

Detection of Food Allergens by Phage-Displayed Produced Antibodies

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Abstract

Phage display is a powerful tool to produce recombinant antibodies against a given antigen without animal immunization. This technology employs libraries of recombinant bacteriophages that display billions of different functional antibody fragments on their surface. They are selected by panning in vitro against the target antigen in search for specific binders. In this chapter, we describe the selection of single chain variable fragment (scFv) antibodies to be used for detection of allergenic proteins from nuts in food products. The artificial libraries TomLinson I+J (MRC Laboratory of Molecular Biology and MRC Centre for Protein Engineering) were employed that resulted in successful phage-ELISA systems for detection of almond and walnut proteins in commercial food products.

Key words Phage display, scFv, Biopanning, Allergen, ELISA

1 Introduction

Immunoassays used to detect allergenic ingredients in foodstuffs generally depend on the availability of antibodies raised in animals. However, current trends toward the replacement of live animals in procedures aim for alternatives not entailing their use, as an attempt to enforce the Three Rs principle [1].

Phage display technology relies on the expression of billions of different peptides or antibody fragments on the surface of filamentous phages [2, 3]. Best phage binders are selected by an in vitro selection procedure called biopanning, which is followed by the recovery of target-bound phage particles [4]. Once specific clones are isolated from the library and their specificity and sensitivity are assessed, antibody-codifying nucleotide sequences can be re-engineered by adding new domains that confer specific properties to the antibody–target complex.

In this work, TomLinson I+J commercial phage libraries were screened in search for phage-antibodies that would specifically recognize proteins from almond [5] and walnut. These synthetic libraries contain over 100 million different scFv fragments (V_H and V_K domains connected to one another by a flexible Glycine-Serine linker) cloned in an ampicillin-resistant phagemid vector pIT2 (HIS, MYC tag), and transformed into TG1 *E. coli* cells [6]. The antibody fragment (scFv) is displayed as a fusion with the terminal phage III protein [7]. TomLinson I+J repertoires are based on a single human V_H framework (V3-23/D47 and J_H4b) paired with a single V_K (O12/O2/DPK9 and J_K1), with diversity introduced in the antigen-binding site [8]. The CDR3 of the heavy chains was designed to be as short as possible yet still able to form an antigen binding surface. It is recommended to use both repertoires in parallel in order to obtain the maximum number of binders.

Specific phage-antibodies that bind to different target molecules can be isolated from the phagemid repertoires following an affinity procedure called “panning”. After each round of panning, the non-binders are washed away and the phagemids bound to the target molecules are eluted and amplified by infection into fresh TG1 cells. The process should be repeated twice or up to four times to ensure that more than half scFv selected bind specifically to the target antigen. The selected phage-scFvs can be screened for binding using a simple ELISA based protocol [5, 9, 10]. Finally, the proteins A and L can be used for detection, purification, or immobilization of the target-specific scFv selected from the TomLinson I+J repertoires [11]. The protocol described in this chapter is based on the work of Lee et al. 2007 [8], but includes some modifications to improve the selection of phage-antibodies specific for the target allergenic food, avoiding cross-reactions with non-target allergens.

2 Materials

Prepare all solutions with ultrapure water and reagents. Prepare and store all reagents at room temperature (RT) unless indicated otherwise.

2.1 Material

1. DynaBeads® M-280 Tosylactivated. Store at 6 °C.
2. Magnet (DynaMag™).
3. Mixer allowing tilting and rotation of tubes (e.g. Hulamixer® sample mixer).
4. Nunc™ MaxiSorb 96-well plates.
5. Nunc™ Immuno Stick.
6. Nunc™ Biobanking and Cell Culture Cryogenic Tubes.

7. 0.45 and 0.2 μ M syringe filters.
8. UV-Vis spectrophotometer.
9. Sterile filter pipette tips.
10. Petri dishes of 90 mm and 140 mm diameter.
11. Incubators at 37 °C and 30 °C with shaker (250 rpm).
12. Centrifuge for conical tube and plate.
13. Water bath.

2.2 Reagents for the Library

1. Protein extraction buffer: 0.035 M Na_2HPO_4 , 1 M NaCl, pH 7.5. Dissolve 12.54 g Na_2HPO_4 in 800 mL of deionized water. Adjust pH to 7.5, then add 58.4 g NaCl and water up to 1 L.
2. Pierce™ BCA Protein Assay Kit.
3. TomLinson I and J scFv libraries (distributed by MR Technology, Cambridge, UK). Check all libraries and stocks are frozen. Keep at -80 °C until needed. Freeze your own stocks at -80 °C.
4. Phagemid antibody libraries I and J.
5. Helper phage KM13.
6. Optimized antibody expression strain HB2151 and controls (anti-bovine serum albumin scFv in *E. coli* TG1TR).
7. Glycerol stocks of *E. coli* TG1 TR strain.
8. Glycerol stock of positive control clones (beta-galactosidase-specific dAb and anti-bovine serum albumin scFv in *E. coli* TG1 TR strain).
9. Glycerol stocks of negative control clone (phagemid).

2.3 General Reagents

1. TYE ampicillin agar plates: Dissolve 15 g Agar, 8 g NaCl, 10 g bacto-tryptone, and 5 g yeast extract for 1 L of double deionized water (ddH_2O). Autoclave. Cool down to 50 °C and add 1 mL of ampicillin solution (*see Note 1*). Pour plates. Plates can be stored at 4 °C for up to 4 weeks.
2. M9 minimal medium plates: Dissolve 15 g agar in 800 mL of ddH_2O . Autoclave. Cool down to 50 °C and add 200 mL 5xM9 salts, 10 mL 20% glucose, 1 mL of 1 M MgSO_4 , 100 μ L of 1 M CaCl_2 , and 1 mL of 1 mg/mL VitB1. Pour plates. Plates can be stored at 4 °C for several months (*see Note 2*).
3. M9 salts (5x solution): Add 64 g Na_2HPO_4 , 15 g KH_2PO_4 , 5 g NH_4Cl , and 2.5 g of NaCl to 1 L of ddH_2O and autoclave.
4. 2xTY medium: Dissolve 16 g bacto-tryptone, 10 g yeast extract, and 5 g NaCl in 1 L of ddH_2O . Autoclave. Cool to RT (25 °C) and add antibiotic solutions and glucose solution as required. Can be stored at 4 °C for up to 4 weeks.

