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Sequential Antigen Panning for Selection of Broadly Cross-Reactive HIV-1-Neutralizing Human Monoclonal Antibodies

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Summary

Many phage display techniques drive selection toward the isolation of highly specific antibodies. However, the identification of monoclonal antibodies that are cross-reactive has implications for the development of diagnostics, therapeutics, and vaccines against pathogens or cancer cells that are able to rapidly generate variants and escape mutants. To identify human monoclonal antibodies with high activity against HIV and broad-spectrum activity, we developed a technique termed sequential antigen panning. This methodology could be used to isolated recombinant antibodies against any antigen that shares epitopes with other antigens.

Keywords

Selection Fab; HIV gp120; gp140; Cross-reaction

1. Introduction

Phage display is a powerful methodology for selection of binders specific for any given target (1–4). After longer than a decade of development, antibody phage display has become a relatively mature methodology and has been successful in various applications (5, 6). The results from panning a phage-displayed antibody library depend largely on antigen property (solubility and purity), antibody library quality (library size and diversity, antibody format, phage fusion partner, etc.), and panning method (solid-phase or solution-phase panning). Monoclonal antibodies with high affinity (nanomolar or even picomolar) for the target of interest can be isolated from phage-displayed antibody libraries (7).

Identification of broadly cross-reactive HIV-1-neutralizing human monoclonal antibodies has implications for the development of HIV-1 therapeutics and vaccines. HIV-1 has evolved various mechanisms for evading immune surveillance (8, 9). The ability of the virus to rapidly generate mutants to escape immune responses and drugs has become a fundamental problem in prevention and treatment of HIV-1 infections. We have aimed to develop antibody-based inhibitors of viral entry that have potential for HIV-1 therapy and prophylaxis. To identify Env-specific human monoclonal antibodies with high affinity and broad-spectrum activity in viral neutralization, we developed a methodology, termed sequential antigen panning (SAP), by sequentially changing antigens during panning and screening (10). Using SAP and recombinant Env ectodomain gp140s from 89.6 and IIIB (clade B) as antigens for panning, and gp140s from 89.6, IIIB and JR-FL (clade B) as antigens for screening, we identified from an HIV-1 immune antibody Fab library a panel of human monoclonal antibodies that are broadly cross-reactive with Env from different clades (10–12). The SAP methodology that was used could also be used for any antigen that shares common epitopes with other antigens. Examples include but are not limited to rapidly

mutating viruses and cancer cells, as well as proteins that share common structural elements. Finally, variations of this methodology can be devised, including strategies to use more antigens in different orders during panning and screening.

2. Materials

2.1. Biotinylation of Antigens

1. Phosphate-buffered saline (PBS): 9.0 g/L NaCl, 144 mg/L KH_2PO_4, 795 mg/L Na_2HPO_4, pH 7.4.
2. 1 M $NaHCO_3$: 84.01 g/L $NaHCO_3$, pH 8.6.
3. 2 M glycine: 150.14 g/L glycine.
4. H_2O: molecular biology grade.
5. Freshly prepared 10 mM biotin stock: weigh 2.2 mg of EZ-Link Sulfo-NHS-LC-Biotin (Pierce) (molecular weight: 556.59), dissolve in 400 μL H_2O (*see* Note 1).
6. Microcon YM-10: regenerated cellulose 10 kDa molecular weight cut-off (Millipore) (*see* Note 2).

2.2. Preparation of Phage Library

1. 2YT medium: 16 g/L Bacto-tryptone, 10 g/L Bacto-yeast extract, 10 g/L NaCl, pH 7.0; autoclaved for 20 min at 15 psi on liquid cycle.
2. *Escherichia coli* TG1: suppressor strain (K12, Δ [lac-pro], supE, thi, hsdD5/FtraD36, $proA^+B^+$, $lacI^q$, lacZ M15) for propagation of phage particles.
3. M13KO7 helper phage (Invitrogen).
4. Ampicillin: 200 mg/mL stock, filter-sterilized through a 0.2 μm filter.
5. 20% (w/v) glucose: filter-sterilized through a 0.2 μm filter.
6. PEG/NaCl: 20% (w/v) polyethylene glycol (PEG) 6000, 117 g/L NaCl. Autoclave for 20 min at 15 psi on liquid cycle (*see* Note 3).
7. 2YTAG agar plates: 1.5% (w/v) agar in 2YT medium, autoclaved for 20 min at 15 psi on liquid cycle. After cooling to 42–45°C, supplement to final concentration of 200 μg/mL with ampicillin and 2% (w/v) glucose, and pour the medium onto 9 cm petri dishes. Keep the plates at 4°C when agar has solidified.
8. Glycerol: autoclaved for 20 min at 15 psi on liquid cycle.

