Chapter 5

Peptides and Anti-peptide Antibodies for Small and Medium Scale Peptide and Anti-peptide Affinity Microarrays: Antigenic Peptide Selection, Immobilization, and Processing

Fan Zhang, Andrea Briones, and Mikhail Soloviev

Abstract

This chapter describes the principles of selection of antigenic peptides for the development of anti-peptide antibodies for use in microarray-based multiplex affinity assays and also with mass-spectrometry detection. The methods described here are mostly applicable to small to medium scale arrays. Although the same principles of peptide selection would be suitable for larger scale arrays (with 100+ features) the actual informatics software and printing methods may well be different. Because of the sheer number of proteins/peptides to be processed and analyzed dedicated software capable of processing all the proteins and an enterprise level array robotics may be necessary for larger scale efforts. This report aims to provide practical advice to those who develop or use arrays with up to ~100 different peptide or protein features.

Key words Proteomics, Peptidomics, Peptide array, Antibody array, Affinity assay, Antigenicity, Immunization, Polyclonal antibodies

1 Introduction

Microarray technology is now well established and used widely for simultaneous measurement of the expression level of many genes or proteins. Applications of peptide microarrays include antibody epitope mapping, a multitude of other protein–protein and protein–peptide interactions studies, various diagnostics and functional analyses and proteomics applications. Unlike nucleic acid arrays, protein molecules normally require all assays and treatments to be under non-denaturing conditions, which introduce strong constraints on the allowed signal-to-noise ratios. The latter translates into the need to have a high binding capacity substrates (e.g., porous or 3D array surfaces) and high affinity of interaction between the sample and capture molecules. Unlike DNA arrays, where affinity depends on the degree of sequence

Marina Cretich and Marcella Chiari (eds.), *Peptide Microarrays: Methods and Protocols*, Methods in Molecular Biology, vol. 1352, DOI 10.1007/978-1-4939-3037-1_5,

complementarity, the length of nucleotide fragments and could be easily predicted and manipulated. Selecting and manipulating affinities of protein–protein or protein–peptide interactions is often impossible or impractical. Protein–peptide interactions are often characterized by low affinity, and this often becomes a bottleneck in devising and using peptide microarrays. Selecting good peptide sequences for generating high affinity antibodies remains largely unresolved problem.

Continuous efforts to predict peptides' and proteins' antigenicity since mid-1970s yielded many useful tools and resources. Advances in recombinant technologies revolutionized protein engineering and the development of recombinant antibody technologies. The explosion of interest in structural proteomics stimulated further research aiming to understand the molecular mechanisms of protein–protein recognition and to reveal the intricate molecular mechanics of macromolecular interactions, including these of antibodies and their antigens. Other major research areas, which are the subject of substantial research effort, are modeling, rational engineering and affinity maturation of protein binding sites. Numerous papers contain data on the role and significance of different amino acids in forming binding sites and molecular docking. The consensus view is that stability and high affinity of protein–protein interactions stem from multiple factors such as precise molecular complementarity, charge complementarity, the presence of multiple hydrogen bonds and van der Waals contacts between the interacting molecules. However, the focus of research in this area has recently moved from predicting antigenic epitopes to improving the affinity of interaction between two given molecules in a given complex. Consequently, the majority of the reported outcomes are computer assisted structure design, mutagenesis and affinity maturation approaches. Predicting of antigenic epitope gave way to high throughput and high cost epitope mapping services, such as provided by PEPperPRINT, GeneScript, or Pepscan, to name a just few suppliers. However, the use of such services becomes prohibitively expensive or impractical if more than one, let alone hundreds of individual antibody–peptide pairs are to be optimized. Limited advice is available on which peptide to use for example for generating anti-peptide antibodies.

The Affinity Peptidomics approach provides a cheaper and simpler alternative to PEPperPRINT peptide array-based method for antibody epitope mapping. The Affinity peptidomics approach to protein arrays also resolved one other major issue of many protein affinity screening applications, including microarrays based multiplex assays, namely protein sample stability and issues related to protein unfolding and denaturing. In the Affinity Peptidomics approaches, samples are first proteolytically digested before the

assays, and then anti-peptide antibodies are used to assay the generated protein digests using a variety of formats, including the microarrays [1–3]. The key advantages of this technique are much reduced heterogeneity of the physical properties of the assayed proteins, the reduced dependence of each individual affinity assays on the individual proteins being tested and the increased multiplexing capabilities, reduced costs (peptide antigens and anti-peptide antibodies are easier and cheaper to produce) and compatibility with array based screenings and mass-spectrometry detection [3]. Another indirect advantage is that experimental protein samples do not any longer required careful storage and preservation (of the original intact protein folding), because the assay is not for an intact protein (as would be any traditional affinity-based assays, such as protein microarrays), but for short peptide fragments of that protein (e.g., tryptic fragments). Samples may be proteolytically digested and thus "preserved" right at the moment of being collected or shortly thereafter or at a later date. The effect of protein degradation and misfolding/denaturation during folding, on the assay performance is therefore greatly reduced or void.