5. H-TOP Agar: Dissolve 10 g Tryptone, 8 g NaCl, 7 g Agar, 5 mM MgCl_2 (1 g of $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$) in 1 L ddH₂O. Autoclave (*see Note 3*).
6. Ampicillin solution: Dissolve ampicillin powder at 100 mg/mL in ddH₂O. Filter through 0.2 μM filter. Aliquot in 1 mL portions. Can be stored at -20°C indefinitely. Thawed aliquots should be freshly diluted 1000-fold into medium or agar.
7. Kanamycin solution: Dissolve kanamycin powder at 50 mg/mL in ddH₂O. Filter through 0.2 μM filter. Aliquot in 1 mL portions. Can be stored at -20°C indefinitely. Thawed aliquots should be freshly diluted 1000-fold into medium or agar.
8. PBS buffer: 1 $\times$ phosphate buffer, pH 7.4. Dissolve 1.44 g Na_2HPO_4 , 0.2 g KCl, 0.24 g KH_2PO_4 , and 8 g NaCl in 1 L of ddH₂O. Adjust to pH 7.4 and autoclave. Can be stored at 4°C for several months.
9. PBS buffer 10 $\times$: 2 g KCl, 2.4 g KH_2PO_4 , 80 g NaCl, 14.4 g Na_2HPO_4 . Dissolve in approximately 800 mL of ddH₂O, adjust pH to 7.4, then fill up to 1 L. Autoclave and store at RT. Dilute tenfold to be used for ELISA immunoassay.
10. MPBS: PBS buffer supplemented with 2% skimmed milk powder (w/v). Filter through 0.45 μM filter. Use directly and discard remaining buffer. Do not store.
11. Trypsin solution: Dissolve trypsin powder at 10 mg/mL in PBS (trypsin stock). Freeze in 20 μL aliquots at -80°C . Can be stored in 100 μL at -20°C for several months. For the experiment, dissolve 50 μL of trypsin stock in 450 μL of PBS.
12. Glucose solution 20%: Dissolve 100 g of glucose (dextrose) in 0.5 L of ddH₂O. Autoclave. Can be stored at 4°C for several months (*see Note 4*).
13. PEG solution 20% PEG- 2.5 M NaCl: Dissolve 100 g of PEG 6000 and 73 g of NaCl in 500 mL of ddH₂O. Filter through 0.2 μM filter or autoclave. Can be stored at RT for up to a year.
14. BSA-PBS: PBS buffer supplemented with 3% (w/v) or 1% (w/v) bovine serum albumin depending on ELISA step. Use directly and discard remaining buffer. Do not store.
15. OVA-PBS: PBS buffer supplemented with 1% (w/v) ovalbumin. Use directly and discard remaining buffer.
16. Glycerol 70%: Add 70 mL glycerol and 30 mL of ddH₂O. Autoclave (*see Note 5*).
17. Buffer A: 0.1 M borate buffer, pH 9.5. Dissolve 6.18 g H_3BO_3 (MW 61.83) in 800 mL ddH₂O. Adjust pH to 9.5 using 5 M NaOH and adjust volume to 1 L with ddH₂O. Autoclave (*see Note 6*).

18. Buffer B: 0.1 M Na-phosphate buffer, pH 7.4. Dissolve 2.62 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ and 14.42 g $\text{Na}_2\text{HPO}_4 \cdot 2 \text{H}_2\text{O}$. Adjust volume to 1 L with ddH_2O . Autoclave (*see Note 6*).
19. Buffer C: 3 M ammonium sulfate in Buffer A or B. Dissolve 39.64 g $(\text{NH}_4)_2 \text{SO}_4$ in Buffer A or B. Adjust pH with NaOH or HCl. Adjust up to 100 mL with Buffer A or B. Autoclave (*see Note 6*).
20. Buffer D or Blocking Buffer: PBS pH 7.4 with 0.5% (w/v) BSA. Add 0.88 g NaCl and 0.5 g BSA to 80 mL 0.01 M sodium-phosphate pH 7.4. Mix thoroughly and adjust volume to 100 mL with 0.01 M sodium-phosphate pH 7.4. Filter through 0.2 μM (*see Note 7*).
21. Buffer E or Washing and storage Buffer: PBS pH 7.4 with 0.1% (w/v) BSA. Add 0.88 g NaCl and 0.1 g BSA to 80 mL 0.01 M sodium phosphate pH 7.4. Mix thoroughly and adjust volume to 100 mL with 0.01 M sodium-phosphate pH 7.4. Filter through 0.2 μM (*see Note 7*).
22. Anti-M13 antibody horseradish-peroxidase (HRP) conjugate. Recognizes phage-antibodies through the M13 major coat protein (pVIII).
23. TMB solution (3,3',5,5'-tetramethylbenzidine; Sigma cat. No. T0440-1L).
24. Stop solution for ELISA: Add 5.5 mL sulfuric acid (96%) to 200 mL of water.
25. My TaqTM Mix (Bioline cat n° 25042).

3 Methods

3.1 Protein Extraction

The same protocol is used to prepare extracts from reference allergenic nuts, and from unknown samples. To avoid cross-contamination, do not handle different allergenic nuts at the same time or in the same working surface. Clean bench thoroughly and change gloves between samples.

1. Grind the sample into small pieces (0.5 mm wide approximately) to facilitate the extraction, using a pestle.
2. Weigh 200 mg of ground sample to extract in a 2 mL tube.
3. Add 1800 μL of protein extraction buffer. Homogenize the mix with a disposable conical pestle (*see Note 8*).
4. Mix thoroughly for 10 min using Hula-Mixer, with vertical rotation, tilting, and vibration.
5. Centrifuge at $15,000 \times g$ for 30 min.
6. Carefully take aqueous phase only (*see Note 9*).

