

Protocol list

Multiple peptide synthesis on pins and peptide scanning (“Pepscan”)

- Peptide synthesis on pins 24
- ELISA using pin-bound peptides 27
- Optimization of secondary antibody conjugate concentration 29
- Regeneration of pins 29
- Epitope mapping with biotinylated peptide ELISA 30

Use of pin peptides in human PBMC proliferation and cell culture assays

- Preparation of peptides and antigens for proliferation assays 59
- Defibrination of whole human blood using glass beads 64
- Preparation of human PBMC from whole blood 65
- Preparation of lymphocytes from whole blood using differential centrifugation on a density gradient 65
- Testing pooled serum to use in T cell epitope mapping assays 69
- Method 1 of the ALLOC algorithm 77
- Method 2 of the ALLOC algorithm 77
- Assay set up procedure 83

Molecular mapping of antigenic and immunogenic epitopes

- Solid phase indirect enzyme-linked immunosorbent assay (ELISA) for peptide antigenicity by simple adsorption of peptides on microplates 93
- Identification of immunogenic T cell epitopes *in vitro* 94
- Immunization using synthetic peptides representing identified epitopes 96
- Detection of *in vivo* anti-peptide antibody production using indirect ELISA 97
- Inhibition ELISA for mapping of the antibody binding site using chimeric peptides and shortened peptides with truncations of the amino or carboxy terminus for mapping of the binding site 99
- Identification of precise T cell epitopes by using a T cell proliferative assay 100
- Cytokine-specific ELISA 100

Indirect methods of CTL epitope identification using synthetic peptides

- Methods for identifying CTL: assays for lytic activity 109
- Methods for identifying CTL: assays for lymphokine secretion 110
- Peptide-binding to class I MHC: Techniques using soluble class I MHC: assembly assay 113

PROTOCOL LIST

- Peptide-binding to class I MHC: Techniques using live cells: surface stabilization of temperature-induced class I MHC molecules 116
- Methods for predicting class I MHC binding sequences 119
- Preparation of immunoaffinity columns 124
- Lysis of cells 125
- Immunoaffinity isolation of class I MHC-peptide complexes 126
- Peptide extraction 127
- Reversed-phase HPLC (RP-HPLC) separation of extracted peptide material 127
- Identification of fractions containing peptide antigen 128
- Preparation of RNA and cDNA 129
- Transfection of COS-7 or HeLa cells 130

Design, synthesis, and characterization of peptoid oligomers (molecular mimetics)

- Resin conditioning prior to synthesis 148
- Synthesis of simple dipeptoid 149
- Cleavage of dipeptoid sequence from resin 150
- Synthesis on Chiron Pins of a dipeptoid comprised of β -amino acid esters as the side chains 152

Immunization strategies

- Immunization 161
- KLH conjugation 163
- Hybridization and subsequent propagation of hybridomas 165
- Monoclonal antibody purification 170
- Horseradish peroxidase (HRP) conjugation of immunoglobulins 171
- Enzyme-linked immunosorbent assay (ELISA) 173
- Haemagglutination 177
- Slot-blotting 179
- HRP immunocytochemistry 181
- Immunofluorescence immunocytochemistry 184
- Carbamylation 186
- UV irradiation 186
- Mild reduction and alkylation 186
- Pepsin digestion 187
- Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) 189
- Electroblotting (tank transfer system) 191
- Electroblotting (semi-dry transfer system) 192
- Probing of membranes 193
- Detection: enzyme-substrate deposition 193
- Detection: radioactive 194
- UV absorption 195
- Fluorescence 196

Measuring carbohydrate-protein interactions

- Haemagglutination reaction 205
- Haemagglutination inhibition (HAI) 206
- Preparation of 'Glycoplate' (**38**): covalent coupling of carboxy-functionalized spacer galabioside (**37**) to a microtitre plate functionalized with secondary amino groups 209
- Competitive ELISA with HB101/pPAP5 bacteria 211
- Competitive ELISA with the periplasmic PapD₉₆PapG₉₆ pre-assembly complex 213

Case study: identification of dominant peptide epitopes

- Affinity purification of antibodies from immunized rabbits for screening of phage peptide library 232
- Panning of phage display peptide library 234
- Phage ELISA 237
- Pepscan ELISA 242
- Construction of a scFv phage display library 245
- Screening of a scFv phage display library 248
- ScFv phage ELISA 250
- Production of soluble scFv fragments from pHEN.1 252

General approaches to mutagenesis in epitope mapping techniques in site-directed mutagenesis

- The Kunkel method of site-directed mutagenesis: template preparation 259
- The Kunkel method of site-directed mutagenesis: primer extension 261
- Megaprimer PCR 267

Chapter 2

Multiple Pin Peptide Scanning (“Pepscan”)

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1 Introduction

The identification of regions of interaction between an antigen and antibody is an important area of research in molecular immunology. The correct identification of epitopes not only allows one to map where the important regions of an antigen are located in its three-dimensional structure but more importantly, it is instrumental in the diagnosis and prognosis of disease, in immunointervention, and in the design of drugs and generation of vaccines where specific peptides are used to induce antibodies to pathogenic organisms.

Various methods have been used to identify epitopes, these include predictive algorithms, which identify possible epitopes, these are then synthesized and screened. One of the prerequisites to use algorithms for epitope prediction is that the amino acid sequence of the protein needs to be known and the major drawback is that the predictions are not always accurate.

Enzymatic and chemical cleavage using cyanogen bromide have also been used to generate peptide fragments which are then screened to locate epitopes on the fragments. Once the fragment is identified, it is sequenced and the epitope characterized. The amino acid sequence of the protein is not required initially for this method.

A popular method for identifying epitopes is epitope mapping using synthetic overlapping peptides spanning the entire sequence of the protein of interest.

The synthesis of peptides can be carried out using resin-based technologies where the peptide is synthesized on the resin and then cleaved. Using this conventional system of protein synthesis, milligram amounts of protein are produced but only one peptide can be synthesized at a time. The bottleneck in epitope analysis therefore arose at the peptide synthesis level. For an epitope mapping method the two considerations of importance were that the method would allow the parallel synthesis of a large number of peptides and subsequent testing of large numbers of samples.

The ability to identify epitopes speeded up enormously by a novel development in solid phase peptide synthesis and testing known as Pepscan technology developed by Geysen *et al.* (1). The Pepscan method utilizes solid phase synthesis

of peptides on polystyrene pins. In this method (1), multiple peptides are synthesized essentially by Merrifield's solid phase protein synthesis method (2) on specially designed polystyrene pins in a 9×12 , 96-well microtitre plate format which are then screened against sera or antibodies of interest using ELISA to identify linear B cell epitopes. This novel development of solid phase peptide synthesis on plastic pins as the support for the synthesis in a 96-well format has permitted easy and efficient synthesis and subsequent screening of large numbers of peptides by ELISA.

The original Geysen method (1) where the peptides were pin-bound has been adapted for T cell assays by developing chemistries that allow cleavage of peptides from the pins (3). Hence, Pepscan can be used for both B and T cell epitope mapping by its versatility in the synthesis of both pin-bound and cleaved peptides. The peptides can also be further modified to incorporate different endings, for example, biotinylated for use in solution phase B cell epitope mapping. The basic technology of the multipin peptide synthesis has also been adapted to allow multi-milligram quantities to be synthesized without sacrificing the advantages of the 96-well format (4). Several other methods of simultaneous multiple peptide synthesis have been reported (5-7).

In this chapter, I shall describe synthesis of peptides, methods for epitope mapping using pin-bound and cleaved biotinylated peptides, applications of Pepscan, use of 'T' (tea-bag) peptide synthesis, synthesis and screening of peptides on membranes and peptide libraries.

1.1 Brief outline of Pepscan

The amino acid sequence of the protein is required. The amino acid sequence of the protein is determined mostly through the DNA sequence. Linear peptides of a given length spanning the entire amino acid sequence of the protein are synthesized on pins which are attached to a plastic support. The pins are incubated with sera or antibodies of interest. The pins are then incubated with species-specific secondary antibody conjugated with enzyme. The complex is visualized using an appropriate substrate and the colour developed read in an ELISA reader. Pepscan has the potential therefore to identify linear epitopes on an antigen (8).

For T cells, peptides are synthesized on pins then cleaved and used in T cell assays for identification of Th cell epitopes and cytotoxic T cell epitopes with Th clones, cytotoxic T cell clones, and PBMC (8).

2 Solid phase peptide synthesis

Before going on to multiple synthesis on pins, I shall describe briefly peptide synthesis on solid phase. In solid phase peptide synthesis (SPPS), peptides are built on an insoluble polymeric support by the sequential addition of amino acids.

Two types of chemistry are available for peptide synthesis, these are the t-Boc and the Fmoc chemistries which use butyloxycarbonyl (t-Boc) and fluorenylmethyloxycarbonyl (Fmoc) as the α -N amino protecting groups.

