Chapter 20

Antibody Affinity Optimization Using Yeast Cell Surface Display

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Summary

Many biosensors depend on molecular recognition reagents to achieve highly sensitive and specific detection levels of an analyte of interest. Although new and improved detection platforms continue to be developed, improvements in the affinity and specificity of the molecular recognition reagents often dictate the ultimate performance level and utility of the instrument. Accordingly, large effort is placed in discovering and characterizing the reagents to be used for a biosensor application. Antibodies, owing to their unparalleled ability to bind a diverse array of antigens with high affinity and specificity, have been widely used as molecular recognition reagents in the biosensor field. The recent advent of recombinant in vitro antibody display technologies, in general, and yeast surface display, in particular, allow specific traits of a given antibody to be discreetly augmented to enhance biosensor performance. Large variegated libraries derived from existing antibodies already employed in a particular biosensor can be created and screened for mutations that confer a desired improved phenotype leading to enhanced biosensor performance. This chapter will provide a protocol for the affinity maturation of a previously isolated monoclonal antibody, the most widely used application of in vitro directed evolution.

Key words: scFv, Recombinant antibody, Directed evolution, Affinity maturation, Yeast display, Mutagenesis, FACS.

1. Introduction

Antibodies, owing to their unparalleled ability to bind a diverse array of antigens with high affinity and specificity, are essential recognition reagents for numerous biosensor platforms. Most diagnostic immunoassays rely upon monoclonal or polyclonal antibodies derived from animals, and this source of recognition

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reagents has served the research community for over three decades. However, one limitation of this approach is the inability to change a specific property of an isolated antibody while leaving others intact. Instead, the only recourse is to initiate another round of immunization with no guarantee of discovering a preferred alternative. The recent advent of in vitro display technologies provides the added flexibility to engineer new or augment specific attributes of interest, such as affinity, specificity, or stability, into previously isolated and otherwise desirable recognition reagents.

A number of different in vitro display systems have been developed including, filamentous phage *(1, 2)*, ribosomal *(3)*, bacterial *(4)*, and yeast *(5)*. Each have their own strengths and weaknesses and is outside the scope of this work but has been recently reviewed *(6)*. However, a central tenet of any in vitro display platform is that the phenotype of the displayed protein must be coupled to the gene encoding the displayed protein. This coupling allows diverse libraries to be sampled and the coding sequence recovered from clones identified with desired properties. Finally, selected products can be expressed in a variety of formats from intact immunoglobulin (Ig), $F(ab)'_2$, Fab, single-chain variable fragment (scFv), to single variable domains depending on the preferred embodiment for application.

Yeast display **(Fig. 1)** has proven to be highly effective platform for various directed evolution applications, including affinity maturation *(7–9)* and changes in specificity *(10, 11)*. Recombinant antibodies are displayed on the yeast surface as a fusion protein to a cell wall component, Aga2 *(5)* and library generation can be facilitated by the homologous recombination system inherent in yeast *(12, 13)*. Coupling fluorescence activated cell sorting (FACS) with cell surface display enables monitoring of both antibody expression on the cell surface and the ability of that antibody to bind antigen **(Fig. 1b)**. The unparalleled resolution offered by FACS during the selection process enables the visualization of discrete populations that differ in affinity and/or epitope specificity and allows the quantitative recovery of desired clones *(14, 15)*. FACS analysis also facilitates the characterization of isolated clones directly on the yeast surface eliminating the need for purified antibody.

A typical affinity maturation project using yeast cell surface display is divided into several steps. First, the variable (VH and VL) domains of the parent hybridoma clone are identified. Second, the VH and VL genes are cloned into a display vector for expression as a scFv fragment on the cell surface. Third, the binding characteristics of the WT scFv are determined. Fourth, diverse variegated populations for selection are created by mutagenesis of the parent clone plasmid DNA. Fifth, clones with improved affinities are enriched from the mutant library after application of selective pressure. Sixth, selected clones are sequenced and

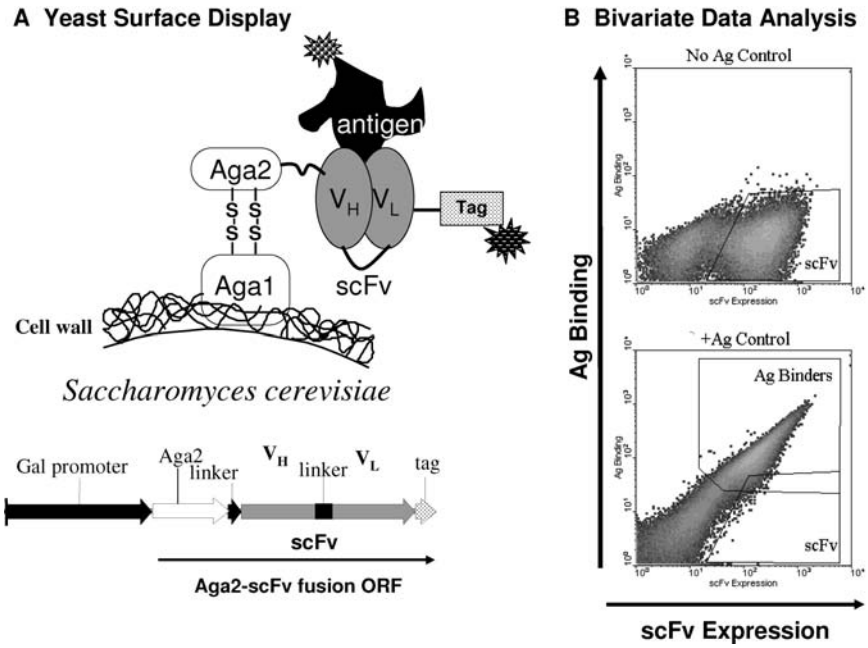

Fig. 1. Yeast surface display. (**A**) The scFv is displayed on yeast cell surface as a translational fusion to Aga2 protein. The VH and VL domains are joined by a flexible peptide $(Gly_4Ser)_3$ linker. Surface expression can be detected using fluorescent labeled antibodies binding to the C-terminal epitope tag. Biotinylated antigen binding can be detected using fluorescent streptavidin/avidin. Expression of the translational fusion in transformed yeast from the pYD1 plasmid is under control of the inducible Gal promoter. (**B**) Dual color flow cytometric analysis. ScFv expression is shown on *X*-axis and antigen-binding on the *Y*-axis. *Top plot* is no antigen control. *Bottom plot* is plus antigen control. Uninduced cells are located in the bottom left quadrant. Only cells expressing scFv are able to bind antigen and move along the *Y*-axis.

binding characteristics characterized. Finally, the VH and VL genes are recovered for cloning into desired expression system. As with any technical approach, many variations exist and may be of benefit for a particular application. This review intends to give the reader a rudimentary protocol that can be augmented and expanded with experience.

2. Materials

2.1. Variable Gene Isolation

2.1.1. mRNA Isolation

1. Oligotex Direct mRNA Minikit (Qiagen cat # 72022) supplied with OL1, ODB, Oligotex beads, OW1, OW2 and OEB buffers.
2. Prepare fresh OL1-BME buffer by adding 30 µL BME (Bio-Rad cat # 161-0710) to 1 mL OL1 buffer.
3. Culture of hybridoma cells producing antibody of interest.

2.1.2. Immunoglobulin cDNA Synthesis

1. cDNA primers supplied with Mouse Ig-Primer set (Novagen cat # 69831-3). MuIgMVH3′-1 (5′-CCCAAGCTTACGAGGGGGAAGACATTTGGGAA-3′). MuIgGVH3′-2 (5′-CCCAAGCTTCCAGGGRCCARKGGATARACIGRTGG-3′), MuIgκVL3′-1 (5′-CCCAAGCTTACTGGATGGTGGGAAGATGGA-3′), MuIgλVL3′-1 (5′-CCCAAGCTTAGCTCYTCWGWGGAIGGYGGRAA-3′).
2. SuperScript III Reverse transcriptase (Invitrogen cat # 18080-044) supplied with 5× First Strand Buffer and 0.1 M DTT.
3. 10 mM dNTP mix (Invitrogen cat # 18427-013).

2.1.3. VH and VL Amplification

1. Mouse Ig-Primer set (Novagen cat # 69831-3).
2. Platinum HiFi Taq polymerase (Invitrogen cat # 11304-011) supplied with 10× HiFi buffer and 50 mM $MgSO_4$.
3. 10 mM dNTP mix (Invitrogen cat # 18427-013).
4. PureLink PCR purification kit (Invitrogen cat # K3100-02).
5. 1% ReadyAgarose minigel with ethidium bromide in 1× TBE buffer (Bio-Rad Cat # 161-3004).
6. *1× TBE running buffer:* Add 100 mL of 10× TBE buffer (Bio-Rad cat # 161-0733) to 900 mL dH_2O. Store at room temperature.
7. 10× loading buffer (Invitrogen cat # 10816-015).

2.1.4. VH and VL Gene Cloning and Identification

1. TOPO-TA cloning kit (Ivitrogen cat # 455-0641) supplied with pCR2.1 TOPO vector, salt solution, and OneShot TOP10 competent *E. coli* aliquots.
2. *Xgal solution:* Prepare 40 mg/mL solution of Xgal by suspending 500 mg of Xgal (Sigma cat # B9146-500) with 12.5 mL DMF (Sigma cat # D4551-250).
3. *1,000× Ampicillin stock:* Prepare 100 mg/mL solution by dissolving 1 g of ampicillin (Sigma cat # A0797) with 10 mL deionized water and sterilize by filtration. Store at –20°C.
4. *LB media:* Dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in deionized water to a volume of 1 L and sterilize by autoclaving.
5. *LB-Amp media:* Dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in deionized water to a volume of 1 L and sterilize by autoclaving. Add 1 mL of 1,000× (100 mg/mL) ampicillin stock to warm media (<50°C).
6. *LB-Amp plates:* Dissolve 10 g tryptone, 5 g yeast extract, 10 g NaCl, and 15 g agar in 1 L deionized water and sterilize by autoclaving. Add 1 mL of 1,000× (100 mg/mL) ampicillin stock to cool (<50°C), mix and pour plates.
7. PureLink Quick Plasmid Miniprep kit (Invitrogen cat # K2100-11) supplied with binding buffer, wash buffer, elution buffer, and spin columns.

8. *EcoRI* restriction endonuclease (Invitrogen cat # 15202-013) supplied with 10× reaction buffer.
9. 10× loading buffer (Invitrogen cat # 10816-015).
10. 1% ReadyAgarose minigel with ethidium bromide in 1×TBE buffer (Bio-Rad Cat # 161-3004).
11. *1× TBE running buffer:* Add 100 mL of 10× TBE buffer (Bio-Rad cat # 161-0733) to 900 mL dH_2O. Store at room temperature.
12. M13 (−20) for sequencing primer (5′-GTAAAACGACGGCCAG-3′).
13. Vector NTI Bioinformatic software (http://www.invitrogen.com/content.cfm?pageid = 10373), which is freely available to academic and nonprofit users.

