Engineering Antibody Affinity by Yeast Surface Display

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Running Title: Antibody Engineering by Yeast Display

Introduction

Yeast surface display (YSD) is a powerful tool for engineering the affinity, specificity, and stability of antibodies, as well as other proteins. Since first described six years ago by Boder and Wittrup,[1] YSD has been employed successfully in engineering a number of antibodies,[2,3] as well as T-cell receptors.[4-6] A recently reported large non-immune single chain antibody library is a good starting point for engineering high affinity antibodies.[7] Cloned variable genes from hybridomas or scFvs or Fabs from phage display libraries are also easily incorporated into a yeast display format. The original YSD protocols were described earlier,[8] but new and refined methods have been developed, in particular improved vectors, mutagenesis methods, and efficient ligation-free yeast transformation procedures. In this chapter, we provide up to date protocols for engineering single chain antibodies by YSD.

Compared to other display formats, yeast surface display offers several advantages. One chief advantage to engineering protein affinity by YSD is that yeast cells can be sorted by Fluorescence Activated Cell Sorting (FACS), allowing quantitative discrimination between mutants.[9] Further, FACS simultaneously gives analysis data, eliminating the need for separate steps of expression & analysis after each round of sorting. Without exception to date, equilibrium binding constants and dissociation rate constants measured for yeast-displayed proteins are in quantitative agreement with those measured for the same proteins *in vitro* by BIAcore or ELISA. Traditional panning methods have also been employed successfully with YSD, including magnetic particle separation.[10] Other advantages

arising from the yeast system include ease of use and presence of the yeast endoplasmic reticulum, which acts as a quality control mechanism and ensures that only properly folded proteins reach the cell surface.

This chapter contains methods for displaying an antibody on yeast, creating mutant libraries, and sorting libraries for improved clones. The constructs and strains required for yeast surface display are described in the first section. The next section contains the method for creating large mutant libraries using homologous recombination, including the precise conditions used for error prone PCR using nucleotide analogues. Finally we include protocols for labeling yeast with fluorophores and sorting by FACS for improved affinity.

The Yeast Surface Display System

As the name implies, yeast surface display involves the expression of a protein of interest on the yeast cell wall, where it can interact with proteins and small molecules in solution. The protein is expressed as a fusion to the Aga2p mating agglutinin protein, which is in turn linked by two disulfide bonds to the Aga1p protein covalently linked to the cell wall (Fig. 1). Expression of both the Aga2p-antibody fusion and Aga1p are under the control of the galactose-inducible GAL1 promoter, which allows inducible over-expression.

In order to use YSD, one must construct a yeast shuttle plasmid with the single-chain antibody of interest fused to Aga2p. This can be derived from the pCTCON vector (Fig. 2) by inserting the open reading frame of the scFv of interest between the NheI and BamHI sites (both of which should be

in frame with the antibody). The yeast strain used must have the Aga1 gene stably integrated under the control of a galactose inducible promoter. EBY100 (Invitrogen) or one of its derivatives are suggested.

Generating Large Mutant Antibody Libraries in Yeast

The most efficient way to make a mutant library in yeast is to use homologous recombination, thereby eliminating the need for ligation and *E.coli* transformation.[11] In brief, cut plasmid and an insert containing the mutated gene are prepared separately, with significant homology (30-50 bp or more) shared by the insert and plasmid at each end. These DNA fragments are then taken up by yeast during electroporation, and re-assembled *in vivo*. Libraries prepared by this method typically include at least 10^7 transformants, and are often over 10^8 in diversity, which approximates the amount that can be sorted by state of the art cell sorters in an hour.

In the following section we describe how to prepare scFv insert DNA with random point mutations by error prone PCR with nucleotide analogues. However, this may be replaced with DNA shuffling with slight modification using one of many published protocols.[12-14]

Preparation of Insert: Error Prone PCR using Nucleotide Analogues

Nucleotide analog mutagenesis allows the frequency of mutation to be tuned based on the number of PCR cycles and the relative concentration of the mutagenic analogues.[15,16] The two analogues, 8-oxo-2'-

deoxyguanosine-5'-triphosphate and 2'-deoxy-p-nucleoside-5'-triphosphate (8-oxo-dGTP and dPTP respectively, TriLink Biotech), create both transition and transversion mutations. In order to ensure that some fraction of the library created is sufficiently mutated to generate improvements, but not so highly mutated as to completely ablate binding, a range of several different mutagenesis levels are used in parallel. The conditions reported here are the ones we typically use to create antibody libraries; these conditions give an error rate ranging from 0.2%-5%.

