

Biopanning for the Characterization of Allergen Mimotopes

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Abstract

Proper understanding of the pathogenesis of type I allergy relies on the identification of allergen epitopes. The phage display technique is a relatively new one to define peptide structures that mimic natural epitopes, including conformational B-cell epitopes. Peptides displayed on the phage recognized by an antiallergen antibody mimic the physicochemical properties of the amino acids and are, therefore, called mimotopes. The main advantage of the biopanning technique described in this chapter is that the structure of the antigen/allergen may be completely unknown; the only material needed is an antibody binding to it. The mimotopes generated by this technique display the features of the antigen/allergen but do not crosslink the mast cell-bound IgE-antibodies. Thus mimotopes could be used as a safe alternative to the commonly applied allergen extracts in immunotherapy of allergic patients and direct the immune response toward the desired allergen epitopes. In the selection procedure called biopanning, phages with the mimotopes best recognized by the selecting antibody are amplified. The titers of phages specifically binding to the selection antibody are checked. In this chapter we describe two alternative methods for colony screening: the immunoblot and ELISA.

Key Words: Biopanning; mimotope; epitope; phage.

1. Introduction

Molecular biology and biochemical techniques have considerably advanced the knowledge of allergens. Until now, descriptions of allergens were based on recombinant DNA technology, chromatography, spectrometry of proteolytic fragments, crystallization, X-ray investigations, and, lately, computational modeling (*1*). These methods supported the characterization of isoforms and the determination of the primary, secondary, and tertiary structures of these proteins. For further understanding of the pathogenesis of type I allergy, allergen epitopes

should be known. Attempts to identify B-cell epitopes of allergens on a molecular basis have been unsuccessful because these epitopes are generally conformational and discontinuous in nature. The phage display technique is a relatively new technique to define peptide structures that mimic natural epitopes, including conformational B-cell epitopes (*1–12*). Peptides displayed on the phage recognized by an anti-allergen antibody do not necessarily reassemble the natural allergen epitopes; they mimic the physicochemical properties of the amino acids and are therefore called mimotopes. There are several advantages of the biopanning technique: (i) the structure of the antigen/allergen may be completely unknown, the only material needed is an antibody binding to it; (ii) the epitopes which this antibody binds to can be either of linear (continuous) or conformational (discontinuous) nature; (iii) the generated mimotopes presented in a monovalent way display the features of the antigen/allergen, but do not crosslink the mast cell-bound IgE-antibodies, thus avoiding side reactions. Mimotopes could therefore be used as a safe alternative to the commonly applied allergen extracts in immunotherapy of allergic patients and direct the immune response toward the desired allergen epitopes. This epitope-specific immunotherapy would further prevent the induction of anaphylactogenic antibodies, which are an additional undesired side-effect of the currently applied hyposensitization therapy. We have successfully performed epitope characterization of respiratory and alimentary allergens (*6,8,13*) and of cancer antigens (*1,14–16*), as well as immunization studies in mouse models (*1,7,17*) in our group.

1.1. Random Phage Display Peptide Libraries

Phage peptide libraries consist of filamentous phages displaying random peptides of defined length on their surface, which can be cloned either into surface protein pVIII (resulting in a high-density display with 1000–2700 copies/phage) or into protein pIII (giving only about 3 copies/phage, but being easily accessible on the phage tip pole) (*7,12*). Alternatively, phagemid libraries can be used for longer peptides, which only contain the genetic information for the assembling of the phage, but are not able to replicate or expulse from bacteria. Therefore, helper phages are needed for phagemid libraries. The procedures described here are for random peptide phagemid libraries, using *Escherichia coli* XL-1 as host and M13 as helper phage (*9,18*). Libraries contain about 10^8 different random peptides from 6 to 15 amino acids, depending on the size of the cloned insert. Some examples including references are given in **Table 1**. In these random peptide phage display libraries, the peptides can be displayed either in linear form or in circular manner, when two flanking cysteins form a disulfide bond and, thereby, a loop (*18*).

