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# Combining Phage and Yeast Cell Surface Antibody Display to Identify Novel Cell Type-Selective Internalizing Human Monoclonal Antibodies

**Scott Bidlingmaier**, **Yang Su**, and **Bin Liu**

## Abstract

Using phage antibody display, large libraries can be generated and screened to identify monoclonal antibodies with affinity for target antigens. However, while library size and diversity is an advantage of the phage display method, there is limited ability to quantitatively enrich for specific binding properties such as affinity. One way of overcoming this limitation is to combine the scale of phage display selections with the flexibility and quantitativeness of FACS-based yeast surface display selections. In this chapter we describe protocols for generating yeast surface antibody display libraries using phage antibody display selection outputs as starting material and FACS-based enrichment of target antigen-binding clones from these libraries. These methods should be widely applicable for the identification of monoclonal antibodies with specific binding properties.

### Keywords

Yeast surface antibody display; Phage antibody display; Human monoclonal antibody; scFv; FACS; Cell surface antigen; Internalization; Cell type-specific binding

## 1 Introduction

Phage antibody display libraries are widely used to generate monoclonal antibodies with affinity for target antigens, and purified recombinant protein antigens and living cells are frequently used as the selection targets for phage antibody display libraries [1–13]. Although very large libraries can be generated and screened in phage antibody display-based selections, it is difficult to fine-tune the selections to select for specific binding properties such as affinity. Additionally, while selection of phage antibody display libraries on living cells yields antibodies that bind to native epitopes on the cell surface, it is difficult to direct the selections towards a specific target. One method of addressing these limitations is to generate yeast antibody display libraries from pre-enriched phage antibody display outputs selected on recombinant antigens, live cells, or tissues [7, 10–13]. Yeast surface display [14] has been widely utilized to screen large libraries for proteins or protein fragments with specific binding properties [15–21], and protocols for the construction and application of yeast surface display libraries have been previously described [21–23]. Using labeled target antigens, yeast antibody display libraries can be coupled with FACS to select high affinity antibodies that bind specifically to the targeted antigen [24, 25]. Use of yeast antibody display combined with FACS allows a more controlled and quantitative selection process

that can be fine-tuned to enrich clones with particular binding properties more effectively than phage display. In this chapter we describe protocols for generating yeast single-chain variable fragment (scFv) display libraries by homologous recombination gap repair cloning using pre-enriched phage scFv display selection outputs as starting material, FACS-based enrichment of target antigen-binding clones from these libraries, and screening and sequencing analysis.

## 2 Materials

### 2.1 Generation of Phage-Derived Yeast scFv Display Library

1. Polyclonal phage scFv display selection outputs (*see* **Note 1**).
2. 2× YT: 16 g tryptone, 10 g yeast extract, 5 g NaCl, bring volume to 1 L with $ddH_2O$, adjust pH to 7.0, and sterilize by autoclaving.
3. 1,000× Tetracycline: 100 mg tetracycline, add 70 % ethanol to 10 mL, and store aliquots at −20 °C.
4. Plasmid spin miniprep kit.
5. Primers for gap repair transfer of scFvs from phage vector to yeast display vector: PhageYD1 Forward 5′-CGGGATCTGTACGACGATGACGATAAGGTACCAGGATCCAGTTTCTATGCGGCCCAGCCG-3′, PhageYD1 Reverse 5′-GAGAGGGTTAGGGATAGGCTTACCTTCGAAGGGCCCTCTAGATGCTAAACAACTTTCAACAGTTTC-3′ (*see* **Note 2**).
6. 10× PCR buffer.
7. dNTPs.
8. Taq polymerase.
9. *S. cerevisiae* strain EBY100 (MATa GAL1-*AGA1*: :URA3 ura3-52 trp1 leu2Δ1 his3Δ200 pep4: :HIS3 prb1Δ1.6R can1 GAL).
10. pYD1 or suitable yeast display vector.
11. EcoRI (*see* **Note 3**).
12. XhoI.
13. YPD: 10 g of yeast extract, 20 g of bacteriological peptone, 20 g dextrose, bring volume to 1 L with $ddH_2O$, and filter sterilize.
14. 1 M Lithium acetate: 33 g lithium acetate, bring volume to 500 mL with $ddH_2O$, and filter sterilize.
15. Electroporation solution (1 M Sorbitol/1 mM $CaCl_2$): 91 g D-Sorbitol, 500 μL 1 M $CaCl_2$; bring volume to 500 μL with $ddH_2O$ and filter sterilize (*see* **Note 4**).
16. 2-Mercaptoethanol.
17. 2 mm Electroporation cuvettes.

