

[7] Phage-Displayed Peptides as Tools for Characterization of Human Sera

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Phage-displayed random peptide libraries have been widely used for the selection of ligands able to bind homogeneous ligates, such as monoclonal antibodies (MAbs) or other proteins.^{1,2}

A large body of information has accumulated to corroborate the conclusion that libraries of small random peptides displayed on phage are sufficiently "complete" to contain specific ligands for almost any given antibody. During the affinity selection process, the potential ligands compete with each other for binding, and the resulting positive clones in theory represent a family of *best* peptide ligands which fit a given antigen-binding site (or portions of it).

Developing the strategy of using sera for the selection of phage-displayed libraries has imparted several very important advantages; when the sera are of pathological interest, it could lead to the discovery of disease-related epitopes, and these could have diagnostic and/or prognostic value and could even be exploited for identifying unknown etiologic agents.

Naturally, when whole sera are used as ligates, the outcome of the selection is a large and heterogeneous population of mimotope-bearing phage clones (phagotopes), most of which are not related to the antigen of interest. Thus, to identify specific phagotopes, affinity-purified antibodies, or elution using the antigen or individual screening of the selected clones through competition would be required; all these procedures would then be feasible only when the original antigen is known and available.

This chapter describes a more general strategy in identifying disease-specific phagotopes, which avails itself only of clinically characterized sera from patients and normal individuals. This strategy involves a three-step protocol: affinity selection with one disease-specific serum, immunoscreening of the selected phage mixture with other patient's sera, and counter-screening using many different sera not related to the specific disease. The phage clones that are not recognized by all or most of the "negative"

¹ R. Cortese, F. Felici, G. Galfrè, A. Luzzago, P. Monaci, and A. Nicosia, *Trends Biotechnol.* **12**, 262 (1994).

² F. Felici, A. Luzzago, P. Monaci, A. Nicosia, M. Sollazzo, and C. Traboni, in "Biotechnology Annual Review" (M. R. El-Gewely, ed.), Vol. 1, Chapter 5. Elsevier, Amsterdam, 1995.

sera and yet react with antibodies contained in patients' sera can thus be identified and characterized. This entire process is therefore workable without the requirement of, or even information about, the etiologic agent or its antigens.

Phage Affinity Selection

Phage peptide libraries with homogeneous ligates, such as MAbs, are generally affinity selected through multiple rounds of selection and amplification,³ which enrich positive phage clones, eliminating most of the background phage.

Two methods can be used for affinity selection: (1) partially purified immunoglobulins are directly coated onto the surface of polystyrene beads⁴ or (2) immunoglobulins are tethered on the surface of magnetic beads through the use of an anti-human Fc secondary antibody covalently linked to the beads.

The latter method has the advantage of orienting the serum antibody molecules, thus maximizing the number of antigen-binding sites free to interact with the target peptides and avoiding the partial denaturation that may occur by directly coating antibodies on beads. The ratio of secondary antibody-coated beads to serum immunoglobulins can be regulated in order to minimize isolating phage clones that interact with the antigen-binding site of the secondary antibody.

Selection I

1. Beads (6.4 mm; Precision Plastic Ball Co., Chicago, IL) are added to 10 $\mu\text{g}/\text{ml}$ of ammonium sulfate-precipitated immunoglobulins in 50 mM sodium carbonate buffer, pH 9.6, and incubated at 4° for 12–16 hr.
2. The beads are washed three times in 1 $\times$ phosphate-buffered saline (PBS), then once in water, for 10 min each time. At this stage beads can be stored frozen at -20°. Beads are subsequently blocked with bovine serum albumin (BSA), 10 mg/ml in 50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% (w/v) Tween 20 (TBS/Tween), and incubated for 4 hr (or longer) at 4°. The beads are briefly washed with TBS/Tween.
3. Incubate 12 coated beads with 1×10^{13} particles per ml of UV-killed M13K07 phage in TBS/Tween/1 mg/ml BSA. Use enough solution to fill the tube and incubate for 4 hr at 4°.

³ J. K. Scott and G. P. Smith, *Science* **249**, 386 (1990).