2.2. Construction of Parental scFv Construct

2.2.1. SOE-PCR to Combine VH and VL Genes

1. Platinum HiFi Taq polymerase (Invitrogen cat # 11304-011) supplied with 10× HiFi buffer and 50 mM $MgSO_4$.
2. 10 mM dNTP mix (Invitrogen cat # 18427-013).
3. VH and VL TOPO clone plasmids from **Subheading 3.1.4**.
4. VH for (GGTGGTGGTTCTGCTAGC + 21 nt of 5′ end of VH gene) primer. VH for primer contains *NheI* restriction site (underlined) for subcloning into vector.
5. VH rev (GGAACCACCACCGCCGCTGCCACCGCCACCGGATCCTAGAA TTCC + 21 nt of 3′ end of VH gene) primer. VH rev primer contains 45 nt of linker between VH and VL genes. Linker region designed to contain 30 bp homology (underlined) with VL for primer described below for overlap extension.
6. VL for primer (GGTGGCGGTGGCAGCGGCGGTGGTGGTTCCGGAGGCGGCGGTAGC + 21 bp of 5′ end of *VL* gene). VL for primer contains 45 nt of linker between VH and VL genes. Linker region designed to contain 30 bp homology (underlined) with VH rev primer described above for overlap extension.
7. VL rev primer (AGACTCGAGCGGCCGC**C** + 21 nt of 3′ of VL gene beginning from the *second base of the penultimate Lys codon*). VL rev primer contains *Not*I restriction site (underlined) for subcloning into vector. It is important to note that the *final* nucleotide position of the *VL* gene *lysine codon* needs to be **G** (sense strand of DNA) to create *Not*I site, encode for final Arg *VL* gene residue and maintain proper reading frame for C-terminal V5 tag sequence (*see* **Note 14**). Lysine codons are AAA and AAG (sense strand of DNA). Therefore, by simply including a **C** nucleotide in the final position of the lysine codon (bold C in VL rev primer sequence above) at the end of the *Not*1 sequence when

designing your VL rev oligo sequence (antisense strand of DNA), the lysine codon heterogeneity is circumvented and proper reading frame maintained.

8. 10× loading buffer (Invitrogen cat # 10816-015).
9. 1% ReadyAgarose minigel with ethidium bromide in 1× TBE buffer (Bio-Rad Cat # 161-3004).
10. *1× TBE running buffer:* Add 100 mL of 10× TBE buffer (Bio-Rad cat # 161-0733) to 900 mL dH_2O. Store at room temperature.

2.2.2. Yeast Display Vector Subcloning

1. pYD1 yeast display vector (Invitrogen cat # V83501). This vector is designed to display recombinant proteins on the surface of *Saccharomyces cerevisiae* by creating an Aga2 translation fusion with the scFv antibody gene. Vector includes the *GAL1* promoter for inducible expression, C-terminal V5 epitope for expression detection, tryptophan auxotrophic marker, *CEN6/ARSH4* origin for selection and maintenance in *S. cerevisiae*, ampicillin resistance gene and pUC-derived origin for selection and maintenance in *E. coli* (*see* **Note 1**).
2. *Nhe*I (NEB cat # R0131S) and *Not*I (NEB cat # R0189S) restriction enzymes supplied with 10× NEB buffer 2 and 100× BSA.
3. Calf intestinal phosphatase (NEB cat # M0290S).
4. 1% ReadyAgarose minigel with ethidium bromide in 1× TBE buffer (Bio-Rad Cat # 161-3004).
5. 1× TBE running buffer as prepared in **Subheading 2.1.4**.
6. 10× loading buffer (Invitrogen cat # 10816-015).
7. PureLink Quick Gel Extraction kit (Invitrogen cat # K2100-12).
8. T4 DNA ligase (Invitrogen cat # 15224-017) supplied with 5× DNA ligase buffer.
9. Max Efficiency DH5α competent cells (Invitrogen cat # 18258-012) supplied with S.O.C. media.
10. LB-Amp liquid media and agar plates as prepared in **Subheading 2.1.4**.
11. pYD1 forward primer (5′-AGTAACGTTTGTCAGTAAT TGC-3′) and pYD1 reverse primer (5′-GTCGATTTTGT-TACATCTACAC-3′).

2.2.3. Yeast Transformation of WT pYD Display Vector

1. EBY100 (Leu^-, Trp^- phenotype) yeast strain (supplied with pYD1 vector from Invitrogen). Strain genotype: *MA*Ta *ur*a3-52 *trp1 le*u2Δ 1 *his*3Δ 200 *pep*4::*HIS*3 *prb*1Δ1.6R *ca*n1 *GAL* (pIU211:*URA*3). EBY100 has genomic insertion of AGA1 regulated by GAL promoter with a URA3 selectable marker.

2. YPD rich nonselective media: Dissolve 20 g peptone and 10 g yeast extract in dH_2O to 0.9 L and sterilize by autoclaving. Dissolve 20 g dextrose in 0.1 L dH_2O, sterilize by filtration, and add to autoclaved solution. Alternately, you can add dextrose prior to autoclaving; however, carmelization may occur, but generally does not cause any problems.
3. Yeast lithium acetate transformation kit (GenomicsOne cat # MRR-GYT 1211) supplied with solution 1 (50%, w/v PEG), solution 2 (1.0 M LiAc), and solution 3 (2.0 mg/mL ssDNA). ssDNA needs to be boiled for 5 min once prior to subsequent use. Aliquots of boiled ssDNA can be made and stored at −20°C obviating the need to repeat boiling for subsequent transformation reactions.
4. SD-CAA plates for selection of pYD transformants. Dissolve 10.19 g Na_2HPO_4–7H20 and 8.56 g NaH_2PO_4-$H_2$0 and 15 g agar in 0.9 L dH_2O and autoclave. Dissolve 26.7 g of 2% dextrose dropout base powder (Q-Biogene cat # 4025-012), 5 g Bacto casamino acids in 0.1 L dH_2O, and sterilize by filtration. Add filter-sterilized solution to cool (<50°C), mix and pour plates. Alternatively, pre-made Synthetic Dextrose (SD)-His-Trp-Ura agar plates are available (Q-Biogen, cat # 4832-124).
5. SD-CAA selective growth media. Dissolve 26.7 g of 2% dextrose dropout base powder (Q-Biogene cat # 4025-012), 5 g Bacto casamino acids (lacks adenosine, uracil and tryptophan) (Becton Dickinson cat # 223050), 10.19 g Na_2HPO_4·7H20 and 8.56 g NaH_2PO_4·$H_2$0, in dH_2O to 1 L and sterilize by filtration (pH ~ 6.5).

2.3. Functional Characterization of Parental WT scFv

2.3.1. scFv Induction

1. SD-CAA selective growth media from **Subheading 2.2.3**.
2. SG/R-CAA selective induction media. Dissolve 36.7 g of 2% gal, 1% raf dropout base powder (Q-Biogene, cat # 4025-912), 5 g Bacto casamino acids (lacks adenosine, uracil and tryptophan) (Becton Dickinson cat # 223050), 10.19 g Na_2HPO_4·7H20 and 8.56 g NaH_2PO_4·$H_2$0 in dH_2O to 1 L and sterilize by filtration (pH ~ 6.5).

2.3.2. Functional Verification of scFv

1. Label antigen with biotin using EZ-link Sulfo-NHS biotinylation kit (Pierce cat # 21425) following manufacturer's instructions. Aim to attach 1–3 biotin molecules per molecule to limit potential epitope interference problems. For alternative labeling strategies *see* **Note 2**.
2. Anti-V5 mAb, IgG2a isotype, (Invitrogen cat # 46-0705) generally supplied around 1 mg/mL.
3. *FACS wash buffer (PBS/1% BSA)*. Add 10 g of BSA to 1 L of phosphate-buffer saline. Mix until dissolved and store at 4°C ≤ 4 weeks.

4. Secondary FACS labeling reagents: Alexa Fluor 488 goat anti-mouse IgG (GaM-488) (Invitrogen cat # A11001), R-phycoerythrin goat anti-mouse IgG (GaM-PE) (Invitrogen cat # P852), and R-phycoerythrin streptavidin (SA-PE) (Invitrogen cat # S866).

2.3.3. Kinetic Analysis

1. Biotinylated antigen (Bt-Ag) from **Subheading 2.3.2**.
2. FACS wash buffer as prepared from **Subheading 2.3.2**.
3. Secondary FACS labeling reagents from **Subheading 2.3.2**.
4. Competition buffer is simply *room temperature* FACS buffer containing suitable amount of *non-biotinylated* antigen (typically 0.1–1 μM) to prevent rebinding of dissociated Bt-Ag.

2.4. Mutagenic Library Construction

2.4.1. Mutagenesis of WT Clone Using Error-Prone PCR

1. Platinum Taq polymerase (Invitrogen cat # 10966-026) supplied with 10× buffer and 50 mM $MgCl_2$.
2. *Nucleotide mixes:* Prepare 2 mM dG/C/TTP mix by adding 40 μL each of 10 mM dGTP (Invitrogen cat # 18254-011), 10 mM dCTP (Invitrogen cat # 18253-013), and 10 mM dTTP (Invitrogen cat # 18255-018) to 80 μL dH_2O. Prepare 2 mM dATP mix by adding 40 μL of 10 mM dATP (Invitrogen cat # 18252-015) to 160 μL dH_2O.
3. Prepare 4 mM $MnCl_2$ solution by adding 40 μL of 0.1 M solution (Sigma cat # 48795) with 0.96 mL dH_2O.
4. pYD1 for and rev primers at 10 μM working stock as in **Subheading 2.2.2**.
5. 1% ReadyAgarose minigel with ethidium bromide in 1× TBE buffer (Bio-Rad Cat # 161-3004).
6. 1× TBE running buffer as prepared in **Subheading 2.1.4**.
7. 10× loading buffer (Invitrogen cat # 10816-015).
8. PureLink PCR purification kit (Invitrogen cat # K3100-02).

2.4.2. Mutagenic Library Yeast Transformation

1. EBY100 yeast strain from **Subheading 2.2.3**.
2. YPD media from **Subheading 2.2.3**.
3. pYD1 plasmid DNA digested with *Nhe*I/*Not*I generated in **Subheading 3.3.2**.
4. Mutagenic scFv insert generated in **Subheading 3.4.1**.
5. Yeast lithium acetate transformation kit (GenomicsOne cat # MRR-GYT 1211) supplied with solution 1 (50%, w/v PEG), solution 2 (1.0 M LiAc), and solution 3 (2.0 mg/mL ssDNA). Aliquots of ssDNA previously boiled in **Subheading 2.2.3** can be used.
6. SD-CAA agar plates and SD-CAA selective media prepared in **Subheading 2.2.3**. 1,000× ampicillin stock prepared in **Sub-**

heading 2.1.4. Prepare 10 mg/mL kanamycin stock by dissolving 1 g of kanamycin sulfate (Calbiochem cat # 420311) in 100 mL dH_2O and sterilize by filtration.

