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Spot-Synthesis: An Easy Technique for the Positionally Addressable, Parallel Chemical Synthesis on a Membrane Support

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Abstract: *Simultaneous parallel syntheses at distinct positions on a membrane support is exemplified with the preparation of series of predefined, short peptide sequences (1). Cellulose paper sheets are used as absorptive membranes. Peptides are assembled by manual or automated spotting of small aliquots of solutions containing the activated amino acid derivatives onto marked positions on the sheets. The application of this method to rapid epitope analysis is demonstrated.*

INTRODUCTION

Exploring the immense diversity of molecular structures accessible to oligo- and polymeric biomolecules such as proteins and nucleic acids by new techniques in molecular biology and chemical synthesis has generated considerable excitement. In particular, the systematic screening of extensive series of short peptide sequences in biological assay systems has become a powerful approach for the investigation of protein-protein interactions. The mechanism of binding between ligands and proteins such as enzymes, receptors and antibodies can be studied at the molecular level and new biologically active sequences are being identified. At present, there are principally two different ways to generate defined sets of peptide sequences for binding experiments: the genetic and the synthetic approach. The first includes cloning and bacterial expression of random DNA fragments as fusion-protein or fusion-phage followed by selection in a binding assay and sequence analysis of positive clones (2,3,4). The second relies on appropriate methods of simultaneous multiple peptide synthesis (SMPS) to cope with the large number of different sequences required. In recent years, several simultaneous synthesis techniques have been developed (for a brief review see Ref. 5). To date, the most efficient synthetic approach is the "pin-method" by Geysen et al. (6) in which peptides are synthesised on the tip of polyacrylate grafted polyethylene rods arranged in an 8x12 microtiter plate array. Recently, Fodor et al. (7) reported on the light directed, spatially addressable, parallel synthesis of a combinatorial set of 1024 peptides on only a 1.6mm x 1.6mm area of a microscope glass slide. In general, small quantities (fmol to μmol) of peptides are sufficient for the functional assays. Soluble as well as immobilised peptides are of equal importance. The synthetic approach offers the additional option to include structural elements other than the natural L-amino acids. Thus, each of the reported techniques has its particular features which makes it specially suited for certain types of experiments, although there is considerable overlap. Ease of handling, availability of devices and degree of automation are other important criteria.

In the following article, a novel technical concept for the solid phase supported simultaneous parallel synthesis is described (8) suitable for both manual as well as automated operation. This technique has been elaborated for the preparation of series of peptide sequences at the 50 nmol scale, utilising conventional

Fmoc/tBu chemistry (5). The method is extremely simple, rapid, economic in the use of reagents, and offers much flexibility with regard to synthesis scale and use of the peptides. It is anticipated that ng to mg quantities of several thousand hexa- to octapeptides can be prepared on one working day.

RESULTS AND DISCUSSION

Principle of the Technique

The basic concept came from the observation that during peptide syntheses on cellulose discs, amino acid coupling reactions can proceed smoothly and to completion, when only enough solution is used as can be taken up by the support matrix. This has been reported by other authors working with cellulose or cotton carriers (9). Instead of using separate pieces (e.g. discs) of paper for individual peptides, this observation suggested that different coupling reactions could be carried out simultaneously on distinct areas of the same sheet of paper. By dispensing of small volumes of solutions circular spots should form resulting from absorption into the matrix; volumes and positions can be calibrated so that neighboring spots do not overlap. Indeed, this turned out to be not only practicable, but a technically very simple approach for the simultaneous parallel assembly of large numbers of different peptides.

The spot size is determined by the volume dispensed, the absorptive properties of the paper material, and the volatility of the solvent. According to the specific functionality of the paper support, the spot size correlates with a particular scale of peptide synthesis. The spot size also directs the minimal distance between spot positions and thus the number of spots that can be distributed onto a given paper sheet (Fig. 1). Some typical data for two types of paper qualities are shown in Table 1. Manual spotting is practically feasible with volumes down to 0.1 μl using an ordinary micro-pipette. Commercial x/y-programmable TLC-spotters or pipetting workstations are perfect devices for automated performance of spot synthesis. Volumes down to 10 nl can be precisely delivered to any position and several thousand peptides may be prepared on a reasonably small sheet. Many more peptides per unit area can be prepared by the photolithographic technique of Fodor et al. (7), this, however, requires extensive instrumentation. Furthermore, even on the smallest spots (10 nl) each peptide is still produced in nmol quantities sufficient for isolation and full characterisation.

Table 1. Relationship of Spotted Volume to Spot Size and Functionality.

A:Whatman 1Chr			B:Whatman 3MM (pre-treated)		
Volume [μl]	Diameter [mm]	Anchor [μmol]	Volume [μl]	Diameter [mm]	Anchor [μmol]
0.1	3	0.02	1	4	0.10
0.5	5	0.07	5	7	0.45
1.0	7	0.13	10	10	0.85
2.0	9	0.25	20	14	1.70
5.0	12	0.60	50	22	4.40

Values are ± 5%. For derivatisation of the paper see next paragraph.

