Chapter 10

Yeast Surface Display for Antibody Isolation: Library Construction, Library Screening, and Affinity Maturation

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Abstract

Antibodies play key roles as reagents, diagnostics, and therapeutics in numerous biological and biomedical research settings. Although many antibodies are commercially available, oftentimes, specific applications require the development of antibodies with customized properties. Yeast surface display is a robust, versatile, and quantitative method for generating these antibodies and is accessible to single-investigator laboratories. This protocol details the key aspects of yeast surface display library construction and screening.

Key words Yeast surface display, Antibody engineering, Antibody fragment, Affinity maturation, Homologous recombination

1 Introduction

In contemporary biological and biomedical research settings, monoclonal antibodies occupy a privileged position as reagents, diagnostics, and—increasingly—therapeutics. The frequent use of antibodies in these settings will likely continue far into the future, as both academic and industrial researchers possess familiarity and expertise in the areas of monoclonal antibody isolation, engineering, and production. Despite the scientific community's increasing adeptness with antibodies, applications involving these proteins are still sometimes limited by specific assay or therapeutic requirements. Each experiment has its own unique considerations, and "one-antibody-fits-all" solutions can be difficult to find.

As a result, methods for isolating and engineering monoclonal antibodies continue to be critical in advancing many lines of research and therapeutic development. The invention of in vitro display methodologies presents single-investigator laboratories with an accessible, powerful alternative to traditional animal immunization and hybridoma-based monoclonal antibody isolation. Most work with display methodologies can be performed at the benchtop or with the aid of increasingly ubiquitous flow cytometry

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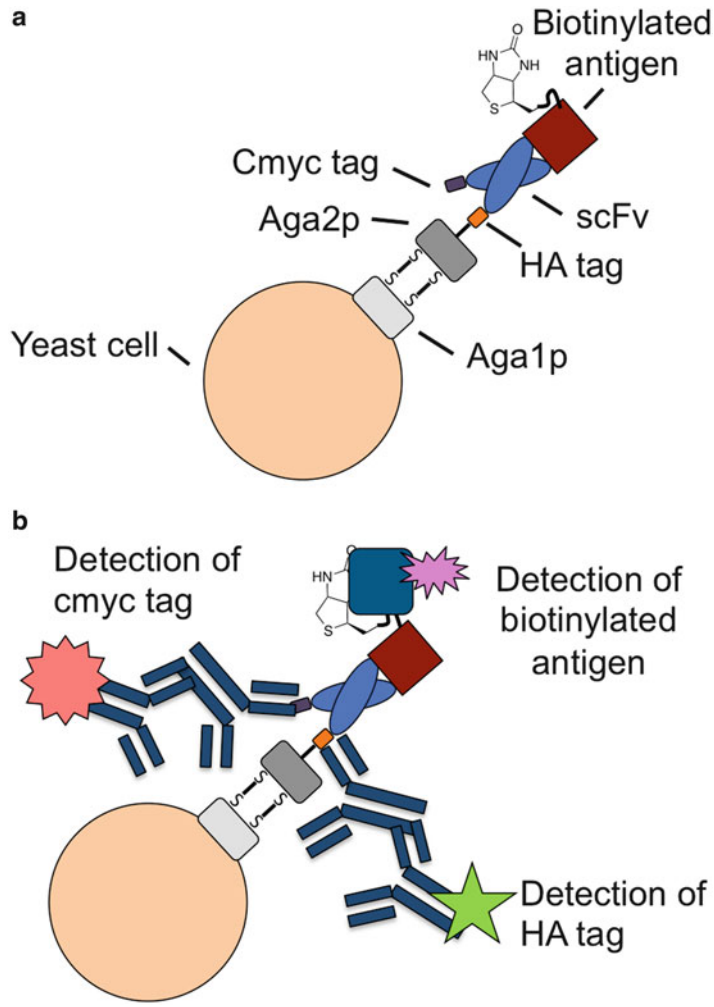

Fig. 1 Schematic representation of yeast surface display and detection strategies. (**a**) Heterologous protein display. A protein of interest such as an scFv is encoded between two epitope tags and fused in-frame with Aga2p. During processing and secretion, Aga2p forms disulfide bonds with Aga1p, resulting in a physical linkage between the protein of interest and the genetic information encoding the protein. (**b**) Detection of protein display and antigen binding. Yeast can be probed for the presence of full-length protein and antigen binding using fluorescent antibodies and streptavidin reagents

instrumentation. In display methodologies, a collection, or a library, of proteins is physically linked to the genetic material encoding these constructs of interest (usually on the surfaces of cells [1, 2] or phage [3, 4], but in some cases directly physically linked to RNA or DNA [5]). Most of these methods are well suited for isolating and engineering antibodies or antibody fragments.

Over the years, yeast surface display (Fig. 1) [6] has proven to be a robust, versatile, quantitative methodology for isolating and engineering antibody fragments [7]. The technique is amenable to

screening naïve antibody fragment libraries for binders to novel antigens as well as the affinity maturation of existing antibody fragments. Some key strengths of yeast surface display are the ease with which a variety of libraries can be constructed and the quantitative, real-time assessment of candidate clones made possible using flow cytometric analysis. Most implementations of yeast surface display utilize proteins containing single domains, although recent reports of the display of proteins such as the dimeric antibody Fc region and intact IgGs suggest that the single-domain constraint can be overcome with the appropriate protein and display constructs [7]. Yeast surface display is now routinely used to screen libraries of antibody fragments with a high degree of success.

This protocol covers the basics of yeast-based construction and screening of antibody fragment libraries for novel binding proteins and high-affinity fragments to a specified target. Previous protocols describe the key methodologies of yeast surface display admirably, and some of the methods described here remain essentially unchanged from these earlier technical references [8, 9]. Other descriptions representing new, straightforward methodologies for constructing libraries, screening large populations of yeast, and reducing the likelihood of finding antibodies recognizing secondary reagents are integrated within the existing tried and true methodologies. The main topics to be described in detail in this protocol are the construction of naïve antibody fragment libraries, bead-based scFv screening of naïve libraries, flow cytometry-based scFv library screening, DNA preparation and evaluation of individual clones, and affinity maturation using error-prone PCR libraries.

2 Materials

2.1 Library Construction and Cell Growth

1. pCTCON2 (yeast surface display shuttle vector available from the authors upon request; contains tryptophan marker for use in yeast and ampicillin marker for propagation in *E. coli*).
2. Restriction enzymes: SalI, NheI, and BamHI.
3. Phusion DNA polymerase, Phusion buffer, and DMSO (provided as a kit; New England Biolabs).
4. dNTPs (10 mM concentration of each deoxynucleotide).
5. Template DNA for library amplification (*see* **Note 1**).
6. Primers for template amplification and homologous recombination. Each scFv library will contain different 5′ and 3′ ends and will need to be designed to accommodate the specific library design. However, for use with pCTCON2, recommended primers for homologous recombination are forward: 5′-GGAGGCGGTAGCGGAGGCGGAGGGTCGGCTAGC (start scFv DNA)-3′ and reverse: 5′-GTCCTCTTCAGAAA TAAGCTTTTGTTCGGAT (end reverse complement of scFv DNA)-3′.

7. Forward and reverse sequencing primers (forward: 5′-GTT CCAGACTACGCTCTGCAGG-3′; reverse: 5′-GATTTTGTT ACATCTACACTGTTG-3′).
8. DNA gel electrophoresis equipment.
9. Nucleic acid gel extraction kit.
10. Spectrophotometer for measuring DNA concentrations.
11. Sterile, doubly distilled water (ddH_2O) chilled to 4 °C.
12. Pellet Paint co-precipitant (EMD Millipore).
13. 70 % ethanol.
14. 100 % ethanol.
15. 100 mM sterile lithium acetate.
16. 1 M dithiothreitol, freshly dissolved in water and sterile filtered immediately prior to use.
17. Sterile 30 % glycerol.
18. 2 mm electroporation cuvettes (chilled on ice prior to use).
19. Biorad Gene Pulser XCell Total System (Biorad).
20. Benchtop vortexer.
21. *Saccharomyces cerevisiae* yeast surface display strain EBY100 (available from the authors upon request).
22. YPD plates: Mix 20 g peptone, 10 g yeast extract, and 15 g agar in 900 mL ddH_2O. Separately, make a solution of 100 mL 20 % glucose (20 g in 100 mL). Autoclave both solutions, cool the solutions to 55–60 °C with stirring, mix them together, and pour plates. The entire mixture can be prepared and autoclaved together, but this tends to lead to caramelization of the glucose.
23. YPD media: Mix 20 g peptone and 10 g yeast extract in 900 mL ddH_2O. Separately, prepare a solution of 100 mL 20 % glucose. Autoclave both solutions, let them cool, and combine the two to make the final product (*see* **Note 2**).
24. Penicillin/streptomycin (Pen/Strep; 10,000 IU/mL and 10,000 μg/mL, respectively, in 100× solution).
25. Sterile glass culture tubes.
26. Sterile baffled flasks for liquid culture growth (*see* **Note 3**).
27. SDCAA medium, pH 4.5: Dissolve 20 g glucose, 6.7 g yeast nitrogen base without amino acids, 5 g casamino acids, and citrate buffer salts (10.4 g sodium citrate, 7.4 g citric acid monohydrate) in 1 L ddH_2O. Filter sterilize the solution and store in autoclaved bottles (*see* **Note 4**).
28. SDCAA plates, pH 6.0: Mix phosphate buffer salts (5.4 g sodium phosphate dibasic, anhydrous, and 8.56 g sodium phosphate monobasic monohydrate), 15 g agar, and 182 g

sorbitol in a final volume of 900 mL with ddH_2O. Autoclave the mixture and cool with stirring at room temperature. At the same time, dissolve 20 g glucose, 6.7 g yeast nitrogen base without amino acids, and 5 g casamino acids in a final volume of 100 mL using vigorous stirring. Once the autoclaved solution has cooled to approximately 60 °C, filter sterilize the glucose/yeast nitrogen base/casamino acid mixture directly into the autoclaved solution, mix briefly, and pour plates.