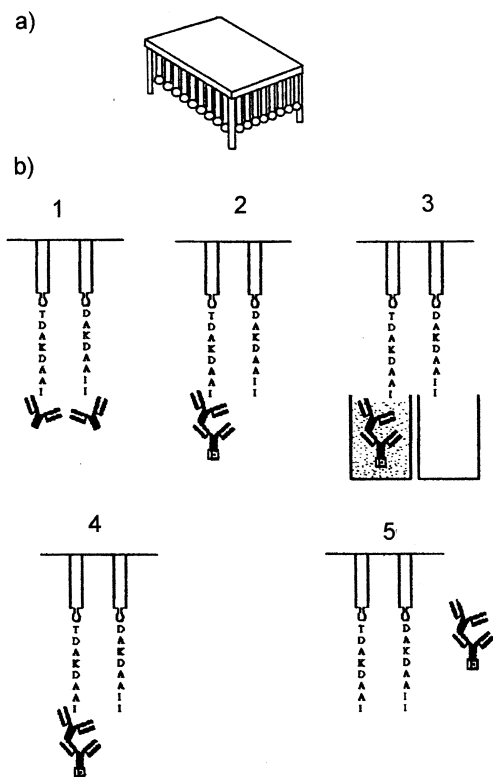


Figure 1 Pepsan assay. (a) Figure showing the plastic block with pins attached to it in a 96-well format. (b) Diagram showing the steps in the Pepsan assay using pins. Step 1: binding of primary antibody (monoclonal or polyclonal antibody or serum) to the peptide on the pin. Step 2: binding of anti-species enzyme-conjugated secondary antibody to the primary antibody. Step 3: visualization of enzyme–antibody complex by immersion of pins in substrate solution. Step 4: regeneration of pins. Removal of antibody complex from the peptide on the pin by sonication. Step 5: regenerated pins ready for the next assay.

Schematically, SPPS is represented in *Figure 2*, showing the attachment, de-protection, chain elongation, and cleavage steps in the synthesis of the peptide.

2.1 Attachment

The peptide chain is synthesized on an insoluble polymeric support by the attachment of the first Boc/Fmoc α -N-protected amino acid via the C-terminus. Synthesis takes place from the C-terminus to N-terminus of the peptide. The linker can vary according to whether a peptide acid or amide is being synthesized. The side chain amino acid also needs protection to prevent unwanted polymerization or side chain reactions. Various side chain protecting groups are used depending on the functional group of the amino acid and the type of chemistry used for the synthesis, i.e. Boc or Fmoc. For example, for serine, threonine, and tyrosine, the functional group being protected is -OH, the protecting group used is t-But for Fmoc chemistry, and benzyl for Boc chemistry.

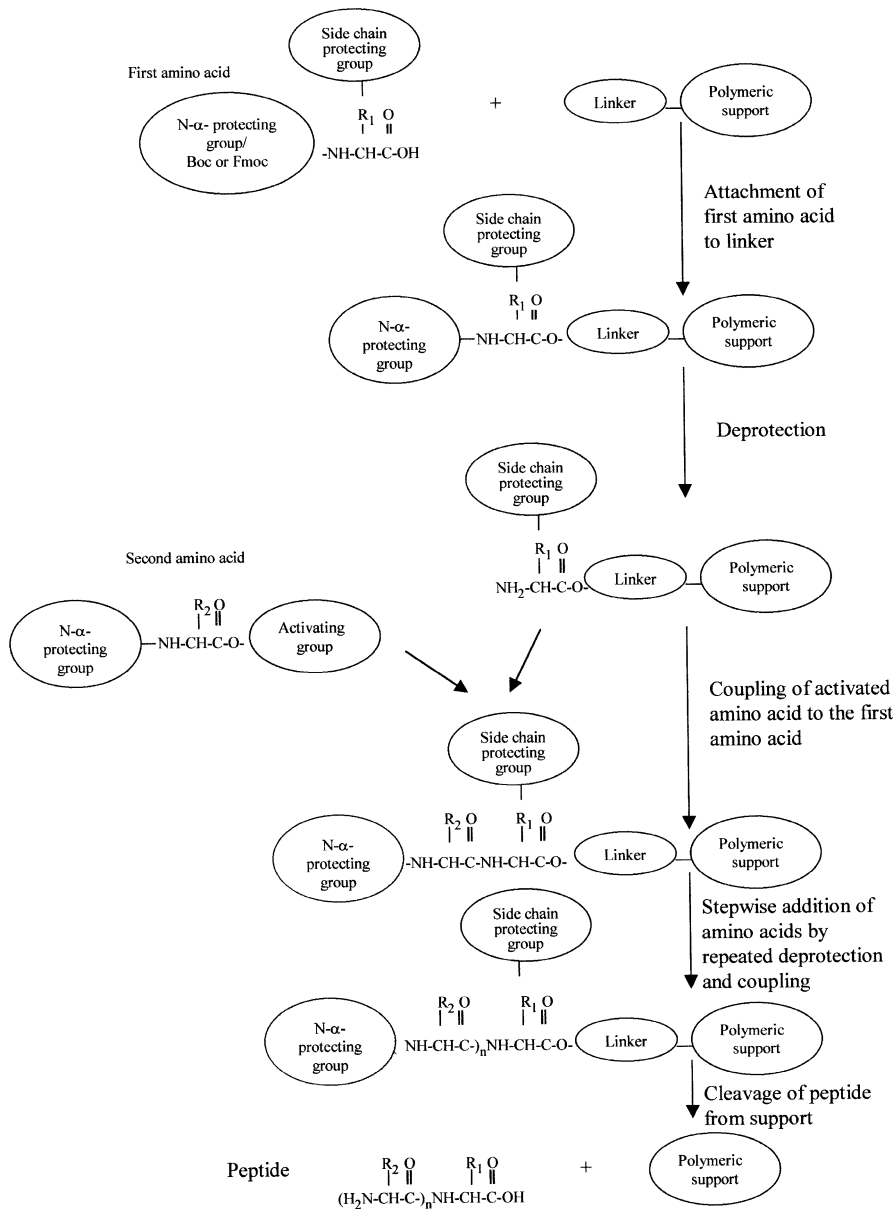


Figure 2 Diagram of solid phase peptide synthesis showing the attachment, N - α -deprotection, coupling, repeated deprotection, and coupling steps in peptide synthesis, and final cleavage of peptide from the support.

2.2 Deprotection and coupling

The α -N-protecting group is removed before the next amino acid is added. Cleavage of the Boc protecting group requires TFA whereas removal of the Fmoc protecting group requires piperidine.

After deprotection the next protected, activated amino acid is coupled to the first amino acid forming a peptide. The coupling process requires the incoming amino acid to be activated at the α carboxyl group so that it can react with the amino group of the growing peptide chain. For resin deprotection, coupling, and washing, dichloromethane and dimethyl formamide are used.

2.3 Elongation of peptide chain

Sequential amino acids are added by repeated deprotection and coupling steps until the required length is achieved.

2.4 Cleavage

The peptide is then cleaved from the resin. Final cleavage of the peptide from the resin and side chain deprotection for Boc chemistry requires strong acid—

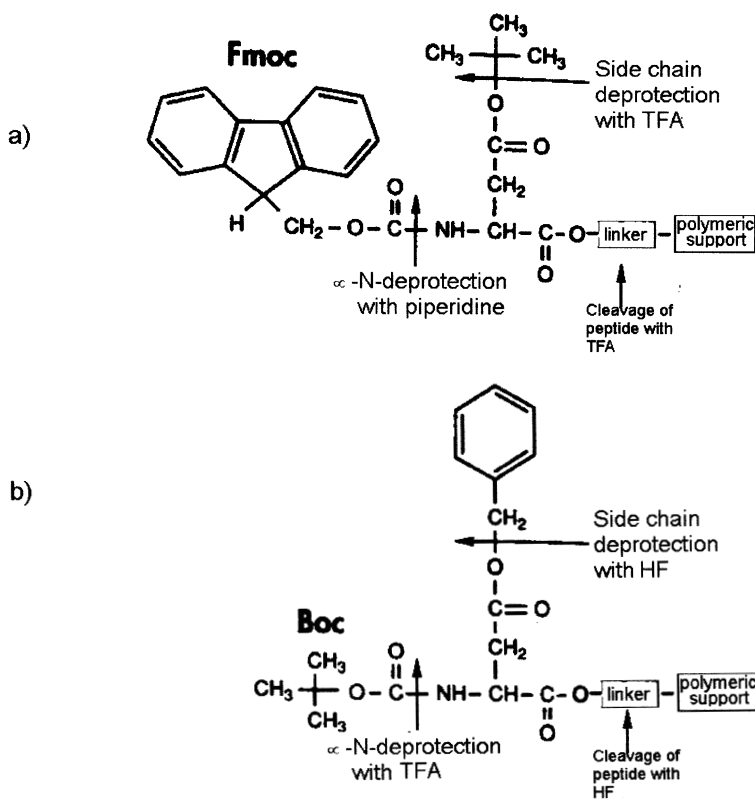


Figure 3 Cleavage of side chain and α -N-deprotecting groups and final cleavage of peptide from the support. (a) Cleavage strategies for Fmoc chemistry. (b) Cleavage strategies for Boc chemistry.

hydrofluoric acid (HF) or trifluoromethanesulfonic acid whereas Fmoc chemistry uses TFA. In general then, Fmoc chemistry is gentler in terms of use of mild base treatment during Fmoc group deprotection and TFA is only used in the final cleavage. Boc chemistry utilizes rather harsh conditions, TFA is used in the Boc group deprotection which is a repetitive cycle and during the final cleavage and deprotection of the final Boc, HF is used. Therefore, Fmoc chemistry is preferred as it uses milder conditions.

