Protocol

PepSpot Peptides

Epitope Mapping with synthetic peptides prepared by SPOT-Synthesis

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Table of content

1 Introduction and experimental Basics

Information about the epitopes of proteins recognized by antibodies or antibody fragments is important for their use as biological or diagnostic tools as well as for understanding molecular recognition events. For many purposes, information about the antigen-antibody interaction at the amino acid level, but not necessarily at the atomic level is needed. Regarding the time required for the experiments and resolution of the data obtained, the use of synthetic peptides derived from the antigen sequence is a recommended strategy (Reineke 1999).

1.1 Peptide scan

Scans of overlapping peptides, so-called peptide scans, derived from the amino acid sequence of the antigens (Geysen 1987) are a widely used tool for epitope mapping. The entire antigen sequence is synthesized as linear 8 to 15meric peptides that are subsequently tested for binding of the antibody (Figure 1).

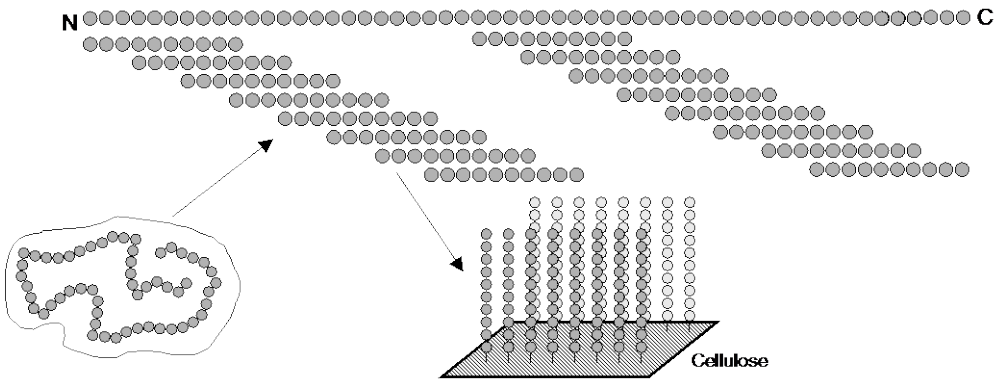

Figure 1: The entire sequence of a protein antigen is synthesized as overlapping linear peptides covalently bound to a continuous cellulose membrane by the SPOT synthesis technique.

For the preparation of the peptides, “SPOT synthesis“ is an easy and very flexible technique for simultaneous parallel chemical synthesis on membrane supports (Frank 1996; Kramer 1998). The peptides are covalently bound to a Whatman 50 cellulose support (Whatman, Maidstone, England) by the C-terminus and have usually an acetylated N-terminus due to (1) a higher stability to degradation, (2) the uncharged N-ac represents better the region in the native antigen than a charged

NH_3^+-group (free amine optional). These solid phase-bound peptides are used for binding studies directly on the membrane (heterogeneous assay).

1.2 Linear and discontinuous epitopes

Two different types of epitopes have to be considered (Figure 2):

- In linear (continuous) binding sites the key amino acids which mediate the contacts to the antibody are located within one part of the primary structure, usually not exceeding 15 amino acids in length. Peptides covering these sequences have affinities to the antibody which are within the range shown by the entire protein antigen.
- In discontinuous (conformational) binding sites the key residues are distributed over two or more binding regions which are separated in the primary structure. Upon folding, these binding regions are brought together on the protein surface to form a composite epitope. Even if the complete epitope forms a high affinity interaction, peptides covering only one binding region, as synthesized in a scan of overlapping peptides, have very low affinities which often cannot be measured in normal ELISA or Biacore experiments. Therefore, detection with directly labelled primary antibody or with a labelled secondary antibody after immobilization of the primary antibody on a PVDF membrane is important (see: Mapping of discontinuous epitopes).