If the gene to be mutated is already in pCTCON, then the following primers may be used to carry out the mutagenesis and subsequent amplification. These primers are designed to have >50 bp of homology to pCTCON for use during homologous recombination.

Forward primer: cgacgattgaaggtagatacccatacgacgttccagactacgctctgcag

Reverse primer: cagatctcgagctattacaagtcttcttcagaaataagcttttgttc

1. Set up six 50 μl PCR reactions

	Final Concentration
10X PCR Buffer (without $MgCl_2$)	
$MgCl_2$	2 mM
Forward Primer	0.5 μM
Reverse Primer	0.5 μM
dNTP's	200 μM
Template	0.1-1 ng
8-oxo-dGTP	2-200 μM
dPTP	2-200 μM
dH20	to final volume

Taq polymerase	2.5 units

Of the six PCR reactions, two should contain 200 μM nucleotide analogues, two should contain 20 μM nucleotide analogues, and two should contain 2 μM nucleotide analogues.

2. Run the PCR for the number of cycles specified below. The cycles should have the following incubation temperatures and times: denature at 94 °C for 45 sec, anneal at 55 °C for 30 sec, extend at 72 °C for 1 min. One should also include a 3 min denaturation step at 94 °C before the cycles begin and a 10 min extension step after the cycles are completed (the 10 min extension may be done on a heating block to run all reactions simultaneously).

Nucleotide Analogue Concentration	Number of PCR cycles
200 μM	5
200 μM	10
20 μM	10
20 μM	20
2 μM	10
2 μM	20

3. Run the entire mutagenic PCR products out on a 1% low melt agarose gel. PCR products cycled 20 times are easily visible on a gel stained with SYBR Gold (Molecular Probes). Reactions cycled 10 times or less may not be visible on the gel; however, it is important to gel purify

anyway to remove the non-mutated template before amplification (next step). Cut out and purify using Qiagen gel purification kit following manufacturer's protocol.

4. Amplify each reaction in the absence of nucleotide analogues to generate sufficient insert DNA for the transformation. Three 100 µl reactions should be set up for each mutagenic reaction, and 1 µl or more of the gel purified product should be used as template in the new reaction. Do not add nucleotide analogues. Cycle 25-30 times as you would for a normal PCR.

5. Optional. Gel purify the PCR products from step 4. Purification will eliminate many PCR artifacts from the library, but may also result in significant loss of PCR product.

6. Concentrate the PCR products using Pellet Paint (Novagen). After pellet has dried, dissolve in water to a final concentration of 5 µg/µl. This protocol typically produces 40-100 µg of PCR product.

Preparation of Vector

1. Miniprep 10 µg or more of pCTCON.

2. Digest with NheI (New England Biolabs) for at least two hours in NEB2 buffer.

3. Adjust salt concentration by adding one-tenth of the total volume of 1 M NaCl.

4. Double digest with BamHI and SalI for two additional hours, to ensure complete digestion of pCTCON and reduce reclosure of the acceptor

vector. (Note that the plasmid is cut in three places to ensure that the vector will not transform yeast cells in the absence of insert.)

5. Use Qiagen nucleotide removal kit to purify DNA from enzymes, keeping in mind that a single column saturates with 10 μg DNA.

6. Concentrate DNA using Paint Pellet reagent. After drying pellet, dissolve in water to 2 μg/μl.

Preparation of Electrocompetent Yeast Cells

This protocol has been adapted from E. Meilhoc et. al.,[17] and generates enough cells for transformation of ~60 μg of insert DNA and ~6 μg of vector, which typically produces ~5 x 10^7 yeast transformants.

1. Inoculate 100 mL of YPD to OD_{600} 0.1 from a fresh overnight culture of EBY100 (or appropriate yeast strain).

2. Grow cells with vigorous shaking at 30ºC to an OD_{600} of 1.3 - 1.5 (about 6 hours).

3. Add 1 mL filter sterilized 1,4-dithiothreitol (DTT, Mallinckrodt) solution (1 M tris, pH 8.0, 2.5 M DTT). DTT is unstable and the solution must be made fresh just before use. Continue to grow with shaking at 30ºC for 20 min.

4. Harvest cells at 3500 rpm, 5 min, 4ºC. Discard supernatant. All centrifugation steps should be carried out in autoclaved centrifuge tubes or sterile Falcon tubes.