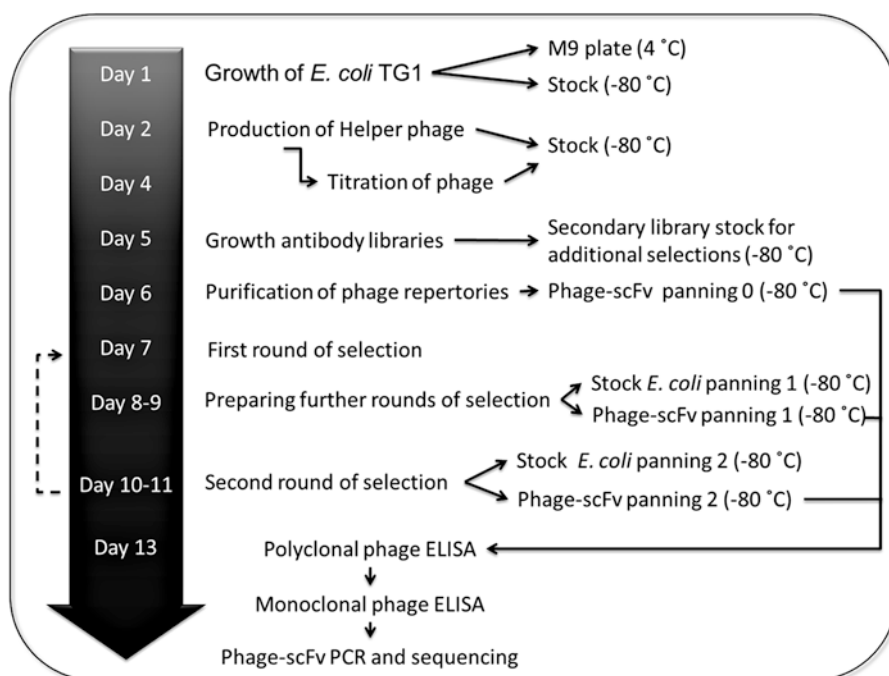


Fig. 1 Overall procedure

7. Filter the aqueous phase through 0.45 μ m syringe filter.
8. Measure protein concentration employing Bicinchoninic acid assay (Pierce™ BCA Protein Assay Kit, Thermo Scientific™).
9. Store in 100 μ L aliquots at -20°C .

3.2 Isolation of Allergen-Specific Phage-scFv from the Libraries

The overall procedure can be completed in 2 weeks (Fig. 1).

DAY 1:

3.3 Growth of *E. coli* TG1 and Production of Large Quantities of Helper Phage

1. Streak *E. coli* TG1 from the glycerol stock on a M9 minimal medium and incubate overnight at 37°C (Only grow *E. coli* 1 day to avoid cilia losing).
2. Pick a single colony from M9 minimal medium plate in a flask with 10 mL 2xTY medium (no antibiotics or glucose) and grow shaking at 37°C 5 h, until OD = 1. (Use 2xTY medium as blank).
3. Plate 50 μ L of the 2xTY culture in a plate of TYE with 100 μ g/mL ampicillin and 50 μ L in a plate with TYE with 50 μ g/mL kanamycin. Incubate at 37°C during 2–3 days to check for the absence of growth.

4. Streak the culture also in M9 minimal medium to have a stock plate with isolated colonies, incubate overnight at 37 °C only for 1 day, and then keep in the fridge.
5. Prepare stock vials of TG1 TR with the remaining 10 mL 2xTY culture, by adding 1.2 mL to a cryotube with 600 µL of glycerol 70%, and freeze at -80 °C.

DAY 2:

6. Pick a single colony from M9 minimal medium plate into 5 mL 2xTY medium, incubate at 37 °C, 250 rpm until OD600 = 0.4.
7. Prepare 14 tenfold serial dilutions of KM13 helper phage in PBS.
8. Add 10 µL of diluted KM13 helper phage to 200 µL TG1 culture each. Incubate in water bath at 37 °C for 30 min without shaking.
9. Take only the last nine phage dilutions in order to get well separated plaques and add 210 µL of each dilution into each vial with 3 mL pre-warmed H-top agar. Mix gently with culture and spread quickly over surface of pre-warmed TYE agar plates with 1% glucose (no antibiotics). H-top agar should be completely melted in microwave distributed in glass vials with 3 mL and cooled to 42 °C in a water bath before use. This step should be performed carefully, one vial at a time, to avoid H-top agar solidification.
10. Incubate overnight at 37 °C.

DAY 3:

11. Pick a single colony of *E. coli* TG1 from M9 minimal medium into 5 mL 2xTY and grow at 37 °C, until OD600 = 0.4.
12. Pick a small plaque with a sterile tip from TYE plates with H-top and place into 5 mL TG1 culture at OD600 = 0.4. Grow for 2 h at 37 °C, 250 rpm.
13. Dilute 100-fold into 500 mL of 2xTY medium. Shake in 2 L flask for 1 h at 250 rpm at 37 °C. Then, add kanamycin to a concentration of 50 µg/mL and grow overnight at 250 rpm at 30 °C.

DAY 4:

14. Spin the culture at $10,800 \times g$ for 15 min at 4 °C in ten 50 mL conical centrifuge tubes.
15. To purify phage by PEG precipitation, add 400 mL supernatant to 100 mL of PEG solution, previously cooled. Incubate on ice for 1 h. In this step a kind of fog can be observed inside the tube corresponding to helper phage.
16. Spin at $10,800 \times g$ for 30 min at 4 °C. Pour away supernatant with PEG. Resuspend the pellets in a total 8 mL PBS and add 2 mL PEG solution. Mix well and leave for 20 min on ice.

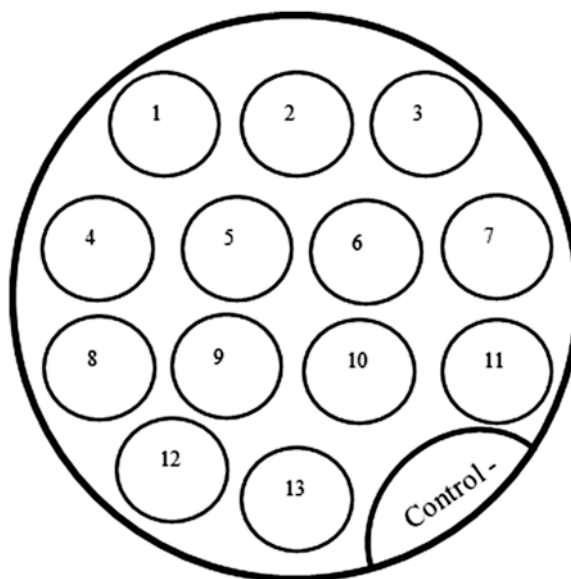


Fig. 2 Representative layout of a phage titration plate

17. Spin at $3300 \times g$ for 30 min. Respin briefly and aspirate any remaining dregs of PEG/NaCl. Resuspend pellet in 5 mL PBS, distribute in microcentrifuge tubes and spin at $11,600 \times g$ for 10 min in a microcentrifuge to remove any remaining bacterial debris. Be careful to take only the supernatant and transfer to a new tube.
18. Store the helper phage at 4°C for short-term storage or in PBS with 15% glycerol for long-term storage at -80°C .

