Isolating and engineering human antibodies using yeast surface display

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This protocol describes the process of isolating and engineering antibodies or proteins for increased affinity and stability using yeast surface display. Single-chain antibody fragments (scFvs) are first isolated from an existing nonimmune human library displayed on the yeast surface using magnetic-activated cell sorting selection followed by selection using flow cytometry. This enriched population is then mutagenized, and successive rounds of random mutagenesis and flow cytometry selection are done to attain desired scFv properties through directed evolution. Labeling strategies for weakly binding scFvs are also described, as well as procedures for characterizing and 'titrating' scFv clones displayed on yeast. The ultimate result of following this protocol is a panel of scFvs with increased stability and affinity for an antigen of interest.

INTRODUCTION

Yeast surface display is a powerful method for isolating and engineering antibodies to increase their affinity, specificity and stability. Yeast display has been used to engineer antibodies to various antigen targets, including T cell receptors[1], huntingtin protein[2], carcinoembryonic antigen[3] and botulinum neurotoxin[4]. In addition, an antibody to fluorescein has been engineered to femtomolar affinity, the highest affinity reported so far[5]. Yeast display has also been used to engineer other proteins with a variety of applications[6–9].

In the yeast surface display system[10] (**Fig. 1**), the antibody is displayed in a single-chain variable fragment (scFv) format in which the heavy and light chains are connected by a flexible polypeptide linker. The scFv is fused to the adhesion subunit of the yeast agglutinin protein Aga2p, which attaches to the yeast cell wall through disulfide bonds to Aga1p. Expression of the Aga2p-scFv is under the control of a galactose-inducible promoter on the yeast display plasmid, which is maintained in yeast episomally with a nutritional marker, whereas Aga1p is expressed from a chromosomally integrated galactose-inducible expression cassette. Each yeast cell typically displays 1×10^4 to 1×10^5 copies of the scFv, and variations in surface expression can be measured through immunofluorescence labeling of either the hemagglutinin or c-Myc epitope tag flanking the scFv. Likewise, binding to a soluble antigen of interest can be determined by labeling of yeast with biotinylated antigen and a secondary reagent such as streptavidin conjugated to a fluorophore.

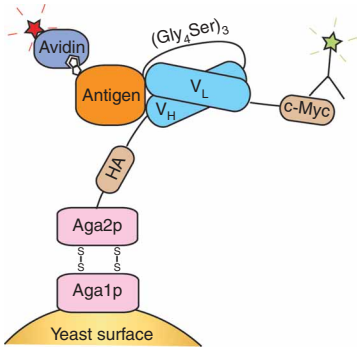

Figure 1 | Yeast surface display. The scFv (cyan) is displayed as an Aga2 (pink) fusion protein on the surface of yeast. Expression can be detected by using fluorescent antibodies binding to the epitope tags (beige), and binding of the scFv to a biotinylated antigen (orange) can be detected using fluorescent avidin (violet). HA, hemagglutinin; V_L, variable light chain; V_H, variable heavy chain; $(Gly_4Ser)_3$, flexible peptide linker.

Yeast surface display offers several advantages for protein-directed evolution. It enables quantitative screening through the use of fluorescence-activated cell sorting, allowing the equilibrium activity and statistics of the sample to be observed directly during the screening process. Furthermore, the antigen-binding signal is normalized for expression, eliminating artifacts due to host expression bias and allowing for fine discrimination between mutants[11]. Antibodies can be engineered for improved stability, as expression is measured directly and has been shown to correlate with the stability of the displayed protein[12]. Using a two-color labeling scheme, with one fluorophore for expression and another for antigen binding, stability and affinity engineering can be accomplished simultaneously. Once maturation is complete, antibody affinity can be conveniently 'titrated' while displayed on the surface of the yeast, obviating the need for expression and purification of each clone. In almost every case for dozens of different antibodies, the binding properties on the yeast cell surface are in quantitative agreement with those measured in solution or by biosensor methods. Finally, the displayed proteins are folded in the endoplasmic reticulum of the eukaryotic yeast cells, taking advantage of endoplasmic reticulum chaperones and quality-control 'machinery'. A theoretical limitation of yeast surface display is the potentially smaller functional library size (about 1×10^7 to 1×10^9) than that of other selection methods (phage display, about 1×10^6 to 1×10^{11}; mRNA-ribosome display, about 1×10^{11} to 1×10^{13}). However, it is difficult to determine the true functional diversity of any display library, and bias-free propagation of yeast libraries has been confirmed over an amplification of 10^{10}-fold (ref. 13). Furthermore, all of these methods greatly undersample the theoretical sequence space of scFv complementary-determining region variation (about 1×10^{80}). To realize the advantages of kinetic

screening and expression normalization, yeast surface display also requires more complex equipment than other display methods. Various technologies for screening recombinant antibody libraries and their relative strengths and weaknesses have been reviewed[14].

Although antibodies can be displayed on yeast in an Fab format[15,16], the protocol presented here details the isolation and subsequent engineering of scFvs starting from an existing non-immune human scFv library[13]. As this library is derived from fully human antibody sequences, the isolated scFvs are potentially suitable for therapeutic development. Once initial candidate scFvs are isolated from this library, directed evolution can be used to obtain scFvs with improved properties, if desired, through several rounds of random mutagenesis and screening. This protocol is also applicable to engineering a previously obtained scFv clone or any protein that can be displayed by yeast for improved binding to a soluble species. If soluble antigen is not available, yeast-displayed scFvs can be 'panned' against mammalian cell monolayers[17] or incubated with mammalian cells in solution and separated by density centrifugation[18], both of which are beyond the scope of this protocol. Although yeast surface display procedures have been described in detail before[19–21], this work includes updated protocols and new methods, as well as a more comprehensive view of the entire antibody isolation and engineering process.