2.3. Panning of Phage Library

1. 5% and 3% MPBS: 5% and 3% (w/v) skim milk in PBS.
2. Freshly prepared 100 mM triethanolamine (TEA): 700 μL triethanolamine (7.18 M original concentration) in 50 mL H_2O, diluted prior to use.
3. 1 M Tris–HCl, pH 7.5: 121.14 g/L Tris base, adjust pH to 7.5 using 1 N HCl.
4. PBST: 0.1% (v/v) Tween-20 in PBS.

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[1]Sulfo-NHS-Biotin reagents are moisture sensitive. Store the vial of biotin reagent at −20°C with desiccant. To avoid moisture condensing onto the product, equilibrate the vial to room temperature before opening.
[2]The molecular cut-off of the Microcon unit used for removing free biotin and buffer exchange into PBS depends on the size of the protein to be biotinylated. For recombinant gp140s, both YM-30 and YM-10 units can be used.
[3]Mix well after autoclaving, and then keep the solution at room temperature.

5. Streptavidin-coated Dynabeads M-280 (Invitrogen).
6. Magnetic separation apparatus: Dynal magnetic stand (Invitrogen).
7. Head-to-tail rotator.
8. Bioassay plates: Bioassay petri dish, 245 mm (VWR).
9. Recombinant $gp140_{89.6}$, $gp140_{IIIB}$, and $gp120_{JR\text{-}FL}$: produced by recombinant vaccinia viruses and purified by a combination of lentil lectin affinity chromatography using Sepha-rose 4B (Pierce) followed by size-exclusion chromatography using a HiLoad 16/60 Superdex 200 prep-grade column (Amersham Bioscience).
10. HIV-1 immune antibody Fab library: constructed using pComb3H phagemid vector and 30 mL of bone marrow obtained from three long-term nonprogressor patients whose sera exhibited the broadest and most potent HIV-1 neutralization among 37 HIV-infected individuals.

2.4. Phage ELISA

1. Coating buffer: 8.401 g/L $NaHCO_3$, 29.25 g/L NaCl, pH 8.3.
2. Horseradish peroxidase (HRP)-conjugated monoclonal mouse anti-M13 antibody (GE Healthcare).
3. 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) peroxidase substrate (Roche).
4. 96 well tissue culture plates: sterile, U bottom (VWR).

2.5. Expression of Soluble Antibody Fragments and ELISA with Soluble Antibodies

1. SB medium: 10 g/L 3-(*N*-morpholino)propanesulfonic acid (MOPS) (Sigma), 30 g/L Bacto-tryptone, and 20 g/L Bacto-yeast extract, pH 7.0. Autoclaved for 20 min at 15 psi on liquid cycle.
2. *E. coli* HB 2151: nonsuppressor strain (K12, ara, [lac-pro], thi/ F$proA^+B^+$, $lacI^q$, lacZΔ M15) for expression of soluble antibodies.
3. 2 M $MgCl_2$: 190.42 g/L magnesium chloride, anhydrous.
4. Isopropyl β-D-1-thiogalactopyranoside (IPTG), 1 M stock: freshly prepared or kept at −20°C.
5. 96 well enzyme-linked immunosorbent assay (ELISA) plates: Nunc Immuno Maxisorp flat-bottom plates (Nunc).
6. HRP-conjugated polyclonal anti-human IgG, $F(ab')_2$ antibodies (Jackson ImmunoResearch).

3. Methods

3.1. Biotinylation the Antigens

1. Take the vial of biotin reagent from the −20°C freezer and let *of* it equilibrate to room temperature before opening the vial in **step 4**.
2. Prepare the antigens. Adjust the concentration of the antigens (recombinant gp140 proteins) to 0.1–1 mg/mL in a suitable buffer (e.g., a phosphate-based buffer). Avoid buffers containing Tris or glycine—if the original buffer contains Tris or glycine, change the buffer to PBS or another suitable buffer before biotinylation.

