Isolating and engineering human antibodies using yeast surface display

Ginger Chao, Wai L Lau, Benjamin J Hackel, Stephen L Sazinsky, Shaun M Lippow & K Dane Wittrup

Department of Chemical Engineering and Biological Engineering Division, Massachusetts Institute of Technology, 77 Massachusetts Avenue E19-563, Cambridge, Massachusetts 02139, USA. Correspondence should be addressed to K.D.W. (wittrup@mit.edu).

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This protocol describes the process of isolating and engineering antibodies or proteins for increased affinity and stability using yeast surface display. Single-chain antibody fragments (scFvs) are first isolated from an existing nonimmune human library displayed on the yeast surface using magnetic-activated cell sorting selection followed by selection using flow cytometry. This enriched population is then mutagenized, and successive rounds of random mutagenesis and flow cytometry selection are done to attain desired scFv properties through directed evolution. Labeling strategies for weakly binding scFvs are also described, as well as procedures for characterizing and 'titrating' scFv clones displayed on yeast. The ultimate result of following this protocol is a panel of scFvs with increased stability and affinity for an antigen of interest.

INTRODUCTION

Yeast surface display is a powerful method for isolating and engineering antibodies to increase their affinity, specificity and stability. Yeast display has been used to engineer antibodies to various antigen targets, including T cell receptors[1], huntingtin protein[2], carcinoembryonic antigen[3] and botulinum neurotoxin[4]. In addition, an antibody to fluorescein has been engineered to femtomolar affinity, the highest affinity reported so far[5]. Yeast display has also been used to engineer other proteins with a variety of applications[6–9].

In the yeast surface display system[10] (**Fig. 1**), the antibody is displayed in a single-chain variable fragment (scFv) format in which the heavy and light chains are connected by a flexible polypeptide linker. The scFv is fused to the adhesion subunit of the yeast agglutinin protein Aga2p, which attaches to the yeast cell wall through disulfide bonds to Aga1p. Expression of the Aga2p-scFv is under the control of a galactose-inducible promoter on the yeast display plasmid, which is maintained in yeast episomally with a nutritional marker, whereas Aga1p is expressed from a chromosomally integrated galactose-inducible expression cassette. Each yeast cell typically displays 1×10^4 to 1×10^5 copies of the scFv, and variations in surface expression can be measured through immunofluorescence labeling of either the hemagglutinin or c-Myc epitope tag flanking the scFv. Likewise, binding to a soluble antigen of interest can be determined by labeling of yeast with biotinylated antigen and a secondary reagent such as streptavidin conjugated to a fluorophore.

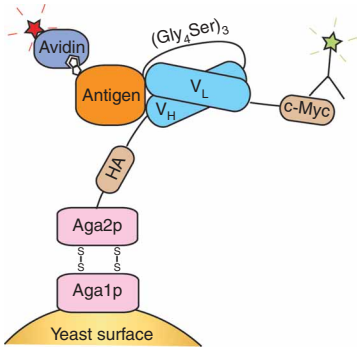

Figure 1 | Yeast surface display. The scFv (cyan) is displayed as an Aga2 (pink) fusion protein on the surface of yeast. Expression can be detected by using fluorescent antibodies binding to the epitope tags (beige), and binding of the scFv to a biotinylated antigen (orange) can be detected using fluorescent avidin (violet). HA, hemagglutinin; V_L, variable light chain; V_H, variable heavy chain; $(Gly_4Ser)_3$, flexible peptide linker.

Yeast surface display offers several advantages for protein-directed evolution. It enables quantitative screening through the use of fluorescence-activated cell sorting, allowing the equilibrium activity and statistics of the sample to be observed directly during the screening process. Furthermore, the antigen-binding signal is normalized for expression, eliminating artifacts due to host expression bias and allowing for fine discrimination between mutants[11]. Antibodies can be engineered for improved stability, as expression is measured directly and has been shown to correlate with the stability of the displayed protein[12]. Using a two-color labeling scheme, with one fluorophore for expression and another for antigen binding, stability and affinity engineering can be accomplished simultaneously. Once maturation is complete, antibody affinity can be conveniently 'titrated' while displayed on the surface of the yeast, obviating the need for expression and purification of each clone. In almost every case for dozens of different antibodies, the binding properties on the yeast cell surface are in quantitative agreement with those measured in solution or by biosensor methods. Finally, the displayed proteins are folded in the endoplasmic reticulum of the eukaryotic yeast cells, taking advantage of endoplasmic reticulum chaperones and quality-control 'machinery'. A theoretical limitation of yeast surface display is the potentially smaller functional library size (about 1×10^7 to 1×10^9) than that of other selection methods (phage display, about 1×10^6 to 1×10^{11}; mRNA-ribosome display, about 1×10^{11} to 1×10^{13}). However, it is difficult to determine the true functional diversity of any display library, and bias-free propagation of yeast libraries has been confirmed over an amplification of 10^{10}-fold (ref. 13). Furthermore, all of these methods greatly undersample the theoretical sequence space of scFv complementary-determining region variation (about 1×10^{80}). To realize the advantages of kinetic

screening and expression normalization, yeast surface display also requires more complex equipment than other display methods. Various technologies for screening recombinant antibody libraries and their relative strengths and weaknesses have been reviewed[14].

Although antibodies can be displayed on yeast in an Fab format[15,16], the protocol presented here details the isolation and subsequent engineering of scFvs starting from an existing non-immune human scFv library[13]. As this library is derived from fully human antibody sequences, the isolated scFvs are potentially suitable for therapeutic development. Once initial candidate scFvs are isolated from this library, directed evolution can be used to obtain scFvs with improved properties, if desired, through several rounds of random mutagenesis and screening. This protocol is also applicable to engineering a previously obtained scFv clone or any protein that can be displayed by yeast for improved binding to a soluble species. If soluble antigen is not available, yeast-displayed scFvs can be 'panned' against mammalian cell monolayers[17] or incubated with mammalian cells in solution and separated by density centrifugation[18], both of which are beyond the scope of this protocol. Although yeast surface display procedures have been described in detail before[19–21], this work includes updated protocols and new methods, as well as a more comprehensive view of the entire antibody isolation and engineering process.

We have provided an outline of the procedure for isolating, engineering and characterizing scFvs against an antigen of interest using yeast surface display (**Fig. 2**). As typical flow cytometry apparatuses can sort 1×10^7 to 1×10^8 cells per hour, magnetic-activated cell sorting (MACS) must first be used to reduce the size of the library from 1×10^9 to about 1×10^7 unique clones (Steps 1–9). The MidiMACS separation protocol described here is adapted from that published by Feldhaus and Siegel[20,22], and an alternative option using Dynal magnetic beads has also been described[23]. If MACS equipment is unavailable, polystyrene or agarose beads conjugated to either the antigen of interest or streptavidin can also be used for initial rounds of sorting in a column format (optimal conditions for a given system should be determined by the user). Steps 11–20 outline a general protocol for labeling yeast cells with fluorescent reagents. This same protocol is used to analyze the display level of the MACS-sorted population (Step 10), to label cell populations for sorting by flow cytometry (Step 21) and to analyze flow cytometry–sorted cell populations (Step 26). Once the MACS-sorted population has been analyzed to ensure scFv expression, the population is further enriched using flow cytometry (Steps 21–25). If the yeast population does not seem to be enriching for antigen binders, a streptavidin preloading protocol (**Box 1**) can be used to increase the antigen avidity and thus the chances of successful enrichment. Next, the flow cytometry–sorted population is analyzed (Steps 26–28). Once it is determined that the scFvs are specific for the antigen of interest, they are engineered for increased affinity and/or stability using random mutagenesis through error-prone PCR with nucleotide analogs (Steps 29–35)[24]. This mutagenesis protocol is preferred because it allows control of the mutation frequency by alteration of the number of PCR cycles and yields both transition and transversion mutations. Other methods of generating diversity, such as DNA shuffling[25,26], are also compatible with the yeast display system, and additional methods of introducing library diversity have been reviewed[27]. For engineering a single scFv or protein sequence, the DNA of interest must first be cloned into a yeast surface display vector, and then the protocol can be followed beginning at Step 29.