29. SGCAA medium, pH 6.0: Dissolve 20 g galactose, 2 g glucose, 6.7 g yeast nitrogen base without amino acids, 5 g casamino acids, and phosphate buffer salts (5.4 g sodium phosphate dibasic, anhydrous, and 8.56 g sodium phosphate monobasic monohydrate) in 1 L ddH_2O. Filter sterilize the solution and store in autoclaved bottles (*see* **Note 5**).
30. 50 mL conical tubes.
31. Refrigerated centrifuge for spinning 50 mL conical tubes.
32. 1.7 mL microcentrifuge tubes.
33. Stationary incubator at 30 °C (for yeast plate incubation).
34. Shaking incubator at 30 °C, 250 rpm (for yeast liquid culture growth).
35. Shaking incubator at 20 °C, 250 rpm (for induction of liquid cultures).
36. Floor centrifuge or other centrifuge for pelleting large volumes (1 L or greater).
37. Autoclavable centrifuge bottles.
38. Microscope (for inspecting bacterial contamination).
39. Zymoprep Yeast Plasmid Miniprep II kit (Zymo Research).
40. Luria-Bertani medium (for *E. coli* growth) (available as premixed powder or use the following recipe: for 1 L, mix 10 g tryptone, 5 g yeast extract, and 10 g sodium chloride in 1 L ddH_2O; autoclave).
41. Ampicillin (1,000× stock: 100 mg/mL dissolved in water and sterile filtered).
42. Luria-Bertani plates (recipe: for 1 L, mix 10 g tryptone, 5 g yeast extract, 10 g sodium chloride, and 15 g agar in 1 L ddH_2O; autoclave, allow media to cool with stirring to 55–60 °C, mix in ampicillin stock, and pour plates).
43. Stationary incubator at 37 °C (for bacterial plate incubation).
44. Shaking incubator at 37 °C (for bacterial liquid culture growth).
45. Competent *E. coli* (*see* **Note 39**).
46. Taq polymerase and PCR buffer.
47. 8-oxo-dGTP.
48. dPTP.

2.2 Bead Sorting

1. Refrigerated benchtop centrifuge for spinning microcentrifuge tubes.
2. Refrigerated centrifuge for spinning plates in swinging bucket rotors.
3. 2.0 mL microcentrifuge tubes (2 mL tubes).
4. 1× PBS, pH 7.4: Mix 8 g sodium chloride, 0.2 g potassium chloride, 1.44 g sodium phosphate dibasic (anhydrous), and 0.24 g potassium phosphate monobasic (anhydrous) in 1 L ddH_2O. Use hydrochloric acid or sodium hydroxide to adjust the pH to 7.4.
5. Sterile PBS + 0.1 % bovine serum albumin (BSA), pH 7.4 (PBSA): Add 1 g BSA to 1 L 1× PBS, pH 7.4, dissolve, and sterile filter. Store in autoclaved bottles.
6. Dynabeads Biotin Binder (Life Technologies).
7. Biotinylated antigen (*see* **Note 6**).
8. Rotary wheels stationed at 4 °C and room temperature.
9. Dynamag-2 magnet with tube holder (Life Technologies).

2.3 Flow Cytometry

1. Primary antibodies.
   (a) Chicken anti-cmyc (Gallus Immunotech).
   (b) Mouse anti-HA antibody 16B12 (Covance).
2. Secondary antibodies.
   (a) Goat anti-chicken Alexa Fluor 647.
   (b) Goat anti-mouse Alexa Fluor 488.
   (c) Streptavidin–Alexa Fluor 488.
   (d) Mouse anti-biotin PE.
3. Flow cytometry tubes compatible with available flow cytometer.
4. 96-well plates (compatible with flow cytometer of choice; U or V bottom preferred for enhanced pelleting).
5. Shaking platforms stationed at 4 °C and room temperature.

3 Methods

3.1 Preparation of Yeast Surface Display Libraries via Homologous Recombination

Steps 1–4 and **steps 5** and **6** can be performed in parallel. **Steps 10** and **11** should be performed concurrently.

1. *Digest pCTCON2 using SalI, NheI, and BamHI over the course of approximately 48 h.* To set up the first portion of the digest, prepare a 100 μL-scale reaction as follows: mix 10 μL 10× restriction buffer, 1 μL 100× BSA, 10 μg backbone vector, and 3 μL SalI. Bring the volume of the reaction to 100 μL using doubly distilled water, mix well, and incubate at 37 °C overnight.

The next day, add 3 μL each of NheI and BamHI to the reaction mixture, mix well, and incubate at 37 °C overnight. The following morning, add 0.5 μL of all three restriction enzymes to the reaction mixture, mix well, and incubate for one final hour (this serves as insurance that the vector has been completely digested). Upon completion of the digest, no further preparation of the vector DNA is required. Store the reaction mixture at −20 °C until it is needed.

2. *Amplify scFv insert DNA using polymerase chain reaction* (*PCR*). To preserve the initial library design and diversity, use Phusion polymerase and set up 200 μL PCR reactions to prepare insert DNA (*see* **Note 1**). Prepare reaction mixtures according to the manufacturer's recommendations using 20–200 ng template DNA per reaction. Cycle the reaction as follows:

   1 cycle of 98 °C for 30 s; 30 cycles of 98 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s; 1 cycle of 72 °C for 10 min; and 1 cycle of 4 °C forever.

   One 200 μL reaction will yield approximately 4–8 μg of DNA. Primers for amplification should contain sufficient homology to the backbone vector to enable efficient recombination in yeast (see recommended primers in Subheading 2.1).

3. *Run a DNA gel to extract properly sized insert DNA*. Run a 1 % gel at 100 V until the insert band (approximately 700–800 base pairs) is well separated from any contaminating bands. Gel extract the DNA and measure its concentration using a spectrophotometer.

4. *Pellet DNA samples for transformation*. Mix 4 μg insert DNA and 1 μg triply digested pCTCON2 DNA (if the digestion protocol of **step 1** was followed, 10 μL ≈ 1 μg) for each transformation in 1.7 mL microcentrifuge tubes and pellet the DNA using Pellet Paint according to the manufacturer's protocol (*see* **Note 7**). In parallel, pellet a sample of digested vector only as a negative control. At the end of the procedure, allow the pelleted DNA to air-dry for approximately 20–30 min. Confirm that the pellet is dry by tapping the tube firmly against the benchtop. A completely dry pellet should be readily dislodged from the bottom of the tube and move upon repeated tapping. Once the pellet is dry, resuspend it in 10 μL sterile ddH_2O. Repeated pipetting will be necessary to completely resolubilize the DNA. If extended storage of pelleted and resuspended DNA is required, the DNA can be stored at −20 °C.

5. *Prepare growing, healthy EBY100 cells to be used for electroporation*. Use a cryogenic stock of EBY100 to streak a YPD plate (*see* **Note 8**). Large colonies should be visible after 2 days of growth at 30 °C in a stationary incubator. Pick a single colony

of EBY100 from the plate and inoculate a 5 mL liquid YPD culture. Grow the cells at 30 °C with shaking overnight. The following day, passage the EBY100 into 5 mL fresh YPD by transferring 100 μL of saturated culture into 5 mL fresh YPD (*see* **Note 9**). Grow the cells at 30 °C with shaking overnight. The next morning, measure the OD_{600} of the EBY100 culture. Dilute the culture to an OD_{600} of 0.2 in a large volume of YPD and grow at 30 °C with shaking until the cells reach an OD_{600} of approximately 1.5 (*see* **Note 10**). In this protocol, 50 mL EBY100 at an OD_{600} of approximately 1.5 will yield enough electrocompetent cells for two transformations; more transformations can be performed if the volume of EBY100 prepared is scaled appropriately.

