Ligand Engineering via Yeast Surface Display and Adherent Cell Panning

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Abstract
High-throughput ligand discovery and evolution – via genotype-phenotype linkage strategies – empower molecularly targeted therapy, diagnostics, and fundamental science. Maintaining high-quality target antigen in these selections, particularly for membrane targets, is often a technical challenge. Panning yeast-displayed ligand libraries on intact mammalian cells expressing the molecular target has emerged as an effective strategy. Herein we describe the techniques used to select target-binding ligands via this approach including the use of target-negative cells to deplete non-specific binders and avidity reduction to preferentially select high-affinity ligands.

Keywords
avidity, cell panning, depletion, ligand, protein engineering, specificity, yeast surface display

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1. Introduction

Protein engineering uses rational design and directed evolution to modulate the function or physical characteristics of proteins. Protein ligands that selectively bind to molecular targets have been successfully applied for clinical diagnostics, such as targeted molecular imaging and the analysis of blood and urine, as well as targeted therapy through inhibition, drug delivery, radioisotope delivery, and immune system engagement [1–5]. Recent advances in genomics, proteomics, and chemical biology have drastically increased our knowledge of disease states on the molecular level, increasing the number of characterized, clinically relevant biomarkers [6]. With this increase, a new demand has emerged for engineered proteins that target these biomarkers.

To meet this demand, a variety of high-throughput screening methods for binding activity have been employed. A key principle of high-throughput protein screening is genotype-phenotype linkage – a method by which the function or biophysical form of a protein can be linked to its corresponding DNA. This facile approach allows million to billions of proteins to be screened for binding activity with simple identification of successful candidates by sequencing of the attached gene. Several common genotype-phenotype linkage strategies - including ribosome display [7–9], mRNA display [10–12], phage display [13–15], yeast display [16,17], and mammalian cell display [18,19] - have been successfully applied for the selection and evolution of engineered binding proteins. While the library sizes of mammalian cell (10^3-10^6 transformants) [18] and yeast cell (10^9 transformants) [16] surface display are smaller than those of mRNA, ribosome, or phage display, both yeast and mammalian cell surface display are of particular interest because of multiple benefits including eukaryotic protein processing [20,21], the efficiency of homologous recombination for library assembly (in yeast) [22], and their ability to express 10^3-10^5 ligand copies per cell [23]. This high multivalency of ligands, coupled with multivalent target expression on the antigen-expressing cells, can provide avidity – the strength of multiple binding interactions – allowing ligands with

very weak affinity to be recovered in a way not possible by monovalent selection techniques (Figure 1). In part due to these advantages, yeast surface display has been extensively applied to the selection and evolution of multiple engineered binding protein scaffolds against a variety of target molecules [16,24,25].

Experimental protein engineering selection strategies require the use of the biomarker of interest to isolate binding proteins. However, many clinically relevant biomarkers possess hydrophobic transmembrane domains, making them difficult to work with in an aqueous system. In lieu of full-length proteins, recombinant extracellular domains are often used as analogs during ligand selections [26–32]. A limitation of this method is that ligands isolated by recombinant selections may not bind to full length protein expressed in intact cells [33,34]. These failures indicate that recombinant analogs may not fully recapitulate the true cellular target. While the issue remains largely unresolved, factors such as protein stability, folding, and modification are likely to blame for these failures. Loss of protein stability can induce misfolding of the protein, whereby the polypeptide is of the proper sequence but adopts an alternate conformation relative to the native protein. The use of such misfolded proteins can induce experimental failure due to a loss of proper biological activity and structure [33,34]. Additionally, non-natural epitopes are often introduced in the form of additional chemical tags required for purification or immobilization, or truncations of the transmembrane domain. In these cases, the isolation of tag-binding ligands can be problematic, even when depletion strategies are employed [28,35]. These factors motivate the need for an alternate selection approach where full-length protein is expressed in its native conformation, which would enhance selection of genuine cell-binding ligands.

To overcome these issues and advance ligand discovery by providing a source of full-length protein in its natural conformation, selection campaigns against intact mammalian cells

have been investigated (Figure 2). These have been employed successfully using both phage and yeast surface display to generate antibody fragments that bind to cancer and blood-brain barrier targets[36–45,46]. Along with these noted successes, limitations on the fractional yield of ligand-displaying yeast [47] and the generation of ligands not specific to the desired cell line or target remain as challenges for the broader use of these methods [35].

Yield, which is strongly correlated to the target expression on the mammalian cell, is severely hindered in these systems, often to the point of ineffectiveness, when using cells with low-to-medium ($\leq 10^5$ targets/cell) expression [47]. Previous efforts to optimize selections against adherent mammalian cells and redesign the yeast surface display construct have improved ligand recovery, but in one thorough study ligands of micromolar affinity were robustly recovered only on cells that expressed on the order of one million targets per cell [47]. This limits the applicability of selections against mammalian cells, as the affinity of a ligand in a naïve library may be weak and most mammalian cell lines do not express sufficient biomarkers to provide effective recovery.