⁴ A. Folgori, R. Tafi, A. Meola, F. Felici, G. Galfrè, R. Cortese, P. Monaci, and A. Nicosia, *EMBO J.* **13**, 2236 (1994).

4. Add the phage peptide library (pVIII-9aa⁵ or pVIII-9aa.Cys,⁶ 10^{11} – 10^{12} phage particles) and incubate for 12–16 hr at 4°.
5. Wash the beads 10 times with TBS/Tween for 10 min each time at 4°.
6. Elute phage by adding 2.4 ml of 0.1 *N* HCl/1 mg/ml BSA, pH 2.2 (adjusted with glycine), for 5 min at room temperature.
7. Neutralize the eluate by adding 240 μ l of 2 *M* Tris, pH 9.0.
8. Titrate eluted phage as transducing units on Amp (ampicillin) X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) IPTG (isopropyl- β -D-thiogalactopyranoside) plates.

Selection II

1. Resuspend magnetic bead (Dynabeads M-450, tosyl-activated, Dynal A.S, Oslo, Norway) stock by mixing vigorously and pipette 500 μ l of suspension into an Eppendorf tube. Insert the tube into the magnetic apparatus and eliminate the supernatant.
2. Wash the magnetic beads by resuspending in 1 ml of borate buffer (50 mM, pH 9.5), inserting in the apparatus, and eliminating the supernatant.
3. Resuspend the magnetic beads in borate buffer containing 75 μ g of anti-human Fc polyclonal antibody (immunopure goat anti-human IgG Fc-specific unconjugated, Pierce, Rockford, IL) in a final volume of 1 ml and incubate by rotating for 24 hr at room temperature.
4. Insert the Eppendorf tube into the magnetic apparatus, discard the supernatant, and resuspend the magnetic beads in 10 ml of 1 $\times$ PBS/0.1% (w/v) BSA, in Falcon tubes. Wash the magnetic beads by rotating at 4° once for 10 min, three times for 30 min, and then (last wash) overnight. After each washing step insert the tubes containing the magnetic beads into the appropriate magnetic apparatus and remove the supernatant. The coated beads can be stored at 4° (adding 0.02% w/v NaN₃); if they need to be stored for more than 2 weeks, wash three times with 10 ml of 1 $\times$ PBS/0.1% BSA (5 min each time) before usage.
5. Transfer magnetic beads into Eppendorf tubes and incubate with 300 μ g of ammonium sulfate-precipitated immunoglobulins in 1 $\times$ PBS/0.1% (w/v) BSA, 0.1% (w/v) Tween 20 in a final volume of 1 ml and put on a rotating wheel at 4° for 12–16 hr.

⁵ F. Felici, L. Castagnoli, A. Musacchio, R. Jappelli, and G. Cesareni, *J. Mol. Biol.* **222**, 301 (1991).

⁶ A. Luzzago, F. Felici, A. Tramontano, A. Pessi, and R. Cortese, *Gene* **128**, 51 (1993).

6. Wash the beads four times for 30 min each time at 4° with 1× PBS/0.1% BSA/0.1% Tween 20 (10 ml for each wash).
7. Resuspend the magnetic beads in 1× PBS/0.1% BSA/0.5% Tween 20 containing 1×10^{13} particles of UV-killed M13K07 in a final volume of 1 ml and put on a rotating wheel for 3–4 hr at 4°.
8. Add the library (pVIII-9aa⁵ or pVIII-9aa.Cys,⁶ 10^{11} – 10^{12} phage particles) and incubate for 12–16 hr at 4°.
9. Wash six to seven times (each wash 30 min) with 10 ml of 1× PBS/0.5% Tween 20 in Falcon tubes.
10. Transfer magnetic beads in Eppendorf tubes and elute phage with 500 μ l of elution buffer for 30 min at room temperature (elution buffer 0.1 N HCl, pH 2.2, pH adjusted with glycine, 1 mg/ml BSA).
11. Neutralize the eluate by transferring into an Eppendorf tube containing 50 μ l of 2 M Tris, pH 9.
12. Titrate eluted phage as transducing units on Amp + X-Gal + IPTG plates.