7. Prepare freezing medium by adding 12.5 mL of 100% glycerol (Sigma cat # G5516-500) to 37.5 mL of SD-CAA selective media, sterilize by filtration, and store at room temperature.

2.5. Selection for Improved Clones

2.5.1. Application of Selective Pressure

1. Mutagenic EBY100 yeast library generated in **Subheading 3.4.2**.
2. Biotinylated antigen (Bt-Ag) from **Subheading 2.3.2**.
3. SD-CAA selective growth media and agar plates prepared in **Subheading 2.2.3**.
4. SG/R-CAA induction media prepared in **Subheading 2.3.1**.
5. Anti-V5 mAb, IgG2a isotype, (Invitrogen cat # 46-0705) generally supplied around 1 mg/mL.
6. FACS wash buffer as prepared from **Subheading 2.3.2**.
7. Secondary FACS labeling reagents from **Subheading 2.3.2**.
8. Competition buffer prepared in **Subheading 2.3.3.**

2.5.2. FACS Enrichment of Improved Clones

1. SD-CAA agar plates and SD-CAA selective media prepared in **Subheading 2.2.3**. 1000× ampicillin stock prepared in **Subheading 2.1.4**. 10 mg/mL kanamycin stock prepared in **Subheading 2.4.2**.
2. Freezing media prepared in **Subheading 2.4.2**.
3. FACSAria (BD Biosciences) high-speed benchtop cell sorter with fixed-alignment cuvette flow cell equipped with at least 488-nm excitation laser and 530/30 and 576/26 bandpass filters to collect Alexa Fluor 488 and R-phycoerythrin fluorescence, respectively.

2.6. Characterization of Individual Improved Clones

2.6.1. Identification of Unique Clones

1. SD-CAA agar plates containing EBY100 colonies with mutant scFv isolated from selections in **Subheading 2.5.2**.
2. SD-CAA selective media prepared in **Subheading 2.2.3**.
3. ZymoPrep yeast plasmid miniprep kit (Zymo Research cat # D20001).
4. Max Efficiency DH5α competent cells (Invitrogen cat # 18258-012) supplied with S.O.C. media.
5. LB-Amp liquid media and agar plates as prepared in **Subheading 2.1.4**.
6. PureLink Quick Plasmid Miniprep kit (Invitrogen cat # K2100-11) supplied with binding buffer, wash buffer, elution buffer, and spin columns.

7. pYD1 forward primer (5′-AGTAACGTTTGTCAGTAATTGC-3′) and pYD1 reverse primer (5′-GTCGATTTTGTTACATCTACAC-3′).
8. Freezing media prepared in **Subheading 2.1.2**.

2.6.2. Kinetic Analysis of Unique Improved Clones

1. Biotinylated antigen (Bt-Ag) from **Subheading 2.3.2**.
2. SG/R-CAA induction media prepared in **Subheading 2.3.1**.
3. Anti-V5 mAb, IgG2a isotype, (Invitrogen cat # 46-0705) generally supplied around 1 mg/mL.
4. FACS wash buffer as prepared from **Subheading 2.3.2**.
5. Secondary FACS labeling reagents from **Subheading 2.3.2**.
6. Competition buffer prepared in **Subheading 2.3.3**.

3. Methods

The affinity optimization process is divided into six major steps. Each step consists of a number of substeps that are required to complete the task. The first three steps construct and characterize the parental scFv clone that will be subsequently modified. Step four introduces random mutations to create a variegated population, and step five isolates clones with improved affinities from that library. Finally, step six characterizes the selected clones to determine whether adequate improvements have been achieved during the affinity optimization process. It should be noted that the degree of improvement depends on many factors, including but not limited to, the diversity of the library and extent of mutagenesis, selective pressure application, the degree of enrichment between rounds of selection and the overall magnitude of desired improvement. However, it will be readily appreciated that this entire process can be reiterated to achieve desired success.

3.1. Isolation of Variable Genes

3.1.1. mRNA Isolation

1. Pellet ~1 × 10^6 hybridoma cells of interest by centrifugation at 110 × g for 5 min at room temperature, aspirate supernatant and resuspend cell pellet in 0.6 mL OL1-BME buffer.
2. Transfer cells to 1.5 mL microcentrifuge tube and lyse cells by passage through syringe with 20-gauge needle ten times. Transfer half of lysate (~300 µL) to second 1.5 mL microcentrifuge tube.
3. Add 0.6 mL ODB buffer to the two tubes and mix by pipetting 3–4 times. Samples were centrifuged at 17,900 × g for 3 min at room temperature and transfer the supernatant to fresh tubes (make sure not to disturb pellet).

4. Add 20 µL of Oligotex suspension (heated to 37°C in water-bath) to each supernatant and mix 3–4 times by pipetting. Incubate samples at room temperature for 10 min, centrifuge at 17,900 × g for 5 min and remove supernatant. You may wish to save supernatant until isolation of mRNA is verified.
5. Combine mRNA-Oligotex pellets into one 1.5 mL microcentrifuge tube with 100 µL (total volume) OL1-BME buffer. Add 400 µL ODB buffer and incubate at 70°C for 3 min followed by 10 min incubation at room temperature.
6. Pellet sample by centrifugation at 17,900 × g for 5 min and remove supernatant. Add 350 µL OW1 buffer and transfer resuspended sample into spin column (provided with kit). Centrifuge sample at 13,000 rpm for 1 min.
7. Transfer spin column with mRNA-Oligotex to new 1.5 mL microcentrifuge tube and add 350 µL OW2 buffer. Centrifuge sample at 17,900 × g for 1 min and discard flow through. Replace spin column in microcentrifuge tube and repeat **step 7** one more time.
8. Transfer spin column with mRNA-Oligotex to new 1.5 mL tube and resuspend with 50 µL OEB buffer (heated to 70°C). Centrifuge spin column at 17,900 × g for 1 min. Eluate will contain isolated mRNA. Repeat **step 8** one more time for total elution volume of 100 µL.
9. Store the mRNA at −80°C.

3.1.2. Immunoglobulin cDNA Synthesis

1. Add 2 pmol (0.4 µL of 10 µM solution) of MuIgMVH3′-1 (VH reactions from IgM), MuIgGVH3′-2 (VH reactions from IgG), MuIgκVL3′-1 (VL reactions from κ light chain), or MuIgλVL3′-1 (VL reactions from λ light chains), 2 µL 10 mM dNTP mix, 18.6 µL of dH_2O, 5 µL of mRNA from **Subheading 3.1.1** in a 200 µL thin-walled PCR tube.
2. Heat mixture to 65°C for 5 min in thermocycler and quick chill on ice.
3. Add 8 µL of 5× First-strand buffer, 4 µL of 0.1 M DTT and 2 µL (400 units) of Superscript III reverse transcriptase to each individual reaction.
4. Incubate the reactions at 55°C for 50 min followed by 70°C for 15 min to deactivate the enzyme.
5. Store cDNA at −70°C until use. The cDNA can now be used as template for PCR. Use only 10% of the first-strand reaction as higher volumes may result in decreased amounts of PCR product.

3.1.3. VH and VL Amplification

1. Combine 5 µL of 10× HiFi buffer, 1 µL of 10 mM dNTP mix, 2 µL of 50 mM MgSO4, 35 µL of dH_2O, 0.5 µL of HiFi Taq (5units/µL), 4 µL of cDNA from **Subheading 3.1.2**,

and 0.5 µL of appropriate 3′ primer (10 pmol/µL) with 2 µL of each appropriate 5′ primer (5 pmol/µL each) to 200 µL thin-walled PCR tube. VH reactions from IgM cDNA use MuIgVH3′-1 with MuIgVH5′-A/B/C/D/E/F primers. VH reactions from IgG cDNA use MuIgVH3′-2 with MuIgVH5′-A/B/C/D/E/F primers. VL reactions from Igκ cDNA use MuIgκVH3′-1 with MuIgkVH5′-A/D/E/F/G primers. VL reactions from Igλ cDNA use MuIgλVH3′-1 with MuIgλVH5′-A primers (*see* **Note 3**). VL primers B and C are often omitted as they will amplify aberrant light chains present in hybridomas derived from Sp2/0 myeloma fusions.

2. Amplify DNA in a thermal cycler for 30 cycles as follows:

   Denature 94°C for 15 s.

   Anneal 50°C (5′ primers A-B) or 55°C (5′ primers C-G) for 30 s.

   Extend 68°C for 30 s.

3. The samples can be stored at –20°C until use.
4. Analyze products by agarose gel electrophoresis. Mix 5 µL of PCR sample with 5 µL of 2× loading buffer and load onto 1% TBE agarose gel. After electrophoresis, visualize by ethidium bromide staining. VH PCR products will be approximately 350 bpin size, and VL PCR products will be approximately 330 bp in size.
5. The amplification reactions that contain appropriately-sized product are purified using commercially available Purelink PCR purification kit for subsequent cloning (*see* **Note 4**).

3.1.4. VH and VL Gene Cloning and Identification

1. Mix 4 µL of the VH or VL PCR with 1 µL of salt solution and 1 µL of pCR2.1 TOPO vector for a total reaction volume of 6 µL. Incubate reactions at room temperature for 5 min for ligation and transfer to ice until used for bacterial transformation.
2. Transform OneShot TOP10 competent *E. coli* with 2 µL of TOPO ligation reaction and inoculated prewarmed LB-Amp/Xgal plate. Incubate plates at 37°C overnight. Colonies containing vectors with inserts will be white.
3. Inoculate 2.5 mL of LB-Amp media with white colony from LB-Amp/Xgal transformation plate. Analyze at least four colonies per clone of interest. Incubate at 37°C with shaking overnight.
4. Isolate plasmid DNA using Invitrogen PureLink Miniprep kit or any other suitable commercially available product. Purified plasmid DNA may be kept at –20°C until use.
5. Digest 5 µL of plasmid DNA with 1–5 units of *EcoR*I restriction endonuclease in a 10 µL volume for at least 1 h at 37°C. Mix

10 µL of digestion reaction sample with 2 µL of 10× loading buffer and analyze on 1% TBE agarose gel. After electrophoresis, visualize by ethidium bromide staining. Correct VH or VL inserts will be approximately 500 bp in size.

6. Individual investigator can either manually perform sequencing reaction or send isolated plasmid DNA containing insert to third party vendor (e.g. SeqWright: http://www.seqwright.com/services/standardsequencing.php). M13 (–20) for primer should be used.
7. Returned sequences from multiple isolates of each clone should be aligned using Vector NTI (Invitrogen) or any other preferred DNA analysis software. Clones containing sequences that match derived consensus sequence should be archived and used for future experiments.
8. Optionally, the variable region germline family and CDRs can be delineated by submitting the derived VH and VL sequences to immunoglobulin bioinformatics websites such as IMGT (http://imgt.cines.fr/IMGT_vquest/share/textes/index.html) or VBASE2 (http://www.vbase2.org/).