Compounding this problem, yeast display selections against mammalian cells often lead to isolation of ligands with activity to an undesired target on the presented cell line, allowing biomarker-specific, off-target specific, and non-target specific ligands to simultaneously enrich. While target-specific ligands have been isolated in several cases, isolated binders are often accompanied by a high frequency of non-target specific binding ligands [35]. Depletions against target-negative mammalian cell monolayers, such as those used to reduce nonspecific binding in phage display cell panning [43], have shown ambiguous efficacy in mitigating this problem. However, preliminary work using disadhered, target-negative mammalian cells has shown promise in providing effective depletion on non-target specific ligands [35,42].

Several techniques presented herein are used to effectively deplete non-target specific ligands and provide the ability to reliably isolate specific ligands from cell-based selections. Cell panning with mammalian cell pre-blocking is recommended when screening for specific ligands to a tissue, cell line, or cell surface target of interest from a naïve library to limit nonspecificity; however, solely enrichment-based cell panning can be used as an alternative in cases where an appropriate negative cell line is not readily available. Sequential pannings can be conducted until a suitable endpoint of diversity and enrichment are reached for the particular application. The use of titratable avidity reduction allows for finer discrimination of recovered ligand affinities and is suited for use after the initial screening of a naïve library to provide a further enrichment advantage to higher affinity clones or in the case of more focused libraries where the expected average affinity of a clone is substantially higher than that of a naïve library.

Collectively, these techniques comprise an effective strategy for discovery and evolution of ligands against biomedically important, yet challenging, cell membrane targets.

2. Materials

2.1 Cell Panning of Yeast Surface Displayed Libraries

Media, Buffers, and Reagents

1. pCT-40 (yeast surface display vector designed for cell panning; available upon request; Figure 3)
2. Growth medium for target-expressing cell line of interest (see **Note 1**).
3. PBSACM buffer: 1x phosphate-buffered saline (PBS), pH 7.4 supplemented with 1% w/w bovine serum albumin, 0.1 mM $CaCl_2$, and 0.1 mM $MgCl_2 \bullet 6H_2O$; sterile filter (**Note 2**).
4. SD-CAA medium: 16.8 g/L sodium citrate dihydrate, 3.9 g/L citric acid, 20.0 g/L dextrose, 6.7 g/L yeast nitrogen base, and 5.0 g/L casamino acids with deionized water; sterile filter.
5. SG-CAA medium: 10.2 g/L sodium phosphate dibasic heptahydrate, 8.6 g/L sodium phosphate monobasic monohydrate, 19.0 g/L galactose, 1.0 g/L dextrose, 6.7 g/L yeast nitrogen base, and 5.0 g/L casamino acids with deionized water; sterile filter.

6. SD-CAA plates: Combine 16.8 g/L sodium citrate dihydrate, 3.9 g/L citric acid, 15 g/L agar with 900 mL/L of deionized water; Autoclave; Separately combine 20.0 g/L dextrose, 6.7 g/L yeast nitrogen base, and 5.0 g/L casamino acids with 100 mL/L of deionized water and filter sterilize into autoclaved contents once they have cooled to 50 °C; Pour 25 mL into each Petri dish)
7. PBS, pH 7.4, sterile filtered
8. Trypsin-EDTA

Equipment and Consumables

1. Stationary incubator with CO_2 control at 37°C (for mammalian cell growth)
2. Biosafety cabinet for mammalian cell culture
3. Shaking incubator at 30°C, 220 rpm (for yeast liquid culture growth)
4. Stationary incubator at 30°C (for yeast plate culture growth)
5. Microcentrifuge with rotor to accommodate 1.7 mL tubes (for preparing yeast for selections)
6. Centrifuge with bucket rotor to accommodate 15 mL or 50 mL conicals (for preparing yeast for selections)
7. Spectrophotometer for measuring optical density at 600 nm (OD600) (see **Note 3**)
8. Hemocytometer or other instrument for counting mammalian cells
9. Mammalian cell culture vessel; *e.g.* T-75 cell culture flask
10. 6-well polystyrene cell culture treated plates
11. 14 mL polystyrene round-bottom tubes (for yeast liquid culture growth)
12. 250 mL baffled flasks (for yeast liquid culture growth)
13. Pipette controller
14. Serological pipettes
15. Pipette tips
16. Cell scrapers
17. 1.7 mL tubes
18. Petri dishes
    (all plates, tubes, flasks, pipettes, tips, scrapers, and dishes must be sterile)

Cells

1. *Saccharomyces cerevisiae* yeast surface display strain EBY100 (available from ATCC) [16]
2. Target-expressing adherent cell line