Immunoscreening Affinity-Selected Phage

The power of the phage display technology is that of allowing a large number of molecules (10^{12} or more) to be rapidly surveyed in small volumes with the ligate of interest. Several rounds of affinity selection are often necessary to eliminate unspecific clones.

This strategy does, however, present a few drawbacks: different phage can be amplified at different rates during each growth cycle that follows the selection step, thus introducing a biological selection unrelated to that obtained by affinity. Furthermore, because polyclonal antibodies have a wide range of concentrations and affinities, multiple rounds of selection could lead to enriching phage recognized only by particular subsets of antibodies. Hence, when sera are used as selectors, it becomes very difficult to predict whether multiple selection steps would effectively favor enrichment of disease-specific versus other phagotopes, as no information on the concentration and affinity of the disease-specific antibodies is available.

These problems can be overcome by reducing the number of selection rounds, and positive phage can be identified by immediately probing a large number of selected clones through immunoscreening phage transferred to nitrocellulose filters.

Generally, between 0.5 and 5 million phage are selected by a given serum starting from 10^{11} to 10^{12} phage particles from pVIII-9aa⁵ or pVIII-9aa.Cys⁶ peptide libraries. Therefore, after one cycle of affinity selection, the number of clones obtained is amenable to filter screening. The specific detection of phage fusion proteins with sera by colony blotting has so far

been hampered by high background.⁷⁻⁹ To reduce this background and to increase the signal to noise ratio, phage clones need to be plated as plaque-forming units (pfu). When using libraries constructed in phagemid vectors,¹⁰ plaque-forming units are obtained by plating phagemid-containing bacteria infected by a helper phage on a competent bacterial lawn (TB cells, prepared as described later).

Because of the polyclonality of the sera, most of the identified phage are recognized by antibodies not related to the disease. However, more than one patient serum can be used in the immunoscreening step, the rationale being that only those phage displaying epitopes recognized by antibodies showing a "common" specificity to the serum used for affinity selection and those used for immunoscreening will be identified. Among the corresponding common phagotopes there is a high percentage of disease-specific ones.

Filter immunoscreening is further exploited at a later stage to reconfirm positivity with many different patients' sera and to assess their specificity for the disease by parallel counterscreening with sera from healthy individuals. This is best performed by plaque purification and arranging single phage clones onto a matrix. This matrix is replica plated in order to obtain many filters bearing the same array of isolated clones which can be immunoscreened in parallel with many different sera.

Phage Rescue and Plating as Plaque-Forming Units

1. Infect 1 ml of XL1-Blue (Stratagene, La Jolla, CA) TB cells with the affinity-selected phage pool. Incubate for 15 min without agitation at 37° and for an additional 30 min with vigorous shaking at 37°.
2. Plate infected cells onto LB + Amp (50 µg/ml) + 1% (w/v) glucose; this is usually 10⁶ cells per 230 × 230-mm plate. Incubate overnight at 37°.
3. The next day, scrape cells from plate surface, resuspending in 50 ml of LB + Amp (50 µg/ml) + 10% glycerol. Store in aliquots at -20°.
4. Inoculate a culture from the scraped cells in LB + Amp (50 µg/ml) at a starting optical density (OD)₆₀₀ of 0.05. Incubate at 37° with vigorous shaking until OD₆₀₀ ≈ 0.2. Leave for a further 15 min without agitation.

⁷ Y. Tsunetsugu-Yokota, M. Tatsumi, V. Robert, C. Devaux, B. Spire, J. C. Chermann, and I. Hirsch, *Gene* **99**, 261 (1991).

⁸ O. O. Minenkova, A. A. Ilyichev, G. P. Kishchenko, and V. A. Petrenko, *Gene* **128**, 85 (1993).

⁹ P. Monaci and A. Nicosia, unpublished data (1994).

¹⁰ L. Dente, G. Cesareni, and R. Cortese, *Nucleic Acids Res.* **11**, 1645 (1983).