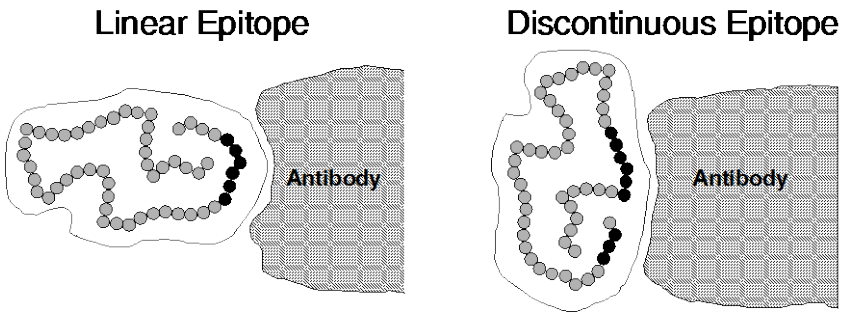

Figure 2: In linear (continuous) epitopes the key residues for binding are located within one stretch of the primary structure of the antigen (left) whereas discontinuous (conformational) epitopes are composed of two or more binding regions which are separated in the primary but adjacent in the tertiary structure (right).

1.3 Substitutional analysis

In many linear epitopes and single binding regions of discontinuous epitopes the amino acids which are in contact with the antibody (key residues) are separated by residues which are buried in the antigen structure, thus not interacting with the binding partner. To identify the residues with critical side chains, substitutional analyses of the epitopes are carried out substituting every position - but only one at a time - by all other L-amino acids (Figure 3). This means all single site substitution analogs are synthesized and tested for binding. Key residues are the ones that may not be exchanged by any other, or only by physicochemically similar amino acids (e. g. leucine/isoleucine) without loss of binding.

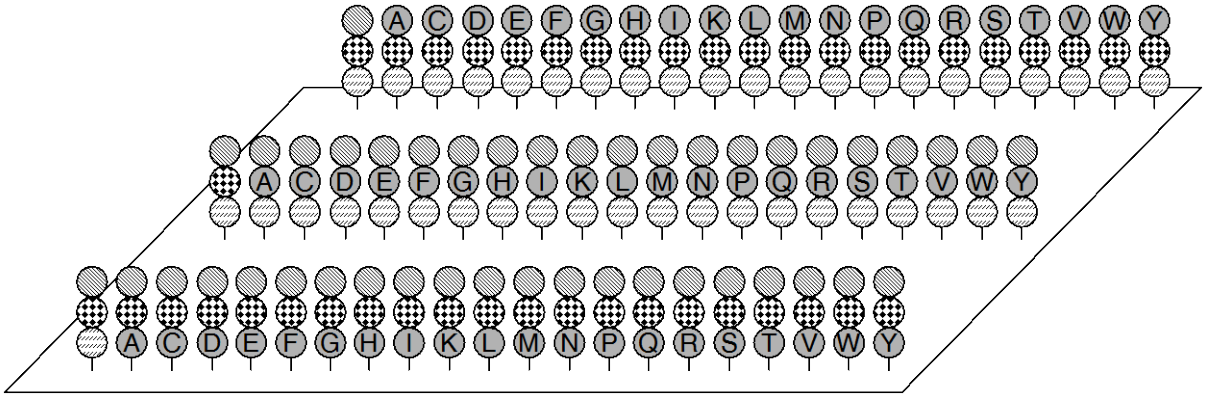

Figure 3: Substitutional analysis for the identification of key residues for binding: Each residue of the wild-type peptide is substituted (rows) by all other L-amino acids. The sequences corresponding to the left column are identical and represent the wild-type peptide. Other spots are single substitution analogs.

2 List of components

Component	Quantity
PepSpots Membrane	Quantity according to order
Datasheet including membrane layout and sequence information	One datasheet per batch
Leaflet including relevant information for storage and handling	One datasheet per batch

3 Storage and Handling

- New membranes should be stored at –20°C until use.
- Incubated membranes which will be used again after only a few days should be washed three times with T-TBS for 10 min and kept with a small volume of T-TBS in a petri dish at 4°C. Drying out of the membrane sometimes leads to poor results in subsequent experiments.
- Incubated membranes which will be stored for a longer period should be regenerated, washed with methanol twice, air dried and kept at –20°C.