2.2 Cell Panning with Depletion by Mammalian Cell Pre-Blocking

Same as 2.1, with the following addition:

Cells

1. Target-negative cell line. *Cell line(s) with similar expression profiles but lacking target are preferable.*

2.3 Cell Panning with Titratable Avidity Reduction

Same as 2.1, with the following additions:

Media, Buffers, and Reagents

1. 10 mM Tris buffer, pH 7.5: 1.24 g/L Tris-HCl and 0.26 g/L Tris base with deionized water. Validate pH. Adjust with 10 mM Tris-HCl or 10 mM Tris base as necessary. Sterile filter.
2. Dithiothreitol (DTT) powder
3. Mouse anti-c-Myc antibody 9E10 (BioLegend)
4. Goat anti-mouse Alexa Fluor 647 antibody (Thermo Scientific)
5. Quantum Simply Cellular anti-mouse IgG kit (Bangs Laboratories)

Equipment and Consumables

1. Flow cytometer
2. Flow cytometry tubes compatible with available flow cytometer

3. Methods

3.1 Cell Panning of Yeast Surface Displayed Libraries

This technique is appropriate for selections of ligands – from both naïve and enriched libraries – that bind to adherent mammalian cells. If available, it is recommended that this protocol be paired with a strategy to deplete non-target specific binding ligands, including mammalian cell pre-blocking (Section 3.2) or ligand selection with purified recombinant antigen. In instances where the antigen is not known (e.g. panning against patient-derived tumors) or no appropriate target-negative cell line exists, this protocol can be used with the understanding that an abundant background of non-target specific ligands will be recovered. Enrichment and yield may vary based on ligand affinity, target expression, and other factors (Figure 4) [47].

3.1.1. *Target-expressing mammalian cell preparation.*

1. Seed target-expressing mammalian cell line in 6-well plates according to their recommended seeding density (see **Notes 4 and 5**). *One well of mammalian cells should be grown per 10^8 yeast to be sorted.*
2. Culture cells at 37 °C in a humidified atmosphere with 5% CO_2 in the appropriate growth medium. Grow cells to 70-90% confluence, as quantified by light microscopy.

3.1.2. *Yeast cell preparation*.

1. Grow the library of plasmid-harboring EBY100 in SD-CAA medium at 30 °C with shaking at 220 rpm for at least two doublings (see **Note 3)**.
2. While the yeast are in logarithmic phase (OD_{600nm}<6), pellet yeast (8,000g for 1 min) and aspirate medium. Resuspend yeast in fresh SG-CAA to an initial

OD_{600nm}≤1. Grow at 30 °C, 220 rpm for at least 8 hours to induce ligand display. Use yeast immediately or store yeast at 4 °C (see **Note 6**).

3.1.3. *Cell Panning Selection.*

1. Measure an appropriate amount of yeast for selection (see **Note 3)**. Wash yeast once with ice cold PBSACM by pelleting at 8,000g for 1 minute followed by aspirating the medium. Resuspend in 1 mL of PBSACM before again pelleting and aspirating the supernatant. Leave yeast as a wet pellet on ice.
2. To prepare the mammalian cells for selection, aspirate the cell culture medium from each well using a serological pipette (see **Note 7**). Wash the cells by gently adding 1 mL ice cold PBSACM down the wall of the well using a serological pipette. Gently tilt plate 5 times (see **Note 8**). Aspirate the buffer using a serological pipette. Visually check the plate surface to ensure mammalian cells remain adhered to the plate surface (see **Note 9**.)
3. Resuspend pelleted yeast using ice cold PBSACM to 10^8 yeast/mL (See **Note 10**).
4. Apply 1 mL of yeast to each well of target-expressing mammalian cells using a 1 mL pipette (see **Note 11**).
5. Incubate this mixture statically for 15 minutes at 4 °C.
6. Aspirate unbound yeast. Gently add 1 mL of ice cold PBSACM to each well of selection using a serological pipette. Gently tilt the plate 25 times and nutate it 5 times by hand (see **Note 12**). Repeat this process 3 times for a total of 4 washes this way. For the fifth wash, add 1 mL of ice cold PBSACM using a serological pipette and nutate the plate 10 times. Aspirate the supernatant.
7. Add 1 mL of SD-CAA using a 1 mL pipette. Remove adhered mammalian cells and bound yeast by thoroughly scraping the plate surface using a cell scraper. Pipette all liquid and place into a 1.7 mL tube.
8. Plate dilutions of the final yeast population on SD-CAA plates to quantify yield (see **Note 13**). Culture final population in 5 mL SD-CAA in a 14 mL polystyrene round-bottom tube overnight at 30 °C with shaking.

3.2 Cell Panning with Depletion by Mammalian Cell Pre-Blocking

This technique is appropriate for selection from both naïve and enriched libraries. Previous experience with this protocol shows that it is effective in lessening the background of non-target specific ligands while not significantly impeding the enrichment of target specific ligands (Figure 5). For preparation of target-expressing mammalian cells and yeast, follow sections 3.1.1 and 3.1.2, respectively.

