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# Epitope Mapping with Membrane-Bound Synthetic Overlapping Peptides

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## Abstract

Epitope mapping with synthetic overlapping peptides is used for identifying epitopes of monoclonal antibodies (mAbs) and antibodies from patient sera (Midoro-Horiuti et al. Mol Immunol 43:509–518, 2006; Ivanciuc et al. J Agric Food Chem 51:4830–4837, 2003; Midoro-Horiuti et al. Mol Immunol 40:555–562, 2003; Wang et al. J Allergy Clin Immunol 125:695–702, 702.e1–702.e6, 2010). When the mAbs recognize epitopes that are also recognized by patients of interest, they may be useful as surrogates for patient antibodies.

### Keywords

Antigen; Epitope; Monoclonal antibody; Overlapping peptides; Patient serum

## 1 Introduction

The peptides are typically synthesized directly on the cellulose membrane starting from their C-termini and thus have free N-termini. The size of peptides used to identify epitopes is typically 8–15 amino acids. These are either linear or can be circularized by oxidizing cysteins added at designated positions in the peptides.

## 2 Materials

1. Overlapping synthetic peptide: Obtain or "print" overlapping synthetic peptides based on the amino acid sequence of the antigen of interest. Store in –20 °C freezer.
2. Specific monoclonal antibodies (mAbs).
3. Secondary antibodies, enzyme (e.g., peroxidase)-labeled antibodies against the first (primary) antibody.
4. MilliQ water (*see* Note 1).

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Commentary
Synthetic peptides on the membrane are used to identify epitopes for mAbs and human serum antibodies.

[1]MilliQ water should be used to make all the buffers in this protocol.

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5. Blocking buffer (GENOSYS Cat. No. SU-07-250): Add and mix 10 mL of Genosys concentrated blocking buffer, 90 mL of Tris-buffered saline/Tween (T-TBS), pH 8.0, and 5 g of sucrose to make 100 mL of blocking buffer. Prepare it just before use. Do not store.
6. Tris-buffered saline (TBS), pH 8.0: Add and mix NaCl (8.0 g), KCl (0.2 g), Tris base (6.1 g), and 800 mL of MilliQ water. Adjust pH to 8.0 with HCl. Make up to 1 L with MilliQ water. Store at room temperature.
7. 0.05 % T-TBS, pH 8.0: Add 0.5 mL of Tween20 to 1 L of TBS. Store at room temperature.
8. PBS (137 mM NaCl, 8.1 mM $Na_2HPO_4 \cdot 12H_2O$, 2.68 mM KCl, 1.47 mM $KH_2PO_4$, pH 7.4): Add and mix 8 g NaCl, 2.9 g $Na_2HPO_4 \cdot 12H_2O$, 0.2 g KCl, and 0.2 g $KH_2PO_4$ with 800 mL MilliQ water. This will be pH 7.2–7.6. If the pH is not within this range, adjust with HCl or NaOH. Make up to 1 L with MilliQ water (*see* Note 2).
9. Regeneration buffer I: Restore Western Blot Stripping Buffer (Thermo Scientific), store in 4 °C [5, 6].
10. Regeneration buffer II (62.5 mM Tris, 2 % SDS, pH 6.7, 100 mM 2-mercaptoethanol): Dissolve 7.57 g Tris base and 20 g SDS in 800 mL MilliQ water. Adjust pH with HCl to 6.7. Make up to 1 L with MilliQ water. Add 70 μL 2-mercaptoethanol per 10 mL regeneration buffer before use (*see* Note 3).
11. Regeneration buffer IIIA (8 M urea, 1 % SDS, 0.1 % 2-mercaptoethanol): Dissolve 480.5 g urea and 10 g SDS in 800 mL MilliQ water. Make up to 1 L with MilliQ water. Store at room temperature. Add 100 μL of 2-mercaptoethanol to 100 mL of regeneration buffer A in a fume hood just before use (*see* Note 4).
12. Regeneration buffer IIIB (50 % ethanol, 10 % acetic acid): Mix 400 mL MilliQ water with 500 mL ethanol and add 100 mL acetic acid. Do not mix ethanol and acetic acid directly. Store at room temperature.
13. Plastic bag and sealer.
14. Transparent plastic film (e.g., Saran wrap).
15. Chemiluminescent substrate (e.g., ECL Western blotting detection reagents, Amersham Pharmacia Biotech).
16. Film and film developer.