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4 Additional Materials required

4.1 Additional Reagents

- Methanol
- Detection reagent: Standard western blotting POD-detection reagents can be applied i.e. Pierce ECL Western Blotting Substrate (ThermoFisher; catalogue number 32109)
- TBS-Buffer (TRIS buffered saline, 1x) (50 mM TRIS; 137 mM NaCl; 2.7 mM KCl; adjust pH to 8.0 with HCl)
- T-TBS-Buffer (TBS-Buffer pH 8.0; 0.05% Tween 20)
- Blocking buffer (Standard western blotting blocking reagents can be applied i.e. Superblock T20 from ThermoFisher; Catalogue number 37536)

Reagents needed for electroblotting experiments (in addition to above listed)

- Cathode buffer (25 mM TRIS; 40 mM 6-aminohexanoic acid; 20% methanol; adjust to pH 9,2)
- Anode buffer I (30 mM TRIS; 20% methanol)
- Anode buffer II (300 mM TRIS; 20% methanol)

Reagents needed for regeneration of peptide membranes

- Regeneration buffer I (62.5 mM TRIS; 2% SDS; adjust pH to 6.7 with HCl)
  - Add 70 microliter 2-mercaptoethanol per 10 ml SDS buffer (= 100 mM)
- Regeneration buffer IIA (8 M urea; 1% SDS; 0.1% 2-mercaptoethanol)
- Regeneration buffer IIB (400 ml water; 500 ml ethanol; 100 ml acetic acid)
- PBS-Buffer (10x) (92 mM Na_2HPO_4 x 12 H2O; 16 mM NaH_2PO_4 x H2O; 1.5 M NaCl, adjust to pH: 7.2 with HCl)

4.2 Additional Hardware

- Orbital shaking system to ease all necessary washing steps
- Standard X-ray film and film cassette, developing machine or a chemiluminescence imaging system i.e. the Fluochem SP Imaging Device (AlphaInnotech, San Leandro, CA, USA)

Hardware needed for electroblotting experiments (in addition to above listed)

- Blotting paper (0.8 mm thick), GB 003 (Schleicher & Schuell, Dassel, Germany).
- Polyvinylene difluoride (PVDF) membrane, Immobilon-P (Millipore, Eschborn, Germany)
- Semi-dry blotter

5 Experimental Protocols

The screening strategy depends on whether the antibody of interest is available in an enzyme-labelled form or if a secondary enzyme-labelled antibody has to be used (Figure 4). In this section we describe the procedure for horseradish peroxidase (HRP or POD)-conjugated antibodies, which is the enzyme of choice, but other enzymes, such as alkaline phosphatase may also be used (Kramer 1998).