3.2.1. *Target-negative mammalian cell preparation.*

1. Seed target-negative mammalian cell line in T-75 flask according to their recommended seeding density (see **Notes 4 and 5**). Culture mammalian cells at 37 °C in a humidified atmosphere with recommended CO_2 in the

appropriate growth medium. Grow to 70-90% confluence, as quantified by light microscopy.

2. Aspirate the cell culture medium from the flask using a serological pipette. Wash the cells by gently adding 5 mL PBS using a serological pipette. Disadhere cells by trypsin-EDTA treatment. Neutralize trypsin using serum-containing cell culture medium. Centrifuge cells at 500g for 3 minutes. Aspirate medium/trypsin mixture. Resuspend cells in serum-containing cell culture medium.
3. Count the disadhered cells using a hemocytometer.
4. Harvest 10^6 cells per 10^8 yeast required. Centrifuge mammalian cells at 500g for 3 minutes at 4°C. Remove supernatant. Resuspend cells in 5 mL of ice cold PBSACM. Repeat twice.
5. Resuspend mammalian cells in ice cold PBSACM to a concentration of 10^6 cells/mL, with a total volume equal to 1 mL/well.

3.2.2. *Cell selection.*

1. Resuspend pelleted yeast to a concentration of 10^8 yeast/mL in PBSACM with 10^6 target negative mammalian cells per mL. Split the yeast and mammalian cell mixture into 1 mL aliquots in 1.7 mL tubes.
2. Allow the yeast and mammalian cells to rotate for 2 hours at 4 °C.
3. Gently apply 1 mL of yeast and mammalian cell mixture to each well of target-expressing mammalian cells using a 1 mL pipette (see **Note 11**).
4. Incubate selection mixture statically for 15 minutes at 4 °C.
5. Aspirate unbound yeast and target-negative mammalian cells.
6. Gently add 1 mL ice cold PBSACM dropwise to each well of selection using a serological pipette. Gently tilt the plate 25 times and nutate it 5 times by hand (see **Note 12**). Repeat this 3 times for a total of 4 washes this way. For the fifth wash, add 1 mL of ice cold PBSACM using a serological pipette and nutate the plate 10 times. Aspirate the supernatant.
7. Add 1 mL SD-CAA using a 1 mL pipette. Remove adhered mammalian cells and bound yeast by thoroughly scraping the plate surface using a cell scraper. Pipette all liquid and place into a 1.7 mL tube.
8. Plate dilutions of the final yeast population on SD-CAA plates to quantify yield (see **Note 13**). Culture final population in SD-CAA overnight at 30 °C with shaking.

3.3 Cell Panning with Titratable Avidity Reduction

This technique is appropriate for selections from enriched pools of target specific ligands with lower binding affinity than desired. Enrichment of ligands with low nanomolar affinity, if present in the unenriched pool, can be expected after multiple rounds of Cell Panning with Titratable Avidity Reduction. This protocol may not be appropriate for disulfide bonded ligands (e.g. antibody fragments) as it relies on reduction with DTT. For these ligands, avidity can be reduced enzymatically through digest with Factor Xa protease

in place of the DTT reaction. For preparation of target-expressing mammalian cells and yeast, follow sections 3.1.1 and 3.1.2, respectively.

3.3.1. *Titratable avidity reduction*.

1. Prepare the appropriate number of yeast in five separate tubes such that each tube contains the full desired library oversampling.
2. Pellet yeast (8,000 g for 1 min) and wash twice with 10 mM Tris buffer pH 7.5. Leave yeast as a wet pellet.
3. Freshly prepare DTT solutions by dissolving DTT powder in 10 mM Tris buffer pH 7.5 and serial diluting in 10 mM Tris buffer pH 7.5, yielding 2.5, 5, 7, and 9 mM DTT (see **Note 14**).
4. Resuspend four yeast library samples in each of the four DTT solutions to a concentration of $5x10^6$ yeast/20 µL DTT solution. Resuspend the fifth yeast library sample in 10 mM Tris buffer pH 7.5 without DTT at the same cell concentration as the DTT samples.
5. Incubate yeast for 20 min in a 30°C static incubator.
6. Pellet yeast (8,000 g for 1 min) and wash twice with PBSACM.
7. Resuspend yeast in 1 mL PBSACM. Remove 20 µL yeast from each sample for assessment of ligand expression by flow cytometry.

3.3.2. *Assessment of ligand expression by flow cytometry*.

Labeling of yeast to assess ligand expression should be completed concurrently with step 3.3.3.