18. Electroporator (*see* **Note 5**).
19. 2× SR-CAA yeast growth media: 20 g raffinose, 14 g yeast nitrogen base, 10 g bacto casamino acids, 5.4 g $Na_2HPO_4$, 7.4 g $NaH_2PO_4$, bring volume to 1 L with $ddH_2O$, and filter sterilize.
20. 10× SD-CAA for making plates: 70 g yeast nitrogen base w/o amino acids, 50 g bacto casamino acids, 100 g dextrose; bring the volume to 500 mL with $ddH_2O$ and filter sterilize.
21. SD-CAA plates: 5.4 g $Na_2HPO_2$, 7.4 g $NaH_2PO_4$, 17 g agar; bring the volume to 900 mL with $ddH_2O$, autoclave to sterilize, let the agar cool until it is cool enough to touch, add 100 mL 10× SD-CAA, mix and pour into plates (100 or 150 mm) and allow to cool at RT, and store the plates at 4 °C until ready to use.

### 2.2 Enrichment of Target Antigen-Binding Clones by FACS

1. Yeast scFv display library generated in previous section.
2. 20 % Galactose: 100 g galactose; bring the volume to 500 mL with $ddH_2O$ and filter sterilize.
3. PBS: 8 g of NaCl, 0.2 g of KCl, 1.44 g of $Na_2HPO_4$, 0.24 g of $KH_2PO_4$, bring the volume to 1 L with $ddH_2O$, adjust pH to 7.4, and sterilize by autoclaving or filtration.
4. Biotinylated or fluorescently labeled target molecules (*see* **Note 6**).
5. Mouse anti-V5.
6. Goat anti-mouse-488.
7. Streptavidin-PE (SA-PE).
8. Streptavidin-488 (SA-488).

### 2.3 Screening and Sequencing of Binding Clones

1. pYD1-specific primers: pYD1 Forward 5′-AGTAACGTTTGTCAGTAATTGC-3′, pYD1 Reverse 5′-GTCGATTTTGTTACATCTACAC-3′ (*see* **Note 7**).
2. Primer for sequencing pYD1: Gap5 5′-TTAAGCTTCTGCAGGCTAGTG-3′ (*see* **Note 7**).

## 3 Methods

The methods detailed below assume that a pre-enriched (either on recombinant antigen or cells) scFv phage display library has been previously generated. It is also assumed that a biotinylated or fluorescently labeled target antigen is available. The methods below are divided into five categories: Generation of Yeast scFv Display Library from Phage Antibody Selection Outputs (Subheading 3.1); Enrichment of Target-binding Clones from Yeast scFv Display Library by FACS (Subheading 3.2); Screening and Sequencing of Binding Clones (Subheading 3.3). The yeast electroporation protocol is adapted from a previously described

protocol [26, 27]. An outline of the selection strategy is shown in Fig. 1 and an example of FACS data from a selection is presented in Fig. 2.

### 3.1 Generation of Yeast scFv Display Library from Phage Antibody Selection Outputs

1. Prepare DNA minipreps from scFv phage display library selection for use as PCR template using standard methods.
2. Set up standard PCR reactions using PhageYD1 Forward and PhageYD1 Reverse primers and 1 ng of the appropriate scFv phage display library selection output plasmid prep as template for each 50 μL reaction. Set up enough reactions to generate 20 μg PCR product.
3. Run PCR reactions on 1.5 % agarose gel, cut out bands (approximately 800 bp) with a clean razor blade, and isolate PCR products using Qiagen gel isolation kit or comparable method following manufacturer's protocols. Elute in $ddH_2O$ and measure concentration by spectrophotometer. If the yield is less than 20 μg, repeat the process until enough PCR product has been purified.
4. Digest 10 μg pYD1 with EcoRI and XhoI (*see* **Note 8**). Run digest on 0.75 % agarose gel, cut out large vector band with clean razor blade, and isolate fragment using Qiagen gel isolation kit or comparable method following manufacturer's protocols. Elute in $ddH_2O$ and measure concentration by spectrophotometer.
5. Inoculate a 5 mL YPD culture with EBY100 and grow overnight with shaking at 30 °C and 200 rpm.
6. Determine concentration of the overnight yeast culture by measuring the $OD_{600}$ of a 1:20 dilution using a spectrophotometer (1 $OD_{600}$ = 2 × $10^7$/mL). Use the overnight culture to inoculate a culture in YPD (the inoculation volume should be at least 50 mL/transformation) at 0.5 $OD_{600}$ and incubate at 30 °C, 200 rpm for 5–6 h (at least two cell divisions).
7. Pellet yeast cells by centrifugation at 1,000 × *g* for 3 min and remove media.
8. Wash yeast pellet twice with 15 mL ice-cold $ddH_2O$ and once with 15 mL electroporation solution.
9. After the electroporation solution wash, resuspend the cell pellet in 20 mL 0.1 M Lithium acetate and add 130 μL 2-mercaptoethanol. Shake at 225 rpm for 30 min at 30 °C (*see* **Note 9**).
10. Pellet the yeast cells by centrifugation, wash once with 15 mL ice-cold electroporation solution, and resuspend the cell pellet in electroporation solution to a final volume of 500 μL for each 50 mL of original culture volume and keep on ice until electroporation.
11. For each electroporation, add 3 μg linearized vector and 9 μg scFv PCR product insert to 400 μL conditioned yeast cells in electroporation solution and mix gently (*see* **Note 10**).