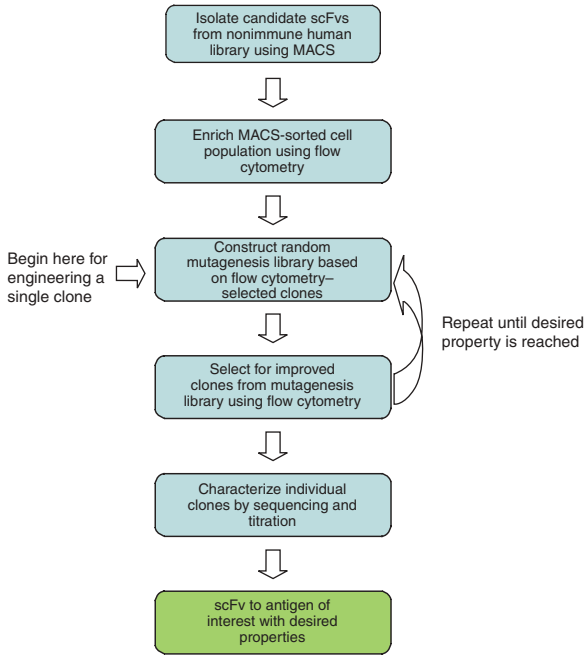

Figure 2 | The antibody isolation and engineering process.

The mutagenized yeast library is created by transformation of yeast with linearized vector and the error-prone PCR product, and *in vivo* homologous recombination occurs between the vector and PCR insert to generate the display plasmid. The protocol for yeast transformation by electroporation described in Steps 36–48 has been adapted from that published by Meilhoc *et al.*[28]. As the PCR insertion products are also homologous to each other, additional recombination events occur between inserts and lead to greater library diversity[26]. If electroporation equipment is unavailable, the yeast can also be transformed using lithium acetate, which may yield slightly lower transformation efficiencies[29]. Rounds of mutagenesis followed by flow cytometry sorting are done until the scFv population reaches the desired property. Finally, individual clones of the population are characterized in depth by titration of scFvs displayed on the yeast surface (Steps 51–63). This protocol is similar to the general protocol for labeling yeast cells described in Steps 11–20.

MATERIALS

REAGENTS

- Nonimmune human scFv yeast library (available by request from Pacific Northwest National Laboratories at http://www.sysbio.org/dataresources/singlechain.stm)
- SDCAA media and plates; SGCAA media (see REAGENT SETUP)
- Yeast strain EBY100 (Invitrogen, cat. no. C839-00; strain information, **Supplementary Methods** online)
- Yeast display vector pYD1 (Invitrogen, cat. no. V835-01) or pCTCON2 (available by request; plasmid map, **Supplementary Fig. 1** online), if engineering a single clone
- PBSM and PBSF buffer (see REAGENT SETUP)

BOX 1 | OPTIONAL PROTOCOL FOR STREPTAVIDIN PRELOADING TO INCREASE ANTIGEN AVIDITY

This protocol is useful for isolating weak antigen binders from the nonimmune human scFv library. It may not be possible to overcome weak scFv binding by simply using higher antigen concentrations because of low antigen availability or excessive nonspecific binding. To circumvent this problem, the biotinylated antigen can be preloaded on a streptavidin-fluorophore conjugate (or neutravidin) to form an antigen-streptavidin fluorescent complex[31]. Streptavidin binds biotin with femtomolar affinity and forms tetramers in physiological conditions, thus generating a tetravalent complex when preincubated with singly biotinylated antigen. This protocol can also be used for antigens with multiple biotins, although all four epitopes in a tetramer may not be accessible because of biotinylated lysines, or a polymer of antigen-streptavidin complex 'crosslinked' by individual antigen molecules may be formed. Streptavidin preloading can increase the effective antigen concentration up to 500-fold.

1. Determine the molar concentration of the streptavidin-fluorophore conjugate. For streptavidin-phycoerythrin from Invitrogen, the concentration is given as 1 mg ml^{-1}. This is equivalent to 3.6 µM streptavidin-phycoerythrin tetramer, based on a molecular mass of 278 kDa (i.e., 53 kDa [streptavidin tetramer] + 225 kDa [phycoerythrin]).
2. Preincubate streptavidin-fluorophore with biotinylated antigen on ice for 30 min, shielded from light, at a streptavidin tetramer/biotinylated antigen molar ratio of 1:4 in as small a volume as possible. The antigen concentration for this incubation is normally in the low micromolar range.
3. Dilute preincubation with PBSF buffer to give an appropriate final concentration and label cells according to Step 13 in the main protocol. For isolation of weak binders from the nonimmune human library, concentrations in the range of 100–500 nM preloaded antigen have been used for the first round of selection.

- Streptavidin magnetic microbeads (Miltenyi, cat. no. 130-048-101)
- Microbeads conjugated to antibody to biotin (anti-biotin microbeads; Miltenyi, cat. no. 130-090-485)
- Penicillin-streptomycin ('pen-strep'; 10,000 units/ml and 10,000 µg/ml; Invitrogen, cat. no. 15140-122)
- Primary anti-c-Myc (chicken anti-c-Myc IgY is recommended; Invitrogen, cat. no. A-21281)
- Antigen of interest, biotinylated (kits available from Invitrogen, cat. no. B30010 or F-2610)
- Secondary reagents, recommended (Invitrogen; see REAGENTS SETUP): Alexa Fluor 488–goat anti-chicken IgG (cat. no. A-11039), streptavidin-phycoerythrin (cat. no. S-866), streptavidin-allophycocyanin (cat. no. S-868) and neutravidin-phycoerythrin (cat. no. A-2660)
- Zymoprep I or Zymoprep II yeast plasmid miniprep kit (Zymo Research, cat. no. D2001 or D2004)
- XLI-blue Supercompetent *Escherichia coli* (Stratagene, cat. no. 200236)
- *Taq* DNA polymerase, including 10× *Taq* buffer and 50 mM $MgCl_2$ (Invitrogen, cat. no. 18038)
- 2′-deoxynucleoside 5′-triphosphates (dNTPs)
- 8-oxo-2′-deoxyguanosine 5′-triphosphate (8-oxo-dGTP; TriLink, cat. no. N-2034)
- 2′-deoxy-*p*-nucleoside-5′-triphosphate (dPTP; TriLink, cat. no. N-2037)
- Oligonucleotide primers
- 10× gel-loading buffer (50% glycerol and 0.1% bromophenol blue)
- SYBR gold nucleic acid gel stain (Invitrogen, cat. no. S-11494)
- QIAquick Gel extraction kit (Qiagen, cat. no. 28704)
- YPD media and plates (see REAGENT SETUP)
- PelletPaint co-precipitant (EMD Biosciences, cat. no. 69049)
- E buffer (see REAGENT SETUP)
- Agarose
- Restriction enzymes (New England Biolabs): *Nhe*I (cat. no. R0131), *Sal*I (cat. no. R0138) and *Bam*HI (cat. no. R0136)
- EZ Yeast Transformation II kit (optional; Zymo Research, cat. no. T2001)
- Tris-dithiothreitol (DTT) buffer (see REAGENT SETUP)

EQUIPMENT

- Incubator shaker at 30 °C
- Incubator shaker at 20 °C (optional, but recommended)
- MACS equipment (Miltenyi): AutoMACS separator (cat. no. 201-01) or MidiMACS separation unit, stand and LS columns (cat. nos. 130-042-401, 130-042-302 and 130-042-303)
- Tube rotator (optional)
- Flow cytometry apparatus with appropriate laser(s) and detectors (see REAGENT SETUP)
- 0.2-cm electroporation cuvettes (BioRad, cat. no. 652086)
- Gene Pulser (BioRad, cat. no. 1652076)

REAGENT SETUP

Fluorophores Appropriate fluorophores should be chosen according to the lasers and filters of the flow cytometer. A discussion of alternative fluorophores and their appropriateness for cytometry is available at http://www.probes.com/handbook and http://www.bdbioscience.com/spectra.

