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Selection of Antibodies Against Biotinylated Antigens

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1. Introduction

Phage antibody (Ab) library selections on peptides or proteins are usually carried out using antigens (Ags) directly coated onto a plastic surface (e.g., Petri dishes, microtiter plate wells, and immunotubes). This straightforward method is easy to perform and has been shown to be successful for a diverse set of Ags (for review, *see ref. 1*). However, phage Ab selections on some proteins and especially on peptides are not always successful, which is often caused by immobilization-associated features. The main problem observed for selection on peptides is the poor coating efficiency of some peptides and the altered availability of epitopes on plastic-coated peptides. The direct coating of proteins on plastic is usually more efficient, but may also be problematic because the passive adsorption on plastic at pH 9.6 is a mechanism of protein denaturation. Under these conditions, 95% of adsorbed proteins are nonfunctional (**2,3**). This problem is not important for a classical enzyme-linked immunosorbent assay (ELISA) mostly because a small fraction of proteins having a native conformation are still detectable. However, this phenomenon can be troublesome for phage Ab library selections because phage Abs binding to epitopes, only present in denatured molecules may be selected.

Several methods have been developed to increase peptide coating, including coupling to bigger proteins (**4**) to amino acid linkers binding plastic (**5,6**) or use of the multiple antigen peptide system (**7**). The most successful method has been the indirect coating of biotinylated Ags via streptavidin: biotinylation of the peptide and immobilization via streptavidin improves the sensitivity in ELISA (**8**) and allows more efficient selection of antipeptide phage Abs (**9,10**).

In the case of phage library selection against proteins, the indirect coating via streptavidin results in higher-density coating, more uniform distribution of Ags on the well surface, and, above all, 60–70% of active molecules (2,3). The use of biotinylated peptide or protein also allows the use of paramagnetic streptavidin-coated microbeads to capture the biotinylated Ags with the phage bound to them. The interaction between the phage particle and the Ag therefore takes place in solution; Ag-bound phage are retrieved via a short incubation with the beads. This technique allows precise control of the Ag concentration and the time of exposure of the Ag to the phage Ab library, two parameters that are useful in affinity selection, e.g., during affinity maturation protocols (11,12). This interaction between Ag and phage Ab in solution leaves a maximum of epitopes available for binding and avoids the selection of scFvs with low affinity, but a high tendency to form dimers (13). The latter will be preferentially selected on Ag-coated surfaces because of their avid binding.

This chapter outlines a protocol for the chemical biotinylation of Ag, followed by the Ab phage library selection against biotinylated Ag in solution. Briefly, the selection procedure is as follows: once specific phage are bound to the Ag, paramagnetic beads, coupled to streptavidin, are added into the solution. The biotinylated Ags with bound phages are captured and the whole complex is drawn out from the suspension by applying a magnet on the side of the tube. The beads are washed several times and specific phages are eluted from the beads (*see Fig. 1*). A sensitive ELISA procedure to monitor selection using the same biotinylated Ag as used during the selection step is also included. In this protocol, the indirect coating of the Ag via streptavidin ensures maintenance of the native structure of the Ag and precoating of the plastic panning surface with biotinylated bovine serum albumin (BSA) is used to circumvent the low adsorption properties of streptavidin (**Fig. 2**).

2. Materials

2.1. Biotinylation of Ag and Selection of Phage Ab

1. Protein/peptide of interest.
2. NHS-SS-Biotin (cat. no. 21331, Pierce, IL) (*see Notes 1–3*).
3. Dialysis tubing or ultrafiltration centrifugation devices (e.g., Centricon 30 or Centricon 10, Amicon, Beverly, MA).
4. 50 mM NaHCO₃, pH 8.5; 1 M Tris-HCl, pH 7.5.
5. Streptavidin Dynabeads (M280, Dynal, Oslo, Norway) and magnetic separation device.
6. Phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBST).
7. Apparatus and buffers for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