12. Transfer electroporation reaction to a pre-chilled 2 mm electroporation cuvette and keep on ice until electroporation.
13. Electroporate the cells at 2,500 V and 25 μF (*see* **Note 11**).
14. Transfer each electroporation reaction to 8 mL of recovery medium (1:1 mix of electroporation solution and YPD) and shake at 225 rpm for 1 h at 30 °C.
15. Pellet electroporated cells by centrifugation and resuspend in 1 mL 2× SR-CAA. Add 1 μL to 1 mL 2× SR-CAA and plate 10 μL on a 150 mm SD-CAA plate for titering. Plate the rest of the transformants on 150 mm SD-CAA plates at 200 μL/plate.
16. Incubate the plates inverted at 30 °C for 3–4 days until colonies grow. Count the number of colonies on the titer plate and estimate the number of transformants by multiplying by $10^5$ (*see* **Note 12**).
17. Recover the library from plates by adding 5 mL of 2× SR-CAA to each plate and scraping with a flame-sterilized spreader and then collect the resuspended cells by pipetting. If there are any minor contaminating bacteria or fungus on the plates, it should be excised with a flame-sterilized scalpel prior to resuspending the cells. Determine cell number by taking an $OD_{600}$ reading of a 1:50-diluted sample of the resuspended cells (1 $OD_{600}$ approximately equals $2 \times 10^7$ yeast cells/mL). Pool transformants in a 50-mL conical using a pipetman. To prepare freezer stocks, mix 5 mL of sterile 50 % glycerol with 5 mL of the collected transformants, pipet 1 mL aliquots into cryotubes, and store at −80 °C.

### 3.2 Selection of Yeast scFv Display Library by FACS

1. Using the resuspended library, start a 100 mL culture at $OD_{600}$ 0.2 in 2× SR-CAA + 2 % galactose. Grow at 30 °C with shaking overnight. The induced library can be stored for several weeks at 4 °C.
2. Check induction of the library by FACS using the mouse anti-V5 antibody. Spin down 100 μL of the induced library at 10,000 × *g* and wash twice with 1 mL PBS. Resuspend cells in 500 μL PBS and add 1 μL mouse anti-V5 antibody. Incubate at RT with rotation for 1 h. Wash three times with 1 mL PBS. Resuspend in 500 μL PBS and add 1 μL goat anti-mouse PE. Incubate at RT with rotation for 30 min. Wash three times with PBS, resuspend in 500 μL PBS, and place on ice.
3. Analyze the cells by FACS. At least 40 % of the population should be V5-positive (*see* **Note 13**). The induced library is now ready for FACS selection experiments.
4. Check the $OD_{600}$ of a 1:10 dilution of the induced library to determine cell density. For the first round of sorting, add $10^8$ yeast cells to six 1.5 mL Eppendorf tubes and wash them twice with 1 mL PBS. For example, if the calculated $OD_{600}$ of the undiluted induced library is 5, then 1 mL of culture will contain approximately $10^8$ cells.
5. Resuspend the yeast in 500 μL PBS containing 200, 100, 50, 25, 12.5, and 0 nM biotinylated target antigen (*see* **Note 14**). Incubate for 1 h at 25 °C.