5. Wash with 25 mL of E buffer (10 mM tris, pH 7.5, 270 mM sucrose, 1 mM $MgCl_2$) at room temperature. Spin down again.

6. Transfer to two 1.5 mL microcentrifuge tubes and wash a second time with 1 mL of E buffer each. Spin down.

7. Resuspend both pellets in E buffer to a final combined volume of 300 μl. Any extra cells that will not be used immediately may be frozen down in 50 μl aliquots for future use. Note that using frozen cells results in a 3-10-fold loss in transformation efficiency.

Electroporation

Electroporation is carried out using a Biorad Gene Pulser device.

1. In a microcentrifuge tube, mix 0.5 μl vector (1 μg), 4.5 μL insert (9 μg), and 50 μL electrocompetent yeast cells. Add the mixture to a sterile 0.2 cm electroporation cuvette (Biorad). Incubate on ice 5 min. Prepare additional cuvettes until all of the DNA is used.

2. Set Gene pulser settings to 25 μF (capacitance) and 0.54 kV (voltage), which gives an electric field strength of 2.7 kV/cm with 0.2 cm cuvettes; time constant should be about 18 ms with 55 μl volumes. The pulse controller accessory is not used.

3. Carry out pulsing at room temperature. Insert cuvette into slide chamber. Push both red buttons simultaneously until pulsing tone is heard, then release.

4. After pulsing, immediately add 1 mL of room temperature YPD media[8] to the cuvette. Incubate at 30 ºC for 1 hour in 15 mL round bottom falcon tubes with shaking (250 rpm).

5. Spin down cells at 3500 rpm in a microcentrifuge. Resuspend in selective media (SD+CAA, [8] 50 mL/electroporation reaction). Plate out serial 10-fold dilutions to determine transformation efficiency. The library may be propagated directly in liquid culture without significant bias, due to repression of scFv expression in glucose-containing medium such as SD+CAA.[7]

Transformation efficiency should be at least 10^5/μg, but is typically around 10^6/μg. In addition to the electroporation mixture described here, one should perform a control where no insert is added and determine the transformation efficiency. This is the background efficiency and should be less than ~1% of that obtained in the presence of insert DNA.

Equilibrium Labeling Protocol

Labeling yeast that are displaying an antibody or antibody library with a fluorescent or biotinylated antigen allows quantification of binding affinity and enables library sorting by FACS. Typically a second fluorophore conjugated to an antibody is used to detect the epitope tag C-terminal to the scFv, which allows for normalization of expression and eliminates non-displaying yeast from quantification. A short protocol follows for labeling with a biotinylated antigen and the 9E10 monoclonal antibody against the C-terminal epitope tag c-myc. This protocol is for

analytical labeling; for labeling large libraries, adjust volumes as describe at the end of the protocol.

1. Grow transformed yeast overnight in SD+CAA. OD_{600} should be greater than one. As a general approximation, OD_{600} =1 represents 10^7 cells/mL.

2. Inoculate a 5 mL culture of SG+CAA[8] (inducing media) with the overnight culture. The final OD_{600} of the new culture should be ~1.

3. Induce at 20 °C with shaking (250 rpm) for at least 18 hrs. Appropriate induction temperature should be tested for each scFv, from 20, 25, 30, or 37 °C.

4. Collect 0.2 OD_{600}-mL of induced yeast in a 1.5 mL microcentrifuge tube. Several such aliquots may be necessary to sample the full diversity of the library, since this aliquot will correspond to approximately 2 x 10^6 cells.

5. Spin down in table top centrifuge for 30 sec at max speed. Discard supernatant.

6. Rinse with PBS/BSA (phosphate buffered saline plus 0.1% BSA). Centrifuge for 10 sec, discard supernatant.

7. Incubate with primary reagents. Add desired concentration of biotinylated antigen and 1 μL 9e10 (1:100, Covance) to a final volume of 100 μL in PBS/BSA. Incubate at desired temperature for 30 min. Larger volumes and longer incubation times are required for very low (<10 nM) antigen concentrations (see notes at end of protocol).

8. Centrifuge, discard supernatant, and rinse with ice cold PBS/BSA. Centrifuge, discard supernatant from rinse.

9. On ice, incubate with secondary reagents. Add 97 μl ice cold PBS/BSA, 2 μl goat anti-mouse FITC conjugate (1:50, Sigma), and 1 μl streptavidin phycoerythrin conjugate (1:100, Molecular Probes). Incubate 30 min.