YPD media Dissolve 20 g dextrose, 20 g peptone and 10 g yeast extract in deionized H_2O to a volume of 1 liter and sterilize by filtration. This medium can be stored for up to 1 month at 25 °C (room temperature) or for up to 2 months at 4 °C.

SDCAA media Dissolve 20 g dextrose, 6.7 g Difco yeast nitrogen base, 5 g Bacto casamino acids, 5.4 g Na_2HPO_4 and 8.56 g $NaH_2PO_4 \cdot H_2O$ in deionized H_2O to a volume of 1 liter and sterilize by filtration. This medium can be stored for up to 6 months at 4 °C.

SDCAA media, pH 4.5 (optional) Prepare as for SDCAA, but use 14.7 g sodium citrate and 4.29 g citric acid monohydrate instead of phosphates.

SGCAA media Prepare as for SDCAA, but use 20 g galactose instead of dextrose.

LB Amp media Dissolve 10 g tryptone, 5 g yeast extract, 10 g NaCl and 50 mg ampicillin in deionized H_2O to a volume of 1 liter and sterilize by filtration. This medium can be stored for up to 4 months at 4 °C.

YPD plates Dissolve 20 g dextrose, 20 g peptone, 10 g yeast extract and 15 g agar in deionized H_2O to a volume of 1 liter, autoclave and pour plates. The plates can be stored for up to 6 months at 4 °C.

SDCAA plate Dissolve 5.4 g Na_2HPO_4, 8.56 g $NaH_2PO_4 \cdot H_2O$, 182 g sorbitol and 15 g agar in deionized H_2O to a volume of 900 ml and autoclave. Dissolve 20 g dextrose, 6.7 g Difco yeast nitrogen base and 5 g bacto casamino acids in deionized H_2O to a volume of 100 ml and sterilize by filtration. Cool autoclaved mixture with stirring until below 50 °C, add filter-sterilized solution, mix and pour plates. Plates can be stored for up to 6 months at 4 °C.

LB Amp plates Dissolve 10 g tryptone, 5 g yeast extract, 10 g NaCl and 15 g agar in deionized H_2O to a volume of 1 liter and autoclave. Dissolve 100 mg ampicillin in deionized H_2O to a volume of 4 ml and sterilize by filtration (prepare fresh solution or store stock solution at –20 °C for up to 1 year). Cool agar mixture with stirring until below 50 °C, add ampicillin solution, mix and pour plates. Plates can be stored for up to 4 months at 4 °C.

Tris-DTT buffer Dissolve 0.39 g 1,4-dithiothreitol in a solution of 1 ml 1 M Tris, pH 8.0 (121.1 g Tris base per liter H_2O, pH 8.0) and sterilize by filtration. This solution must be prepared fresh or can be stored for up to 6 months at –20 °C.

E buffer Dissolve 1.2 g Tris base, 92.4 g sucrose and 0.2 g $MgCl_2$ in deionized H_2O to a volume of 1 liter, adjust the pH to 7.5 and sterilize by filtration. This solution can be stored at room temperature.

PBSF buffer Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 and 1 g bovine serum albumin in 1 liter of deionized H_2O, adjust the pH to 7.4 and sterilize by filtration. This solution can be stored for up to 6 months at 4 °C.

PBSM buffer Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, 5 g bovine serum albumin and 744 mg EDTA in 1 liter of deionized H_2O, adjust the pH to 7.4 and sterilize by filtration. This solution can be stored for up to 6 months at 4 °C.

Tris-acetate-EDTA buffer (50× TAE) Dissolve 242 g Tris base in 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA (186.1 g disodium EDTA••2 H_2O per liter of H_2O; pH 8.0) and add deionized H_2O to a volume of 1 liter. Dilute with deionized H_2O and use at a concentration of 1×. This solution can be stored at room temperature.

PROCEDURE

1| Thaw frozen aliquots of human scFv yeast library at room temperature and grow overnight (about 20 h) in at least 1 liter of SDCAA media in an incubator shaker set to 30 °C and 250 r.p.m. (**Supplementary Table 1** online lists preferred yeast culture vessels). The yeast typically grow overnight to an absorbance of about 6–8 at 600 nm (an absorbance of 1 at 600 nm is about 1×10^7 cells per ml). Passage the cells by starting a new culture with 1×10^{10} cells in fresh SDCAA media to eliminate dead cells. If desired, the viability of the library can be tested by plating serial dilutions on SDCAA plates to ensure adequate library diversity. All subsequent yeast growth and induction steps should be done with shaking at 250 r.p.m.

■ **PAUSE POINT** Yeast cells grow overnight. Full-grown cultures can be stored for about 2 weeks at room temperature and for about 1 month at 4 °C. Frozen aliquots can be made for long-term storage (**Supplementary Methods** online). Cells should be freshly passaged before induction.

2| Pellet at least 1×10^{10} cells from a freshly passaged library culture at 2,500*g* for 5 min in 50-ml conical tubes and resuspend cells in SGCAA media to an absorbance of about 0.5–1 at 600 nm to induce expression of scFvs. Induce the nonimmune human library at 20 °C for at least 36 h. For single clones and smaller libraries, cells should be induced for at least 20 h. Induction of cells during exponential growth (an absorbance of 2–5 at 600 nm) has been shown to increase scFv expression. The addition of 2 g l^{-1} dextrose to SGCAA media can also improve surface display amounts. The induction temperature can be raised to 30 °C or 37 °C to select for more stable clones, but the temperature is usually not raised until after the first MACS selection.

■ **PAUSE POINT** Yeast cells induce overnight.

▲ **CRITICAL STEP** Typically, at least tenfold oversampling of any yeast population is used to reduce the probability of losing unique clones.

? TROUBLESHOOTING

Isolating candidate scFv clones using MACS

3| Pellet 1×10^{10} freshly induced yeast cells at 2,500*g* for 5 min and aspirate the supernatant. To wash, resuspend cells in 25 ml PBSM buffer, repellet cells and discard supernatant.

4| To label yeast, resuspend cells in 10 ml PBSM buffer. Add biotinylated antigen to a final concentration of 1 μM and mix by gentle inversion. Initial antigen labeling concentrations for enrichment from the scFv library vary with the antigen of interest. A starting point of 1 μM is generally sufficient to enrich scFvs that bind weakly to human antigens, and subsequent sorting by flow cytometry at more stringent concentrations can be used to obtain clones that bind with higher affinity.

? TROUBLESHOOTING

5| Incubate cell suspension at room temperature with gentle rotation on a tube rotator for 60 min, followed by 10 min on ice. If a tube rotator is unavailable, keep cells in suspension throughout incubation by periodic inversion or vortexing.

6| Isolate antigen-specific scFvs using magnetic sorting; the following separation steps are for use with the Miltenyi microbead system using an automated bead capture system (AutoMACS; A) or manually with a column attached to a magnet (MidiMACS; B). All of these steps should use ice-cold PBSM buffer and should be done on ice or at 4 °C.

▲ **CRITICAL STEP** Completion of secondary labeling at 4 °C minimizes antigen dissociation from yeast-displayed scFvs.

(A) AutoMACS separation

(i) Turn on autoMACS and run the 'Clean' program. Parameters chosen for yeast separation are described below. For detailed instructions on using the autoMACS system, consult the user's manual.

(ii) Transfer 5 ml of the cell suspension to a new tube. Continue incubating the other half of the sample with antigen with rotation at 4 °C. Separation is divided into two runs to avoid overloading the separation column (approximate capacity of 5×10^9 cells).

(iii) Pellet cells at 2,500*g* for 5 min at 4 °C, aspirate supernatant and wash (rinse, repellet and aspirate) cells with 25 ml PBSM buffer.