1. In addition to the yeast recovered from step 3.1.2, prepare a sample of $1x10^5$ yeast that are not treated with DTT but are also not labeled with mouse anti-c-Myc antibody (secondary only control) by pelleting yeast (8,000 g for 1 min) and washing once with 1 mL PBSACM.
2. Pellet yeast and aspirate the supernatant.
3. Resuspend yeast with 20 µL of mouse anti-c-Myc antibody (diluted 1:100, 5 µg/mL final concentration).
4. Incubate for 20 min at room temperature.
5. Pellet yeast, aspirate supernatant, and wash with 1 mL PBSACM.
6. Resuspend yeast with 20 µL of goat anti-mouse Alexa Fluor 647 antibody (diluted 1:1,000, 10 µg/mL final concentration).
7. Incubate for 15 min at room temperature in the dark.
8. Pellet yeast, aspirate supernatant, and wash with 1 mL PBSACM.
9. Resuspend yeast in 200 µL of PBSACM and analyze fluorescence of at least 10,000 events using an appropriate flow cytometer.

3.3.3. *Generating a fluorescence calibration curve to assess ligand expression*.

Labeling of the Quantum Simply Cellular anti-mouse IgG beads should be performed concurrently with labeling of yeast in step 3.3.2.

1. In a single tube, pool 5 µL each of Beads B, Beads 1, Beads 2, Beads 3, and Beads 4. Add 1 mL PBSACM to the bead pool. Pellet the beads (2,500 g for 2.5 min) and carefully aspirate the supernatant.

2. Resuspend beads in 20 µL mouse anti-c-Myc antibody (diluted 1:100, 5 µg/mL final concentration).
3. Incubate for 20 min at room temperature.
4. Pellet the beads, aspirate the supernatant, and wash with 1 mL PBSACM.
5. Resuspend beads in 20 µL of goat anti-mouse Alexa Fluor 647 (diluted 1:1,000, 10 µg/mL final concentration).
6. Incubate for 15 min at room temperature in the dark.
7. Pellet the beads, aspirate the supernatant, and wash with 1 mL PBSACM.
8. Resuspend the beads in 100 µL of PBSACM and analyze fluorescence of at least 5,000 events using the appropriate flow cytometer.

3.3.4. *Analysis of ligand expression data*.

The fluorescence standard curve and absolute quantification of ligand expression are generated using a Microsoft Excel spreadsheet specific to each individual bead lot provided by the manufacturer of the Quantum Simply Cellular anti-mouse IgG kit.

1. With flow cytometry analysis software, generate a dot plot for the Quantum Simply Cellular beads of forward scatter (FSC) vs side scatter (SSC). Draw a gate around the singlet beads (the most abundant population) as in Figure 6B.
2. Apply this gate to a histogram of Alexa Fluor 647 fluorescence.
3. Using a histogram gate, assess the median fluorescence of each individual peak as in Figure 6C.
4. Insert each individual fluorescence value into the spreadsheet corresponding to each individual bead population. This will generate the standard curve. *The R^2 value should be at least 0.99 to continue.*
5. Generate a density plot for the secondary-only control yeast sample of FSC vs SSC. Draw a gate around the most abundant population (corresponding to singlet yeast) as in Figure 6D.
6. Apply this gate to a histogram of Alexa Fluor 647 fluorescence as in Figure 6E.
7. Assess the median fluorescence of all yeast.
8. Repeat this process for each of the treated yeast samples.
9. In an individual row of the spreadsheet, enter the median fluorescence of a single yeast sample, subtracting the median fluorescence of the secondary-only control. The spreadsheet will automatically generate an absolute ligand expression level for each treated sample.
10. Continue to cell selection with a yeast sample expressing 3,000-6,000 ligands/cell following the protocol from step 3.1.3.

4. Notes

1. Appropriate growth medium will depend upon the cell line. Please refer to the medium formula recommended by your cell line provider for optimal results.
2. All reagents must be sterile filtered (0.2 µm) into autoclaved glassware prior to use.