10. Centrifuge, discard supernatant, and rinse with ice cold PBS/BSA. Centrifuge, discard supernatant from rinse.

11. Resuspend cells in 500 μl ice cold PBS/BSA and transfer to tubes for flow cytometry or FACS sorting.

An important consideration when labeling high affinity antibodies (<30 nM) is depletion of antigen from the labeling mixture. This results in a lower than expected concentration of soluble (free) antigen, and hence a lower signal. Sorting libraries under depletion conditions can reduce the difference in signal observed for improved clones compared to their wild-type counterparts. The equivalent concentration of yeast surface-displayed proteins when 0.2 OD_{600}-mL of yeast is added to a 100 μl volume is approximately 3 nM or less. To avoid depletion, always use at least a 10-fold excess of antigen by adjusting the total volume and/or reducing the number of yeast added (as little as 0.05 OD_{600}-mL can be used).

Note that for labeling large libraries, it is advisable not to scale up directly. Instead use 1 mL volume per 10^8 cells labeled, keeping the reagent dilutions constant. Depletion can be especially severe with such high cell densities, however, and the experiment must be designed to avoid such conditions.

Analyzing Clones and Libraries by Flow Cytometry

Once a yeast population is labeled, it can be analyzed by flow cytometry. This allows quantification of binding affinity by titrating antigen concentration. In addition to the samples that one wishes to analyze, a negative control (no fluorophores) and two single positive controls (just one fluorophore in each) should be prepared. With standard filters installed, FITC will be detected in the FL1 channel, while PE will be detected by FL2 for the settings on most flow cytometers. However, some "bleed over" or spectral overlap will be present in each channel, which must be compensated out. One should use the negative control to set the voltage and gain on each of the detectors so that the negative population has order of magnitude intensity of one to ten. The single positive controls are used to adjust compensation so that no FITC signal is detected in FL2 and no PE signal in FL1.

In a titration, one generally sets a gate on cells that express the antibody (i.e. FITC positive cells if the preceding labeling protocol is used) to eliminate non-expressing cells from quantification.

For sorting or analyzing a library, it is helpful to also prepare a labeled sample of the wild-type antibody and saturated library for comparison and to aid in drawing sort windows.

Sorting Yeast Surface Display Libraries by FACS

FACS is the most efficient and accurate way to sort yeast surface display libraries, although magnetic particle strategies have also been

employed.[1,2] To sort a library by FACS, one labels cells according to the protocol above, taking into consideration the notes that follow the protocol. Equations describing the optimum labeling concentration for a first library sort are available,[3] or one can simply choose a concentration that results in a weak signal (say, one fourth of the K_d value). One should typically screen 10-100 times the number of independent clones that are in the library. When drawing a gate for collecting cells, it is advisable to use a window with a diagonal edge to normalize for expression, if a double positive diagonal is present (Fig. 3). If no diagonal is observed (little or no binding), the entire double positive quadrant should be collected. Cells should be sorted directly into SD+CAA with antibiotics such as penicillin and streptomycin to diminish the risk of bacterial contamination. Cells will grow to saturation in one (if $>10^5$ cells are collected) or two ($<10^5$) days.

The very first time a library is sorted, gates are drawn conservatively (0.5% to 1% of the library is collected) to minimize the likelihood that an improved clone is missed. After the first sort, care should be taken to note the number of cells collected, as this is the maximum number of independent clones remaining in the library. In subsequent sorts, when the library size has been reduced and the amount of sorting time necessary decreases, one should bring several samples labeled under different conditions for sorting. These samples should be sorted at increasing stringency to rapidly isolate the best clones. Sort gates should cover the range of 0.01% of cells collected to 0.5%. All samples should be analyzed and the one with the greatest improvement should be chosen for further sorting. Typically the

single best clone, or clones containing a consensus mutation, are isolated within 4 sorts.

The cells collected in the final sort are plated out for clonal analysis. The mutant plasmids may be recovered from yeast using the Zymoprep kit (Zymo Research). The following primers may be used for sequencing:

Forward Sequencing Primer: gttccagactacgctctgcagg

Reverse Sequencing Primer: gattttgttacatctacactgttg

Summary

The protocols and methods described here enable engineering of scFv's by yeast surface display. Each protocol is up to date, and has been verified and optimized through several years of application. The directed evolution process is often applied iteratively until the desired affinity is achieved. A single round of mutagenesis and screening typically results in 10- to 100-fold improvement in the K_d value, with largest improvements obtained when the wild-type affinity is low (say, low micromolar binding constant). A complete cycle of mutagenesis and screening, from wild-type clone to improved mutant clone, requires conservatively approximately 3-6 weeks.