3 Multiple peptide synthesis on pins

There are some excellent protocols on multiple peptide synthesis and synthetic peptides in epitope mapping mainly by Rodda, Tribbick, Maeji, and Bray (8–12).

Custom-made peptides can also be purchased from various suppliers. Chiron Technologies Pty supply custom-made peptides or customers can purchase peptide synthesis kits with full instructions for the users to synthesize their own peptides. According to Geysen *et al.* (1) peptide synthesis on pins is simple and does not require an extensive knowledge of organic chemistry.

The kind of peptides one uses depends very much on what assay the peptides are being used for; for B cell epitope mapping, both pin-bound and cleaved peptides can be used, in the latter case it is preferable to use biotinylated cleaved peptides.

For T cell epitope mapping, cleaved peptides are used, again the peptide endings can vary depending on whether one is looking at Tc cells, Th, cells or T cell clones.

Table 1 shows the different kind of kits that are available from Chiron Technology.

In all cases, the initial synthesis is on pins and then the peptides can stay pin-bound (non-cleaved) or cleaved as shown in Figure 3. The pin technology uses the milder Fmoc chemistry.

3.1 Pins

The pins are polyethylene rods onto which 6% acrylic acid has been polymerized as polyacrylic acid. The pins are mounted on a plastic holder with a 8×12 format similar to the ELISA plates, thus facilitating not only the synthesis of multiple peptides but also the screening of peptides for epitopes.

3.2 Choice of peptide length

Identification of sequential epitopes can be achieved by using a complete set of overlapping peptides spanning the entire amino acid sequence of the antigen.

Detailed studies of antibody binding peptides have led to the general consensus that the size of sequential epitopes is between five to eight residues long (1, 13). To scan all the peptides through a protein, one needs to consider the cost and effort in making and scanning all the peptides as well as ensuring that no epitopes are missed due to insufficient overlap between successive peptides.

Table 1 Kits for peptide synthesis using the pin technology (9, 10)

Kit name	Amount of peptide	Peptide produced	Used for (10)
Non-cleavable peptides			
Non-cleavable multipin peptide synthesis kit (NCP)	50 nmol	N-peptide-linker-pin ^a	Antibody epitope scanning using pin-bound peptides
Cleavable peptides			
Cleavable peptide kit, diketopiperazine group at the C-termini (DKP)	1 μ mol	N-peptide-DKP ^b	(i) Producing biotinylated peptides for antibody scanning (ii) Th cell epitope scanning
Cleavable peptide kit, glycine acid or amide at the C-termini (GAP)	1 μ mol	N-peptide-glycine acid/amide ^c	Tc cell epitope scanning
Multipin multiple peptide synthesis kit (MPS)	7–8 μ mol	N-peptide-acid ^c	General peptide synthesis
Multipin multiple peptide synthesis kit (MPS)	7–8 μ mol	N-peptide-amide ^c	General peptide synthesis

^a Acetyl group at the N-termini.

^b Can have biotin group at the N-termini.

^c Can have acetyl, biotin, or free amine at the N-termini.

One approach would be to make overlapping octapeptides stepping through the sequence one residue at a time, i.e. octamers offset by 1. Therefore for a protein of n residues, one would require the synthesis and testing of $(n - 7)$ octapeptides (1). A protein of 400 residues would require the synthesis and screening of $(400 - 7)$ 393 octamers.

However, as a starting point Rodda *et al.* (8) suggest that a set of overlapping peptides should not drop below 8-mers as the longest peptide length for which all possible sequences are present in the scan such that 9-mers offset by 2, 10-mers offset by 3, 11-mers offset by 4, and so on up to 20-mers offset by 13 can be used. The number of peptides that need to be synthesized is affected by the peptide length and offset, e.g. for a protein of 100 amino acids, synthesis of 8-mer peptides offset by 1 would require 93 peptides, whereas synthesis of 9-mers offset by 2 would reduce this number to 47, and for 10-mers offset by 3, the number of peptides required to be synthesized would be 31. For 11-mers, offset by 4, the number of peptides would fall to 24, approximately a quarter of those for 8-mers offset by 1 (14).

Similar considerations apply to designing a peptide set for T cell epitope mapping, i.e. peptide length, offset, quantity, and purity of peptide. Solubility of the peptides is important, hence the peptides need to be screened for hydrophobicity, this can be done using various computer programs and the peptide length adjusted to make the peptides more soluble (8).

For T cell epitopes the maximum continuous sequence length for which no sequence within the protein is missing is 9 residues or more. For Th epitopes the peptide set consists of 16-mers offset by 4, hence the number of peptides needed to be synthesized is around a quarter of the number of amino acid residues in

the protein. For preliminary Tc epitope location use 12-mers and upwards, however for accurate Tc mapping, 9-mers offset by 1 are used and in this case, the number of peptides approximates the number of amino acids in the protein.

Naturally processed Tc peptides are usually 8, 9, or 10 amino acids whereas naturally processed Th peptides are between 13–18 amino acids.

Protocol 1

Peptide synthesis on pins^a

Having decided on the type of peptide ending, order the appropriate pin synthesis kit. The synthesis kit from Chiron comes with pins, plastic baths, reaction trays, technical manual, software, pins with pre-synthesized control positive and negative peptides, and corresponding monoclonal antibody for the positive control.

Equipment and reagents

Make sure all reagents are AR or the highest quality.

- Computer
- Computer controlled display unit called Pin Aid which aids in the dispensing of reagents (Chiron or its distributors): consists of an 8×12 array with LED display which lights up the wells that require a particular activated amino acid solution to be added at the coupling step in peptide synthesis
- Pipettors (solvent resistant)
- Activating agent (diisopropylcarbodiimide, DIC), catalyst (1-hydroxybenzotriazole, HOBT) (Sigma, Fluka, Merck, Aldrich)
- Fmoc protected amino acids with the appropriate side chain protection (Chiron Mimotopes, Bachem, Novabiochem, Sigma)
- Acetic anhydride, diisopropylethylamine
- Piperidine for deprotection (Aldrich, Fluka, Merck)
- Solvents: dimethyl formamide (DMF) tested to have only low levels of amine, methanol (Merck, Fluka, Sigma, Aldrich)
- Indicator: 0.7% bromophenol blue in DMF (Merck)
- Side chain deprotection: TFA (Merck, Aldrich, Fluka)
- Scavengers and reducing agents: ethanedithiol (EDT), anisole, mercaptoethanol (Merck, Aldrich, Fluka)
- Biotin or long chain biotin if biotinylation of peptides is required
- Appropriate solvents for cleavage or washing dried peptide include HPLC grade acetonitrile, ether, petroleum ether

A. Peptide synthesis

- 1 Select appropriate kit type and design of peptide set.
- 2 Generate a peptide synthesis schedule by entering the peptide sequence into the kit software. This will produce a printed schedule showing:
 - (a) The layout of the peptides including controls on each 'block' which holds 96 pins.
 - (b) The location and amount of amino acids to be added for each coupling cycle of synthesis and the amount of catalysts and activation reagent required.

Protocol 1 continued

- 3 Following the synthesis schedule produced in step 2, put the required pins onto the holder. All 96 pins may not be required depending on the number of peptides being synthesized and whether the peptides are the same length or not.

B. Deprotect the pins in readiness to accept the first amino acid

- 1 To Fmoc deprotect the α amino acid on the pins, add 20% piperidine/DMF to the bath and place the block of pins in the bath ensuring the tips are covered. Cover and leave for 20 min at room temperature (RT).
- 2 After 20 min, wash the pins in DMF for 2 min, air dry for 2 min followed by a 2 min methanol wash, and air dry for 2 min.
- 3 Repeat methanol washes three times and air dry the block.
- 4 Prepare and activate the required amount of each activated Fmoc protected amino acid.
- 5 Put the required volume of each amino acid into the correct wells of the reaction tray, checking the computer generated synthesis print-out for the well positions. It may help if two people performed this step, one person reads the position and the other dispenses. Alternatively, use the Pin Aid if available, the appropriate well position is shown by the lit up LEDs.

C. Coupling

- 1 Lower the pins into the solutions in the wells of the reaction tray. Make sure the block of pins is in the right orientation. Incubate for 2 h or longer at 20–25°C in a sealed polythene bag or polyethylene box. Coupling is complete when the blue staining from the reactive pin surface disappears.
- 2 Wash the pins in DMF and methanol and air dry.
- 3 Start the next cycle of amino acid addition. Repeat part B, and part C, steps 1 and 2 until all the peptides are synthesized.
- 4 Deprotect the final Fmoc amino acid by following part B and part C, steps 1 and 2. Then proceed to part F for side chain deprotection unless N-terminal acetylation or biotinylation are required. For pin-bound peptides, N-terminal acetylation is recommended (9). Carry out N-terminal acetylation according to part D and biotinylation according to part E.