Table 1
Libraries of Random Peptides

Amino acid insert	Diversity	Ref.
Libraries of random linear peptides		
6 mer	2×10^8	(2)
6 mer	2×10^7	(24)
9 mer	1×10^9	(9)
9 mer	Unknown	(25)
10 mer	4×10^8	(26)
15 mer	Unknown	(2)
Libraries of random peptides constrained by flanking cysteins		
7 mer	4×10^9	(27)
9 mer	1×10^9	(18)
10 mer	1×10^9	(25)

In the selection procedure called biopanning, phages bind via their displayed peptides to a specific (selection) antibody and can be isolated and amplified. Several rounds of biopanning (three to four usually) are done, starting with the original library. The phage eluents from the preceding round are used for a new selection. This procedure renders an amplification of the phages with the mimotopes best recognized by the selecting antibody. First, the outcome of biopanning is controlled by determination of the total phage titer. An increase in the phage titer from one to the subsequent round is usually an indication for successful biopanning. Second, the increase of the titers of phages specifically binding to the selection antibody has to be checked by immunodotting or ELISA. The selection antibody and an isotype control antibody are dotted and incubated with the amplified original library and phage eluents from the different biopanning rounds. The extent of binding to the selection antibody will ideally increase from round 1 to the last. To identify certain positive single phage clones, a colony screening assay is performed. In this chapter, we describe two alternative methods for colony screening, first the immunoblot according to Scott et al. (2) and second the detection via ELISA (19,20). Individual clones of interest are amplified and can be further analyzed by DNA sequencing.

2. Material

2.1. Biopanning

1. Microtiter plates (Maxisorp, NUNC, Roskilde, Denmark).
2. Bicarbonate buffer: 0.1 M bicarbonate, pH 9.6.

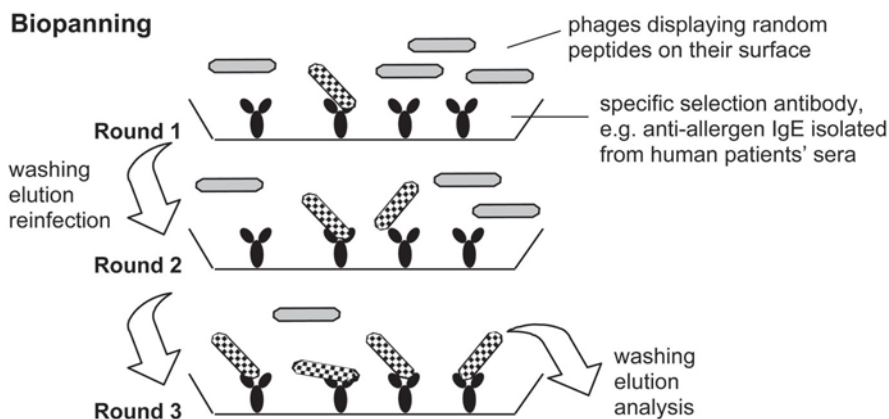


Fig. 1. Principle of the biopanning procedure for selection of specific ligands for an antibody. A solid phase is coated with the selection antibody. After each round of biopanning, specifically bound phages (squared pattern) are eluted and amplified, whereas nonbinding phages (gray) are washed away. Usually three to four rounds of biopanning are necessary for an enrichment of specifically binding phage clones. Using an antiallergen antibody for coating, the selected phages will display peptides on their surface, which mimic the natural allergen epitope (mimotope) (28), reprint kindly permitted by Allergo Journal.