6. *Prepare EBY100 for electroporation*. Upon reaching an OD_{600} of 1.5, use sterile technique to transfer the culture to 50 mL conical tubes. Pellet the cells by spinning them at 2,000 × *g* for 5 min. Decant the supernatant and resuspend each tube in 25 mL sterile 100 mM lithium acetate (half of the pelleted culture volume) by shaking the tubes vigorously. Add 0.25 mL freshly prepared, sterile-filtered 1 M DTT to each tube. Loosen the cap on each tube, tape the lid on to ensure adequate oxygenation, and then incubate the cells at 30 °C in the shaking incubator for 10 min. Following the incubation, the cells should remain at 4 °C or on ice for the remainder of the preparation. After incubation, tighten the lids on the tubes and pellet the cells at 2,000 × *g* for 5 min. Decant the supernatant and resuspend the cells in 25 mL/tube chilled, sterile ddH_2O with vigorous shaking. Pellet the cells at 2,000 × *g* for 5 min. Decant the supernatant and resuspend the cells in 0.25 mL/tube sterile, chilled ddH_2O using repeated pipetting. Be sure to examine the bottom of the tube to confirm that all of the cells are resuspended. The cells are now electrocompetent.

7. *Transform electrocompetent EBY100 with library DNA*. Take 250 μL electrocompetent cells and add them to the 10 μL preparations of library DNA (or control). Mix cells and DNA well using a pipette and transfer the cells to prechilled 2 mm electroporation cuvettes. Dry the outside of a single electroporation cuvette, place the cuvette in the shock pad of the Biorad Gene Pulser XCell, and shock the cells using a square wave protocol with the following parameters: 500 V, 15 ms pulse duration, 1 pulse only, and 2 mm cuvette. Immediately after the shock is completed, remove the cuvette from the shock pad, add 1 mL YPD to the cuvette (this does not need to be performed under sterile conditions), and mix the YPD and cells multiple times. Dump the contents of the cuvette into a sterile glass test tube, then add 1 mL YPD to the cuvette, rinse the cuvette, and dump the contents into the glass tube.

Repeat the electroporation for all cuvettes and then incubate the glass tubes at 30 °C without shaking for 1 h. At this time, warm SDCAA plates to 30 °C (one plate per transformation).

8. *Take samples of transformed cells to estimate the number of transformants in each tube*. Remove the glass tubes containing the transformed cells from the incubator after the incubation is complete, vortex each tube (Gently! Use the lowest vortex setting on a benchtop vortexer), remove 10 μL, and dilute the cells into 990 μL SDCAA medium (100× dilution). Set the dilutions aside briefly at room temperature while completing **step 9**.
9. *Prepare transformed cells for overnight growth in selective media*. Take the tubes of transformed cells and pellet them in glass tubes at 900×*g* for 5 min. After aspirating the supernatant, resuspend the cells in the tube with approximately 5 mL SDCAA. Dilute the resuspended cells into a total of 100 mL SDCAA for each set of transformed cells. If numerous transformations of the same genetic diversity have been performed, these transformed cells can be pooled and grown in the same, larger amount of media (100 mL/transformation is a good rule of thumb). Grow the cells at 30 °C in baffled culture flasks for approximately 24 h.
10. *Dilute*, *plate*, *and grow cells to estimate the total number of transformants from each electroporation*. Take the samples obtained in **step 8** (100× dilutions), vortex, and transfer 10 μL into Eppendorf tubes containing 90 μL SDCAA (1,000× dilutions). Repeat dilutions into fresh media twice more to obtain 10,000× and 100,000× dilutions, using a fresh tip for each dilution to avoid the possibility of cells sticking to tips in one dilution and becoming dislodged in future dilutions. Remove the prewarmed SDCAA plates from the incubator and divide each plate into quadrants using a permanent marker. Take each diluted sample from a transformation, vortex vigorously, remove 20 μL, and deposit into one quadrant of the plate. Use the pipette tip to spread the cells evenly throughout the area. Repeat the process for each dilution, using a fresh tip for each deposition and spreading. Incubate plates at 30 °C for 3 days, and count colonies to determine the number of transformants. A single colony in the 100×, 1,000×, 10,000×, and 100,000× quadrants corresponds to 1×10^4, 1×10^5, 1×10^6, and 1×10^7 transformed cells, respectively.
11. *Passage the library and prepare frozen stocks*. After 24 h of growth, check the OD_{600}s of the growing library cultures. Typically, saturation OD_{600}s in SDCAA range from 8 to 12. If the cultures are not yet saturated, continue growing the cells until saturation is reached. Once the cultures are saturated, pellet the cultures in conical tubes (to remove cellular debris) at 2,000×*g* for 5 min.

Decant the supernatants and resuspend the pelleted cells in SDCAA to a final OD_{600} of approximately 1. To passage a 100 mL culture, resuspend the entire pellet in 1 L SDCAA. For passaging pooled transformations with a number of transformants totaling less than or equal to 1×10^9, pellet 1×10^{10} cells and resuspend the cells in 1 L SDCAA (*see* **Note 11**). Allow the passaged yeast to grow to saturation. For immediate induction and bead-based screening, use a portion of the saturated culture in Subheading 3.2, **step 1** (*see* **Note 12**). Take a small sample (2–3 μL), place on a microscope slide with a cover slip, and examine the culture under a microscope to confirm the absence of bacterial contamination (*see* **Note 13**). Transfer the remainder of the culture into sterile centrifuge bottles and pellet the cells using a floor centrifuge. Decant the supernatant and resuspend the pellet in a total of 10 mL 30 % sterile glycerol. The combination of the resuspended pellet and glycerol should lead to an approximate total volume of 20 mL and final glycerol concentration of 15 %. Aliquot the cells into 2 mL cryogenic vials and freeze the vials at −80 °C. These library fractions can be stored indefinitely (*see* **Note 14**).

12. *Perform library quality control to sample library genetic diversity and verify that the majority of clones are of full length* (*optional but recommended*). Using the methods described in Subheading 3.4, **steps 1–3**, isolate the DNA from individual library members and submit these clones for sequencing (10 gives a good coarse-grain assessment of quality). Verify that the sequences are consistent with the library construction, the clones isolated are distinct, and most clones are free of stop codons (the method of library construction should dictate the probability of encountering a stop codon in a given sequence). Using the small-scale induction method described in Subheading 3.2, **step 11**, and the flow cytometry preparation and methods from Subheading 3.2, **steps 12–14**, and Table 1, determine the percentage of clones in the library displaying full-length scFvs (*see* **Note 15**).

3.2 Bead-Based Enrichments of Yeast Surface Display Libraries

This section assumes a library size of no greater than 1×10^8. Notes in the section explain how to scale the screening procedure to accommodate libraries of larger size.

1. *Thaw a library vial and induce cells for bead sorting.* Remove a vial of cells from the freezer and let it thaw on the benchtop. Once thawed, place the contents of the vial in 1 L SDCAA in a baffled flask and grow the cells overnight at 30 °C (*see* **Note 16**). The next morning, measure the OD_{600} of the culture and pellet 1×10^9 yeast cells (e.g., with an OD_{600} of 10, 10 mL) at $2{,}000 \times g$ for 5 min (in case of flocculation, *see* **Note 12**).

Table 1
Yeast surface display specimen labeling

Specimen name	Cells used	Primary label(s)	Dilution/ concentration	Secondary label(s)	Dilution
Negative	Any	None		None	
488 primary and secondary	Library fraction	Mouse anti-HA	1:200	Goat anti-mouse 488	1:100
488 primary and secondary	Single clone	Biotinylated antigen	Varies	Streptavidin 488	1:100
647 primary and secondary	Any displaying cells	Chicken anti-cmyc	1:250	Streptavidin 647	1:100
PE primary and secondary	Single clone	Biotinylated antigen	Varies	Mouse anti-biotin PE	1:100
Library validation sample	Portion of naïve library	(1) Mouse anti-HA (2) Chicken anti-cmyc	(1) 1:200 (2) 1:250	(1) Goat anti-mouse 488 (2) Goat anti-chicken 647	(1) 1:100 (2) 1:100
Library secondary binder analysis sample	Sorted library	Chicken anti-cmyc	1:250	(1) Streptavidin 488 (2) Goat anti-chicken 647	(1) 1:100 (2) 1:100
Library secondary binder analysis sample	Sorted library	Chicken anti-cmyc	1:250	(1) Mouse anti-biotin PE (2) Goat anti-chicken 647	(1) 1:100 (2) 1:100
Library analysis/sorting sample	Sorted library	(1) Biotinylated antigen (2) Chicken anti-cmyc	(1) Varies (2) 1:250	(1) Mouse anti-biotin PE (2) Goat anti-chicken 647	(1) 1:100 (2) 1:100
Library analysis/sorting sample	Sorted library	(1) Biotinylated antigen (2) Chicken anti-cmyc	(1) Varies (2) 1:250	(1) Streptavidin 488 (2) Goat anti-chicken 647	(1) 1:100 (1) 1:100

Resuspend the cells in 100 mL SDCAA and grow the cells at 30 °C with shaking. This step results in a culture at an initial OD_{600} of 1. Grow the cells until the OD_{600} of the culture is between 2 and 5, the logarithmic growth phase of yeast (*see* **Note 17**). Pellet 1×10^9 cells at $2{,}000 \times g$ for 5 min in 50 mL conical tubes, decant the supernatant, and resuspend the cells in 100 mL SGCAA (again, resulting in a culture at an OD_{600} of 1). Incubate the cells at 20 °C in a shaking incubator for 18–24 h to induce the cells (*see* **Note 18**).

