METHODS IN MOLECULAR BIOLOGY™

Volume 263

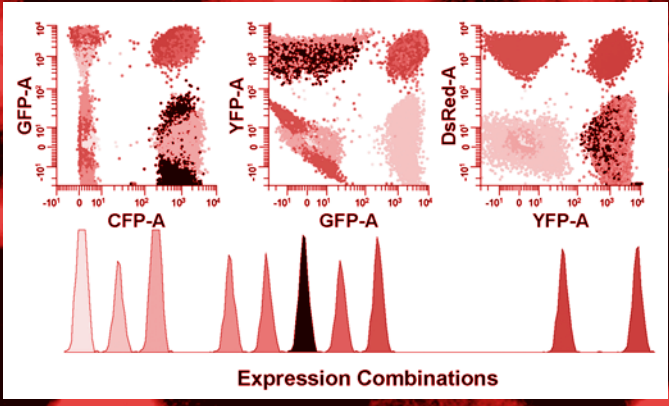
Flow Cytometry Protocols

SECOND EDITION

Edited by

Teresa S. Hawley

Robert G. Hawley



HUMANA PRESS

17

Flow Cytometric Screening of Yeast Surface Display Libraries

Michael Feldhaus and Robert Siegel

Summary

A method to screen and isolate antigen specific clones from a library of single-chain antibodies expressed on the surface of yeast cells is presented. Two rounds of magnetic bead enrichment before flow cytometric sorting enables one to screen libraries of far greater diversity than can be screened by just flow cytometry. The strength of flow cytometric sorting is the ability to follow the selection in real time and to isolate easily the highest affinity antigen-specific clones. A major strength of yeast display as a discovery platform is the ability to characterize the binding properties, the affinity of a clone without the need for subcloning, expression, and purification of the scFv. The methodology for directed evolution of single-chain antibodies to increase the affinity of a clone is also described.

Key Words

Affinity, antibodies, "directed evolution," scFv, scFv libraries, selections, yeast display.

1. Introduction

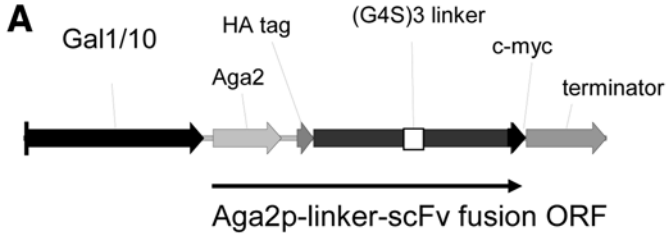
Creating affinity reagents to important biomolecules is one of the most critical, yet one of the most challenging tasks facing biologists. This chapter describes the implementation of a yeast surface display library of scFv (*s*ingle-*c*hain *F*ragment *v*ariable) antibodies as a method to solve part of this problem. The methodology was originally described by Feldhaus et al. ***(1)***. The scFv library is specifically designed to display full-length scFv antibodies whose expression on the yeast cell surface can be monitored with either N-terminal HA and C-terminal c-myc epitope tags. These epitope tags allow monitoring of clones or libraries of scFv clones for surface expression of full-length scFv by flow cytometry. The extra cellular surface display of scFv by *Saccharomyces*

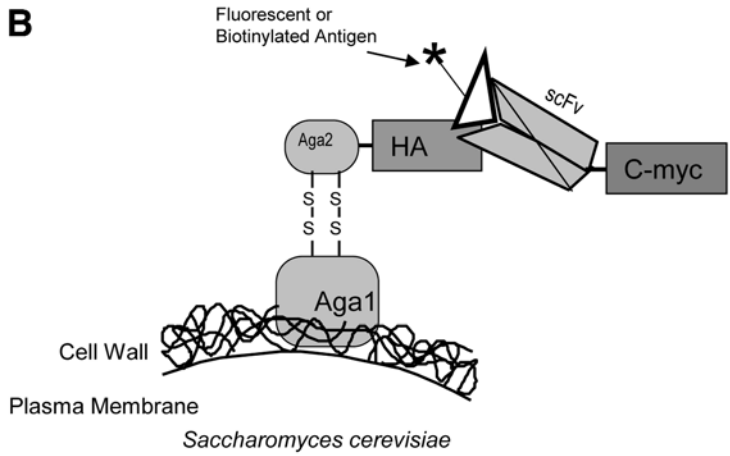
From: *Methods in Molecular Biology: Flow Cytometry Protocols, 2nd ed.*
Edited by: T. S. Hawley and R. G. Hawley

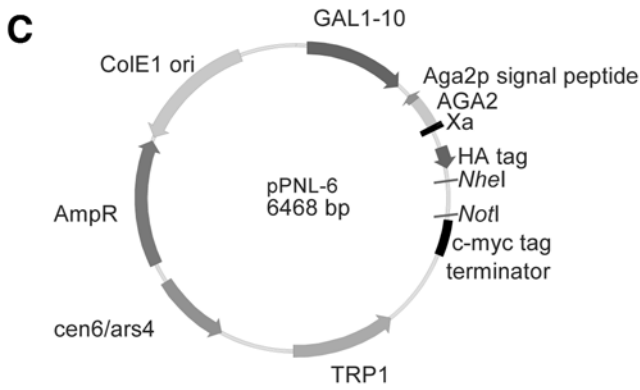
cerevisiae also allows the detection of appropriately labeled antigen–antibody interactions by flow cytometry. As a eukaryote, *S. cerevisiae* offers the advantage of posttranslational modifications and processing of mammalian proteins and, therefore is well suited for expression of human derived antibody fragments. In addition, the short doubling time of *S. cerevisiae* allows the rapid analysis and isolation of antigen-specific scFv antibodies.

Yeast display, based on the platform created by Dane Wittrup at Massachusetts Institute of Technology, uses the a-agglutinin yeast adhesion receptor to display recombinant proteins on the surface of *S. cerevisiae* ***(2,3)***. In *S. cerevisiae*, the a-agglutinin receptor acts as an adhesion molecule to stabilize cell–cell interactions and facilitate fusion between mating "a" and α haploid yeast cells. The receptor consists of two proteins, Aga1 and Aga2. Aga1 is secreted from the cell and becomes covalently attached to β-glucan in the extra cellular matrix of the yeast cell wall. Aga2 binds to Aga1 through two disulfide bonds, presumably in the Golgi, and after secretion remains attached to the cell via Aga1. The yeast display system takes advantage of the association of Aga1 and Aga2 proteins to display a recombinant scFv on the yeast cell surface. The gene of interest is cloned into the pYD1 vector (Invitrogen, Carlsbad, CA), or a derivative of it, in frame with the *AGA2* gene. The resulting construct is transformed into the EBY100 *S. cerevisiae* strain containing a chromosomal integrant of the *AGA1* gene. Expression of both the Aga2 fusion protein from pYD1 and the Aga1 protein in the EBY100 host strain is regulated by the *GAL1* promoter, a tightly regulated promoter that does not allow any detectable scFv expression in absence of galactose. On induction with galactose, the Aga1 protein and the Aga2 fusion protein associate within the secretory pathway, and the epitope-tagged scFv antibody is displayed on the cell surface (**Fig. 1A**,**B**). **Figure 2**

Fig. 1. *(see facing page)* Features of the pPNL6 vector, the *E. coli*/yeast shuttle vector for the selection and induced expression of an scFv Aga2 fusion protein. *(Note: pYD1 is very similar but has modified epitope tags and restriction sites.)* The pPNL6 vector (or pYD1) containing a nonimmune scFv library offers several key features that make it easy to display proteins of interest on *S. cerevisiae*. These include: (1) *GAL1/10* promoter for strong inducible expression following the addition of galactose. The tight regulation of this promoter during growth on glucose allows for large expansion of the library without worry of clone biased growth. (2) N-terminal HA and C-terminal c-myc (only in pPNL6) epitopes for detection of the displayed scFv antibody with an anti-c-myc (9E10) or anti-HA (12CA5) antibody. (3) *TRP1* auxotrophic marker for yeast selection and ampicillin for *E. coli* selection. (4) *CEN/ARS* origin for selection and maintenance in *S. cerevisiae*. **(A)** The *GAL1/10*-regulated scFv surface expression construct. **(B)** The scFv Aga2 fusion protein surface expression. **(C)** Plasmid map of pPNL6.