(iv) Resuspend pellet in 2.5 ml PBSM buffer, add 100 μl streptavidin microbeads to the suspension and mix by gentle inversion.

(v) Incubate suspension on ice for 10 min, with gentle inversion every 2 min. (For anti-biotin microbeads, incubate for 30 min at 4 °C with rotation.)

(vi) Add 22.5 ml PBSM buffer to the suspension and gently mix by inversion. Under the 'pos1' port of the instrument, place a 50-ml tube filled with 18 ml SDCAA media to collect eluted cells. Under the 'neg1' port, place an empty 50-ml tube to collect the column flow-through. Place the cell suspension under the intake port and choose separation protocol 'Possel_S' to begin the separation. Two main separation options are available with autoMACS: 'Possel' allows for rapid separation (4 ml min^{-1}), whereas 'Possel_S' allows for slower, more sensitive separation (1 ml min^{-1}). With the Possel_S protocol, the volume described above should require about 30 min for sorting.

(vii) Repeat Steps iii–vi for the remainder of the sample.

(B) MidiMACS separation

(i) Place an LS column onto the magnet and stand assembly. Wash the column with 3 ml PBSM buffer to equilibrate. It is preferable to place the magnet assembly in a cold room or on a shelf in a 4 °C refrigerator.
(ii) Pellet cells at 2,500*g* for 5 min at 4 °C, aspirate supernatant and wash (rinse, repellet and aspirate) with 50 ml PBSM buffer.
(iii) Resuspend pellet in 5 ml PBSM buffer, add 200 µl streptavidin microbeads to the suspension and mix by gentle inversion.
(iv) Incubate suspension on ice for 10 min, with gentle inversion every 2 min. (For anti-biotin microbeads, incubate for 30 min with rotation at 4 °C.)
(v) Pellet cells at 2,500*g* for 5 min at 4 °C, aspirate supernatant and resuspend pellet in 50 ml PBSM buffer.
(vi) Vortex the cell suspension and apply 7 ml to the column. When the cells have passed through the column, briefly remove the column from the magnet and immediately place back on the magnet to reorient the magnetic beads in the column, allowing unlabeled cells trapped in the column to flow through. Add 1 ml PBSM buffer and let it flow through. Repeat until all cells have been loaded.
(vii) Wash the column with 3 ml PBSM buffer. To elute cells, remove the column and place over a collection tube. Add 7 ml SDCAA media to the column and use the plunger supplied with the column to push the remaining cells through.

7| Plate serial dilutions of the sorted cell suspension onto SDCAA plates and incubate at 30 °C to estimate the number of cells captured by MACS. Yeast typically require 2 d of incubation to form visible colonies.

8| Propagate eluted yeast for subsequent rounds of sorting. Add SDCAA media to the eluted cell suspension to a final volume of 500 ml, add pen-strep solution (1:100 dilution) and grow culture overnight at 30 °C.
▲ **CRITICAL STEP** To suppress bacterial contamination after magnetic sorting, grow yeast cultures in SDCAA media with pen-strep or another antibiotic, or in SDCAA media, pH 4.5.
■ **PAUSE POINT** Yeast cells grow overnight.
? TROUBLESHOOTING

9| Induce cells as described in Step 2 and repeat Steps 3–8 for subsequent MACS selection until the number of unique clones in the library is about 1×10^7. Alternate the use of streptavidin and anti-biotin microbeads during successive MACS selections to decrease enrichment for clones that bind the secondary reagent.
■ **PAUSE POINT** Yeast cultures may be treated as described in the pause point after Step 1.
▲ **CRITICAL STEP** Alternating secondary reagents reduces the chance of isolating scFvs specific for the reagents.

10| To ensure that the population sorted by MACS has been enriched for yeast cells displaying scFvs, it is advisable to label a fraction of this population and analyze its scFv expression using flow cytometry before further enrichment. Label the population sorted by MACS by following Steps 11–20, adding only anti-c-Myc and not antigen at Step 13.
? TROUBLESHOOTING

Labeling yeast cells for flow cytometry

11| If sorting a population, induce at an excess of least tenfold of library size of freshly grown cells in SGCAA media as in Step 2. If analyzing a subset of a population, inducing 5×10^7 cells in 5 ml SGCAA media is sufficient.
■ **PAUSE POINT** Yeast cells induce overnight.

12| Pellet an appropriate number of induced cells at 14,000*g* for 30 s in a 1.5-ml microfuge tube, aspirate the supernatant, and wash with 1 ml PBSF buffer (rinse, repellet and aspirate supernatant). If labeling a library population for flow cytometry selection, label a tenfold excess of population diversity. For characterization of a population or clone, it is convenient to work with a quantity of cells that forms a visible pellet after centrifugation, usually 1×10^6 cells.
? TROUBLESHOOTING

13| Label yeast with chicken anti-c-Myc IgY (1:250 dilution) and an appropriate concentration of biotinylated antigen in an appropriate final volume of PBSF buffer. Vortex to resuspend.
Antigen concentrations are chosen based on the expected dissociation constant (K_d) of the population and are discussed further in Step 49. Typical labeling volumes are 50 µl for 1×10^6 cells and 0.5–1 ml for 1×10^8 cells. The labeling volume must be large enough to allow yeast to stay in suspension and should be chosen such that antigen binding to yeast-displayed scFv is not under depleting conditions. This can be accomplished by maintaining at least a tenfold of antigen over scFv. In this step, for example, assuming 5×10^4 scFv fusions per yeast cell, the scFv concentration in the sample is calculated as follows: (5×10^4 scFvs per cell) × (1×10^6 cells per 50 µl) = 1.7 nM. Therefore, the lowest recommended antigen-labeling concentration is 17 nM for this volume, and the volume should be increased accordingly for lower antigen concentrations. Maintaining an antigen excess of tenfold may not be important in early rounds of sorting, when many scFv clones may not bind antigen to a large degree. However, as the scFv affinity increases, the tenfold molar excess becomes essential. The sample can also be incubated with rotation to keep cells in suspension.

14| Incubate cells at room temperature for an appropriate amount of time (usually 30 min). Resuspend cells as necessary during incubation.

If the antigen concentration is in molar excess of scFv fusions, the time constant (τ) of the approach to equilibrium is defined as $\tau = (k_{on}[Ag]_0 + k_{off})^{-1}$, where $[Ag]_0$ is the concentration of antigen at time zero and k_{on} and k_{off} are the on and off rates, respectively; scFv binding has reached 95% of equilibrium binding at time 3τ and 99% at 4.6τ. For typical scFvs isolated from the nonimmune library, 30 min at room temperature is sufficient for antigen concentrations that are nanomolar and higher. For deviations from those conditions, the equation above should be used to calculate the expected time to reach equilibrium. Typical scFv k_{on} values are about 1×10^5 M^{-1} s^{-1}, and $k_{off} = K_d \times k_{on}$ can be calculated from the estimated K_d of the sample. However, k_{on} can increase substantially during subsequent scFv affinity maturation. Although we describe protocols for equilibrium screening here, it is also possible to do kinetic screens as described by Boder and Wittrup[19].

15| Pellet cells at 14,000*g* for 30 s at 4 °C and wash with 1 ml ice-cold PBSF buffer. This and all subsequent steps for yeast labeling should use ice-cold PBSF buffer and should be done on ice or at 4 °C.

▲ **CRITICAL STEP** Completing secondary labeling at 4 °C minimizes antigen dissociation from yeast-displayed scFvs.

16| Label yeast with appropriate secondary reagents, such as Alexa Fluor 488–goat anti-chicken IgG (1:100 dilution) and streptavidin-phycoerythrin (1:100 dilution), in an appropriate final volume (usually 50 µl for 1×10^6 cells). Vortex cells to resuspend. Typical dilutions for other secondary reagents are 1:50 for neutravidin-phycoerythrin and 1:100 for streptavidin-allophycocyanin. A working dilution for other secondary reagents should be determined by titration.