3. Phosphate-buffered saline (PBS): 137 mM NaCl, 3 mM KCl, 6.5 mM Na_2HPO_4 , 1.7 mM KH_2PO_4 .
4. Blocking buffer: PBS, containing 3% (w/v) BSA.
5. PBS/casein buffer: PBS, containing 0.15% (w/v) casein, dissolved in 56°C water bath. This buffer may be kept for several weeks at 4°C when autoclaved.
6. PBS-T: PBS, containing 0.1% (v/v) Tween 20.
7. Elution buffer: 0.1 M HCl, adjusted to pH 2.2 with solid glycine.
8. Neutralization buffer: 2 M Tris.
9. Use aerosol-resistant tips when pipeting phage preparations to avoid cross-contamination via the pipet.
10. Because of the danger of infections the phage-contaminated waste may potentially pose, it must be disposed of according to local safety regulations, e.g., decontaminated in hypochlorite.
11. Perform experiments with phages under half-sterile conditions (best in a laminar flow). Clear bench from contaminating phages by using UV light for 30 min.

2.2. Amplification of Phages

1. SB medium: 30 g/L peptone from casein, 20 g/L yeast extract, 10 g/L MOPS (3-(*N* morpholino) propane sulfonic acid), add deionized H_2O and shake until the solutes have dissolved. Adjust the pH to 7.0 and the volume of the solution to 1 l with deionized H_2O . Sterilize by autoclaving for 20 min.

2. Antibiotics stocks:
Carbenicillin (100 mg/mL H₂O)
Kanamycin (25 mg/mL H₂O)
Tetracycline (10 mg/mL 70% (v/v) ethanol).
3. Bacterial strain *E. coli* XL-1 Blue.
4. LB-agar plates:
 - a. First prepare liquid media (LB medium): 10 g/L bacto trypton, 5 g/L yeast extract, 5 g/L NaCl, add deionized H₂O and shake until the solutes have dissolved. Adjust the pH to 7.0 with 5 M NaOH before adding 15 g/L bacto-agar. Sterilize by autoclaving for 20 min on liquid cycle. When the medium is removed from the autoclave, swirl it gently to distribute the melted agar throughout the solution (*see Note 1*). Allow the medium to cool to 50°C. To avoid producing air bubbles, mix the medium by swirling.
 - b. Medium can then be poured directly from the flask into sterile Petri dishes; allow about 30–35 mL of medium per 90-mm plate. When the medium has hardened completely, invert the plates to prevent contamination by condensation water, and store them at 4°C until needed. The plates should be removed from storage 1–2 h before usage (*see Note 2*).
5. Helper phage M13 (10¹² plaque forming units (pfu)/mL PBS) (VSCM13 from Stratagene or M13 K07 from Pharmacia).
6. PEG/NaCl: 160 g/L polyethylene glycol 8000, 40 g/L NaCl.
7. PBS/casein buffer (*see above*).
8. A bent glass rod is used for plating bacteria on LB-agar plates. Decontaminate rod using a Bunsen burner, let it shortly cool down, and only then use for plating another sample.
9. In all culturing steps seal bottles/tubes with foil or cap tight enough to prevent contamination, but also guaranteeing sufficient aeration of the bacterial culture during incubation.

2.3. Determination of Total Phage Titer

1. SB medium.
2. Antibiotics stocks:
Carbenicillin (100 mg/mL H₂O)
Tetracycline (10 mg/mL 70% (v/v) ethanol).
3. LB/carb plates: First prepare LB media containing 10 g/L bacto-agar as described above. When the medium has cooled down to 50°C, add 1 mL/L carbenicillin stock solution (100 mg/mL H₂O) and swirl gently to distribute the antibiotic throughout the solution. Medium can then be poured into plates as described above.

2.4. Monitoring of Specific Phage Titer: Immunodot Assay

1. Nitrocellulose with appropriate pore size (4 µm). Membranes with printed raster of 0.5 cm² are recommended for precise dotting (Millipore Corp., Bedford, MA, USA).