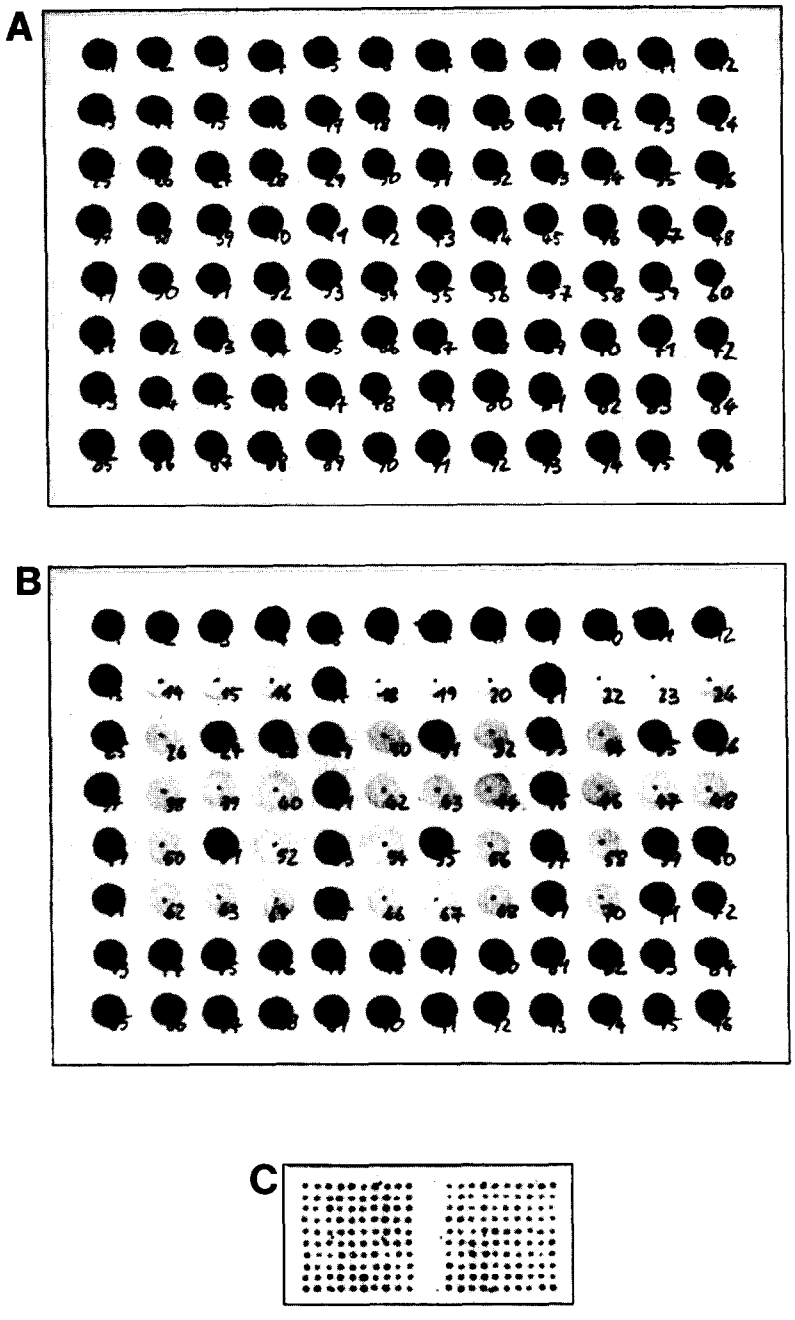


Fig. 1. Some typical arrangements of spots on paper sheets (original size). Spots represent ßAla-ßAla anchors stained with BPB. A: the microtiter plate adapted format of 96 spots; B: A, after reaction of selected spots with Fmoc-Gly-OPfp; C: two arrays of 100 spots each on a 2x2 cm area.

The particular features of cellulose paper (10,11) enabled the realisation of this technique although other chemically resistant membranes might work equally well. The easy and stable marking with a pencil helps to unambiguously address any position during manual assembly of sequences. The peptides can be used immobilised on the support in a solid phase binding assay. Alternatively, the spots can be punched out and the peptides cleaved for use in solution. The ready availability of many paper qualities permits the choice of the necessary properties (mechanical stability, thickness) according to the special requirements of the assay.

Preparation of the Support

In a first attempt, the spot technique was developed as an approach for rapid antibody binding studies with peptides immobilised at the spot positions. For this analytical purpose, a support material is required with only a low functionality per unit of area but which does not interfere with the binding assay, thus a thin paper with optimal washing characteristics such as a pure cellulose chromatography paper. The size of the paper sheet as well as the arrangement of the spots can be chosen to suit the investigators own particular requirements. Immunologists, for example, may prefer a microtiter plate format with 8x12 spots (Fig. 1A,B). An appropriate sheet of paper is cut out and the positions for the spots marked with a pencil at a distance according to the chosen spot size (see Table 1).

The paper sheet needs to be chemically derivatised to introduce suitable anchor functions for peptide synthesis at the spot positions. These anchors also serve as spacer arms to improve the accessibility of the immobilised peptides. One possible route is shown in Figure 2. In a first step (A) an even distribution of more reactive amino functions is generated by the esterification of an αN-protected amino acid derivative to the hydroxyl groups of the cellulose followed by the cleavage of the protecting group. In a second step (B), another amino acid is coupled to these amino functions, but now only small aliquots as chosen from Table 1 of a solution containing the corresponding activated derivative are pipetted onto the pencil points. NMP (b.p. 203°C) is used as solvent because of its low volatility. All residual amino functions on the sheet are then blocked by acetylation and the N-terminus of the dipeptide anchor is released. Free amino functions on the paper are visualised by staining with the bromophenol blue indicator (BPB) dissolved in DMF and appear as distinct blue spots (Fig. 1A). Initially, Fmoc-glycine was used in both derivatisation steps. However, a

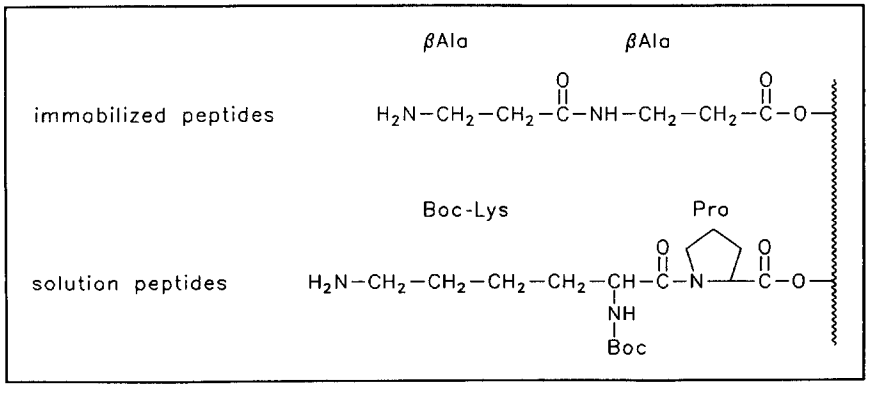


Fig. 2. Anchors used for spot-synthesis on paper sheets.

considerable portion of the Gly-Gly anchor were lost upon Fmoc deprotection with piperidine in DMF and storage, most probably via diketopiperazine formation. Furthermore, the Gly-Gly tail of the peptides seems to interfere with antibody binding and some sera bind non-specifically to Ac-Gly-Gly. Fmoc-ßAla is a better choice (Fig. 2): ß-alanine is a non-proteinogenic amino acid, the ßAla-ßAla anchor is not prone to self-catalysed hydrolysis, and the spacer arm is longer. Carbodiimide mediated esterification of an Fmoc-amino acid in the presence of NMI as chosen for step A is the most critical reaction of the derivatisation process described. Yields can vary considerably depending on the quality of reagents and technical performance. With respect to the use in antibody binding assays, functionality of the paper should be homogeneous and in an optimal window between 10 to 20 μmol/g. Higher loadings may cause aggregation of certain peptide sequences and thus impair the access by the antibody, while lower loadings will reduce the sensitivity of the assay. A special membrane optimised and tested for antibody binding studies is commercially available.

To allow for cleavage of the peptides from the support a cleavable handle may be incorporated into the anchor. A large variety of anchor configurations known from the literature are available, the choice of which depending on the final form of the peptides (5). In order to avoid chromatographic purification of the peptides, the linkage to the support is cleaved only after all chemicals from the deprotection step have been washed off. In the case of the Boc-Lys-Pro anchor described by Bray et al. (12), the peptide is released into neutral phosphate buffer via diketopiperazine formation. This anchor is similarly introduced as spots on the paper when using Fmoc-Pro in step A and Boc-Lys(Fmoc) in step B (Fig. 2).

