Washing buffer PBSTMH: PBSTM with heparin at 2.5 mg/ml
Elution buffer PEB20: PBS with 20 m*M* EDTA

Protocol for Ribosome Display in Vitro Translation and Affinity Selection. Sterilized 12% (v/v) milk is combined with 10× PBSM to obtain 10% (v/v) milk in 1× PBSM, and this solution can be stored on ice for several weeks. In an ice–water bath, a tube containing 200 μl of PBSTMH buffer and 62.5 μl of 10% (v/v) sterilized milk in PBSM is prepared. On ice, the following ice-cold solutions are combined: 0.8 μl of 2.5 *M* KCl, 0.2 μl of 200 m*M* methionine, 1 μl of 1 m*M* each amino acid except methionine, 33 μl of Flexi rabbit reticulocyte lysate, and sterile, double-distrilled water is added up to 40 μl. The amino acid mix (1 m*M* each) without methionine is included in the translation kit, as is KCl. The library mRNA should be thawed only directly before use, and the remainder should be immediately frozen. Five micrograms (approximately 1×10^{13} molecules) of ice-cold library mRNA, either capped or uncapped, in 10 μl is added to the mixture, gently vortexed, and immediately placed in a 30° water bath. After 20 min of *in vitro* translation, the mixture is pipetted out of the reaction tube, immediately added to the tube containing buffer PBSTMH and milk, briefly and gently vortexed, and placed in the ice–water bath. We did not find it necessary to centrifuge the translation mixture prior to selection. Selection is carried out as described for the *E. coli* system, except that buffers WBT and EB20 are replaced by buffers PBSTM and PEB20, respectively. mRNA purification, reverse transcription, and PCR are performed as described for the *E. coli* system, except that primers SDA and T7B are replaced by primers SDA-RRL and T7RR-EN.

[25] Yeast Surface Display for Directed Evolution of Protein Expression, Affinity, and Stability

By ERIC T. BODER and K. DANE WITTRUP

Background

Many platforms are available for the construction of peptide and polypeptide libraries, allowing directed evolution or functional genomics studies.[1] Currently, the two most widely used polypeptide library methods are phage display and the yeast two-hybrid method. However, neither of these methods is effective for complex extracellular eukaryotic proteins, because

[1] E. V. Shusta, J. J. Van Antwerp, and K. D. Wittrup, *Curr. Opin. Biotechnol.* **10,** 117 (1999).

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0076-6879/00 $30.00

of the absence of such posttranslational modifications as glycosylation and efficient disulfide isomerization. We have developed a yeast surface display method that addresses this deficiency by utilizing the yeast secretory apparatus to process cell wall protein fusions.[2] Yeast surface display is well suited to engineer extracellular eukaryotic proteins such as antibody fragments, cytokines, and receptor ectodomains.

A further advantageous characteristic of yeast surface display is that soluble ligand-binding kinetics and equilibria may be measured in the display format, and as a result quantitatively optimized screening protocols may be designed.[3] Using such optimal screening conditions, numerous mutants with small improvements may be finely discriminated with high statistical certainty, and further recombination may be used to achieve greater improvements.[4]

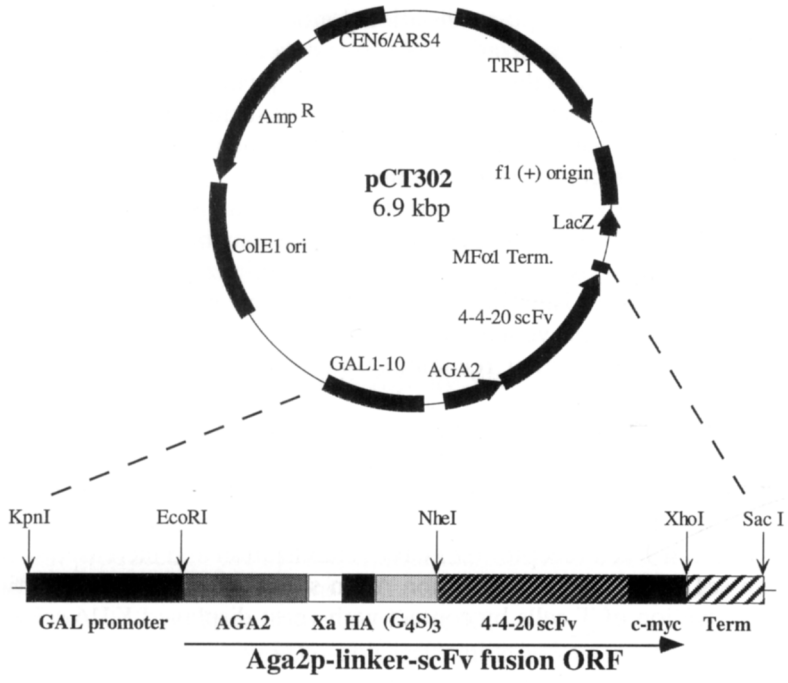
To date, we have applied yeast display in the following studies: affinity maturation of the 4-4-20 anti-fluorescein single-chain antibody (scFv) to femtomolar affinity[5]; affinity maturation of the KJ16 anti-T cell receptor scFv[6]; affinity maturation of the D1.3 anti-lysozyme scFv[7]; display of a single-chain T cell receptor (scTCR)[8]; stabilization and increased secretion of an scTCR[9]; affinity maturation of an scTCR against a superantigen[10]; and activation of T cells by contact with yeast-displayed KJ16 scFv.[11]