6. Wash each of the incubations twice with PBS and incubate with 500 μL of 1:500 diluted SA-PE (*see* **Note 15**) for 10 min at 25 °C. Wash the cells three times with PBS and resuspend each reaction in 3 mL PBS. Keep the cells on ice in the dark until sorting.

7. Analyze all six reactions by FACS. Compare the signal from the different target antigen concentrations to the negative control with no target antigen. If high affinity antibodies are desired, choose the lowest concentration of target antigen that gives significantly more signal than the negative control for sorting. If preservation of diversity is a priority, a higher concentration incubation should be sorted to reduce the stringency. Placement of sort gate can be similarly modulated to suit the desired stringency, but gating should not be extremely stringent in the first round. Analyze at least $5 \times 10^7$ cells from the chosen target antigen selection incubation and sort PE-positive cells into an Eppendorf tube containing 100 μL 2× SR-CAA. Make a note of the total number of cells analyzed and the number sorted.

8. Plate the sorted cells on one or several large pre-dried SD-CAA plates by gently spreading. Incubate the plates in an inverted position at 30 °C until colonies are formed (3–4 days).

9. Add 3 mL 2× SR-CAA to the plate(s) and recover the cells by scraping with a sterile cell spreader. To prepare a freezer stock, add 500 μL of 50 % glycerol to 500 μL cells in a cryotube and store at −80 °C.

10. To induce the first-round output, inoculate a 10 mL culture at 0.5 $OD_{600}$ in 2× SR-CAA + 2 % galactose using the remaining cells from the round-one output and grow at 30 °C with shaking overnight.

11. Wash approximately $5 \times 10^7$ cells from the induced first-round output with PBS. Set up control and selection incubations using the biotinylated target antigen in the same manner as the first round and incubate for 1 h at 25 °C. If enrichment for higher affinity antibodies is desired, the target antigen concentration range can be lowered.

12. Wash the cells twice with PBS and incubate with 500 mL of 1:500 diluted SA-488 for 10 min at 25 °C. Alternating use of different secondary detection agents is suggested to minimize the chance of enriching antibody clones that may have some affinity for the secondary reagents. Wash the cells three times with PBS and resuspend in 1 mL PBS. Keep the cells on ice in the dark until sorting.

13. Analyze the reactions by FACS and again compare the signal from the different target antigen concentrations to the negative control with no target antigen. Choose the incubation that best suits your objectives for sorting. Analyze at least $10^7$ cells from the chosen target antigen selection incubation and sort 488-positive cells into an Eppendorf tube containing 100 μL 2× SR-CAA. Again, make a note of the total number of cells analyzed and the number sorted, and plate the sorted cells on one or several large SD-CAA plates. If the output is ready to be screened at this point, some plates can be seeded at a lower density (500 cells/plate) based on the sorting

data to facilitate the picking of individual colonies for screening. Incubate at 30 °C until colonies form.

14. Add 3 mL SR-CAA to plate(s) and recover cells by scraping. Prepare a frozen stock as previously described and store at −80 °C. Induce the second-round (if more rounds of sorting are necessary) output by inoculating a 10-mL culture at 0.5 $OD_{600}$ in SR-CAA + 2 % galactose using the remaining cells and grow at 25 °C with shaking overnight.

15. Prepare incubations as described for the previous rounds, reducing the biotinylated target antigen concentration as appropriate if enrichment for higher affinity antibodies is desired. Use a secondary detection reagent different from the one used in the previous round and analyze and sort as described for the second round. Plate the sorted cells on one or several large SD-CAA plates. If the intention is to screen individual clones from this sort output, some plates can be seeded at a lower density (500 cells/plate) for screening. After the colonies have grown, make a glycerol freezer stock of the third-round sort output as described previously (*see* **Note 16**).

### 3.3 Screening and Sequencing of Binding Clones

1. Inoculate 2 mL 2× SR-CAA + 2 % galactose cultures with a yeast colony from the SD-CAA sort output plates and grow for at least 16 h with shaking at 30 °C (*see* **Note 17**).

2. Pellet 200 μL of the overnight cultures using a tabletop microfuge and wash twice with 1 mL PBS.

3. Incubate yeast for 1 h at room temperature with 100 μL of an appropriate concentration of biotinylated or fluorescently labeled target molecule and mouse anti-V5 antibody (1/1,000 dilution of stock solution) in PBS.

4. Wash yeast twice with 1 mL PBS and incubate with 100 μl 1/1,000 SA-PE and 1/1,000 Alexa fluor 647-conjugated anti-mouse secondary antibody in PBS for 20 min at room temperature.