2. *Prepare antigen-coated beads for positive enrichment.* Mix together 10 μL resuspended biotin binder beads (coated with streptavidin), 100 μL PBSA, and 33 pmol biotinylated antigen in a 2 mL tube (*see* **Notes 19** and **20**). Incubate at 4 °C for 2 h to overnight on a rotary wheel. After the completion of incubation, add 1 mL of ice-cold PBSA to the tube and place the tube on a Dynamag-2 magnet for 2 min. Remove the supernatant completely with a pipette, and resuspend the beads in 1 mL ice-cold PBSA to wash the beads, pipetting the beads up and down. Place the beads on the magnet for 2 min and remove the supernatant. Remove the washed beads from the magnet, resuspend the beads in 100 μL ice-cold PBSA, and place them on ice until needed.
3. *Prepare cells for negative and positive sorts.* Measure the OD_{600} of the induced culture and remove a culture volume containing 2×10^9 cells (*see* **Note 21**). Pellet the cells in 50 mL conical tubes at $2{,}000 \times g$ for 5 min, decant the supernatant, and resuspend the cells in a total volume of 1 mL sterile, ice-cold PBSA using repeated pipetting. Transfer the cells to a 2 mL tube and pellet the cells at $12{,}000 \times g$ for 1 min. Remove the supernatant with a pipette tip and resuspend the cells in 1 mL sterile, ice-cold PBSA. Repeat the pelleting, supernatant removal, and resuspension. The cells are now ready for magnetic bead sorting (*see* **Notes 22** and **23**).
4. *Perform a negative bead sort on the cells* (*see* **Note 24**). All sorting should be performed at 4 °C. Add 100 μL resuspended biotin binder beads to the cells. Incubate cells on a rotary wheel at 4 °C for 2 h (longer incubation times are also acceptable). Upon completion of the incubation, place the tube on the magnet, taking care to transfer any liquid lodged in the cap of the tube to the bottom portion of the tube. After 2 min, carefully remove the supernatant from the tube and transfer it into a fresh 2 mL tube. The supernatant will serve as the input for the next sort. Resuspend the beads in 1 mL ice-cold PBSA with a pipette (at this stage, do not invert the tube) and place the tube on the magnet for 2 min. Remove the supernatant of the washed beads and discard it. Resuspend the beads in 1 mL ice-cold PBSA and set aside the sample to enable estimation of the number of cells captured in the negative sort in **step 8**.

5. *Perform a second negative sort on the cells.* Repeat **step 4** using the supernatant from the previous step and then proceed to the following step with the depleted supernatant.
6. *Perform a positive sort on the cells.* This step should also be performed at 4 °C. Mix the supernatant from **step 5** with the antigen-coated beads from **step 2** and incubate on the rotary wheel at 4 °C for 2 h. Upon completion of the incubation, place the tube on the magnet, taking care to transfer any liquid lodged in the cap of the tube to the bottom portion of the tube. Incubate the cells and the beads on the magnet for 2 min, then carefully remove the supernatant from the tube and discard it. Resuspend the beads in 1 mL ice-cold PBSA using a pipette and place the beads on the magnet for 2 min. Remove the supernatant and discard it. The beads should contain a population enriched for binding to the target.
7. *Rescue the enriched population*. Resuspend beads and cells from the previous step in 1 mL SDCAA and transfer the cells to a sterile glass culture tube containing 4 mL SDCAA (5 mL SDCAA + beads + cells in total). Vortex the tube gently and then remove a sample of 5 μL from the culture. Dilute the sample into 995 μL SDCAA (200× dilution) and set aside for **step 8**. Grow the cells and beads at 30 °C overnight with shaking.
8. *Plate fractions of beads from negative and positive sorts to estimate the number of cells recovered in each step*. Vortex the saved beads from the negative sorts and transfer 100 μL beads into 400 μL fresh SDCAA. Take these diluted samples, vortex, and transfer 5 μL of each sample into 995 μL SDCAA (200× dilution). Vortex the 200× dilutions of the negative sort and the positive sort (from the previous step) and transfer 10 μL from each population into 190 μL SDCAA (4,000× dilution). Divide an SDCAA plate into six regions using a permanent marker. Vortex each dilution and plate 20 μL. Grow the plate at 30 °C for 3 days and count the colonies. One colony in the 200× and 4,000× dilutions represents 5×10^4 and 1×10^6 cells recovered, respectively.
9. *Prepare sorted cells for further sorts by removing the beads from the first sort*, *expanding the cells*, *and inducing a portion of the cells.* After the overnight growth, measure the OD_{600} of the cells. If the OD_{600} is still low, continue to allow the cells to grow; another day of growth is acceptable in case of especially low OD_{600}s (*see* **Note 25**). Once the culture approaches saturation, pellet the cells (at 900 × *g* for 5 min) and aspirate the supernatant. Resuspend the pellet in 1 mL SDCAA and transfer the cells to a 2 mL tube. Place the tube on the magnet for 2 min. Recover the supernatant and dilute the cells into two cultures for further expansion. Dilute 2.5×10^8 cells into 25 mL SDCAA medium for growth and induction and dilute the

remaining cells into 25 mL SDCAA for overnight growth and temporary storage at 4 °C in case the first sorted population needs to be induced and sorted again (for revival recommendations, *see* **Note 14**). Once the 2.5×10^8 cell culture reaches an OD_{600} between 2 and 5, pellet 5×10^8 cells and resuspend in 50 mL SGCAA. Incubate the cells at 20 °C with shaking for 18–24 h to induce.

10. *Perform an additional series of negative and positive bead sorts.* Prepare more antigen-coated beads according to **step 2**. Once the cells are induced, take an OD_{600} to enable estimation of the total number of induced cells. Take between 0.5 and 2×10^9 cells and perform **steps 3–8**, yielding beads and cells growing in a 5 mL SDCAA culture.
11. *Perform a small-scale induction to enable initial flow cytometry analysis.* Once the twice-enriched population from **step 10** reaches saturation, pellet the cells and remove the beads as in **step 9**. Take 5×10^7 cells and dilute them to an OD_{600} of 1 in 5 mL SDCAA. Grow the cells at 30 °C until the OD_{600} is between 2 and 5. Take 5×10^7 cells, transfer them to a new glass culture tube, pellet them at $900 \times g$ for 5 min, remove the supernatant, and resuspend the cells in 5 mL SGCAA. Grow the cells at 20 °C with shaking for 18–24 h to obtain induced cells.
12. *Prepare the twice-sorted population for flow cytometry analysis.* For this step, the preparation of control samples from a single clone known to display a full-length, functional binding protein is highly encouraged and will be required for fluorescence-activated cell sorting in Subheading 3.3 (*see* **Note 26**). The labeling procedures described in this step assume that only the sorted sample is available, but if the control construct is available, use the induced control construct for negative, 488 primary and secondary, and 647 primary and secondary samples. Measure the OD_{600} of the induced cells and remove 5×10^6 cells for the following samples: negative, 488 primary and secondary, 647 primary and secondary, streptavidin detection only, and detection of antigen. Pellet the cells at $12{,}000 \times g$ for 30 s, aspirate the supernatant, and wash the cells by resuspending the cells in 1 mL sterile PBSA, pelleting ($12{,}000 \times g$ for 30 s), and aspirating the supernatant. Resuspend the washed cells in 250 μL PBSA and aliquot 50 μL into each of the five 1.7 mL microcentrifuge tubes. Label the tubes and set the negative sample aside on ice. Add primary labels to the cells according to Table 1, vortex the cells briefly (*see* **Note 27**), and incubate the cells at room temperature on a rotary wheel for 30 min. For the sample in which antigen will be detected, be sure to use a high concentration of antigen (1 μM is ideal) to ensure that cells displaying weak binding proteins appear positive on the flow cytometer. After completion of the incubation,

pellet the cells using a refrigerated tabletop centrifuge at 4 °C (12,000 × *g* for 30 s for the remainder of this step), aspirate the supernatant, resuspend the cells in 1 mL ice-cold PBSA, pellet, aspirate, then resuspend the cells in 50 μL ice-cold PBSA and add secondary labeling reagents according to Table 1. Vortex the cells briefly, place the cells on ice, and protect them from light while incubating with the secondary reagents for 10 min (up to 30 min in the secondary reagents is acceptable). Pellet the cells, wash them once in ice-cold PBSA, and leave the aspirated pellets on ice until immediately prior to analysis.