Yeast Display of a Protein of Interest

A given protein may be displayed on the surface of yeast by expression as a protein fusion to the Aga2p mating agglutinin protein. We have constructed the pCT302 plasmid (Fig. 1) for expression of such fusions under control of the GAL1,10 galactose-inducible promoter. Because the Aga2p protein is tethered in the cell wall via disulfide bridges to the Aga1p protein, we have constructed yeast strain EBY100 (**a** *GAL1-AGA1::URA3 ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS2 prb1Δ1.6R can1 GAL*), in which Aga1p

[2] E. T. Boder and K. D. Wittrup, *Nature Biotechnol.* **15,** 553 (1997).
[3] E. T. Boder and K. D. Wittrup, *Biotechnol. Prog.* **14,** 55 (1998).
[4] W. P. Stemmer, *Nature (London)* **370,** 389 (1994).
[5] E. T. Boder and K. D. Wittrup, *Proc. Natl. Acad. Sci. U.S.A.,* in press (2000).
[6] M. C. Kieke, B. K. Cho, E. T. Boder, D. M. Kranz, and K. D. Wittrup, *Protein Eng.* **10,** 1303 (1997).
[7] J. J. Van Antwerp and K. D. Wittrup, unpublished data (1999).
[8] M. C. Kieke, E. V. Shusta, E. T. Boder, L. Teyton, K. D. Wittrup, and D. M. Kranz, *Proc. Natl. Acad. Sci. U.S.A.* **96,** 5651 (1999).
[9] E. V. Shusta, M. C. Kieke, E. Parke, D. M. Kranz, and K. D. Wittrup, *Nature Biotechnol.* **18,** 754 (2000).
[10] M. C. Kieke, K. D. Wittrup, and D. M. Kranz, unpublished data (1999).
[11] B. K. Cho, M. C. Kieke, E. T. Boder, K. D. Wittrup, and D. M. Kranz, *J. Immunol. Methods* **220,** 179 (1998).

A



N-terminal flanking sequence:

```
ATAAACACACAGTATGTTTTTAAGGACAATAGCTCGACGATTGAAGGTAGATACCCATAC
I  N  T  Q  Y  V  F  K  D  N  S  S  T  I  E  G  R  Y  P  Y   -
          Aga2p  <-|->  Linker  <- |Factor Xa^|-> HA

                  PstI/
GACGTTCCAGACTACGCTCTGCAGGCTAGTGGTGGTGGTGGTTCTGGTGGTGGTGGTTCT
D  V  P  D  Y  A  L  Q  A  S  G  G  G  G  S  G  G  G  G  S   -
epitope tag <-|->          Linker

           NheI/    AatII/
GGTGGTGGTGGTTCTGCTAGCGACGTCGTTATGACTCAAACACCACTATCACTTCCTGTT
G  G  G  G  S  A  S  D  V  V  M  T  Q  T  P  L  S  L  P  V   -
                  <-|-> VL of 4-4-20 scFv
```

C-terminal flanking sequence:

```
                                        XhoI/   /BglII
TCCTCAGAACAAAAGCTTATTTCTGAAGAAGACTTGTAATAGCTCGAGATC
S  S  E  Q  K  L  I  S  E  E  D  L  *              -
<-|   |->  c-myc epitope tag <-|
```

FIG. 1

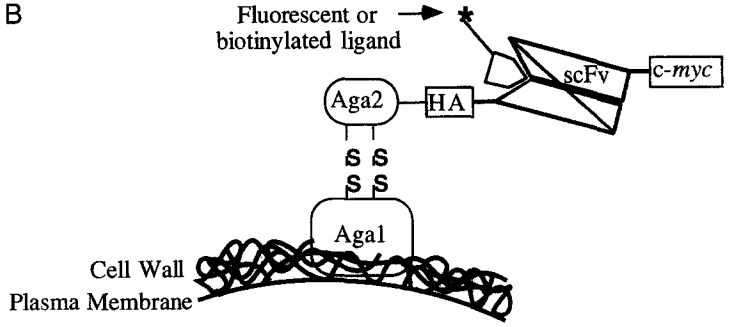


FIG. 1. (A) Map of plasmid pCT302 for expression of Aga2p fusion proteins. Flanking sequences are shown. We have generally inserted new ORFs as *NheI–XhoI* fragments. (B) Schematic of fusion topology on the cell surface. Aga1p is covalently attached to the cell wall via a phosphatidylinositol glycan tail, and Aga2p is disulfide bonded to Aga1p. The N-terminal HA and C-terminal c-Myc epitope tags enable detection of a fusion protein independent of its ligand-binding characteristics. For purposes of illustration, an scFv antibody fragment is shown as the protein of interest. Objects are not drawn to actual proportionate scale. Steric hindrance by cell wall components is not generally an issue, because an Aga2p–scFv fusion is accessible for labeling with 2×10^6 kDa fluorescein dextran.[2]

expression is inducible from a single, integrated open reading frame (ORF) downstream of the *GAL1,10* promoter.