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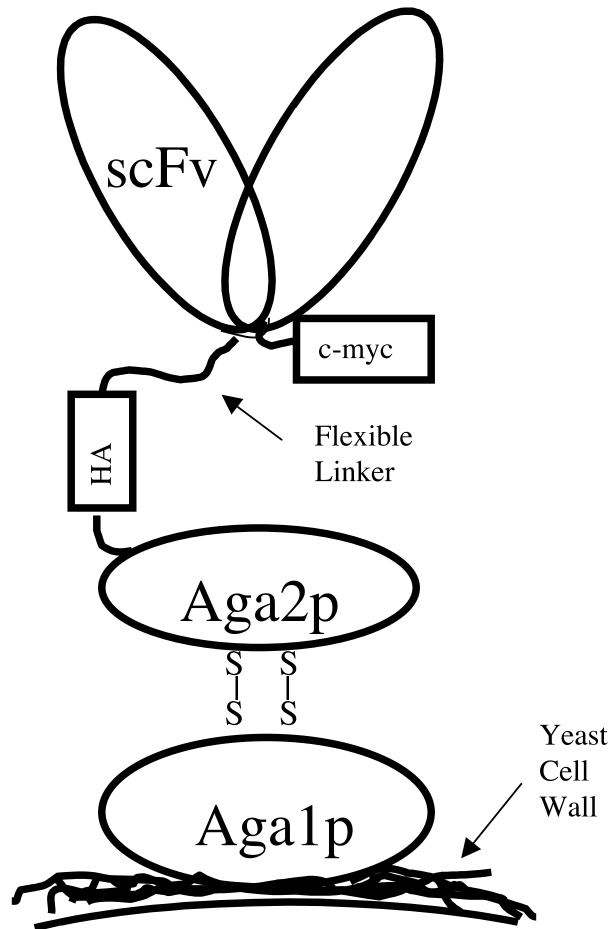
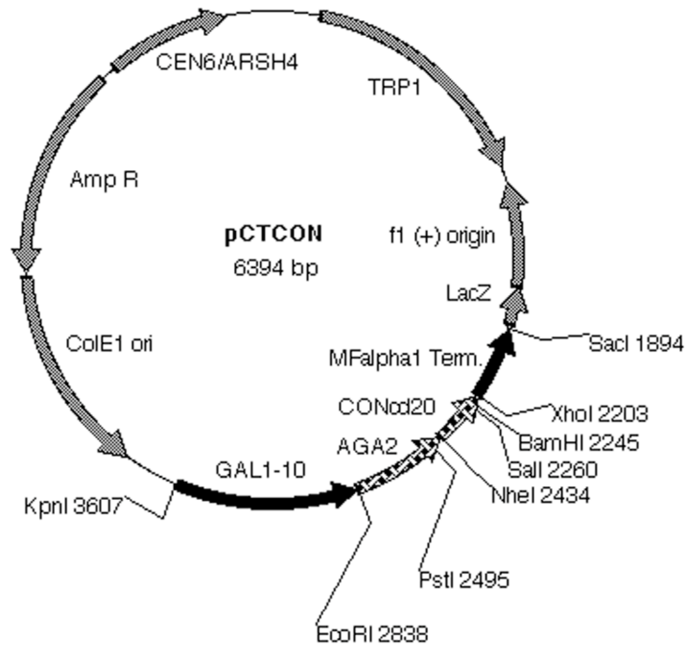


Figure 1. Yeast surface display schematic. The single chain antibody is expressed as a fusion to the Aga2 mating protein. C-myc and HA epitope tags are present to quantify expression by immunofluorescence.

A



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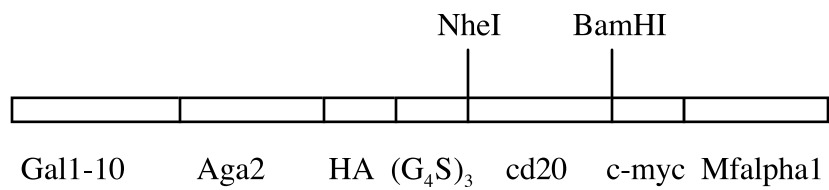

Figure 2. Plasmid map of pCTCON. A. CON cd20 is expressed from the plasmid as a fusion to the yeast mating protein Aga2. B. The CON cd20 gene can be replaced with an scFv of interest using the NheI and BamHI sites.