3. The number of yeast used in a selection should be at least 10-fold of the library diversity for all steps. Reference [48] gives equations to calculate the amount of oversampling needed to expect to screen a percentage of your library as well as the amount of oversampling required to have degree of certainty that you have sampled all possible variants. Briefly, three-fold oversampling is required to expect to sample 95% of your initial library, while roughly five-fold oversampling is required to expect to sample 99% of your initial library. Small libraries ($<10^7$ variants) can be grown in 5 mL SD-CAA in 14 mL polystyrene round-bottom tubes. Large libraries ($>10^7$ variants) can be grown in 50-100 mL SD-CAA in 250 mL baffled flasks.
4. Culture plates can be treated with poly-L-lysine prior to seeding to encourage mammalian cell adhesion without significantly increasing the background binding of yeast cells.
5. The yield of bound yeast is strongly correlated to target expression on the cell surface in this assay. Expression levels greater than 10^4 targets per cell are recommended to see positive enrichment of binding yeast.
6. Depending on ligand stability, induced yeast can be stored for multiple weeks
7. Aspiration is conducted by tilting the plate until liquid pools on one side of the well. The liquid is slowly drawn into the serological pipette to minimize shear on the mammalian cell monolayer and prevent the removal of adhered mammalian cells.
8. A single tilt is conducted by rotating the plate around its middle axis about 30° from level in either direction.
9. In cases where mammalian cell lines fail to remain adhered, gentler pipetting and tilting may reduce the shear experienced by the cell surface and lower the probability of cell detachment. Poly-L-Lysine treatment of culture plates may enhance cell attachment while not significantly affecting the background retention of nonbinding yeast.
10. Fresh tips must be used when drawing clean buffer and tips must be changed between cell lines or yeast libraries when conducting parallel selections to minimize cross-contamination.
11. When adding liquid to the wells, place plate on a level surface and put the pipette tip against the well wall. Slowly inject liquid to minimize shear on the mammalian cell surface.
12. A single nutation is conducted similar to the motion of a laboratory rocker, causing the liquid to gently swirl along the wall of the plate.
13. Calculate dilutions based upon the expected yield of your sort (on the order of 0.01-0.1% for initial sorting of naïve libraries and up to the order of 1-10% depending upon the expression level and average affinity of your library) so that colonies are countable (<100 colonies). Do not dilute more than 100x at a time.
14. DTT must be prepared fresh daily as it will oxidize in solution over time (half-life at pH 7.5 and 20°C is 10 hours) [49].

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Figure Legends

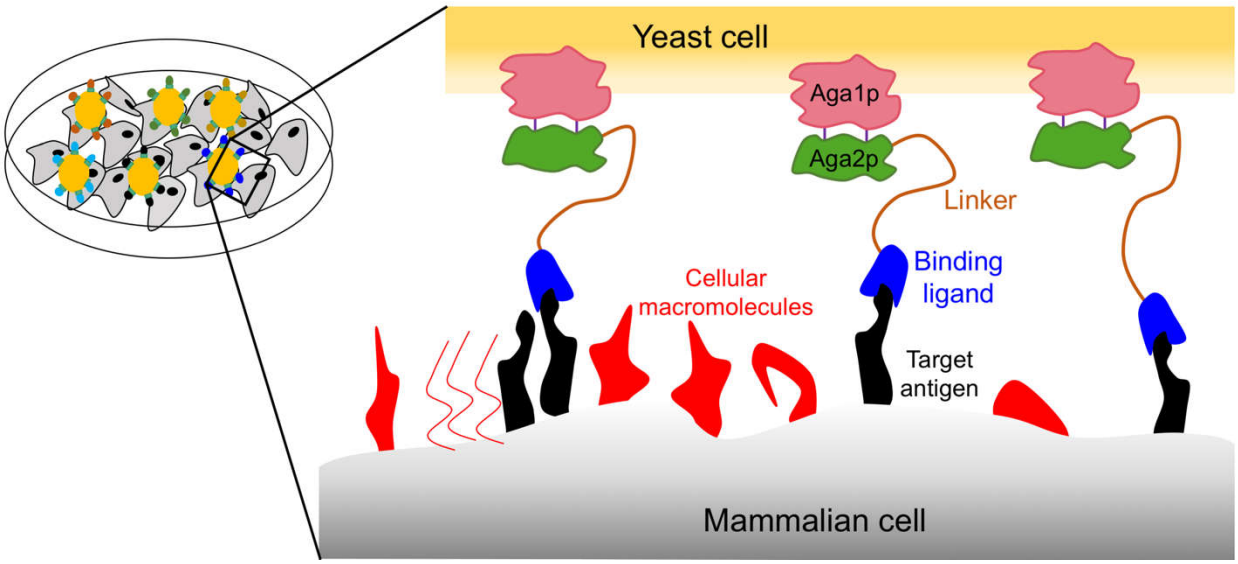

Figure 1. *Schematic of cell panning with yeast surface display*. A candidate binding ligand (blue) is produced as a fusion to yeast mating protein agglutinin 2 (Aga2p, green) linked by a flexible polypeptide (orange). This fusion is secreted and anchored to the yeast cell wall by disulfide linkage to yeast mating protein agglutinin 1 (Aga1p, pink). When brought into contact with target-

expressing cells, the displayed ligands can interact with their binding partners (black) in an avid fashion. Additional cellular macromolecules (red) are also present, which can also interact with displayed ligands.