The presence of the fusion protein on the cell surface may be detected independently of its ligand-binding activity by immunofluorescent labeling of epitope tags. Quantitative detection of fusion polypeptide surface levels via epitope tag labeling is important to normalize for random variation in surface expression levels when measuring ligand-binding kinetics and equilibria. Two epitope tags are used in the fusion, one N terminal (hemagglutinin, HA) and one C terminal (c-Myc) to the protein of interest. If the 3′ *Xho*I site is used to subclone the ORF into pCT302, the c-Myc coding sequence must be reconstructed. Both tags are of value; immunofluorescent detection of the c-Myc epitope on the cell surface confirms the presence of the full-length polypeptide, and the quality control apparatus of the endoplasmic reticulum (ER) provides high confidence that the protein on the surface is correctly folded.[12] The HA tag serves as a useful internal control should the fusion be displayed in a partially proteolyzed form.[9] The availability of two epitope tags also allows one to alternate their use, thereby circumventing artifactual isolation of epitope tag mutations during library screening.[6]

Once the ORF of interest is subcloned in-frame with Aga2p in pCT302,

[12] C. Hammond and A. Helenius, *Curr. Opin. Cell Biol.* **7**, 523 (1995).

the expression plasmid may be transformed into the yeast strain EBY100 by any of a number of standard yeast transformation methods, with selection on SD + CAA medium [yeast nitrogen base (6.7 g/liter), Na_2HPO_4 (5.4 g/liter), $NaH_2PO_4 \cdot H_2O$ (8.56 g/liter), dextrose (20 g/liter), Bacto Casamino acids (CAA, 5 g/liter; Difco, Detroit, MI)]. This allows selection of EBY100 and pCT302 under Trp– and Ura– conditions, and yeast generally grow faster and to higher density in this semidefined medium. The transformants should be maintained on glucose as a carbon source in order to repress Aga1p and Aga2p fusion expression, and thus repress consequent counter-selection against potentially toxic heterologous expression effects. The ORF of interest should be examined for Lys–Arg sequences; if the expressed protein contains this sequence, an alternative *Saccharomyces cerevisiae* strain containing a deletion of the chromosomal Kex2 protease ORF may be required to prevent endoproteolytic processing within the *trans*-Golgi network.[13,14]

The Aga2p protein fusion is expressed on the cell surface by induction in galactose-containing medium. From plates (SD + CAA containing 1 *M* sorbitol), 3- to 5-ml liquid test tube cultures (SD + CAA) are inoculated and grown at 30° for 12–48 hr. Length of the lag phase is highly variable and depends on the age of the colony and the size of the inoculum. The time of growth on glucose is not directly significant; however, best surface expression usually results when exponentially growing cells are switched to galactose. For EBY100 transformants grown under these conditions, this corresponds to an OD_{600} of 2.0–5.0; generally, overnight growth from a fresh colony will yield a culture in this density range in approximately 12–20 hr. Cultures grown to stationary phase (OD_{600} >10) may be induced for surface expression; however, a smaller fraction of cells display the protein of interest and lower expression levels per cell are typical. Stationary-phase liquid cultures may be reprepared for optimal surface display by pelleting cells, resuspending in fresh SD medium, and grown for ~6–10 hr at 30°. To induce fusion expression, the culture is centrifuged for 10–20 sec at maximum speed in a microcentrifuge, and resuspended in SG + CAA medium, which is identical to SD medium except that glucose is replaced by galactose as the carbon source. Induction of surface expression must be performed at 20° for best results for single-chain antibodies; at >25° surface expression levels vary substantially with induction time, and lower surface levels are generally found. However, other mammalian proteins are better displayed by induction at 30 or 37°. Cells should be induced in SG at starting densities of 0.2–2.0 OD_{600}. Cultures may be induced

[13] N. C. Rockwell and R. S. Fuller, *Biochemistry* **37,** 3386 (1998).
[14] A. Bevan, C. Brenner, and R. S. Fuller, *Proc. Natl. Acad. Sci. U.S.A.* **95,** 10384 (1998).

from 16 to 48 hr, most typically 20–24 hr. The culture usually undergoes approximately one doubling during induction at 20°, resulting in a final culture density of approximately 0.3–3.0 OD_{600}. The additional stability afforded by expression at 20° may be a potential advantage for unstable proteins such as single-chain antibodies, by comparison with bacterial or mammalian expression platforms.