3.4 Titration of the Helper Phage

1. Test trypsin cleavage: Add 5 μL of trypsin solution to 45 μL phage, and incubate for 30 min at 37°C in a water bath.
2. Dilute 10 μL of trypsin-treated phage to 90 μL of PBS and make 100-fold serial dilutions of this in 1 mL aliquots of PBS. Perform the same dilution series using 10 μL of non-trypsin-treated phage.
3. Take 10 μL of each dilution series and infect 200 μL each of TG1 bacteria with $\text{OD}_{600} = 0.4$ (prepared as Subheading 3.3, step 6). Incubate in a water bath for 30 min at 37°C .
4. Spot 10 μL from each phage dilution, treated and not treated with trypsin, on TYE plates supplemented with kanamycin 50 $\mu\text{g}/\text{mL}$ and incubate at 37°C overnight (Fig. 2). As negative control, plate 10 μL of not infected TG1. The number of colonies obtained from trypsin-treated phage should be at least 10^6 -fold lower than that non-treated phage. If not, discard helper phage preparation and start from a different plaque.

- To calculate the concentration of Helper phage:

$$\frac{PFU}{mL} = \frac{Colonies\ number \times Dilution\ factor}{Plated\ volume\ (mL)}$$

- Phage can be frozen in liquid nitrogen (add 20% glycerol) and stored at $-80\ ^\circ\text{C}$ for many years.

DAY 5:

3.5 Growth of Phage-Antibody Libraries (See Note 10)

- Add the frozen antibody library stock into 200 mL 2xTY medium supplemented with 1% glucose and 100 $\mu\text{g/mL}$ ampicillin. The presence of 1% glucose allows the effective suppression of antibody expression during bacterial growth. Measure OD600 in a UV-Vis spectrophotometer. It should be approximately 0.1.
- Grow culture at $37\ ^\circ\text{C}$, 250 rpm in a 2 L glass flask until OD600 = 0.4 (approximately 1–2 h).
- Take 100 mL of this culture and add 2×10^8 [11] KM13 helper phages. Use the rest of the culture to make secondary bacterial stock of the library following Subheading 3.6.
- Incubate in a water bath at $37\ ^\circ\text{C}$ for 30 min.
- Spin the culture at $3200 \times g$ for 10 min. Resuspend in 200 mL of 2xTY medium with 0.1% glucose, 100 $\mu\text{g/mL}$ ampicillin, and 50 $\mu\text{g/mL}$ kanamycin.
- Incubate at $30\ ^\circ\text{C}$ with shaking overnight in a 2 L glass flask.

3.6 Make Secondary Stock of Libraries.

- Grow the remaining 100 mL (from Subheading 3.5, step 3) for a further 2 h shaking at $37\ ^\circ\text{C}$.
- Spin down the cells at $10,800 \times g$ for 10 min. Resuspend in 10 mL of 2xTY containing 15% glycerol.
- Store this secondary stock in $20 \times 500\ \mu\text{L}$ aliquots at $-80\ ^\circ\text{C}$. Use one aliquot for each phage preparation if you wish to perform additional selections.

DAY 6:

3.7 Purification of Phage Repertoire by PEG Precipitation

- Spin the overnight culture from Subheading 3.5, step 6 at $3200 \times g$ for 30 min at $4\ ^\circ\text{C}$.
- Add 20 mL PEG solution to 80 mL supernatant. Mix well and leave for 1 h on ice.
- Spin $3200 \times g$ for 30 min at $4\ ^\circ\text{C}$. Pour away supernatant with PEG/NaCl. Respin briefly and aspirate any remaining dregs of PEG/NaCl.
- Resuspend the pellet in 1 mL PBS and spin at $12,000 \times g$ for 10 min in a microcentrifuge to remove any remaining bacterial debris.

5. Store the phage at 4 °C for short-term storage or in PBS with 15% glycerol for long-term storage at –80 °C.
6. **To titer the phage stock** dilute 10 µL phage in 90 µL PBS, and repeat serial dilutions, until there are 13 dilutions in total. Add 200 µL of TG1 at an OD 600 of 0.4 to each tube and incubate at 37 °C in a water bath for 30 min. Spot 10 µL of each dilution on a TYE plate containing 100 µg/mL ampicillin and 1% glucose and grow overnight at 37 °C. Phage stock should be 10^{12} – 10^{13} /mL, enough for at least ten selections. Store a part of this eluted phage (around 200 µL) to be used as “Panning 0” in ELISA, and use the remaining for panning 1.

3.8 First Round of Selection on Immuno Stick Paddles (See Note 11)

1. Coat Nunc™ Immuno Stick paddles overnight at 4 °C with 100 µg/mL of the required antigen in 1.5 mL PBS. The efficiency of coating can depend on the antigen concentration, the buffer, and the temperature. Usually, 10–100 µg/mL antigen in PBS is used (*see* **Note 12**).

DAY 7:

2. Wash paddles in the tube three times with PBS (fill the tube with PBS, screw and unscrew the paddle several times, and pour PBS out. Repeat three times).
3. Fill tubes with 3% BSA in PBS to block paddles. Incubate standing on the bench for 1 h at 37 °C.
4. Wash paddles in the tubes three times with PBS.
5. Add 1 mL phage (10^{12} phages) from Subheading 3.7, **step 5** (or from **step 6** stock) in 500 µL of 6% BSA in PBS. Add this mix to the tube containing the paddle coated with the non-target allergen. Incubate for 60 min at 25 °C rotating in the Hula-Mixer. Discard the paddle coated with non-target allergen and change it by the paddle coated with the target allergen. Incubate for 60 min at 25 °C rotating in the Hula-Mixer and for a further 60 min standing on the bench. Discard supernatant.
6. Wash paddle in the tube ten times with PBS.
7. Transfer the paddle to a new tube containing 500 µL of trypsin-PBS (50 µL of 10 mg/mL trypsin stock solution added to 450 µL PBS) and incubate rotating for 10 min at RT using Hula-mixer to elute the phage bound to the paddle.
8. Add 250 µL of the eluted phage to 1.75 mL of TG1 at an OD 600 of 0.4 (Prepared as in Subheading 3.3, **step 6**). The remaining eluted phage should be stored at 4 °C in case it is necessary to repeat infection. Incubate for 30 min at 37 °C in a water bath without shaking.
9. Take 10 µL of TG1 infected with eluted phage and dilute in 990 µL of PBS to obtain 10^2 dilution, and make two 100-fold serial dilutions of this to obtain 10^4 and 10^6 dilutions.

Pour 50 μL of each dilution on TYE plates containing 100 $\mu\text{g}/\text{mL}$ ampicillin and 1% glucose and grow overnight at 37 °C to titer the phage. Incubate at 37 °C overnight (*see Note 13*).