The majority of antigenic prediction tools available to date rely on protein structural information or are limited to epitopes based on the protein surface and are not suitable for use with Affinity peptidomics approach. Such tools have only limited usability in the analysis of tryptic peptides for their antigenicity and may miss sequences which are "antigenic" but are not fully solvent exposed. We previously described the preferred formats for such multiplex antibody–peptide affinity assays (on microarrays) and reported the key principles of selecting peptides for antibody detection. There is some limited yet clear correlation between the key physical and chemical properties of tryptic peptides and their ability to yield high titre anti-peptide antibodies capable of capturing proteolytic peptides in a MALDI-TOF-MS assay or a microarray formats. The original parent protein structure, folding and fragment solvent exposure play no role in determining *tryptic* peptides' antigenicities, thus making the majority of existing antigenicity prediction tools useless. The approach detailed below could be useful for ranking and selecting the best tryptic peptide sequences for anti-peptide antibody development in situations when a choice of peptide epitopes from a single protein target is available. We use this approach for selecting antigenic peptides for generation of anti-peptide antibodies for use in Affinity Peptidomics assays or a variety of similar microarray assays. Here we provide a simple practical guide for selecting the best antigenic proteolytic peptide for developing anti-peptide antibodies.

2 Materials

2.1 Selection of Peptides for Anti-peptide Antibody Development

1. Protein or nucleotide databases. There are many; the service currently provided by NCBI appears to offer the most comprehensive search facilities. Use FASTA (text) display option for extracting multiple entries:

   http://www.ncbi.nlm.nih.gov/protein
   http://www.ncbi.nlm.nih.gov/gene

2. *PeptideMass* (*see* **Note 1**) online service for predicting all proteolytic peptides from specified proteins using a wide range of proteases. This tool is also used to predict potential posttranslational modification sites in these peptides and therefore indicates which peptides might be preferred or avoided when selecting sequences for anti-peptide antibody generation.

   http://web.expasy.org/peptide_mass/

2.2 Affinity Peptidomics: Antibody Microarrays

1. A microarray printer. Whilst we are suing Flexys contact microarray gridding robot (from Genomic Solutions Inc.), many other contact and non-contact microarrayers exist (*see* **Note 2**).
2. A microarray scanner. Whilst we are suing BioChip microarray Scanner (Packard Bioscience) with 16 bit TIFF readout, many other suitable scanners exist (*see* **Note 3**).
3. ArrayIt® SuperNylon Microarray Substrate or Biodyne® Positively charged nylon membrane (0.45 μm) or a similar membrane (*see* **Note 4**).
4. Anti-peptide antibodies and control antibodies (*see* **Note 5**).
5. Size exclusion chromatography (SEC) setup: Waters 600E pump and system controller (Waters) and Spectroflow 757 Absorbance detector (Applied Biosystems); Sephadex® G-25 column (5 mL bed volume) (*see* **Note 6**).
6. Microarray reaction cassette with optional compression fit silicone gasket to make multiple wells on any standard glass microarray (*see* **Note 7**).
7. Proteins: Bovine serum albumin (BSA): 9 % (w/v) in water.
8. Trypsin inhibitor: 10 mM phenylmethanesulfonylfluoride (PMSF) in isopropanol, store at −20 °C.
9. Complete Mini EDTA-free Protease Inhibitor Cocktail Tablets (Roche Applied Science). Prepare 25× stock solution by dissolving one tablet in 400 μL of water, store at −20 °C.
10. Size Exclusion Chromatography (SEC) running buffer (use PBS): 10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4.
11. Microarray blocking and assay buffer: 9 % BSA, 0.1 % Tween 20 in PBS (*see* **Note 8**).

12. Microarray washing buffer: 0.1 % BSA, 0.02 % Tween 20 in PBS (*see* **Note 8**).
13. COOMASSIE® Brilliant Blue G-250 for making colored spots on arrays to facilitate manipulation and identification (*see* **Note 9**).
14. Rhodamine B isothiocyanate (RITC) (*see* **Note 10**).
15. Sequencing grade Trypsin.

3 Methods

3.1 Selection of Peptides for Anti-peptide Antibody Development

Although the common trend is to focus on anti-protein antibodies, custom development of such is often very expensive, takes long time and is not practical or affordable when antibody–antigen pairs are for use in a microarrays format (often 100+ individual features). Furthermore, these are often generated against recombinant expressed fragments, rather than original protein antigens and would require extensive validation efforts. This section aims to describe how to select peptide sequences for use as antigens for antibody production; methods of antibody production are outside the scope of this paper. Many commercial providers are now offering polyclonal antibody services.