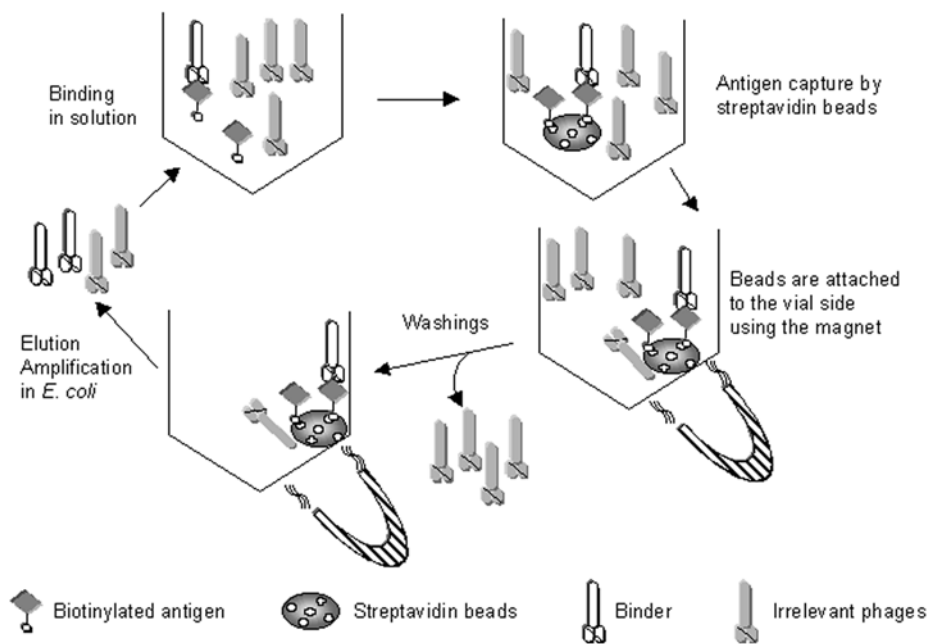


Fig. 1. Principle of phage antibody selection on paramagnetic beads.

8. Ab-phage display library, freshly amplified and titered (colony-forming U/mL).
9. 2% (w/v) and 4% Dried skimmed milk powder (e.g., Marvel) in PBS (PBSM).
10. PBS containing 5% dimethyl sulfoxide (DMSO) (*see Note 4*).
11. 2% Marvel, 2% Tween-20 in PBS (PBSMT).
12. 10 mM Dithiothreitol (DTT).
13. *Escherichia coli* TG1 and medium, helper phage, and so on, required for amplification of phage-Ab.

2.2. ELISA

1. Biotinylated Ag at a concentration of 1–5 $\mu\text{g/mL}$ in PBSM–5% DMSO (*see Note 4*). For inhibition ELISA (IE), the concentration of biotinylated Ag should be 1 $\mu\text{g/mL}$. IE also requires nonbiotinylated Ag at 1 mg/mL in 2% PBSM.
2. Selected phage-Ab clones, expressed as (*myc*-tagged) soluble Ab fragments.
3. 0.1% (v/v) Tween-20 in PBS (PBST).
4. Biotinylated BSA stock solution: 2 mg/mL in PBS. Working solution per microtiter plate: add 10 μL stock solution to 10 mL PBS.
5. Streptavidin solution: 1 mg/mL H_2O . Working solution per microtiter plate: add 100 μL stock solution to 10 mL PBS–0.5% gelatin.

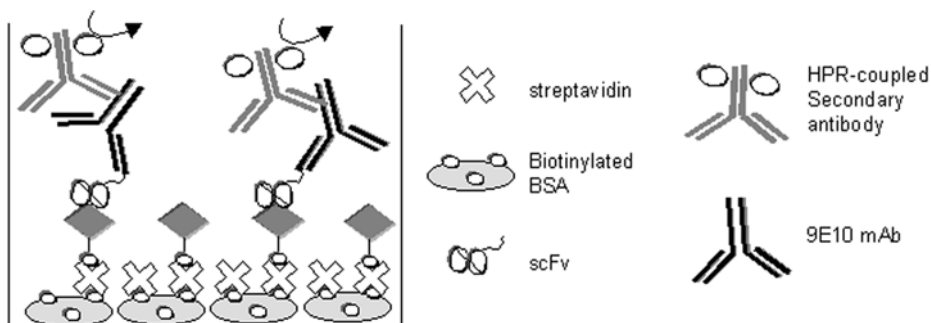


Fig. 2. ELISA using biotinylated antigen and soluble antibody fragments.