2. Dot buffers:
 - a. Tris-buffered saline (TBS) washing buffer: 2 M Tris, 1 M HCl, 2 M NaCl, pH 7.0.
 - b. TBS/casein buffer for blocking and antibody dilution: TBS, containing 0.15% (w/v) casein, dissolved in 56°C water bath. This buffer may be kept for several weeks at 4°C when autoclaved.
 - c. Developing stock solution: Prepare 4-chloro-1-naphthol (carcinogenic, wear gloves!) 3 mg/mL methanol (light sensitive, wrap with foil). Can be kept at 4°C for 3–4 weeks.
3. Dotting 50- μ g syringe with 1- μ L drop dispenser (Hamilton, Bonaduz, Switzerland).
4. Plastic reservoirs, eight-well (Labystems, Finland).
5. Antibody HRP/anti-M13 conjugate (Pharmacia).

2.5. Colony Screening Assay (Two Alternative Methods)

The colony screening can be performed either by the immunoblot method (**Section 2.5.1.**) or by the ELISA method (**Section 2.5.2.**).

2.5.1. Immunoblot Method

2.5.1.1. RANDOM SELECTION OF PHAGE COLONIES AND TRANSFER ON FILTER MEMBRANES

1. LB/carb plates: Prepare the same number as single clones will be tested by selecting and control antibodies, respectively.
2. Wooden tooth sticks sterilized at 180°C for 3 h or sterile pipet tips.
3. Nitrocellulose filters (use square diameter of 82 mm for 100-mm plates) (Schleicher & Schuell BA 85).
4. Filter paper (Whatman).
5. Induction reagents: 5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) in sterile H₂O.
6. Chloroform atmosphere (work in fume hood): Place several small flat bottom glass dishes containing chloroform in larger glass Petri dish (square diameter 20 cm and above), cover with glass lid.

2.5.1.2. COLONY SCREENING IMMUNOBLOT

1. Basic buffer for colony screening immunoblot: 50 mM Tris, 150 mM NaCl, 5 mM MgCl₂, pH 8.0.
2. Blocking buffer: basic buffer, containing 3% (w/v) BSA.
3. Lysozyme buffer: basic buffer containing, 400 μ g lysozyme/mL and 20 U DNase/mL.
4. PBS/casein blot buffer: PBS, containing 0.15% (w/v) casein.
5. PBS-T washing buffer: PBS, containing 0.1% (v/v) Tween 20.

2.5.2. ELISA Method

2.5.2.1. RANDOM SELECTION OF PHAGE COLONIES AND TRANSFER ONTO MICROTITER PLATES

1. SB medium.
2. Microtiter plates (Maxisorp, NUNC).

2.5.2.2. COLONY SCREENING ELISA

1. Selection antibody.
2. Coating buffer: 50 mM NaHCO_3 , pH 9.6.
3. Microtiter plates.
4. Blocking buffer: PBS-T, containing 1% BSA.
5. Antibody HRP/anti-M13 conjugate.
6. ABTS solution: 10 mg 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) substrate (ABTS; Sigma) in 10 mL citrate buffer (100 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 6 mM citric acid, pH 4.0) and 10 μL H_2O_2 .

3. Methods

3.1. Biopanning

1. Coat four wells of a microtiter plate with 50 μL antibody solution each (20–50 $\mu\text{g}/\text{mL}$) in bicarbonate buffer.
2. Incubate overnight at 4°C.
3. Remove the coating solution and wash twice with water by filling wells with 150- μL volumes from a multidispenser microliter pipet and emptying again.
4. Block the wells by completely filling with blocking buffer (200 μL each) and incubate at 37°C in a humidified container for 1 h.
5. Remove the blocking solution and add 50 μL of the phage library (about 10^{11} pfu/well) in PBS/casein buffer. For determination of the library's phage titer, see **Section 3.3**.
6. Incubate in a humidified container at 37°C for 2 h.
7. Remove the phages by pipeting thoroughly and wash the plate once with water.
8. Wash with PBS-T by completely filling the wells and resuspending. Wait 5 min, then remove PBS-T. In the first round, wash in this fashion 3 $\times$, in the second round 5 $\times$, and in subsequent rounds 10 $\times$.
9. Wash once more with ddH_2O and empty wells.
10. For eluting the phages, add 50 μL of elution buffer to each well and incubate at room temperature for 10 min.
11. Pipet the elution buffer several times up and down to collect all eluted phages (do not aspirate into filter of pipet tip, phages will be lost!). Remove and transfer phages to microliter tubes. Immediately, neutralize solution by adding 3 μL neutralizing buffer per 50 μL of elution buffer used. Ideally use eluents freshly for further biopanning or store overnight at 4°C.