D. N-terminal acetylation (9)

- 1 Place the pins in a mixture of DMF/acetic anhydride/diisopropylethylamine in a ratio of 193:6:1 in 200 ml.
- 2 Leave for 90 min at RT.
- 3 Wash the pins in a methanol bath and air dry.
- 4 Proceed to part F.

Protocol 1 continued**E. N-terminal biotinylation (9)**

- 1 Make a 125 mM solution of biotin in DMF and activate it with 10 × concentrate solutions of activating agent (158 mg DIC in 1 ml DMF) and catalyst (192 mg HOBt in 1 ml DMF) in a ratio of 80 biotin :10 DIC:10 HOBt (by vol.).
- 2 Dispense appropriate amount of activated biotin (150 μl for 1 μmole scale and 450 μl for 5 μmole scale) into the wells in the reaction tray and incubate the pins (after part C) in the tray for 2 h or more.
- 3 Wash the pins in methanol.
- 4 Proceed to part F.

F. Side chain deprotection and cleavage

- 1 Immerse the pins in TFA/ EDT/ anisole solution (38:1:1) for 2.5 h at RT. For 1 μmole scale and 5 μmole scale use 0.3 ml and 1.5 ml respectively per pin. At this stage the side chain deprotection also simultaneously cleaves off the peptides from the pins on the MPS kits while for the non-cleavable peptides (NCP), DKP, and GAP kits, the peptides are still attached on the pins. For MPS kits go to part F, step 5.
- 2 For the DKP, GAP, and NCP kits, wash the pins in methanol (0.5% acetic acid in 1:1 methanol/water) for 1 h. Follow with two more washes in methanol. The NCP peptides (non-cleavable) on pins are ready for testing.
- 3 Cleavage of peptides from the GAP kit. Cleave the peptides directly into a rack of 1 ml polypropylene tubes. Add 0.7 ml of 0.1 M NaOH (or 0.1 M NaOH in 40% (w/v) acetonitrile to solubilize hydrophobic residues) to each tube and place the pins in the tubes for 0.5–1 h. Sonication reduces the cleavage time. Neutralize the peptides immediately after cleavage. The peptides are ready for use.
- 4 Cleavage of peptides from DKP kit. Cleave the peptides in a neutral or alkali buffer. Immerse each pin in 0.8 ml of cleavage buffer like 0.1 M sodium phosphate pH 7.6 or 0.05 M Hepes pH 7.6 (40% (w/v) acetonitrile/water can be included to solubilize hydrophobic residues) for 16 h or 1 h if the pins in buffer are sonicated during cleavage. After cleavage, the peptides are ready for use.
- 5 Further processing of cleaved peptides from the MPS kits from part F, step 1. The cleaved peptides are in TFA solution. Dry down the TFA solution containing the peptide to ~0.1 ml using a gentle stream of dry nitrogen in a good chemical fume hood. Extract each peptide with 8 ml of cold ether/petroleum ether/mercaptoethanol^b in a ratio of 1:2:0.003 for 30 min. Spin at 3200 g in a flame-proof centrifuge and collect the precipitate by decanting the supernatant. Wash the precipitated peptide with 4 ml of cold 1:2 ether/petroleum ether^b and collect precipitate as above. Dry the pellet with a gentle stream of nitrogen.

^a Modified from ref. 8 by kind permission from Oxford University Press.

^b Take great care as these are highly flammable.

In a conventional day three amino acid couplings can be done, making it possible to synthesize a set of 15-mer peptides in two weeks.

Once the peptides have been synthesized, the non-cleavable peptides on pins are stored dry in the refrigerator, in the presence of desiccant, the peptides are stable for months (9). Cleaved peptides, can be stored frozen in aliquots at -20°C or lower or as lyophilized powders.

4 Testing of antibody epitopes

4.1 Pin-bound non-cleavable peptides

Pin-bound peptides have a peptide coating of around 50 nmol/pin enough for 50 tests. They can be used with monoclonal and polyclonal antibodies and allow high sensitivity of detection of antibody epitopes. However, one block of peptides can only be used for one test/day after which the pins have to be regenerated (see protocol for regeneration). The reproducibility of the assay decreases with the use of the pins and the quality of synthesis of the peptide cannot be verified easily although control peptides are included in the synthesis cycle. Rodda *et al.* (8) recommend pin-bound peptides be made in duplicate to test for reproducibility and to allow for test and control sera to be run in parallel if required. Although control peptides are synthesized to check the efficiency of synthesis and purity, the individual pin-bound peptides cannot be checked for purity, therefore once a peptide has been identified as an epitope, it should be re-synthesized by conventional methods and screened to validate the result.

Protocol 2

ELISA using pin-bound peptides^a

Equipment and reagents

- Non-cleavable peptides on pins
- Epitope mapping kit: includes a control antibody which binds the control peptide PLAQ and does not bind the negative control peptide with the sequence GLAQ
- 96-well flat-bottom microtitre plates
- Shallow plastic baths when all the pins are incubated in the same solution
- Horizontal shaker run at 80–100 r.p.m.
- Elisa reader (e.g. Anthos, Lab systems Multiskan)
- Computer and software for storing and analysing data
- Phosphate-buffered saline (PBS)
- PBST: PBS containing 0.1% Tween 20
- PBSTA (sample diluent): PBST with 0.1% sodium azide
- Substrate solution: 50 mg ABTS (Sigma tablets, Cat. No. A-9941) in 100 ml citrate phosphate buffer (citric acid anhydrous 2.16 g and sodium phosphate 4.59 g disodium hydrogen orthophosphate 12 H₂O in 500 ml), pH 4.0 and 30 μl of 30% hydrogen peroxide
- Conjugate diluent: PBST with 1% sheep, goat, or rabbit serum (v/v), depending on the source of the antibody and 0.1% sodium caseinate (w/v)
- Anti-species horseradish peroxidase (HRP)-conjugated IgG antibody (Sigma, Dako)

Protocol 2 continued**Method**

- 1 Check that there is no binding of conjugate to the pin-bound peptides, i.e. conjugate blank test, follow step 2 and then 5–8.
- 2 Blocking of the peptide pins. Fill the wells of a microtitre plate with PBST^b (0.2 ml/well) and place the pins into the plate. Incubate the pins in the PBST for 1 h at RT. This step reduces non-specific binding to the pins.
- 3 Incubation with primary antibody. Dilute the test sample (monoclonal antibody, polyclonal antibody, or serum sample) in PBSTA,^c dilute hyperimmune sera 1/5000 and patient and control sera at 1/000 to start with as it is best not to overload the pins with excess antibody. Add 0.2 ml of diluted sample per well in a microtitre plate. Take the pins out of the blocking solution, shaking off any excess, and place the pins in the sample solution in the plate. Incubate overnight at 4 °C on a horizontal shaker at 100 r.p.m.
- 4 Wash in PBS four times.
- 5 Secondary antibody. Dilute the secondary antibody in conjugate diluent using the predetermined dilution arrived at in *Protocol 2a* and place 0.2 ml/well in a microtitre plate. Incubate the pins in the secondary antibody for 1 h at RT on a shaking platform at 100 r.p.m.
- 6 Development of colour. Wash the pins three times in PBST, and finally wash the pins once with PBS to remove traces of Tween from the pins. Place substrate solution in a microtitre plate (0.2 ml/well) and incubate the pins in the substrate for 30–60 min.
- 7 Read the absorbance of the coloured product in the microtitre plate at 405 nm using a reference filter at 492 nm. The reaction stops as soon as the pins are removed from the substrate, and it re-starts as soon as the pins are put back in the substrate. The pins can be reinserted in the substrate. Store the data for further analysis (Section 5).
- 8 Regenerate the pins (*Protocol 3*).

^a Modified from ref. 8 by kind permission from Oxford University Press.

^b Rodda *et al.* (8) use PBST for blocking and PBSTA for sample dilution. Worthington and Morgan (14) use PBS, 0.1% Tween containing 1% ovalbumin, and 1% BSA as blocking buffer, sera diluent, and conjugate diluent. Follow manufacturer's instructions.

^c Avoid azide in any solutions for horseradish peroxidase as HRP is sensitive to azide.

The optimum concentration of the conjugate is determined by assaying with different concentrations of the conjugate against different concentrations of the test antibody (14). The sensitivity of the conjugate will tend to plateau with increasing concentration (12).

The antibody complexed to peptide needs to be removed from the pins before the next assay can be performed on the same set of pins.

Protocol 2a

Optimization of secondary antibody conjugate concentration^a

Equipment and reagents

- See *Protocol 2*
- Test antibody, 0.1 M bicarbonate buffer pH 9.6

Method

- 1 Coat a microtitre plate with test antibody serially diluted (1/50–1/10 000) in bicarbonate buffer. Incubate at room temperature for 1 h.
- 2 Wash plates three times in PBST.
- 3 Add blocking buffer and incubate for 1 h at RT.
- 4 Take off the blocking buffer and add serially diluted (1/100–1/10 000) secondary antibody. Incubate for 1 h at RT.
- 5 Wash three times with PBST and add substrate solution. Monitor colour development and read at 405 nm with reference filter at 492 nm.
- 6 Using this checked approach where both the test antibody and conjugate are serially diluted, take the dilution of the conjugate before a major drop in OD occurs at a coating antibody dilution where the absorbance is on scale as the working strength of the conjugate (14).