6. Anti-tag monoclonal Ab (e.g., 9E10 for *myc*-tagged Abs). Dilute in 2% PBSM according to the supplier's recommendations.
7. Rabbit anti-mouse peroxidase (RAMPO). Dilute in 2% PBSM at a concentration recommended by the supplier.
8. 10X Tetramethylbenzidine buffer (TMB). Dissolve 37.4 g Na acetate-3H₂O in 230 mL of H₂O. Adjust the pH with saturated citric acid (92.5 g citric acid-50 mL H₂O) and adjust the volume to 250 mL.
9. TMB stock. Dissolve 10 mg TMB in 1 mL DMSO.
10. TMB staining solution. Mix 1 mL 10X TMB buffer with 9 mL H₂O/microtiter plate. Add 100 μ L TMB and 1 μ L 30% hydrogen peroxidase. Make this solution fresh and keep it in the dark.
11. 96-Well, flat-bottomed ELISA microtiter plates (2 plates to screen 96 colonies).
12. For IE: microtiter plates with low coating efficiency (2/96 colonies).
13. Microtiter plate reader (for optical density 450 nm [OD₄₅₀] measurements).

3. Methods

3.1. Biotinylation of Ag

This method describes chemical biotinylation, which is the most common way to obtain a biotinylated Ag. For other alternatives, see **Notes 1–3**.

3.1.1. Chemical Biotinylation of Ag

1. Dissolve the peptide/protein of interest at a concentration of 1–10 mg/mL in 50 mM NaHCO₃, pH 8.5. If the peptide/protein is in another solvent, dialyze for at least 4 h against 1 L 50 mM NaHCO₃, changing the buffer 2–3 $\times$.
2. Calculate the amount of NHS-SS-Biotin required using a molar ratio of biotin:protein between 5 and 20:1 (see **Note 5**).
3. Dissolve the required amount of NHS-SS-Biotin in dH₂O (see **Note 6**) and immediately add to the protein sample, or, alternatively, when using larger amounts of protein, add NHS-SS-Biotin directly to the protein solution.

4. Incubate for 30 min at room temperature or for 2 h on ice if the protein is temperature-sensitive.
5. Add 1 *M* Tris-HCl, pH 7.5, to a final concentration of 50 *mM* and incubate for 1 h on ice to block any free NHS-SS-Biotin.
6. To remove the free NHS-SS-Biotin, dialyze for at least 4 h (to overnight) at 4°C against PBS, changing the buffer. Alternatively, follow **steps 7–9** below. For small peptides (<20 amino acids), alternative separation protocols (e.g., affinity chromatography, high-performance liquid chromatography) should be followed.
7. Alternative to **step 6**: spin the solution at 1000–5000*g* in an ultrafiltration device (e.g., Centricon 10 or 30) to concentrate the sample in 100 μ L.
8. Dilute the sample in PBS to dilute out free NHS-SS-Biotin left after concentration.
9. Repeat **steps 6** and **7** twice more.
10. Add Na azide to a final concentration of 0.1%.
11. Store in small aliquots at –20°C or at 4°C. Storage conditions should be tested for individual proteins.

3.1.2. Determination of Biotinylation Efficiency

It is important to determine the percentage of protein that has actually been biotinylated. If the Ag has to be used for selection in solution, the nonbiotinylated part of the preparation will be detrimental to selection, blocking specific phages, and impairing their binding to the biotinylated fraction. Hence, this nonbiotinylated fraction must represent less than 10–15%. This protocol is also used to determine the amount of biotinylated peptide captured by a certain amount of magnetic beads. Extrapolation of the results can be used for determining the concentration of Ag and amount of beads to be used during phage library selection.

1. Resuspend the streptavidin Dynabeads with gentle shaking.
2. Make five dilutions of the biotinylated protein/peptide between 5 and 50 *nM* in 200 μ L PBS.
3. Transfer 50 μ L beads into a tube that fits into the magnetic separator and add an excess of PBS; shake gently to mix.
4. Put the tube into the magnetic separation device for 2 min and pipet off the PBS.
5. Add 0.5 mL PBST and incubate for 60 min.
6. Remove the PBST as in **step 4** and resuspend the beads in 50 μ L of PBST.
7. Aliquot 10 μ L of the beads into 5 tubes and add 100 μ L diluted peptide/protein to each tube. Seal the tubes and incubate for 30 min at room temperature in an end-over-end rotator. The remaining 100 μ L of each dilution (fraction 0) will be used to evaluate the percentage of biotinylation.
8. Place the tubes into the magnet for 2 min, remove, and store 100 μ L of the supernatants (fraction 1).