The scale of synthesis can be easily increased to the μmol range using a thicker paper such as Whatman 3MM. Following the same procedure as above, preswollen (dilute TFA in DCM, 10) 3MM paper was aminated in step A to 1.2 μmol per cm^2. Spot sizes and functionalities are given in Table 1. Up to threefold higher loadings can be achieved by employing more efficient coupling reagents such as 1-mesitylenesulfonyl-3-nitro-1,2,4-triazolide MSNT (11,14).

Assembly of Peptides

Correct and rapid performance of the many pipetting operations during assembly of large numbers of peptides requires the help of an appropriate computer program. A comfortable window-driven SPOTs™ software has been written (15) which allows easy retrieval and editing of sequence files and offers several options to generate series of peptide sequences (Fig. 3). Peptides to be synthesised are ordered under consecutive numbers which refer to the corresponding numbers of the spots on the paper sheet. The program calculates the sum of all amino acid additions (amino acid usage) which helps to estimate the volumes of stock solutions needed to complete the whole synthesis. For each synthesis cycle (simultaneous elongation of all peptides by one amino acid residue) a listing of spot numbers to which a particular amino acid derivative has to be delivered is then given. This listing is used as pipetting scheme for manual spotting or may control the pipetting automat.

An appropriate part of the original sheet, which carries the number of spots required, may be cut out. The spots are marked with the peptide numbers with a pencil (see Fig. 1A); any other code may be written onto the margin or back of the sheet for identification of the experiment. Following the pipetting scheme, aliquots of solutions of Fmoc-amino acid Pfp-esters in NMP are spotted (Fig. 4). These aliquots are kept slightly larger than those applied in the derivatisation step B in order to cover the spot completely. For the slow coupling derivatives of valine, isoleucine and threonine the respective Fmoc-amino acid N-carboxy anhydrides (NCAs) may be used. The BPB staining on the spots enables the operator to visually monitor the

time course of individual coupling reactions by a color change from blue to yellow (13, Fig. 1B). In case of incomplete couplings, an additional aliquot may be added after 15 min. Coupling reactions are stopped by rapid washing with dilute acetic anhydride in DMF. The steps comprising one full cycle of amino acid addition are listed in Table 2. Cycle time can be less than 1 h and hexa- to octapeptides can be prepared on one working day. When peptides of different length are to be prepared, some chains are finished earlier than others because all peptides on one sheet are started together. If a larger group of these is located on a distinct part of the sheet, this may simply be cut off after completion of these chains. Otherwise, these spots may be blocked in the following cycle by spotting acetic anhydride in NMP. If a free terminal amino group of the

```
SPOTscan - Wed May 06 18:29:42 1992

Sequence name: cmv26.seq
Sequence length: 58
Peptide length: 6
Offset: 1
53 spots on 1 membrane

Amino acid usage:
   A=12 D=24 E=8 G=137 H=7 I=1 K=11
   L=42 M=12 N=18 P=12 R=10 S=24

Spot  Mol.Wt.  Peptide sequence
----  -------  ----------------
   1      658   IEGRGK
   2      632   EGRGKS
   3      659   GRGKSR
   4      659   RGKSRG
   5      560   GKSRGG
   6      560   KSRGGG
   7      489   SRGGGG
   8      459   RGGGGG
   9      360   GGGGGG
  10      360   GGGGGG
  11      390   GGGGGS
  12      446   GGGGSL
  13      476   GGGSLS
  14      506   GGSLSS
  15      562   GSLSSL
  16      576   SLSSLA
  17      603   LSSLAN
  18      561   SSLANA
  19      531   SLANAG
  20      501   LANAGG
  21      501   ANAGGL
  22      567   NAGGLH
  23      568   AGGLHD
  24      612   GGLHDD
  25      612   GLHDDG
  26      652   LHDDGP
  27      596   HDDGPG
  28      572   DDGPGL
  29      572   DGPGLD
  30      571   GPGLDN
  31      629   PGLDND
  32      645   GLDNDL
  33      719   LDNDLM
  34      720   DNDLMN
  35      734   NDLMNE
  36      717   DLMNEP
  37      733   LMNEPM
  38      677   MNEPMG
  39      659   NEPMGL
  40      602   EPMGLG
  41      530   PMGLGG
  42      546   MGLGGL
  43      472   GLGGLG
  44      472   LGGLGG
  45      416   GGLGGG
  46      416   GLGGGG
  47      416   LGGGGG
  48      360   GGGGGG
  49      360   GGGGGG
  50      360   GGGGGG
  51      431   GGGGGK
  52      502   GGGGKK
  53      582   GGGKKH
```

```
cmv26.seq - Pipetting Schedule for Cycle 1
A: 16 18
D: 23 24 29 31
E: 35
G: 4 5 6 7 8 9 10 19 20 25 27 38 40 41 43 44 45 46 47 48 49 50
H: 22 53
K: 1 51 52
L: 12 15 21 28 32 39 42
M: 33 37
N: 17 30 34
P: 26 36
R: 3
S: 2 11 13 14
```

```
cmv26.seq - Pipetting Schedule for Cycle 2
A: 17 19
D: 24 25 30 32
E: 36
G: 1 5 6 7 8 9 10 11 20 21 26 28 39 41 42 44 45 46 47 48 49 50 51
H: 23
K: 2 52 53
L: 13 16 22 29 33 40 43
M: 34 38
N: 18 31 35
P: 27 37
R: 4
S: 3 12 14 15
```

```
cmv26.seq - Pipetting Schedule for Cycle 5
A: 20 22
D: 27 28 33 35
E: 1 39
G: 2 4 8 9 10 11 12 13 14 23 24 29 31 42 44 45 47 48 49 50 51 52 53
H: 26
K: 5
L: 16 19 25 32 36 43 46
M: 37 41
N: 21 34 38
P: 30 40
R: 3 7
S: 6 15 17 18
```

```
cmv26.seq - Pipetting Schedule for Cycle 6
A: 21* 23*
D: 28* 29* 34* 36*
E: 2* 40*
G: 3* 5* 9* 10* 11* 12* 13* 14* 15* 24* 25* 30* 32* 43* 45* 46* 48* 49* 50*
   51* 52* 53*
H: 27*
I: 1*
K: 6*
L: 17* 20* 26* 33* 37* 44* 47*
M: 38* 42*
N: 22* 35* 39*
P: 31* 41*
R: 4* 8*
S: 7* 16* 18* 19*
```

Fig. 3. Abstract of a spot-synthesis protocol form for the assembly of 53 hexapeptides derived from the 58 amino acid residues long CMV26 sequence (16). Numbers marked with an asterisk indicate peptide chains that are finished after the respective cycle.