1. Enter protein sequence or database accession number of the protein of interest into the *PeptideMass* program (*see* **Notes 11** and **12**).
2. Select "reduced" option for Cysteines, select no acrylamide adducts, no Methionine oxidation, $(M+H)^+$ and monoisotopic masses. Select "Trypsin", choose "no missed cleavages" and select to display all peptides (i.e., larger than 0 Da). Choose to sort peptides by peptide masses (*see* **Note 13**).
3. Choose to display all posttranslational modification, database conflicts, all polymorphisms and splice variants (*see* **Note 14**).
4. Perform the analysis; the *PeptideMass* program will display a list of predicted tryptic peptides, their masses and any information on splice variants, isoforms, and database conflicts. Peptide ranging between 10 and 20 amino acids in length might become useful in developing anti-peptide antibodies. For the ease of use, copy the table and paste for example into EXCEL datasheet.
5. Select a subset of peptides suitable for *chemical synthesis* (*see* **Note 15**). Selection criteria:
   (a) Peptide lengths should be between 5 and 30 amino acids.
   (b) Avoid multiple Prolines, Serines, Aspartic Acids, and Glycines.

Table 1
Amino acid preferences for selecting peptides for immunizations

	Desired amino acids	Allowed amino acids	Make little difference	Should be avoided if possible
Polar	Lys, Arg	His, Asn	Gln, Ser, Thr	Asp, Glu
Nonpolar	Leu, Ile, Val	Met, Gly	Ala, Pro, Trp	Tyr, Phe

(c) Avoid internal Cysteines.

(d) Avoid the following duplets of amino acids: Ser-Ser, Asp-Gly, Asp-Pro.

(e) Avoid the following triplets of amino acids: Gly-Asn-Gly, Gly-Pro-Gly.

(f) Avoid charge clustering and fewer than one in five charged amino acid side chains.

6. Select a subset of peptides suitable for *antibody generation*. Peptides containing 10–15 amino acids make good and economical antipeptide epitopes. Peptide ranking criteria are listed below and summarized in Table 1 (*see* **Notes 16–18**).

(a) Peptides must contain basic amino acids, the total number of these is not limited.

(b) Peptides must contain large aliphatic amino acids, the total number of these is not limited.

(c) Amino acids with acidic side chains should be avoided if at all possible.

(d) Aromatic nonpolar amino acids should be avoided if at all possible.

(e) The presence of polar non-charged amino acids is often necessary to maintain the overall hydrophilicity and solubility of the peptide (Gln, Asn).

(f) Small numbers of these amino acids are allowed (Met, Pro, Gly).

3.2 Affinity Peptidomics: Antibody Microarrays

In a traditional direct binding affinity assay, a labeled antigen or antibody is added to the immobilized antibody or antigen, respectively, and the detected signal is proportional to the concentration of antigen. In a competitive binding assay, where the amount of bound labeled antigen is reduced (displaced) by binding of the unlabeled antigen, the signal detected will generally be inversely proportional to the concentration of the assayed unlabeled antigen. Microarray experiments often employ two-color assay systems (two samples, two different fluorescent dyes, the sample is mixed and the array scanned twice to measure each of the two analytes).

The result is typically a ratio of two signals measured for each spot. Two-color detection is a competitive affinity assay. Affinity peptidomics microarrays are most suitable for use with competition based detection (either one or two colors).

To simplify affinity microarray experiments, we prefer using single label competitive assays rather than traditional direct binding two-color assays. The justification of the choice can be found here [4, 5]. Briefly, our approach allows to avoid repetitive labeling of the experimental samples and compensates for the heterogeneity of the antibody affinities. Our protocols were originally devised for use with recombinant scFv anti-peptide antibodies developed using Phage display [6], but were later adapted for use with traditional anti-peptide polyclonal antibodies. Such peptide affinity assays are widely applicable to the detection and quantification of the proteolytic or naturally occurring peptides. The protocol below exemplifies a single color detection approach (the simplest); with minor modifications it can be also used for two-color detection.

3.3 Proteolysis and Labeling of Serum Protein Samples

1. Use 100 µL aliquots of each of the serum samples to be tested. Add a few microliters of 1 M K_2HPO_4 or 1 M Tris pH 9 to bring the pH of the sample to pH 8, check pH by spotting the buffered serum onto pH paper (*see* **Note 19**).
2. Make one pooled serum sample by mixing equal volumes from all serum samples being tested (*see* **Note 20**).
3. Add Trypsin to each sample, including the pooled serum sample: use 1 µg per ~20–50 µg of the total serum protein and incubate at 37 °C overnight (*see* **Note 21**).
4. Stop proteolysis by adding 20 µL of 10 mM PMSF (*see* **Note 22**).
5. To fluorescently label the pooled serum sample add 80 µL PBS to a 20 µL aliquot of the digested serum, then add 100 µL of 1 % RITC. Incubate at room temperature for 30–60 min (*see* **Note 23**).
6. Stop labeling reaction by adding 20 µL of 1 M Tris pH 8.5.
7. Purify labeled peptides using size exclusion chromatography (SEC) setup (*see* **Note 24**).