3.2. Construction of Parental scFv Construct

3.2.1. SOE-PCR to Combine VH and VL Genes

1. Dilute VH and VL TOPO plasmid DNA by adding 1–99 µL of dH_2O.
2. Combine 78 µL dH_2O, 10 µL of 10× HiFi buffer, 4 µL of 50 mM $MgS0_4$, 2.5 µL of 10 mM dNTP mix, 0.5 µL HiFi Taq (5 u/µL), 1 µL of diluted VH or VL plasmid with 2 µL of 10 µM VH for and 2 µL of 10 µM VH rev primers or 2 µL of 10 µM VL for and 2 µL of 10 µM VL rev primers, respectively, in 200 µL thin-walled PCR tube.
3. Amplify DNA in a thermal cycler as follows:

   Denature 94°C for 30 s.

   Anneal 55°C for 30 s.

   Extend 68°C for 30 s.

   Repeat for 30 cycles followed by final cycle 68°C for 5 min.
4. Analyze products by agarose gel electrophoresis. Mix 5 µL of PCR sample with 5 µL of 2× loading buffer and load onto 1% TBE agarose gel. After electrophoresis, visualize by ethidium bromide staining.
5. Amplified VH and VL DNA is purified using commercially available PureLink PCR purification kit and eluted in 100 µL volume.
6. Combine 77 µL dH_2O, 10 µL of 10× HiFi buffer, 4 µL of 50 mM $MgSO_4$, 2.5 µL of 10 mM dNTP mix, 2 µL of 10 µM VH for and 2 µL of 10 µM VL rev primers with 1 µL of the purified VH *and* VL amplified DNA from the previous step in 200 µL thin-walled PCR tube.

7. Amplify DNA as in **step 3** to create scFv construct.
8. Analyze products as in **step 4**. Amplified scFv DNA should be around 800 bp in length.
9. Purify amplified scFv DNA using commercially available PureLink PCR purification kit prior to subsequent steps and store DNA at –20°C until use (*see* **Note 5**).

3.2.2. Yeast Display Vector Subcloning

1. Prepare pYD1 yeast display vector for subsequent subcloning by simultaneously digesting 10 µg of vector with ten units (1 µL) of *Nhe*I and 10 units (1 µL) of *Not*I restriction enzymes with 5 µL of 10× NEB buffer 2, 0.5 µL of 100× BSA and adjust to 50 µL total reaction volume with dH_2O. Incubate at 37°C from 2 h to overnight (*see* **Note 6**).
2. Remove 5′ phosphate groups by adding 10 units (1 µL) of calf intestinal phosphatase directly to restriction digestion reaction and continue incubation at 37°C for at least 1 h.
3. Gel purify vector fragment by adding 10 µL of 10× loading buffer and loading reaction sample onto 1% TBE agarose gel and electrophoresis at 100 V for 45 min. Remove 4.9 kb fragment of interest with clean razor blade or scalpel into fresh 1.5 mL microcentrifuge tube after visualization with ethidium bromide staining.
4. Recover gel purified vector fragment using commercially available PureLink gel extraction kit. Quantitate recovered DNA by measuring absorbance at 260 nm. 1.0 A_{260} unit equals 50 µg/mL of dsDNA. Store material at –20°C until further use.
5. Prepare amplified scFv DNA from **Subheading 3.2.1** for subcloning by simultaneously digesting 50 µL of amplified DNA with ten units (1 µL) of *Nhe*I and 10 units (1 µL) of *Not*1 restriction enzymes with 10 µL of 10× NEB buffer 2, 1 µL of 100× BSA and adjust to 100 µL total reaction volume with dH_2O. Incubate at 37°C from 2 h to overnight.
6. Purify digested scFv PCR DNA using commercially available PureLink PCR purification kit and quantitate as in **step 4**.
7. Ligate digested scFv DNA into pYD1 display vector by adding threefold molar excess of scFv insert to 100 ng of pYD1 × *Nhe*I/*Not*I vector in 20 µL reaction volume containing 1 unit of T4 DNA ligase and 4 µL of 5× ligase reaction buffer. Incubate reaction at room temperature (22–25°C) for 1 h. Be sure to include vector only negative control reaction.
8. Dilute ligation reactions fivefold in TE buffer and transform 1 µL of the diluted reaction (1–10 ng DNA) into 100 µL aliquot of maximum efficiency DH5α *E. coli* competent cells, inoculate LB-Amp agar plates and incubate at 37°C overnight.
9. Isolate plasmid DNA from multiple transformed colonies as in **Subheading 3.1.4**.

10. scFv DNA inserts should be sequence verified using pYD1 forward and pYD1 reverse primers to ensure construct is correct and in-frame with Aga2 ORF (*see* **Note 15**).

3.2.3. Yeast Transformation of WT pYD Display Vector

1. Inoculate 10 mL of YPD rich growth media with EBY100 yeast strain. Incubate at 30°C overnight with shaking
2. Pellet 1 mL of overnight EBY100 culture at 17,900 × g for 30 s and resuspend cell pellet in 1 mL of dH_2O. Re-pellet cells and remove supernatant.
3. Mix 240 µL of solution 1 (50%, w/v PEG), 36 µL of solution 2 (1.0 M LiAc), 25 µL of solution 3 (ssDNA 2 mg/mL), 45 µL of dH_2O, and 5 µL of plasmid DNA (100 ng–5 µg) in separate 1.5 mL microcentrifuge tube and add to top of cell pellet. Vortex cell pellet for at least 1 min to resuspend cells.
4. Incubate cells at 42°C for 40–60 min.
5. Pellet cells as above and discard supernatant. Gently resuspend cell pellet in 200 µL of dH_2O. Spread 10% (20 µL) and 90% (180 µL) of cells onto SD-CAA selective plates. Incubate plates at 30°C for 2–3 days until colonies are evident. Pick individual colonies for functional analysis.

3.3. Functional Characterization of Parental WT scFv

3.3.1. scFv Induction

1. Inoculate a fresh 10 mL SD-CAA culture with WT scFv EBY100 glycerol stock or colony from agar plate. Incubate overnight at 30°C with shaking. Cultures typically saturate at densities of 3–8 OD_{600} (1 OD_{600} is approximately 2×10^7 cells/mL) (*see* **Note 7**).
2. Pellet approximately 5 OD_{600} of cells in a benchtop microcentrifuge at 17,900 × g for 30 s and resuspend cells in 10 mL of SG/R-CAA media to a final density of approximately 0.5 OD_{600}/mL. Incubate at 20°C with shaking for 16–24 h. Induction time should result in no more than three cell doublings. *See* **Note 8** to increase scFv expression levels.
3. Determine OD_{600} of induced culture. Cultures can be stored at 4°C for approximately 2 weeks without substantial degradation of scFv prior to analysis.

3.3.2. Functional Verification of scFv

1. Remove 0.3 OD of induced yeast cells from **Subheading 3.3.1** for six 0.05 OD (~1×10^6 cells) reactions. Pellet cells at 17,900 × g for 30 s, aspirate supernatant, and wash cells with 0.5 mL of FACS wash buffer. Pellet cells as above and resuspend in 0.3 mL FACS wash buffer (1 OD_{600} cells/mL). Reactions to test include: V5 tag detected with GaM-488, V5 tag detected with GaM-PE, no Ag control, and 1–10–100 nM titers of biotinylated antigen (Bt-Ag). The V5 control reactions will verify cell surface expression of the

WT scFv by virtue of the C-terminal epitope tag and also be used to properly compensate the flow cytometer for spectral overlap between the Alexa Fluor 488 and phycoerythrin fluorescent dyes used for two color analysis. The no Ag control reaction ensures scFv is not interacting with SA-PE in absence of Bt-Ag. The Bt-Ag titer reactions should produce a dose-dependent response and confirm activity of the displayed scFv.

2. Transfer 50 µL aliquot (~0.05 OD_{600} cells) into individual reaction tubes containing anti-V5 mAb (2.5 µg/mL reaction concentration) and appropriate concentration of Bt-Ag in a total reaction volume of 0.1 mL.
3. Incubate reactions at room temperature for 30 min followed by 10 min incubation on ice. All subsequent steps should be performed using cold buffer and on ice to minimize antigen dissociation.
4. Pellet cells at 17,900 × g at 4°C for 30 s, aspirate supernatant, resuspend with 0.5 mL of cold FACS wash buffer and repellet cells.
5. Label yeast with 0.1 mL final volume of appropriate secondary reagents and incubate on ice for 15–20 min shielded from direct light. (1) GaM-PE (diluted 1:100) for one V5 control reaction, (2) GaM-488 (diluted 1:100) for the second V5 control reaction, and (3) GaM-488 (diluted 1:100) and SA-PE (diluted 1:200) for the no antigen control and Bt-Ag titer reactions.
6. Pellet cells at 17,900 × g at 4°C for 30 s, aspirate supernatant, resuspend with 0.5 mL of cold FACS wash buffer. Pellet cells again and resuspend in 0.5 mL cold FACS wash buffer.
7. Load reaction samples onto flow cytometer and develop analysis template **(Fig. 1b)**. The yeast population is normally gated on forward and side-scatter channels. Yeast scFv expression and antigen-binding populations are normally gated on Alexa Fluor 488 and PE channels, respectively, and visualized in a bivariate plot. Antigen-binding gate should be determined from no antigen control reaction. Collect 10,000 events per reaction to calculate percentage of population and the mean fluorescence intensity (MFI) of both the GaM-488 (scFv expression) and SA-PE (antigen binding) channels. Compensate the flow cytometer for spectral overlap between the Alexa Fluor 488 and phycoerythrin fluorescent dyes used for two color analysis.
8. Analyze flow cytometry data. Typically 50–80% of clonal yeast population will express scFv. The remaining nonexpressing cells naturally occur due to yeast physiology during the induction process and can be used as negative control population for setting analysis gates. Only the scFv expressing population should respond to Bt-Ag in a dose-dependent manner, which confirms correct sequence and conformation of the cloned WT scFv.