5. Wash twice with 1 mL PBS and analyze the yeast by FACS. Plate small patches of binding clones on SD-CAA plates and incubate overnight at 30 °C.

6. The scFv sequences of the binding clones can be determined by colony PCR followed by sequencing of the PCR products (*see* **Note 18**). From each patch, scrape about 5 μL of yeast with a sterile pipet tip and spread in the bottom of a PCR tube. Cap the tubes and microwave on high for 2 min. Prepare a PCR master mix as follows:

| | |
|---|---|
| 10× buffer | 2.5 μL/reaction |
| dNTPs | Final concentration 200 μM each |
| Taq | 1 μL/reaction |
| pYD1 Forward and pYD1 Reverse primers | Final concentration 0.5 μM each |
| $ddH_2O$ | Up to 25 μL/reaction |

Add 25 μL PCR mix on top of microwaved yeast cells and run the following PCR cycle (*see* **Note 19**):

| | |
|---|---|
| 3 min at 95 °C | |
| 30 s at 95 °C | |
| 30 s at 51 °C | ×30 cycles |
| 90 s at 72 °C | |
| 5 min at 71 °C | |

7. Run the PCR reactions on an agarose gel, excise bands (should be approximately 1,200 bp) with a clean razor blade, and purify using the Qiagen gel isolation kit or other suitable protocol. Sequence the purified PCR products using the Gap5 primer.

## 4 Notes

1. We have used polyclonal phage scFv display selection outputs enriched for binding to living cells and recombinant protein target antigens as starting material for making yeast scFv display libraries. Our protocols are designed for phage scFv display libraries in the Fd vector [7, 28]. Before transfer to yeast, it is necessary to verify that the phage selection was successful by testing the polyclonal selection output for binding to the target (cells or purified target antigen). Protocols for the generation and use of phage scFv display libraries to select on purified target antigens, living cells, and tissues have been described previously [6, 10, 29–32].
2. These primers were designed to be compatible with the Fd phage display vector and the pYD1 yeast display vector. If other vectors will be used, then new primers will need to be designed. Gap repair cloning in yeast works most efficiently when greater than 40 base pairs of homology with the linearized vector are designed into the primer. Care must be taken when designing primers to ensure that the scFv is cloned in frame with the phage protein.
3. The EcoRI and XhoI restriction sites are designed for use with the pYD1 yeast display vector. If a different or modified vector is used, then different enzymes may have to be chosen. The sites chosen for linearizing the vector must be compatible with the scFv PCR gap repair primers.
4. Gentle heating may be required to help the sorbitol go into solution.
5. E.g., Eppendorf 2510 or Bio-Rad Gene Pulser II
6. We usually use biotinylated target antigens. We typically use the EZ-Link Sulfo-NHS-Biotin reagent for biotinylating purified recombinant protein targets. If possible some form of quality control (binding or activity) should be performed on the labeled target antigen.
7. Different primers may need to be designed if a vector other than pYD1 was utilized.
8. Two enzymes are used to minimize the uncut vector background.

9. Shaking can be done in a small flask or 50 mL conical.

10. In order to not dilute the electroporation reaction, the total DNA volume should be less than 50 μL. If the DNA concentration is not high enough to achieve this, it will need to be concentrated using a suitable method.

11. The time constant should be 3–5 ms, although we have observed lower time constants that still resulted in acceptable transfection efficiencies. If "popping" occurs, sharply tap the electroporation cuvette on a hard horizontal surface several times to eliminate air bubbles and repeat the electroporation. We use an Eppendorf 2510 electroporator. Other electroporators should work similarly using equivalent settings.

12. In our hands the transformation efficiency usually varies between 0.5 and $2 \times 10^7$ colonies/transformation, which is more than sufficient for the generation of yeast scFv display libraries from pre-enriched phage scFv display selection outputs. The number of transformations can be scaled up to suit the desired library size.

13. The described strategy generates a c-terminal V5 epitope tag on the yeast-displayed scFv. There is always a display-negative population after induction when analyzed by FACS, and the maximum induction will vary from experiment to experiment. This is seen even when using pYD1-transformed EBY100 as a positive control.

14. We have provided a range that based on our experience usually covers most libraries generated from phage scFv display selection outputs. This range may need to be adjusted depending on the binding activity of different libraries.

