[27] Engineering Antibody Affinity by Yeast Surface Display

By DAVID W. COLBY, BRENDA A. KELLOGG, CHRISTILYN P. GRAFF, YIK A. YEUNG, JEFFREY S. SWERS, and K. DANE WITTRUP

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 in 1997 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 nonimmune single chain antibody library serves as 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 incorporated easily 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. This article provides 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 and analysis after each round of sorting. Without exception to date, equilbrium-binding constants and dissociation rate constants measured for yeast-displayed proteins are in quantitative agreement with those measured for the same proteins *in vitro* using BIAcore or ELISA.

[1] E. T. Boder and K. D. Wittrup, *Nature Biotechnol.* **15,** 553 (1997).

[2] M. C. Kieke, B. K. Cho, E. T. Boder, D. M. Kranz, and K. D. Wittrup, *Protein Eng.* **10,** 1303 (1997).

[3] E. T. Boder, K. S. Midelfort, and K. D. Wittrup, *Proc. Natl. Acad. Sci. USA* **97,** 10701 (2000).

[4] P. D. Holler *et al.*, *Proc. Natl. Acad. Sci. USA* **97,** 5387 (2000).

[5] M. C. Kieke *et al.*, *Proc. Natl. Acad. Sci. USA* **96,** 5651 (1999).

[6] M. C. Kieke *et al.*, *J. Mol. Biol.* **307,** 1305 (2001).

[7] M. J. Feldhaus *et al.*, *Nature Biotechnol.* **21,** 163 (2003).

[8] E. T. Boder and K. D. Wittrup, *Methods Enzymol.* **328,** 430 (2000).

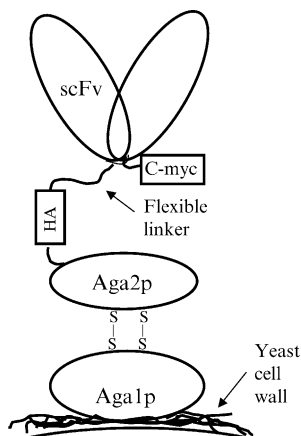[9] J. J. VanAntwerp and K. D. Wittrup, *Biotechnol. Prog.* **16,** 31 (2000).

Fig. 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.

Traditional panning methods have also been successfully employed with YSD, including magnetic particle separation.[10] Other advantages arising from the yeast system include ease of use and the presence of the yeast endoplasmic reticulum, the later of which acts as a quality control mechanism, ensuring that only properly folded proteins reach the cell surface.

This article 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 polymerase chain reaction (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 linked covalently to the cell wall (Fig. 1).

[10] Y. A. Yeung and K. D. Wittrup, *Biotechnol. Prog.* **18,** 212 (2002).

Expression of both the Aga2p–antibody fusion and Aga1p are under the control of the galactose-inducible GAL1 promoter, which allows inducible overexpression.

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 *Nhe*I and the *Bam*HI 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 as hosts.
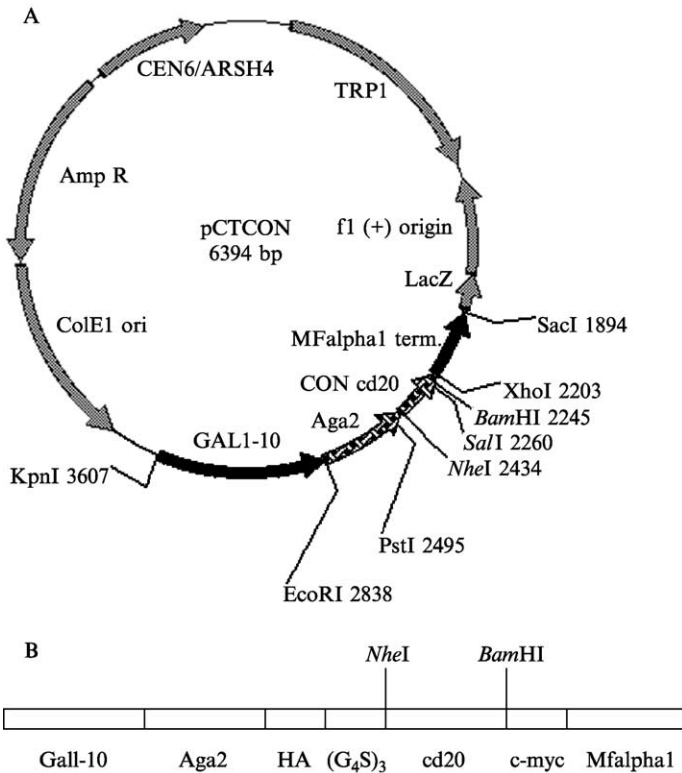

FIG. 2. Plasmid map of pCTCON. (A) CON cd20 is expressed from the plasmid as a fusion to the yeast mating protein Aga2. (B) The CON cd20 gene can be replaced with an scFv of interest using *Nhe*I and *Bam*HI sites.

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 *Escherichia 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 are reassembled *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.