3.4 Microarrays for Fluorescent Detection and Quantification of Peptides (See Note 25)

1. Set up microarray spotting instrument. The Flexys microarray gridding robot allows for three washing buffers to be used for cleaning the pins and the washing program should be set as follows: 1 % Tween 20 wash for 30 s; followed by PBS wash for 10 s, followed by another wash in 1 % Tween 20 for 30 s and PBS wash for 10 s. The final wash is in 0.1 % BSA in PBS with 0.1 % Tween 20 for 30 s (*see* **Note 26**).
2. To check pins quality and to match the pins, perform a trial run by spotting the same fluorescently labeled protein and scan the slides to determine the efficiency of protein transfer for each individual pin (*see* **Notes 27–29**).

3. To measure sample volumes required for spotting, add an even number of identical ~20 μL aliquots of any sample to a microwell plate, and insert it in the robotic spotter. Samples should have the same protein concentration and buffer as that in the antibody samples to be spotted. Choose the wells (or pins) such that half of the samples are transferred to the membrane, and half are not used. Run a number of transfers (e.g., ~100). Remove the plate from the robot and measure the remaining sample volumes, compare volume in the used and unused wells, average the difference and divide by the number of transfers (*see* **Note 30**).
4. Transfer the required volumes of antibodies to microwell plates, insert them into the robot holder and run the spotting program using the parameters specified and tested in previous steps (*see* **Note 31**).
5. Remove slides from the robot and transfer them into a sealed chamber containing a few milliliters of 37 % formaldehyde. Incubate overnight in a fume hood at room temperature (*see* **Note 32**).
6. Block the membranes using large volume of Microarray blocking and assay buffer for at least 2 h.
7. Assemble the assay mixtures as follows (exemplified for 200 μL final volume sample): use ~10 μL of the unlabeled serum digest (or the equivalent amount of the purified proteolytic peptides), add 1 μL of the 25× Protease Inhibitor Cocktail, incubate for 15 min at room temperature. Add 50 μL of the labeled and purified pooled sera digest and 140 μL of fresh Microarray blocking and assay buffer. Assemble an individual assay mixture for each of the tested sera samples (*see* **Notes 33** and **34**).
8. Transfer Microarrays to reaction cassettes with optional compression fit silicone gasket. Add the assay mix and complete the assembly of the cassette. Incubate at room temperature in the dark for at least 2 h. Arrays must not be allowed to dry out (*see* **Note 35**).
9. To wash the arrays transfer them to a flask containing ~50 mL of the Microarray washing buffer for 10 s, change buffer and incubate for 5 min, change buffer again and incubate for 10 min (*see* **Note 36**).
10. Dry the arrays (arrayed side up) in darkness overnight.
11. Scan arrays using a suitable instrument. We use a BioChip microarray Scanner. The scanner settings (focus, laser intensity, and photomultiplier attenuation) should be adjusted to the 3D slides used, but should not be changed between the slides (*see* Fig. 1).