## 3 Methods

Solution volumes indicated below are for about 3 × 8 cm membrane. This size of membrane can contain about 120 peptides.

[2]Other PBS can be used. Avoid $NaN_3$. This will inactivate peroxidase.
[3]Store 2-mercaptoethanol in 4 °C and add just before each use.
[4]Appropriate concentration and incubation time vary depending on the antibody. Adjust these based on the strength of signals from the first experiment results.

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### 3.1 Testing the Nonspecific Antibody Binding

1. Remove the membrane from the freezer, allow to warm to room temperature, and rinse with 5 mL of methanol in polypropylene container for 1 min.
2. Wash the membrane three times with 10 mL TBS for 10 min with shaking. The membrane should be covered by the solution.
3. Block the membrane with 1 mL blocking buffer in the sealed plastic bag overnight at room temperature with gentle shaking. Plastic container with lid can be used, instead of plastic bag. You need 10 mL blocking buffer if you use a plastic container. Do not stack membranes (*see* Note 5).
4. Wash the membrane in the plastic container once with 10 mL T-TBS for 10 min with shaking.
5. Incubate the membrane with 1 mL peroxidase-labeled second antibody (antibody directed against first antibody) in blocking buffer (1:1,000–1:2,000 dilution) for 2 h at room temperature with shaking.
6. Wash the membrane three times with 10 mL T-TBS for 10 min with shaking.
7. Incubate the membrane with 1 mL ECL solution for 1 min in the plastic container in the darkroom. Make sure that the ECL reagent covers the membrane.
8. Wrap the membrane in the transparent plastic film, and insert the membrane, with the peptide side facing the film in the film cassette. Expose the membrane to the film for 15 s, 30 s, 1 min, 5 min, and 30 min.
9. Develop the film.
10. If you see spots, you will need to use a other secondary antibody system to avoid nonspecific antibody binding. If you see no spots, go to the epitope mapping experiments.

### 3.2 Epitope Mapping

1. Remove the membrane from the freezer, allow to warm to room temperature, and rinse with 5 mL of methanol in polypropylene container for 1 min.
2. Wash the membrane three times with 10 mL TBS for 10 min with shaking. The membrane should be covered by the solution.
3. Block the membrane with 1 mL of blocking buffer in the plastic bag overnight at room temperature with shaking. Plastic container with lid can be used instead of plastic bag. You will need 10 mL blocking buffer if you use a plastic container. Do not overlay membranes.
4. Wash the membrane in the plastic container once with 10 mL T-TBS for 10 min with shaking.

[5]You can put two membranes in the same bag back to back to avoid overlaying them on their surface.

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5. Incubate membrane with first antibody diluted in blocking buffer for 2 h to overnight (concentration is about 1 ng/mL–1 μg/mL) (*see* Note 4).
6. Wash the membrane three times with 10 mL T-TBS for 10 min with shaking.
7. Incubate the membrane with 1 mL peroxidase-labeled second antibody (antibody directed against first antibody) in blocking buffer (typically 1:1,000–1:2,000 dilution) for 2 h at room temperature with shaking (*see* Note 4).
8. Wash the membrane three times with 10 mL T-TBS for 10 min with shaking.
9. Incubate the membrane with 1 mL ECL solution for 1 min in the plastic container in the darkroom. Make sure that the ECL reagent covers the membrane (*see* Note 6).
10. Wrap the membrane in the transparent plastic film, and insert the membrane with the peptide side facing the film in the film cassette. Expose the membrane to the film for 15 s, 30 s, 1 min, 5 min, and 30 min (*see* Note 7).
11. Develop the film.