3.3.3. Kinetic Analysis

1. To determine dissociation rate constant of clone of interest, plan to use 10–15 dissociation timepoints for analysis. Each timepoint will use 0.05 OD_{600} (~1 × 10^6) cells; therefore, 0.75 OD_{600} of induced cells will be required for each experimental run.
2. Pellet cells at 17,900 × g for 30 s and aspirate culture supernatant. Wash cells by resuspending in 0.5 mL of FACS wash buffer, pellet cells as above and resuspend in 0.75 mL FACS wash buffer (1 OD_{600} cells/mL).
3. Remove 50 µL aliquot (~0.05 OD_{600} cells) for no antigen control reaction. Keep cells on ice until **step 7** and process sample separately from remainder of cells.
4. Saturate displayed scFv by incubation with 0.1–1 µM biotinylated antigen (Bt-Ag). Reaction volume (0.5–1.0 mL) should be large enough for yeast to remain in suspension and to ensure antigen binding to displayed scFv is under nondepleting ligand conditions. As a general rule, this can be accomplished by maintaining at least tenfold molar excess of antigen relative to scFv. Assuming, 5 × 10^4 scFv/cell and 2 × 10^7 cells/OD_{600}, an aliquot of 0.75 OD_{600} would contain 1.5 × 10^7 cells with 1.2 × 10^{-12} total moles of scFv or 1.7 nM at 0.75 mL.
5. Incubate reactions at room temperature for 30 min followed by shift to ice for 10 min to minimize dissociation during sample processing.
6. Remove unbound Bt-Ag by pelleting cells at 17,900 × g at 4°C for 30 s, aspirate supernatant, resuspend with 0.5 mL of cold FACS wash buffer and repellet cells.
7. Resuspend cells in 0.7 mL of secondary labeling reagent mixture (SA-PE diluted 1:100 in cold FACS buffer), vortex cells to resuspend and incubate on ice for 20 min. No antigen control reaction from **step 3** should be resuspended in 0.1 mL of secondary labeling reagent mixture. SA-PE will bind to Bt-Ag on cell surface and act as reporter signal for flow cytometry. *See* **Note 9** for alternative detection strategies.
8. Remove 50 µL aliquot (~0.05 OD_{600} cells) secondary labeling cell mixture for saturated binding control reaction. Proceed directly to **step 11** and process sample separately from remainder of cells. No antigen control reaction should also proceed directly to **step 11**.
9. Wash as in **step 6**; however, labeled cells should be resuspended with 0.65 mL of *room temperature* competition buffer containing 0.1–1 µM of *non-biotinylated* competitor antigen. Room temperature reaction conditions will allow normal kinetics of scFv–Ag interaction. The competition mixture will prevent clones that dissociate bound antigen from rebinding Bt-Ag.

10. Remove 50 µL aliquots into 1.5 mL microcentrifuge tubes at various timepoints, immediately pellet cells and aspirate supernatant as above and place tube with cell pellet on ice for subsequent analysis with flow cytometry. Initial dissociation time points include: 2.5 min, 5 min, 10 min, 15 min, 20 min, 30 min, 1 h, 2 h, 3 h, 4 h and overnight, if needed, until fluorescence approaches background levels (**Fig. 3**). Time points may have to be adjusted depending on the dissociation kinetics of the particular system under investigation.
11. Measure amount of retained Bt-Ag by determining level of fluorescence with flow cytometer. Resuspend cell pellet aliquot in 0.5 mL cold FACS buffer immediately before analysis and load sample on instrument. Collect 10,000 events per aliquot to calculate the PE MFI using analysis template developed above in **Subheading 3.3.2**. It is often convenient to group the initial time points for analysis (e.g. 2.5, 5, and 10 min).
12. Determine dissociation rate constant by fitting PE MFI data for all timepoints to first-order exponential decay (**Eq. 1**) shown below:

$$F = F_{sat}^{(\square k_{off} t)} + F_{bkg} \quad (1)$$

where F is observed fluorescence, F_{sat} is fluorescence at saturation prior to competitor addition, F_{bkg} is background fluorescence of cells, k_{off} is dissociation rate constant, and t is time in seconds. Determine the dissociation half-life ($t_{1/2}$) using the derived dissociation rate using (**Eq. 2**):

$$t_{1/2} = \ln 2 / k_{off} \quad (2)$$

3.4. Mutagenic Library Construction

3.4.1. Mutagenesis of WT Clone Using Error-Prone PCR

1. To obtain enough material for library construction, prepare five to ten 100 µL PCR in 0.2 mL thin wall tubes as outlined in table below. It is helpful to make a master mix and aliquot individual reactions. *See* **Note 10** for alternative mutagenesis strategies.

Volume (µL)	R× component	R× concentration
10	10× Taq Buffer (-$MgCl_2$)	1×
3	50 mM $MgCl_2$	1.5 mM
5	4 mM $MnCl_2$	1.5 mM
10	2 mM dG/C/TTPs	0.2 mM
5	2 mM ATP	0.1 mM

(continued)

Volume (μL)	R× component	R× concentration
5	10 μM pYD1 For primer	0.5 μM
5	10 μM pYD1 Rev primer	0.5 μM
0.2	Platinum Taq	1 unit
	WT pYD plasmid Template	10–1 μg
≤56.8	dH_2O	

2. Amplify DNA in thermal cycler using *low fidelity* Taq Polymerase as follows: (1) 95°C 5 min, (2) 92°C 30 s, (3) 50°C 30 s, (4) 72°C 1 min, (5) repeat **steps 2–4** 39 times, (6) 72°C 5 min. Completed reactions can be stored at 4°C.
3. Pool all reactions and remove primers and other reaction components with commercially available PCR purification kit. Elute amplified DNA in 100 μL TE. *See* **Note 11**.
4. Quantitate mutagenic DNA by measuring absorbance at 260 nm. Analyze purified mutagenic DNA by loading 1 μL on 1% TBE agarose gel and electrophoresis at 100 V for 45 min. Visualize with ethidium bromide staining. Amplified mutagenic DNA should be approximately 1 Kb in size and yield should be > 3 μg. Mutagenic DNA can be stored at –20°C.

3.4.2. Mutagenic Library Yeast T ransformation

1. Homologous recombination will be used to insert the mutagenic scFv PCR products into the pYD1 yeast display vector. This obviates the need to digest PCR DNA, ligate into vector, and transform into bacteria only to have to subsequently transform into yeast. The mutagenic scFv library contains ≥35 bp of overlap with pYD1 vector on both the 5′ and 3′ ends of amplified DNA. Cotransformation of the mutagenic scFv DNA pool with linearized pYD1 plasmid results in efficient reconstitution of intact plasmid containing mutagenic scFv insert. *See* **Note 12** for additional information on yeast homologous recombination.
2. Inoculate 10 mL of YPD media with EBY100 colony and grow overnight at 30°C with shaking.
3. Inoculate 50 mL of YDP media to density of 0.25 OD_{600}/mL (~5 × 10^6 cells/mL) using the overnight culture from **step 1** and incubate cells at 30°C with shaking until density of 1.0 OD_{600}/mL (2 × 10^7 cells/mL) is reached. This usually takes 4–6 h and provides enough cells for ten 1× scale transformation reactions. Highest transformation efficiency is achieved with log phase cells that have undergone *at least* two cell divisions.

4. Pellet cells 2,000 × g for 5 min at room temperature and discard supernatant.
5. Resuspend cells in 20 mL of dH_2O by vortexing and pellet as above.
6. Resuspend cell pellet in 1 mL of dH_2O and transfer to 1.5 mL microcentrifuge tube. Pellet cells at 17,900 × g for 30 s and discard supernatant.
7. Resuspend cells in *final volume* of 0.5 mL dH_2O (2×10^9 cells/mL = 10^8 cells/50 µL aliquot).
8. Transfer sufficient number of aliquots to new tube(s), pellet cells as above, and aspirate supernatant. To efficiently generate the desired number of transformants, reactions should be scaled rather than simply adding more DNA. Mutagenic libraries with 10^6–10^7 diversity are often sufficient for initial affinity improvements. A 1× transformation scale typically yields 10^6 transformants/µg of DNA/10^8 cells. Mix the reaction components listed (minus the cells) in the table below and pipette into the tube on top of the cell pellet. Please note that the ss-DNA must be denatured by boiling for 5 min prior to use. The total volume of DNA (insert and vector) and dH_20 needs to equal 50 µL for 1× transfection scale. Mutagenic scFv PCR insert should be present three to fivefold molar excess relative to linearized pYD1 vector. Use pYD1 vector digested with *Nhe*I/*Not*I from **Subheading 3.2.2** above. Remember to include linearized vector only control (1× scale is sufficient).

Reaction component	1× scale	5× scale
Cells (2×10^9/mL)	50 µL (10^8) – aspirate	250 µL (5×10^8) – aspirate
Solution 1 (50% PEG)	240 µL	1.2 mL
Solution 2 (1 M LiAc)	36 µL	180 µL
Solution 3 (ss-DNA)	25 µL	125 µL
pYD1 × *Nhe*I/*Not*I	1 µg (~3×10^{-13} mol)	5 µg
Mutagenic insert	1 µg (~1.6×10^{-12} mol)	5 µg
dH_2O	≤50 µL	≤250 µL
Total volume	351 µL	2.255 mL

9. Vortex for at least 1 min until the cell pellet is suspended in transformation mix.

10. Heat shock in 42°C water bath for 40–60 min. Gently invert tube several times during incubation to keep cells in suspension.
11. Pellet cells at 17,900× g for 30 s and aspirate transformation mix.
12. Add 1 mL of dH_2O to cell pellet and gently resuspend after incubation at room temperature for 5 min.
13. Inoculate dilution plates to determine transformation efficiency and library diversity. Transfer 1/200th aliquot of transformed cells (5 µL from 1 mL) to 0.495 mL dH_2O. Create 10^3–10^4–10^5 total dilution plates by spreading 100 µL–10 µL – 1 µL, respectively, of 1/200th aliquot on SD-CAA agar plates and incubate 2–4 days at 30°C. Calculate library diversity by multiplying number of colonies by total dilution factor. Calculate transformation efficiency by dividing total number of transformants/amount of linear vector DNA (in µg) used for transformation. The vector only control transformation should have about 1% efficiency compared with vector plus insert reaction.
14. Propagate the mutagenic library by inoculating 250 mL SD-CAA selective growth media supplemented with ampicillin (100 µg/mL final) and kanamycin (30 µg/mL final) with the remaining transformed cell suspension from **step 12**. Incubate at 30°C with shaking for 1–2 days until culture is saturated (3–5 OD_{600}/mL). Transformed cells are enriched by auxotrophic selection using the tryptophan synthetase gene on the reconstituted pYD1 plasmid in media lacking tryptophan. Untransformed cells are still viable but do not grow in selective media.
15. Passage transformed cells at least once to reduce the number of untransformed cells. Inoculate a fresh 250 mL SD-CAA selective growth media supplemented with ampicillin (100 µg/mL final) and kanamycin (30 µg/mL final) culture with *at least* tenfold over-representation of library diversity (determined from dilution plates in **step 13**) and incubate at 30°C with shaking until culture is saturated. Culture should be visually inspected with a microscope to confirm absence of bacterial contamination in the library.
16. Archive mutagenic library. Pellet enough cells for 5–10 aliquots with each aliquot containing with *at least* tenfold over-representation of library diversity. Suspend cell pellet in 10 mL freezing solution (25% glycerol in SD-CAA media) and transfer 1 mL aliquots to cryogenic vials. Transfer library aliquots to –70°C or liquid nitrogen for long-term storage.
17. It is good practice to sequence the scFv gene from a number of library clones to gauge degree of mutagenesis. Recover

pYD1 plasmid from yeast and sequence scFv gene as outlined in **Subheading 3.6.1** below.