13. *Perform flow cytometry analysis on labeled cells.* Resuspend the labeled cell populations and transfer them to tubes compatible with the flow cytometer to be used (*see* **Note 28**). Run samples one by one, starting with the negative, 488 primary and secondary, and 647 primary and secondary samples first. Adjust detector voltages so that stained cells appear on scale for the corresponding detector. If some cells have especially high fluorescence values, continue to decrease the voltage at the expense of the negative cells, which may appear below the detection limit. Finish running all samples on the cytometer, making sure to record data on all samples after the detector voltages have been finalized. Figure 2 depicts typical histograms of a bead-sorted library population and a control clone (*see* **Note 29**).
14. *Analyze data and determine future experiments.* Examine the flow cytometry data using two-dimensional dot plots comparing display and antigen binding levels as in Fig. 2. Sorted library populations will vary greatly in the number of antigen-positive cells present after two rounds of bead sorting;

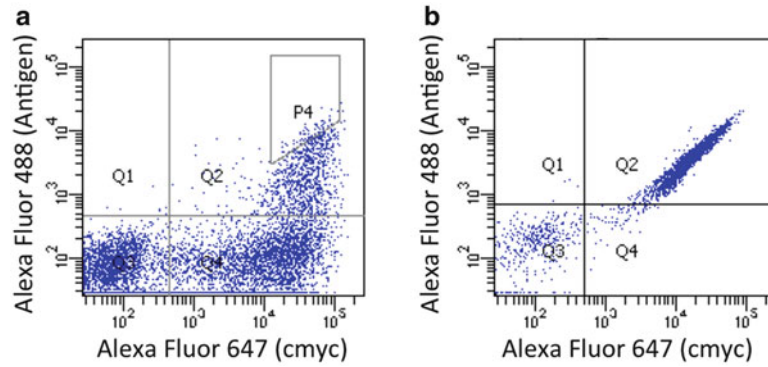

Fig. 2 Flow cytometry setup. (**a**) Gating strategy for enrichment of high-affinity antigen binders. A library subjected to two rounds of bead sorting appears enriched for antigen binders. Full-length clones binding only the highest amounts of antigen will be collected in an aggressive attempt to isolate the highest affinity binders. (**b**) The typical fluorescence profile of a single clone. Note the high degree of correlation between display levels and antigen binding levels

this will be largely dependent upon library quality. The "secondary only" labeling will reveal the presence of streptavidin-binding clones. Enriched populations should be carefully monitored for secondary binders due to their occasional persistence even after repeated library depletion. If the sorted population reveals even a small percentage of antigen-positive, full-length clones (as low as 0.1 %), use flow cytometry to enrich the population for antigen-specific clones using Subheading 3.3. If more than 1–2 % of cells are antigen positive, perform additional flow cytometry experiments to determine if a substantial antigen-binding population persists at lower concentrations of antigen (e.g., 100 nM, 10 nM). If the data reveal no evidence for antigen-positive clones, consider performing additional rounds of bead-based enrichments or constructing a library with a different diversification strategy.

Steps 1 and **2** can be performed concurrently.

3.3 Flow Cytometric Enrichments Using Equilibrium Conditions

1. *Prepare a control population of induced cells to be used in setting up sorting conditions.* Using a plasmid encoding a functional, surface-displayed binding protein (*see* **Note 26**), transform zymocompetent EBY100 according to the manufacturer's protocol. Plate the cells on SDCAA plates and allow the cells to grow for 2–3 days at 30 °C. Pick a single colony from the plate and use it to inoculate a 5 mL SDCAA culture. Grow the culture at 30 °C with shaking until saturation is reached (1–2 days). Perform a 5 mL-scale induction using the steps outlined in Subheading 3.2, **step 11**. Measure and record the OD_{600} of the induced cells and store the cells at 4 °C. These induced cells can be used as controls for flow cytometry experiments for at least 1 month without significant loss of the quality of display or the physical properties of the cells. Uninduced cells can also be stored at 4 °C for at least 1 month without loss of viability; these cells can be used to prepare more induced cells when necessary or proliferate yeast containing the construct for future use.
2. *Prepare populations for flow cytometric enrichment using antigen labeling under equilibrium conditions.* Use the instructions of Subheading 3.2, **step 1** or **11**, to grow and induce cells for flow cytometric cell sorting. The choice of large- or small-scale induction will depend on the estimated diversity of the population to be sorted (*see* **Note 30**). Upon completing induction, label cells displaying the control construct and cells displaying the population to be sorted. For populations to be sorted, label at least 1×10^7 cells per sample (regardless of population diversity) and try as best as possible to oversample the population diversity by tenfold. Also, bear in mind that flow cytometers can usually sort $0.5–1 \times 10^8$ cells in an hour;

do not expect to process billions of cells in a day unless several machines are available. All cells can be labeled using the general procedure outlined in Subheading 3.2, **step 12**, with three minor modifications: (1) For the first round of flow cytometric enrichment, use the secondary label mouse anti-biotin PE in place of streptavidin 488 in order to avoid enrichment for any remaining streptavidin binders in the population (*see* Table 1 for labeling of samples and controls). (2) The sorted population of cells will likely need to be labeled in a larger volume. When antigen is present at very high concentrations, the highest recommended cell density is 1×10^7 cells per 50 μL volume. When cells are exposed to lower concentrations of antigen, ensure that the antigen concentration exceeds ten times the effective concentration of binding protein in the solution. Assume that each cell displays approximately 5×10^4 scFvs (*see* **Note 31**). (3) Ensure that the antigen labeling approaches equilibrium prior to moving to secondary labeling using the formula $\tau = (k_{on}[Ag]_0 + k_{off})^{-1}$, where τ is the equilibrium time constant; k_{on} and k_{off} are the on and off rates of antigen–scFv binding, respectively; and $[Ag]_0$ is the initial concentration of antigen in solution. The binding has reached 95 % of equilibrium at 3τ and 99 % equilibrium at 4.6τ. A typical scFv k_{on} is 1×10^5 M^{-1} s^{-1}, and the k_{off} can be determined using the relationship $K_d = k_{off}/k_{on}$, where K_d is the equilibrium dissociation constant (*see* **Note 32**) [8]. In all cases, perform labeling with primary antigen for at least 30 min. Upon completion of labeling, leave cells to be sorted as pellets on ice until immediately prior to sorting. The labeling will remain stable for long periods of time under these conditions. In cases where a long sort (>10–20 min) or labile antigen binding is anticipated, divide the labeled population into several tubes and prepare each tube as the sorting requires in the following step (1×10^7 cells/sample is a good place to start).

3. *Run flow cytometry controls and sort populations of interest.* Run the negative, PE-only, and Alexa Fluor 647-only control populations, adjusting voltages as in Subheading 3.2, **step 13** (*see* **Note 33**). Use these samples to set up a two-dimensional plot with Alexa Fluor 647 and PE as the axes and draw a quadrant that places negative, PE-positive, and Alexa Fluor 647-positive cells in separate quadrants. Resuspend the first sample to be sorted and obtain data from a few thousand cells. Draw a polygon gate that captures full-length, antigen-positive cells (*see* **Note 34**). Set up a glass culture tube containing 1 mL SDCAA in the collection area, specify the population to be collected, and initiate the sort. Be sure to examine the fluorescence values as a function of time. If the fluorescence of the population is changing rapidly, frequently adjust the gating or change tubes. In a single collection tube, collect no more than 1 mL

volume of sorted cells to avoid flocculation issues associated with most phosphate-based flow cytometry buffers (*see* **Note 12**). When a collection tube contains 1 mL sorted cells or a new population will be sorted, remove and cap the collection tube and vortex briefly. Leave the collection tube on ice, and proceed to **step 4** once all sorting is complete. Be sure to record the number of cells collected in each tube to ensure that population diversity can be preserved in subsequent steps by carrying on an appropriate number of cells.

4. *Rescue sorted cells.* Take the collection tubes and wash down the sides of the tubes with 4 mL additional SDCAA. Grow the cells at 30 °C with shaking until the cells reach saturation (if speed is an issue, *see* **Note 25** regarding induction of cells prior to saturation). The number of collected cells will determine the time needed to reach saturation. Tubes containing only a few thousand collected cells will almost certainly take 2 days to reach saturation OD_{600}, while tubes containing higher numbers will reach saturation in 1–1.5 days.

5. *Assess sort quality and continue sorting library until sufficiently enriched.* Using Subheading 3.2, **steps 11–13**, perform inductions and flow cytometry analysis on the sorted populations (*see* **Note 35**). If a high percentage of antigen binding is anticipated, prepare samples in which cells are labeled with decreasing concentrations of antigen to determine the concentration of antigen to be used for future sorting. Also, be sure to prepare samples in which binding to mouse anti-biotin PE is assessed (Table 1). Run the samples on the flow cytometer and determine the percentage of cells displaying full-length clones that recognize the antigen and the percentage recognizing secondary reagents. If the population appears to be enriched for antigen-specific binding, continue with flow cytometry-based enrichment until a majority of cells exhibit binding to the antigen, decreasing antigen concentration if higher affinity antibodies appear to be present in the population. If the population exhibits some antigen recognition and some mouse anti-biotin PE recognition, use the methods of this section to sort the population again using streptavidin–Alexa Fluor 488 for secondary detection (*see* **Note 36**). Do not use the same secondary reagent for antigen detection for more than two rounds of sorting in a row to minimize the possibility of isolating secondary reagent binders. Continue sorting the library until the population has become enriched for binders of the highest affinity possible (*see* **Note 37**). If the population appears to contain clones with the desired affinities, or if individual clones are to be affinity matured, evaluate the properties of individual clones using the methods of Subheading 3.4. If the entire population is to be affinity matured, use Subheading 3.4, **steps 1** and **2**, to prepare DNA suitable for use in Subheading 3.5.