A
Gal1/10
HA tag
(G4S)3 linker
c-myc
Aga2
terminator
Aga2p-linker-scFv fusion ORF


B
Fluorescent or
Biotinylated Antigen
scFv
Aga2
HA
C-myc
S
S
S
S
Aga1
Cell Wall
Plasma Membrane
Saccharomyces cerevisiae


C
GAL1-10
ColE1 ori
Aga2p signal peptide
AGA2
Xa
HA tag
NheI
NotI
c-myc tag
terminator
pPNL-6
6468 bp
AmpR
cen6/ars4
TRP1

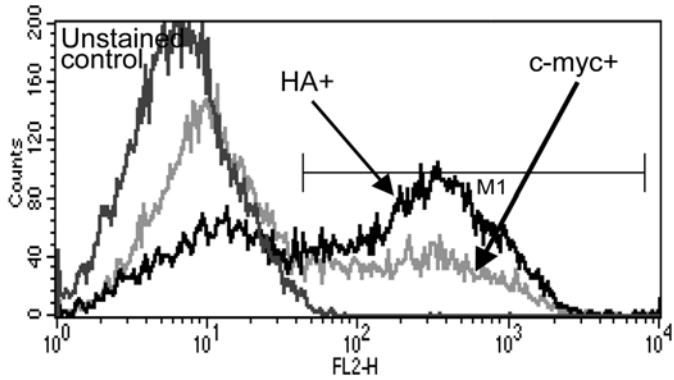


Fig. 2. Flow cytometric analysis of a nonimmune scFv library surface expression on yeast. Typically, 60–70% of the yeast in the scFv library will be HA epitope tag positive and 35–40% c-myc positive.

shows the HA and c-myc epitope staining patterns for an scFv antibody library. Molecular interactions with the scFv antibody can be easily assayed by incubating the cells with a ligand of interest **(Fig. 1C)**. **Figure 3** graphically depicts a generalized scheme for enriching and identifying antigen-specific binders within a nonimmune scFv library. A combination of two rounds of selection using magnetic particles followed by two rounds of flow cytometric sorting will generally allow recovery of clones of interest (*see* **Table 1**).

Yeast surface display of scFv antibodies has also been successfully utilized to isolate higher affinity clones from small (~1×10^6) mutagenic libraries generated from a unique antigen binding scFv clone ***(4)***. Mutagenic libraries are constructed by amplifying the parental scFv gene one wants to obtain higher affinity variants of using error-prone PCR to incorporate three to seven point mutations/scFv ***(5,6)***. The material is cloned into the surface expression vector using the endogenous homologous recombination system present in yeast, known as "gap repair" ***(7)***. Gap repair that allows gene insertion in chromosomes or plasmids at exact sites by utilizing as little as 30 basepair regions of homology between your gene of interest and its target site. This allows mutated libraries of 1–10 $\times$ 10^6 clones to be rapidly generated and screened, in about 2 wk, by selecting the brightest antigen binding fraction of the population using decreasing amounts of antigen relative to the K_D of the starting parental clone. The screening involves three to four rounds of flow cytometry sorting, however. The flow cytometric sorting protocol is slightly different for a library based on a mutagenized clone than for a nonimmune library, and each is described separately in **Subheadings 3.6.** and **3.4.**, respectively.

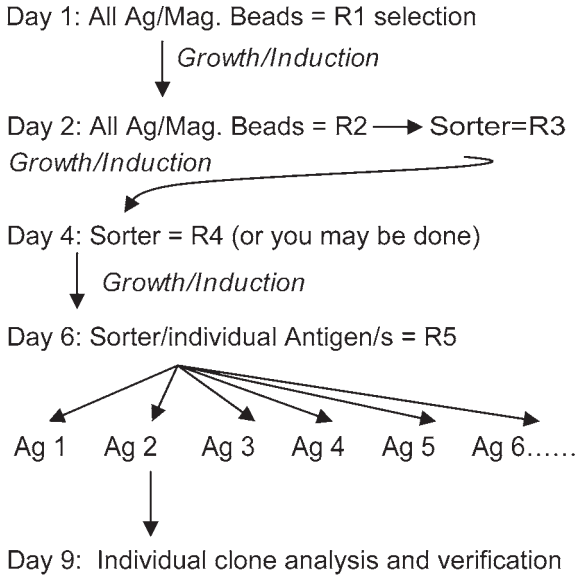


Fig. 3. Isolation of scFv through sequential enrichment of a nonimmune scFv library. Schematic of generalized scFv library screen.

One of the real strengths of the yeast display system is the speed of characterizing the binding affinity of the clones ***(8)***. A brief and greatly simplified version is described in **Subheading 3.6.** Additional useful resources to complement the protocols in this chapter include some yeast-specific protocols. For yeast transformation, *see* http://www.umanitoba.ca/faculties/medicine/biochem/gietz/Trafo.html. Also *see* **refs. *9*** and ***10***.

2. Materials

2.1. Media and Agar Plates

In making the media we add the following components in the following order, which appears to help with solubility of some components. Add amino acids to water, then the sugars, then add a 10X solution of buffer. The pH should be approx 6.25. We filter-sterilize all selective media (*see* **Note 1**).

1. Rich nonselective media (YEPD or YPD [yeast extract peptone dextrose]): 10 g/L of yeast extract, 20 g/L of peptone, and 20 g/L of dextrose. Filter sterilize or autoclave.
2. Selective growth media (SD + CAA): 5 g/L of casamino acids (-ade, -ura, -trp), 20 g/L of dextrose, 1.7 g/L of YNB (yeast nitrogen base) without ammonium sulfate and amino acids (Difco brand, cat. no. 233520, Becton Dickinson, Franklin

Table 1
Enrichment Factor and Recovery of Antigen-Specific Clones Using a Combination of Magnetic Bead Enrichment and Flow Cytometric Cell Sorting

Selection round and type	Complexity	Clone frequency = Ag-specific complexity	Library coverage screened	Ag-specific cells/total cells	Recovery of Ag-specific cells based on 50% recovery per round of selection	Selection output	Enrichment = original complexity × output complexity
R1 magnetic beads	10^9	$1/10^9$	10X	$10/10^{10}$	5	1×10^7	$1/10^9 \times 5/10^7$ = 500X
R2 magnetic beads	1×10^7	$5/10^7$	1000X	$1000/10^{10}$	500	1×10^7	$5/10^7 \times 5/10^5$ = 500X
R3 cell sorter	1×10^7	$5/10^5$	0	$500/1 \times 10^7$	250	1×10^4	$5/10^5 \times 2.5/10^2$ = 2000X
R4 cell sorter	250×10^4	2.5/100	1000X	$25{,}000/10^6$	12,500	1000	$2.5/10^2 \times 1000/1000$ to 1000/2000 about 50X
R4 output sorter-analysis	5×10^4	5–9/10					

It is important to use at least 10-fold coverage of the starting complexity library to screen the library fully. After two rounds of enriching using magnetic beads the 500 Ag-specific cells would theoretically be present in a total of 1×10^7 nonspecific cells. Using the Miltenyi MACS system, we find that from 10^{10} cells loaded onto the column, the background is consistently approx $0.5–2 \times 10^7$ cells (based on OD_{600}), regardless of scFv expression or number of antigen-binding cells. The cell sorter greatly reduces the complexity from 10^7 to 10^4 –10^5 total cells depending on the stringency of sort gates and total number of cells sorted. By the second round of flow cytometric sorting, it is usually apparent if an antigen-specific clone is present at a frequency greater than 1/1000. It is always important to have a sample prepared that has been stained with all secondary regents, but without antigen (i.e., anti-c-myc/GaM–488 and streptavidin–PE).