3.2. Amplification of Phages

1. Inoculate 10 mL SB medium containing 10 $\mu\text{g}/\text{mL}$ tetracycline with a colony picked from a fresh plate of *E. coli* XL-1 Blue cells and grow by shaking overnight at 37°C (= overnight culture).
2. Infect 10 mL SB medium containing 10 $\mu\text{g}/\text{mL}$ tetracycline with 100 μL of the overnight culture. Incubate at 37°C until an OD at 600 nm of 0.7 is reached.
3. Incubate the eluted phages (200 μL) with 2 mL of prepared *E. coli* XL-1 Blue cells ($\text{OD}_{600\text{nm}} = 0.7$) for 15 min at room temperature. Before doing so, remove aliquots of eluted phages for determination of the titers in this round of biopanning (see **Section 3.3**).

4. Add 10 mL of 37°C prewarmed SB medium containing 20 µg/mL carbenicillin and 10 µg/mL tetracycline.
5. Shake tube at 300 rpm for 1 h at 37°C.
6. Transfer to 100 mL of SB medium containing 50 µg/mL carbenicillin and 10 µg/mL tetracycline and shake at 300 rpm for 2 h at 37°C.
7. Add 1 mL of helper phage M13 (10¹² pfu/mL) and shake at 300 rpm for 2 h at 37°C.
8. Add 70 µg/mL kanamycin and incubate at 300 rpm overnight at 37°C.
9. Centrifuge at 4000g for 20 min at 4°C.
10. Transfer the phage supernatant to a clean bottle and add 35 mL of PEG/NaCl to 100 mL phage supernatant, mix gently.
11. Precipitate the phage on ice for 30 min.
12. Centrifuge at 10,000g for 20 min at 4°C and remove supernatants.
13. Resuspend the phage pellet in 2 mL PBS/casein, centrifuge for 5 min at 13,000 rpm (Eppendorf microcentrifuge), and store the supernatant at 4°C (see **Note 3**).
14. For subsequent rounds of biopanning reapply 50 µL of eluted phage suspension from point 13 above in PBS/casein to antibody-coated ELISA plate wells and repeat as described above in **Section 3.1.**, point 5.

3.3. Determination of Total Phage Titer

1. Prepare fresh *E. coli* XL-1 Blue cells with an OD_{600nm} = 0.7 as above (**Section 3.2.**, points 1 and 2).
2. Prepare dilutions (10⁻³, 10⁻⁶, and 10⁻⁸) of the PBS/0.15% (w/v) casein resuspended phages from **Section 3.2.**, point 13, eluted in different rounds of biopanning in 1 mL SB medium. Alternatively, use original library.
3. Use 5 µL of each dilution to infect 50 µL of fresh *E. coli* XL-1 Blue cells (OD_{600nm} = 0.7).
4. Incubate at room temperature for 15 min.
5. Directly apply samples onto LB/carb plates with a microliter pipet and plate infected cells evenly on the agar plate using a glass rod. Incubate overnight at 37°C (plates in regular position).
6. Count colonies and calculate pfu/mL, taking the dilutions into account.
7. Alternatively, prepare different dilutions of phages in 1 mL SB medium and measure concentration in a glass cuvet in the photometer at 269 and 320 nm, distracting empty SB medium as background. Calculate particles per mL (p/mL) according to

$$\text{p/mL} = \frac{(\text{ABS}_{269\text{nm}} - \text{ABS}_{320\text{nm}}) \times \text{dilution} \times 6 \times 10^{16}}{3488}$$