^a Modified from ref. 14 by kind permission from Oxford University Press.

Protocol 3

Regeneration of pins

Equipment and reagents

- Disruption (sonication) buffer: 0.1 M sodium phosphate buffer pH 7.2, containing 0.1% 2-mercaptoethanol and 0.1% SDS
- Sonication bath (~ 7 kW power)
- Methanol, water

Method

- 1 Sonicate the pins in sonication buffer at 60 °C for 10 min.
- 2 Wash the pins in water at 60 °C and the rinse in warm methanol.
- 3 Air dry the pins. They are now ready for use in a further ELISA test. If not using immediately, store dry in the cold.

4.2 Biotinylated peptides

The peptides are synthesized with biotin at either the carboxy or the amino terminus, a spacer is incorporated between the biotin and the peptide. The general format of the peptide is biotin-SGSS-peptide, SGSS being the spacer. The C-terminal can be amidated or have a diketopiperazine group.

Biotinylated peptides are synthesized as cleaved peptides and stored as lyophilized powders. Once the peptides are reconstituted and diluted they should be aliquoted and frozen at -20°C or lower to avoid degradation.

The synthesis range is $1\ \mu\text{mol}$ per pin which is enough for thousands of assays. The assays are reproducible as a fresh aliquot of peptides is used each time and many samples can be processed in one day depending on the user. The sensitivity using biotinylated peptides is lower than on pins as the density of peptide on the pin is higher. The biotinylated peptides are best captured on streptavidin coated plates to ensure uniformity of peptide on the plate. Using streptavidin to capture of biotinylated peptide requires less than $1\ \mu\text{g/ml}$ of biotinylated peptide.

Protocol 4

Epitope mapping with biotinylated peptide ELISA

Equipment and reagents

- Microtitre plates (Nunc, maxisorp F96 Cat. 4204-4)
- Computer for data analysis
- ELISA reader (e.g. Anthos)
- Multichannel pipette
- Biotinylated peptide set (biotin-SGSS-peptide-DKP)
- Streptavidin (Sigma, Cat. No. S-4762)
- Bovine serum albumin (BSA) (Sigma, No. A-7030)
- Blocking buffer:^a PBS, 0.1% Tween, 2% BSA
- Sample diluent: PBS, 0.1% BSA, 0.1% azide
- Secondary antibody: anti-species HRP-conjugated IgG (Sigma)
- Substrate buffer: as in *Protocol 2* (a ready to use TMB (tetramethylbenzidine) substrate for HRP is available from various suppliers)

Method

- 1 Coat microtitre plates with streptavidin at $5\ \mu\text{g/ml}$ ($100\ \mu\text{l/well}$). Leave in a 37°C oven till the plates are dry. (At this stage, the plates can be stored dry at 4°C for up to four weeks. Place the coated plates in a plastic bag with desiccant like silica gel and store.)
- 2 Wash the plates four times with PBST. When processing a lot of plates together it is better to use a well washer (e.g. Well Wash 4). When washing the plates manually, flood the plates with buffer, then flick the plates upside down to remove the buffer, and finally remove excess buffer by slapping the plates down onto a wodge of paper towels on the bench top.
- 3 Block the plates with blocking buffer. Dispense $200\ \mu\text{l/well}$ and incubate for 1 h at RT on a horizontal shaker.

Protocol 4 continued

- 4 Wash the plates four times as in step 2.
- 5 Reconstitute the biotinylated peptides in 200 μ l of a pure solvent like DMSO or DMF or solvent/water mixture. Dilute the required amount of peptides 1/1000 in PBS/BSA/azide. The amount of diluted peptide should be about 1 μ g/ml. A 1/5000 dilution of peptides (0.2 μ g/ml) can be used but a loss in sensitivity may occur. The dilutions are best done in numbered racked polypropylene tubes from Bio-Rad (Cat. No. 223-9390). The racks are numbered in a 9×12 ELISA format and can be racked for use with multichannel pipettes which makes it easy to dispense the diluted peptides into the corresponding wells in step 6. Aliquot the rest of the peptide solutions and freeze at -20°C or lower. Again in our experience, the reconstituted aliquoted peptides are best stored in the racked numbered tubes thus facilitating dispensing for further dilutions.
- 6 Dispense 100 μ l of diluted biotinylated peptides in duplicate into the corresponding wells and incubate for 1 h at RT on a horizontal shaker.
- 7 Wash the plates four times as in step 2. At this stage, the peptided plates can be used immediately or dried at 37°C and stored dry at 4°C . Several batches of plates can be prepared and stored for later use.
- 8 Dilute the serum/antibody to be tested in sample diluent, the dilution of the sample varies depending on the sample and the amount of antibody present. For sera from hyperimmunized animals and ascitic fluid from hybridomas start with 1/1000 while for human convalescent and control sera start with 1/500.
- 9 Add 100 μ l/well of diluted sample in duplicate. Include a conjugate blank plate, this is where the plate is processed in the same way as the sample plate but the sample is omitted and instead at this stage sample diluent added to the peptide plate. Incubate for 1 h at RT on a horizontal shaker, sensitivity can be increased by incubating overnight at 4°C .
- 10 Wash the plates as in step 2.
- 11 Add 100 μ l/well of conjugate solution diluted in blocking buffer. Incubate the plates for 1 h at RT on a horizontal shaker.
- 12 Wash the plates as in step 2. Then wash the plates twice with PBS to remove the last traces of Tween.
- 13 Prepare the substrate solution, and dispense 100 μ l/well. Incubate at RT for up to 45 min. Read at 405 nm using a reference filter at 492 nm. If using the ready to use TMB substrate solution, decant the required amount of solution in a clean container and bring it to room temperature, keeping it in the dark until use. Dispense 100 μ l/well and incubate the plate for up to 45 min at RT. A blue colour will develop. Stop the reaction with 0.5 M sulfuric acid and read the bright yellow colour produced at 450 nm with 620 nm as a reference.

^a This blocking buffer is used normally, however Rodda *et al.* (8) recommend the use of sodium caseinate as an effective blocker (1% sodium caseinate/0.1% Tween in PBS).

5 Results and data analysis

Figure 4 shows the scan for a polyclonal rabbit antiserum raised against a peptide sequence of p43, a 43 kDa protein from *Mycobacterium paratuberculosis*. The antiserum was screened against 15-mer biotinylated peptides spanning the entire sequence of p43. The antiserum used at 1/1000 dilution showed reactivity with the peptide sequence it was raised against. A parallel run using pre-immune sera was run. The same biotinylated peptides were screened against sera from Crohn's patients (Figures 5a, 5b) and non-convalescent sera from normal healthy controls (Figure 5c). A peptide sequence at the carboxy terminus of p43 was identified in Crohn's sera by this method (15).

Since not all sequences in the protein are identified as epitopes, the non-reactive sequences can act as control peptides. However, once an epitope is identified, it is better to synthesize a scrambled sequence of the epitope and test it against the sera of interest. Serum samples tend to give higher backgrounds compared to monoclonal and peptide antibodies and the interpretation of data can be difficult in terms of what constitutes an epitope and where the background cut-off should lie. A test background can be established by ranking the absorbance values and averaging the lowest 25% of the values and then adding three times the standard deviation (1, 14). All values above this cut-off are viewed as significant. This does not always work with serum samples as quite high back-

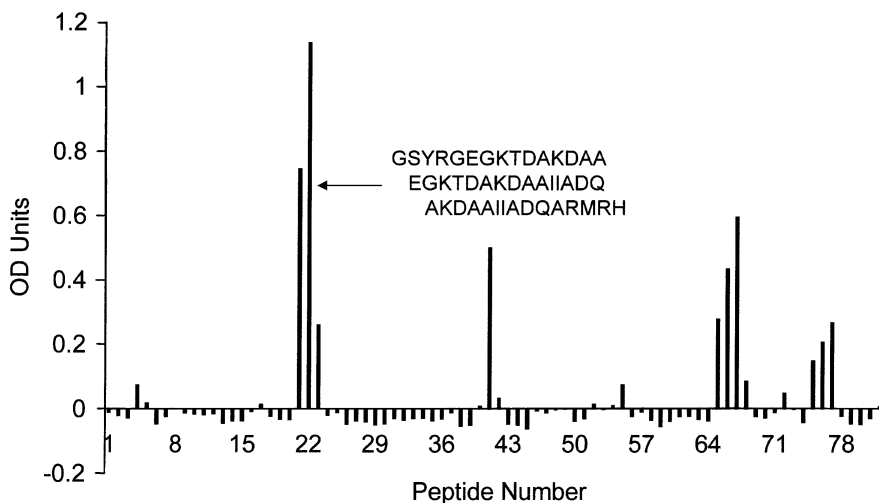


Figure 4 Scan showing the binding of an anti-peptide antibody to 15-mer biotinylated peptides spanning the entire sequence of protein p43 from *Mycobacterium paratuberculosis*. The peptide number is on the horizontal scale while the binding of the antibody is represented on the vertical scale in optical density units (OD) at 405 nm. The antibody was raised to a 18-mer peptide with the sequence, YRGEKTDAAKDAIIADQ, binding occurs at overlapping peptides 23, 24, and 25. The maximum binding is at peptide 24 which has most of the peptide sequence to which the antibody has been raised.

grounds are often obtained with some sera (cf. *Figures 5a* and *5b*). With serum samples, data can be analysed by plotting a consensus plot for the sera analysed.