5. Infect 0.5 ml of this culture with M13K07 helper phage at a multiplicity of infection (m.o.i.) of 30. Incubate for 15 min at 37° without agitation and for an additional 45 min with vigorous shaking.
6. Spin for 5 min in a microfuge. Discard the supernatant and resuspend the bacterial pellet in 1 ml of LB. Repeat three times to get rid of all nonadsorbed helper phage.
7. Mix 30 μ l of a 10^{-2} dilution of the resuspended bacteria in LB with 0.6 ml of XL1-Blue TB cells, add 15 ml of melted-top agar, and plate onto LB plates (15 cm diameter).
8. Wet nitrocellulose filters in LB + 35 μ g/ml IPTG, let filters air dry for 10 min, lay them on the plates, and incubate at 37° overnight.
9. To measure the titer and percentage of blue clones, plate a dilution of the superinfected bacteria (usually 10^{-6}) with 0.2 ml of XL1-Blue TB cells and 5 ml of top agar onto LB + IPTG + X-Gal plates (10 cm diameter).

Filter Immunoscreening

1. Using a needle, mark the filters when still on the plate and peel them off. Place filters between two sheets of 3Chr paper (Whatman, Maidstone, England) and roll them to remove excess bacteria. If screening is not required immediately, plates with filters can be stored for a few days at 4°.
2. Block the filters in I-buffer [$1\times$ PBS, 0.1% (w/v) Nonidet P-40 (NP-40), 5% (w/v) nonfat dry milk, 0.05% (w/v) NaN_3] four times for 30 min at room temperature with gentle agitation.
3. Assemble the serum mix (typically use 20 ml of incubation mix/filter): 1:50–1:100 serum dilution in I-buffer, 10^{11} particles of wild-type phage, and 400 μ l of XL1-Blue bacterial extract (prepared as described later).
4. Preincubate the mix for 2 hr at room temperature on a rotating wheel.
5. Add the preincubated serum mix to the filters and leave it overnight at 4° with gentle agitation.
6. Wash the filters 10 times for 10 min at 4° in W-buffer ($1\times$ PBS, 0.1% NP-40) with agitation.
7. Add the AP-conjugated secondary antibody in 20 ml of I-buffer (for human sera use a 1:5000 dilution of goat anti-human IgG Fc-specific; Sigma, St. Louis, MO) to each filter and incubate for 4 hr at 4° with gentle agitation.
8. Wash the filters 10 times for 10 min at 4° in W-buffer with agitation.

9. Wash the filters once in substrate buffer (100 mM Tris-Cl, pH 9.6, 100 mM NaCl, 5 mM MgCl₂) and then incubate the filter with developing solution (prepared by adding 66 μ l of a 50-mg/ml stock solution of nitro blue tetrazolium (NBT) in 70% (v/v) *N,N*-dimethylformamide and 33 μ l of a 50-mg/ml stock solution of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 100% *N,N*-dimethylformamide, to 10 ml of substrate solution) at room temperature (typically 15 ml/filter).
10. Stop reaction by washing with H₂O.

Recovery of Positive Clones and Replica Plating

1. Mark positive clones on filters by making holes with a needle.
2. Position the filter under the plate (or carefully lay it on the agar surface) and collect phage clones by aspirating a portion of top agar in correspondence to the holes using a Pasteur pipette connected to a flask under vacuum.
3. Add 20 ml of LB and transfer collected phage plaques to 30-ml Corex tubes.
4. Spin for 10 min at 8000 rpm at 4° and then transfer supernatant into a new tube.
5. Bacteria are killed by heat treatment at 70° for 20 min and are removed by centrifugation.
6. Infect 0.6 ml of XL1-Blue TB cells with a suitable dilution and incubate for 10 min at 37° without shaking and then for 30 min at 37° with shaking.
7. Superinfect cells with M13K07 phage at a m.o.i. of ≈ 30 , incubate for 10 min at 37° without shaking, then 30 min at 37° with shaking, and plate onto LB + Amp (50 μ g/ml) + Kan (kanamycin 20 μ g/ml) + 1% glucose plates (15-cm diameters). Incubate overnight at 37°. Because isolated colonies need to be obtained, repeat plating by changing the amount of infecting phage in order to obtain the desired number of clones to be screened.
8. With a toothpick, transfer Amp^r/Kan^r colonies onto a new LB + Amp (50 μ g/ml) + Kan (20 μ g/ml) + 1% glucose 15-cm plate according to an ordered grid (grids can be more easily generated and replicated using a custom-made stainless steel multipin matrix). Add positive and negative controls to the grid in an orderly way to make plaque identification easier on the filter. Incubate overnight at 37°.
9. Plate a lawn of XL1-Blue TB cells (0.6 ml) with 15 ml of BBL top agar onto LB plates (15 cm diameter).