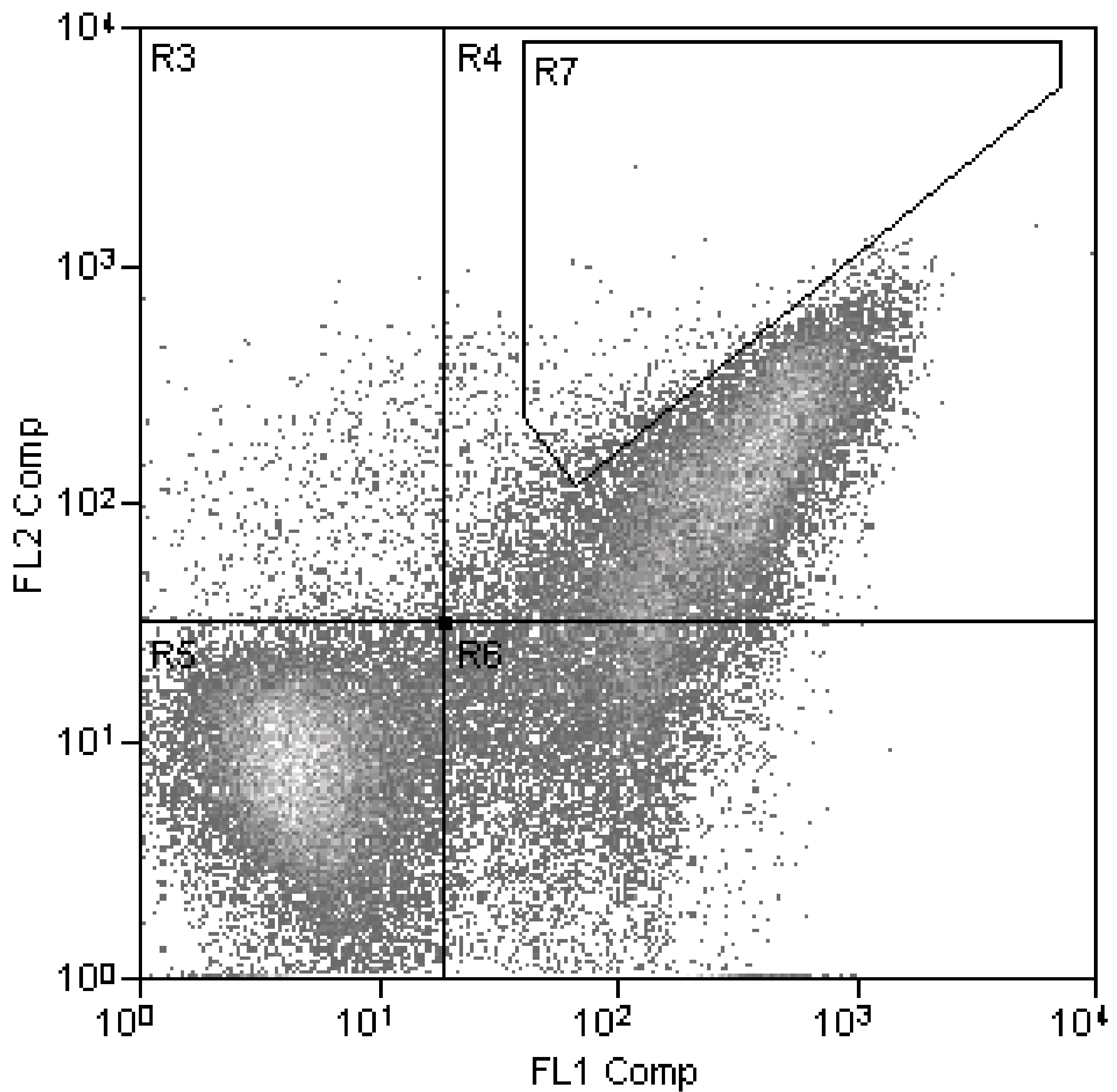

Figure 3. Sort gate. If a diagonal population is present in the library, a sort gate such as the one labeled R7 should be drawn to take full advantage of expression normalization.