Yeast cultures may be fluorescently labeled for examination by flow cytometry, by the following procedures.

1. Centrifuge a volume of induced culture equivalent to 0.2 OD_{600} ml (e.g., 0.2 ml of a culture with OD_{600} of 1.0; OD_{600} of 1.0 corresponds to $\sim 10^7$ cells/ml for EBY100) in a microcentrifuge at maximum speed for ~10–20 sec. Remove and discard the supernatant.

2. Resuspend the cell pellet by vortexing in ice-cold buffered saline solution (BSS) containing bovine serum albumin (BSA, 1 mg/ml; Sigma). BSA is present to serve as a carrier protein for ligands added in subsequent labeling steps, to minimize adsorptive losses on plasticware. Either PBS [NaCl (8 g/liter), KCl (0.2 g/liter), Na_2HPO_4 (1.44 g/liter), KH_2PO_4 (0.24 g/liter), pH 7.4] or TBS (50 m*M* Tris-HCl, 100 m*M* NaCl, pH 8.0) can be used. Pellet the culture again by microcentrifuge.

3. Labeling of the c-Myc and HA epitope tags may be performed after or during certain steps of the more specific ligand-binding protocols described in the next section. For instance, we have commonly combined ligand and epitope tag primary antibody labeling in a single step. Likewise, we have combined secondary epitope tag labeling with ligand labeling in one step. The 12CA5 monoclonal antibody (MAb) labeling step has also been performed during 25° kinetic library screening steps (see below).

4. Resuspend the cell pellet in 100 μl of BSS containing either primary antibody against the epitope tags, or biotinylated or fluorescently labeled ligand for the protein of interest. Specific conditions of interest follow:

HA epitope tag: 12CA5 monoclonal antibody (Boehringer Mannheim, Indianapolis, IN) at 10 μg/ml final concentration. Incubate 30 min on ice

c-Myc epitope tag: 1 : 100 dilution of the 9E10 monoclonal antibody ascites fluid (Berkeley Antibody Company, Richmond, CA) for 30 min on ice

Biotinylated ligand detection: 1 : 100 streptavidin–phycoerythrin (PharMingen, San Diego, CA) 30 min on ice

5. After incubation with the primary antibody, pellet the cells, remove and discard the supernatant, and wash once with 0.5 ml of ice-cold BSS.

6. Label the cells with fluorescently labeled ligand or fluorescently labeled secondary antibody [e.g., fluorescein isothiocynate (FITC)- or phy-

coerythrin-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO) at titers of ~1:25, for 12CA5 or 9E10 MAb]. Incubate on ice for 30–45 min.

7. Pellet the cells and wash at least with 0.1 ml of ice-cold BSS. Resuspend the cells in ice-cold BSS at a density suitable for microscopy (~10^7 cell/ml) or flow cytometry (~1–5 × 10^6/ml). Cultures should be kept on ice after all primary and secondary antibody labeling steps, prior to and during flow cytometry analysis.

8. Immunofluorescently labeled yeast may be examined by flow cytometry to obtain data of the type shown in Fig. 2. Setting of the instrument's gains should be chosen to allow detection of the background fluorescent peak.

One feature worthy of note in the flow cytometry data is the presence of a peak with background fluorescence equivalent in intensity to that obtained with negative controls. This negative peak is present for all proteins examined to date, and represents at least 15% of the cells examined in every case. Nonfluorescent cells appear to contain pCT302 vector and intracellular HA-tagged proteins[15]; the nonfluorescent fraction of the population is a strong function of the stability of the protein fusion expressed,[8] as well as of the temperature of induction.[16] This leads us to speculate that the nonfluorescent cells represent those cells for which the secretory apparatus has become saturated and blocked with misfolded protein,[17] a phenomenon that has now been well documented for solubly expressed and secreted proteins.[17–19] In any case, in more than 20 library screens to date we have found that the presence of the unlabeled cell population does not interfere with library screening for improved protein properties.

Construction of Yeast Display Libraries

If the protein of interest is displayed on the cell surface in active form, one may proceed directly to library construction in order to identify mutants with improved properties. We have also found that if the original protein is not displayed, it is possible to isolate "displayable" mutants of the protein[8] to enable further directed evolution.

The full range of available mutagenesis methods is consistent with yeast surface display library construction. We have had particular success with

[15] E. T. Boder, S. M. Elliott, and K. D. Wittrup, unpublished data (1999).
[16] M. C. Kieke, E. T. Boder, D. M. Kranz, and K. D. Wittrup, unpublished data (1999).
[17] A. S. Robinson and K. D. Wittrup, *Biotechnol. Prog.* **11,** 171 (1995).
[18] R. N. Parekh and K. D. Wittrup, *Biotechnol. Prog.* **13,** 117 (1997).
[19] E. V. Shusta, R. T. Raines, A. Pluckthun, and K. D. Wittrup, *Nature Biotechnol.* **16,** 773 (1998).