17| Incubate cells on ice and shielded from light for 10–20 min. For the reagents described above, a substantial fluorescence signal is seen after 5 min of incubation. Longer incubation may increase the signal but that is balanced by the rate at which antigen dissociates from the scFv.

18| Pellet cells for 14,000*g* for 30 s at 4 °C and wash with 1 ml PBSF buffer. Keep the cell pellet on ice.

19| Before analysis, set up a flow cytometry protocol using control yeast samples. Prepare a negative control sample with secondary only (**Fig. 3a**) and positive control samples with Alexa Fluor 488–anti-c-Myc only (**Fig. 3b**) and a phycoerythrin signal only. The yeast population is normally gated on forward- and side-scatter channels to remove debris and aggregated cells (usually less than 5% of the sample). In addition, the flow cytometry apparatus should be properly compensated to reject crosstalk between the Alexa Fluor 488 and phycoerythrin channels of the fluorescence detector, as compensation is necessary when there is substantial overlap between the emission spectra of the two fluorophores.

20| Resuspend cell sample in PBSF buffer immediately before flow cytometry. Optimal resuspension volumes vary according to the flow cytometry apparatus used; typically, the volume should be at least 500 µl and the cell concentration should not exceed 1×10^8 cells per ml. Load the cell sample into the flow cytometry apparatus using an appropriate tube.

■ **PAUSE POINT** Yeast cultures may be treated as described in the pause point after Step 1.

Enrichment of candidate scFv clones using flow cytometry

21| After the steps outlined above have been done to verify scFv expression for the MACS-sorted population, this population can now be further enriched by labeling the cells with antigen followed by flow cytometry selection. Repeat Steps 11–20 for labeling of the entire MACS-sorted population, this time also labeling with antigen at a concentration of approximately 1 µM or less.

22| Draw an appropriate sort gate in the double-positive quadrant to isolate cells that are positive for both scFv expression and antigen binding. In the first flow cytometry round, it is customary to use a very conservative selection and to gate approximately the top 5% of the population of cells to avoid loss of unique clones (**Fig. 3c**). As the population becomes enriched, more diagonal sort windows can be drawn (**Fig. 4a**), collecting the top 0.1–1%.

23| Collect sorted cells in 1 ml SDCAA media.

24| Propagate collected yeast for subsequent rounds of sorting. Add SDCAA media to the eluted cell suspension to a final volume of 5 ml, add pen-strep (1:100 dilution) and grow the culture overnight at 30 °C.

▲ **CRITICAL STEP** To suppress bacterial contamination after flow cytometry, grow yeast cultures in SDCAA media with pen-strep or another antibiotic, or in SDCAA media, pH 4.5.

? TROUBLESHOOTING

25| Repeat Steps 21–24 until the population is enriched for double-positive cells. Typically, this requires three to five rounds of flow cytometry. It should be noted that neutravidin-phycoerythrin usually produces a much weaker fluorescence signal than does streptavidin-phycoerythrin or streptavidin-allophycocyanin. However, it is important to alternate the use of streptavidin-allophycocyanin and neutravidin-phycoerythrin during successive flow cytometry selections to decrease enrichment for clones

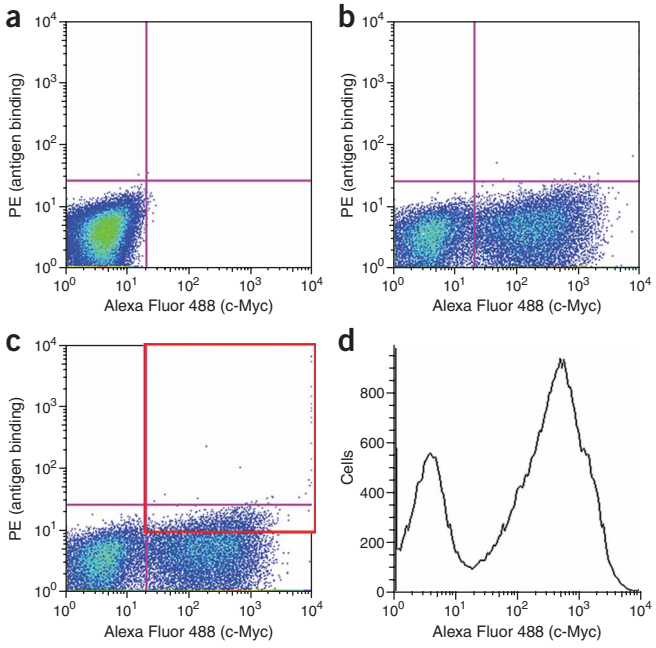

Figure 3 | Representative flow cytometry data. (**a**) Unlabeled yeast cells. (**b**) Yeast cells labeled with chicken anti-c-Myc IgY followed by Alexa Fluor 488–conjugated goat anti-chicken (Alexa Fluor 488 control), compensated to reject crosstalk between the Alexa Fluor 488 and phycoerythrin channels. (**c**) MACS-sorted population labeled for the initial round of flow cytometry sorting. Cells are double-labeled with anti-c-Myc IgY as in **b** plus biotinylated antigen followed by streptavidin-phycoerythrin. The thick red outline indicates a typical sort gate. (**d**) Histogram of Alexa Fluor 488 signal for cells labeled in **c**, indicating yeast surface expression of scFvs. The left peak represents the nondisplaying fraction of yeast due to plasmid loss. PE, phycoerythrin.

that bind the secondary reagent. If antibodies to a particular antigen epitope are not desired, preexisting antibodies or proteins that bind to the undesired epitopes can be used to 'mask' their presence to the yeast-displayed scFv library[22]. However, the population must be tested after each flow cytometry selection to ensure that scFvs binding to the masking agents have not been isolated.

■ **PAUSE POINT** Yeast cultures may be treated as described in the pause point after Step 1.

▲ **CRITICAL STEP** Alternating secondary reagents reduces the chance of isolating scFvs that are specific for the reagents.

? TROUBLESHOOTING

Characterization of enriched scFv clones

26| At this point, it is advisable to ensure that the sorted population has been enriched for the desired properties. This is done by using cell labeling followed by flow cytometry analysis to confirm that the selected scFvs are specific for the antigen of interest and not the secondary reagents. This also aids in estimating the affinities of the scFvs. Follow Steps 11–20 to label the sorted yeast population at a few representative concentrations of antigen. Also prepare a sample without antigen to test for the presence of secondary reagent binders.

■ **PAUSE POINT** Yeast cultures may be treated as described in the pause point after Step 1.

? TROUBLESHOOTING

27| Isolate plasmid DNA from the selected population of yeast using a Zymoprep kit according to the manufacturer's instructions. Ensure that the amount of yeast used for the Zymoprep kit is at least tenfold larger than the expected population diversity. This will yield a heterogeneous DNA sample containing display plasmids from each of the different yeast clones in the selected population.

■ **PAUSE POINT** Store sample prepared with the Zymoprep kit at –20 °C.

28| Transform 1–5 µl of DNA prepared with the Zymoprep kit in Step 27 into XLI-blue *E. coli* or an alternative *E. coli* strain of similar subcloning efficiency, spread on LB Amp plates and incubate overnight at 37 °C. Inoculate and grow multiple colonies (typically about ten) overnight at 37 °C in LB Amp media, then prepare plasmid DNA by miniprep and sequence the scFv. Sequencing primers for pCTCON2 are forward, 5′-GTTCCAGACTACGCTCTGCAGG-3′, and reverse, 5′-GATTTTGTTACATCTACACTGTTG-3′. This step ensures that true scFvs, not truncation products, have been selected.

■ **PAUSE POINT** Store DNA prepared by miniprep at –20 °C.

