafyllou, B., Tribbick, G., and Geysen, H.M. 1990. Proceedings of the fifteenth annual conference on protein structure and function. Lorne, Australia.
9. Stover, C.K., Marana, D.P., Carter, J.M., Roe, B.A., Mardis, E., and Oaks, E.V. 1990. *Infection and Immunity* **58**:2076.
10. VanAlbert, S., Lee, J., Lyon, J.A., and Carter, J.M. (1991) Amino acid indexer for synthesis of Geysen peptides. Patent application #07/679,990 (pending). For further information contact CRACO, Inc., Biomedical Division, 2519 Meredith Drive, Vienna, VA 22181-4037.
11. van der Zee, R., van Eden, W., Meloen, R.H., Noordzij, A., and van Embden, J.D.A. 1989. *Eur. J. Immunol.* **19**:43.
12. Wang, J., DiPasquale, A., and Geysen, H.M. 1991. Proceedings of the sixteenth annual conference on protein structure and conformation. Lorne, Australia.