error-prone polymerase chain reaction (PCR)[20] of the entire ORF of the protein of interest, as well as mutagenesis of the entire plasmid by an *Escherichia coli* mutator strain (e.g., XL1-Red; Stratagene, La Jolla, CA). We provide an example of error-prone PCR mutagenesis of an scFv ORF. Primers were first designed to amplify the scFv gene with >100bp of Aga2p sequence 5′ of the *Nhe*I cloning site and 50 bp of sequence 3′ of the *Xho*I cloning site (see Fig. 1). Primer sequences are 5′-GGCAGCCCCATAAACACACAGTAT-3′ and 5′-GTTACATCTACACTGTTGTTAT-3′. Alternatively, standard T7 and T3 primers may be used to amplify the entire expression cassette from outside the promoter and transcriptional terminator. These large overhangs improve the efficiency of restriction digestion and dramatically increase the number of recombinants generated when subcloning the PCR products. The PCR is made mutagenic by the presence of 0.3–0.375 m*M* manganese chloride along with 2.25 m*M* magnesium chloride.[20,21] Under these conditions we obtain error rates of from 0.5 to 1.0%. Four 75-μl PCR are pooled and purified electrophoretically on a 1% (w/v) low melting agarose gel. The product band is excised from the gel and DNA is eluted in TAE buffer with a Bio-Rad (Hercules, CA) Electroeluter model 422, following the manufacturer recommended protocol. To reduce the EDTA concentration before restriction digestion, eluted products are diluted to 2 ml in doubly distilled H_2O, concentrated to ~60 μl in a Centricon-30 cartridge (Amicon, Danvers, MA), diluted to 0.5 ml in doubly distilled H_2O, and concentrated to ~10–20 μl in a Microcon-50 cartridge (Amicon). Final products are digested with *Nhe*I and *Xho*I and again gel purified with low melting point agarose. The appropriate band is cut out with a razor blade under illumination from a hand-held high-UV lamp, and the DNA is recovered from the gel slices with a Wizard PCR Prep kit (Promega, Madison, WI). This mutagenized insert is then ligated into a similarly gel-purified pCT302 backbone at an insert-to-vector ratio of ~2:1. Multiple 40-μl ligation reactions are performed to make use of as much mutagenized DNA as possible. One key to large library size is maximization of amount of DNA in each step (>1 μg).

We have also used DNA shuffling.[4,22] Key variables for optimization of that protocol are choice of DNase concentration and/or incubation time, and use of primers for final PCR amplification that are nested within the amplified region of those used for the original template amplification.

The ligation mixture is transformed into maximum-competency *E. coli* cells (e.g., XL10-Gold; Stratagene). Alternatively, to achieve maximal li-

[20] D. W. Leung, E. Chen, and D. V. Goeddel, *Technique* **1,** 11 (1989).
[21] R. C. Cadwell and G. F. Joyce, *PCR Methods Appl.* **3,** S136 (1994).
[22] H. Zhao and F. H. Arnold, *Nucleic Acids Res.* **25,** 1307 (1997).

brary size, multiple ligation reactions may be combined, diluted to 0.5 ml in doubly distilled H_2O to reduce salt concentration, concentrated to 10 μl in a Microcon-50 cartridge (Amicon), and transformed into electroporation-competent *E. coli* in multiple parallel transformation (e.g., DH10B ElectoMAX cells; Life Technologies, Bethesda, MD). This method has yielded DNA libraries containing $>10^7$ recombinants. The *E. coli* culture is maintained in liquid LB medium with carbenicillin (100 μg/ml) and ampicillin (50 μg/ml). Aliquots are plated to determine transformation efficiency. The liquid culture is then inoculated into 200-ml cultures and grown for 16–20 hr at 30°. Plasmid DNA is purified from the 200-ml culture by Qiagen Maxiprep kit or similar method.

Finally, the mutagenized plasmid pool is transformed into yeast by the high-efficiency protocol essentially as described by Gietz and Schiestl.[23] Multiple parallel transformations are performed; after gentle resuspension of cells in doubly distilled H_2O, cells are pelleted at 6000 rpm, resuspended in 100 μl of doubly distilled H_2O, and pooled. The transformed culture is amplified directly in SD + CAA liquid culture without plating, after plating of aliquots to determine yeast library size. Particularly important variables are the time of heat shock, quality of the single-stranded DNA (ssDNA) preparation, and gentle resuspension of cells after pelleting. For EBY100, a heat shock time of 30–35 min is optimal.[24]

Screening of Yeast Display Libraries

Successful engineering of proteins by directed evolution depends not only on a suitably diverse library from which to select altered phenotypes, but also critically on a quantitatively designed screening and isolation methodology. Specific parameters important in screening and sorting of yeast displayed libraries are ligand concentration, kinetic competition time, thermal denaturation time, and fluorescence-activated cell sorting (FACS) stringency. Mathematical estimation of these parameters enhances the utility of the surface display approach.[3]