3.4. Monitoring of Specific Phage Titer: Immunodot Assay

Procedure essentially described elsewhere in this volume, with slight modification:

1. Dilute selection antibody which was used for biopanning and, for control purposes, nonrelevant isotype control antibody to 1 mg/mL PBS for dotting.

2. Prepare 1 μL dots in triplicates per sample or dilution.
3. Air-dry membrane at least 2 h at RT before starting the immunoblot or store ready-to-use dot strips dry at 4°C for several weeks.
4. Number and cut strips from membrane. Perform all of the following steps on a rocking table.
5. Saturate with blocking buffer for 30 min at RT.
6. Dilute phage from original library or phage eluents from round 1, 2, 3, and so on to a final concentration of 10^7 pfu/mL (total phage titer) in PBS/casein and incubate dot strip overnight at RT. For negative control, incubate with buffer only.
7. Rinse well with sufficient volume of washing buffer, 3×10 min, changing the buffer each time.
8. Dilute detection antibody (HRP/anti-M13 conjugate) 1:2000 in dilution buffer and incubate for 2 h at RT.
9. Wash with washing buffer 3×10 min.
10. Freshly prepare the immunodot-developing buffer: 2 mL developing stock solution, add 10 mL TBS and 5 μL H_2O_2 . Put immediately 1 mL per blot strip and develop color reaction for approximately 30 min in the dark under constant agitation. Stop the reaction by washing with ddH₂O and allow strips to air-dry.

3.5. Colony Screening Assay

3.5.1. Immunoblot Method

3.5.1.1. RANDOM SELECTION OF PHAGE COLONIES AND TRANSFER ON FILTER MEMBRANES

1. Use plates from the last panning round (or another round of interest) which were used for determination of phage titers. Select single bacterial clones randomly and streak approximately 50 single clones on an LB/carb plate, using in each case a new sterilized tooth stick or sterile pipet tip. Prepare two identical plates. Number the clones on the back of the two identical plates to guarantee later identification.
2. Grow colonies on both plates overnight at 37°C.
3. Seal copy colony plate with parafilm and store reversed at 4°C. Use the other plate for the following procedure.
4. Chill plate at 4°C for 30–60 min.
5. Wet nitrocellulose filters 30 min prior to use by placing them in Petri dishes with IPTG solution and then place them on filter paper to air-dry (determine the number of filters according to planned, following immunoassay) (*see Note 4*).
6. Apply IPTG-treated nitrocellulose filter on the colony plate, wait 1 min, then remove the filter, and place it on a new LB/carb plate overnight at 30°C (several copies can be done, *see Note 5*). Store original colony plate at 4°C for later identification of clones.
7. Chill all the plates at 4°C for 30–60 min.
8. The following procedures can be done on a normal bench.

9. Chill shock: Carefully remove the nitrocellulose filters from the agar using a forceps and put them (colony side up) for 10 min onto aluminum foil separating them from dry ice (see **Note 6**).
10. Transfer filters into a chloroform atmosphere for 30 min.
11. The filters may now be used for the immunoblot (see **Section 3.5.1.2.**) or stored separated by in-between paper in a plastic Petri dish at -20°C until usage.