3.5. Selection for Improved Clones

An overview of a generic selection process is depicted in **Fig. 2**. A selective pressure designed to isolated mutants with improved characteristics is applied to an initial mutagenic library. Clones with enhanced binding properties are enriched and propagated for additional rounds of selection and/or analysis. A kinetic dissociation selection (**Fig. 3**) is often used with clones with high initial affinity (single to subnanomolar K_D) and the duration of dissociation (i.e., the selective pressure) can be calculated after determining the WT scFv binding characteristics. The duration of dissociation is chosen to maximize differences between clones with large to modest improvements from WT *(16)*. Individual clones containing enhanced binding properties are then sequenced to identify the location and identity of the mutations.

3.5.1. Application of Selective Pressure

1. Induce at least 10 to 100-fold over-representation of mutagenic library diversity. Inoculate SG/R-CAA induction media to a density of 0.5 OD_{600}/mL with EBY100 library cells from SD-CAA culture at midlog growth. Incubate at 20°C with shaking for 16–24 h. Induction time should result in no

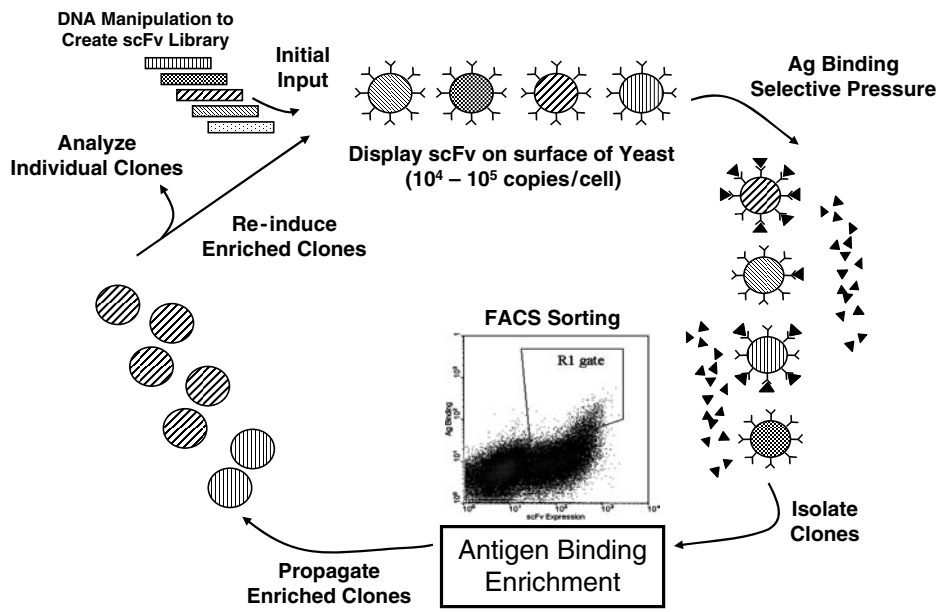

Fig. 2. The reiterative selection cycle. An initial library (10^6–10^7 complex) is constructed and induced for scFv expression. The library is allowed to bind to antigen under a selective pressure (e.g. antigen concentration, time, competitor) before clones with improved properties are isolated by FACS. Enriched clones are propagated and reinduced for scFv expression and the round of selection is repeated, using the enriched rather than the initial library. Individual clones can be analyzed and saved between rounds of selection.

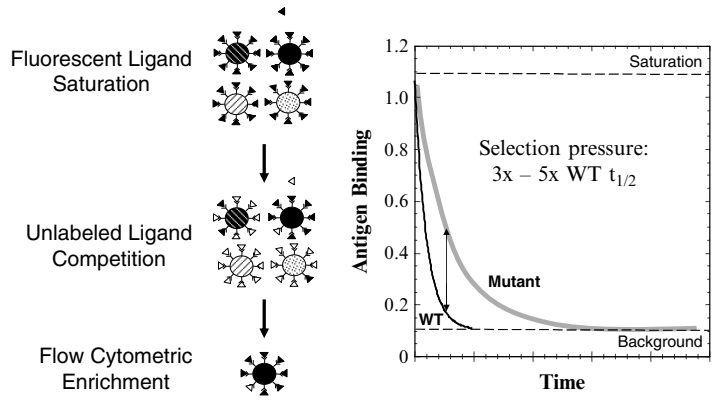

Fig. 3. Kinetic screening strategy. An initial library (10^6–10^7 complex) is incubated with biotinylated (or other suitable label) antigen (*solid triangles*) to saturate binding. The library is then incubated for a specified period of time in presence of excess unlabeled antigen (*open triangles*). FACS is used to visualize and isolate clones with the highest fluorescence having the slowest dissociation rate. The duration of the dissociation step (generally 3× – 5× the WT $t_{1/2}$ calculated from **Eq. 2**) is adjusted to maximize the differentiation of the mutant(s) relative to the WT clone (*see* **ref. 16** for greater detail).

more than three cell doublings. Reduced temperature aids correct scFv folding and decreases cell-doubling time. *See* **Note 8** to increase scFv expression levels.

2. Remove at least tenfold representation of library diversity aliquot from induction culture. Pellet cells at 17,900 × g for 30 s and aspirate culture supernatant. Wash cells by resuspending in 1 mL of FACS wash buffer, pellet cells as above, aspirate and suspend cell pellet in 1 mL FACS wash buffer. An aliquot of induced WT scFv cells should also be included for controls (0.05 OD_{600}/reaction).
3. Label yeast for scFv expression by adding 10 µL anti-V5 mAb (250 µg/mL) to 1 mL of induced cells. Remove 0.05 OD_{600} (~1 × 10^6 cells) aliquot for no antigen control reaction and proceed directly to **step 5** processing sample separately from remainder of cells.
4. Saturate displayed scFv by incubation with 0.1–1 µM biotinylated antigen (Bt-Ag). Reaction volume (0.5–1.0 mL) should be large enough for yeast to remain in suspension and to ensure antigen binding to displayed scFv is under non-depleting ligand conditions. As a general rule, this can be accomplished by maintaining at least tenfold molar excess of antigen relative to scFv. A tenfold representation of a typical library with 10^7 diversity would contain 10^8 induced yeast. Assuming, 5 × 10^4 scFv/cell, an aliquot of 10^8 cells would contain 8 × 10^{-12} mol of scFv for a reaction concentration of 8 × 10^{-9} M at 1 mL.

5. Incubate reactions at room temperature for 30 min followed by shift to ice for 10 min to minimize dissociation during sample processing. Remove 0.05 OD_{600} (~1 × 10^6 cells) aliquot for saturated binding control reaction and proceed directly to **step 8** processing sample separately from remainder of cells.
6. Remove unbound Bt-Ag and V5 mAb by pelleting cells at 17,900 × g at 4°C for 30 s, aspirate supernatant, resuspend with 0.5 mL of cold FACS wash buffer and repellet cells.
7. Apply selection pressure to mutagenic library. Suspend labeled cells in 1 mL of *room temperature* competition buffer containing 0.1–1 μM of *non-biotinylated* competitor antigen. Room temperature reaction conditions will allow normal kinetics of scFv–Ag interaction. The competition mixture will prevent clones that dissociate bound antigen from rebinding Bt-Ag. The length of dissociation will determine the stringency of selection. In general, a dissociation period that is 3×–5× the $t_{1/2}$ calculated in **Subheading 3.3.3** produces maximum separation of improved clones from WT (*see* **Note 13**). For example, a WT clone having a dissociation rate of 1 × 10^{-4}/s would have a $t_{1/2}$ of 115 min. Therefore, a suitable selection dissociation time would be 346 min (5.75 h). Cells should be kept in suspension by continual mixing or occasional manual vortexing.
8. Wash cells as in **step 6** using cold FACS buffer and keeping samples on ice for remainder of experiment.
9. Resuspend cells in 1 mL of secondary labeling reagent mixture (SA-PE diluted 1:200 and GaM-488 diluted 1:100 in cold FACS buffer), vortex cells to resuspend and incubate on ice for 20 min shielded from direct light. The various control reactions (0.05 OD_{600} aliquots) should be resuspended in 0.1 mL of secondary labeling reagent mixture. SA-PE will bind to Bt-Ag on cell surface and act as reporter signal for flow cytometry. GaM-488 will bind anti-V5 mAb that is bound to the C-terminal V5 tag for scFv expression detection.
10. Wash cells as in **step 6**. Resuspend library cells in 1–2 mL cold FACS buffer. Resuspend control reactions in 0.5 mL cold FACS buffer. Keep samples on ice, shielded from direct light and proceed directly to FACS enrichment.

3.5.2. FACS Enrichment of Improved Clones

1. Measure amount of retained Bt-Ag and scFv expression of the various control and mutagenic library reactions with fluorescence activated cell sorter instrument (e.g., FACSAria from Becton Dickinson). Load samples from **Subheading 3.5.1** onto instrument and collect 50,000–100,000 events per reaction using analysis template developed above

in **Subheading 3.3.2** to calculate percentage of population and the MFI of both the GaM-488 (scFv expression) and SA-PE (antigen binding) channels. The dual labeling strategy will allow scFv expression to be normalized and remove expression bias from subsequent selections. Analysis of an increased number of cells will allow clones present at a lower frequencies in the population to be visualized and aid in placement of sort gate used to isolate improved binders. Compensate the flow cytometer for spectral overlap between the Alexa Fluor 488 and phycoerythrin fluorescent dyes used for two color analysis.