3.4 DNA Preparation and Evaluation of Individual Clones

1. *Isolate plasmid DNA from yeast population.* Take up to 1×10^8 yeast from a growing culture with an OD_{600} below approximately 2 and perform a zymoprep according to the manufacturer's recommendations with the following modifications: (1) use double the amount of zymolyase recommended and increase the digestion time to at least 2 h with mixing every hour; (2) following the pelleting of cellular debris, decant the supernatant into a silica column with high binding capacity and complete the procedure (*see* **Note 38**). In the last step, elute the DNA into 40 μL ddH_2O.
2. *Transform E. coli with zymoprepped DNA, grow transformed E. coli, and perform minipreps.* Using chemically competent or electrocompetent *E. coli* (*see* **Note 39**) according to the manufacturer's instructions, transform the cells with 1–10 μL of the zymoprepped DNA population of interest. Rescue the cells for 1 h at 37 °C in 0.5–2 mL SOC medium. If individual clones are to be isolated, plate 20–200 μL of the rescued cells on prewarmed LB plates containing 100 μg/mL ampicillin (LB-amp) and incubate the plates at 37 °C for 12–18 h. Use single colonies from the plates to inoculate 5 mL cultures in LB media containing 100 μg/mL ampicillin (*see* **Note 40**). If the DNA from the entire population is needed in bulk (e.g., for affinity maturation), dilute the rescued cells into 50 mL LB medium containing 100 μg/mL ampicillin (*see* **Note 41**). Grow liquid cultures for 12–18 h or until the cultures reach saturation. Pellet the cells and perform minipreps according to the manufacturer's instructions. If DNA is to be sequenced, elute the DNA using ddH_2O, not buffer. If DNA will be immediately mutagenized, proceed to Subheading 3.5.
3. *Sequence individual clones.* Because highly enriched populations are oftentimes dominated by a few clones, identifying unique clones can cut down on labor in subsequent evaluation of scFv properties. Submit DNA for sequencing using the forward sequencing primer (*see* Subheading 2). In many cases, the sequencing read quality will remain high throughout the length of the scFv. If a reverse sequence is needed, use the reverse primer (*see* Subheading 2). Confirm that clones of interest are of full length and select unique clones for further evaluation.
4. *Transform zymocompetent EBY100 with individual clones and prepare induced samples.* Use the instructions of Subheading 3.3, **step 1**, and Subheading 3.2, **step 11**, to prepare induced samples of EBY100 displaying the clones of interest. Note that more than one transformation can be plated on the same SDCAA plate.
5. *Confirm antigen-specific binding using flow cytometry* (*optional, but recommended*). Before thoroughly evaluating the binding properties of individual clones, verify that each clone recognizes

the antigen of interest. Perform flow cytometry analysis on cells displaying individual clones stained with secondary antigen detection reagents only and antigen plus secondary (Table 1) to validate the antigen specificity of each clone.

6. *Titrate antigen with individual clones and perform flow cytometry.* With antigen-specific clones in hand, titrate the antigen concentration on a series of cell samples and perform flow cytometry to enable estimation of the equilibrium dissociation constant (K_d). For crude estimations, fourfold dilutions two orders of magnitude above and below the anticipated K_d are sufficient, while fine K_d determination can be obtained with twofold dilutions in triplicate. The large numbers of samples necessary to obtain K_ds are most efficiently obtained by performing staining in 96-well plates (*see* **Note 42**). For these experiments, a greatly reduced number of cells and lower concentrations of anti-cmyc and secondary labels can be used. Prepare the plate samples in parallel with negative and single-color controls in tubes (as described in Subheading 3.2, **step 12**) for voltage adjustment on the flow cytometer. Wash induced cells in PBSA and resuspend the cells to a final concentration of 1.5×10^6 cells/mL in PBSA. Remove a large enough volume to enable distribution of 10 μL of cells (1.5×10^4) into each of 8 wells (fourfold dilutions) or 16 wells (twofold dilutions) and place in a new 1.7 mL microcentrifuge tube. Add chicken anti-cmyc to a final dilution of 1:200 and vortex the cells briefly. If sufficiently low concentrations of antigen will be reached, the antibody-displaying cells will need to be reduced in order to maintain a tenfold excess of effective antibody concentration. In these cases, use bare EBY100 to dilute the antibody-displaying cells to a lower concentration while maintaining a sufficient number of cells to establish a pellet. Prepare bare EBY100 in the same way as the antibody-displaying cells (including addition of chicken anti-cmyc) and mix with cells displaying scFvs in appropriate ratios (e.g., 4:1 or 9:1 bare:displaying). Set aside the cells in tubes while preparing the titrations on the plate. Add 40 μL PBSA to each well of a 96-well plate to be used in the experiment except the well that will contain the highest concentration of antigen in each titration. Add antigen diluted in PBSA to the first well for each titration at a concentration of 1.25 times the final highest desired concentration and a volume of 53.33 μL (fourfold dilutions) or 80 μL (twofold dilutions). Complete the titration by diluting 13.33 μL (fourfold) or 40 μL (twofold) antigen into the well intended to have the next highest concentration of antigen and so forth until 7 out of 8 (fourfold) or 15 out of 16 (twofold) wells to be used for a sample have had antigen diluted into them. Remove 13.33 μL (fourfold) or 40 μL (twofold) from the 7th or 15th wells, respectively, and discard.

Leave the 8th or the 16th wells blank to serve as a secondary-only staining reference. Add 10 μL cmyc-labeled cells to each well, taking care to add cells diluted with EBY100 to wells that contain especially low concentrations of antigen as required. This lowers the antigen concentration in each well by a factor of 1.25. Seal the plate with adhesive foil and incubate the cells on a shaking platform at 150 rpm until near-equilibrium conditions are reached as described in Subheading 3.3, **step 2**. Upon completion of the primary incubation, add 150 μL/well ice-cold PBSA to the plate and spin the cells at 1,300 × *g* for 5 min in a swinging bucket rotor. Dump the supernatant of the pelleted wells out by inverting the plate and using quick shaking motions, then place the plate on ice. Resuspend each well in 50 μL of secondary labeling reagent diluted in ice-cold PBSA. In this case, 1:500 dilutions of each detection reagent are sufficient to conserve labeling reagents. Seal the plate with adhesive foil and incubate it at 4 °C on a shaking platform for 10 min. Add 150 μL/well ice-cold PBSA and pellet the cells. Dump the supernatant and resuspend the cells in 200 μL/well ice-cold PBSA. Run the samples on the flow cytometer, starting with controls (prepared in tubes) to set detector voltages, followed by the plate.

7. *Process flow cytometry data and estimate K_ds of individual clones.* Take the raw flow cytometry data and gate out the population of cells positive for the cmyc label. Process the data to obtain the median fluorescence values corresponding to antigen display in all samples using FlowJo or BD FACSDiva software. Fit the fluorescence data as a function of antigen concentration to the equation $MF_{high} = MF_{low} + (MF_{observed} \times [Ag])/([Ag] + K_d)$ using a least squares approach in a program such as Microsoft Excel or Matlab. In the equation, MF_{high} equals the mean fluorescence value of the sample exposed to the highest antigen concentration, MF_{low} equals the mean fluorescence obtained in the absence of antigen, and $MF_{observed}$ equals the fluorescence observed at a specific antigen concentration. To average multiple experiments, average the K_ds obtained in individual experiments and report the average and standard deviations or 95 % confidence intervals of the values. These data can be used to determine if further affinity maturation is desired. An approach to affinity maturation is described in Subheading 3.5.

3.5 Affinity Maturation Using Error-Prone PCR Libraries

1. *Introduce mutations into DNA encoding scFvs to be affinity matured.* Using the DNA encoding an individual clone, several pooled clones, or an entire population (*see* **Note 43**), perform error-prone PCR at 50 μL scale using the following recipe:

   1.0 μL 0.7 ng/μL template DNA (roughly 1×10^8 plasmids).

   2.5 μL 10 μM forward amplification primer (*see* **Note 44**).

2.5 μL 10 μM reverse amplification primer.

5.0 μL 10× ThermoPol buffer.

1.0 μL Taq enzyme.

1.0 μL 10 mM dNTP mix.

5.0 μL 20 μM 8-oxo-dGTP.

5.0 μL 20 μM dPTP.

27 μL ddH_2O.

Cycle the reaction as follows:

1 cycle of 95 °C for 3 min; 12–16 cycles of 95 °C for 45 s, 60 °C for 30 s, and 72 °C for 90 s; 1 cycle of 72 °C for 10 min; and 1 cycle of 4 °C forever.

Running the PCR with the mutagenic dNTPs (8-oxo-dGTP and dPTP) under these conditions will introduce, on average, one or more amino acid mutations per gene [10].

2. *Purify the mutagenized DNA*, *amplify the DNA further*, *and purify the amplified DNA*. Run a DNA gel and purify the full-length PCR product using a gel extraction kit. Elute the DNA in 40 μL ddH_2O. Amplify the DNA further in a 200 μL PCR reaction using Taq polymerase according to the following recipe:

   10 μL template (from previous PCR).