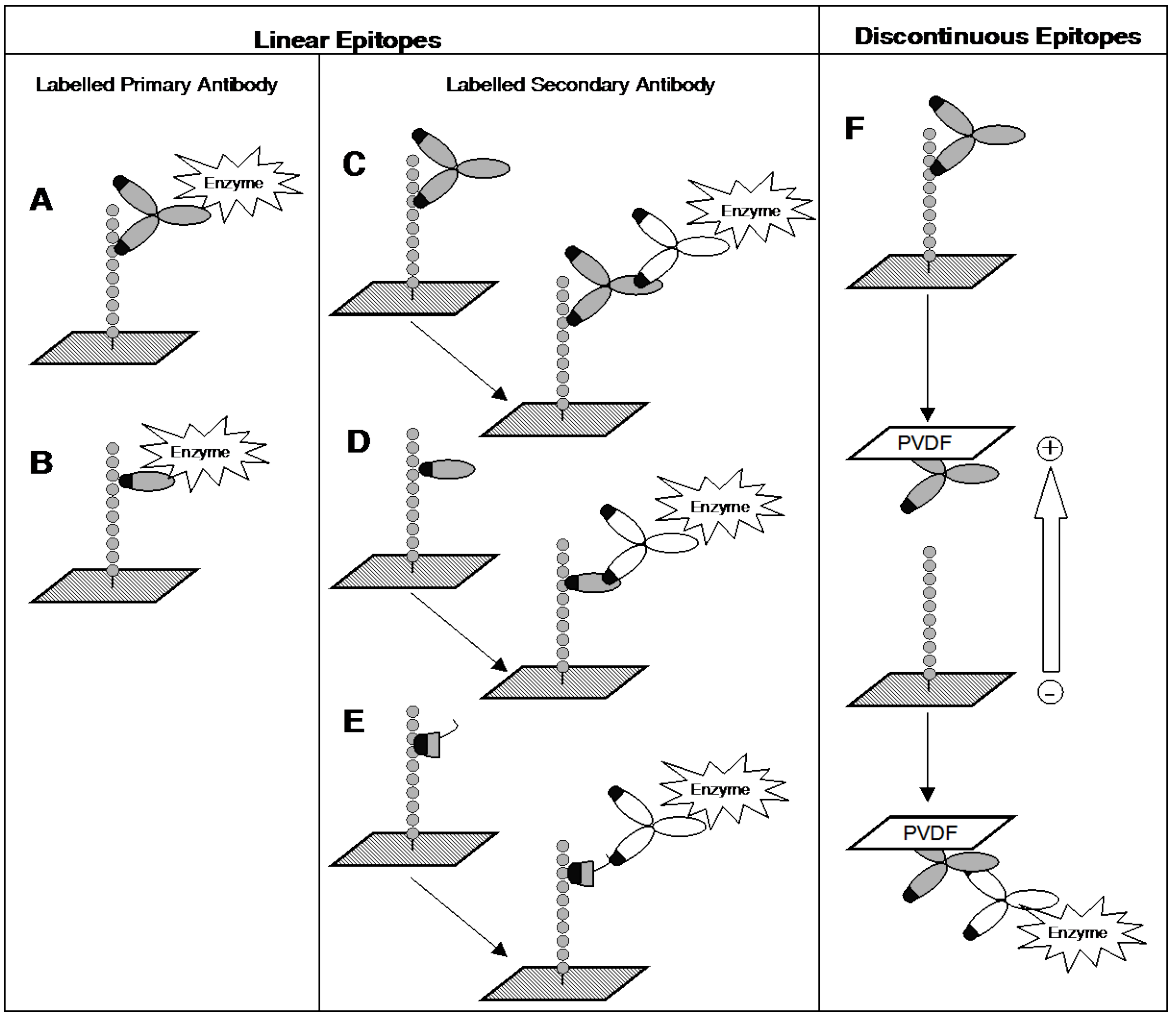

Figure 4: Binding assays are carried out directly on the membrane. Linear epitopes are detected either with labelled primary antibodies (A) and antibody fragments (B) or with labelled secondary antibodies directed against these antibodies (C and D) or affinity purification tags (E). For mapping discontinuous epitopes the primary antibody has to be immobilized on a polyvinylene difluoride (PVDF) membrane by electrotransfer prior to detection due to the very low affinities of separated epitope binding regions to the antibody (F).

Note: Results with directly labelled antibodies are often much better, especially for low-affinity binding antibodies, and may even be essential for the mapping of discontinuous epitopes. Conjugation of antibodies with peroxidase is easy to perform and has been described (Wilson 1978).
The following procedure is practical for the detection of both peroxidase-conjugated primary antibodies and labelled secondary antibodies.

	Do not use sodium azide as a preservative for buffers with peroxidase as it is an inhibitor of the enzyme. The presence of azide will greatly reduce or eliminate the signal.

	**Control experiments are essential to avoid false positive bindings of enzyme-labelled secondary antibody or the enzymes themselves! We strongly recommend doing the control experiments prior to incubation with the primary antibody under investigation, since in some cases the membranes cannot be regenerated completely.**

5.1 Mapping of linear epitopes

In order to show that the antibody binds the peptides at the paratope the whole procedure, either with enzyme-labelled first or secondary antibody, has to be performed with an antibody of the same subclass but different specificity. Sequences that also bind this control antibody most likely interact with the constant regions of the probed antibodies.

5.1.1 Enzyme-labelled primary antibody

The membrane has to be incubated according to the protocol below (5.1.3) omitting steps 5 and 6.

	It has often been observed that alkaline phosphatase alone binds to peptide spots depending on their sequences whereas horseradish peroxidase in almost all cases does not interact with the peptides

5.1.2 Enzyme-labelled secondary antibody

The membrane has to be incubated according to the protocol below (5.1.3)

	If recombinant antibody fragments are detected using antibodies directed against the purification tag (Fig. 4 E), binding of the detection antibodies to the peptides on the membrane can be observed. These peptides contain, or mimic the key residues for binding of the anti-tag antibody.

5.1.3 Membrane incubation

1. Rinse the membrane with a small volume of methanol for 5 min. This is done to avoid the precipitation of hydrophobic peptides during the following TBS-T washing procedure.
2. Wash the membrane with an appropriate volume of TBS-T for 3x3 min. The volume depends on the membrane and the vessel size. The membrane should be sufficiently covered by the solution.
3. Block the membrane with the same volume of blocking buffer for at least 2 hours at room temperature with shaking (blocking over night is also possible).