10. Sterilize the multipin matrix by leaving it for 5 min in CHCl_3 and then by flame, wetting with ethanol, and burning it five times. Finally, cool the multipin matrix on an LB plate.
11. Transfer colonies using the multipin matrix (or one by one, with toothpicks) from the LB + Amp (50 $\mu\text{g/ml}$) + Kan (20 $\mu\text{g/ml}$) + 1% glucose master plate to the LB plates with XL1-Blue lawn (up to seven or eight plates can be sequentially replicated).
12. Layer nitrocellulose filters on the picked plates and incubate them at 37° overnight (filters are prewetted in IPTG/LB solution as described earlier).
13. Proceed to immunoscreening as described earlier.

Bacterial Extract Preparation

1. XL1-Blue bacteria are grown in TB (20 $\mu\text{g/ml}$ tetracycline) overnight at 37°.
2. Bacteria are centrifuged and resuspended in 1/100 of the original volume of 1× PBS + 0.05% NaN_3 .
3. Cells are broken by French press lysis or sonication (or through other means) and stored in aliquots at -20°.

Preparation of Terrific Broth XL1-Blue Cells (TB Cells)

1. Inoculate 20–30 ml of terrific broth (TB) containing 20 $\mu\text{g/ml}$ tetracycline with XL1-Blue cells in a 250-ml flask. Start from fresh bacterial cells grown on a minimal medium + 10 $\mu\text{g/ml}$ tetracycline plate.
2. Grow with vigorous shaking at 37° up to $\text{OD}_{600} \approx 1.7$ (measured on a 1:10 dilution in TB).
3. Use these cells within 1 hr. The concentration of viable cells is about $5 \times 10^9/\text{ml}$.

ELISA Using Phage Supernatant and Human Sera

The third and final step of the procedure for selecting disease-specific phagotopes involves testing isolated clones for their reactivity against a large number of positive and negative sera. This analysis is carried out by ELISA¹¹ on a large number of clones and is more sensitive and quantitative than plaque immunoscreening.

Phage particles from crude supernatants of infected bacteria are tethered by an antiphage MAb onto the surface of a multiwell microplate. The

¹¹ L. Dente, G. Cesareni, G. Micheli, F. Felici, A. Folgori, A. Luzzago, P. Monaci, A. Nicosia, and P. Delmastro, *Gene* **148**, 7 (1994).

MAB, which specifically recognizes the N terminus of protein III(pIII), links the phage to the plate, leaving peptides displayed as a fusion to the major coat protein free to interact with the serum antibodies. The binding of serum antibodies to phage-borne peptides can be detected via anti-human secondary antibodies conjugated to alkaline phosphatase.

In these conditions, background resulting from the interaction of serum antibodies with bacterial proteins and other contaminants is very low.

Preparation of Phage Supernatant

1. Phage from a single plaque are picked and transferred to microtubes containing 200 μ l of phage buffer (22 mM KH_2PO_4 , 50 mM Na_2HPO_4 , 85 mM NaCl, 1 mM MgSO_4 , 0.1 mM CaCl_2 , 0.1% w/v gelatin).
2. Bacteria are killed by heat treatment at 70° for 20 min and removed by centrifugation.
3. The phage-containing supernatant is recovered and stored at 4°, and dilutions are used to infect XL1-Blue cells.
5. Infected bacteria are plated onto LB agar plates added with 50 μ g Amp/ml.
6. Single colonies are inoculated into 2 ml of LB containing 50 μ g Amp/ml and are grown at 37° with vigorous shaking (>350 rpm) until $\text{OD}_{600} \approx 0.25$.
7. Bacterial cells are incubated for 10 min with very gentle agitation to allow pili regeneration, superinfected with helper phage M13K07 (m.o.i. ≈ 30) and added with IPTG (0.1 mM final concentration), mixed gently for 10 min, and then incubated for an additional 5 hr with strong agitation, always at 37°.
8. Bacteria are eliminated by centrifugation, and NaN_3 (0.05% final concentration) is added to the phage supernatant, which is stored at 4°. Usually titers are above 1×10^{10} TU/ml.