2. Analysis of control and library reactions from **Subheading 3.5.2**. The mutagenic library should not bind to the secondary reagent used to detect Bt-Ag in the absence of antigen. If substantial background binding is observed, evaluate a panel of reagents to minimize/eliminate effect (e.g., neutravidin-PE, streptavidin-allophycocyanin, anti-biotin mAb). The library will likely have a lower percentage of cells expressing full-length scFv relative to the WT control and may also have a lower overall GAM-488 (scFv expression) MFI. A smattering of cells (fraction of percent) above the main antigen-binding population may be evident **(Fig. 4)**. These are cells of interest as they have a higher PE MFI relative to the main population presumably due to decreased dissociation kinetics.
3. Draw an appropriate sort gate in a diagonal line parallel to the main antigen-binding population in the double-positive

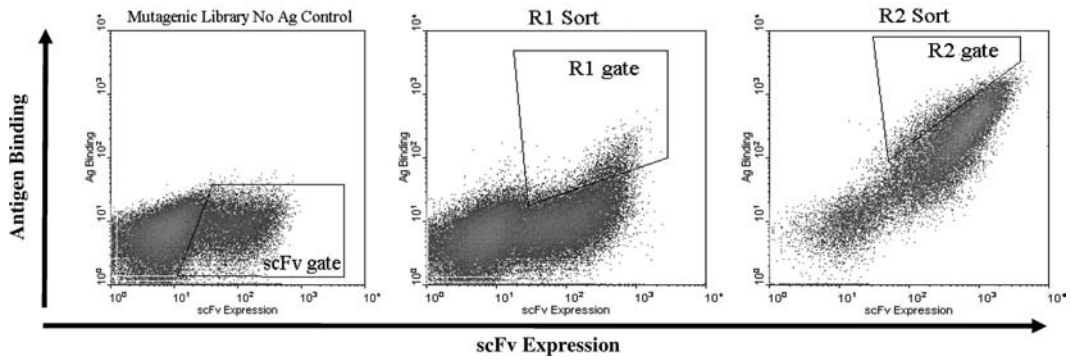

Fig. 4. Affinity optimization selection progression. (*Left*) Mutagenic library stained for antibody expression and incubated with SA-PE in the absence of antigen. (*Center*) Round 1 sort of mutagenic library. Bulk of library has low level of antigen binding after dissociation; however, small percentage of cells show increased fluorescence indicating slower rate of dissociation. (*Right*) Round 2 sort of mutagenic library. Bulk of enriched library has increased level of antigen binding after dissociation relative to initial sort. Cells with the highest level of fluorescence are once again sorted. scFv expression was detected using anti-V5 mAb/GaM-488 and is indicated with arrow on *X*-axis. Antigen binding was detected using biotinylated-Ag/SA-PE and is indicated with *arrow* on *Y*-axis. The scFv expression gate and respective sort gates are indicated in each plot.

quadrant to isolate cells with improved binding characteristics. Typically the brightest 0.1–1.0% of scFv-expressing and antigen-binding cells are sorted (**Fig. 4**). Sort the entire mutagenic library collecting sorted cells in 1.5 mL tube containing 0.2 mL SD-CAA media.

4. Propagate sorted yeast for subsequent rounds of selection. Inoculate 1–5 mL SD-CAA selective growth media with sorted cells from **step 3**. Add ampicillin (100 µg/mL) and kanamycin (30 µg/mL) to suppress bacterial contamination. Incubate 30°C at with shaking for 1–2 days until culture is saturated. Expand culture to larger volumes once cells are in midlog growth if desired.
5. Create dilution plates to estimate selection diversity by spreading appropriate aliquot of *initial* culture inoculated with sorted cells on SD-CAA agar plates. Incubate 2–4 days at 30°C until colonies are evident. The estimated diversity will be used to determine amount of cells needed for subsequent rounds of selection. The diversity will decrease with each round of selection. Typical diversity after first round of selection is 1–5 × 10^4 (0.5% gate used with library having 10^7 initial complexity). The dilution plates will also serve as source of clonal segregation for analysis of individual clones (*see* **Subheading 3.6.1**).
6. Archive selection output: Pellet enough cells for 2–3 aliquots with each aliquot containing *at least* tenfold over-representation of diversity present after round of selection (often aliquots will be >1,000-fold diversity). Suspend cell pellet in 2–3 mL freezing solution (25% glycerol in SD-CAA media) and transfer 1 mL aliquots to cryogenic vials. Transfer library aliquots to −70°C or liquid nitrogen for long-term storage.
7. Repeat the steps in **Subheading 3.5.1** and **3.5.2** for 2–3 rounds of selection. Each successive round of selection should yield a population of cells with increased antigen-binding fluorescence (**Fig. 4**). Analyze individual clones once incremental improvements are no longer observed between rounds of selection and/or apply increased selective pressure to isolate clones with the highest degree of improvement. However, sequence diversity of isolated mutants will be decreased.

3.6. Characterization of Individual Improved Clones

3.6.1. Identification of Unique Clones

1. Inoculate several 2–5 mL SD-CAA cultures with individual colonies from dilution agar plates from last round of selection from **Subheading 3.5.2**. Incubate overnight at 30°C with shaking. Cultures can be grown in individual tubes or in individual wells in a 2 mL 96-well plate for ease of handling. Typically 8–48 clones are initially characterized to assess sequence diversity of improved clones. Often 1–4 consensus mutants are identified at the end of 2–3 rounds of selection. If a consensus is not observed, then more clones should be analyzed and/or

additional rounds of selection should be performed. One can also use this particular protocol to analyze a few clones from each round of selection to observe the progression of mutation enrichment.

2. Isolate plasmid DNA from individual clones using commercially available yeast plasmid DNA miniprep kit (Zymoprep), according to manufacturer's instructions. Unlike typical pUC-based *E. coli* plasmids, only 1 copy of the *CEN6/ARSH4*-based pYD1 plasmid will be present per yeast cell. *See* **Note 14** for alternative strategies to recover gene of interest for sequence analysis.
3. Transform 5 µL of DNA prepared in **step 2** into max efficiency DH5α *E. coli* competent cells, spread entire transformation reaction onto LB-Amp agar plates and incubate at 37°C overnight.
4. Isolate plasmid DNA from individual colonies using Invitrogen PureLink Miniprep kit or any other suitable commercially available product. Purified plasmid DNA may be kept at −20°C until use.
5. Individual investigator can either manually perform sequencing reaction or send isolated plasmid DNA to third party vendor. pYD1 for and pYD1 rev primers should be used for sequencing reactions.
6. Sequences from multiple mutant clones should be aligned and compared against the parental WT scFv sequence using Vector NTI (Invitrogen) or any other preferred DNA analysis software. Given the random nature of the mutagenesis process, mutations can be distributed throughout the VH or VL genes. However, experience has shown that scFv derived directly from preexisting monoclonal antibodies will often contain mutations in or near the CDR regions. However, framework mutations can also be important and should not be discarded out of hand, especially if rare amino acids are replaced with consensus germline residues.
7. Each unique mutant clone identified should be preserved. At this point, it is convenient to simultaneously archive the yeast culture, transformed *E. coli* culture, and purified plasmid DNA for each mutant of interest.

3.6.2. Kinetic Analysis of Improved Clones

1. Inoculate 9 mL of SG/R-CAA induction media by directly adding approximately 1 mL of fresh SD-CAA culture from clones of interest identified in **Subheading 3.6.1**. Incubate at 20°C with shaking for 16–48 h. At this point, it is not necessary to be extremely vigilant about inoculation densities as each culture is monoclonal and loss of rare clones within the population is not of concern. Monoclonal cultures will typically have 50–80% scFv expression.

2. Determine OD_{600} of induced culture. Cultures can be stored at 4°C for approximately 2 weeks without substantial degradation of scFv prior to analysis.
3. Confirm scFv expression and specific antigen binding of each individual mutant as outlined previously in **Subheading 3.3.2**.
4. Determine dissociation rate constant of clone(s) of interest exactly as outlined previously in **Subheading 3.3.3**. Keep in mind that mutant clones should hopefully have slower dissociation kinetics and time points will likely have to be extended to observed complete or near-complete decay of antigen-binding fluorescence. It is also good practice to include the parental WT scFv clone for comparison and to confirm reproducibility with previous results.
5. Dissociation rate improvements of two to tenfold are typical for selections from initial mutagenic library. Two options are available if greater improvements are desired. The entire pool of mutant clones isolated from the original selection can be combined and used as template for another round of mutagenesis followed by selections. The mutations from individual clones can be combined in a site-specific manner to determine whether additive or synergistic effects are obtained.
6. Selected mutant VH and VL genes are ready to be transferred to expression construct of choice. Antibodies can be produced in scFv, Fab, or IgG formats depending on biophysical properties of the antibody and the biosensor requirements. The scFv format is perhaps the quickest and easiest to produce in either *E. coli* or yeast *(17)*; however, stability can be an issue *(18)*. The Fab format is widely used as it is generally more stable than scFv, but expression levels in *E. coli* can be low and purification more difficult due to the heterodimeric nature of the fragment *(19)*. The IgG format is the most stable and widely used; however, the tetrameric quaternary structure dictates mammalian expression system, which can be time consuming and burdensome for the uninitiated *(20)*.

4. Notes

1. Recent inquiries to Invitrogen indicate that the yeast display kit may not be available for future purchase. Alternative contacts to obtain both display vectors and EBY100 yeast strains include Dr. K. Dane Wittrup's laboratory at MIT (http://web.mit.edu/kdw-lab/) and Dr. Cheryl Baird at Pacific Northwest National Laboratory (http://www.sysbio.org/dataresources/singlechain.stm).