9. Resuspend the Dynabeads in 1 mL PBST, place the tubes into the magnet, and discard the supernatant. Repeat 4×.
10. If the protein measurements are to be performed by SDS-PAGE, resuspend the beads in 110 μL 1X reducing SDS-PAGE sample buffer and incubate for 10 min (fraction 2a; eluted protein). Alternatively, the protein concentration can be measured by UV 280 nm. In this case, resuspend the beads in PBS containing 10 mM DTT (fraction 2b).
11. For SDS-PAGE measurements, add 10 μL 10X reducing loading buffer to fractions 0 and 1, and load samples (e.g., 10 μL and 50 μL) of fractions 0, 1, and 2a to a gel of suitable acrylamide percentage for the protein of interest. Perform SDS-PAGE, stain gel with Coomassie blue, and destain.
12. Alternatively, dilute fractions 0, 1, and 2b in an amount of PBS suitable for the quartz cuvet. Measure UV₂₈₀ absorption.
13. The percentage of protein found in fraction 2 is the percentage of biotinylation. The proteins in fraction 1 are not biotinylated.
14. If the biotinylation was efficient, check the maximum amount of biotinylated protein able to bind 10 μL streptavidin dynabeads (the highest concentration for which there is almost no protein in fraction 1). Extrapolate this amount to phage-selection conditions (e.g., a maximum of 30 nM can be bound at >85% to 10 μL beads: therefore, for 500 mM Ag used during the selections, 166 μL of magnetic beads should be used).

3.2. Selection of Abs by Means of Phage Display

1. Mix equal volumes of the phage library and 4% PBSM in a total volume of 0.5 mL. During the first selection, the number of phage particles should be at least 100× higher than the library size (e.g., 10^{12} cfu for a library of 10^{10} clones). Diversity drops to 10^6 after the first round and is thus not limiting in the next rounds.
2. Incubate on a rotator at room temperature for 60 min.
3. While preincubating the phage, wash 100–200 μL streptavidin Dynabeads/Ag sample in a tube with 1 mL PBST using the magnetic separation device as described in **Subheading 3.1.2**. The minimal amount of beads for selection can be calculated as described in **Subheading 3.1.2**.
4. Resuspend the beads in 1 mL 2% PBSM.
5. Equilibrate the beads at room temperature for 1–2 h using a rotator.
6. Add the biotinylated Ag (100–500 nM) diluted in 0.5 mL PBS (+ 5% DMSO if the Ag solubility is an issue, e.g. for certain peptides) directly into the equilibrated phage mix. Incubate on a rotator at room temperature for 30 min–1 h.
7. Using the magnet, draw the equilibrated beads to one side of the tube and remove the PBSM.
8. Resuspend the Dynabeads in the phage–Ag mix and incubate on a rotator at room temperature for 15 min (*see Note 7*).

9. Place the tubes in the magnetic separator and wait until all the beads are bound to the magnetic site (1 min).
10. Tip the rack upside down and back again with the caps closed, which will wash down the beads from the cap. Leave the tubes in the rack for 2 min, then aspirate the tubes carefully, leaving the beads on the side of the tube.
11. Using the magnet, wash the beads carefully 6× with 1 mL PBSMT.
12. Transfer beads to a new Eppendorf tube and wash the beads 6× with 1 mL PBSMT.
13. Transfer the beads to a new Eppendorf tube and wash the beads 2× with 1 mL PBS.
14. Transfer the beads to a new tube and elute the phage from the beads by adding 200 µL 10 mM DTT and rotate the tube for 5 min at room temperature (*see Note 8*). Place the tubes in the magnetic separator and transfer the supernatant containing the phages to a new tube.
15. Infect a fresh exponentially growing culture of *Escherichia coli* TG1 with the eluted phage and amplify according to standard protocols (*see Chapter 9*) to perform further rounds of selection (*see Notes 9 and 10*). Store any remaining phage eluate at 4°C.
16. Express soluble Ab fragments from the selected phage clones using standard protocols for the particular expression system.