Two screening approaches exist for identifying desirable affinity mutants within a surface displayed library. The most suitable method depends on the values of the affinity and kinetic constants of the wild-type protein–ligand binding interaction. Mutants may be distinguished by equilibrated binding with low concentrations of fluorescently labeled ligand in cases of fairly low affinity interactions ($K_d > 1$ n*M*, or no affinity if the library is being screened to isolate a novel binding specificity). However, for applications

[23] R. D. Gietz and R. H. Schiestl, *Methods Mol. Cell. Biol.* **5,** 255 (1995).
[24] E. T. Boder, B. G. Goekner, and K. D. Wittrup, unpublished data (1999).

designed to evolve tight-binding proteins, excessively large volumes of dilute ligand solutions are necessary to maintain molar ligand excess, complicating handling of samples. In such cases, improvements in binding affinity may be approximated by changes in dissociation kinetics. Kinetic competition for a stoichiometrically limiting ligand can be used to identify improved clones within the population[25]; however, this method eliminates the quantitative predictability of the screening approach and is not recommended in general. General strategies for equilibrium or kinetic screening of yeast displayed libraries are outlined below.

Quantitative Equilibrium Binding Screen

To verify the protein–ligand dissociation constant K_d within the surface display context a titration of the wild-type protein is performed by flow cytometric analysis.[6] A useful procedure for this analysis is as follows.

1. Grow and induce yeast cultures as described above, and harvest multiple samples containing $\sim 2 \times 10^6$ cells (i.e., $\sim$0.2 OD_{600}-ml). If necessary the number of cells may be reduced to 1×10^6.
2. Label samples with 12CA5 MAb and biotinylated or fluorescently labeled ligand as described above. Use 10 or more dilutions of ligand such that the expected K_d of the interaction is effectively spanned. For example, for an expected K_d of 100 n*M*, ligand concentrations from $\sim$10 n*M* to 1 μM should yield adequate results. Importantly, a 10-fold or greater molar excess of ligand must be maintained at all dilutions. A conservative estimate of the displayed protein concentration can be made by assuming $\sim 10^5$ copies/cell. Thus, 2×10^6 cells per sample yields $\sim$0.33 pmol of displayed protein per sample, and incubation volumes should be adjusted to ensure >3 pmol of total ligand at the desired concentration. Volumes up to 50 ml have been used successfully. Note also that lower ligand concentrations may require longer incubations to ensure equilibrium.
3. Label with secondary antibodies and/or streptavidin–phycoerythrin as described previously.
4. Analyze cell populations by flow cytometry. Gate on only the displaying fraction of the population (observed by 12CA5 labeling). Determine the mean fluorescence intensity (i.e., the arithmetic mean) due to ligand binding of the displaying population. Note that geometric mean fluorescence (i.e., mean logarithmic histogram channel of fluorescence) is not useful for equilibrium or kinetic analysis. Therefore, alternative statistics such as peak or median fluorescence should be used if the instrument reports only geometric mean.

[25] R. E. Hawkins, S. J. Russell, and G. Winter, *J. Mol. Biol.* **226,** 889 (1992).

5. Plot (fluorescence intensity)/(ligand concentration) versus fluorescence intensity and apply Scatchard analysis to determine K_d. Deviations from linearity at higher ligand concentrations reflect saturation binding of surface protein, and data points beyond the saturating concentration should be ignored. An alternative and more rigorous procedure is to use a nonlinear least-squares routine to fit the binding equilibrium equation.

Once the K_d of the wild-type interaction has been measured, the optimum ligand concentration for discriminating mutants improved by a defined increment may be calculated from the following equation[3]:

$$\frac{[L]_{opt}}{K_d^{wt}} = \frac{1}{(S_r K_r)^{1/2}}$$

where $[L]_{opt}$ is the concentration of ligand yielding the maximum ratio of mutant to wild-type fluorescence, S_r is the maximum signal-to-background ratio for yeast saturated with fluorescent ligand, and K_r is the minimum affinity improvement desired (e.g., $K_r = 5$ if mutants improved fivefold in affinity are desired). S_r is the ratio of fluorescence of yeast saturated with fluorescent ligand over autofluorescence of unlabeled yeast, and is dependent on the particular flow cytometer and efficiency of protein expression. Our experience suggests a ligand concentration of ~ 0.05–$0.1 \times K_d$ of the wild-type interaction should generally yield adequate discrimination of mutants improved 3- to 10-fold.

Quantitative Kinetic Binding Screen

Screening by dissociation rate is achieved by labeling yeast to saturation with fluorescently labeled ligand followed by incubation in the presence of excess nonfluorescent ligand competitor. Prior to screening a displayed library for improved dissociation kinetics, the K_{off} of the wild-type protein–ligand reaction must be obtained. A protocol for determining K_{off} by flow cytometry of yeast displaying the protein of interest follows.