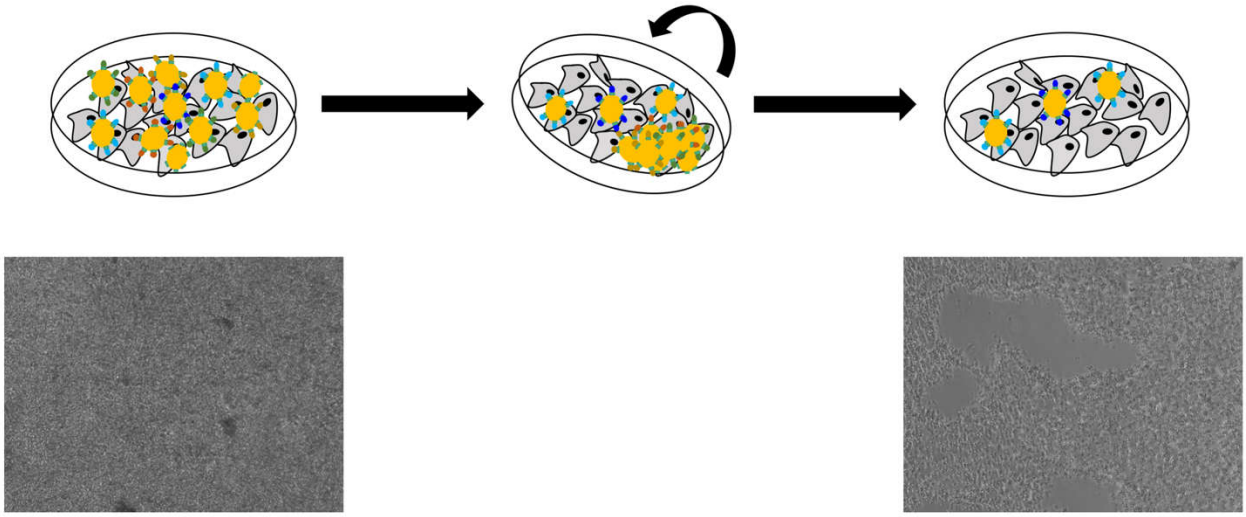

Figure 2. *Schematic of the cell panning selection process*. A yeast display library is introduced to a mammalian cell monolayer. After sufficient incubation, non-binding yeast are washed away, leaving only bound yeast on the mammalian cell monolayer. Sequential application of this approach will further enrich binding clones.

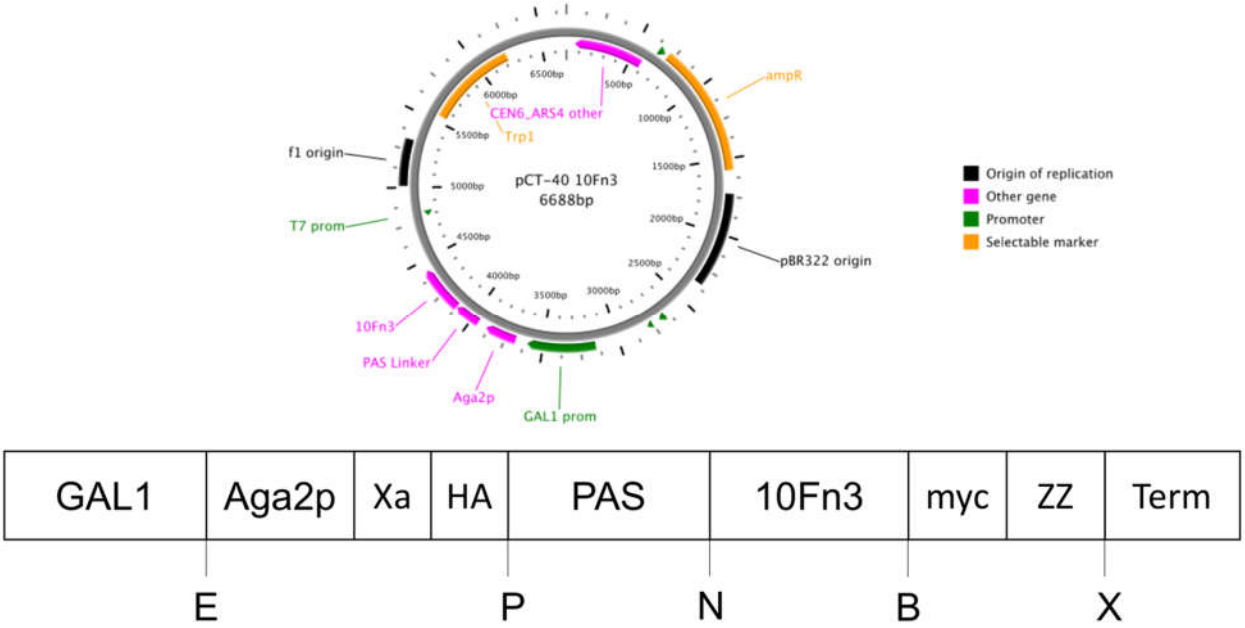

Fig. 3 pCT-40 vector. *CEN6-ARS4* centromeric sequence 6 and autonomously replicating sequence H4, *amp prom* ampR promoter, *ampR* ampicillin resistance selection marker, *pBR332 origin* PBR332 origin of replication, *lac prom* lac promoter, *T3 prom* T3 promoter, *GAL1* GAL1 promoter, *Aga2p* agglutinin 2p protein subunit, *Xa* factor Xa cleavage site, *HA* hemagglutinin epitope, *P* PstI site, *PAS* flexible PAS-motif linker, *N* NheI site, *10Fn3* wild-type tenth human fibronectin domain 3 gene, *B* BamHI site, *myc* c-Myc epitope, *ZZ* TAATAG stop codons, *X* XhoI