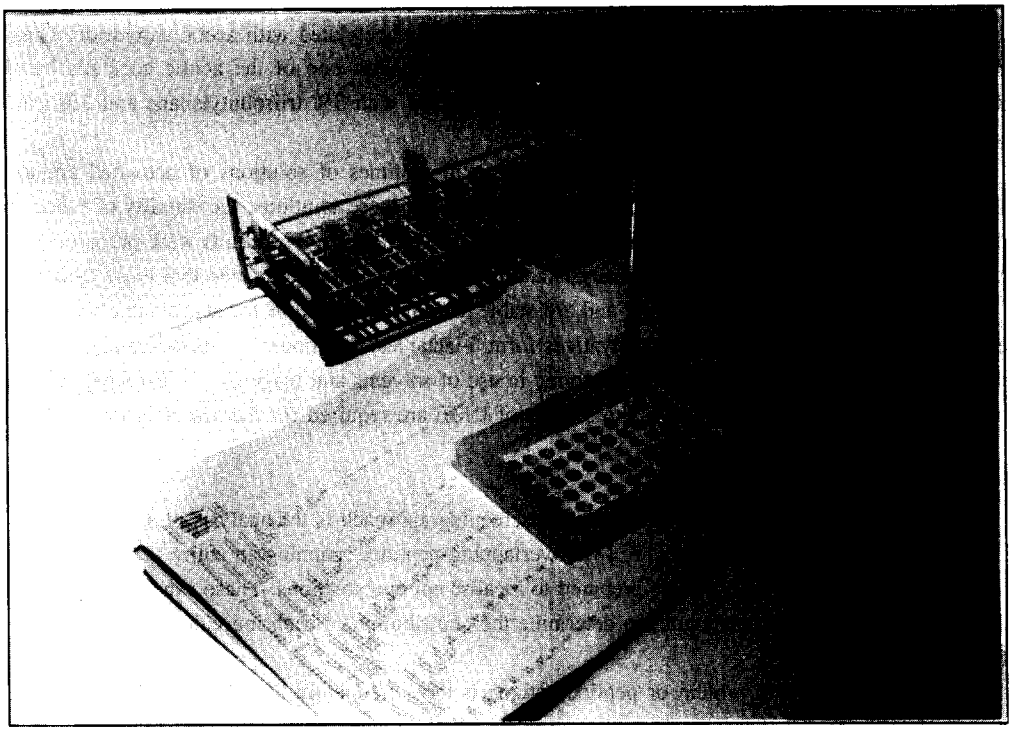


Fig. 4: Manual performance of spot-synthesis.

Table 2. Steps Comprising One Cycle of Amino Acid Addition

Step	Operation
1	spotting of activated Fmoc-amino acid derivative in NMP, leave for 15 min
(2)	respotting (optional), leave for another 15 min
3	wash with 2 % acetic anhydride in DMF (0.5 min)
4	wash with 2 % acetic anhydride in DMF (3 to 5 min)
5	2 washes with DMF (each 2 min)
6	Fmoc-cleavage, 20% piperidine in DMF (5 min)
7	4 washes with DMF (each 2 min)
8	staining, 0.01% (w/v) BPB in DMF (2 min)
9	2 washes with ethanol (each 2 min)
10	drying with cold air from a hair dryer between 3MM paper

peptides is required, the last amino acids of the shorter chains are coupled as their αN-Boc protected derivatives. After the final cycle, peptides can be N-terminally acetylated with acetic anhydride. Cleavage of side chain protecting groups is accomplished by treatment with one of the acidic cocktails based on TFA/DCM (1:1). Particularly good results have been obtained with 3% triisobutylsilane and 2% water as scavengers.

Syntheses at the nmol scale require only very small volumes of solutions of activated amino acid derivatives. In order to minimise the effort in the preparation of these solutions, the stability of Fmoc-amino acid Pfp-esters and NCAs in NMP solution was investigated. 0.3 M stock solutions were prepared and 100 µl aliquots stored frozen at -70°C. TLC analysis and coupling efficiency confirmed that these frozen stocks can be used safely for at least one month and are stable at room temperature for at least one working day. The only exception is Arginine whose derivatives form δ-lactames. Solutions of Arg-derivatives are freshly prepared. SPOT synthesis is particularly economic in use of solvents and reagents. On average, some 50 to 90 mg of amino acid derivatives and 2,5 to 3 liters of DMF are required for the assembly of 96 15mers.

Antibody Binding Assay

The most frequent application of the peptide screening approach is the analysis of sequential antibody binding sites, or continuous epitopes (17, 18). Overlapping peptides spanning an entire protein sequence or region of known immune reactivity are prepared to localise epitope positions. These can be further analysed with substitution or deletion analogues to determine the contribution of each individual amino acid residue to the recognition process.

Antibody binding to a protein or peptide antigen is most frequently identified by an enzyme-linked-immunosorbent-assay (ELISA, Fig. 5), although other labelling techniques employing radioactive isotopes or fluorescent dyes are adequate (19). In the case of peptides immobilised as spots on a paper sheet, standard enzyme-conjugate/chromogen combinations which form water insoluble colored products can be used. As a result, colored spots appear at the positions of binding. The dye and adsorbed proteins on the sheet can be removed by washing with organic solvents and detergents (stripping) which allows the peptides to be reacted

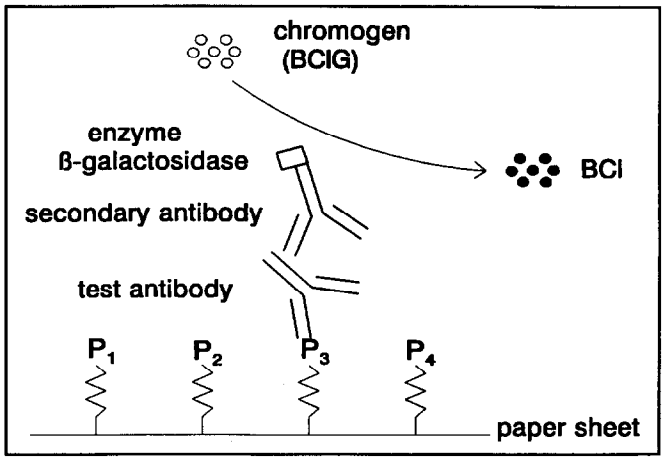
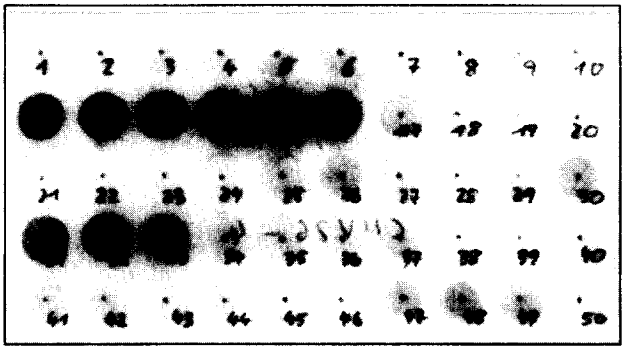


Fig. 5. Schematic representation of the antibody binding assay.

several times with different antisera. On occasions, sheets have been re-used more than ten times. Although less sensitive, a ß-galactosidase conjugated second antibody and 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (BCIG or XGAL) as substrate (chromogen) were chosen because of the mild, non-destructive reaction conditions which improve multiple re-use of paper-bound peptides.