10. Spin the remaining TG1-infected culture at $11,600 \times g$ in a microcentrifuge for 5 min. Resuspend the pelleted bacteria in 1 mL of 2xTY and plate 500 μL on two large Petri dishes (140 mm) containing TYE, 100 $\mu\text{g}/\text{mL}$ ampicillin, and 1% glucose. Grow plates at 37 °C overnight.

DAY 8:

3.9 Preparing Further Rounds of Selection

1. After growing overnight culture in two large plates, add 1 mL of 2xTY medium to each one. Scrape cells from the agar using a glass spreader, and mix them thoroughly in a microcentrifuge tube.
2. Prepare 50 mL of 2xTY medium with 100 $\mu\text{g}/\text{mL}$ ampicillin and 1% glucose and inoculate with 50 μL of the scraped TG1 culture. Grow shaking at 37 °C until OD600 = 0.4. Store the remaining bacteria at -80 °C making aliquots of 500 μL in 15% glycerol (stock *E. coli* panning 1).
3. Take 10 mL of this culture and add 5×10^{10} [10] helper phage. Incubate without shaking in a 37 °C water bath for 30 min.
4. Centrifuge at $3200 \times g$ for 10 min. Resuspend the pellet in 100 mL of 2xTY with 100 $\mu\text{g}/\text{mL}$ ampicillin, 50 $\mu\text{g}/\text{mL}$ kanamycin, and 0.1% glucose. Incubate overnight at 30 °C with shaking.

DAY 9:

5. Centrifuge the overnight culture at $3200 \times g$ for 15 min.
6. Purify phage by PEG purification as described in Subheading 3.7 (Purification of phage repertoire by PEG precipitation).
7. Once resuspended in 1 mL PBS (Subheading 3.7, step 4), the precipitated phage can be stored at 4 °C for up to 1 week or used immediately for the second round of selection. Keep an aliquot (150 μL) of this precipitated phage, at -80 °C with 15% glycerol, labeled as “phage-scFv panning1”, to be used in pools ELISA.

3.10 Preparation of Paramagnetic Beads for the Second Round of Selection

This protocol is based on use of 5 mg (165 μL) Dynabeads® M-280 Tosylactivated. It is not recommended to couple <5 mg beads at a time.

1. Wash of paramagnetic beads by resuspending the beads in a vial. Vortex for >30 s.
2. Transfer 165 μL of beads (5 mg) to a 2 mL screw cap tube.
3. Add the same volume of Buffer A or B and resuspend.
4. Place the tube in a magnet for 1 min and discard the supernatant.

5. Remove the tube from the magnet and resuspend the washed beads in the same volume of Buffer A or B as the initial volume of beads (**step 3**)
6. Transfer 165 μL of washed beads to a new screw cap tube. Place the tube in a magnet for 1 min and discard the supernatant.
7. Resuspend the beads in 100 μg of ligand for each 5 mg of beads (*see Note 14*). Add buffer A or B to a total volume of 150 μL . Mix thoroughly by vortex or pipetting. Add 100 μL Buffer C and mix by vortexing or pipetting.
8. Incubate in Hula-Mixer at 37 °C for 12–18 h (total volume = 250 μL).

DAY 10:

9. After incubation with the protein extract, place the tube on a magnet for 2 min and remove the supernatant.
10. Remove the tube from the magnet and add 1 mL Buffer D (blocking buffer), vortex 30 s and incubate at 37 °C for 2 h in Hula-mixer.
11. First spin the tube and then place it on a magnet for 2 min and remove the supernatant.
12. Remove the tube from the magnet and add 1 mL Buffer E (washing buffer), vortex for 5–10 s. Place the tube on a magnet for 2 min and remove the supernatant.
13. Repeat **step 12** once more.
14. Resuspend and dilute the beads in 1 mL of Buffer E. Vortex and split in two tubes (500 μL each). For the Panning it is enough to use 2.5 mg of beads. Store the remaining 2.5 mg of beads at 4 °C.

3.11 Second Round of Selection on Paramagnetic Beads

1. Take 500 μL (2.5 mg) of the beads coated with non-target allergen, place the tube on the magnet 2 min and remove the supernatant.
2. To perform the negative panning add 1 mL of eluted phage-antibodies from Subheading 3.9, **step 7** (10^{12} pfu) together with 500 μL BSA 6% to the tube. Incubate rotating in Hula-mixer for 1 h at 25 °C.
3. Place the tube on the magnet 5 min and collect the supernatant (from beads coated with non-target allergen or negative panning). The phage-antibodies not bound to these beads are used for positive panning in the tube containing beads coated with target allergen.
4. Place the tube containing 500 μL (2.5 mg) of beads coated with the target allergen on the magnet for 2 min, and remove the supernatant. Add the supernatant collected from

the previous step (negative panning) to the beads. Incubate in Hula-mixer for 1 h at 25 °C, and without shaking for a further hour at 25 °C.

5. Place the tube on the magnet 5 min and discard the supernatant.
6. Continue to wash with Buffer E. Repeat 20 times: Add 1 mL Buffer E, vortex the tube, place the tube on the magnet 1 min, discard supernatant.
7. Transfer the beads into a new tube to avoid eluting phage-antibodies stuck on the tube wall.
8. Add 500 µL of trypsin solution with PBS (50 µL of Trypsin with 450 µL of PBS). Incubate for 10 min in Hula-mixer at 25 °C.
9. Place the tube on the magnet 5 min and gather the supernatant with the target-specific phage-antibodies.
10. Repeat the same process with trypsin-eluted phages as described above for round 1 (from Subheading 3.10, steps 8–10).

DAY 11–12:

11. Obtain large quantities of phage-antibodies from round 2 using protocol described in Subheading 3.9, to continue with the third panning. Store infected bacteria (from Subheading 3.9, step 2) as “stock *E. coli* panning 2”, and precipitated phage (from Subheading 3.9, step 7) as “phage-scFv panning 2”.

3.12 Third Round of Selection on Immune Stick Paddles (Optional)

Exactly proceed again with the same protocol as is described in Subheading 3.8. After each round of selection, check the specificity of eluted phage-antibodies by ELISA.

DAY 13:

3.13 Screening Phage Particles by ELISA

Population of phage produced at each round of selection can be screened by ELISA for binding to the target and non-target allergen in order to identify “polyclonal” phage-antibodies with increased affinity to the target. Also, phages from single colonies can be screened by ELISA in order to identify “monoclonal” target-specific phage-antibodies. In general, we have found that 1% powder milk in PBS is best for blocking during monoclonal phage ELISA whereas 1% Ovalbumin in PBS is best for blocking in polyclonal phage ELISA.