   2 μL 100 μM forward primer.

   2 μL 100 μM reverse primer.

   20 μL 10× ThermoPol buffer.

   4 μL Taq enzyme.

   4 μL 10 mM dNTP mix.

   158 μL ddH_2O.

   Use the following cycling:

   1 cycle of 95 °C for 3 min; 30 cycles of 95 °C for 45 s, 55 °C for 30 s, and 72 °C for 90 s; 1 cycle of 72 °C for 10 min; and 1 cycle of 4 °C forever.

   Run a DNA gel of the amplified product, extract the properly sized band, and purify it. This PCR reaction will usually yield between 4 and 8 μg of purified product (*see* **Note 45**).

3. *Create a library*. Using the techniques outlined in Subheading 3.1, construct a library in yeast using a single electroporation cuvette per library. Given the limited throughput of flow cytometers, no benefit will be obtained from having libraries larger than approximately 1×10^7 clones, although there is no harm in having a library of larger size, either. Induce the library using the techniques described in Subheading 3.1.

4. *Screen the library using equilibrium screening* (*if appropriate*). If the K_ds of the majority of the clones in the library remain of

low to medium affinity (weaker than 5–10 nM), equilibrium screening remains the most feasible and straightforward method to search for clones with improved affinity for the antigen. To maximize the probability of finding clones with higher affinities, label the induced library with a concentration of antigen resulting in weak but perceptible binding for the majority of clones in the library (*see* **Note 46**). Large volumes (50–100 mL) may be required to enable sorting with antigen in the low nanomolar range. If this is the case, do not label the cells with cmyc during incubation with the antigen. Instead, after completing antigen labeling, pellet the cells, wash once, and resuspend cells in ice-cold PBSA containing a 1:250 dilution of chicken anti-cmyc, keeping the concentration of cells at 1×10^7 cells/50 μL or lower. Vortex the cells briefly and incubate on ice for 10 min before proceeding with washing and secondary labeling as described in Subheading 3.2, **step 12**. Sort the library using the techniques described in Subheading 3.3 until no further enrichment can be obtained. If the enriched population remains bright upon labeling with subnanomolar concentrations of antigen, screen the library using the kinetic screening approach described in the next step. Otherwise, isolate the DNA from the enriched population, evaluate individual clones, and initiate construction of a second error-prone PCR library.

5. *Screen the library using kinetic sorting.* If the library contains clones with K_ds at or below the low nanomolar range, equilibrium screening becomes impractical due to the large volumes required to label cells with antigen. Kinetic sorting can be used to circumvent this issue. To set up kinetic sorting, label cells with a concentration of biotinylated antigen that will allow all of the antibody-displaying cells to become saturated with antigen (i.e., tenfold higher than the K_d of the lowest affinity antibody in the population). Pellet the cells and wash them once in 1 mL ice-cold PBSA. After pelleting a second time, resuspend the cells in a large excess of nonbiotinylated antigen (at least 10–100-fold higher than the effective antibody concentration in solution) and allow biotinylated antigen to dissociate from the cells at room temperature. Rebinding of biotinylated antigen will be prevented by the presence of the nonbiotinylated antigen. In an analogous situation to equilibrium screening, allow the biotinylated antigen to dissociate from cells until the majority of the clones in the library have a weak but perceptible amount of antigen binding when analyzed on the flow cytometer (*see* **Note 47**). After a sufficient length of time, pellet the cells and wash them once in ice-cold PBSA. As in **step 4**, resuspend the cells with 1:250 cmyc in ice-cold PBSA to provide a primary label for the detection of scFv display, and then proceed with secondary labeling and

sorting as in Subheading 3.3. Sort the library until sufficient enrichment is obtained and then determine whether to characterize individual mutants or move directly on to more affinity maturation.

4 Notes

1. Numerous methods exist for preparing antibody fragment libraries or for obtaining DNA for construction of a library. For example, the Sidhu laboratory at the University of Toronto has described one accessible approach to the de novo assembly of minimal antibody fragment libraries in a recent book chapter [11]. Many antibody libraries have been made for phage display and can easily be adapted for yeast surface display. Some existing antibody fragment libraries include Tomlinson I and J libraries [12] and the ETH-2 human antibody library [13]. Construction of nonimmune antibody libraries from human antibody repertoires is also a well-established approach [14].
2. Typically, YPD media should not be supplemented with antibiotics, especially when preparing libraries. However, in cases where contamination poses a significant threat, supplement the media with 100× Pen/Strep solution.
3. Whenever possible, grow liquid cultures in flasks in which no more than 40 % of the culture volume is occupied by liquid. One exception: 2 L flasks seem to work well for growing 1 L cultures.
4. Growth of yeast at pH 4.5 discourages bacterial growth. Addition of Pen/Strep to all SDCAA and SGCAA medium is also recommended to prevent contamination. If contamination appears, supplement the culture with 50 μg/mL kanamycin and continue growing the culture to attempt to eliminate the bacteria.
5. A pH of 6.0 increases the probability that antibodies will remain properly folded when displayed on the surface of yeast.
6. Some proteins are sold commercially as biotinylated proteins. A good positive control antigen is lysozyme, sold in biotinylated form by Sigma (product #L0289). Biotinylation of antigens can also be accomplished via chemical [15] or enzymatic [16] means.
7. Each transformation performed using this protocol will yield roughly 1×10^7–1×10^8 transformants. Thus, if a billion-member library is to be constructed, preparations should be made for approximately 20 transformations.
8. A freshly streaked plate of cryogenically frozen EBY100 helps to ensure that the cells retain their tryptophan auxotrophy.

Cryogenic stocks can be prepared by mixing 1 mL saturated EBY100 culture with 1 mL sterile 30 % glycerol in a 2 mL cryogenic vial and freezing the vial at −80 °C.

9. Two passages should be performed prior to electroporation to ensure that the EBY100 are as healthy as possible. For especially large-scale electroporation efforts, the second passage should be performed into a larger volume of YPD. This volume can be estimated based on the observation that the saturation OD_{600} of EBY100 in YPD is typically between 8 and 16.
10. The doubling time of EBY100 in YPD media is approximately 1.5–2 h.
11. The OD_{600} measurement of liquid yeast cultures allows for an estimate of the number of yeast in a given culture. An OD_{600} of 1 corresponds to approximately 1×10^7 cells in 1 mL media. At the time of passaging, the number of transformants in the library (also known as the library size) will not yet be known due to the slow rate of colony formation on SDCAA plates. Thus, the library diversity must be preserved by assuming the highest possible number of transformants in the library and passaging sufficient numbers of cells. The electroporation procedure described here typically yields 1×10^7–1×10^8 transformants per electroporation. Thus, if five electroporations are pooled, the maximum expected library size would be approximately 5×10^8. Allowing for an additional safety factor of 2, the library should be assumed to contain 1×10^9 members. In order to preserve library diversity in this case, a number of cells equal to or greater than ten times the maximum library size should be passaged (based on Poisson statistical calculations [17]).
12. Occasionally, when yeast cells are grown to saturation or experience other conditions of stress, they become flocculent, forming clumps of cells that tend to sediment quickly. This does not appear to affect plasmid retention, but flocculation can impact the ability to accurately read OD_{600}s, aliquot cells for different experiments, or screen cells individually in a flow cytometry experiment. Therefore, if a culture is observed to be flocculent, pellet a portion of the offending culture and resuspend the pellet to an OD_{600} of 1 in SDCAA medium containing 4 % glucose instead of 2 %. Dilution of the cells and growth in richer medium tend to reverse flocculation.
13. Under a microscope, yeast (~3–5 μm in diameter) will appear stationary, while any contaminating bacteria (~1 μm in diameter) will be quite motile.
14. For libraries in which repeated use of frozen fractions is anticipated, expand the culture to a greater volume, prepare a large number of stocks (40–100), and deem this collection of vials

the master cell bank. To obtain the maximum possible number of stocks out of the library, upon thawing a member of the master bank (to be described in Subheading 3.2, **step 1**), expand the culture to a large volume and create 10–100 frozen vials of a working bank. Use all of the vials in the working bank, and when it is spent, make a new working bank from a single vial of the master bank. For libraries to be used for short-term purposes only (≤1 month), these libraries can be stored at 4 °C. Revival can be accomplished by resuspending the stored culture, pelleting 1×10^{10} cells, resuspending the pellet in fresh medium, and initiating sorting following the steps laid out in Subheading 3.2 starting with **step 1**.