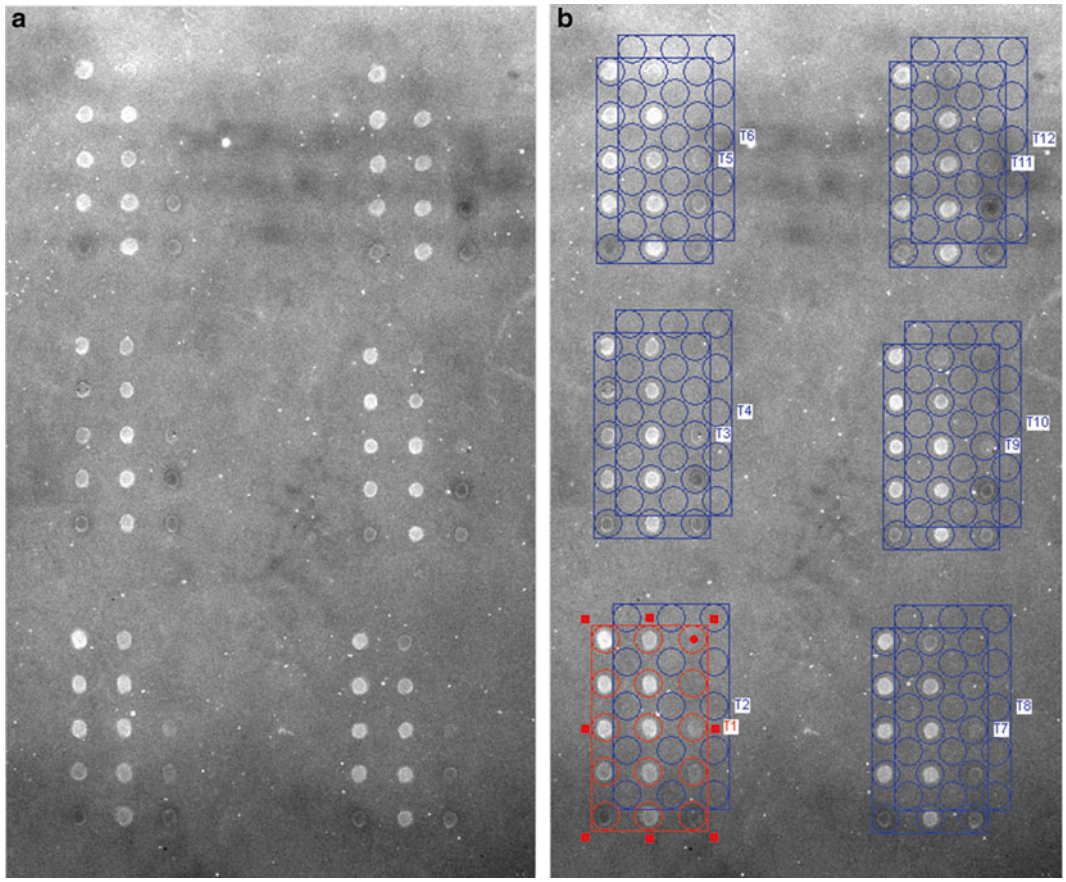

Fig. 1 A typical anti-peptide antibody array. (**a**) Fluorescent readout of a fragment of a microarray following a competitive binding experiment. Six spotted sections are shown with the total of 17 anti-peptide antibodies. Each section is spotted three times for reproducibility. First and the last few spots in each section contain Coomassie dye and are therefore not easily distinguishable on the fluorescent scan (550 nm). (**b**) The same as in panel (**a**) but with the readout grids (T1 to T12) shown. Each section has one grid for reading the fluorescent signal intensities and one identical grid to red background signal for each individual spot

12. Data analysis depends on whether competitive or non-competitive assay was used and also on the set of normalization spots used. In most cases, however, readouts should be normalized pin-to-pin and array-to-array (*see* **Note 37**).

4 Notes

1. Many online tools are available for predicting proteolytic digestion sites, the *PeptideMass* (maintained by Swiss Institute of Bioinformatics and available form SIB ExPASy Bioformatics Resources Portal) provides a wide choice of proteolytic enzymes, including many native digestive enzymes and a good

range of display options generating conveniently formatted data suitable for further processing [7].

2. A range of suitable hardware is very large and extends from a handheld MicroCaster™ Arrayer to enterprise scale NanoPrintTM workstation for printing up to 17,000 microarrays with complete automation. For the methods described here, a smaller scale desktop microarray instruments would be the most suitable. For example SpotBot® 2 Personal Microarrayer from Arrayit corporation which is capable of printing up to 384 samples onto the maximum of 14 slides.
3. A range of suitable hardware is very large. The user may want to consider for example SpotLight™ Personal Two-Color Fluorescence Microarray Scanner or ArrayIt® SpotWare™ Scanner, the latter capable of reading multiple[le slides and enables the use of colorimetric kits based on alkaline phosphatase (AP) and horseradish peroxidase (HRP).
4. 3D porous substrate provide with very high protein binding capacity and are therefore preferred over flat 2D substrates. Ready-made and commercially available membrane substrates such as immobilized Nylon or immobilized Nitrocellulose are available from multiple suppliers, e.g., SuperNylon or SuperNitro from ArrayIt® or FAST™ and CAST™ slides from Schleicher and Schuel. ArrayIt® SuperNylon Microarray Substrates are 25×76 mm ($W \times D$) and have a 150 μm thick immobilized nylon membrane. In addition to their use with proteins or peptides, SuperNylon Microarray Substrates can be used with DNAs, carbohydrates and any other molecules that bind nylon. The binding capacity is 2 μg protein per mm^2. SuperNylon is compatible with most of microarray scanners and can be used with fluorescent, colorimetric, radioactive, and chemiluminescent labels. ArrayIt® SuperNitro Microarray Substrate slides or supported nitrocellulose membrane may provide a good alternative to nylon based supports. These are similar in their performance to the SuperNylon Microarray Substrates, but there might be buffer compatibility issues. Ordinary membranes provide cheaper alternative but are more fiddly to use. These have to be attached to a glass slide during printing, e.g., with a small piece of tape. The advantage is that these can be later treated as soft membranes, rather than rigid and large microscope slides.
5. Antibody sample purity and the protein binding capacity of the microarray substrate material will affect the amount of retained antibodies and therefore the maximum signal obtainable. We typically use total IgG fraction of antisera to spot on the arrays; these require supports with higher protein binding capacities to ensure that sufficient amount of the specific antibody is attached to the membrane. Surfaces with lower binding capacities may be used with purified antibodies.