3.3. Inhibition ELISA

The purpose of this ELISA is to identify binders among phages retrieved after each selection round. The setup of this ELISA is similar to the setup used for selection. It uses the same biotinylated Ag and an indirect coating via streptavidin, to ensure maintenance of the native structure of the Ag and precoating of the plastic panning surface with biotinylated BSA is used to circumvent the low adsorption properties of streptavidin. This ELISA uses an anti-tag (*myc*) Ab to detect soluble Ab bound to biotinylated Ag. The use of other Ab expression systems will necessitate the use of a different detection Ab.

An optional competition step (IE) allows one to ensure that the Ag is also recognized in solution by the binders. These extra steps are in parentheses at the end of some of the following steps.

1. Add 100 µL biotinylated BSA to each well of the microtiter plate. For screening colonies in 96-well plates, coat two plates (negative control and positive plates). Incubate for 1 h at 37°C or overnight at 4°C.
2. Discard the coating solution and wash the plates 3× in PBST for 5 min by submerging the plate into the wash buffer and removing the air bubbles by rubbing the plate. Following the final wash, remove any remaining wash solution from the wells by tapping on paper towels.

3. Add 100 μL /well of streptavidin to both plates. Incubate for 1 h at room temperature while shaking gently.
4. Wash the plates as described in **step 2**.
5. Add 100 μL biotinylated Ag diluted in PBS (1–10 $\mu\text{g/mL}$) to each well of the positive plate and add 100 μL of 2% PBSM to the wells of the negative control plate. Incubate for 1 h at room temperature. (For IE only: add the biotinylated Ag to both plates.)
6. Wash the plates 3 $\times$ with PBST (+ DMSO) (*see Note 4*) as described in **step 2**.
7. Block the plates with 200 μL /well 2% PBSM–DMSO and incubate for at least 30 min at room temperature.
8. Discard the blocking solution and add 50 μL /well 4% PBSM–DMSO to all the wells of both plates. (For IE only: this step must be done in two other noncoated plates with low coating efficiency. It will be used to incubate the Abs and the nonlabeled Ag).
9. Add 50 μL /well culture supernatant containing soluble Ab fragment and mix by pipeting. (For IE: add also 10 μL /well PBSM to one of the plates from **step 8** [positive] and add 10 μL /well nonbiotinylated Ag to the other plate from **step 8** [negative]. Mix by pipeting and incubate for 30 min. Discard the blocking agent of plates from **step 7**. Add 100 μL positive mix to one plate and 100 μL negative mix to the other.)
10. Incubate for 1.5 h at room temperature with gentle shaking.
11. Wash 3 $\times$ with PBST as described in **step 2**.
12. Add 100 μL /well diluted detection Ab (e.g., 9E10) to all of the wells and incubate for 1 h at room temperature with gentle shaking.
13. Wash as in **step 2**.
14. Add 100 μL /well RAMPO solution to all of the wells and incubate for 1 h at room temperature with gentle shaking.
15. Wash as in **step 2**.
16. Develop the ELISA by adding 100 μL /well TMB substrate solution. Incubate for 10–30 min in the dark until sufficient color has developed. Stop the reaction by adding 50 μL /well 2 *M* H_2SO_4 .
17. Measure the optical density at 450 nm. If the optical density of a clone on the positive plate is higher than 2 $\times$ the optical density of the same clone on the negative plate, it can be considered positive and should be tested further.

4. Notes

1. There are many commercially available reagents that can be used for biotinylation using a variety of chemistries. For most biotinylations, we prefer to use the chemical reagent NHS-SS-Biotin (sulfo-succinimidyl-2-[biotinamido]ethyl-1,3-dithiopropionate, mol wt 606.70). This molecule is a unique biotin analog with an extended spacer arm of approx 24.3 Å in length, capable of reacting with primary amine groups (lysines and NH_2 termini). The long chain reduces

steric hindrances associated with binding of biotinylated molecules to avidin or streptavidin and should not interfere with the structure of the protein/peptide involved.