We have provided an outline of the procedure for isolating, engineering and characterizing scFvs against an antigen of interest using yeast surface display (**Fig. 2**). As typical flow cytometry apparatuses can sort 1×10^7 to 1×10^8 cells per hour, magnetic-activated cell sorting (MACS) must first be used to reduce the size of the library from 1×10^9 to about 1×10^7 unique clones (Steps 1–9). The MidiMACS separation protocol described here is adapted from that published by Feldhaus and Siegel[20,22], and an alternative option using Dynal magnetic beads has also been described[23]. If MACS equipment is unavailable, polystyrene or agarose beads conjugated to either the antigen of interest or streptavidin can also be used for initial rounds of sorting in a column format (optimal conditions for a given system should be determined by the user). Steps 11–20 outline a general protocol for labeling yeast cells with fluorescent reagents. This same protocol is used to analyze the display level of the MACS-sorted population (Step 10), to label cell populations for sorting by flow cytometry (Step 21) and to analyze flow cytometry–sorted cell populations (Step 26). Once the MACS-sorted population has been analyzed to ensure scFv expression, the population is further enriched using flow cytometry (Steps 21–25). If the yeast population does not seem to be enriching for antigen binders, a streptavidin preloading protocol (**Box 1**) can be used to increase the antigen avidity and thus the chances of successful enrichment. Next, the flow cytometry–sorted population is analyzed (Steps 26–28). Once it is determined that the scFvs are specific for the antigen of interest, they are engineered for increased affinity and/or stability using random mutagenesis through error-prone PCR with nucleotide analogs (Steps 29–35)[24]. This mutagenesis protocol is preferred because it allows control of the mutation frequency by alteration of the number of PCR cycles and yields both transition and transversion mutations. Other methods of generating diversity, such as DNA shuffling[25,26], are also compatible with the yeast display system, and additional methods of introducing library diversity have been reviewed[27]. For engineering a single scFv or protein sequence, the DNA of interest must first be cloned into a yeast surface display vector, and then the protocol can be followed beginning at Step 29.

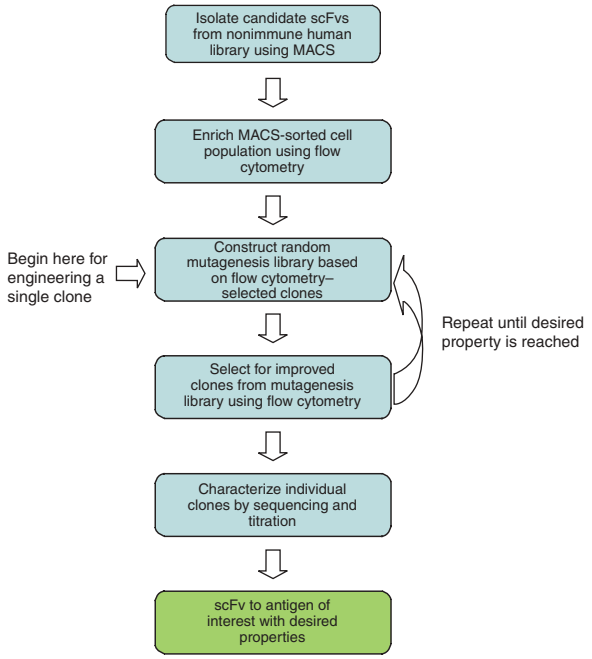

Figure 2 | The antibody isolation and engineering process.

The mutagenized yeast library is created by transformation of yeast with linearized vector and the error-prone PCR product, and *in vivo* homologous recombination occurs between the vector and PCR insert to generate the display plasmid. The protocol for yeast transformation by electroporation described in Steps 36–48 has been adapted from that published by Meilhoc *et al.*[28]. As the PCR insertion products are also homologous to each other, additional recombination events occur between inserts and lead to greater library diversity[26]. If electroporation equipment is unavailable, the yeast can also be transformed using lithium acetate, which may yield slightly lower transformation efficiencies[29]. Rounds of mutagenesis followed by flow cytometry sorting are done until the scFv population reaches the desired property. Finally, individual clones of the population are characterized in depth by titration of scFvs displayed on the yeast surface (Steps 51–63). This protocol is similar to the general protocol for labeling yeast cells described in Steps 11–20.

MATERIALS

REAGENTS

- Nonimmune human scFv yeast library (available by request from Pacific Northwest National Laboratories at http://www.sysbio.org/dataresources/singlechain.stm)
- SDCAA media and plates; SGCAA media (see REAGENT SETUP)
- Yeast strain EBY100 (Invitrogen, cat. no. C839-00; strain information, **Supplementary Methods** online)
- Yeast display vector pYD1 (Invitrogen, cat. no. V835-01) or pCTCON2 (available by request; plasmid map, **Supplementary Fig. 1** online), if engineering a single clone
- PBSM and PBSF buffer (see REAGENT SETUP)