Lakes, NJ), 5.3 g/L of ammonium sulfate, 10.19 g/L of $Na_2HPO_4 \bullet 7H_2O$, and 8.56 g/L of $NaH_2PO_4 \bullet H_2O$ (*see* **Note 2**).

3. Selective scFv induction media (SG/R + CAA): Same as selective growth media except substitute 20 g/L of galactose, 20 g/L of raffinose, and 1 g/L of dextrose for dextrose.
4. Agar plates: Add 20 g/L of agar to YEPD; SDA-HUT (synthetic defined agar, -histidine, -uracil, -tryptophan). Buy or make them yourself (our supplier is Teknova, Half Moon Bay, CA).

2.2. Strains and Plasmids

1. EBY100 (Invitrogen): (Leu^-, Trp^-) BJ5465 is MATa. It has the auxotrophic: ura3-52 (a Ty element insertion with no detectable background reversion frequency), trp1 (an amber point mutation), leu2δ200, his3δ200, pep4:HIS3, prbd1.6R, can1, GAL. EBY100 has genomic insertion of AGA1 regulated by GAL promoter with a URA3 selectable marker. The scFv library is displayed in this strain.
2. Plasmids: pYD1 (Invitrogen) is the plasmid that our scFv library is cloned into as a fusion protein to AGA2. Selects for ability to grow in the absence of tryptophan in yeast and on ampicillin in *E. coli.* pPNL6 is based on this plasmid with minor differences such as a C-terminal c-myc epitope tag instead of a V5 epitope tag.

2.3. Antigens

Biotinylated antigens can be generated in a variety of ways. We find the NHS Biotinylation kit and the 2-hydroxyazobenzen-4′-carboxylic acid (HABA) system to quantify the number of biotin/molecule of protein to be robust and easy (both from Pierce Biotechnology, Rockford, IL). We strive to obtain two or three biotin per protein molecule. More biotin/protein is not desirable because of concerns about blocking the epitope/antibody interaction site.

2.4. Flow Cytometry and Magnetic Bead Enrichment

1. Miltenyi magnetic-activated cell sorting (MACS) LS columns and streptavidin magnetic beads (and antibiotin magnetic beads) with manual magnetic separator (Miltenyi Biotech, Auburn, CA).
2. Anti-HA (12CA5) antibody.
3. Anti-c-myc antibody (clone 9E10) (Amersham Biosciences, Piscataway, NJ; or Covance, Babco, Cumberland, VA), 200 µg/mL for pPNL6-derived libraries; or anti-V5 mAb for pYD1 libraries.
4. Goat antimouse (GaM) Alexa 488-conjugated (Molecular Probes, Eugene, OR).
5. Goat antimouse (GaM) Alexa 633-conjugated (Molecular Probes).
6. Goat antimouse (GaM) PE-conjugated (Molecular Probes).
7. Streptavidin–R-phycoerythrin (SA–PE) (Molecular Probes).
8. Streptavidin–Alexa 633 (SA–633) (Molecular Probes).
9. Streptavidin–Alexa 647 (SA–647) (Molecular Probes).
10. Neutravidin–fluorescein isothiocyanate (FITC) (NA–FITC) (Molecular Probes).

11. Neutravidin–PE (NA–PE) (Molecular Probes).
12. Biotinylated purified antigens, 2 µg/kDa of antigen (*see* **Subheading 2.3.** for preparation).
13. Penicillin/streptomycin, 100X tissue culture grade (Gibco™ Invitrogen Corporation).
14. Wash buffer/buffer: Phosphate-buffered saline (PBS), 0.5% bovine serum albumin (BSA), and 2 m*M* EDTA.

3. Methods

The methods below are divided into six categories: (1) growth and induction for scFv expression of libraries or clones, (2) magnetic bead enrichment of biotinylated antigen binding yeast, (3) fluorescent staining for both antigen binding and scFv expression of the library, (4) flow cytometric sorting of library, (5) clone validation and subsequent affinity determination, and (6) sorting of mutagenic scFv library for isolation of higher affinity clones. Library construction is not covered in this chapter. Only selections of scFv clones of interest are described.

Many of the techniques, growth and induction conditions, and staining protocols are very similar between nonimmune libraries, immune libraries, or mutagenized scFv clone libraries. The major differences between the various library types are both the diversity of the libraries (number of different antibody clones present) and the frequency of antigen-binding clones within that diversity.

*3.1. Growth and Induction of Surface Expression of scFv (***see** ***Note 3)***

3.1.1. Libraries of scFv

1. Start a SD + CAA culture that contains a minimum of a 10X representation of the diversity of your library. For a 10^9 diverse library, 10^{10} yeast are needed to start the culture. The culture would be 1 L of 0.5 OD_{600}/mL. Plating a dilution plate is highly recommended to verify colony-forming units and lack of bacterial contamination.
2. Grow at 30°C with shaking overnight (*see* **Note 4**). Take a 10X representation of library diversity from the culture and pellet. If you diversity is 10^9 clones, then a 10X representation would be 10^{10} yeast. That means at 2×10^7 yeast/OD_{600} unit you need 500 OD_{600}. For example, if your OD is 5.0 OD_{600}/mL then you need 100 mL of the culture to obtain 10^{10} yeast. Generally, the culture should be freshly saturated or be <4 OD_{600}/mL.
3. Resuspend the cell pellet in SG/R + CAA at 0.5 OD_{600}/mL and incubate at 20°C with shaking for one to two doublings as determined by OD_{600}. This will generally take 12–16 h.
4. Wash the yeast once in wash buffer. The yeast are now ready to be labeled with biotinylated antigen and enriched on a magnetic column. You can also store the cells by placing the flask at 4°C for up to a week with minimal degradation of

c-myc expression level or positive cells as determined by flow cytometry. However, the yeast do become sticky and tend to clump more, which may affect selections, and viability is often reduced by 50%.

3.1.2. Individual scFv Clones

1. Start a 1- to 5-mL SD + CAA culture from a single isolated yeast colony.
2. Grow at 30°C with shaking overnight.
3. Pellet enough cells to start a 1- to 5-mL culture at 0.5 OD_{600}/mL in induction media, SG/R + CAA.
4. Resuspend cell pellet in SG/R + CAA at 0.5 OD_{600}/mL and incubate at 20°C with shaking for one to two doublings as determined by OD_{600}. This will generally take 12–16 h.
5. Wash cells once in wash buffer before staining for flow cytometry, or store the cells by placing the flask at 4°C for up to a week. *See* **Subheading 3.1.1., step 4** for notes about 4°C storage of induced cultures.