- (a) The geometric mean antibody titre and range are plotted for the peptides binding antibodies from the sera tested.
- (b) A frequency profile is plotted to show the number of sera reacting with each peptide.
- (c) The reactivity of each individual serum to each peptide is plotted. In this way, epitopes masked by high background can be revealed (16).

One way to confirm binding to the sequential epitope identified is to elute the serum antibody bound to the pin by using buffers of low or high pH and testing the eluted antibody for specific binding to the peptide by standard ELISA. Tribbick *et al.* (17) have used this elution method to investigate the specificity of many antibodies in polyclonal serum.

6 Further analysis

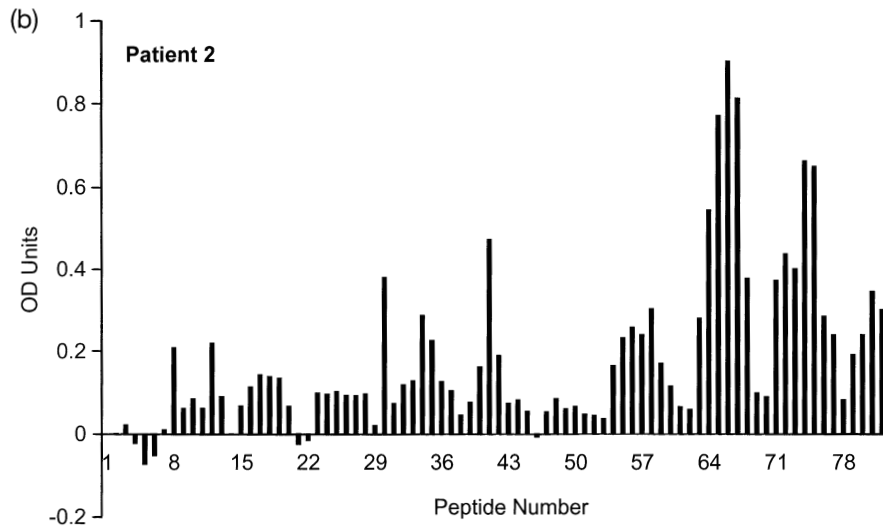
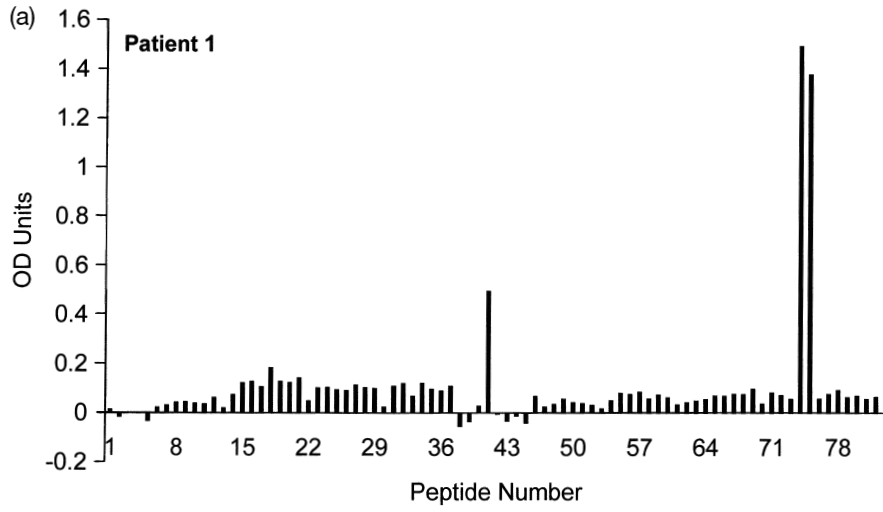
An epitope identified by a primary scan using overlapping peptides of constant length called General Net, can be further characterized by Window net and Replacement net analysis (*Figure 6*).

6.1 Window net analysis

This allows the minimal (optimal) antibody binding peptide to be defined. Peptides of different lengths representing the epitope are synthesized, the sera re-tested against the peptides, and the minimum length recognized by the antibody defined (1). Amino acid additions and deletions should be carried out at both the amino and carboxy termini.

6.2 Replacement net analysis

Replacement net analysis can be carried out to determine the amino acid residues critical to the binding. This is done by synthesizing a set of peptides comprising the core binding fragment, with each amino acid replaced in turn by the 19 amino acids. Both D- and L-amino acids can be used for replacement. A critical residue is one whose replacement abrogates binding. If binding is reduced or lost compared to the parent residue, then the parent residue is considered to contribute directly to the binding. There are many studies in the literature highlighting the effects of single amino acid changes in the epitope (18–21). Geysen *et al.* (22) using the Pepscan method identified an immunogenic epitope of foot and mouth disease virus to a resolution of a single amino acid. In this study, 205 overlapping hexapeptides were synthesized covering the total 213 amino acid sequence of VP1 of foot and mouth disease virus. Antigenic profiles were plotted



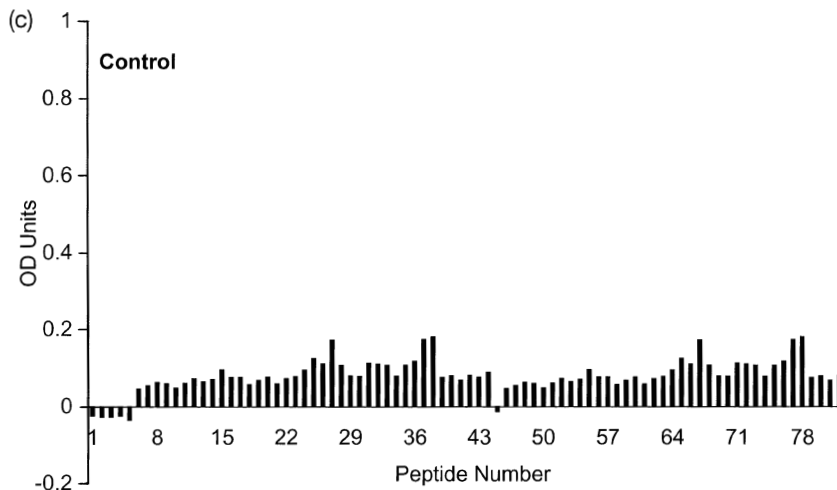


Figure 5 (see ref. 15) Scan showing the binding of two sera from Crohn's patients and one from a normal control to 15-mer biotinylated peptides spanning the entire sequence of protein p43 from *Mycobacterium paratuberculosis*. The peptide number is on the horizontal scale while the binding of the antibody is represented on the vertical scale in optical density units (OD) at 405 nm. (a) Scan of serum from patient 1, showing low background and a clear binding to two carboxy terminal epitopes. (b) Scan of serum from patient 2, showing a much higher background and less defined epitopes. (c) Scan of serum from a normal healthy control.

as peptide number against absorbance (OD). An antigenic peptide was defined as one giving an OD significantly above background level of the test. Peptides 146 (GDLQVL) and 147 (DLQVLA) were identified as epitopes. To further determine whether the epitope was the common sequence DLQVL or GDLQVLA a **replacement net analysis** was carried out. Each of the 6 amino acids in the peptide sequence 146 was substituted in turn by the 19 common amino acids. Analysis of binding was carried out with the 120 hexapeptides and the particular amino acids critical for antibody binding were determined. In the testing of replacement nets, the binding was expressed relative to the binding of the parent peptide. Leucine at positions 148 and 151 essential, while glutamine and alanine at 149 and 152 were not so important. The contribution of alanine of peptide 147 to the binding was also investigated by synthesizing 20 peptides with the complete sequence of peptide 146 with one of the 19 amino acids added to the carboxy terminus of the peptide. This detailed analysis demonstrates the identification of an epitope at high resolution. Similarly Geysen *et al.* (23) have demonstrated the use of window net and replacement net analysis for the model protein myohaemerythrin. Pepsan analysis has led to a vast number of publications on the identification of epitopes, all of which are too numerous to list. Some of the Pepsan application are given in Section 7.

Identification of epitopes within a protein region.