3. Add 1/10 (v/v) of 1 M $NaHCO_3$ to the antigen solution.
4. Prepare 10 mM biotin stock: gently open the biotin bottle, weigh 2.2 mg biotin reagent, and dissolve it in 400 μL of molecular biology-grade water.
5. Add a 20-fold molar excess of biotin to the antigens.
6. Incubate at room temperature for 30 min.
7. Add 1/20 (v/v) of 2 M glycine to the mixture to terminate the reaction.
8. Dialyze the mixture against PBS (change the buffer three to four times) or use Microcon YM-10 to remove free biotin if dealing with a small volume.
9. Check the efficiency of the biotinylation reaction by dot blotting.

3.2. Preparation of Phage Antibody Library

1. Inoculate 500 μL of a glycerol stock of *E. coli* TG1 carrying an HIV-1 immune human antibody Fab library (*see* Note 4) into 500 mL 2YT medium supplemented with 200 μg/mL ampicillin and 2% glucose, grow at 37°C for approximately 3 h with shaking at 250 rpm till the optical density at 600 nm reaches 0.6.
2. Transfer 25 mL of culture into a 50 mL centrifuge tube (*see* Note 5), add 4 mL M13KO7 helper phage (>1 × 10^{11} cfu/ mL) to the tube, and gently mix by inverting the tube twice (*see* Note 6). Keep the tube stationary at 37°C for 30 min. Keep the remaining 475 mL of the culture growing for 3 h, centrifuge at 3,300 × *g* for 20 min, resuspend the cell pellet in 2YT medium containing 15% glycerol, and store the glycerol stock at −80°C for future use.
3. Centrifuge the helper phage-infected culture at 3,300 × *g* for 10 min, resuspend the cell pellet in 500 mL 2YT medium supplemented with 200 μg/mL ampicillin and 35 μg/mL kanamycin (2YTAK medium) (*see* Note 7), and grow the cells at 30°C for 16–20 h with shaking at 250 rpm.
4. Spin down the cells at 11,000 × *g* for 10 min (*see* Note 8), divide the supernatant between two 500 mL centrifuge bottles, add 1/5 (v/v) of PEG/HCl (50 mL) to each bottle, and mix well. Keep the bottle at 4°C for 1 h or more.
5. Centrifuge the solution at 11,000 × *g* for 30 min, remove the supernatant (*see* Note 9), resuspend the phage pellet in each bottle in 20 mL sterile H_2O, and combine. Add 1/5 (v/v) PEG/HCl (8 mL) to the phage suspension, mix well, and keep the mixture at 4°C for 20 min or more.
6. Centrifuge the phage solution at 11,000 × *g* for 10 min, remove the supernatant, centrifuge again for 2 min, and remove the residual supernatant.
7. Resuspend the pellet in 5 mL PBS in 15 mL tubes, and centrifuge at 11,600 × *g* for 10 min to remove cell debris. Transfer the supernatant containing phage particles to a fresh tube.

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[4]The optical density at 600 nm should be below 0.1 after inoculation. The minimum cell number of the initial inoculum should be equal to the size of the library. For example, if the library size is 1 × 10^{10}, the inoculum should contain at least 1 × 10^{10} *E. coli* TG1 cells.

[5]Try to use disposable containers if possible when handling phage particles; 1 OD_{600nm} *E. coli* TG1 culture = 8 × 10^8 cells per mL.

[6]The ratio of phage to cells for infection should be approximately 20 to 1.

[7]The cell pellet can be resuspended in 10 mL 2YTAK medium and then transferred to a 2 L flask containing 490 mL 2YTAK medium.

[8]All centrifuge steps should be done at 4°C except where indicated.

[9]All waste solutions from phage preparation need to be decontaminated by adding 10% Clorox bleach (final concentration) to the solutions and incubating for at least 2 h before disposal. This minimizes the risk of cross-contamination from one selection to another.

8. Titer the phage library. Prepare serial ten-fold dilutions of the phage in PBS, then take 1 μL of 10^{-6}, 10^{-7}, and 10^{-8} dilutions and infect each to 1 mL of log-phase *E. coli* TG1 cells by incubating at 37°C for 30 min without shaking. Spread 50 μL of cells of each infection mix onto 2YTAG plates, incubate the plates at 30°C overnight, count the colonies, and calculate the phage titer (*see* Note 10). Alternatively, the phage titer can be estimated by measuring the optical density at 280 nm (1 OD_{280nm} = 2.33×10^{12} cfu/mL).

9. Keep the phage library at 4°C for short-term storage or add 15% glycerol and keep it at −80°C for long-term storage (longer than a year).