3.2. Magnetic Bead Enrichment Using Miltenyi MACS LS Columns for Two Rounds of Magnetic Bead Enrichment

Process for enrichment of antigen-specific clones from a nonimmune library of 10^9 diversity:

1. Resuspend 1×10^{10} yeast (from induced culture) in 10 mL of wash buffer.
2. Add one or more biotinylated antigens at 100 n*M* final concentration. Incubate at 25°C (room temperature) for 30 min followed by 5–10 min on ice. **All subsequent steps should be done with ice-cold buffer or at 4°C.**
3. Pellet cells (3000*g* for 3 min) and wash three times with 50 mL of wash buffer.
4. Resuspend the cell pellet in 5 mL of wash buffer with 200 µL of magnetic-activated cell sorting (MACS, Miltenyi) streptavidin magnetic microbeads (or antibiotin magnetic microbeads). Alternating between these beads during subsequent rounds of selection decreases the chance of obtaining secondary reagent-specific clones.
5. Incubate on ice for 10 min with gentle mixing by inversion every 2 min.
6. Pretreat Miltenyi LS column, loaded into magnet, by flowing 3 mL of ice-cold wash buffer through it using gravity.
7. Add 40 mL of wash buffer, pellet cells and resuspend in 50 mL of wash buffer. Make sure it is a single-cell suspension by vortex-mixing and passing cells through a cell strainer cap tube (Falcon brand, cat. no. 352235, BD Biosciences, San Jose, CA) immediately prior to loading onto Miltenyi LS column.
8. Add the 7-mL cell suspension that was just put through the cell strainer cap to the column. After each 7 mL of cells have entered the column and the flow has stopped, remove the column from magnet and immediately put back into magnet. This rearranges the iron beads in the column and allows the cells that are physically trapped between the beads to pass through. With the column back in the magnet, add 1 mL of wash buffer and let flow through, then another 7 mL of

cells onto column. Repeat the column removal procedure between each loading of cells. It will take about 30 min to load all 50 mL of cells.

9. Once all of the cells have been loaded on the column, wash the column with 3 mL of wash buffer. Make sure the upper loading chamber is washed of all residual cells. This wash removes the cells in the void volume of the column. Remove column from magnet and immediately replace as before. Repeat this wash two additional times.
10. Once the column has stopped dripping, remove the column from magnet and then add 7 mL of wash buffer and use the plunger to push all remaining cells out into a 15-mL conical tube. We generally elute approx $1–3 \times 10^7$ cells. Pellet cells and resuspend as follows:
    a. If round 1 selection: Resuspend yeast in 200 mL of selection media (SD + CAA with pen/strep) for overnight growth to saturation (10^{10} yeast). Plate a dilution to obtain an accurate number of yeast isolated. This is the R1output diversity. Induce as described previously in SG/R + CAA. This allows maximal expansion of library and of your clone of interest for subsequent repeat of magnetic bead enrichment. Use antibiotin magnetic beads as above for second round of selection. This is the R2output.
    b. If round 2 selection (second enrichment on magnetic column): Resuspend cells in 500 µL of buffer for subsequent staining for sorting by flow cytometry.

3.3. Fluorescent Staining of Cells From the MACS Column Before Flow Cytometric Cell Sorting (see Note 5)

It is important to note that at the start of each round of selection you should stain your library for anti-HA (12CA5), anti-c-myc (9E10), and secondary only controls. This establishes several important baselines. (1) Were my yeast properly induced? (2) What percent are expressing c-myc (i.e., full length scFv)? (3) Do my secondary reagents bind nonspecifically? (4) Am I enriching for secondary reagent binders? Therefore, if you are going to use SA–PE to label antigen bound cells, you should add just SA–PE to your induced cells to see if it binds. The control for GaM reagents should be stained and analyzed (no anti-c-myc antibody should be added). We generally see very little labeling of the aliquot, <0.1%. If you see labeling try other secondary reagents (i.e., neutravidin, streptavidin or antibiotin monoclonal antibody [MAb]) to minimize enriching for secondary reagent binders.

1. Add 5 µL of 9E10 anti-c-myc antibody (200 µg/mL) to the 500 µL containing $1–10 \times 10^7$ yeast eluted from the magnetic column. You can also add 100 nM biotinylated antigen at this point. However, this is unnecessary if cells were selected on magnetic column as they should already have antigen bound.
2. Incubate for 30 min on ice. Pellet in microfuge on high for 10 s. Wash two times with 500 µL of buffer.

3. Add a 1:200 dilution of secondary reagents (GaM-Alexa 488 for c-myc and SA–PE for the biotinylated antigen) (*see* **Note 6** and **Fig. 4**).
4. Incubate for 30 min on ice, pellet the cells, and wash two times with 500 µL of ice-cold wash buffer.
5. Resuspend the cells in 1 mL of buffer and keep on ice in the dark until sorting.

3.4. Flow Cytometry Sorting of Antigen-Specific scFv Clones From Nonimmune Library

3.4.1. Sort Gate Decision

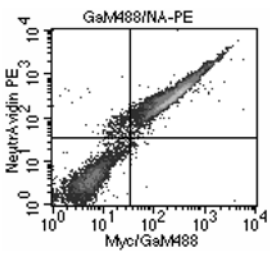
Sorting from a nonimmune library that has gone through two rounds of enrichment on magnetic beads should allow you to see antigen binding yeast at a frequency of 1/1000 to 1/5000 cells. The sort gate is set in one of the following two manners:

1. The first method is based on sorting the top 0.1% of the brightest antigen binders that are also c-myc positive. These may or may not be an obvious or distinct population. Generally for the first sort (round 3 of selection), most or all of the cells coming off the magnetic column should be sorted. In **Fig. 5**, top panel, the population is obvious; however, this is not always the case (depending on enrichment ratios) and sometimes antigen binding cells must be sorted on "faith."
2. The second method relies on staining the same sublibrary in the presence and absence of antigen. It is often very clear where to set the sort gate at this point. An example of sorting is presented in **Fig. 5**, bottom panels.

The sort criteria are usually less stringent for the first flow cytometric sort. Sort more slowly (less coincidence aborts) and on an enrich mode. The rate of "more slowly" is dependent on the sorter you are using. A skilled operator should be able to provide guidance.

3.4.2. First Sort, Round 3 Selection

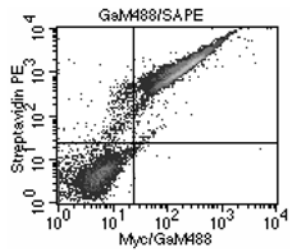
1. Sort yeast into Eppendorf tubes containing 100 µL of YPD media. The YPD helps the yeast recover. We let them sit in the YPD media for about an hour before growth on selectable media.
2. After sorting, plate the cells on SD + CAA pen/strep and an appropriate dilution plate (*see* **Note 7**).
3. Incubate plates at 30°C for 24–48 h.
4. Scrape colonies together and then grow for several hours in SD + CAA media.
5. Subculture at least a 10X representation of sublibrary diversity (determined from the dilution plate of sorted cells) into SG/R + CAA induction media.
6. Make glycerol stock of at least 10X representation of the sublibrary diversity from the SD + CAA culture for storage in case subsequent steps need to be repeated.