3.13.1 Polyclonal Phage ELISA

1. Coat a Nunc™ MaxiSorb 96-well plate overnight with 100 µL per well of 1/200 protein extract in PBS (*see Note 15*). Incubate for 1 h at 37 °C.
2. Wash wells five times with PBS. You should check that all wells fill with wash solution (if they do not you may create false positives during further washes). Discard liquid by flipping plate

over and then shaking it. Add 200 μL per well of 1% MPBS (1% skimmed milk powder in PBS) or 1% OVA (1% ovalbumin in PBS) to block, and incubate for 2 h at RT, or 1 h at 37 °C, or overnight at 4 °C.

3. Wash wells five times with PBS. Add 1 μL PEG precipitated phage from the end of each round of selection in 100 μL 1% MPBS (or 1% OVA-PBS) to each well.
4. Incubate for 1 h at RT. Discard phage solution and wash ten times with PBS (*see* **Note 16**).
5. Add 1 in 5000 dilution of HRP-anti-M13 in 1% MPBS (or 1% OVA-PBS). Incubate for 1 h at RT. Wash 5 times with PBS.
6. Add 100 μL of TMB solution to each well and leave at RT for 2–15 min. A blue color should develop in positive wells.
7. Stop the reaction by adding 50 μL 1 M sulfuric acid. The blue color should turn yellow.
8. Read the absorbance at 450 nm.

Figure 3 shows representative polyclonal ELISA results for selection of almond-specific and walnut-specific phage-scFv.

3.13.2 Monoclonal Phage ELISA

1. Inoculate individual colonies from the titration plates from each round of selection (Subheading 3.8, step 9) into 200 μL 2xTY containing 100 $\mu\text{g}/\text{mL}$ ampicillin and 1% glucose in 96 cell-well plates. Grow shaking (250 rpm) two or more hours at 37 °C.
2. Transfer a small inoculum (about 2 μL) from this plate to a second 96 cell-well plate containing 200 μL of 2xTY with 100 $\mu\text{g}/\text{mL}$ ampicillin and 1% glucose per well. Grow shaking (250 rpm) at 37 °C for 2 h. (Make glycerol stocks of the original 96-well plate, by adding glycerol to a final concentration of 15%, and then storing the plates at –80 °C).
3. After 2 h incubation (of the second plate) add 25 μL 2xTY containing 100 $\mu\text{g}/\text{mL}$ ampicillin, 1% glucose and 10^9 helper phage.
4. Shake (250 rpm) at 37 °C for 1 h. Spin $1800 \times g$ for 10 min, then aspirate off the supernatant.
5. Resuspend pellet in 200 μL 2xTY containing 100 $\mu\text{g}/\text{mL}$ ampicillin and 50 $\mu\text{g}/\text{mL}$ kanamycin. Grow shaking (250 rpm) overnight at 30 °C.
6. Spin at $1800 \times g$ for 10 min and use 50 μL of the supernatant (instead of the PEG precipitated polyclonal phage) in phage ELISA as detailed above.

Figure 4 shows the phage-ELISA results of individual clones after Planning 2 in the selection of walnut-specific phage-antibodies.

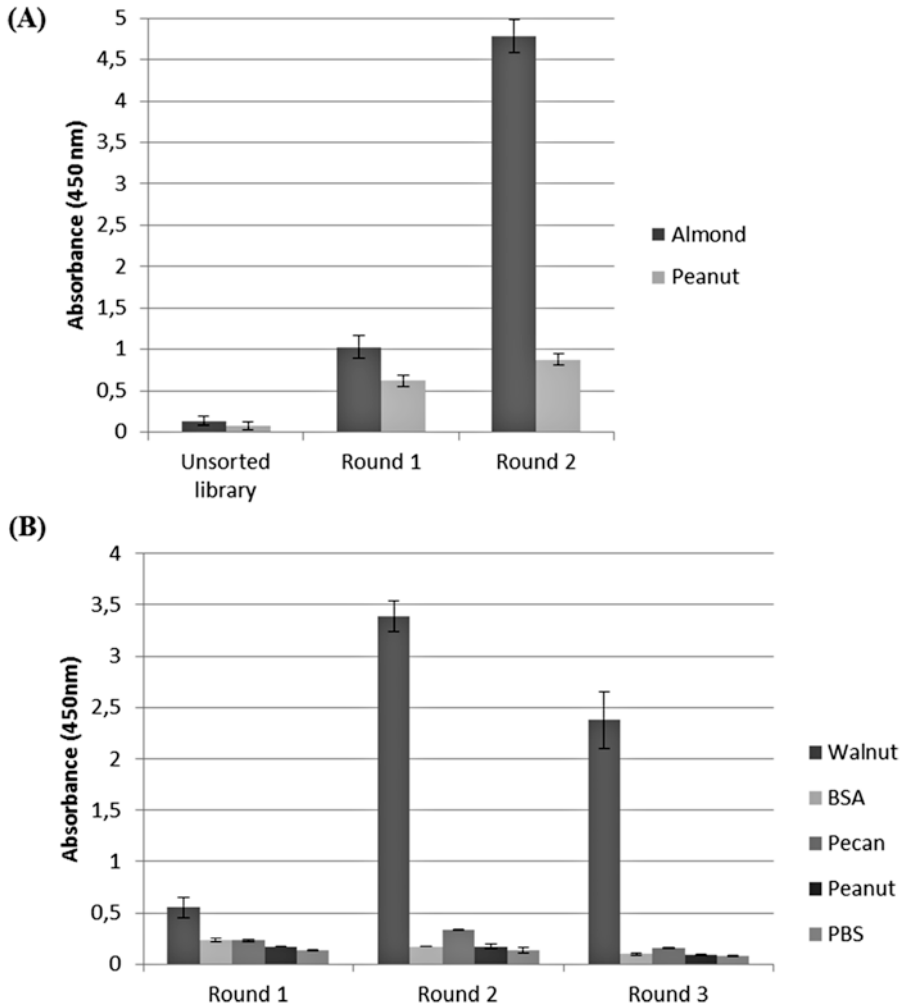


Fig. 3 Indirect phage-ELISA results obtained with polyclonal phages rescued at each round of selection against almond (a) and walnut (b) extract. Precipitated phages from each round of selection were analyzed by ELISA. Absorbance values are the mean of three independent determinations. Error bars show the standard deviation for each set of data

3.14 PCR Screening Selected Clones

Individual clones selected by ELISA can be further characterized through a PCR screening to check the presence of full length V_H and V_k insert. Primers used and expected sizes depending on the fragments to be amplified are shown in Table 1.