   General note: Do not overlay two or more peptide membranes in one vessel.
4. Incubate the membrane with the same volume of 0.1-10 µg/ml antibody solution (in blocking buffer) for 3 h. If the primary antibody is peroxidase-conjugated proceed with step 7, if not proceed with the following steps:

5. Wash the membrane three times with the same volume of TBS-T for 5 min each (reduction of background)
6. Incubate the membrane with an appropriate volume of peroxidase-labelled secondary antibody solution (antibody directed against the primary antibody; 0.5–2 µg/ml, diluted in blocking buffer) for 2 h with agitation. Dilute standard anti-sera 1 : 10,000 in blocking buffer).
7. Wash the membrane three times with the same volume of TBS-T for 5 min.

The following steps should be carried out in a darkroom unless a LumiImager™ or another chemiluminescence imager is available:

8. Incubate the membrane with at least 100 µl/cm^2 of detection reagent for about 1 min. Make sure that each part of the membrane is covered with detection solution.
9. Wash the reagent over the membrane repeatedly with a pipette or shake the vessel gently.
10. Insert the membrane antibody side up into a film cassette or the chemiluminescence imaging device according to manufactorers instructions.
11. If needed, switch off the light, place a sheet of film onto the membrane and close the cassette. Expose for 60 s (in the case of working with a chemiluminescence imaging device expose 1 min).
12. Immediately do another exposure for a suitable time (up to 30 min) estimated from the signal intensity of the first trial.

5.2 Mapping of discontinuous epitopes

- For the mapping of discontinuous epitopes peptide-antibody interaction with low affinities have to be detected. Detection with directly labelled primary antibodies (see above) or with a labelled secondary antibody after immobilization of the primary antibody on a PVDF membrane (see below; Rüdiger 1997) is important since direct detection of molecules binding to the peptides with low affinities is extremely difficult. This is due to the shifting of the binding equilibrium towards the non-complexed antibody during incubation with the detection antibody.

  Note: Detection with directly labelled primary antibodies usually result in more precise images in comparison to the electrotransfer technique.

  In this section a procedure is described in which the pattern of the peptide-bound antibody on the peptide membrane is transferred and immobilized onto a PVDF membrane. For a better performance the electrotransfer should be carried out in a fractionated manner. Subsequently, the antibody is detected on the PVDF membranes with an enzyme-labelled secondary antibody.

  Note: For the following procedure an ether-linked oligo-ethyleneglycol modified cellulose peptide membrane (Ast 1999) is recommended, since these membranes have peptide-cellulose linkages which are stable at basic pH values as found in the blotting buffers. If membranes with ester-linked peptides are used, the peptides themselves can be transferred onto the PVDF membranes and then the control experiments as described for linear epitopes have to be performed after blotting.

5.2.1 Preparations

1. Cut out six pieces of blotting paper per peptide membrane and blotting step. The blot sandwich should be a few millimeters larger than the peptide membrane.
2. Cut out a PVDF membrane with the same size as the blotting paper.
3. Rinse the PVDF membrane shortly with methanol.
4. Equilibrate the PVDF membrane in anode buffer I for at least 10 min.

5. Equilibrate two blotting papers in cathode buffer, two in anode buffer I, and the remaining two in anode buffer II.

5.2.2 Membrane incubation

6. Rinse the peptide membrane with a small volume of methanol for 1 min. This is done to avoid the precipitation of hydrophobic peptides during the following TBS washing procedure. General note: Do not overlay two or more peptide membranes in one vessel.
7. Wash the membrane three times with an appropriate volume of TBS for 10 min. The volume depends on the membrane and the vessel size. The membrane should be sufficiently covered by the solution.
8. Block the membrane with the same volume of blocking buffer for 3 h at room temperature with shaking.
9. Wash the membrane with the same volume of T-TBS for 10 min.
10. Incubate the membrane with the same volume of 1-5 µg/ml antibody solution in blocking buffer overnight at room temperature.
11. Wash the membrane three times for 1 min with the same volume of T-TBS. Turn the membrane over repeatedly during this procedure.