The following section describes how to prepare scFv insert DNA with random point mutations by error-prone PCR using nucleotide analogues. However, this mutagenesis procedure 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 analogue mutagenesis allows the frequency of mutation to be tuned based on the number of PCR cycles and the relative concentration of the mutagenic nucleotide analogues used during PCR.[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 so as to completely ablate binding, a range of several different mutagenesis levels are used in parallel. The conditions reported here are the ones typically used to create antibody libraries; these conditions give an error rate ranging from 0.2 to 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

[11] C. K. Raymond, T. A. Pownder, and S. L. Sexson, *Biotechniques* **26,** 134 (1999).
[12] W. P. Stemmer, *Nature* **370,** 389 (1994).
[13] W. P. Stemmer, *Proc. Natl. Acad. Sci. USA* **91,** 10747 (1994).
[14] A. A. Volkov and F. H. Arnold, *Methods Enzymol.* **328,** 447 (2000).
[15] M. Zaccolo and E. Gherardi, *J. Mol. Biol.* **285,** 775 (1999).
[16] M. Zaccolo, D. M. Williams, D. M. Brown, and E. Gherardi, *J. Mol. Biol.* **255,** 589 (1996).

1. Set up six 50-μl PCR reactions

	Final concentration
10× PCR buffer (without MgCl$_2$)	
MgCl$_2$	2 mM
Forward primer	0.5 μM
Reverse primer	0.5 μM
dNTPs	200 μM
Template	0.1–1 ng
8-Oxo-dGTP	2–200 μM
dPTP	2–200 μM
dH$_2$0	to final volume
Taq polymerase	2.5 units

Of the six PCR reactions, two should contain 200 μM nucleotide ana-
logues, 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° for 45 s, anneal at 55° for 30 s, and extend at 72° for 1 min.
One should also include a 3-min denaturation step at 94° 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 (μM)	Number of PCR cycles
200	5
200	10
20	10
20	20
2	10
2	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 nonmutated template before amplification (next
step). Cut out and purify using the Qiagen gel purification kit following the
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 a 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 the pellet has dried, dissolve in water to a final concentration of 5 $\mu g/\mu l$. This protocol typically produces 40–100 μg of PCR product.

Preparation of Vector

1. Miniprep 10 μg or more of pCTCON.
2. Digest with *Nhe*I (New England Biolabs) for at least 2 h in NEB2 buffer.
3. Adjust salt concentration by adding one-tenth of the total volume of 1 *M* NaCl.
4. Double digest with *Bam*HI and *Sal*I for another 2 h 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 the 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 the Paint Pellet reagent. After drying the pellet, dissolve in water to 2 $\mu g/\mu l$.

Preparation of Electrocompetent Yeast Cells

This protocol has been adapted from Meilhoc *et al.*[17] and generates enough cells for the transformation of ~60 μg of insert DNA and ~6 μg of vector, which typically produces ~5×10^7 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 30° to an OD_{600} of 1.3–1.5 (about 6 h).
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 30° for 20 min.

[17] E. Meilhoc, J. M. Masson, and J. Teissie, *Biotechnology* **8,** 223 (1990).

4. Harvest cells at 3500 rpm, 5 min, 4°. Discard supernatant. All centrifugation steps should be carried out in autoclaved centrifuge tubes or in 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 not used immediately may be frozen down in 50-μl aliquots for future use. Note that using frozen cells results in a 3- to 10-fold loss in transformation efficiency.

Electroporation

Electroporation is carried out using a Bio-Rad 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 (Bio-Rad). 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; the 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 the cuvette into the slide chamber. Push both red buttons simultaneously until a pulsing tone is heard and then release.

4. After pulsing, immediately add 1 ml of room temperature YPD media[8] to the cuvette. Incubate at 30° for 1 h 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 the 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 the normalization of expression and eliminates nondisplaying 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 described at the end of the protocol.

1. Grow transformed yeast overnight in SD+CAA. OD_{600} should be greater than 1. 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° with shaking (250 rpm) for at least 18 h. The appropriate induction temperature should be tested for each scFv, from 20, 25, 30, or 37°.

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, as this aliquot will correspond to approximately 2×10^6 cells.

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

6. Rinse with phosphate-buffered saline (PBS) plus 0.1% bovine serum albumin (BSA). Centrifuge for 10 s and 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 and discard supernatant from rinse.

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

10. Centrifuge, discard supernatant, and rinse with ice-cold PBS/BSA. Centrifuge and 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 the 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 the 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, whereas 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 an order of magnitude intensity of 1 to 10. 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 nonexpressing 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
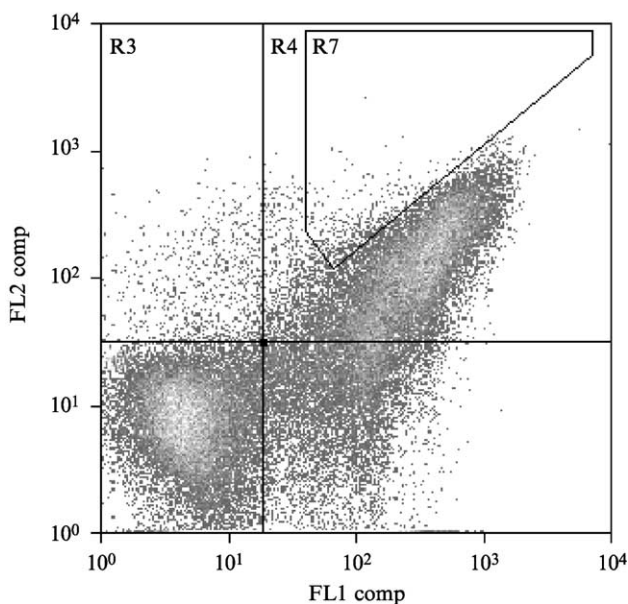
FIG. 3. Sort gate. If a diagonal population is present in the library, a sort gate such as the one labeled R7 should be drawn to take full advantage of expression normalization.

protocol given earlier, 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 1 (if $>10^5$ cells are collected) or 2 ($<10^5$) 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, is isolated within four sorts.

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 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 the 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.

Acknowledgments