Sample ID: GaM488/NA-PE

Total Events: 20000
X Parameter: FL1-H Myc/GaM488
Y Parameter: FL2-H NeutrAvidin PE

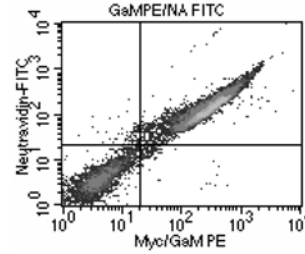
Quad	% Total	X Mean	Y Mean
UL	1.58	21.76	111.83
UR	77.33	282.69	374.02
LL	20.94	6.61	5.35
LR	0.14	168.10	



Sample ID: GaM488/SAPE

Total Events: 20000
X Parameter: FL1-H Myc/GaM488
Y Parameter: FL2-H Streptavidin PE

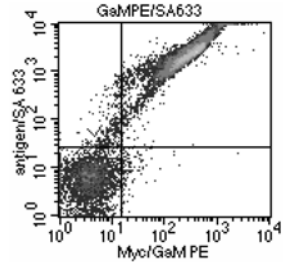
Quad	% Total	X Mean	Y Mean
UL	2.11	13.76	217.47
UR	76.73	234.55	1384.01
LL	21.04	5.96	5.59
LR	0.10	249.55	17.69



Sample ID: GaMPE/NA FITC

Total Events: 20000
X Parameter: FL2-H Myc/GaM PE
Y Parameter: FL1-H Neutravidin-FITC

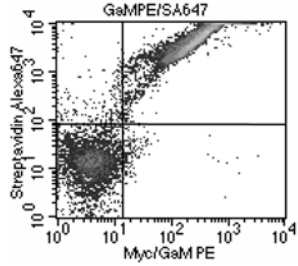
Quad	% Total	X Mean	Y Mean
UL	0.65	15.02	36.80
UR	77.09	319.88	161.59
LL	21.81	5.02	4.58
LR	0.46	75.22	12.91



Sample ID: GaMPE/SA633

Total Events: 20000
X Parameter: FL2-H Myc/GaM PE
Y Parameter: FL4-H antigen/SA633

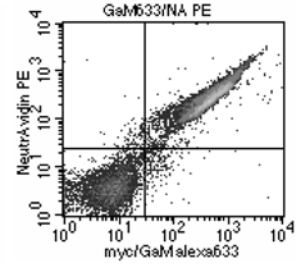
Quad	% Total	X Mean	Y Mean
UL	1.93	9.27	142.58
UR	77.75	310.47	2521.77
LL	19.71	4.22	6.43
LR	0.60	43.96	10.68



Sample ID: GaMPE/SA647

Total Events: 20000
X Parameter: FL2-H Myc/GaM PE
Y Parameter: FL4-H Streptavidin Alexa647

Quad	% Total	X Mean	Y Mean
UL	1.28	7.36	602.13
UR	77.37	310.67	4801.63
LL	20.65	4.46	16.41
LR	0.70	107.39	25.90



Sample ID: GaM633/NA PE

Total Events: 20000
X Parameter: FL4-H myc/GaM Alexa633
Y Parameter: FL2-H NeutrAvidin PE

Quad	% Total	X Mean	Y Mean
UL	0.60	19.71	75.69
UR	76.70	554.27	330.34
LL	21.79	7.98	4.61
LR	0.92	1319.72	11.27

Fig. 4. Different fluorescent reagents that can be employed to detect c-myc expression and biotinylated antigen binding.

The glycerol stock is the yeast in a 15% glycerol solution and can be stored at –80°C. The cell concentration can be from 1 OD_{600}/mL up to 100 OD_{600}/mL.

3.4.3 Second Sort, Round 4 Selection

At this point your diversity is generally under 100,000. Therefore, staining 2×10^7 yeast gives you a 200-fold coverage of your diversity. You also expect

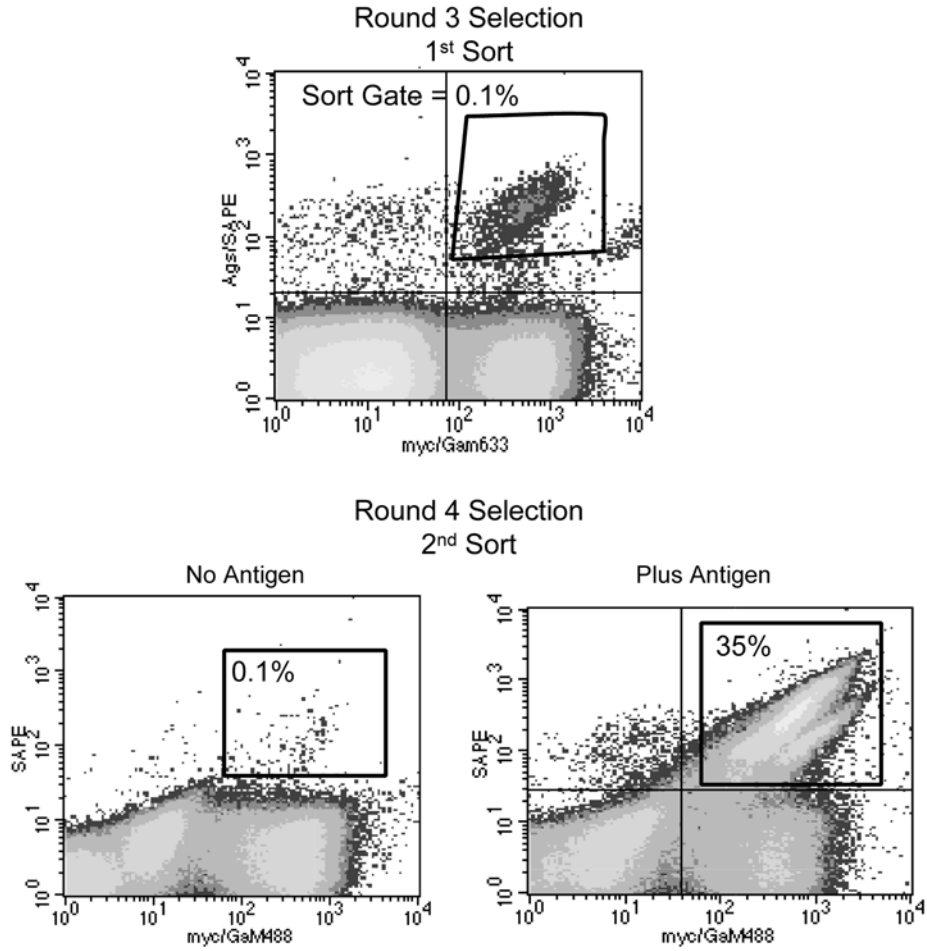


Fig. 5. Sorting of R2 output from second magnetic bead enrichment column to create R3 output and then R4 output. The "no-antigen" control shows that when antigen is present there are many more dual positive yeast and provides confidence that what you are sorting is actually binding your antigen and not the detection reagent.

to see clones that bind your antigen at a frequency of 1–10%. Cells are stained as before, with all controls:

Control 1 = Unstained
Control 2 = GaM–Alexa 488 only
Control 3 = SA–PE only
Control 4 = anti-myc + GaM–Alexa 488
Control 5 = anti-myc + GaM–Alexa 488 + SA–PE
Sample = anti-myc/GaM–Alexa 488 + antigen/SA–PE

A number of controls (listed above) should be done with the induced R3 sublibrary output prior to the fourth round of selection. Control 5 will help distinguish antigen detection reagents specific binders from true antigen binders. Analyze at least 100,000 events to allow patterns to emerge in the bivariate plot. SA–PE-positive clones that do not have the c-myc tag can be easily removed during the subsequent sort by setting the selection gate to isolate only c-myc-positive/antigen binding cells.

If the SA–PE-positive clones from control 5 are also c-myc positive, then careful examination of control 5 to the antigen containing sample may allow a different subpopulation to be visualized in the dual-positive quadrant of the bivariate plot. The population that is present in the antigen containing sample and not in the no-antigen control can be specifically isolated. However, you may also see the percentage dual-positive population change from 2% in the no-antigen control to 4% or more in the antigen-containing sample. These antigen binders are able to be identified in one of two manners. First, after sorting with SA–PE, individual clones can be subsequently screened for antigen binding specificity. The number of clones to analyze will be dictated on the relative differences in the percentage positive between control 5 and the antigen-containing sample. Second, the secondary reagent used to detect antigen-positive cells can be changed to antibiotin MAb or neutravidin, both labeled with an appropriate fluorescent dye. This prevents those clones in the library that specifically bind streptavidin from contaminating the selection process altogether. However, make sure to repeat control 5 with whatever secondary reagent you intend to use to detect antigen binding.