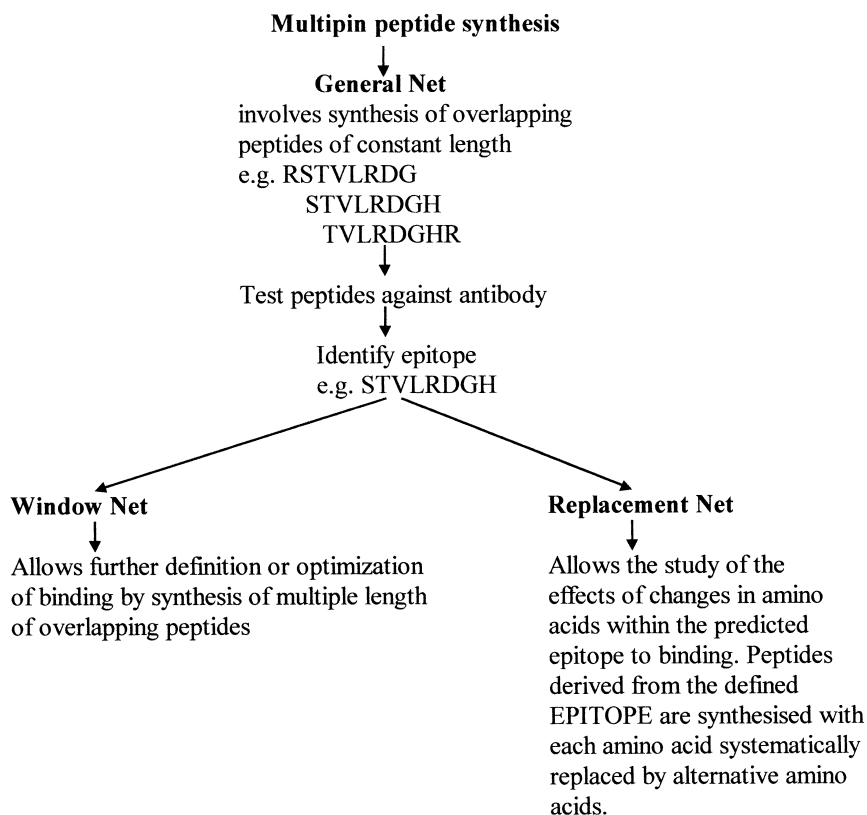


Figure 6 Identification of epitopes within a protein region.

7 Applications of Pepsan

Pepsan has been used in a wide range of applications from the study of antigen-antibody interactions (18, 22) to identification of epitopes on monoclonal antibodies (23-25) and in the study of autoantibodies (19, 26), to detection of epitopes on toxins (20, 21), viruses (27-34), bacteria (35-39), parasites (40), tumour suppressor protein (41), enzymes (42, 43), hormones (44, 45), in the design of vaccines (46-52), and even used for the location of the epitopes/sweet taste determinants (active site) of sweet tasting protein thaumatin (53). Rodda (18) studied the antibody response to myoglobin using Pepsan. A hexapeptide epitope was identified and detailed analysis of the epitope carried out by replacement net analysis showed the N-terminal leucine to be crucial to the binding of antibody to the peptide.

Using the Pepsan method, Koshy *et al.* (51) studied the binding of monoclonal antibody to chicken riboflavin carrier protein (RCP). A novel neutralization site

conserved in all known bovine respiratory syncytial virus (BRSV) and human RSV strains was identified by Pepsan analysis of monoclonal antibodies raised against fusion protein complex of BRSV. This linear conserved epitope may be a potential candidate for a peptide based vaccine which can produce neutralizing antibodies to all groups and subgroups of RSV (52).

Cross-reactive idiotypes have been detected on anti-DNA autoantibodies in SLE. Autoantibodies associated with SLE include those that bind to SmB/B' peptides. Overlapping octapeptides of SmB/B' were tested by Pepsan analysis against sera from normal and SLE patients and an octapeptide sequence identified (18). Using a set of overlapping peptides representing the VH and VL regions of monoclonal antibodies derived from mouse models of SLE and reacting these against normal and lupus mice sera, a mosaic of antibody V-region idiotypes were identified (26).

Pepsan has been used to identify a major B cell epitope within the immunodominant nucleoprotein amino subregion of the hepatitis C virus nucleocapsid protein (23).

Epitope mapping of scorpion neurotoxin II from *Androctonus australis* by the Pepsan method identified a new antigenic region for AaH II antibodies, fine analysis showed residues lysine, aspartic acid, and glycine to be important in binding (20).

Using the Pepsan method, the binding of α -bungarotoxin to Torpedo acetylcholine receptor was studied. The epitopes of 12 MCAs against the cytoplasmic side of acetylcholine receptor (AChR) α -subunit were mapped using 300 sequential peptides, a very immunogenic cytoplasmic epitope (VICE- α) was identified (25).

Epitope characterization of monoclonal antibodies against mucin protein core identified a short hydrophilic region in the MUC 1 mucin core as immunodominant in the induction of antibodies (54).

Epitope mapping of a novel fimbrial protein, Pg II from *Porphyromonas gingivalis* revealed seven immunodominant regions which reacted with sera from patients with periodontal diseases (39).

Interesting information was obtained by Pepsan analysis of horseradish peroxidase (HRP isoenzyme C). Epitopes detected in loops and folds of the HRP peptide chain with irregular shapes contained functionally important residues like Arg-38 of the active site of the enzyme and Phe-142 and 143 which form a channel allowing aromatic residues to reach the active site while amino acid residues which formed calcium binding sites did not form part of the epitope (43).

An amino acid sequence (PLITHVLPFEKINE) from alcohol dehydrogenase was studied and a pentamer (HVLFPF) binding to a monoclonal antibody identified (42). Potential vaccine candidates have been elucidated by Pepsan studies on the glycoprotein E of *Varicella zoster* virus (47).

Pepsan has also provided the methodology for the analysis of humoral responses induced by vaccination (48, 49) and the study of the fine-specificity of antibodies induced by HIV candidate vaccines (50). Pepsan provides a methodology for the design, evaluation, and selection of candidate vaccines.

8 Other systems for epitope analysis

8.1 Simple precision original test system (SPOTs)

Another method for multiple peptide synthesis and epitope analysis is SPOTs (14) available from Genoyis. In this system, up to 96 peptides are synthesized in parallel using the Fmoc chemistry on a derivatized membrane (instead of pins) and the membrane probed with antibody. The SPOTs has an 8 × 12 cm format with 96 spots allowing 96 peptides to be simultaneously synthesized (one on each spot). The membrane is pre-activated ready for the first amino acid to be added, coupling takes 15–30 minutes, after deprotection the second amino acid is added, each complete cycle taking 90 minutes. Up to a maximum of 15 cycles can be performed.

The membrane with the completed peptides can be probed with antibody. The membrane is blocked, incubated with test antibody, washed, and incubated with species-specific enzyme-conjugated secondary antibody, followed by a wash, and finally incubated with enzyme substrate. Peptide epitopes recognized by the antibody light up as spots on the membrane. As with the pins, the membrane can be cleaned and re-probed allowing for optimization of antibody dilutions. In the SPOTs the peptides can also be cleaved off the membrane, lyophilized, and used in different assays. The coupling efficiencies of three different coupling methods have also been studied by Molina *et al.* (55).

8.2 Tea-bag synthesis

An alternative to Pepscan is the use of T bag synthesis where multiple peptides are simultaneously synthesized on resin (5) or on paper discs (7) as the solid phase contained in separate solvent permeable packets similar to tea-bags. Various methods for multiple peptide synthesis on solid phase have been described. Houghten's T bag synthesis (5) method followed shortly after the peptide synthesis method on pins, this was then followed by synthesis on paper (7, 56), cotton (57–59). In 1990, Krachnak *et al.* (60) described solid phase multiple peptide synthesis on paper discs followed by determination of antibody binding to the disc-bound peptides.

8.2.1 'T' bag (tea-bag) synthesis using resin

In the 'T' bag synthesis method described by Houghten (5), resin is used as the solid phase. The method for peptide synthesis is essentially that described by Merrifield (2) and all resins commonly used for SPPS can be employed.

50–100 mg (0.2–0.8 meq/g) of standard Boc amino acid resin is placed into 15 × 20 mm polypropylene (mesh size 74 μm) pouches. Each of the bags is numbered inside with indelible black ink and the bag permanently sealed. This ensures that each bag is easily identifiable. The bags are now ready for use for simultaneous synthesis of a large number of different peptides as well as for concurrent synthesis of multiple analogues of an individual peptide. More than 100 peptides can be synthesized at a time with quantities of more than 10 mg each.

All the bags are washed, deprotected, and neutralized in a reaction vessel. The bags are then taken out and reacted with individual solutions of protected amino acids for coupling. All the bags requiring a certain amino acid can be processed in the same vessel. After coupling, the bags are put in the reaction vessel for washing, deprotection, and neutralization ready for the addition of the next amino acid. Additional cycles of the washing, deprotection, neutralization, and coupling are carried out until the synthesis is complete. Any variations, e.g. single residue replacement/omissions, shorter chain length can be carried out by removing the bag at the appropriate step completion, modified separately, and added back to the reaction vessel for completion.

After completion of synthesis, protected peptide resins still within the bags are cleaved and the peptides extracted from the resins. The average purity of the peptides obtained was 84% (70–94%). The purity of the peptides synthesized by this method was found to be good or better than the purity of peptides prepared by free resin synthesis.