2. It is also possible to efficiently biotinylate proteins using an enzymatic reaction. *E. coli* possesses a cytoplasmic enzyme, BirA, which is capable of specifically recognizing a sequence of 13 amino acids, and adding a biotin on a unique lysine present on this sequence (14). If this sequence is fused as a tag to the N- or C-terminal part of a protein, the resulting fusion will also be biotinylated. The chief advantage of this system is that the protein remains fully intact. Conversely, chemical biotinylation randomly modifies any accessible lysine. Overbiotinylation often leads to inactivation of the protein of interest, especially if a lysine is present in the active site of the protein. The use of a low ratio of biotin:protein may reduce this problem, but this may lead to poor yield of biotinylation. The enzymatic biotinylation avoids this drawback, leading to a 100% active protein, but also to a high yield of biotinylation (typically 85–95%). The “tagged” enzymatic method of biotinylating Ag has another important advantage: it allows an ideal orientation of the protein during the selection or the ELISA analysis. In both instances, the tag will be bound to streptavidin and will thus be directed toward the solid surface (beads or plastic); the rest of the molecule is perfectly oriented, available for interaction with the phage-Ab. This allows a uniform presentation of the Ag, whereas chemical biotinylation will lead to a number of Ags having the epitope of interest directed toward streptavidin and thus not available for phage-Ab binding.
3. It is also possible to perform enzymatic biotinylation in vivo if the Ag is produced in the cytoplasm of *E. coli*. In this case, the only requirement is to overexpress *birA* and add free biotin to the culture medium. The biotinylation is also efficient on intracellularly expressed proteins that form inclusion bodies. However, if the Ag has to be produced in the periplasm of *E. coli*, the biotinylation yield is poor (0.1–1%) (Chames et al., unpublished). In this case, and when the Ag is produced in another expression system, the biotinylation of the tag can still be performed in vitro on the purified protein using purified commercially available BirA. The main drawbacks of the enzymatic methods are that they cannot be applied on nonrecombinant proteins, and that the link between biotin and the Ag cannot be broken using DTT. In addition, failure to obtain good yields of biotinylation may occur because of degradation of the biotinylation tag caused by the presence of proteases co-purified with the protein of interest. Therefore, protease inhibitors must be included.
4. Check whether the Ag is water-soluble in the buffers used. If the Ag (peptide) is too hydrophobic, one must find alternative buffer conditions in which it remains in solution and use these conditions for the selection. We have, for example, successfully used 5% DMSO in all solutions.
5. Although the amount of NHS-SS-Biotin required depends on the number of lysines present within the protein, a ratio of 5:1 protein:biotin usually works

well. When enough protein is available, it is advised to test different ratios of protein:biotin. Overbiotinylation often results in nonfunctional protein (aggregation, and so on), therefore, the best molar ratio of biotin:protein must be determined empirically. Ideally, 1–2 biotinylated residues should be present per molecule. To determine the number of biotin molecules per protein/peptide, the HABA method can be used (*see* www.piercenet.com) (NHS-SS-Biotin, mol wt 606.70; NHS-LC-Biotin, mol wt 556.58).

6. Avoid buffers containing amines (such as Tris-HCl or glycine) since these compete with peptide/protein during the biotinylation reaction. In addition, reducing agents should not be included in the conjugation step to prevent cleavage of the disulfide bond within NHS-SS-Biotin.
7. If a significant proportion of the peptide/protein is not labeled, one can incubate the Ag first with the streptavidin beads, taking into account the molarity of the biotinylated peptide/protein and wash away the nonbiotinylated peptide. The beads are then used directly for the selection.
8. The presence of the S-S linker in NHS-S-S-Biotin enables the use of a reducing agent (DTT, DTE, β -mercaptoethanol) to separate the Ag and all phage-Abs bound to it from the beads. This feature allows a more specific elution, which is useful when unwanted streptavidin binders are preferentially selected from a phage-Ab repertoire. For other biotinylation chemistries, elute the bound phage with 1 mL 100 mM triethylamine, then transfer the solution to an Eppendorf tube containing 0.1 mL 1 M Tris-HCl, pH 7.4, and mix by inversion. It is necessary to neutralize the phage eluate immediately after elution.
9. For the selection of high-affinity Abs, it is advisable to perform further rounds of selection with a decreasing Ag concentration. For example, use 100 nM biotinylated Ag for the first round, 20 nM for the second round, 5 nM for the third round, and 1 nM for the fourth round.
10. The use of 10 mM DTT as elution buffer should avoid the preferential selection of streptavidin phage binders. However, if this still occurs (which may be the case when using nonimmunized or synthetic Ab libraries), deplete the library by incubating for 1 h (from round 2 on, and later) with 100 μ L streptavidin-Dynabeads before adding the biotinylated Ag to the depleted library.

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