1. Sort on "purity mode" if an antigen specific population is obvious. Sorting a few hundred to a thousand yeast cells at this point will generally suffice to give an antigen positive clone.
2. Sort yeast into Eppendorf tubes containing 100 µL of YPD media.
3. After sorting, plate the cells on SD + CAA pen/strep. No dilution plate is required when <1000 yeast are plated. We generally see a 50–80% plating efficiency.
4. Incubate plates at 30°C for 24–48 h.
5. Pick individual colonies into SD + CAA media. We will generally pick 10 different clones. These may all be the same or ten unique clones. The number of unique clones will be determined either by sequencing or BstN1 fingerprinting of polymerase chain reaction (PCR)-amplified scFv genes ***(11)***.
6. Subculture 100 µL into 1 mL of SG/R + CAA. Grow at 20°C for 16 h to induce expression of scFv.

3.5. Clone Validation and Affinity Determination

This verifies the individual clones from the previous sort are true antigen binders and that they express full-length scFv as determined by c-myc expression. Each sample needs to be stained with antigen and without antigen:

No Ag = anti-c-myc + GaM–Alexa 488 + SA–PE
Plus antigen = anti-c-myc/GaM–Alexa 488 + antigen/SA–PE

3.5.1. Flow Cytometric Staining of Individual Clones

1. Prepare 1–2 × 10^6 yeast by washing the induced yeast one time with 1 mL of buffer.
2. Resuspend yeast in 100 µL containing 1 µL of anti-c-myc MAb and for plus antigen stain biotinylated antigen (generally 100 n*M*).
3. Incubate for 30 min at room temperature followed by 5 min on ice. Pellet in microfuge on high for 10 s. Wash two times with 500 µL of ice-cold buffer.
4. Resuspend pellet in 100 µL of a 1:200 dilution of secondary reagents (GaM–Alexa 488 and SA–PE). **Figure 4** shows several combinations of fluorescent reagents that can be used.
5. Incubate for 30 min on ice, pellet the cells, and wash two times in cold buffer.
6. Resuspend cells in 1 mL of buffer and keep on ice in the dark until analysis by flow cytometry.

3.5.2. Affinity Determination Using Equilibrium Binding Titration Curves to Determine Equilibrium Dissociation Constants K_D

Because the initial selections are performed at 100 n*M*, the vast majority of clones identified from a nonimmune library will be in the 1–100 n*M* affinity range. The lower the concentration of antigen in the initial selection, the lower the affinity range will be and fewer unique clones are generally isolated. Once antigen binding has been verified, we determine clone uniqueness by utilizing a DNA fingerprint of the PCR-amplified scFv restriction digested with *BstN*1 ***(11,12)***. This limits the number of clones we need to determine the K_D for, as many clones may be identical as demonstrated by identical *BstN*1 fingerprints obtained as described previously. This range of affinities can be determined on the yeast surface by measuring the amount of antigen bound at different concentration at equilibrium. The technique relies on measuring the mean fluorescence intensity (MFI) of the bound antigen, at a variety of concentrations of antigen, on the c-myc positive yeast. K_D is measured by determining at what concentration of antigen is half of the scFv on the surface of the yeast cell bound to antigen. Therefore measuring the MFI of the yeast when no antigen is bound and determining the concentration of antigen that gives the maximal MFI is needed. This is easily accomplished by setting up a series of antigen concentrations in which to label the yeast with and then measuring the MFI of the antigen binding population by flow cytometry. The MFI, obtained using flow cytometry with each of the antigen concentrations tested, is then plotted against the antigen concentration; using a nonlinear least-squares curve to fit the data, the K_D is determined (*see* **Figs. 6** and **7**).

Staining 10^5 yeast/antigen concentration represents approx 10^9–10^{10} antigen binding sites (scFv) in the sample. We assume 10^5 scFv/yeast and 50% express

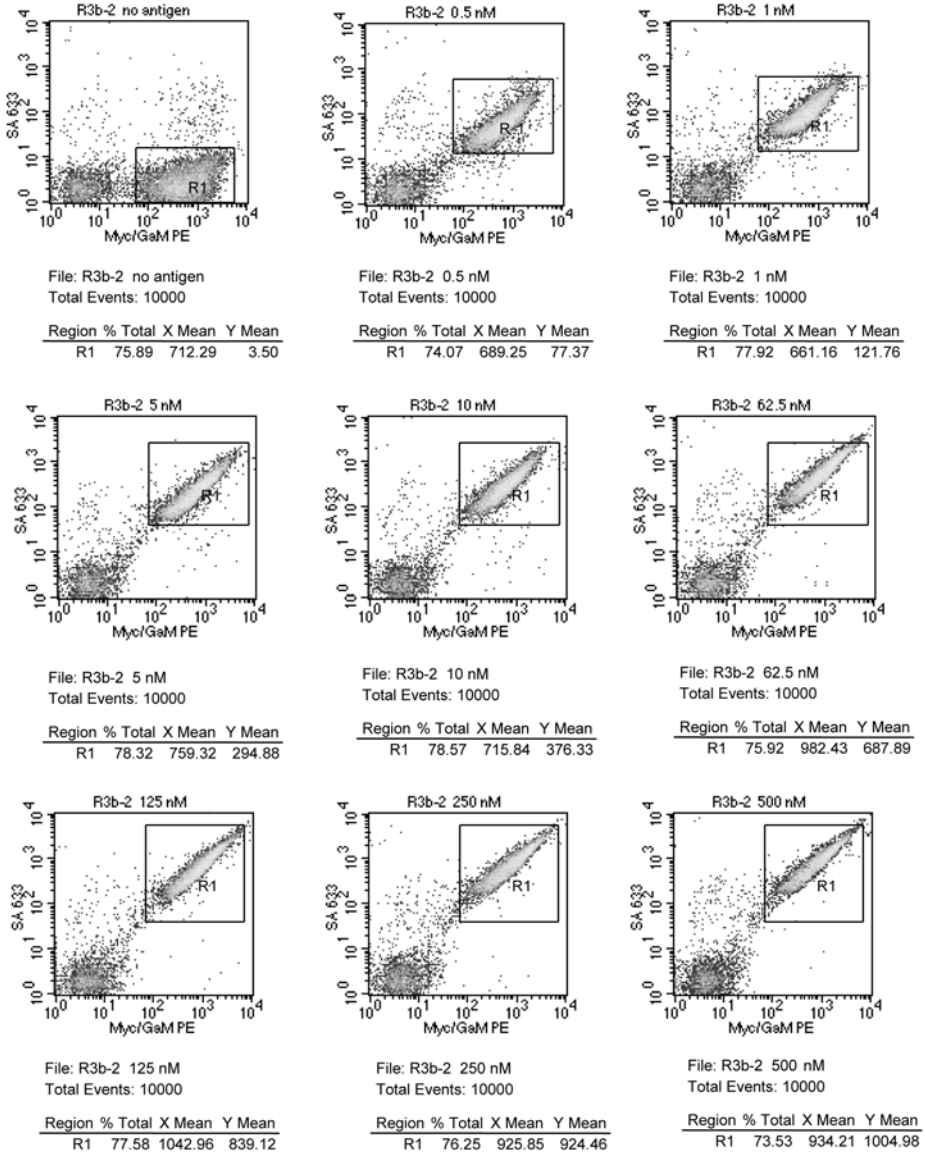
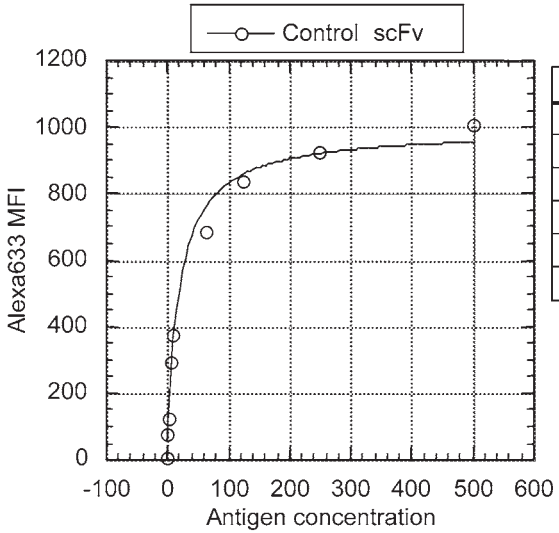