2. Antigen can also be directly labeled using a fluorochrome of choice; however, the use of biotin-streptavidin system enables signal amplification to maximize signal to noise ratio. Alternatively, antigen bound by the surface-displayed scFv can be detected using a second antigen-specific mAb that binds a nonoverlapping epitope relative to the scFv of interest. See **refs. 9** and **11** for greater detail.
3. Amplification reactions can either be done by using one primer pair at a time or using pools of 5′ primers. Typically primers A-B are pooled (anneal at 50°C) and the remaining primers are pooled (anneal at 55°C). Remember that VL primers B and C often amplify aberrant light chains present in hybridomas derived from Sp2/0 myeloma fusions. These primers should only be included in primer pools if non-Sp2/0 myeloma was used for generating hybridoma.
4. Gel purification of amplified VH and VL genes may be necessary if many bands are observed from the PCR.
5. Gel purification of amplified scFv gene may be necessary if many product bands are observed from the PCR. VL for and VH rev oligos are designed to introduce GlySer linker between VH and VL genes. The linker sequence is:

```
EcoRI        Bam HI                        VL for
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Gly Ile Leu Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
GGA ATT CTA GGA TCC GGT GGC GGT GGC AGC GGC GGT GGT GGT TCC GGA GGC GGC GGT AGC
CCT TAA GAT CCT AGG CCA CCG CCA CCG TCG CCG CCA CCA CCA AGG CCT CCG CCG CCA TCG
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                          VH rev
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The *EcoR*I and *BamH*I sites and sequence can be omitted from linker leaving 15 residue GlySer repeat sequence. Please note that the linker between Aga2 protein and start of scFv protein is also GlySer linker. However, the codons of the GlySer scFv linker have been changed to prevent possible recombination.

6. Choice of restriction sites will depend on absence from sequenced VH and VL genes. For example, *Nhe*I site is only present in 1 murine VH germline gene, whereas no *Not*I sites are found in murine VH or VL germline genes. If restriction sites used for cloning are changed, be sure to verify the correct reading frame is maintained when designing cloning strategy.
7. Cell numbers are generally calculated using the optical density (OD) at 600 nm determined with a spectrophotometer. EBY100 typically has 2×10^7 cells/mL per OD_{600} unit. This

should be determined by plating yeast on YPD agar plates and/or using a hemacytometer to calibrate your spectrophotometer.

8. Induction of cells during exponential growth (1–3 OD_{600}/mL) will increase expression. scFv expression can also be enhanced by having 0.1–0.2% dextrose in the induction media. Often cells can be simply diluted tenfold from the SD-CAA growth culture. For example 9 mL of SG/R-CAA induction media can be directly inoculated with 1 mL of SD-CAA culture.
9. Alternatively, antigen bound by the surface-displayed scFv can be detected using a second antigen-specific mAb that binds a nonoverlapping epitope relative to the scFv of interest. See **refs. 9** and **11** for greater detail. The order of secondary labeling can also be changed and performed *after* removing a dissociation time point aliquot from the room temperature competition buffer. This will minimize any impact dissociation of the reporter system might have on the results, but is usually only a concern with a system other than biotin-streptavidin or very long dissociation periods (>12 h) are used.
10. Mutant libraries can be constructed by a variety of means. The simplest format has been described in this protocol. Other approaches include the use of nucleoside analogues *(21)* or commercially available kits (Stratagene GeneMorphII cat # 200550) that allows the mutation frequency to be tuned without bias to the identity of the mutation. Also, mutagenic DNA can always be amplified under normal conditions to increase the amount of mutagenic product.
11. The amplified mutagenic DNA can be gel purified to remove the original template from mutagenized PCR product. Practical experience has shown contamination of WT scFv pYD1 plasmid has not been problematic due to minimal amount of intact plasmid and relatively low transformation frequency of yeast. However, gel purification should be used if PCR does not generate a discrete product band of appropriate size or one wants to eliminate all WT plasmid.
12. Extensive information about yeast lithium acetate transformation is available. Refer to reference *(22)* or http://www.umanitoba.ca/faculties/medicine/biochem/gietz/Trafo.html.
13. Refer to (*14*, *16*) for extensive discussion and application of optimal kinetic selection strategies. As an alternative selection strategy, an equilibrium selection in which the concentration of antigen is reduced (generally 1/10th – 1/20th) relative to the WT K_D can be utilized (*see* ref. *(23)* for detailed protocol including alternative mutagenesis strategy). However, most WT scFv derived from existing hybridomas will have

affinities in the single to subnanomolar range, which makes this approach more difficult requiring extremely large reaction volumes (see above mentioned references).

14. The mutant scFv gene can also be directly sequenced after amplification from the yeast colony. Simply transfer a portion of the colony (or cell pellet from an aliquot of liquid culture) into 20 μL of 0.1% SDS and boil for 5 min. Pellet lysate and use 1–2 μL of supernatant as template for PCR amplification reaction as described in **Subheading 3.2.1** using pYD1 for and rev primers. Remove primers and unincorporated nucleotides from PCR using Exo-SAP (USB cat # 78200) following manufacturer's directions and use as template for sequencing reaction. However, this approach is not the most robust and one will want to eventually recover the plasmid with the mutant scFv as described in **Subheading 3.6.1**.
15. The entire Aga2-scFv translation coding sequence is shown below. Pertinent features are highlighted in the sequence. Representative VH and VL genes are from GenBank accession number AF003725. The third position of the penultimate lysine codon in the VL gene included in the VL rev primer is denoted with a box on the antisense DNA strand.

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                                                     Aga2
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
MetGlnLeu LeuArgCys PheSerIle PheSerVal IleAlaSer ValLeuAla GlnGluLeu ThrThrIle CysGluGln IleProSer
ATGCAGTTA CTTCGCTGT TTTTCAATA TTTTCTGTT ATTGCTTCA GTTTTAGCA CAGGAACTG ACAACTATA TGCGAGCAA ATCCCCTCA
TACGTCAAT GAAGCGACA AAAAGTTAT AAAAGACAA TAACGAAGT CAAAATCGT GTCCTTGAC TGTTGATAT ACGCTCGTT TAGGGGAGT
                                                  Aga2
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
ProThrLeu GluSerThr ProTyrSer LeuSerThr ThrThrIle LeuAlaAsn GlyLysAla MetGlnGly ValPheGlu TyrTyrLys
CCAACTTTA GAATCGACG CCGTACTCT TTGTCAACG ACTACTATT TTGGCCAAC GGGAAGGCA ATGCAAGGA GTTTTTGAA TATTACAAA
GGTTGAAAT CTTAGCTGC GGCATGAGA AACAGTTGC TGATGATAA AACCGGTTG CCCTTCCGT TACGTTCCT CAAAAACTT ATAATGTTT
                                             Aga2
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
   pYD1 forward primer
  ~~~~~~~~~~~~~~~~~~~~~~~~
SerValThr PheValSer AsnCysGly SerHisPro SerThrThr SerLysGly SerProIle AsnThrGln TyrValPhe LysLeuLeu
TCAGTAACG TTTGTCAGT AATTGCGGT TCTCACCCC TCAACAACT AGCAAAGGC AGCCCCATA AACACACAG TATGTTTTT AAGCTTCTG
AGTCATTGC AAACAGTCA TTAACGCCA AGAGTGGGG AGTTGTTGA TCGTTTCCG TCGGGGTAT TTGTGTGTC ATACAAAAA TTCGAAGAC
                                      Linker
           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                     VH for
                                              ~~~~~~~~~~~~~~~~~~~~                    VH gene
                                                          NheI  ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
                                                          ~~~~~~
GlnAlaSer GlyGlyGly GlySerGly GlyGlyGly SerGlyGly GlyGlySer AlaSerGln ValLysLeu GlnGlnSer GlyAlaGlu
CAGGCTAGT GGTGGTGGT GGTTCTGGT GGTGGTGGT TCTGGTGGT GGTGGTTCT GCTAGCCAG GTGAAGCTG CAGCAGTCA GGGGCTGAG
GTCCGATCA CCACCACCA CCAAGACCA CCACCACCA AGACCACCA CCACCAAGA CGATCGGTC CACTTCGAC GTCGTCAGT CCCCGACTC
                                                 VH gene
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
LeuValArg ProGlyAla SerValLys LeuSerCys LysAlaSer GlyTyrSer LeuThrSer TyrTrpMet AsnTrpVal LysGlnArg
CTGGTGAGG CCTGGAGCT TCAGTGAAG CTGTCCTGC AAGGCTTCT GGCTACTCC CTCACCAGC TACTGGATG AACTGGGTG AAGCAGAGG
GACCACTCC GGACCTCGA AGTCACTTC GACAGGACG TTCCGAAGA CCGATGAGG GAGTGGTCG ATGACCTAC TTGACCCAC TTCGTCTCC
                                                 VH gene
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
ProGlyGln GlyLeuGlu TrpIleGly MetIleHis ProSerAsp SerAspThr ArgPheAsn GlnLysPhe GluAspLys AlaThrLeu
CCTGGACAA GGCCTTGAG TGGATTGGC ATGATTCAT CCTTCCGAT AGTGACACT AGGTTCAAT CAGAAGTTC GAGGACAAG GCCACATTG
GGACCTGTT CCGGAACTC ACCTAACCG TACTAAGTA GGAAGGCTA TCACTGTGA TCCAAGTTA GTCTTCAAG CTCCTGTTC CGGTGTAAC
```

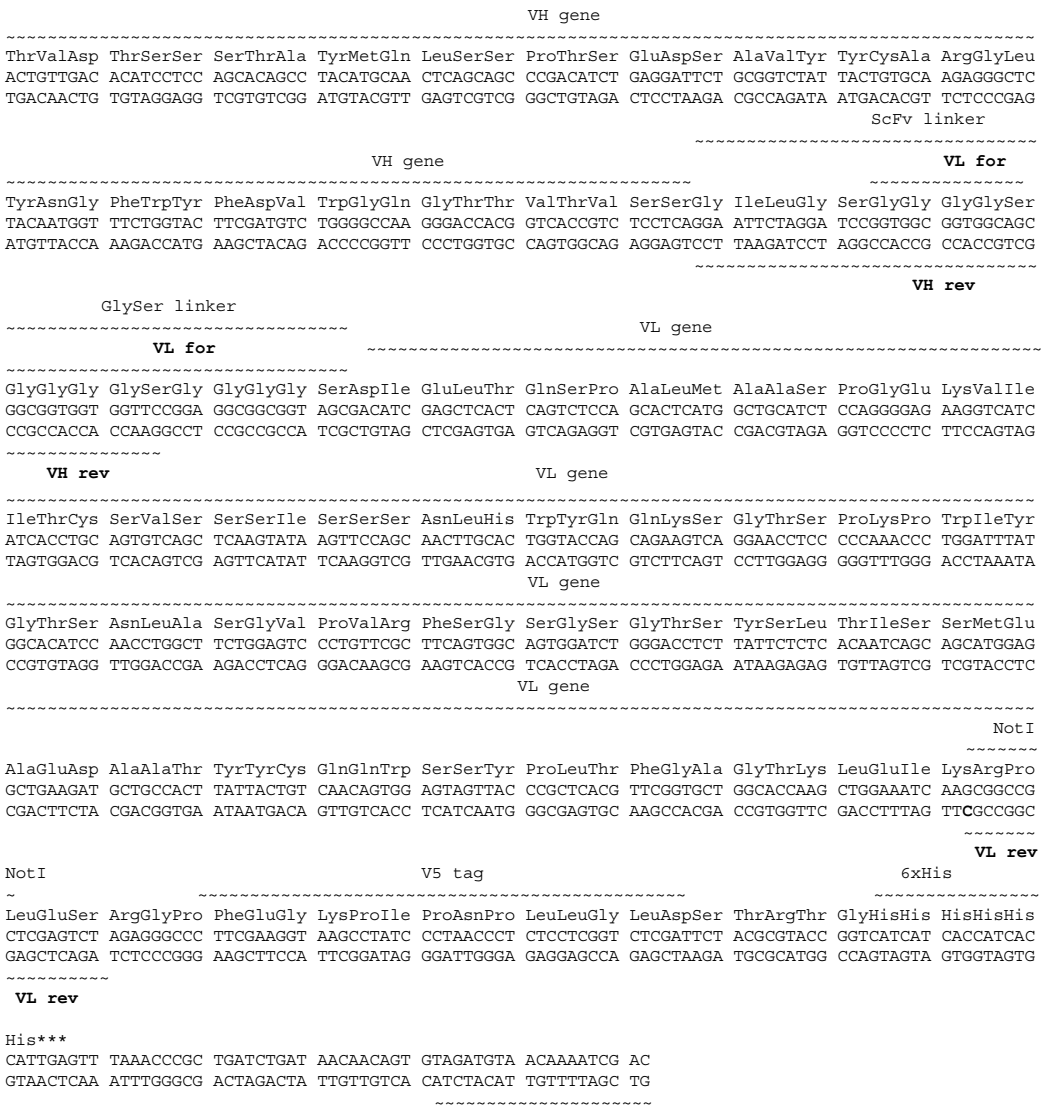

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