The analysis of epitopes recognised by a polyclonal serum that was raised in rabbits against a ß-galactosidase-CMV26 fusion protein (16) is given as an example. The CMV26 sequence is an immunogenic region of the human cytomegalovirus 36/40K protein recognised by sera of several patients seropositive to the whole virus and was isolated from a random expression library of open reading frames in a pEXStuI-vector. The CMV26-sequence was devided into 49 decapeptides with an offset of one amino acid residue and these were synthesised, as described above, on a ßAla-ßAla derivatised sheet with an array of 10x5 spots.



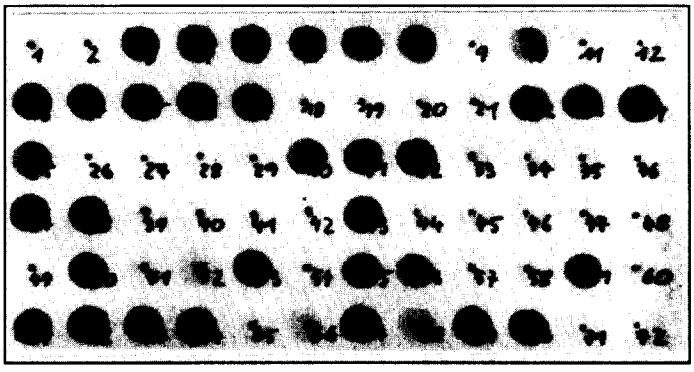
CMV26-decapeptide scan

```
          10        20        30        40        50
  IEGRGKSRGGGGGGGSLSSLANAGGLHDDGPGLDNDLMNEPMGLGGLGGGGGGGGKKH
1.IEGRGKSRGG       21.ANAGGLHDDG       41.PMGLGGLGGG
 2.EGRGKSRGGG       22.NAGGLHDDGP       42.MGLGGLGGGG
  3.GRGKSRGGGG       23.AGGLHDDGPG       43.GLGGLGGGGG
   4.RGKSRGGGGG       24.GGLHDDGPGL       44.LGGLGGGGGG
    5.GKSRGGGGGG       25.GLHDDGPGLD       45.GGLGGGGGGG
     6.KSRGGGGGGG       26.LHDDGPGLDN       46.GLGGGGGGGG
      7.SRGGGGGGGS       27.HDDGPGLDND       47.LGGGGGGGGK
       8.RGGGGGGGSL       28.DDGPGLDNDL       48.GGGGGGGGKK
        9.GGGGGGGSLS       29.DGPGLDNDLM       49.GGGGGGGKKH
        10.GGGGGGSLSS      30.GPGLDNDLMN
         11.GGGGGSLSSL      31.PGLDNDLMNE
         12.GGGGSLSSLA      32.GLDNDLMNEP
          13.GGGSLSSLAN      33.LDNDLMNEPM
           14.GGSLSSLANA      34.DNDLMNEPMG
           15.GSLSSLANAG      35.NDLMNEPMGL
            16.SLSSLANAGG      36.DLMNEPMGLG
             17.LSSLANAGGL      37.LMNEPMGLGG
              18.SSLANAGGLH      38.MNEPMGLGGL
               19.SLANAGGLHD      39.NEPMGLGGLG
                20.LANAGGLHDD      40.EPMGLGGLGG
```

Fig. 6. Analysis of a series of overlapping decapeptides derived from the CMV26 sequence for binding to a polyclonal anti-CMV26 rabbit antiserum. Reactive peptides are displayed in bold; the common epitope sequence is underlined.

Figure 6 shows the result obtained when probing the peptides with the rabbit anti-CMV26 serum in an ELISA as described above. Two series of peptides reacted most strongly, involving decapeptides 11 to 16, with the common sequence motif SLSSL and decapeptides 30 to 33, with the common sequence motif LDNDLMN. These two dominant epitopes had been identified before by independent methods (20): 1. binding to overlapping octapeptides synthesised on cellulose discs (11) and blotted onto a nylon membrane; 2. binding to immobilised overlapping decapeptides synthesised on the tips of glass rods.

The SLSSL epitope region was then analysed in more detail by synthesising peptide series of different length including alanine substitution analogues (Fig. 7). The decapeptides No. 1 to 10 are identical to those of No. 9 to 18 of Figure 6 and give the same result. Spot No. 10 displays a yellow to reddish color and thus is no false positive signal. Such signals were frequently observed with peptides having basic residues, histidine in this case. This could be attributed to a non-specific side reaction in the presence of potassium ferricyanide(III) which has been used initially as a mild oxidant for the formation of the indigo dye from the indoxyl precursor. Many other oxidants have been tested to replace the ferricyanide and avoid this side reac-



Size-scan: overlapping peptides, start position=1, offset=1

		spot No.	positives
length 10	GGGGGGGSLSSLANAGGLH	1-10	3-8
length 9	GGGGGGSLSSLANAGGL	11-19	13-17
length 8	GGGGGSLSSLANAGG	20-27	22-25
length 7	GGGGSLSSLANAG	28-34	30-32
length 6	GGGSLSSLANA	35-40	37,38
length 5	GGSLSSLAN	41-45	43
length 4	GSLSSLA	46-49	-

epitope sequence underlined

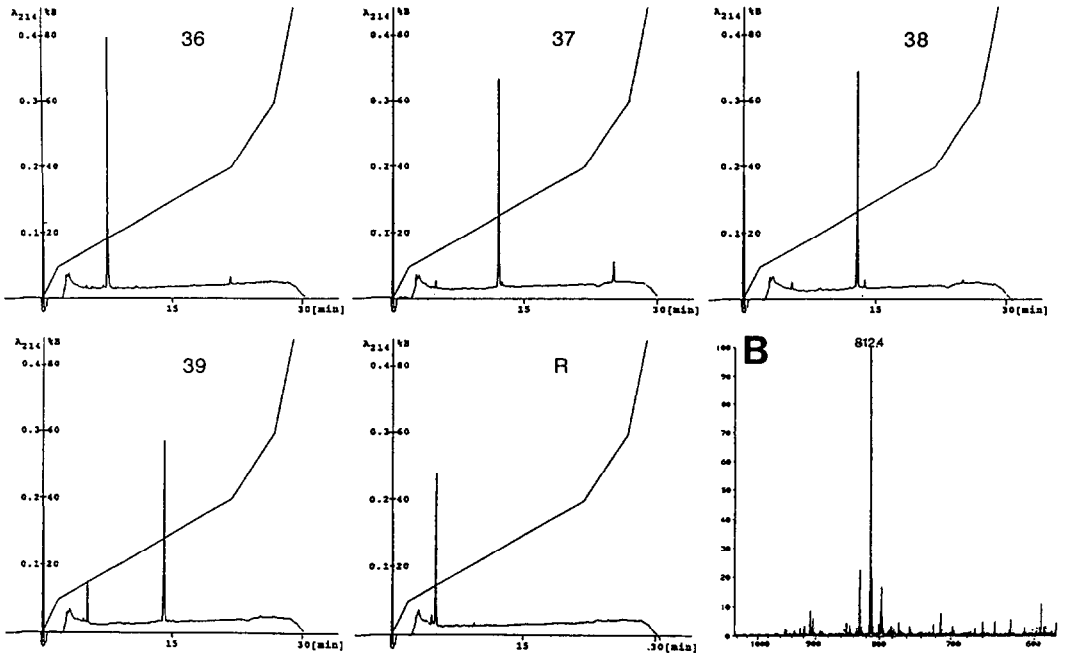
Analogue-scan: single Ala substitution at position 1 to n