Fig. 6. K_D determination using analysis of MFI from bivariate plots obtained on a flow cytometer looking at c-myc expression (*x*-axis) and antigen binding (*y*-axis).



y = m1 + m2*M0/(m3+m0)		
	Value	Error
m1	59.065	30.611
m2	934.13	43.673
m3	21.096	5.2127
Chisq	14715	NA
R	0.99389	NA

Antigen concentration	Alexa633 MFI
0.0000	3.5000
0.50000	77.370
1.0000	121.70
5.0000	294.90
10.000	376.30
62.500	687.90
125.00	839.10
250.00	924.50
500.00	1005.0

Fig. 7. K_D determination using flow cytometry and nonlinear least-squares fit.

scFv. You want to maintain at least 10X excess of number of molecules of antigen over the number of scFv molecules. This is nondepleting ligand conditions.

We routinely prepare 12 clones using a 96-well microtiter plate format choosing eight concentrations of antigen, which include a "no-antigen" control for background.

1. Label 10^6 induced yeast with 100 µL of a 1:100 dilution on the c-myc MAb (200 µg/mL) for 30 min on ice.
2. Add 10 µL (10^5 yeast) directly into the antigen concentrations (*see* **step 3** below). This uses very little c-myc antibody, 1 µL/100 µL of 10^6 cells.
3. The following concentrations and volumes will allow for an accurate K_D to be measured for affinities between 1 and 100 n*M*. 10^5 yeast/sample = 5×10^9 scFv. If you have an idea of what your affinity is you can omit the first or last two antigen concentrations. For example, for a 100 n*M* affinity you do not need the 1 n*M* or 0.5 n*M* antigen concentrations. For a 5 n*M* affinity you do not need the 500 n*M*

or 100 n*M* concentrations. You are striving to do a series of two- to fivefold dilutions at 5–10X over K_D to 5–10X below K_D.

Concentration of antigen	Volume of antigen solution	No. of molecules of antigen
500 n*M*	100 µL	3×10^{13}
100 n*M*	100 µL	6×10^{12}
50 n*M*	100 µL	3×10^{12}
25 n*M*	100 µL	1.5×10^{12}
10 n*M*	100 µL	6×10^{11}
5 n*M*	100 µL	3×10^{11}
1 n*M*	500 µL	3×10^{11}
0.5 n*M*	500 µL	1.5×10^{11} = 30X excess
0.1 n*M*	1 mL	6×10^{10} = 10X excess

4. Incubate the samples at room temperature for 1 h. This allows ample time for the binding reaction to reach 90% equilibrium for antibody clones with off-rates in the 10^{-3}. An off-rate of 10^{-3} is what is generally seen for clones with 10 n*M* K_D, an affinity representing the majority of clones in the library. For clones with slower off-rates, 10^{-4} or 10^{-5}, 10- to 24-h incubations with antigen would be required to reach 90% equilibrium. These represent nanomolar and subnanomolar affinity antibodies.
5. Place samples on ice for 5 min. The decrease in temperature greatly decreases the off-rate. For a 25°C decrease in temperature, a 50- to 100-fold decrease in the off-rate is seen ***(13)***.
6. Wash the sample two times with 500 µL of ice-cold buffer.
7. Resuspend the sample in 100 µL of a 1:200 dilution of secondary reagents, GaM-Alexa488 and SA–PE.
8. Incubate on ice for 30 min. Pellet cells and wash one time with 500 µL of ice-cold buffer.
9. Resuspend in 500 µL of buffer and store on ice in dark until analyzed by flow cytometry.
10. Gather 10,000 events for a bivariate plot using the fluorescence channels that detect Alexa 488 (emission ~525 nm, usually FL1 on Becton Dickinson and Coulter brand flow cytometers) and phycoerythrin (emission 575 nm, usually FL2 on Becton Dickinson and Coulter brand flow cytometers).
11. Collect the statistical information from the dual c-myc+/antigen+ population.
12. Plot the MFI against the concentration of antigen and use a nonlinear least-squares to fit the curve (such as found in Kaleidgraph or GraphPad) and determine the K_D using the following equation:

$$y = m1 + m2^* \, m0/(m3 + m0)$$

where $m1$ = MFI of no antigen control, $m2$ = MFI at saturation, and $m3 = K_D$. If "R" values are generated, values of 0.998 and greater will give K_D values accurate within 30%.

3.6. Sorting of Mutagenic scFv Library for Isolation of Higher Affinity Clones

Boder et al. developed a methodology for generation and isolating higher affinity mutants of a specific scFv. This was demostrated by the maturation from nanomolar to femtomolar binding K_D for an scFv ***(4)***. The construction of a mutagenized library can be done several ways and is not covered here ***(6,14,15)***. However, there are several guidelines one should follow. The number of mutations per clone will affect the number of mutants that can display functional full length scFv. The number of mutations per clone will also affect the likelihood of finding a clone of increased affinity ***(5)***. A library of 10^6–10^7 different mutants can easily be screened using the methodology below.

1. Grow and induce library with a minimum of 10X coverage.
2. Stain no more than 10^8 cells (it takes ~3 h to sort at 10,000 events/s).
3. Label yeast with anti-c-myc to identify full-length scFv antibody clones. The mutagenesis will induce a larger number of stops and truncated proteins. At the same time, add antigen at the K_D of the parental clone. Incubate for 30 min at room temperature followed by 5 min on ice before pelleting and washing cells twice. Proceed to **step 4**.
   An alternative approach that is more productive for affinity maturation of clones that are already subnanomolar affinities is to select clones by a combination of fast on-rate and slow off-rate by the following steps:
   a. Label with biotinylated antigen for 1 h at 25°C. Do *not* label with anti-c-myc.
   b. After washing away unbound antigen, resuspend cells in 100 mL of 25°C wash buffer. Incubate sample at 25°C for 1–24 h (depending on off-rate of the parental antibody from which the library was made). Change buffer twice during incubation.
   c. Pellet cells and stain for c-myc, 30 min, 25°C. Wash three times.
   d. Resuspend cells in secondary reagents, GaM–Alexa 488 and SA–PE, 30 min, 4°C.
   e. Proceed to **step 5**.
4. Resuspend the cell pellet in secondary reagents. Incubate on ice in the dark for 30 min. Wash once and resuspend.
5. Run the sample on the sorter. Sort the entire sample for the brightest 1.0% of c-myc-positive antigen-binding population. Expect 5–20% of the cells to express c-myc-positive scFv, and of these 10–20% will bind antigen.
6. Cells will either be plated on selective plates that contain antibiotics or grown in selective liquid media (about 5 mL of SD + CAA). The antibiotics can be any or all of the following: pen/strep (10 mg/mL) used at 100 µg/mL or ampicillin or kanamycin (10 mg/mL) used at 10–20 µg/mL.
7. Plates are incubated at 30°C for 1–2 d. Colonies are pooled and then induced. Liquid media grown cells are pelleted and induced. An aliquot is generally frozen as well.