To perform PCR analysis we used My TaqTM Mix (ready-to-use 2x mix for fast and highly-specific PCR) for a total volume of 25 μ L per reaction (Table 2). All reactions must be set-up on ice and then subjected to temperature cycles for amplifications of the target sequences (Table 3).

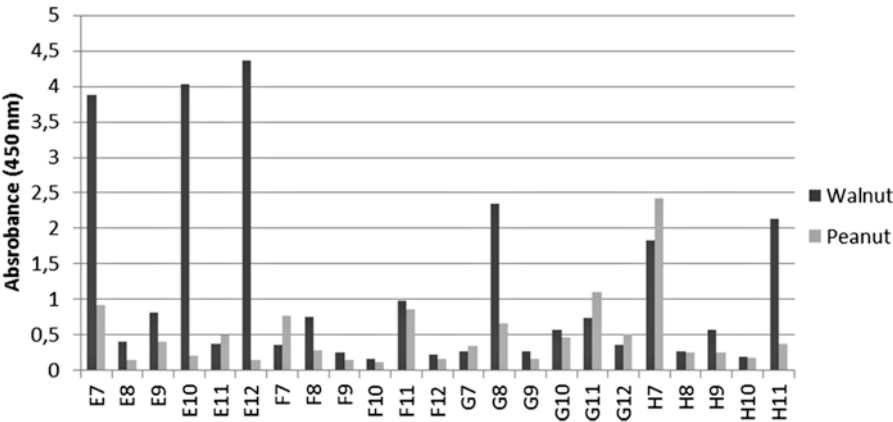


Fig. 4 Indirect phage-ELISA results obtained with monoclonal phages (phages obtained from individual colonies) against walnut extract after the second panning. Clone E12, later renamed as JR35, was selected after ELISA and sequencing characterization

Table 1
Primers used for PCR screening of individual clones

Fragment	Primers (5'-3')	Expected size
Whole insert VH + Vκ	Fw- LMB3: CAG GAA ACA GCT ATG AC Rev.- pHEN seq: CTA TGC GGC CCC ATT CA	With whole insert: 935 bp Without insert: 329 bp With only V _H : 625 bp With only V _κ : 635 bp
VH only	Fw- LMB3: CAG GAA ACA GCT ATG AC Rev.- link seq new: CGA CCC GCC ACC GCC GCT G	With insert: 527 bp Without insert: 227 bp
Vκ only	Fw-DPK9 FR1: CAT CTG TAG GAG ACA GAG TC Rev.- pHEN seq: CTA TGC GGC CCC ATT CA	With insert: 368 bp Without insert: no band

Table 2
Preparation of PCR reactions for screening of individual clones

Component	Concentration	Volume per sample (μL)
Template	Culture from Subheading 3.13.2, step 2 or pick a colony from Subheading 3.8, step 9	2
Primers (Fw and Rev)	20 nmol/mL each	0.5
My Taq™ Mix		12.5
Water (ddH2O)		Up to 25
Final volume PCR mix		25

Table 3
PCR cycling conditions for screening of individual clones

Step	Temperature	Time	Cycles
Initial denaturation	95 °C	1 min	1
Denaturation	95 °C	15 s	30
Annealing	User determined (usually 2–5 °C below the lower T _m of the primer pair)	15 s	
Extension	72 °C	10 s	

3.15 Sequencing- Selected Clones

For sequencing of selected clones, the following primers are recommended.

For VH use link seq new 5'-CGA CCC GCC ACC GCC GCT G-3'.

For Vk use pHEN seq 5'-CTA TGC GGC CCC ATT CA-3'.

The nucleotide sequences obtained for the almond- and walnut-specific scFv (Fig. 5) are shown as an example, and also comparison of their deduced amino acid sequences (Fig. 6).

4 Notes

1. TYE plates are used in this protocol without, with 1%, and with 0.1% of glucose. For 1 L you must add 50 mL or 5 mL of glucose solution 20%, respectively. Add the glucose together with the antibiotic. Depending on required final volume, you must add the quantity of water necessary to maintain the glucose concentration. Plates should be dried in flow-bench before use.
2. MgSO₄, CaCl₂, and Vitamin B1 (Thiamine Hydrochloride) must be filtered through 0.2 µM filter in a laminar flow cabinet and used fresh that day.
3. Pour H-Top agar plates and store the excess for weeks at 4 °C. This medium solidifies very easily. It is important to warm up in a water bath (55 °C) before use.
4. It is advisable to separate the solution into smaller bottles before sterilization to avoid contamination.
5. To handle glycerol it is advisable to use a micropipette for viscous liquids.
6. Buffers A, B, and C are used for prewashing and coupling of Dynabeads® M-280 Tosylactivated. Buffer A is the recommended buffer for coupling. For pH labile ligands, use Buffer B. Do not add any protein (apart from your specific protein ligand), sugar, etc., to these buffers.