15. Other fluorophore combinations can be used to match the capabilities of your FACS instrument.

16. Two or three rounds usually provide sufficient enrichment for monoclonal screening. More rounds of sorting can be done, but are likely to greatly reduce the diversity of the output.

17. The yeast can also be induced in 96-well plates for screening. We use round bottom polystyrene plates, inoculate 200 μL/well, and shake on a platform shaker at 120 rpm.

18. Alternatively the plasmids can be rescued into bacteria, miniprepped, and sequenced. A protocol for this has been described by us previously [23].

19. Although we have had good success with the simple microwave method, yeast colony PCR can be temperamental. Yeast minipreps followed by plasmid rescue into bacteria, miniprepping, and sequencing is an alternative method (*see* **Note 18**). This method also has the advantage of providing you with the plasmid containing the scFv, which can be used in downstream procedures such as PCR or cloning. We routinely use the colony PCR method for rapid screening and then rescue the plasmids from clones we wish to do additional work with.

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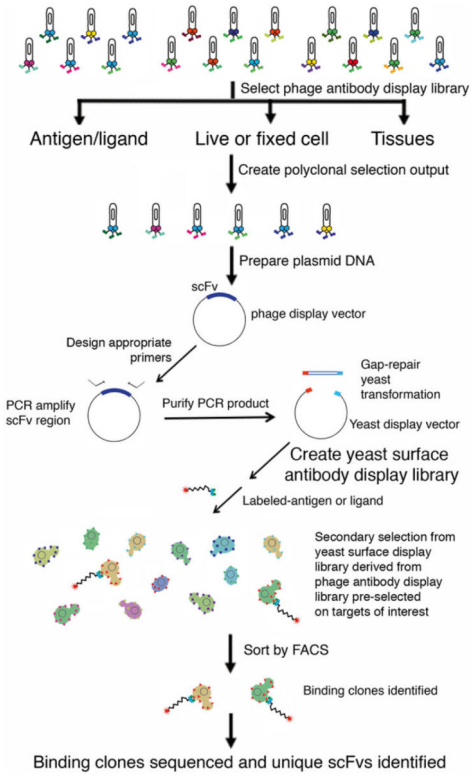


**Fig. 1.**

Outline of the combination selection strategy utilizing both phage and yeast surface antibody display. Naïve phage antibody display libraries will be first selected on target antigens or ligands, live or fixed cells, or tissues (fresh, frozen, or formalin-fixed paraffin-embedded). Selection condition can be manipulated to enrich for desired antibody property such as internalization. Following two to three rounds of selection, plasmid DNA from the polyclonal phage output will be purified and used as template to amplify the scFv genes with flanking primers designed for gap-repair-based yeast transformation (*see* text for details). The resulting yeast antibody display libraries will be subjected to FACS-based selection against fluorescence-labeled antigens or ligands to identify high affinity binders. The figure is adapted from our original drawings in [13] and [16]

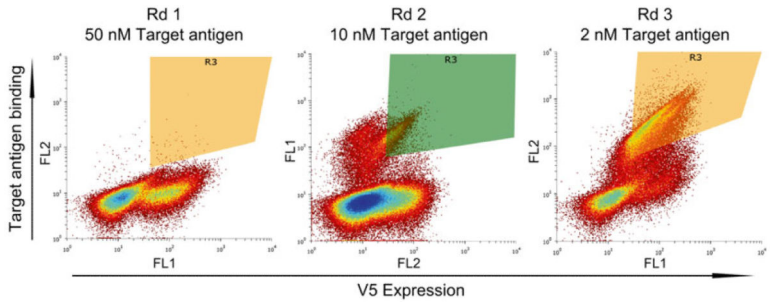


**Fig. 2.**

Example selection of target antigen-binding scFv clones from a yeast surface scFv display library by FACS. The yeast scFv display library was generated from polyclonal phage scFv display selection outputs enriched for binding to epitopes expressed on the surface of living cells. The yeast scFv display library was induced, incubated with biotinylated target antigen, and subjected to FACS as described in the methods. Enrichment of target antigen-binding scFv clones was achieved through three rounds of FACS with decreasing concentrations of biotinylated target antigen. The *vertical axis* represents target antigen binding, while the *horizontal axis* represents display of the V5 epitope tag. The *shaded regions* (R3) are the sorted populations for each round. Secondary detection reagents were alternated between PE (FL2) and FITC (FL1). Screening of the third-round output yielded clones with sub-nanomolar affinity for the target antigen