1. Grow and induce yeast cells as described above. Harvest $\sim 2 \times 10^7$ cells (2 OD_{600}-ml) and label for two-color fluorescence with anti-HA peptide MAb and fluorescently labeled ligand. Label the cells with a saturating amount of fluorescent ligand for a sufficient time to saturate labeling.
2. Pellet, remove, and discard the supernatant, wash with ice-cold BSS, pellet by centrifugation, and keep on ice until ready to begin flow cytometric analysis.
3. Add 2 ml of nonfluorescent ligand preequilibrated to room temperature (or other temperature of interest). The concentration of nonfluorescent ligand should be adjusted to yield a 10 to 100-fold excess over saturated

displayed protein complexes. A conservative estimate of this value may be calculated by assuming $\sim 10^5$ receptors per cell.

4. Analyze the fluorescence of the displaying population (i.e., gated by anti-HA epitope labeling) as a function of time. This may be performed by analysis of aliquots taken at time points and quenched on ice, or kinetic data may be taken on-line with some flow cytometers. Arithmetic mean, median, or peak fluorescence values may be used to extract K_{off}.

After determination of wild-type dissociation rate, time of competition with nonfluorescent ligand yielding the maximal fluorescence discrimination of mutants improved by a defined increment can be calculated from the following equation[3]:

$$k_{off,wt}t_{opt} = 0.293 + 2.05 \log k_r + \left(2.30 - 0.759\frac{1}{k_r}\right)\log S_r$$

where t_{opt} is the optimal duration of competition, S_r is the signal-to-background ratio of flow cytometrically analyzed yeast, and k_r is the minimum fold improvement desired in k_{off}. S_r is best calculated as the ratio of fluorescence of displaying yeast saturated with fluorescent ligand over that of displaying yeast following competition to complete dissociation. Alternatively, mathematical analysis and experience suggest competition times of $\sim 5/k_{off}$ of the wild-type interaction should allow discrimination of mutants improved threefold under most experimental conditions.

Stability Screen by Thermal Denaturation Kinetics

A convenient method for evolving improved stability in a protein makes use of the protein denaturation rate at temperatures up to 50°. Viability of yeast may be maintained at these temperatures by pretreatment at 37° to induce stress response proteins. Prior to screening for improved denaturation kinetics, the wild-type denaturation rate should be measured by the following or similar methods.

1. Grow and induce yeast as described above. Harvest $\sim 2 \times 10^6$ cells per sample in six samples of 100 μl each.
2. Heat shock the samples for 50 min at 37°.
3. Incubate the cells at 50° for various times up to complete denaturation of the protein of interest, and then quench by adding 1 ml of ice-cold BSS.
4. Label the cells for two-color fluorescence as described with 12CA5 MAb and fluorescent ligand or conformation-sensitive antibody.
5. Analyze ligand-associated fluorescence intensity of the displaying fraction (as observed by 12CA5 labeling) as a function of time. Arithmetic mean, peak, or median fluorescence (but not geometric mean) may be fit

as a first-order exponential decay to determine k_{den}, the rate for constant for denaturation.

After determination of k_{den} of the wild-type protein, the optimal duration of 50° incubation for screening libraries can be calculated by using the t_{opt} kinetic equation given above, substituting k_{den} for k_{off}.

Library Sorting

The yeast display library should be oversampled by at least 10-fold to improve the probability of isolating rare clones (e.g., analyze ~10^8 cell from a yeast library with 10^7 clones). At typical flow cytometry sorting rates of 10^3–10^4 cells/sec, screening of 10^8 yeast may be performed in a full work day.

Diagonal sorting windows as shown in Fig. 2A should be drawn to take advantage of quantitative normalization by surface expression level. Trial windows should be drawn until the desired fraction of the population falls within the sort window. Labeled wild-type control cultures should be prepared each day for assistance in setting sort windows and confirmation of progress in library enrichment. In the first sort of a library, it is best to isolate the top 5% of the population in high-recovery mode (enrichment), to ensure retention of rare clones. Ensuing rounds of screening should use windows set to the top 0.1–1% of the population in purifying mode, with stringency increasing each sorting round. Sorted yeast remain viable in buffered sheath fluid for the duration of the sort. Sorted cells should be inoculated into SD + CAA, containing kanamycin (25 mg/ml) and adjusted to pH 4.5 with citrate buffer [sodium citrate (14.7 g/liter), citric acid monohydrate (4.29 g/liter)], to discourage growth of bacterial contaminants. The sorted cells may be passaged in liquid culture directly to another round of induction, labeling, and sorting. If necessary, these SD + CAA liquid cultures may be stored in the refrigerator for several weeks prior to revival at 30° and subsequent induction. We have found substantial enrichment of clones within the sorting window as early as the second screen, consistent with a frequency of approximately 1% of improved clones in the library. More typically, substantial enrichment (i.e., appearance of a minor, flow cytometrically observable population) is obtained by the third screen of a given library. If no enrichment is evident in the sort window by the fourth sort, there would be little justification for progressing to a fifth screen, as single clones from the original library should be enriched by that point. This has not been an issue to date, as we have isolated improved clones from each library screened.