3.5.1.2. COLONY SCREENING IMMUNOBLOT

1. Immerse each filter in 25 mL blocking buffer and put on a shaker for 30 min.
2. Remove the blocking buffer, add 25 mL of lysozyme buffer on each filter, and agitate for 1 h.
3. Remove the buffer, add PBS/casein (10 mL per Petri dish), and agitate for 1 h.
4. Incubate each filter with 10 mL of the antibody used for biopanning diluted in PBS/casein and one filter with a control antibody.
5. Wash each filter 3×10 min using 10 mL PBS-T.
6. Add second antibody binding to the selection antibody conjugated with HRP for 1 h, and thereafter develop each filter with developing solution.
7. Align positive clones on the colony screening filter with the clone number on plate and pick the corresponding clone from the copy plate for amplification and further analysis (see **Note 7**).

3.5.2. ELISA Method

3.5.2.1. RANDOM SELECTION OF PHAGE COLONIES AND TRANSFER ONTO MICROTITER PLATES

Procedure is the same as in **Section 3.5.1.1.**, points 1–4, for selection of random colonies.

1. Place 270 μL SB medium per well onto microtiter plates.
2. Transfer single colonies from plates in **Section 3.5.1.1.**, point 4, by sterile pipet tips into the wells.
3. Incubate overnight at 37°C , sealed with parafilm, agitating at 160 rpm.
4. Centrifuge plates at 3000 rpm, 30 min, 4°C .
5. Use supernatants for detection of specificity in **Section 3.5.2.2.**

3.5.2.2. COLONY SCREENING ELISA

1. Coat fresh microtiter plates with selection antibody in coating buffer, 100 μL /well, overnight at 4°C (negative control: no coating and coating with irrelevant antibody, positive control: direct coating of phages).
2. Wash $5 \times$ with PBS-T, 200 μL /well.
3. Block plates with blocking buffer, 120 μL , 1 h at 37°C , and 1 h at 4°C .
4. Wash $5 \times$ with PBS-T, 200 μL /well.
5. Add supernatants with phages from ELISA plates I (**Section 3.5.2.1**, point 5), 80 μL /well in duplicates (on negative controls: SB medium or XL-1 supernatant without phages, on positive control: SB medium).

6. Incubate overnight at 4°C.
7. Collect supernatants with phages and store at -20°C for further usage. (see **Note 7**).
8. Wash plates 5× with PBS-T, 200 µL/well.
9. Apply antibody HRP/anti-M13 conjugate, diluted 1:1000 in PBS-T/0.1% BSA, 100 µL/well and incubate 1 h at 37°C and 1 h at 4°C.
10. Wash 5× with PBS-T, 200 µL/well.
11. Apply ABTS solution and measure optical density at 405–490 nm.

4. Notes

1. Be careful! The fluid may be superheated and may boil over when swirled, use protection gloves.
2. Fresh plates will “sweat” when incubated at 37°C. This allows bacterial colonies to spread across the surfaces of the plates and increases the risk of cross-contamination. To avoid this, the plates should be incubated 1–2 h at 37°C in an inverted position before they are used. Moreover, any condensation from the lids of the plates should be wiped off. The liquid can be removed by shaking the lid with a single, quick motion. To minimize the possibility of contamination, hold the open plate in an inverted position while removing the liquid from the lid.
3. Longer storage of phages in PBS/casein or in glycerol can be done at -20 or -70°C.
4. This assay is primarily a means for the identification of phages specifically selected by the antibody used for biopanning. However, filters with phage clones may also be tested with other antibodies suspected to crossreact with this epitope. This is a very direct method for proof of crossreactive epitopes (e.g., IgG vs IgE epitope of allergen).
5. On the same plate four or more filters can be applied, but each filter should be fixed on the plate for about 5 min before removal and then placed on a new LB/carb plate overnight at 30°C.
6. If dry ice is not available, ice for chilling can be treated with NaCl salt achieving a temperature of approximately -25°C.
7. Further procedures with single phage clones may for instance comprise DNA sequence analysis and alignment of the deduced amino acid sequence of the mimotopes with the natural antigen's or allergen's sequence (**6,16,21**), immunological cross-inhibition assays for determination of crossreactive allergen epitopes (**22**), or immunization experiments (**7,17,23**).

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