3.3. Sequential Antigen Panning of Phage Library

1. Take 5×10^{12} to 1×10^{13} cfu phage particles from the phage library to a sterile 1.5 mL microfuge tube, add 500 μL of 5% MPBS and 100 μL pre-washed streptavidin-M280-Dynabeads, and bring the final volume up to 1 mL by adding PBS. Incubate the solution at room temperature for 1 h with gentle mixing on a head-to-tail rotator.

2. Separate the Dynabeads from the solution by letting the tube sit in a magnetic separator for a few minutes. Transfer the solution to a fresh 1.5 mL microfuge tube.

3. To the 1 mL solution of phage, add biotinylated recombinant $gp140_{89.6}$ to a final concentration of 50 nM, and incubate the antigen/phage mixture at room temperature for 2 h with gentle mixing on a head-to-tail rotator.

4. Add 100 μL prewashed streptavidin-M280-Dynabeads to the mixture, and keep rotating for 30 min.

5. Separate the Dynabeads from the solution as described in **step 2**; wash the beads ten times with PBST and ten times with PBS, using the magnetic separator to recover the biotinylated $gp140_{89.6}$with any attached phage after each wash.

6. Elute bound phage from the Dynabeads by adding 1 mL of freshly prepared 100 mM TEA and incubating the beads at room temperature for 10 min with gentle shaking on a head-to-tail rotator. Separate the beads from solution, transfer the eluted phage to a 50 mL centrifuge tube containing 0.5 mL of 1 M Tris HCl, pH 7.5, and vortex the eluted phage for 10 s.

7. Add 10 mL log-phase *E. coli* TG1 culture to the eluted phage, keep the tube still, and incubate the phage/cell mixture at 37°C for 30 min.

8. Centrifuge the mixture at 3,300 × *g* for 10 min, remove the supernatant, and resuspend the cell pellet in 1 mL 2YT medium. Titer the recovery of phage from the first round of panning by preparing serial ten-fold dilutions from an aliquot of the mixture, and spreading 50 μL from the 10^{-3}, 10^{-4}, and 10^{-5} dilutions onto 2YTAG plates and incubating the plates at 30°C overnight. Spread the rest of the bacterial mixture onto a bioassay plate containing 2YTAG agar, and incubate the bioassay plate at 30°C overnight.

9. Prepare phage for the second round of panning. Follow the methods in **Subheading 3.2**, but with decreased scale and a simplified procedure. Briefly, add 5 or 6 mL 2YT medium containing 15% glycerol to the bioassay plate and scrape the colonies of bacteria into a suspension. Take 100 μL, inoculate into 100 mL 2YTAG medium (*see* Note 11), and grow to log-phase (OD_{600nm} = 0.5–0.6) (~2 h). Take 10 mL of

[10]The phage library should have a titer of 10^{13}–10^{14} cfu/mL. If the titer is too high or too low, repeat the preparation of phage.

the culture and infect with 1.6 mL M13KO7 helper phage. Centrifuge the cells, resuspend the cell pellet in 100 mL 2YTAK, and grow at 30°C overnight. Centrifuge the culture, precipitate phage particles from the supernatant by adding 1/5 (v/v) PEG/HCl, and recover by centrifuging at 11,000 × *g* for 10 min. Resuspend the phage in 2 mL PBS and centrifuge at 11,600 × *g* for 10 min to remove bacterial debris. Keep the amplified phage at 4°C.

10. Second round of panning against 10 nM biotinylated $gp140_{89.6}$ (*see* Note 12): repeat **steps 1–8** except that the input of phage can be reduced to 1×10^{11}–1×10^{12} cfu and the antigen concentration can be reduced to 10 nM. In addition, the washing stringency is increased to 20 washes of captured Dynabeads with PBST and 10 washes with PBS.
11. Prepare phage for the third round of panning by repeating **step 9**.
12. Third round of panning against 50 nM biotinylated $gp140_{IIIB}$: Repeat **steps 1–8**. The input of phage can be maintained at that used for the second round of panning.
13. Prepare the phage library for the fourth round of panning. Repeat **step 9**.
14. Fourth round of panning against 10 nM biotinylated $gp140_{IIIB}$: Repeat **step 10**, except that the antigen is 10 nM biotinylated $gp140_{IIIB}$ and the washing stringency is increased to 20 washes with PBST and 10 washes with PBS.
15. Prepare the phage library for the fifth round of panning. Repeat **step 9**.
16. Fifth round of panning against 2 nM biotinylated $gp140_{89.6}$: Repeat **step 10** except that the antigen concentration is decreased to 2 nM.
17. Prepare the phage library for the sixth round of panning. Repeat **step 9**.
18. Sixth round of panning against 2 nM biotinylated $gp140_{IIIB}$: Repeat **step 16** except that the antigen is 2 nM biotinylated $gp140_{IIIB}$ (*see* Note 13).