15. In this analysis any cell exhibiting positive staining for both cmyc and HA tags will be assumed to be of full length. Given the limited throughput of the flow cytometer, only a small amount of the total library can be investigated practically. Thus, while a small-scale induction of the library will not contain the entirety of the library diversity, it will contain enough of the library to determine the percentage of full-length clones.
16. In general, approximately 70 % of frozen cells will be viable upon thawing. For especially large libraries, multiple aliquots may need to be thawed in order to adequately preserve diversity. To validate a freezing procedure, plate serial dilutions of the thawed cells on SDCAA plates and count colonies to estimate the number of viable cells in the vial.
17. The doubling time of yeast in SDCAA is approximately 3–4 h.
18. Larger libraries (up to 1×10^9 members) can be thawed and induced on a similar scale. After thawing the cells and letting the initial culture grow to saturation, carry a number of cells equal to ten times the library diversity on to each subsequent step. Freshly diluted cultures should have an OD_{600} of no greater than 1, a criteria that can be satisfied by using the rule of thumb that 100 mL media should be used per 1×10^9 cells (or per 1×10^8 library members).
19. The incubation step can be performed as induction of the yeast cells is occurring. Final washing of the beads for positive enrichment should be performed within 1–2 h of adding the beads to the cells after negative selections. The value of 33 pmol represents sufficient antigen to saturate the available streptavidin-binding sites present in 10 μL biotin binder beads according to the manufacturer's information. If biotinylated antigen is especially precious, as few as 6.7 pmol antigen can be used. However, in this case, the negative selections are especially important to avoid enriching the library for streptavidin or magnetic particle binders.

20. In the case of larger libraries, use 10 μL beads for every 2×10^9 cells to be sorted for up to 1×10^{10} cells. For cell numbers between 1×10^{10} and 1.5×10^{10}, 50 μL beads can be used.
21. In general, the use of more cells in a bead sort will lead to a lower probability of nonspecific bead binding, with the ratio of 2×10^9 cells to 10 μL biotin binder beads providing a high level of consistency. Induction of 1×10^9 cells will almost always yield more than two billion cells after 18–24 h. Typically, induced cultures reach an OD_{600} between 2.5 and 4.
22. Strict sterile technique is not necessary during bead preparation and sorting. Minimal instances of contamination will be observed as long as all reagents and pipettes are sterile and tubes are open for a short time.
23. Resuspend cells to be used in sorts in ice-cold PBSA. For cell numbers less than 2×10^9, use 1 mL PBSA. For cell numbers in between 2×10^9 and 1×10^{10}, use 1 mL PBSA per 2×10^9 cells. For cell numbers between 1×10^{10} and 1.5×10^{10}, use 5 mL.
24. For libraries that will be used for routine binder isolation, consider depleting the library of bead binders repeatedly and storing this depleted library in cryogenic vials. This will result in a library that can be immediately enriched for binding to antigens. To deplete, perform three negative depletions on the first depletion day, recover the supernatant from the third depletion, pellet the cells, and resuspend them in 1 L SDCAA. Grow, passage, and induce the cells, perform a second day of three depletions, and expand the cells for pelleting and cryogenic freezing as described in Subheading 3.1. To perform bead sorts with the library, thaw and induce the cells as outlined in Subheading 3.2, **step 1**; prepare antigen-coated beads as in Subheading 3.2, **step 2**; and proceed immediately to Subheading 3.2, **step 6**.
25. When speed is especially crucial, cells can be induced as soon as they reach logarithmic phase after a bead sort. Remove the beads from the cells, and resuspend the cells in SGCAA at a final OD_{600} of 1 and induce at 20 °C for 18–24 h. The one drawback to this accelerated approach is that fewer cells are used in the second round of sorting, which can potentially lead to more nonspecific binding and lower enrichment factors. In general, the bead sorting seems to work efficiently when at least 5×10^8 cells are used as input.
26. Numerous plasmids encoding functional, high-affinity binders to different targets now exist. Antibody fragment and fibronectin lysozyme binders are especially easy to use given the ready availability of biotinylated lysozyme and include the scFv D1.3 [18] and the fibronectin L7.5.1 [19].

Many other displayed binding proteins suitable as controls have also been described in the literature [7].

27. Vortex all samples after flow cytometry reagent additions in order to improve the uniformity of labeling.
28. Preferred tubes will be different for distinct models of flow cytometers, but most instrument manufacturers recommend sample filtration with devices such as tubes topped with a mesh filtration cap such as Falcon 12×75 mm polystyrene test tubes (Falcon #352235, VWR #21008-948).
29. When a population of yeast is induced, a certain percentage (roughly 30–50 %) fails to display the protein of interest, even in a genetically uniform population.
30. In general, populations smaller than 1×10^7 members should be induced at the 5 mL scale.
31. For example, in a volume of 50 μL, 1×10^7 cells displaying approximately 5×10^4 antibodies per cells will have an effective concentration of $(10^7 \text{ cells}/0.00005 \text{ L}) \times (5 \times 10^4 \text{ antibodies}/\text{cell}) \times (1 \text{ mol}/6.02 \times 10^{23} \text{ antibodies}) = 16.6$ nM. Thus, cell labeling with antigen concentrations lower than approximately 170 nM should be performed in larger volumes.
32. For example, for a population in which the highest expected binders have K_ds of roughly 10 nM and the solution is being labeled at 100 nM, 95 % equilibrium will be reached in $3\tau = 3((1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}) \times (100 \times 10^{-9} \text{ M}) + 1 \times 10^{-3} \text{ s}^{-1})^{-1} = 273$ s or roughly 5 min. For most naïve libraries, the highest K_ds encountered will not fall below 1 nM. The apparent K_ds of populations of cells can also be determined using the titration methods outlined in Subheading 3.4.
33. Because detection of antigen binding tends to yield lower levels of cell staining than the detection of N- or C-terminal display (Fig. 1), the signal of antigen-binding cells may not be easily maximized without raising the apparent fluorescent values of negative cells to inappropriate levels. In this case, use the negative cells as a guide to set the PE voltage so that these cells fall within the lowest decade of fluorescence values.
34. In cases where only high-affinity binders are deemed important, this gate can be quite stringent, encompassing a region substantially separated from the borders of the quadrants (Fig. 2) and as few as 0.1 % or less of the total events. Regardless of antigen binding stringency, be sure to gate only on cells that are clearly of full length to minimize the isolation of truncated clones.
35. This analysis can be performed in parallel with additional sorting.
36. If streptavidin binders were present after bead enrichments, perform analysis of the population using streptavidin–Alexa

Fluor 488 to ensure that these binders are now absent from the population. Furthermore, if secondary reagent binding becomes a severe problem at any point, sort the population for full-length clones that do not recognize the secondary reagents before sorting again for antigen binders.

37. Most naïve libraries can be enriched using equilibrium sorting methods alone, but some special cases (library with exceptional properties, bi- or multivalent antigens) may require kinetic sorting as described in Subheading 3.5, **step 5**.
38. The columns accompanying the Zymoprep kit do not always bind the plasmid DNA efficiently, so alternatives such as Qiagen Qiaprep columns or Epoch Life Sciences EconoSpin columns are preferred.
39. Either chemically competent or electrocompetent *E. coli* can be transformed with zymoprepped DNA. In some cases, the zymoprep procedure gives low yields of DNA. In these cases, use electrocompetent cells for transformation. The *E. coli* strains DH10B and DH5α are excellent cloning strains, and competent versions of these cell strains are readily available from companies such as Life Technologies (DH10B: electrocompetent: ElectroMAX DH10B Cells; chemically competent: MAX Efficiency DH10B Competent Cells; DH5α: electrocompetent: ElectroMAX DH5α-E Competent Cells; chemically competent: MAX Efficiency DH5α Competent Cells).
40. For routine evaluations, ten colonies per transformed DNA population are sufficient to determine basic information about the genetic diversity of a population such as the number of distinct clones.
41. If bulk DNA is to be used for affinity maturation purposes only, the number of transformants is not critical as long as the population contains only a few dominant clones. On the other hand, if library coverage is crucial, take a portion of the freshly inoculated 50 mL culture and plate it on an LB-Amp plate and grow the plate at 37 °C for 12–18 h. Use the number of colonies that appear on the plate to estimate the total number of transformants; ensure that this number is greater than or equal to ten times the number of yeast cells recovered in the final sort.
42. Tube-based titration setup has been described previously [8].
43. If multiple scFvs are used, this enables recombination within scFv sequences to occur, leading to potentially faster affinity maturation [20]. DNA shuffling [21] or other approaches to sequence diversification can also be considered for the rapid affinity maturation of antibodies.
44. Use the same primers in the previous amplification of library DNA for homologous recombination. This will exclude roughly

nine to ten residues at the start and end of the protein from mutagenesis. If these residues are suspected to play an important role in the structure or the function of the scFv of interest, use primers that bind outside of the coding sequence of the scFv [8].

45. If between 1 and 4 μg DNA is obtained from the reaction, proceed on to Subheading 3.5, **step 3**, and create a library. The resulting library will likely contain enough diversity (1×10^6 or more transformants) to reliably yield improved scFvs.
46. If the library was created from a single clone, experiments with the single clone can be used to empirically determine an appropriate antigen concentration. If the K_d of the clone is known, an optimal value can be determined by using theories described in the literature [22].
47. As in the case of equilibrium screening, if the library was created from a single clone, the single clone can be used to empirically determine an appropriate length of competition prior to preparing library samples for sorting. If the dissociation rate (k_{off}) of the clone is known, theory can also be used to determine suitable lengths of competition [22].

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