? TROUBLESHOOTING

Mutagenesis of clones using error-prone PCR

29| For PCR, combine the following components in a 200-µl thin-walled PCR tube:

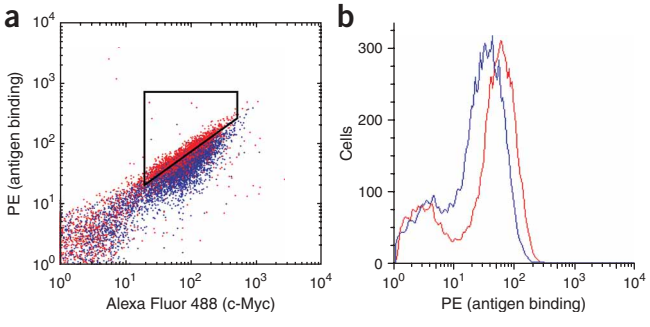

Figure 4 | Overlay of representative flow cytometry data for two scFv clones. Data for wild-type scFv are blue; data for a mutant with twofold higher affinity are red. Yeast cells were labeled as described in **Figure 3** with antigen at a concentration equal to the wild-type K_d. (**a**) A diagonal sort window (black) can be drawn to capture even slightly improved mutants efficiently. (**b**) Phycoerythrin (PE) fluorescence. The large degree of overlap between these histograms emphasizes the importance of two-color labeling to enable better differentiation between yeast populations.

Volume	Component	Final concentration
5.0 µl	10× Taq buffer (without $MgCl_2$)	1×
2.0 µl	$MgCl_2$ (50 mM)	2 mM
2.5 µl	Forward primer (10 µM)	0.5 µM
2.5 µl	Reverse primer (10 µM)	0.5 µM
1.0 µl	dNTPs (10 mM each)	200 µM
6.7 µl	Template DNA (100 pg μl^{-1})	13.3 pg μl^{-1}
5.0 µl	8-oxo-dGTP (20 µM)	2 µM
5.0 µl	dPTP (20 µM)	2 µM
19.8 µl	ddH_2O	–
0.5 µl	*Taq* DNA polymerase (5 U μl^{-1})	0.05 U μl^{-1}

The forward and reverse primers should be selected to amplify the gene of interest and to provide approximately 50 base pairs of homology to the vector used for homologous recombination. For the pCTCON2 system, effective primers are forward, 5′-CGACGATTGAAGGTAGATACCCATACGACGTTCCAGACTACGCTCTGCAG-3′, and reverse, 5′-CAGATCTCGAGCTATTACAAGTCCTCTTCAGA AATAAGCTTTTGTTC-3′. For clones isolated from the nonimmune human library, the reverse primer should be modified to 5′-CGAGCTATTACAAGTCTTCTTCAGAAATAAGCTTTTGTTCTAGAATTCCGGA-3′. Use DNA prepared with the Zymoprep kit in Step 27 as the template DNA to 'carry over' all clones from the selected population to the next round of mutagenesis.

30| Amplify DNA in a thermal cycler using the following conditions:

Cycles	Denaturation	Annealing	Polymerization
1	94 °C for 3 min	–	–
2–11	94 °C for 45 s	60 °C for 30 s	72 °C for 90 s
12	–	–	72 °C for 10 min

Experimental and theoretical analyses have identified the mutagenesis composition and cycling conditions described above as optimal, yielding one to nine amino acid mutations per scFv gene. If desired, doubling the number of PCR cycles to 20 results in three to fourteen mutations per gene.

■ **PAUSE POINT** Store PCR sample at 4 °C overnight or –20 °C indefinitely.

31| Gel-purify the PCR products to separate the mutagenized PCR product from the original template DNA. Add 5.6 µl of 10× gel-loading buffer to PCR products and apply PCR products to a 1.5% agarose gel. Run the gel at 100 V for 45 min and stain with 1× SYBR gold nucleic acid gel stain in TAE buffer. Cut the PCR product of about 900 bp from the gel and extract using the QIAquick gel extraction kit. DNA recovery can be increased by reapplying the flow-through to the column.

■ **PAUSE POINT** Store purified PCR product at –20 °C.

? TROUBLESHOOTING

32| Combine the following components in a 200-µl thin-walled PCR tube to amplify the mutagenized PCR insert. This amplification is necessary to yield sufficient DNA for yeast transformation.

Volume	Component	Final concentration
10.0 µl	10× Taq buffer (without $MgCl_2$)	1×
4.0 µl	$MgCl_2$ (50 mM)	2 mM
5.0 µl	Forward primer (10 µM)	0.5 µM
5.0 µl	Reverse primer (10 µM)	0.5 µM
2.0 µl	dNTPs (10 mM each)	200 µM
4.0 µl	Extracted PCR product	–
69.0 µl	ddH_2O	–
1.0 µl	*Taq* DNA polymerase (5 U μl^{-1})	0.05 U μl^{-1}

Multiple tubes may be prepared to yield more DNA for increased transformation yield. Typically, eight tubes are used to yield a total of about 40 µg DNA.

33| Amplify DNA in a thermal cycler using the following conditions:

Cycle	Denaturation	Annealing	Polymerization
1	94 °C for 3 min	–	–
2–31	94 °C for 45 s	60 °C for 30 s	72 °C for 90 s
32	–	–	72 °C, 10 min

34| Gel-purify the PCR product as described in Step 31. Gel purification of the final PCR product is recommended to eliminate expression of PCR artifacts in the library. However, this typically results in DNA losses of 25–50%.
■ **PAUSE POINT** Store the purified PCR product at –20 °C.

35| Follow the restriction enzyme manufacturer's instructions to digest 6 µg of pCTCON2 with *Nhe*I, *Bam*HI and *Sal*I. If digestion is done with these enzymes, it is unnecessary to purify the digested vector further. Analogous digestion should be done with alternative vectors, selecting digestion locations to allow homology of about 50 bp with the PCR insert.
■ **PAUSE POINT** Store digested DNA vector at –20 °C.

Yeast transformation

36| Inoculate 5 ml YPD media with an EBY100 colony (freshly streaked on a YPD plate) and grow overnight at 30 °C.
■ **PAUSE POINT** Yeast cells grow overnight.

37| Inoculate a 50-ml culture in YPD media to an absorbance of 0.1 at 600 nm using the overnight culture from Step 36 and grow cells at 30 °C to an absorbance of about 1.3–1.5 at 600 nm (about 6 h).
▲ **CRITICAL STEP** Cells must be in early to mid-log growth phase. Using cells in late log or stationary phase substantially decreases transformation efficiency.

38| While cells are growing, precipitate DNA for transformation using PelletPaint according to the manufacturer's protocol. Typically, DNA to be put into four electroporation cuvettes, each with 5 µg of insert from Step 34 and 1 µg of cut vector from Step 35, is used to generate a library of about 5×10^7. Also prepare a backbone-only control. Leave DNA in pellet form. Insert/backbone ratios can be varied from 5:1 to 1:1 with at least 1 µg backbone per cuvette. The amounts described above have been optimal in our laboratory.

39| Once cells have reached an absorbance of about 1.3–1.5 at 600 nm, add 500 µl Tris-DTT buffer to the culture. Incubate in a shaker at 30 °C for 15 min.
▲ **CRITICAL STEP** Transformation efficiency is relatively constant for DTT incubation times of 10–20 min, but decreases considerably for incubation over 20 min.

40| Pellet cells at 2,500*g* for 3 min at 4 °C and wash with 25 ml ice-cold E buffer (rinse, repellet and aspirate supernatant). Wash cells again with 1 ml ice-cold E buffer.

41| Resuspend cells in E buffer to a total volume of 300 µl. Resuspend DNA and control pellets from Step 38 using the appropriate volume of cell suspension (50 µl per cuvette). Keep cells on ice.

42| Aliquot 50 µl of resuspended cell-DNA mixture per prechilled electroporation cuvette. Keep electroporation cuvettes on ice until pulsed.

43| Load cuvette into gene pulser and electroporate at 0.54 kV and 25 µF without a pulse controller. Immediately add 1 ml of warm (30 °C) YPD media to the cuvette. Typical time constants for electroporation range from about 15 ms to 40 ms without greatly affecting transformation efficiency.