6. Liquid chromatography setups vary and any suitable equipment and properly sized columns could be used. Gravity flow may also be used for peptide purification, but care should be taken to properly calibrate the elution times of the protein (Trypsin) fraction, the peptides and the unincorporated RITC. The flow rate will vary if gravity flow is used, so calibration should be done by the volume eluted (weigh each tube containing each sample and subtract the weight of the tube), rather than the elution time.
7. Membranes can be assayed in small Petri dishes or sticky gaskets may be used to create small assay/hybridization chambers on the surface of the slides. A small strip of Parafilm or another similar laboratory film may be used to cover a drop of assay buffer on the surface of the slide and might provide sufficient barrier to stop evaporation. Placing array face down into a drop of assay buffer in a small Petri dish works fine too.
8. A 5 % solution of dry fat-free milk powder in 10 mM Na_2CO_3 pH 9 works well for blocking membranes. Alternatively, suitable array buffers are available from Arrayit for a range of proteins, antibodies, and peptide arrays. The Arrayit Protein Microarray Buffer Kit contains the following components: Microarray Activation Buffer, Protein Microarray Reaction Buffer, Protein Microarray Wash Buffer, Protein Microarray Rinse Buffer.
9. Alternatively, Ponceau S may be used. This dye is fully reversible and can be added to printing buffer to all antibodies, proteins, or peptides to facilitate visualizing the spotted array position.
10. A multitude of fluorescent dyes is now available. Amino group-reactive dyes will provide most useful when labeling tryptic digests and thiol group-reactive dyes when labeling synthetic peptides containing cysteine.
11. Entering UniProtKB, Swiss-Prot, or TrEMBL accession numbers is the preferred option, since this would allow to also include in the analysis posttranslational modification, database sequence conflicts, alternative splicing variants and polymorphisms.
12. This tool is convenient for the analysis of individual or small sets of proteins. We created a simple proteolytic digestion tool using EXCEL, which we use for in silico digestion and comparison of individual or groups of proteins. Other existing tools for predicting proteolytic peptides can be used; the choice of the method should not affect the outcome of the predictions. *PeptideCutter* (http://web.expasy.org/peptide_cutter/) is another convenient tool for predicting proteolytic digestion sites. Use the "Table of sites, sorted sequentially by amino acid number" display option.
13. Although mass calculations are not critical at this point, it is worth selecting this and other options, as these would become useful later.

14. Having this information handy will help prevent errors in the subsequent anti-peptide antibody generation program, which may be costly and which may cause very long delays, e.g., if an antibody has to be remade. Posttranslational modifications sites are best being avoided if an anti-peptide antibody is to be generated.
15. The selection of the subset of suitable peptide can be achieved simply by selecting the range of lengths 10–15 amino acids in the EXCEL file, containing the output of the *PeptieMass* program, followed by a quick check for any of the unwanted amino acids. We have entered the above rules into a Visual Basic Macro which is run in Excel, making the selection easy even if multiple proteins are analyzed. Sorting the *PeptieMass* results by mass allows to very easily select a range of peptides of suitable size. We also used truncated tryptic sequences for antibody generation (i.e., just partial tryptic peptide sequence, if the native predicted fragments were too long).
16. Cysteine is not included in the selection criteria because it is often the amino acid which is added to the peptide sequence to provide sulfhydryl for peptide cross-linking and conjugation.
17. Peptides generated using Trypsin, will often contain only one Lysine or Arginine, therefore presence of histidines may become an important selection criterion. Basic peptides are less likely to yield good antibodies and are also usually less suitable for use with positive mode MALDI-MS and thus should be avoided.
18. Much has been published on the prediction of antigenic epitopes from protein sequences [8–16]. Most of the tools however aim to identify linear epitopes in the larger protein sequence. None of these address the affinity of the predicted antibody–antigen pairs. Previously reported tools are based on the amino acid propensity scales, which take into account hydrophilicity, surface accessibility and segmental mobility of amino acids and are not therefore applicable for selecting best peptides for anti-peptide antibody generation. We use our own simple peptide selection and ranking tool (a Macro run within EXCEL).
19. This amount (~100 μL) should be sufficient for more than one assay, but the choice should depend on the volume and the number of assays by the user.
20. The pooled serum is used for fluorescent labeling and as a reference sample in a competitive binding assay. We first make a pooled sample and then proteolytically digest it. Alternatively, individually digested samples cane be pooled after the proteolysis.