### 3.3 Regeneration of Peptide Membrane (Stripping Antibodies from the Membrane) Protocol I

1. Wash the membrane with 10 mL MilliQ water for 10 min with shaking.
2. Incubate the membrane in Restore Western Blot Stripping Buffer for 5–15 min at room temperature with shaking or incubate at 37 °C for high-affinity antibodies.
3. Wash the membrane with 10 mL T-TBS for 10 min with shaking.
4. If the membrane was incubated with directly labeled antibody, check the success of the regeneration by incubating the membrane with ECL and exposing to the film at least as long as the original exposure.
5. If the membrane was incubated with a primary antibody followed by labeled second antibody, incubate the membrane with an enzyme-labeled secondary antibody, ECL, and expose to the film at least as long as the original exposure.
6. If the membrane has antibody signal after procedure 4 or 5, repeat **steps 1–3** and test the membrane as in **step 4** or **5**. Alternatively, regeneration protocol II or III can be used at this step (*see* Note 8).

### 3.4 Regeneration of Peptide Membrane (Stripping Antibodies from the Membrane) Protocol II

1. Wash the membrane three times with 10 mL water for 10 min with shaking.

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[6]Add 0.5 mL each of ECL solution A and B in the darkroom just before use, mix well, and add the membrane.

[7]Alternatively a bioimaging system (e.g., ChemiDoc-It®TS2 Imager, UVP) can be used.

[8]The strength of regeneration protocol is I < II < III. You should try protocol I first, II next, and then III for most of the antibodies to avoid damaging the peptides on the membrane. If one procedure does not work, you can repeat the same procedure or try the next procedure.

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2. Wash the membrane at least four times with 10 mL regeneration buffer II for 30 min at 50 °C with shaking.
3. Wash the membrane at least three times with 10 mL PBS for 20 min at room temperature with shaking.
4. Wash the membrane three times with 10 mL T-TBS for 20 min at room temperature with shaking.
5. Wash the membrane three times with 10 mL TBS for 10 min at room temperature with shaking.
6. If the membrane was incubated with directly labeled antibody, check the success of the regeneration by incubating the membrane with ECL and exposing to the film at least as long as the original exposure.
7. If the membrane was incubated with a primary antibody with labeled second antibody, incubate the membrane with an enzyme-labeled secondary antibody, ECL, and expose to the film at least as long as the original exposure.
8. If the membrane has antibody (luminescent) signal after procedure 6 or 7, repeat **steps 1**–**5** and test the membrane as in **step 6** or **7**. Alternatively, regeneration protocol I or III can be used at this step.

### 3.5 Regeneration of Peptide Membrane (Stripping Antibodies from the Membrane) Protocol III

1. Wash the membrane three times with 10 mL water for 10 min with shaking.
2. Incubate the membrane three times with regeneration buffer IIIA for 10 min with shaking.
3. Incubate the membrane three times with regeneration buffer IIIB for 10 min with shaking.
4. Wash the membrane with water for 10 min with shaking.
5. Wash the membrane three times with water for 10 min with shaking.
6. If the membrane was incubated with directly labeled antibody, check the success of the regeneration by incubating the membrane with ECL and exposing to the film at least as long as the original exposure.
7. If the membrane was incubated with a primary antibody with labeled second antibody, incubate the membrane with an enzyme-labeled secondary antibody, ECL, and expose to the film for at least as long as the original exposure.
8. If the membrane has antibody signal after procedure 6 or 7, repeat **steps 1**–**5** and test the membrane as in **step 6** or **7**. Alternatively, regeneration protocol I or II can be used at this step.

### 3.6 Storage of Peptide Membrane

1. New membrane should be stored at −20 °C until use.

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2. Used membrane, which will be used again within a few days, should be washed three times with T-TBS for 10 min and kept with a small volume of T-TBS in the plastic bag at 4 °C. Avoid drying out.

3. Used membrane 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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