3.4. Polyclonal Phage ELISA

1. Coating. Prepare 1 μg/mL $gp140_{89.6}$ and $gp140_{IIIB}$ in coating buffer, then coat each well on MaxiSorp plates with 100 μL of solution (*see* Note 14). Incubate the plates at 4°C overnight.
2. Blocking. Wash the plates four times with PBST, then add 200 μL of 3% MPBS per well and incubate the plates at 37°C for 1 h.
3. Polyclonal phage. Take aliquots of the phage stocks prepared after each round of panning and dilute into 3% MPBS. Remove the blocking buffer from the plates, then add 100 μL containing 1×10^9 cfu phage from each stock to each well. Incubate the plates at 37°C for 2 h.
4. Detecting antibody. Remove the phage solutions, wash the plates four times with PBST, and add 100 μL per well of a 1:5,000 dilution of an HRP-conjugated anti-M13 monoclonal antibody. Incubate the plates at 37°C for 1 h.

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[11] Keep the rest of the glycerol stock at −80°C.

[12] Antigen can be altered in the second round of panning. For example, the second round of panning can be done against 50 nM $gp140_{IIIB}$, the third round of panning against 10 nM $gp140_{89.6}$, and the fourth round of panning against 10 nM $gp140_{IIIB}$. In our experience, sequentially changing antigens is more efficient than alternating antigens, which means that panning against the first antigen for two to three rounds followed by panning against the second antigen for another two to three rounds is more efficient in selecting antibodies that are cross-reactive with both antigens.

[13] Keep the titering plates after the fifth and sixth rounds of panning. They will be used to prepare monoclonal phages for screening.

[14] MaxiSorp plates enhance the coating of gp140s/120s since they are envelope glycoproteins. Standard ELISA plates may be used for other antigens.

5. Substrate. Remove the detecting antibody solution, wash the plates four times with PBST, and add 100 μL per well ABTS. Allow color to develop at room temperature over 10–20 min, and then measure the optical density at 405 nm.

3.5. Preparation of Monoclonal Phage (see Note 15)

1. Inoculate well-isolated single colonies from titration plates into 100 μL 2YTAG medium in a sterile 96 well plate ("seed plate"). Grow at 37°C overnight with shaking at 220 rpm.
2. Transfer 20 μL overnight culture from each well to 180 μL 2YTAG medium containing 10^9 cfu/mL M13KO7 helper phage in a sterile U-bottom 96 well plate (*see* Note 16). Grow at 37°C for 2 h with shaking at 220 rpm to allow the bacteria to reach log phase. Keep the seed plate at 4°C for short-term storage (less than a month) or −80°C after adding 15–20% glycerol for long-term storage.
3. Spin down the bacteria in the U-bottom plate at 1,800 × *g* for 10 min, pipette off the supernatants, add 200 μL 2YTAK medium to each well, and resuspend the bacterial pellets.
4. Grow the bacteria at 30°C overnight with shaking at 200 rpm.
5. Spin down the bacteria in the U-bottom plate at 1,800 × *g* for 10 min. The supernatant can be used directly in monoclonal phage ELISA **(step 3** in **Subheading 3.6)**.

3.6. Monoclonal Phage ELISA

1. Coating. Dilute recombinant $gp140_{89.6}$, $gp140_{IIIB}$, and $gp120_{JR\text{-}FL}$ to 1 μg/mL in coating buffer, and apply 100 μL per well to MaxiSorp plates. Incubate the plates at 4°C overnight.
2. Blocking. Wash the plates four times with PBST, and add 200 μL of 3% MPBS to each well. Incubate the plates at 37°C for 1 h.
3. Monoclonal phage. Remove the blocking buffer, and add 50 μL of 3% MPBS to each well. Transfer 50 μL of monoclonal phage-containing culture supernatant to each well and incubate the plates at 37°C for 2 h.
4. Detecting antibody. Remove the solutions containing monoclonal phage and wash the plates four times with PBST. Add 100 μL of a 1:5,000-diluted HRP-conjugated anti-M13 monoclonal antibody and incubate the plates at 37°C for 1 h.
5. Substrate. Remove the detecting antibody solution, wash the plates four times with PBST, and add 100 μL per well ABTS. Allow color to develop at room temperature over 10–20 min and then measure the optical density at 405 nm (*see* Note 17).