Using this method a series of 247 replacement analogues of an antigenic peptide sequence (aa 98–110) of influenza HA1 and 13 controls were synthesized (in two weeks). These peptides were probed with monoclonal antibody using a standard ELISA to determine the importance of individual amino acids in antibody binding. This “T” bag synthesis approach has been used for synthesizing synthetic peptide combinatorial libraries (61, 62) using MBHA resin, t-Boc chemistry, and polypropylene mesh pouches (see Section 8.3.2).

8.2.2 T bag synthesis using paper discs

In this method (7) peptides were simultaneously synthesized on paper discs using Fmoc chemistry. Discs of filter paper (Schleicher and Schull) with 0.6 cm diameter were punched out, activated, and derivatized to get free amino groups on them. 100 of these derivatized discs (200 mg) were then placed in T bags made of 75 μm polypropylene mesh, the bags indelibly marked, and sealed. N-terminal Fmoc protected and side chain protected amino acid was then coupled on the disc, followed by side chain deprotection and washing. Cycles of coupling, deprotection, and washing were carried out until the synthesis was complete. Finally side chain deprotection was carried out. The peptides were ready to be tested for epitope mapping in solid phase immunological procedures without detachment from the paper discs. This method allows the simultaneous synthesis and subsequent immunological testing of large numbers of peptides.

Using this method, epitope mapping was carried out on the feline major allergen Fel d I. A total of 15 000 paper disc-bound peptides (146 nonapeptides overlapping by 8 amino acid residues) were synthesized simultaneously with 100 discs per bag, thereby permitting 100 epitope mapping tests. The tests were carried out using radioimmunoassay and the method was compared to Pepsan where the same peptides were synthesized and binding of each serum tested on pins. Results of paper disc RIA and pin ELISA showed binding of peptides 41–49 to 45–53 and peptides 43–51 and 44–52 in chain 2 of Fel d I respectively.

Studies on the structural requirement for ligand binding to the neuropeptide Y receptor from rat cerebral cortex have been carried out using analogues of the neuropeptide Y model peptide synthesized by the 'T' bag method (63). The T bag peptide synthesis approach using Fmoc chemistry and simultaneous multiple peptide synthesis has been used for antigenic mapping of viral proteins (64). The role of individual amino acids in binding human and macaque antibodies was determined in the human immunodeficiency virus type 1 (HIV-1) gp 41, residues 594–613. Using decapeptides with 9 amino acid overlap, amino acids 599–603 were found to be the main recognition site for 19 human anti-HIV positive sera.

8.3 Peptide epitope libraries

Epitope libraries provide a method for identifying peptide ligands for antibodies, receptors, or other binding proteins and provide a tool to rapidly identify lead ligands in the drug discovery process (65).

An alternative to mapping epitopes via the antigen is to employ combinatorial methods whereby a library of peptides is generated. The library of peptides can be generated by chemical synthesis (1, 61, 62, 66, 67) or biologically by phage display systems (68). Only chemically synthesized peptide libraries will be dealt with here. The library is a vast collection of all theoretically possible peptides consisting of 4, 5, 6, and so on amino acid residues where the total number of variations is calculated as 20^n , n being the number of residues and 20 is the number of optional amino acids. Therefore to synthesize a library of penta-peptides, the total number of variants (i.e. complexity of the library) would be $20^5 = 3.2 \times 10^6$ peptides whereas for a library of hexapeptides the total number of peptides would be $20^6 = 6.4 \times 10^7$ (69).

8.3.1 Peptide libraries on pins

Geysen *et al.* (22, 70), using replacement net analysis showed that for significant binding to an antibody, three amino acid residues within the peptide sequence should have both the correct identity and position and at least two of the three amino acids should be adjacent to one another. If two amino acid positions are fixed, 400 peptide mixtures would be required to synthesize all possible hexapeptides. These 400 hexapeptide mixtures are assayed for binding by a monoclonal antibody. The best binding peptide mixture is identified and subsequent re-synthesis and screening of the peptide mixture with first pair of amino acids extended to three defined residues, followed by four, and so on. Geysen *et al.* (71) using this strategy concluded that what is important is that the peptide should have complementarity between antigen binding site and the surface of the antibody with respect to shape and charge. This led to the term 'Mimotope' which is defined as the optimum binding peptide that mimics the binding of the epitope without necessarily bearing any relationship to the primary sequence. Elucidation of such a binding peptide can be achieved by:

- (a) Synthesizing peptide mixtures, e.g. libraries as described above or in Section 8.3.2.
- (b) Starting with a dipeptide and building a defined peptide on it, the ‘dipeptide strategy’ (Section 8.3.3).

8.3.2 Combinatorial libraries using ‘T’ bags

Houghten *et al.* (61) have developed synthetic peptide combinatorial libraries (which uses an iterative selection and enhancement process to define the most active sequence) composed of free peptides in quantities which can be used in virtually all existing assays). This method essentially uses the same principle as the Geysen *et al.* (71) library approach except that the synthesis is carried out in T bags as described in Section 8.2.1 and the peptides can be used in solution. There is no limitation to the number of peptides that can be synthesized. A hexapeptide library (with N-terminal acetylation and C-terminal amidation) was synthesized, starting with the first two amino acids positions defined in each peptide and the last four positions of equimolar ratios of 18 of the 20 natural L-amino acids (cysteine and tryptophan were left out in the initial library for ease of synthesis). The initial starting sequence can be represented as Ac-O₁O₂XXXX-NH₂, where O₁ and O₂ are the defined amino acids and X is the equimolar mixture of the 18 amino acids used, hence 324 hexapeptides mixtures were synthesized in the first round. In this method the assay used for testing was the ability of the peptide mixtures to inhibit of binding of a monoclonal antibody to a 13-residue peptide. The optimum defined amino acid mixture that gave maximum inhibition was noted and new peptide mixtures were synthesized with the third amino acid defined (at this stage tryptophan was included in the X position). The iterative process was carried out for the three remaining positions. Using this method a library composed of over 34 million hexapeptides was synthesized and used to identify an antigenic determinant of a monoclonal antibody. The same approach was used in the development of new antimicrobial peptides. A conceptually different approach is the positional scanning synthetic peptide combinatorial library (PS-SPCL) (62) where ten positional decapeptide libraries were synthesized. Each of the ten decapeptide positional peptide libraries is made up of 20 peptide mixtures with a **single** amino acid position defined and the other 9 positions composed of the 18 amino acid mix as described above.

8.3.3 Dipeptide net

In this method, all possible dipeptides are synthesized, each on a separate support, and screened against the antibody. The sequence of the dipeptide that binds best is then extended by re-synthesizing 20 analogues of the dipeptide with only one of the 20 amino acids for the third residue, then taking the best of the tripeptide and synthesizing its analogues with one of the 20 amino acids as the fourth residue and so on. This eventually leads to the identification of an optimum binding peptide. There is a possibility with this approach that a low binding dipeptide which is discarded might actually be as a high binder in a longer peptide.

8.3.4 Peptide libraries on beads

Lam *et al.* (72) have described a method for the synthesis of a peptide library consisting of a set of all possible peptides on resin beads using a 'one-bead, one-peptide' approach. This method involves the synthesis of a large library comprising millions of beads, each bead containing a single representing the universe of possible random peptides in roughly equimolar ratios. Different amino acids have different coupling rates and therefore use of a random mixture of amino acids in a peptide synthesis protocol would lead to unequal representation. To circumvent this, a 'split synthesis' approach was used. A pool of resin beads was distributed into separate reaction vessels containing a single amino acid. This first amino acid was coupled to the resin and after the first coupling cycle, the resins were pooled, split, and distributed into reaction vessels. The process of coupling the second amino acid was carried out. This randomizing and splitting process was continued till the required length of peptides was reached. Using this method, each bead should only contain a single peptide species. Standard solid phase Boc and Fmoc chemistries are applied.

For tripeptides starting with three reaction vessels and three cycles of coupling, 27 tripeptides each with a different sequence would be produced. On a larger scale, starting with 19 reaction vessels and synthesis of pentapeptides, would produce a library of up to 19^5 , i.e. 2 476 099 individual peptides of differing sequence. Such a synthesis would take a few days.

Screening of the peptide library, which may seem a daunting task was quite cleverly achieved. The peptide beads were put in a Petri dish and reacted with enzyme labelled or fluorescein labelled acceptor molecule. Where there was binding of the acceptor to the peptide bead, staining was visible which could be easily visualized under a low power microscope. The reactive beads were then removed and microsequenced after removal of the acceptor molecule. Screening of a library of several million beads could be accomplished in an afternoon using 10–15 Petri dishes.

This method has been used to study the binding of a monoclonal antibody to β endorphin specific for a pentapeptide YGGFL. Six reactive beads were retrieved from two million beads screened from the pentapeptide library, the affinities of the ligands were better than those obtained by a phage library method. Using the same pentapeptide library, the peptides binding sequence for streptavidin was found to be have a consensus sequence of HPQ (72).

This method of synthesis and screening of peptide libraries is simple and identifies ligands with affinities virtually identical to the natural ligand. Both D- and L-amino acids can be used and most importantly the peptides sequences on the beads do not need to be predetermined as only those beads that light up need to be sequenced.

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MULTIPLE PIN PEPTIDE SCANNING (“PEPSCAN”)

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