5.2.3 Blotting

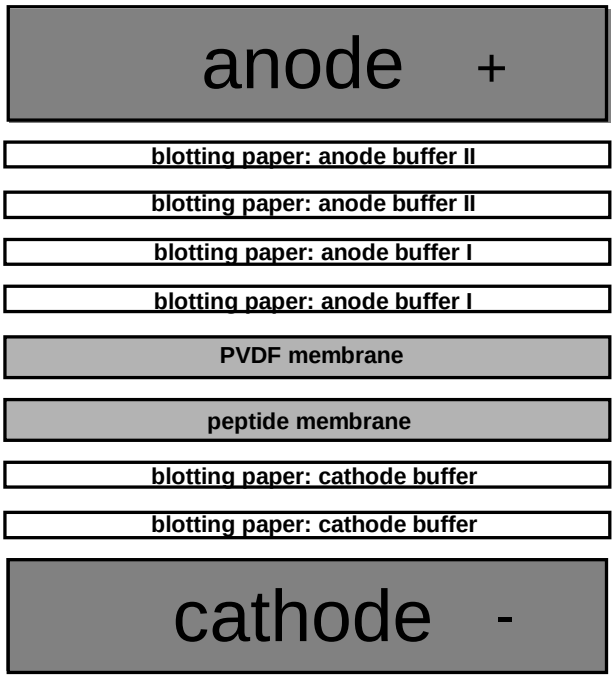

Figure 5: Electrotransfer and immobilization of peptide-bound antibody onto a PVDF membrane.

12. Prepare the blot sandwich as shown in Figure 5. Depending on the orientation of the electrodes of the semi-dry blotter the whole blot-sandwich can be inverted. Avoid air bubbles between the layers and place the sandwich in the center of the electrodes to obtain a homogeneous field. Remove excess buffer with a paper towel before lying on the upper electrode.
13. Blot at 1.0 mA/cm^2 for 30 min. The voltage can change slightly during the process.
14. Remove the first blotting sandwich and prepare the secondary with new blotting paper and a new piece of PVDF membrane. Make sure that the peptide membrane remains wet during this process. Blot again at 1.0 mA/cm^2 for 30 min.
15. Repeat step 14 and blot for the third time for 1 h.
16. Collect the PVDF membranes in T-TBS. Wash the membranes in T-TBS twice for 10 min.
17. Block the PVDF membranes with blocking buffer for 3 h.

18. Continue with steps 7 to 12 from the procedure for Mapping of linear epitopes (5.1.3).

- **Note:** Very often the first PVDF membrane shows a relatively high background. Therefore, fractional blotting is recommended. Step 14 and 15 can be preceded as one step. Doing so the current can be highered slightly to 2.0 mA / cm2. Avoid warming of the semi-dry blotter!

6 Regeneration of peptide membranes

Two different regeneration protocols can be carried out. Start with protocol I and proceed with protocol II if it was not successful.

Note: Usually the peptide membranes can be used several times, but in a few cases regeneration fails due to strong binding of native or denatured antibodies to the peptides or the cellulose.

6.1 Regeneration protocol I

1. Wash the membrane three times with water for 10 min.
2. Wash the membrane at least four times for 30 min with regeneration buffer I at 50 °C.. **Note:** Temperatures above 50 °C can harm the membrane and/or the peptides.
3. Wash at least three times for 20 min with PBS (10 x) at room temperature.
4. Wash the membrane three times for 20 min with T-TBS at room temperature.
5. Wash the membrane three times for 10 min with TBS at room temperature.
6. If the membrane was incubated with a directly labelled antibody check the success of the regeneration by rinsing the membrane in substrate solution and then exposing it at least as long as in the original exposure. If spots are still detected repeat the protocol.
7. If the membrane was incubated with a primary antibody in combination with an enzyme-labelled secondary antibody check the regeneration as described in step 6 of regeneration protocol. If no signals are observed, then no secondary antibody remains on the membrane. Re-incubate the membrane with the secondary antibody and substrate solution and expose at least as long as in the original exposure to show that the primary antibody is also completely removed. If spots are still detected repeat the protocol.
8. If the indirect detection method for discontinuous epitopes was used check the regeneration by blotting the membrane at 1.0 mA/cm^2 for 2 h and proceed with

step 17 of the procedure for mapping discontinuous epitopes (5.2.3). If spots are still detected repeat the protocol.