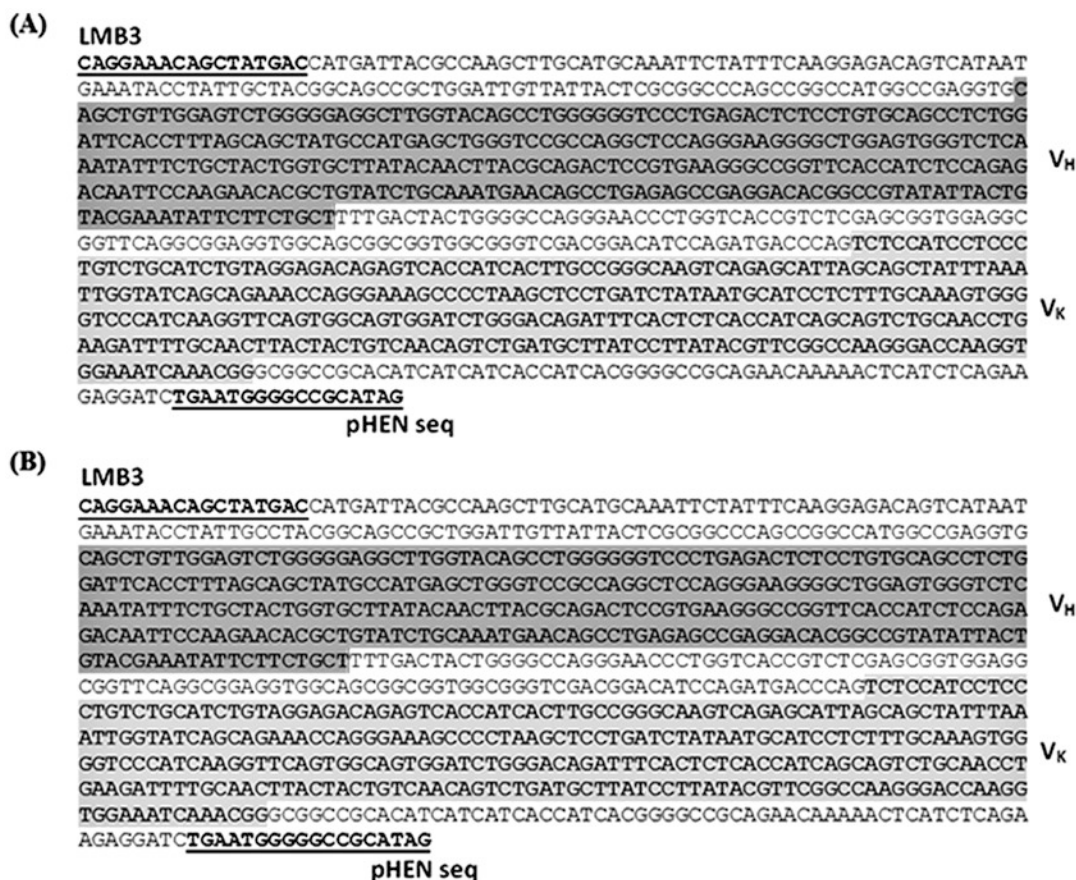


Fig. 5 Sequencing of pIT2 vector with primers LMB3 and pHEN. Sequence of V_H and V_K region specific to almond (a) and walnut (b). In yellow primers LMB3 and pHEN. In pink the sequence of V_H region and in grey V_K region

7. Buffers D and E are used for washing of all ligand-coupled beads. Buffer E can also be used for storage of ligand-coupled beads. Do not use these buffers or any buffer containing protein or aminogroups (e.g. glycine, Tris) for pre-washing or coupling to these beads. Protein blocking is recommended as it reduces aggregation and non-specific binding. If a preservative is needed for the coupled beads, a final concentration of <0.1% (w/v) sodium azide may be added to Buffer E.
8. The disposable pestle can be fabricated in the lab by sealing a glass Pasteur pipette or a micropipette tip with a flame.
9. Three layers are usually observed after centrifugation: a sediment, intermediate aqueous phase with proteins and DNA, and some floating particles.
10. It is advisable to run through the protocols using the control clones before you use the library: Streak the controls on TYE plates containing 100 µg/mL ampicillin and 1% glucose.

		H-CDR1		H-CDR2	
Almond	QPGGSLRLSCAAS	GFTFSSY	AMSWVRQAPGKGLEWVSAIT	SYGSDTY	ADSVKGRFTISR
Walnut	QPGGSLRLSCAAS	GFTFSSY	AMSWVRQAPGKGLEWVSNISAT	GAYTTY	ADSVKGRFTISR
			H-CDR3		Linker
Almond	DNSKNTLYLQMNSLRAEDTGVYYCAK	SAYDFD	YWQGTLTVSSGGGGSGGGGSGGGGST		
Walnut	DNSKNTLYLQMNSLRAEDTAVYYCTK	YSSAFD	YWQGTLTVSSGGGGSGGGGSGGGGST		
		L-CDR1		L-CDR2	
Almond	DIQMTQSPSSLSASVGDRVTIT	CRASQSI	SNWYQQKPGKAPKLLIYSA	SALQ	SGVPS
Walnut	DIQMTQSPSSLSASVGDRVTIT	CRASQSI	SNWYQQKPGKAPKLLIYNASSL	Q	SGVPS
			L-CDR3		Hys-Tag
Almond	RFSGSGSGTDFTLTISLQPEDFATYYC	QQGASD	PTTFGQGTKVEIKRAAAHHHHHHGAA		
Walnut	RFSGSGSGTDFTLTISLQPEDFATYYC	QSDAYP	TFGQGTKVEIKRAAAHHHHHHGAA		
Almond	EQKLISEEDLNGAA				
Walnut	EQKLISEEDLNGAA				

Fig. 6 Amino acid sequences of the almond [5] and walnut scFvs deduced from the nucleotide sequences by Expasy Website. Positions of the respective complementary determining regions for the variable domains of heavy (H-CDR 1-3) and light (L-CDR 1-3) chains are indicated

After overnight growth at 37 °C in an incubator pick a single colony from each and grow these overnight (shaking at 37 °C) in 5 mL 2xTY containing 100 µg/mL ampicillin and 1% glucose. Make phage for the positive and negative controls separately (use 500 µL of overnight in Subheading 3.9). Use a 1:100 mixture of phage produced from the positive and the negative controls and perform one round of selection (Subheading 3.8) using 100 µg/mL of BSA in PBS for coating. Check for enrichment of BSA binders (should be over 50% after one round of selection) by monoclonal phage ELISA (Subheading 3.12.2).

11. The first round of selection (enrichment of antigen-specific phage by binding to immobilized antigen) is the most important one. Any errors or loss of diversity made at this point will be amplified in subsequent rounds of selection.
12. To increase specificity of the phage selection for the target allergen, we first perform a negative panning with a non-target allergen. Thus, it is necessary to coat one immuno stick with the target allergen and a second one with a non-target allergen (e.g., for selection of phage-antibodies against almond, one immune stick is coated with 100 µg/mL of almond extract and the other with 100 µg/mL of peanut extract).
13. This method of phage titration allows recovery of isolated colonies to be analyzed by the monoclonal phage ELISA, after the second round of selection. After each round you should obtain at least 100 infective phages. If not, you must repeat the process, using the remaining 250 µL of eluted phage from

Subheading 3.8, step 8. If you obtain less than 100 phages again, repeat the selection starting at Subheading 3.8, step 1.

14. The concentration of protein extract must be calculated for each specific quantity of beads, for example, using 5 mg of beads, calculate the necessary volume of sample/protein extract to have a final concentration of 100 µg in a total volume of 150 µL with buffer A. To increase specificity of the phage selection for the target allergen, we perform a negative panning on paramagnetic beads in a similar way as explained in panning 1 with immuno sticks. Thus, it is necessary to prepare two different batches of coated beads, 5 mg of beads are coated with the target allergen in a screw cap tube (almond or walnut extract in our case) and 5 mg with the non-target allergen in a second tube (peanut extract).
15. To check specificity, include appropriate controls: wells coated with the target allergen, with non-target allergen(s), and without allergen (just PBS).
16. We do not recommend to include Tween 20 in phage ELISA, as we have observed a higher background.

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