		spot No.	positives
length 5	sLSsL	50-54	50,53,
length 7	gsLSsLa	55-61	55,56,59,61
length 9	ggsLSsLan	62-70	62,63,64,67,69,70

replaceble positions in lower case

Fig. 7. "Peptide size" and "alanine substitution" analysis of the SLSSL epitope region identified in Figure 6.

A



C

Peptide No.	Sequence	Molecular Mass[a] (theor.)	[M+H]$^+$	Amino Acid Analysis[b] D	S	G	A	L	P	K	Quantity [nmol]
36	Ac-G-G-S-L-S-S-DKP	755.4	756.4	-	2.88	1.98	-	1.00	x	x	66
37	Ac-G-S-L-S-S-L-DKP	811.4	812.4	-	2.90	0.97	-	2.00	x	x	52
38	Ac-S-L-S-S-L-A-DKP	825.4	826.4	-	2.90	-	0.99	2.00	x	x	54
39	Ac-L-S-S-L-A-N-DKP	852.4	853.4	0.89	1.98	-	1.00	2.00	x	x	57
R	Ac-DKP	268.2	267.2	-	-	-	-	-	x	x	ca. 75

a) The Ac-DKP was only detected as [M-H]$^-$ in negative ion mode FAB-MS.

b) The DKP moiety was not completely degraded under standard hydrolysis conditions. x means, Pro and Lys are present in equal amounts, however, not equimolar to amino acid residues in the peptide chain.

Fig. 8. Analytical data of four CMV26 related hexapeptides that were spot-synthesised on a Boc-Lys-Pro derivatised paper sheet and cleaved from the support via diketopiperazine formation. A: HPLC traces of crude peptides; B: Positive ion mode FAB-MS spectrum of peptide No. 37; C: Tabulated relevant data of the peptides.

tion. The best results were obtained with thiazolyl blue tetrazolium bromide (MTT) which, in addition, forms a dark violet formazan that also improves the sensitivity of the assay. All shorter peptide series clearly confirm that SLSSL is the shortest sequence recognised by the antibody. The signal response is independent of the length of the peptide and the position of the epitope within the peptide. Thus, the ßAla-ßAla spacer is long enough to allow efficient binding of the antibody, even when the epitope sequence is directly attached to it (Fig. 7, spot No. 43). This is also true for the alanine substitution analogues. All three series give the same result, e.g. the first and fourth position of the epitope sequence can be replaced by Ala.

In order to prove that differential color development is a result of specific binding and not due to a variation of peptide quality, the CMV26 hexapeptide series (Fig. 7, spots No. 36 to 39) was repeated on a Boc-Lys-Pro derivatised sheet and the peptides were eluted carrying a C-terminal diketopiperazine moiety (12). Figure 8 gives some analytical data of these peptides. All were nearly homogeneous by HPLC, correct in their sequences as proven by FAB-MS and amino acid analyses, and obtained in almost equal yields.

Conclusions

The spot-synthesis is a technically very easy and straightforward method. The quality of the peptides prepared by this method is remarkably good and sufficient for most biological studies. Peptides up to 20 residues in length have been successfully prepared. In antibody binding assays, identical results to other established methods are obtained. The development of the spot-method was very much inspired by the successful PEPSCAN method of Geysen et al. (6) which uses arrays of polyethylene rods with polyacrylate grafted tips. The spot-method provides a simpler, more economic and rapid access to large numbers of short peptide sequences at the nmol to μmol scale for biological screening purposes. Moreover, it offers a high degree of flexibility concerning arrangement, scale and number of syntheses per area of supporting paper sheet. Miniaturisation should allow to accomodate up to a hundred spots per 1 cm^2 and several thousand peptides may be screened on a reasonably small sheet. Like other parallel SMPS techniques, it lends itself to automation. Work in this direction is in progress. At present, the application of spot-synthesis in other areas of peptide research is being tested, such as in the analysis of the sequence specificity of protein modifying enzymes, the screening of hormone derived peptide mimetica etc. In principle, the spot technique should also be applicable to the stepwise parallel assembly of other oligomers, e.g. oligonucleotides, provided that the chemistry allows such a simple handling of reagents.

EXPERIMENTAL

General Methods

Chemicals and solvents are of the highest available purity and purchased from various commercial sources. Bromophenol blue (E. Merck, Darmstadt) is dissolved in DMF to 10 mg/ml and this solution is used as a stock for staining of amino functions. NMI was distilled from solid NaOH and stored in a tightly closed flask over molecular sieve 4Å (NMI is hygroscopic!). NMP was treated with molecular sieve (4Å) until a 1 ml aliquot yielded a bright yellow color upon the addition of 5 μl BPB stock. Cellulose paper (1Chr, 3MM) was obtained as 46 x 57 cm sheets from Whatman (Maidstone, England). Fmoc/tBu protected amino acid derivatives are from Novabiochem (Läufelfingen, Switzerland) and the corresponding HOBt-esters are prepared in-situ by reaction with 1.5 eq. HOBt and 1.2 eq. DIC in NMP for 60 min. Fmoc-amino acid Pfp- or Dhbt-esters (Thr and Ser) are from Cambridge Research Biochemicals (Northwich, England), and Fmoc-

amino acid NCAs from Propeptide (Vert-le-Petit, France). They are dissolved in NMP at a concentration of 0.3 M and stored frozen at -70°C as 0.1 ml aliquots in screw-cap plastic tubes. Solutions of arginine derivatives were always freshly prepared. Special side chain protection: Arg(Pmc), Cys(Trt) or Cys(Acm), His(Boc). The goat-anti-rabbit ß-galactosidase conjugated secondary antibody was obtained from Southern Biotechnology Associates Inc. (Birmingham, USA) and used as recommended by the supplier.