44| Transfer cells from pulsed cuvettes to a 15-ml Falcon tube. Wash each cuvette with an additional 1 ml of YPD media to recover the remaining cells from the cuvettes.

45| Shake cells from Step 44 for 1 h at 30 °C.

46| Pellet cells at 2,500*g* for 5 min and remove supernatant. Resuspend in 10 ml SDCAA media. Plate serial dilutions on SDCAA plates to determine transformation efficiency. The backbone-only control should have an efficiency of less than 1% that of the backbone-plus-insert transformations.
? TROUBLESHOOTING

47| Transfer cell suspension to a flask with 100–1000 ml SDCAA media plus pen-strep (1:100 dilution). Incubate at 30 °C for 24–48 h.

48| Passage library at least once before use to reduce the number of untransformed cells (see Step 1).

Selection for improved clones using flow cytometry

49| Repeat Steps 21–25 for the newly mutagenized library from Step 48. The antigen concentration should be reduced in each successive round of mutation and screening. A good 'rule of thumb' for antigen labeling concentration is 5–10% of the wild-type or starting equilibrium K_d, and simple formulae are available to calculate optimal labeling conditions[30]. For each flow cytometry selection, tubes labeled at different antigen concentrations can be prepared, and sorting the sample that gives the best differentiation between the starting and desired affinity will aid in accelerating the selection process.
■ **PAUSE POINT** Yeast cultures may be treated as described in the pause point after Step 1.

50| Repeat Steps 26–49 for each round of mutagenesis and screening until scFvs have reached desired affinity and/or stability.
■ **PAUSE POINT** Yeast cultures may be treated as described in the pause point after Step 1.

Characterization of clones by titration

51| Inoculate a 5-ml SDCAA culture with a clone of interest and grow overnight at 30 °C. Single clones can be obtained by plating a population of yeast on an SDCAA plate or transforming EBY100 with display plasmid for a single scFv clone (the EZ Yeast Transformation II kit is recommended).
■ **PAUSE POINT** Yeast cells grow overnight.

52| Inoculate a 5-ml SGCAA culture with 5×10^7 cells from Step 51 and induce at 20 °C for at least 20 h.
■ **PAUSE POINT** Yeast cells induce overnight.

53| Plan to use 10–14 tubes of varying antigen concentration. The concentration range should ideally span two orders of magnitude both above and below the K_d of the clone being measured, but practical considerations of tube volumes or reagent usage may limit the ability to achieve this goal. If the K_d is unknown, use several concentrations across a wide range and then focus the range in a subsequent experiment. As in Step 13, the volume must be adjusted such that the antigen is in molar excess of the number of scFv fusions. Although 1×10^6 cells are typically used for labeling, the number of cells can be decreased to 1×10^5 to reduce the required volume. An example experimental setup is in **Table 1**.

54| Calculate the total number of cells needed for all tubes. Pellet the total volume of cells at 14,000*g* for 30 s, aspirate the supernatant and wash the cells with 1 ml PBSF buffer (rinse, repellet and aspirate). Resuspend cells to a density of 1×10^7 cells per ml.

55| To each tube, add the appropriate volumes of buffer, cell solution and antigen, making sure to add the buffer first. Mix by tapping or vortexing.

56| Place the tubes in a water bath of the temperature of interest (e.g., 25 °C) and wait as the binding approaches equilibrium (typically 3 h).
See Step 14 for a discussion on the kinetics of the approach to equilibrium; 30 min is usually sufficient, and 3 h is common practice. At concentrations below the K_d, it is k_{off} that controls τ and therefore the time to reach equilibrium. High-affinity antibody-antigen interactions (K_d of 1 nM or less) typically have k_{off} values in the range of 10^{-3} s^{-1} and lower, necessitating longer incubation times.

57| Pellet cells at 14,000*g* for 30 s at 4 °C, aspirate supernatant and wash cells with 1 ml PBSF buffer. This and all subsequent titration steps should use ice-cold PBSF buffer and should be done on ice or at 4 °C. For 15- or 50-ml tubes, move cells to 1.5-ml microcentrifuge tubes for secondary incubation.
▲ **CRITICAL STEP** Completing cell labeling at 4 °C minimizes antigen dissociation from yeast-displayed scFvs.

58| Add 100 µl PBSF buffer with streptavidin-phycoerythrin (1:100 dilution) to each tube. Resuspend the cells and mix by pipetting or vortexing.

59| Incubate on ice for 10–20 min, shielding from light.

TABLE 1 | Example setup for K_d determination.

Tube	[Ag]	Buffer volume to add	Cells[a]	Antigen volume to add[b]				Ag/Ab ratio[c]
				25 µM	5 µM	1 µM	200 nM	
1	0	150 µl	50 µl					
2	1 µM	150 µl	50 µl	8.33				5016.7
3	316 nM	150 µl	50 µl		13.50			1625.8
4	100 nM	150 µl	50 µl		4.08			491.4
5	31.6 nM	500 µl	50 µl			17.96		432.5
6	10 nM	500 µl	50 µl			5.56		133.8
7	3.16 nM	1 ml	50 µl				16.87	81.2
8	1 nM	1 ml	50 µl				5.28	25.4
9	316 pM	10 ml	50 µl				15.92	76.6
10	100 pM	10 ml	50 µl				5.03	24.2
11	31.6 pM	40 ml	50 µl				6.33	30.5
12	10 pM	40 ml	50 µl				2.00	9.6

[a]Cells are at a concentration of 1×10^7 cells per ml. [b]Columns list antigen volume to add for each concentration. [c]Assuming 5×10^4 scFvs per cell. Ag, antigen; Ab, antibody.

60| Pellet cells at 14,000*g* for 30 s at 4 °C, aspirate supernatant and wash with 1 ml PBSF buffer. Keep cell pellets on ice.

61| Analyze cells of each tube using a flow cytometry apparatus (see Steps 19–20).
■ **PAUSE POINT** Data can be analyzed at any time.

62| Use the total mean fluorescence from the phycoerythrin channel (MFU_{tot}) versus antigen concentration ([Ag]) to fit the equilibrium dissociation constant (K_d). The data should fit the following equation: $MFU_{tot} = MFU_{min} + (MFU_{range} \times [Ag]) / ([Ag] + K_d)$. Compute the sum of the square of the differences between MFU_{tot} measured and MFU_{tot} as calculated from the equation and minimize this sum as a function of the three free parameters (K_d, MFU_{min} and MFU_{range}). [Ag] is assumed to be constant, as it is in large excess relative to the scFv and therefore is equal to the initial antigen concentration. The Solver tool in the program Excel offers a sufficient nonlinear optimization method. To plot the data as normalized fraction bound, subtract from each MFU_{tot} data point the fit MFU_{min} and then divide by the fit MFU_{range}.
An alternative fitting procedure uses the mean fluorescence of only the fraction of cells that display antibody (MFU_{disp}) rather than the total mean fluorescence (MFU_{tot}). The alternative method will yield an identical fit K_d value, but MFU_{min} and MFU_{range} will change. The utility of the alternative method is being able to use MFU_{range} as an indicator of the cell display without having to label the c-Myc epitope tag explicitly. To deduce MFU_{disp}, you must determine the autofluorescence of the nondisplaying yeast cells and subtract that from the total fluorescence. Use high concentration points at which the displaying fraction is distinctly separated from the nondisplaying fraction to measure the nondisplaying fraction (f_{non}) and its mean fluorescence (MFU_{non}) and average those values. Next, calculate the mean fluorescence of the displaying fraction (MFU_{disp}) using the following equation: $MFU_{disp} = (MFU_{tot} - f_{non} \times MFU_{non}) / (1 - f_{non})$.
? TROUBLESHOOTING

63| Repeat Steps 51–62 multiple times and report the average and standard deviation of the fit K_d value from these independent experiments. Once individual scFv clones have been characterized, the most desirable scFvs can then be cloned into the expression vector of choice for secretion from bacteria or yeast to yield soluble scFv preparations.