Preparation of Anti-pIII MAb

57D1 is a monoclonal antibody generated by immunization of rats with a suspension of M13 Phage.¹¹ The monoclonal (IgG_{γ1} subclass) recognizes an epitope localized in the N-terminal portion of the minor capsid protein III of the filamentous phage M13. The supernatant from the 57D1 hybridoma is precipitated by 50% $(\text{NH}_4)_2\text{SO}_4$, resuspended in 1× PBS, dialyzed, and purified on protein G–Sephadex. The optimal MAb dilution for coating the ELISA plate is determined experimentally for each batch of purified antibody.

Carrier Phage

XL1-Blue cells are grown at 37° with vigorous shaking in TB + 20 μ g of tetracycline/ml until OD₆₀₀ $\approx$ 0.25. Cells are then infected with f1-11.1 mutant phage¹¹ at an m.o.i. of $\approx$ 30 and are grown overnight at 37° with vigorous agitation. The phage supernatant is cleared by centrifugation, precipitated by polyethylene glycol (PEG) 6000/NaCl (4% and 0.5 M final concentration, respectively), resuspended in 1/10 of the original volume of 1 $\times$ PBS + 0.05% NaN₃, and stored in aliquots at 4°.

ELISA

1. One hundred microliters of purified MAb 57D1 diluted in coating buffer (50 mM NaHCO₃, pH 9.6) is aliquoted in each well of multiwell plates (Immunoplate Maxisorp, Nunc, Denmark) and incubated for 12 hr at 4°.
2. After washing several times with washing buffer (1 $\times$ PBS/0.05% Tween 20), 250 μ l/well of blocking buffer (5% nonfat dry milk/0.05% Tween 20/0.02% NaN₃ in 1 $\times$ PBS) is added and plates are incubated at 37° for 1 hr.
3. The blocking solution is then discarded and coated plates can be used immediately or stored at -20° for several weeks.
4. A mixture of 50 μ l of blocking buffer and 50 μ l of cleared phage supernatant is added to each well.
5. Phage particles are allowed to bind to the coated MAb for 1 hr at 37°.
6. Preincubate 1 μ l of serum in 100 μ l of blocking buffer containing 2.5 μ l of XL1-Blue bacterial extract, about 5×10^{10} pfu of PEG-concentrated f1-11.1 phage, and 5 μ l of supernatant from unrelated rat hybridoma cells for 1 hr at room temperature. In this step, serum-contained antibodies directed against wild-type phage, bacterial contaminants, and rat anti-pIII MAb are titrated.
7. Discard the phage supernatant from the coated plate and wash wells several times with washing buffer.
8. Add 100 μ l/well of a preincubated 1 : 100 serum dilution in blocking buffer to each well and incubate plate at 4° for 12 hr. Serum dilution and incubation times can both vary according to the desired assay sensitivity and background to noise ratio.
9. Discard the serum dilution and wash the plate several times with washing buffer.
10. Add 100 μ l/well of alkaline phosphatase-conjugated, goat anti-human IgG (Fc-specific) Ab (Sigma, St. Louis, MO), diluted 1 : 5000 in blocking buffer, and allow to incubate at room temperature for 150 min (or at 4°C for 4 hr).

11. Wash plates several times with washing buffer and one more time with substrate buffer (10% w/v diethanolamine, 0.5 mM MgCl_2 , 0.05% w/v NaN_3 , adjusted at pH 9.8 with HCl).
12. Reveal the alkaline phosphatase reaction by adding 100 μl /well of a 1-mg/ml solution of *p*-nitrophenyl phosphate in substrate buffer.
13. The results are recorded as the difference between A_{405} and A_{655} by an automated ELISA reader (Labsystems Multiskan, Finland).