Solutions of Fmoc-amino acid Pfp-esters are routinely checked by TLC on plates of silica gel 60 F-254 from E. Merck (Darmstadt, FRG) with ethyl acetate as solvent and should contain no baseline material. - HPLC-separations were carried out on an analytical reversed-phase silica gel column (Nucleosil 300-7C18, 4x250 mm, Macherey-Nagel, Düren, FRG) with gradients of acetonitrile/0.1% TFA in water/0.1% TFA; flow was 1 ml/min. - Mass spectra were recorded on a Kratos MS 50RF with a high field magnet (mass range ca. 10.000 at 8 kV) and a Kratos FAB source using a beam of neutral xenon atoms at 8-9 kV and thioglycerol as matrix. Peak fractions from HPLC separations containing 5 to 10 μg peptide were evaporated to dryness, dissolved in 3 μl DMSO and aliquots thereof were injected into the thioglycerol matrix. - Amino acid analyses were carried out on a Biotronik LC200. - Sonication was carried out in a water bath from Bandelin electronic (Berlin, FRG) type Sonorex Super RK510H. - UV/VIS extinction coefficients were measured with a Zeiss PMQII spectrophotometer. - Amino functions on paper sheets were quantified by a modified ninhydrin assay as described (11).

Derivatisation of Paper Sheets

Step A: Paper sheets of required size and property are cut out from larger commercial sheets and the spot positions are marked with a pencil at a distance according to the chosen spot size (Table 1). The sheets are dried overnight under vacuum in a desiccator and then reacted for 3 h in a closed container with a solution containing 0.2 M Fmoc-amino acid, 0.24 M DIC, and NMI in DMF. The solution should be completely taken up by the paper (e.g. 2 ml/100 cm^2 for 1Chr, 4 ml/100 cm^2 for 3MM). The quality and concentration of NMI largely determine the yield (area specific functionalisation) of this reaction. For analytical antibody binding experiments the required low functionality (0.1 to 0.2 μmol/cm2 on 1Chr) is achieved when reacting Fmoc-ß-alanine in the presence of an equal concentration of NMI. For preparative synthesis of solution phase peptides Fmoc-proline is reacted in the presence of the double concentration of NMI which yields a functionality of 0.4 to 0.6 μmol/cm^2 on 1Chr and 1.2 to 1.4 μmol/cm2 on 3MM. After 3 washes with DMF, treatment with 20% piperidine in DMF for 20 min, three more washes with DMF, and two washes with ethanol, the sheets are dried under vacuum in a desiccator.

Step B: (typical procedure of the manual version for a 96 spot microtiter format on 1Chr) All chemical steps are carried out with a paper sheet from step A placed in a flat, 10 x 14 cm wide and 1 cm deep, inert plastic tray. A glass plate is used as a cover. Liquids are aspirated with a polypropylene pipette tip mounted on a polypropylene tubing and connected via a 2 l suction flask to a vacuum line. Unless otherwise stated, 15 ml portions of solvents and solutions are used and the tray is gently agitated on a rocker plate for 2 min. A solution (100 μl per sheet) containing 0.3 M HOBt-ester of Fmoc-ß-alanine in NMP is prepared and 0.5 μl aliquots spotted, with a pipette, onto each of the 96 pencil points onto a ß-Ala derivatised sheet from step A. After 20 min, the sheet is washed twice with 2% acetic anhydride in DMF and residual amino functions are further acetylated by treatment with 2% acetic anhydride, 1% diisopropylethylamine in DMF for 30 min. Further washings and Fmoc cleavage are carried out as listed in Table 2. Free amino functions on the sheet are stained with 1 % BPB stock in DMF and appear as distinct blue spots (Fig. 1a). Similarly, Lys-Pro

derivatised sheets are prepared using Pro-loaded sheets of 1Chr or 3MM from step A and spotting a solution of 0.3 M HOBt-ester of Boc-Lys(Fmoc)-OH in NMP. For the experiment described in Figure 8, an array of 10 x 7 spots generated by spotting 1 μl aliquots at a distance of 1 cm on 1Chr paper has been used.

For other paper sizes and spot arrangements volumes of solvents and solutions as well as dimensions of the tray should be adapted in such a way as to keep consumption of chemicals to a minimum. Stability of anchor functions on derivatised paper was tested after incubation over prolonged times in sealed plastic bags at various temperatures by determining the amount of paper bound amino groups with the ninhydrin assay.

Assembly of Peptides

Technical details are as above in step B and all steps comprising one cycle of amino acid addition are listed in Table 2. Following a pipetting scheme as shown in Figure 3, e.g. 0.7 to 0.9 μl aliquots of 0.3 M solutions of activated Fmoc-amino acid derivative in NMP are pipetted onto the blue spots of a 96 microtiter formatted sheet. This corresponds to about a 3-fold excess over amino groups on the spots. Coupling reactions are followed by color change of the spots from blue to yellow (Figure 1b). With incomplete couplings, an additional aliquot may be added after 15 min or a double spotting may be performed routinely. N-terminal capping of shorter peptide chains within a normal elongation cycle is achieved by spotting 0.4 M acetic anhydride in NMP onto the respective spots. The numbers of these are listed in an extra line of the pipetting schedule. After the final cycle, peptides may be acetylated with 2% acetic anhydride in DMF for 20 min following step 8 of Table 2. Complete acetylation is indicated by the disappearance of the blue BPB color. The paper sheet is then washed twice with DMF, twice with ethanol, and dried. Cleavage of side chain protecting groups is accomplished by immersing the dry sheet into 20 ml of 50% TFA, 3% triisobutylsilane and 2% water in DCM (all V/V) for 2 h with the tray tightly closed to avoid evaporation. Chemicals are washed out with DCM (four times), DMF (three times) and ethanol (twice). The sheet may be dried, sealed in a plastic bag and stored at -20°C or is further processed as below under "antibody binding assay".

Peptides synthesised on ϵ-Lys-Pro anchors are eluted by a modified procedure of Bray et al. (12). After the DCM washes following the TFA deprotection step (above), the sheet is further washed by sonicating three times for 15 min in methanol/water (1:1) containing 0.1% HCl and twice in 1 M acetic acid. The sheet is then dried under vacuum in a desiccator, the spots are cut or punched out and transferred individually to appropriately sized polypropylene tubes (Eppendorf). The peptides are cleaved from the supports by shaking overnight in 0.5 ml 0.05 M triethylammonium acetate (pH 7) containing 20% dioxane. The supernatants are transferred to another tube and lyophilised. The residues were dissolved in 0.5 ml water containing 10% acetonitrile and 0.1% TFA. Aliquots were injected onto a RP-HPLC column and peak fractions collected for FAB-MS and amino acid analyses.