8. Therefore, from 10^7 cells you might sort out a total of 10^3–10^4 cells. Typically, 50% of the cells will form colonies or will be viable. For subsequent sorts, 70% of the cells will be c-myc-positive, with the vast majority being binding antigen.
9. After regrowth and induction of the yeast from the round 1 selection, 10^8 are labeled with antigen at a concentration of 0.5–0.1X the concentration at K_D. The addition of anti c-myc and the staining with secondary reagents is as described in **Subheading 3.5.1.**
10. Sort on the brightest 0.1% of antigen-binding c-myc positive clones. *Note:* The sublibraries overall K_D can be determined using an approach similar to that used for determining the K_D of an individual clone as outlined in **Subheading 3.5.2.** We will generally do our first sort with antigen concentration at K_D and then drop the concentration by 2- to 10-fold each subsequent sort.
11. Repeat **steps 6–10** for two additional rounds.
12. Individual clones can be assayed for K_D and sequencing of the insert can determine number of unique clones and where particular changes from the parental clone occur. Generally, by the fourth round of selection 80% of the clones in the sublibrary will be improved mutants (*see* **Note 8**).

3.7. Frequently Asked Questions (FAQS)

1. Some proteins we use seem to stick to all the yeast. Why?
   Answer: Before starting any selection we routinely check the protein of interest for binding to a single scFv clone that is not induced, the library not induced and the library in the induced state. You should see no labeling in any case. If there is labeling it could be binding to the yeast surface or the scFv in a nonspecific fashion. We have the most reproducible results and experience with peptides and secreted proteins. However, we have also obtained antigen specific scFv to a variety of cytoplasmic proteins. We recommend trying different selection buffers to decrease or eliminate the nonspecific binding. For example, 0.1% Tween–PBS, no EDTA, and so forth.
2. I have gone through five rounds of selection and all I got were secondary reagents binders or nothing. What's wrong?
   Answer: It could be a large number of possibilities. The most common are enumerated below.
   a. Did you check that the sorter was set up properly? Did the flow cytometer operator show you what was sorted by rerunning that sample or doing a test sort of labeled beads? Did you set up a dilution plate from the sorted cells? Was the number what you expected? We generally see about a 40–70% plating efficiency from the number of cells sorted.
   b. Do you know your antigen is biotinylated? How? We use the Pierce HABA methodology to determine moles of biotin per mole of antigen and strive for a 1–2 mol of biotin/mol of antigen.
   c. Try increasing the concentration of antigen up to 1 μM.
   d. There is also the possibility there are no binders to your protein (*see* **Note 9**).

3. Can I just use flow cytometric sorting or just use magnetic beads to get my antigen specific clones?
   Answer: Yes; however, we believe combining the two is the most powerful way to screen the complete diversity of the library ***(1)***. In our paper, HEL was isolated using the MACS system only.

4. Notes

1. YPED cultures saturate between 8 and 10 OD_{600}/mL, while SD + CAA cultures saturate between 3 and 5 OD_{600}/mL.
2. For 2X SD + CAA we add 10 g of casamino acids, 3.4 g of yeast nitrogen base, and 10.6 g of ammonium sulfate. This formulation can give greater cell numbers for growing the library up and freezing it down, generally yielding cell densities between 6 and 8 OD_{600}/mL.
3. Cell numbers are generally determined by OD_{600} reading on a spectrophotometer. We find about 2×10^7 cells/mL per OD_{600} unit. This should be determined by plating yeast on agar plates or using a hemacytometer to calibrate your spectrophotometer.
4. Fisher Scientific (Pittsburg, PA) supplies Pyrex brand "DeLong culture flask" with three baffles on flask bottom. It works best to increase aeration and growth. However, it is not essential.
5. This protocol is for staining up to 5×10^7 cells in 500 µL of wash buffer. To stain less yeast, decrease the volume proportionally, yet keep the reagent concentration the same. Most secondary reagents should be titered in your laboratory for the most appropriate dilutions. **Figure 4** show several combinations of secondary reagents that work well to label biotinylated antigens and anti-c-myc MAb.
6. It is important to use different secondary antigen-labeling reagents (streptavidin–Alexa 633 or neutravidin–PE) between flow cytometric sorts to eliminate or reduce secondary reagent-specific scFv. **Figure 4** shows several combinations of fluorescent reagents that can be used if secondary reagent binders are obvious.
7. Yeast do not grow very well in liquid culture when grown at low cell densities ($<10^4$/mL). Therefore, when we sort out <10,000 yeast, we generally will plate them.
8. It is often useful to run the original clone side by side with your new mutants to verify even slight differences in K_D, threefold differences in K_D are easily seen.
9. Note, we have never *not* obtained a streptavidin binder if we go through five rounds of selection.

References

1. Feldhaus, M. J., Siegel, R. W., Opresko, L. K., et al. (2003) Flow-cytometric isolation of human antibodies from a nonimmune Saccharomyces cerevisiae surface display library. *Nat. Biotechnol.* **21,** 163–170.
2. Boder, E. T. and Wittrup, K. D. (1998) Optimal screening of surface-displayed polypeptide libraries. *Biotechnol. Prog.* **14,** 55–62.

3. Boder, E. T. and Wittrup, K. D. (1997) Yeast surface display for screening combinatorial polypeptide libraries. *Nat. Biotechnol.* **15,** 553–557.
4. Boder, E. T., Midelfort, K. S., and Wittrup, K. D. (2000) Directed evolution of antibody fragments with monovalent femtomolar antigen-binding affinity. *Proc. Natl. Acad. Sci. USA* **97,** 10,701–10,705.
5. Daugherty, P. S., Chen, G., Iverson, B. L., and Georgiou, G. (2000) Quantitative analysis of the effect of the mutation frequency on the affinity maturation of single chain Fv antibodies. *Proc. Natl. Acad. Sci. USA* **97,** 2029–2034.
6. Stemmer, W. P. (1994) Rapid evolution of a protein in vitro by DNA shuffling. *Nature* **370,** 389–391.
7. Orr-Weaver, T. L. and Szostak, J. W. (1983) Yeast recombination: the association between double-strand gap repair and crossing-over. *Proc. Natl. Acad. Sci. USA* **80,** 4417–4421.
8. VanAntwerp, J. J. and Wittrup, K. D. (2000) Fine affinity discrimination by yeast surface display and flow cytometry. *Biotechnol. Prog.* **16,** 31–37.
9. (1993) *Saccharomyces cerevisiae*, in *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., and Struhl, K., eds.), John Wiley & Sons, Inc., New York.
10. (2002) Guide to Yeast Genetics and Molecular Cell Biology, in *Methods in Enzymology*, Vol. 350 (Guthrie, C. and Fink, G. R., eds.), Academic Press, San Diego.
11. Marks, J. D., Hoogenboom, H. R., Bonnert, T. P., McCafferty, J., Griffiths, A. D., and Winter, G. (1991) By-passing immunization. Human antibodies from V-gene libraries displayed on phage. *J. Mol. Biol.* **222,** 581–597.
12. Griffiths, A. D., Malmqvist, M., Marks, J. D., et al. (1993) Human anti-self antibodies with high specificity from phage display libraries. *EMBO J.* **12,** 725–735.
13. Mummert, M. E. and Voss, E. W. Jr. (1996) Transition-state theory and secondary forces in antigen-antibody complexes. *Biochemistry* **35,** 8187–8192.
14. Lorimer, I. A. and Pastan, I. (1995) Random recombination of antibody single chain Fv sequences after fragmentation with DNaseI in the presence of Mn2+. *Nucleic Acids Res.* **23,** 3067–3068.
15. Zhao, H. and Arnold, F. H. (1997) Optimization of DNA shuffling for high fidelity recombination. *Nucleic Acids Res.* **25,** 1307–1308.