Once the analyzed population exhibits a substantial fraction (>10%)

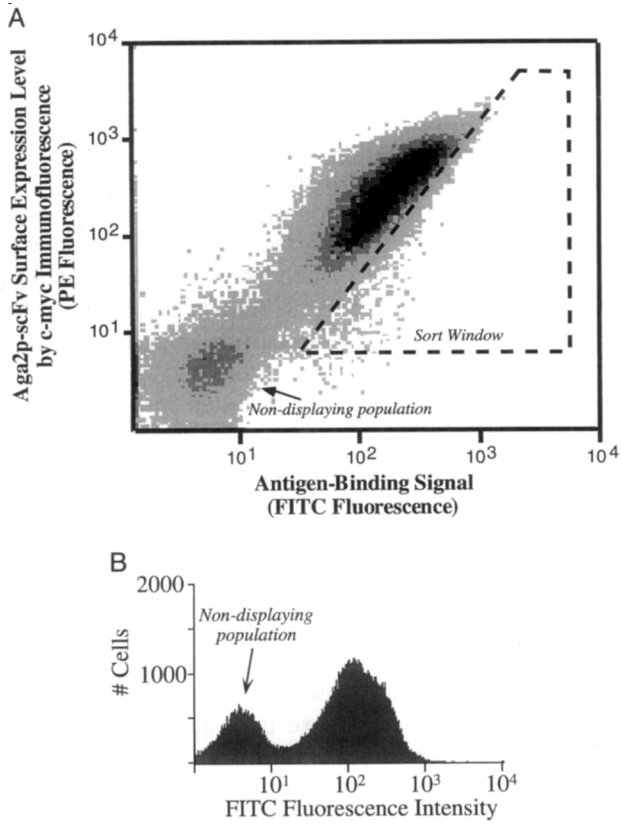


FIG. 2. (A) Flow cytometric analysis of a culture expressing an Aga2p-4-4-20 scFv fusion protein. In the dot plot, each dot represents a single analyzed cell. On the *y* axis, cell surface levels of the HA epitope are shown, while levels of binding to a fluorescein–dextran conjugate are shown on the *x* axis. The ratio of these two signals allows detection of ligand-binding activity normalized by the number of fusion proteins on the cell, enabling sort windows to be set as shown for isolation of improved mutants. (A) More common fluorophore pair is streptavidin–phycoerythrin to detect biotinylated ligand, and FITC-labeled secondary antibody to detect the 12CA5 or 9E10 MAb. (B) The projection of the single-cell fluorescence intensity histogram on of the FITC axis is shown.

within the sort window, the sorted culture may be plated to isolate individual clones. For simplicity, these monoclonal cultures can be analyzed individually by flow cytometry for improved affinity, dissociation rate, or stability. In our experience, the precision of equilibrium constant measurements by flow cytometry is ±40%, and ±10% for the dissociation rate. It is convenient to perform this screening process without the necessity of subcloning, expressing, and purifying the mutant proteins.

Plasmids may be recovered from yeast by rescue to *E. coli* either by use of a commercial kit (Zymoprep; Zymo Research, Orange, CA), or essentially as described previously.[26] Briefly, sorted cells are inoculated into SD–CAA medium and grown overnight at 30°. Cells are pelleted and resuspended in lithium chloride buffer containing Triton X-100, mixed with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1, v/v/v), and mechanically disrupted with zirconium oxide beads. The aqueous phase is collected and further purified using the Wizard DNA Cleanup kit (Promega). Eluted plasmids are transformed into competent *E. coli.* For recovery of a sorted library, it is important to use high-competency *E. coli,* maintain the entire transformed *E. coli* culture in liquid medium, and plate an aliquot to determine the total transformants.

Mutant genes of interest may be subcloned into expression/secretion vectors for yeast in order to solubly express the mutant proteins for further analysis. *Saccharomyces cerevisiae* expression systems for secretion of single-chain antibodies at 20 mg/liter in shake flask culture have been developed.[19]

Summary

The described protocols enable thorough screening of polypeptide libraries with high confidence in the isolation of improved clones. It should be emphasized that the protocols have been fashioned for thoroughness, rather than speed. With library plasmid DNA in hand, the time to plated candidate yeast display mutants is typically 2–3 weeks. Each of the experimental approaches required for this method is fairly standard: yeast culture, immunofluorescent labeling, flow cytometry. Protocols that are more rapid could conceivably be developed by using solid substrate separations with magnetic beads, for instance. However, loss of the two-color normalization possible with flow cytometry would remove the quantitative advantage of the method.

Yeast display complements existing polypeptide library methods and opens the possibility of examining extracellular eukaryotic proteins, an important class of proteins not generally amenable to yeast two-hybrid or phage display methodologies.

Acknowledgments

Helpful comments on the manuscript were provided by C. Graff, M. Kieke, E. Shusta, and J. VanAntwerp.

[26] A. C. Ward, *Nucleic Acids Res.* **18,** 5319 (1990).