Antibody binding assay

The sheet from the last wash of the deprotection procedure, still in ethanol, is placed into a clean polystyrene tray (e.g. cover of a microtiter plate). 10 ml portions of solutions are used and removed by decantation. The tray is covered with a glass plate and gently agitated on a rocker plate for ten min or as indicated. Buffer solutions used were: TBS (8 g NaCl, 0.2 g KCl, 6.1 g Tris·base per liter, adjusted to pH 7 with HCl); T-TBS (TBS plus 0.05% Tween 20); PBS (8 g NaCl, 0.2 g KCl, 1.43 g $Na_2HPO_4 \cdot 2H_2O$, 0.2 g KH_2PO_4 per liter, adjusted to pH 7 with HCl); a suitable blocking buffer (pH 7-7.5). Efficient blocking of unspecific reactive sites on the membrane and spots is essential for the unambiguous detection of specific

antibody binding. The paper membrane generally gives no background but some peptides on the spots may show a strong crossreactivity with the secondary antibody-enzyme conjugate. For each series of peptides and secondary antibody conjugate the optimal blocking conditions have to be evaluated. There are many formulations available for blocking buffers (19); best results have been obtained with two commercial recipes: membrane blocking reagent from Cambridge Research Biochemicals (Northwich, UK) or SuperBlock Blocking Buffer-Blotting from Pierce (Rockford, USA).

The following series of incubations is carried out: TBS (3-times), blocking buffer (overnight), T-TBS (once), the test antibody or antiserum diluted in blocking buffer (2 to 4h), T-TBS (twice), a ß-galactosidase conjugated second antibody diluted in blocking buffer (1 to 2h), T-TBS (twice), and PBS (twice). The sheet is then transferred to a glass tray and treated with the chromogen solution (3 mg BCIG, 3 mM potassium ferricyanide(III) or 4.5 mg MTT, 5 mM $MgCl_2$ in 10 ml BPS, 10 to 30 min). (CAUTION: MTT is toxic and may cause heritable genetic damage!) Positive spots develop a blue/violet color. Staining is stopped by washing with PBS. The sheet is stored at 4°C in PBS containing 0.05% sodium azide. It is recommended to assay first only the enzyme conjugated secondary antibody for unspecific binding!

Removal of bound antibodies and dye (stripping) is achieved by successive washing with the following solutions: water (twice), DMF (three times, the second wash under sonication as long as needed to completely dissolve the dye), water (three times), solution A [8 M urea, 1% SDS, 0.1% mercaptoethanol adjusted to pH 7 with acetic acid] (three times with sonication at 40 °C), solution B [20% acetic acid, 50% ethanol in water] (three times), ethanol (twice). The sheet may then be re-used for antibody binding as described above or dried with an air dryer, sealed in a plastic bag and stored at -20°C. Because of the lability towards hydrolysis of the ester linkage between the cellulose and the dipeptide anchor, the pH of all buffer solutions used for antibody binding and stripping should be $\leq$ 7.5.

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REFERENCES

1. Preliminary accounts on this method were presented as posters at the 21. European Peptide Symposium in Platja d'Aro, Spain, September 1990 and at the 12. American Peptide Symposium in Boston, USA, June 1991.
2. Stanley, K. K.; Herz, J. *EMBO J.* **1987**, *6*, 1951-1957.
3. Scott, J. K.; Smith, G. P. *Science* **1990**, *249*, 386-390; Devlin, J. J.; Panganiban, L. C.; Devlin, P. E. *Science* **1990**, *249*, 404-406.

4. Cwirla, S. E.; Peters, E. A.; Barrett, R. W.; Dower, W. J. *Proc. Natl. Acad. Sci. U. S. A.* **1990**, *87*, 6378-6382.
5. Fields, G. B.; Noble, R. L. Int. J. Peptide Protein Res. **1990**, *35*, 161-214.
6. Geysen, H. M.; Meloen, R. H.; Barteling, S. J. *Proc. Natl. Acad. Sci. U. S. A.* **1984**, *81*, 3998-4002.
7. Fodor, S. P. A.; Read, J. L.; Pirrung, M. C.; Stryer, L.; Lu, A. T.; Solas, D. *Science* **1991**, *251*, 767-773.
8. Abbreviations used: Ac, acetyl; BCIG, 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside; BCI, 5-bromo-4-chloro-indigo; BPB, bromophenol blue; DCM, dichloromethane; DIC, diisopropylcarbodiimide; Dhbt, 3-hydroxy-2,3-dihydro-4-oxo-benzotriazine; DKP, diketopiperazine; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; ELISA, enzyme linked immunosorbent assay; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; MTT, thiazolyl blue tetrazolium bromide; NCA, N-carboxy anhydride; NMI, N-methylimidazole; NMP, N-methylpyrrolidinone; Pfp, pentafluorophenyl; SDS, sodium dodecyl sulfate; SMPS, simultaneous multiple peptide synthesis; TFA, trifluoroacetic acid.
9. Lebl, M.; Stierandova, A.; Eichler, J.; Bienert, M. In *Peptides 1990*; Giralt, E.; Andreu, D. Eds.; ESCOM Science Publishers, B. V.: Leiden, **1991**; pp. 153-155.
10. Frank, R.; Heikens, W.; Heisterberg-Moutses, G.; Blöcker, H. *Nucleic Acids Res.* **1983**, *11*, 4365-4377.
11. Frank, R.; Döring, R. *Tetrahedron* **1988**, *44*, 6031-6040.
12. Bray, A. M.; Maeji, N. J.; Geysen, H. M. *Tetrahedron Letters* **1990**, *31*, 5811-5814.
13. Krchnák, V.; Vágner, J.; Safár, P.; Lebl, M. *Collect. Czech. Chem. Commun.* **1988**, *53*, 2542-2548.
14. Blankemeyer-Menge, B.; Frank, R. *Tetrahedron Letters* **1990**, *31*, 1701-1704.
15. This software was written by D. N. Lincoln at the GBF and is available from Cambridge Research Biochemicals Ltd. (Northwich, England) as part of a SPOTs™ synthesis kit.
16. Lindenmaier, W.; Necker, A.; Krause, S.; Bonewald, R.; Collins, J. *Arch. Virol.* **1990**,
17. Atassi, M. Z. *Eur. J. Biochem.* **1984**, *145*, 1-20.
18. Geysen, H. M.; Tainer, J. A.; Rodda, S.J.; Mason, T. J.; Alexander, H.; Getzoff, E. D.; Lerner, R. A. *Science* **1987**, *235*, 1184-1190; Getzoff, E. D.; Geysen, H. M.; Rodda, S.J.; Alexander, H.; Tainer, J. A.; Lerner, R. A. *Science* **1987**, *235*, 1191-1196.
19. Harlow, E.; Lane, D. *Antibodies: A Laboratory Manual*; Cold Spring Harbor: Cold Spring Harbor. **1988**.
20. Krause, S. *Epitopkartierung und chemische Synthese antigener Determinanten des humanen Cytomegalievirus*, Universität Oldenburg (FRG) 1991.