● **TIMING**
Steps 1–2, 3 days; Steps 3–8, 3 h; Step 10, 2 h; Steps 12–20, 2 h; Steps 22–24, 2 h; Steps 27–28, 2 h; Steps 29–35, 8 h; Steps 37–47, 8 h; Steps 53–61, 4 h.

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

STEP	PROBLEM	POSSIBLE REASON	SOLUTION
2	Low scFv surface display.	Induction time may be too short.	Induction time can be increased up to 48 h.
4	Biotinylated antigen not available or perturbs an epitope of interest.		Although it is preferable to use biotinylated antigen, it is also possible to detect an antigen of interest using a biotinylated secondary reagent. For example, a biotinylated antibody to epitope tag can be used to bind the tag on protein antigens.
8	Bacterial contamination persists.		Cells can be eluted and grown in SDCAA media, pH 4.5, with pen-strep and 25 μg ml^{-1} kanamycin.
10	MACS-sorted cells show poor c-Myc display (<5% cells positive).	Poor scFv induction, error in MACS protocol, or selected clones are V_H only. There are some frameshifts in the nonimmune library which result in heavy chain–only antibodies lacking the c-Myc tag.	See troubleshooting for Step 2 to improve induction conditions. Repeat MACS protocol if an error possibly occurred. Heavy-chain-only binders can be detected by labeling yeast with anti-hemagglutinin (**Fig. 1**). If full scFvs based on these heavy chains are desired, homologous recombination in yeast (Steps 36–48) can be used to add light chains to the selected heavy chains.
12	Yeast cells have difficulty pelleting.	Yeast sticking to sides of tubes.	Tubes can be pre-rinsed, or the yeast mixture can be co-pelleted, with PBSF buffer to block nonspecific binding sites.
24	See troubleshooting for Step 8.		
25	Population does not seem to be enriching for antigen binders.	Affinity of scFv too low to see a signal at 1 μM antigen.	Increase antigen concentration or use streptavidin preloading protocol described in **Box 1**.

TABLE 2 | Troubleshooting table (continued).

26	Secondary reagent binders.		Ensure secondary reagents are being alternated in each successive flow cytometry selection. Additional secondary reagents may be alternated. If a population contains both antigen-specific and secondary-specific scFvs, depletion sorting can be done to remove the secondary binders. Label the population without adding antigen and isolate cells that express scFv but do not bind to the secondary reagents.
28	No or few *E. coli* transformants.		Switch from Zymoprep I to Zymoprep II or vice versa. Although the Zymoprep II manufacturer's protocol should yield sufficient transformants, two modifications will substantially increase transformation efficiency: centrifuge the neutralized cell lysate supernatant a second time before application to the spin column, and clean the eluted product on a Qiagen DNA spin column.
31	PCR product not visible in gel.	Limited amplification yields a small amount of PCR product that can be difficult to see without a sensitive stain.	A DNA ladder can be used to indicate the expected location of the desired PCR product, or a control product amplified with 25 cycles can be generated and used as a standard.
46	Low yeast transformation efficiency.	Improper cell temperature.	Maintain cells at 4 °C from post-DTT treatment to electroporation, avoid touching metal electrodes of electroporation cuvettes and immediately dilute cells in warm YPD media after electroporation.
		Impurities.	Use highest-quality water for E buffer preparation to maintain proper conductivity; de-salt DNA samples if they have a high salt content.
62	Data points for lower concentrations lie systematically below the fit curve.	Antigen-scFv binding has not reached sufficiently close to equilibrium.	Label cells for a longer period of time (see Step 14 for a discussion of time to reach equilibrium).
	High concentration points do not 'level off' and instead increase in mean fluorescence.	Nonspecific sticking of antigen to yeast surface.	Ignore these points and fit curve using remaining data; alternatively, a first-order concentration-dependent term can be added to account for nonspecific binding.
	Data are very 'noisy'.	Antigen-scFv complexes are dissociating during subsequent steps.	Reduce time for secondary labeling or eliminate one or more wash steps (except for final wash).

ANTICIPATED RESULTS

MACS selection from the nonimmune library typically requires one to two 'sorts' to reduce the size of the population to approximately 1×10^7. Then, the scFv expression of the MACS-selected cells is measured using flow cytometry. Our representative flow cytometry control data show an unlabeled yeast population (**Fig. 3a**) and a population labeled with anti-c-Myc and Alexa Fluor 488 (**Fig. 3b**). In any flow cytometry yeast display data, there are always two distinct populations (**Fig. 3b-d**), with the lower negative peak or population representing the nondisplaying yeast cells due to plasmid loss. The negative population is usually around 15% of the total cell population, but can be as high as 50% depending on growth and induction conditions. Once scFv expression has been confirmed, the MACS-selected cells are further enriched using flow cytometry (initial flow cytometry selection example, **Fig. 3c**). In these early selections, it may be difficult to see a phycoerythrin signal in the double-positive quadrant, as antigen-binding scFvs may be rare in the total population. However, after approximately three to five flow cytometry selections, a double-positive population should begin to appear. If this does not occur, increasing the antigen concentration or streptavidin preloading should be considered. Typically, scFvs isolated from the library against human antigens have affinities in the low micromolar range, as the library is nonimmune and is derived from human mRNA. To increase this affinity, each successive round of sorting consists of the creation of a mutagenic library followed by three to five flow cytometry selections, with labeling at decreasing antigen concentrations in each round and possibly in each flow cytometry selection. As the scFvs increase in affinity for the antigen, a diagonal population should appear in which

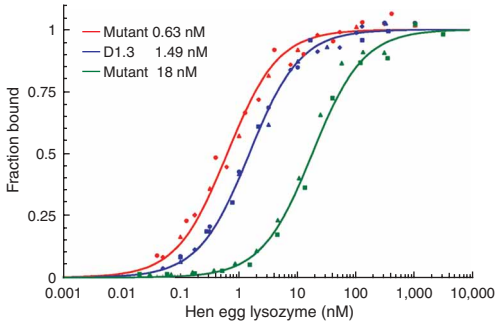

Figure 5 | Titration curves. The scFv D1.3 is displayed on the surface of yeast, and binding to soluble lysozyme is detected with biotinylated rabbit polyclonal antibodies to lysozyme, followed by labeling with streptavidin-phycoerythrin (polyclonal antibodies that bound yeast nonspecifically were removed before use). For wild-type D1.3 and each mutant, 10- or 12-point curves were obtained on separate days and are plotted using different symbols for each. Four fits to wild-type give a K_d of 1.49 ± 0.09 nM, and three fits to the high-affinity mutant give a K_d of 0.63 ± 0.13 nM, distinctly distinguishing the 2.4-fold difference in affinity.

antigen binding signal increases with yeast surface expression (**Fig. 4a**). Even when there is considerable overlap between the phycoerythrin histograms of two yeast populations, normalization for yeast surface expression allows the populations to be distinguished distinctly in the two-dimensional dot plot (**Fig. 4**). This enables isolation of clones with higher antigen-binding signals with similar amounts of expression. The number of rounds of mutagenesis and selection required to obtain a desired scFv will depend on the antigen concentrations used and nature of the antigen. Typically, three to six rounds of mutation and selection may be needed. Once the target scFv properties have been met, the scFv clones can be 'titrated' conveniently while displayed on the surface of yeast, where the range of K_d values that can be measured with this protocol is 10 pM to 1 μM. Our example titration of three different clones (**Fig. 5**) shows that mutants with similar affinities can be distinguished easily.

Note: Supplementary information is available via the HTML version of this article.

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Erratum: Isolating and engineering human antibodies using yeast surface display

Ginger Chao, Wai L Lau, Benjamin J Hackel, Stephen L Sazinsky, Shaun M Lippow & K Dane Wittrup

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In the version of this article initially published, the *x*-axis of Figure 4b should have been labeled "PE antigen binding", not "Alexa Fluor 488 (c-Myc)". The figure has been corrected in the HTML and PDF versions of the article.