Characterization of Disease-Specific Phagotopes

It is generally important to identify the natural epitope mimicked by a selected mimotope-bearing phage (phagotope): if the etiologic agent of the disease is unknown, this analysis can lead to its identification, or when the natural antigen has been already identified, it allows its immunogenic determinants to be mapped.

The sequence of the displayed peptide often shows no similarity with the sequence of the natural antigen. This is consistent with the fact that phagotopes mimic the binding properties of the natural epitope, but do not necessarily use the same primary sequence. However, the phagotope can be used to immunopurify from a positive serum the antibodies binding to it, which can then be utilized in experimental protocols to identify the mimicked determinant.

For example, purified antibodies can be tested for their reactivity against tissue specimens through *in situ* immunohistochemistry. Their reactivity against antigens expressed in eukaryotic cells and/or against recombinant proteins expressed in cells through transfection can also be tested. Binding can be detected by immunofluorescence.

Phagotope-purified antibodies can also be tested for their reactivity against purified recombinant proteins expressed in bacteria using a standard ELISA protocol in which the plate is coated with the purified antigen. The same experimental approach can be exploited in identifying phagotopes mimicking the same epitope.

Phage Growth and Purification

1. A single phagemid-containing colony is inoculated into 5 ml of LB containing 50 μg Amp/ml, 1% glucose and is grown overnight at 37° (glucose represses the expression of the pVIII gene under the control of the lac promoter).
2. The starting culture is diluted in 400 ml of LB + Amp (50 μg /ml) to obtain an OD_{600} at ≈ 0.05 and is grown up in a 2-liter flask with vigorous shaking (at least 300 rpm) until $\text{OD}_{600} \approx 0.25$.

3. The culture is very gently agitated for 15 min to allow pili regeneration, added with IPTG (0.1 mM final concentration) to induce the pVIII expression from the lac promoter. At the same time, cells are infected with helper phage M13K07 (m.o.i. ≈ 30), mixed gently, and left for 15 min at 37° with no agitation to allow infection. Culture is then incubated for 5 hr with strong agitation, always at 37°.
4. After this step, bacteria are eliminated by centrifugation for 30 min at 5000 rpm at 4° and the supernatant is recovered.
5. Phage particles are precipitated by adding PEG 6000/NaCl (4% and 0.5 M final concentrations, respectively) and incubating on ice for at least 4 hr at 4°. The phage pellet is recovered by centrifugation for 40 min at 5000 rpm at 4° and is resuspended in one-tenth of the original volume with 1× PBS.
6. The phage suspension is then incubated for 30 min at 70° in a water bath to denature contaminating bacterial proteins that are removed by centrifugation for 30 min at 10,000 rpm at 4°.
7. PEG 6000/NaCl is again added to the resulting phage supernatant; phage particles are allowed to precipitate for 2 hr at 4° and are then recovered by centrifugation for 30 min (10,000 rpm, 4°).
8. The phage pellet is then dissolved in 1/100 of the original volume with 1× PBS, the suspension is treated for 30 min at 70° in a water bath, and then cleared by centrifugation for 30 min at 10,000 rpm at 4°. Phage concentration is determined as Amp-transducing units.

Affinity Purification

1. A 60-mm-diameter polystyrene petri dish (Falcon) is coated with 4 ml of a solution of 1×10^{11} purified phage particles/ml in NaHCO_3 , pH 9.6, for 12 hr at 4°.
2. The phage solution is discarded, the dish is washed several times with washing buffer (1× PBS/0.05% Tween 20), 6 ml of blocking buffer (5% nonfat dry milk, 0.05% Tween 20/0.02% NaN_3 in 1× PBS) is added, and the dish is incubated at 37° for 1 hr.
3. Antibodies directed against phage and bacterial contaminants are titrated by preincubating the serum with bacterial extract and carrier phage. We usually preincubate 40 μl of serum in a total volume of 4 ml of blocking buffer containing 100 μl of bacterial extract and about 4×10^{12} pfu of PEG-concentrated M13 phage for 1 hr at room temperature.
4. The blocking solution is discarded and the petri dish is washed several times with washing buffer. Four milliliters of preincubated serum mix is added to the plate and incubated at 4° for 12 hr (both

serum dilution and incubation times can vary according to the affinity of the phagotope and specific antibody concentration in the serum).