21. One may assume that the total serum protein concentration is below 10 %, hence 100 µL of serum should not contain more than 10 mg protein. Hence 0.2–0.5 mg Trypsin should be sufficient.
22. PMSF will inactivate Trypsin irreversibly. PMSF will hydrolyse in water, especially at high pH, and may not work at high salt concentrations, so if in doubt, samples should be diluted and the pH shall be adjusted to pH 7 prior to adding PMSF. Alternatively trypsin may be inactivated by boiling. However, the high total protein concentration in the sample could result in the formation of protein precipitate which will complicate the extraction of peptides.
23. Fluorescence dye NIR-664-iodoacetamide may be used to label peptides through cystine side chains. Such approach would be more suitable for synthetic peptide mixtures where all peptides contain Cysteines.
24. Crude Tryptic digests may be used for affinity assays with or without additional purification (as long as Trypsin is inactivated). Fluorescently labeled peptides must be purified from the unincorporated fluorescent molecules. We use SEC on Sephadex® G-25 to separate the labeled peptides from both Trypsin and the unincorporated RITC. The same procedure can be applied to unlabeled tryptic digests. There is a large choice of commercially available SEC or reverse phase C18 cartridges and purification tools.
25. Irrespective of the type of spotting instrument used (even if using a hand-held "MicroCaster" spotter, Whatman/Schleicher and Schuell), similar key principles have to be followed:
    (a) Spotting should be done at least in triplicate for each individual antibody. The number of replicates is usually not a limiting factor (hundreds or thousands of spots can be made on each array), we found that having six replicates is sufficient in most cases.
    (b) Careful consideration must be given to the array layout: replicates should be spread over the whole array area to minimize staining and scanning artifacts. Our instrument (Flexys robotic spotter) produces blocks of densely arranged spots (grids, having from 5×5 to 12×12 spots each) whilst each grid is well separated from each other. In such a case each grid may contain only a single copy of any antibody, but the patterns should be replicated at least three (better six) times and be spread over the whole array area.
    (c) Relevant negative controls must be included. For example if polyclonal rabbit anti-peptide antibodies are used, pre-immunization sera or just total rabbit IgGs would make a suitable negative control. IgG concentration should be

ideally the same as in other (specific) antibody samples and at least the same number of replicates should be made. These will provide an important reference point for the data analysis; any errors in determining the nonspecific background may affect quantification.

(d) Reference spots (fluorescently labeled protein) should be added to each array, we have at least one reference spot per each grid of spots. These are needed for signal normalization during scanning and for pin calibration.

(e) Colored spots should be added to ease array handling. These can be for example Coomassie Brilliant Blue or Coomassie-stained protein or a just add a low concentration Ponceau S to printing buffer to all proteins or peptides spotted. Colored spots will help to determine the correct membrane surface, distinguish front from the back of the membrane and identify array borders.

(f) If using contact spotting, pins should be either matched or calibrated.

26. Pin washing and reconditioning is very important for the avoidance of carry-over contaminations and for achieving high reproducibility of spotting. Pin washing procedures and buffers differ significantly from DNA gridding protocols.

27. If a large number of pins is available to the user, the simplest way would be to select those which result in the identical efficiency of protein transfer from the microwell plates to the membrane (array). If this is not possible, pins should be calibrated (by measuring the fluorescence in each spot), from multiple replicates and the values should be taken into account when interpreting the main assay results. Alternatively, calibration controls (fluorescence reference spots) should be included for each individual pin when spotting the antibodies

28. Multiple transfers should be made for each spot (i.e., the material spotted repeatedly onto the same spot on the membrane). This will dramatically increase the reproducibility of antibody transfer and increase the amount of the spotted antibodies (leading to the stronger and more reproducible signals and lesser variability between spots). We routinely use between six and ten transfers per spot. Lengthy transfer procedures should be avoided to prevent sample evaporation issues.

29. High humidity should be maintained inside the robot whilst spotting, especially for longer runs.

30. When using contact spotting, the volume transferred by the pins will depend on many parameters, such as sample viscosity, surface tension, cleanliness of the pins, contact time, and the material and porosity of the membrane. These are difficult to predict but easy to measure. We typically have values of ~20 nL per single transfer per pin.