6.2 Regeneration protocol II

1. Wash the membrane twice with water for 10 min.
2. Incubate the membrane three times with regeneration buffer IIA for 10 min.
3. Incubate the membrane three times with regeneration buffer IIB for 10 min.
4. Wash the membrane with water for 10 min.
5. Wash the membrane three times for 10 min with T-TBS.
6. If the membrane was incubated with a directly labelled antibody check the success of the regeneration by rinsing the membrane in substrate solution and exposing it at least as long as in the original exposure. If spots are still detected repeat regeneration protocol II.
7. If the membrane was incubated with a primary antibody in combination with an enzyme-labelled second antibody check the regeneration as described in step 6 of the regeneration protocol I. If no signals are observed, then no second antibody remains on the membrane. Re-incubate the membrane with the second antibody and substrate solution and expose at least as long as in the original exposure to show that the primary antibody is also completely removed. If spots are still detected repeat regeneration protocol II.
8. If the indirect detection method for discontinuous epitopes was used check the regeneration by blotting the membrane at 1.0 mA/cm2 for 2 h and proceed with step 17 of the procedure for mapping discontinuous epitopes. If spots are still detected repeat regeneration protocol II.

7 Troubleshooting

7.1 Mapping of linear epitopes

- No signals
  - Increase the antibody concentrations.
  - Prolong the incubation time with primary antibody to overnight at 4° C.
  - Perform a simultaneous incubation of first and secondary antibody.
  - Shorten the washing times. Use washing buffer without Tween 20.
  - Shorten the blocking time to 3 h.
  - Use a supersensitive chemiluminescence substrate (e. g. SuperSignal®Ultra, Pierce).
  - Check the antibodies and enzymes in an alternative system.
- High background
  - Increase the detergent concentration in washing buffer.
  - Increase the washing times and/or the washing volumes.
- Clear spots on dark background (In this case the primary and/or secondary antibody concentrations may be too high. On the spots a high amount of antibody conjugate results in all the substrate being used up before the X-ray film can be exposed on the membrane or the imaging system can be started, resulting in clear spots.
  - Wash extensively with T-TBS and re-detect
  - Regenerate the membrane starting with regeneration protocol I and incubate with lower concentrations of proteins.
- “Ring-spots”
  - If spots have a white center and a dark ring a peptide membrane with a lower peptide density, in the range of 20 to 40 nmol/cm^2 is recommended (standard membranes have 150 to 200 nmol/cm^2; Kramer 1999).

7.2 Mapping of discontinuous epitopes

- No signals
  - Increase the antibody concentrations.
  - Prolong the incubation time with primary antibody to overnight at 4° C.
  - Shorten the washing time before electrotransfer.
  - Use blocking buffer without Tween 20 or no blocking buffer.
  - Wash with TBS (no Tween 20).
  - Perform only one blotting step for 2 h.
  - Increase the current.
  - Check the antibodies and enzymes in an alternative system.
- High background
  - Usually the background of the first blot is higher in comparison to the following blots. If the background of the last blot is still too high prolong the washing times before the electrotransfer.
  - The transfer is only sufficient if the isoelectric point of the antibody is below pH 8.5. In very few cases higher values can occur depending on the amino acid composition of the paratope. This can be checked experimentally, or if the antibody sequence is known from prediction programs.
  - If by the secondary blot there is no background, it is sufficient to do only two fractionated blotting steps. The spot pattern can change between subsequent blots. This is probably due to different peptide-antibody affinities.
- Correlation between signals and spot number (sequence)
  - A long exposure results in a background that includes the edge of the membrane. The spot position relative to the edge gives the spot number and the sequence.
  - The rows and columns of the peptide membrane can be marked with a pencil. This is also visible on the chemiluminescence exposure of the PVDF membrane.
  - The rows and columns of the peptide membrane can be marked on the overlayed PVDF membrane when preparing the blot sandwich.

8 Applications

The techniques described are primarily used for the mapping of monoclonal antibody epitopes. Analogous procedures can be applied for the characterization of polyclonal antibodies or patients sera (Valle 1999). In these cases it cannot be decided if the antibody binding peptides are derived from linear epitopes or from different binding regions of discontinuous epitopes.

Although antibody epitope mapping was the first application of the SPOT synthesis technique, it has also been used extensively for characterization of protein-protein interactions in general, such as cytokine/receptor, chaperone/substrate and protein-domain/ligand contacts (Reineke 1999).

Another approach for the identification of antibody binding peptides is the use of synthetic combinatorial peptide libraries. Peptides identified by this procedure either resemble the epitope sequence of the natural protein antigen or have a novel sequence mimicking a natural epitope called a mimotope (Geysen 1986, Kramer 1997).

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