5. The serum dilution is then discarded and the dish is washed several times with washing buffer. Bound antibodies are eluted by a 10-min incubation at room temperature with 4 ml of elution buffer (0.1 M glycine hydrochloride, pH 2.7, added with 10 μ g/ml BSA).
6. The buffer is then collected and neutralized with 100 μ l of 2 M Tris-HCl, pH 9.4. Antibodies can be concentrated by Centriscart (10,000 molecular weight cutoff; Sartorius, Germany).

Phage Competition in ELISA

One piece of information that is essential to quicken mapping of the disease-specific phagotopes is the classification of the selected phage on the basis of their ability to react with the same antibodies. This can be done by phage competition in ELISA.

Because of the complexity of the library used and the possibility that a certain epitope can be mimicked by different peptidic structures, it is possible that different phagotopes are isolated which mimic the same antigenic determinant.

A sensitive method to assess this is to use the first phagotope to immunopurify antibodies binding to it from a positive serum (as described in the previous section), and then test these affinity-purified antibodies for their reactivity against different phagotopes. For this experiment the same protocol described in ELISA Using Phage Supernatant and Human Sera can be adopted, eliminating the preincubation step of the affinity-purified antibodies with carrier phage and bacterial extract.

A much faster way to accomplish this information would be to use nonpurified supernatants directly for phage competition, but it presents the drawback that the presence of the inhibiting phage would (in any case) reduce the signal due to competition for the binding to the coated MAb. This limitation can be overcome by using phage f1-11.1 as the helper in the preparation of the competing phage. f1-11.1 is a mutant of f1 which bears a substitution of the glutamic acid at position 5 of pIII with a glycine, which impairs binding of the MAb 57-D1 to pIII.¹¹ Thus, phagotopes prepared using f1-11.1 do not displace the phage tethered on the surface of the plate via MAb 57-D1, but can still compete for the serum antibodies directed at the pVIII-displayed mimotopes. The phage supernatant is then prepared according to the procedure reported in ELISA Using Phage Supernatant and Human Sera, but using the mutant f1-11.1 as a helper. Subsequently, ELISA is performed according to the previously described

procedure, but 20 μ l of supernatant from the competing phagotome is included in the serum mixture.

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[8] Phage Display Methods for Selecting Zinc Finger Proteins with Novel DNA-Binding Specificities

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Introduction

DNA-binding proteins play critical roles in cell biology, and the design of proteins with novel sequence specificities or functions may have important applications in research, biotechnology, and medicine. Recent reports have highlighted some of the potential of these design efforts. For example, proteins with designed DNA-binding specificities have been used to regulate the transcription of specific genes.^{1,2} DNA-binding domains also have been attached to other proteins, such as nucleases³ and general transcription factors,⁴ to create hybrid proteins with interesting properties. These designed DNA-binding proteins offer great promise as research tools and may eventually be used in gene therapy. However, many potential applications will require binding to novel target sites, and so the utility of these proteins may depend on our ability to design or select DNA-binding domains with desired DNA sequence specificities.

The zinc finger motif, which was first recognized in transcription factor IIIA (TFIIIA),⁵ appears to offer an especially attractive framework for constructing proteins with novel DNA sequence specificities. The zinc finger is one of the most common eukaryotic DNA-binding motifs,^{6,7} and this family of proteins can recognize a diverse set of DNA sequences (see

¹ J. L. Pomerantz, P. A. Sharp, and C. O. Pabo, *Science* **267**, 93 (1995).

² Y. Choo, I. Sánchez-García, and A. Klug, *Nature (London)* **372**, 642 (1994).

³ Y. Kim and S. Chandrasegaran, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 883 (1994).

⁴ S. Chatterjee and K. Struhl, *Nature (London)* **374**, 820 (1995).

⁵ J. Miller, A. D. McLachlan, and A. Klug, *EMBO J.* **4**, 1609 (1985).

⁶ G. R. Pellegrino and J. M. Berg, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 671 (1991).

⁷ G. H. Jacobs, *EMBO J.* **11**, 4507 (1992).