31. Making small batches of arrays (up to ten arrays per batch) works best in our hands. Increasing the number of arrays further increases variations in the efficiency of transfer. This is probably due to the buildup of dry residue on the pins, which causes the changes. As a rule keep the total number of transfers between pin washes below ~50.
32. Because protein cross-linking with formaldehyde vapor occurs slowly, long incubation time is necessary. This will also ensure better reproducibility of the cross-linking. Alternatively, transfer spotted arrays (or membranes) into 0.003 % solution of freshly prepared glutaraldehyde and incubate overnight. Blocking the unreacted groups with glycine or Tris buffer is optional; we found no clear evidence for including this step, perhaps because blocking might be accomplished during the subsequent steps during incubation of the membranes in the blocking and assay buffers containing amino groups.
33. Because of the competitive nature of the assay, higher concentration of unlabeled peptide (test sample) will yield weaker fluorescent staining (higher degree of displacement of the labeled reference). At least two samples should be assayed, so relative concentrations of the assayed peptides can be compared between the two samples, or between one unknown sample and one known or polled reference sample. Labeled peptides' concentrations may be high, ideally should be above their K_D values. A typical individual assay should have approximately 1:1 (50 %) displacement. One control assay mixture should contain only labeled peptides with no unlabeled peptide added (no displacement array). Another control assay mixture (complete displacement array) should contain a 100-fold excess of the unlabeled peptides. The unlabeled and labeled peptides should be mixed prior to the incubation with the arrays.
34. The protocol described here is most suitable for running a number of different affinity assays and for relative quantification of the peptide levels. The pooled serum sample will serve as a reference sample. Alternatively any one of the samples can be used, e.g., any normal serum sample. The concentration (or dilution) of the unlabeled proteolytic peptides should be approximately equivalent to the concentration of pooled labeled peptides. This will result in the most accurate measurements (50 % displacement). Before running large series, it is worth running a pilot experiment to check that addition of the unlabeled test sample does not reduce the fluorescent signal more than twice on average. Use two identical slides, make the assay mixture for two arrays, but only add unlabeled serum to one of the arrays (use equivalent volume of 9 % BSA in PBS for the other array).

35. Alternatively, transfer 100 μL of the assay mix to a small petri dish, place the microarray face down on top of the drop of incubation mix. Close the Petri dish; Incubate at room temperature in the dark
36. We use 50 mL Falcon tubes for washes. For convenience and to prevent handling mistakes, we use sets of three tubes for each array, filled with 50 mL of the washing buffer. The arrays are transferred from one flask to another at pre-set intervals. Optionally membranes can be further rinsed in deionised water prior to the next step.
37. In competitive assays a higher readout would indicate lower competition for the immobilized binding site from the unlabeled sample and therefore lower concentration of the competing unlabeled peptide. Lower fluorescence would indicate indicates increased competition for binding sites (higher concentration of the matching peptide in the test sample).

References

1. Soloviev M, Finch P (2006) Peptidomics: bridging the gap between proteome and metabolome. Proteomics 6:744–747
2. Soloviev M, Finch P (2005) Peptidomics, current status. J Chromatogr B 815:11–24
3. Scrivener E, Barry R, Platt A et al (2003) Peptidomics: a new approach to affinity protein microarrays. Proteomics 3:122–128
4. Soloviev M, Terrett J (2005) Practical guide to protein microarrays: assay systems, methods and algorithms. In: Schena M (ed) Protein microarrays. Jones and Bartlett, Sudbury, MA, pp 43–56
5. Barry R, Diggle T, Terrett J, Soloviev M (2003) Competitive assay formats for high-throughput affinity arrays. J Biomol Screen 8:257–263
6. Zhang F, Dulneva A, Bailes J et al (2010) Affinity peptidomics: peptide selection and affinity capture on hydrogels and microarrays. Methods Mol Biol 615:313–344
7. Artimo P, Jonnalagedda M, Arnold K et al (2012) ExPASy: SIB bioinformatics resource portal. Nucleic Acids Res 40:W597–W603
8. Dietrich JB (1985) Antibodies against synthetic peptides—their interest in biology. Annee Biol 24:129–152
9. Hopp TP, Woods KR (1981) Prediction of protein antigenic determinants from amino-acid sequences. Proc Natl Acad Sci U S A 78: 3824–3828
10. Westhof E, Altschuh D, Moras D et al (1984) Correlation between segmental mobility and the location of antigenic determinants in proteins. Nature 311:123–126
11. Hofmann HJ, Hädge D (1987) On the theoretical prediction of protein antigenic determinants from amino acid sequences. Biomed Biochim Acta 46:855–866
12. Pellequer JL, Westhof E (1993) PREDITOP: a program for antigenicity prediction. J Mol Graph 11:204–210
13. Ponomarenko JV, Bourne PE (2007) Antibody-protein interactions: benchmark datasets and prediction tools evaluation. BMC Struct Biol 7:64
14. Flower DR (2007) Immunoinformatics and the in silico prediction of immunogenicity. An introduction. Methods Mol Biol 409:1–15
15. Ponomarenko J, Bui HH, Li W et al (2008) ElliPro: a new structure-based tool for the prediction of antibody epitopes. BMC Bioinformatics 9:514
16. Hancock DC, O'Reilly NJ (2005) Synthetic peptides as antigens for antibody production. Methods Mol Biol 295:13–26