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[15] Prepare monoclonal phage after the fifth and sixth rounds of panning if they show enrichment in polyclonal phage ELISA for recombinant $gp140_{89.6}$ and $gp140_{IIIB}$. If there is no enrichment after the sixth round of panning, perform seventh and eighth rounds of panning by repeating the fifth and sixth rounds of panning. If there is still no enrichment after the eighth round of panning, it is unlikely that cross-reactive clones will emerge from screening.

[16] To avoid phage cross-contamination, U-bottom plates are recommended.

[17] Identify phage clones that cross-react with all the antigens tested in the monoclonal phage ELISA (OD_{405nm} ≥1.0), and use corresponding cultures in the seed plate as inocula for the preparation of plasmid DNA. Send the plasmid DNA for sequencing and analyze the sequences. Express clones with unique sequences as soluble Fab fragments.

3.7. Expression and Purification of Soluble Fab Fragments

1. Prepare phagemid DNA from clones chosen from ELISA results. Transform DNA into electrocompetent *E. coli* HB2151, plate the transformed cells onto 2YTAG agar plates, and incubate at 37°C overnight.
2. Pick single colonies from fresh transformation plates and inoculate into 3- to 5 mL aliquots of 2YTAG medium. Grow at 37°C for 6–8 h with shaking at 250 rpm.
3. Transfer each culture to SB medium (inoculation: 1–5% [v/v]) supplemented with 200 μg/mL ampicillin and 20 mM $MgCl_2$. Grow the bacteria at 37°C with shaking at 250 rpm until OD_{600nm} = 0.6–0.9.
4. Induce each culture by adding IPTG to a final concentration of 0.5 mM (range: 0.1–1.0 mM) and keep growing the bacteria at the reduced temperature of 30°C with shaking at 250 rpm for 12–20 h (*see* Note 18).
5. Centrifuge the culture at 3,300 × *g* for 15 min at 4°C. Resuspend the pellet in 10 mL PBS containing protease inhibitors.
6. Sonicate the bacteria on ice in a sonic disrupter for 180 s, pulsing at 50% duty cycle, with the output control set at 5.
7. Pellet the cellular debris by centrifuging at approximately 48,000 × *g* for 30 min at 4°C. Transfer the supernatant to a clean tube. The lysate can be stored for up to 1 month at −20°C.
8. Purify the Fab fragments by protein G affinity purification.

3.8. Immunoassay of Interaction Between Soluble Fabs and Recombinant gp140s or gp120s

1. Coating. Dilute recombinant $gp140_{89.6}$, $gp140_{IIIB}$, and $gp120_{JR\text{-}FL}$ to 1 μg/mL in coating buffer, and coat at 100 μL per well to MaxiSorp plates. Incubate the plates at 4°C overnight.
2. Blocking. Wash the plates four times with PBST, then add 200 μL of 3% BSA in PBS to each well. Incubate the plates at 37°C for 1 h.
3. Primary antibody. Remove the blocking buffer and add 100 μL per well of serial three-fold dilutions of the soluble Fab antibodies, starting at a concentration of 20 μg/mL. Incubate the plates at 37°C for 2 h.
4. Secondary antibody. Remove the Fab solutions, wash the plates four times with PBST, then add 100 μL per well of a 1:2,500-diluted solution of HRP-conjugated goat anti-human IgG, F(ab′)2 polyclonal antibody. Incubate the plates at 37°C for 1 h.
5. Substrate. Remove the secondary antibody solution, wash the plates four times with PBST, and add 100 μL per well ABTS. Allow the reaction to proceed at room temperature for 10–20 min then measure the optical density at 405 nm.

[18]The final OD_{600nm} before induction, IPTG concentrations, and time for induction can vary between antibodies and should be optimized. If the Fab is toxic to the bacterial host, glucose should be used to suppress Fab expression while cultures are growing. The glucose needs to be removed before adding IPTG for induction; this can be achieved by harvesting the cells by centrifugation and resuspending the cells in fresh medium lacking glucose but containing IPTG at the desired concentration.

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