site, *Term* alpha mating factor terminator, *f1 origin* f1 origin of replication, *Trp1* Trp1 gene. The lower representation is not to scale.

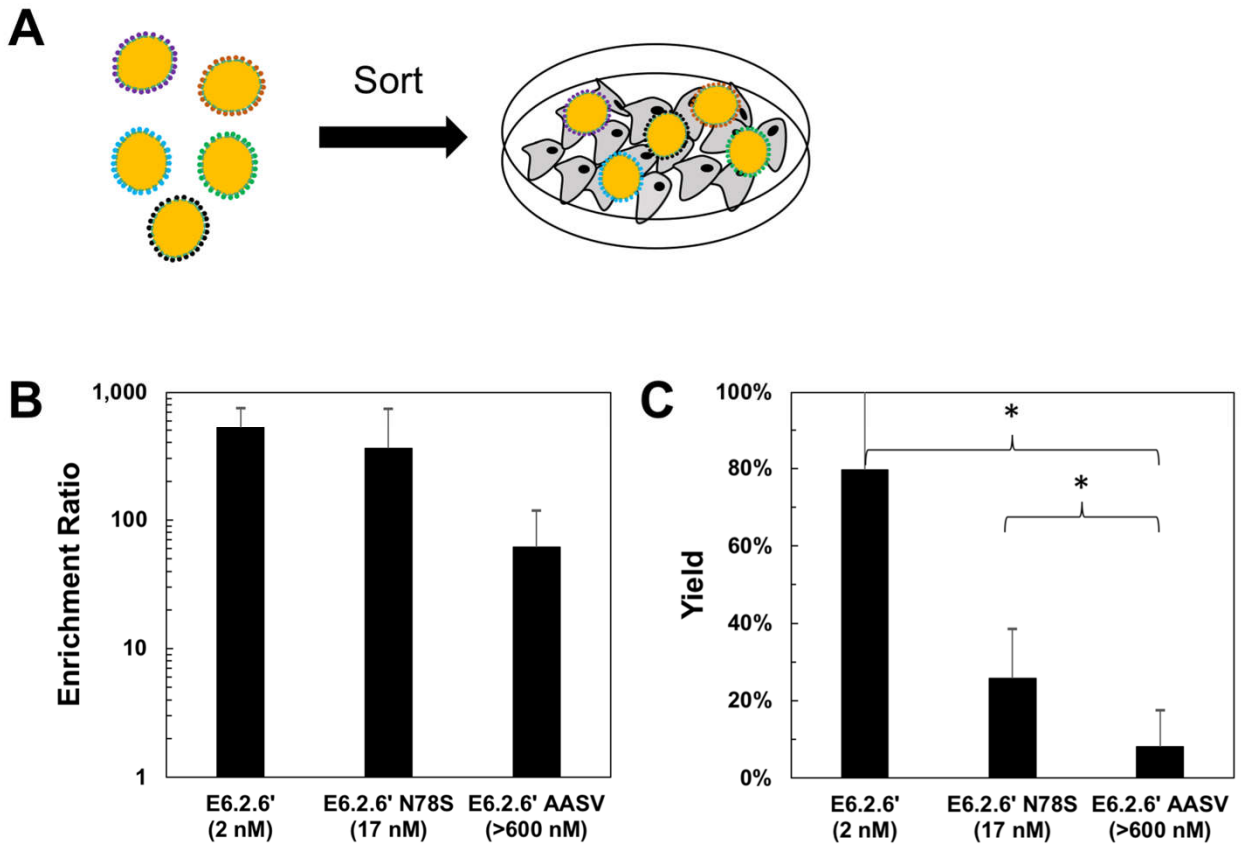

Figure 4. *Cell panning enrichment of yeast surface displayed binding ligands.* Yeast expressing a ligand library are introduced to a target-expressing mammalian cell monolayer (A). Previous experience with an affinity series of EGFR-binding fibronectin domains panned against EGFR-high MDA-MB-468 breast cancer informs experimental expectations. Binding clones with affinities of low nM to μM were effectively enriched from a dilute pool (1:1,000 ligand-displaying yeast : non-displaying yeast) (B). High- and mid-affinity binding resulted in significantly higher yield ($p<0.05$) than low-affinity binding (C).[47]

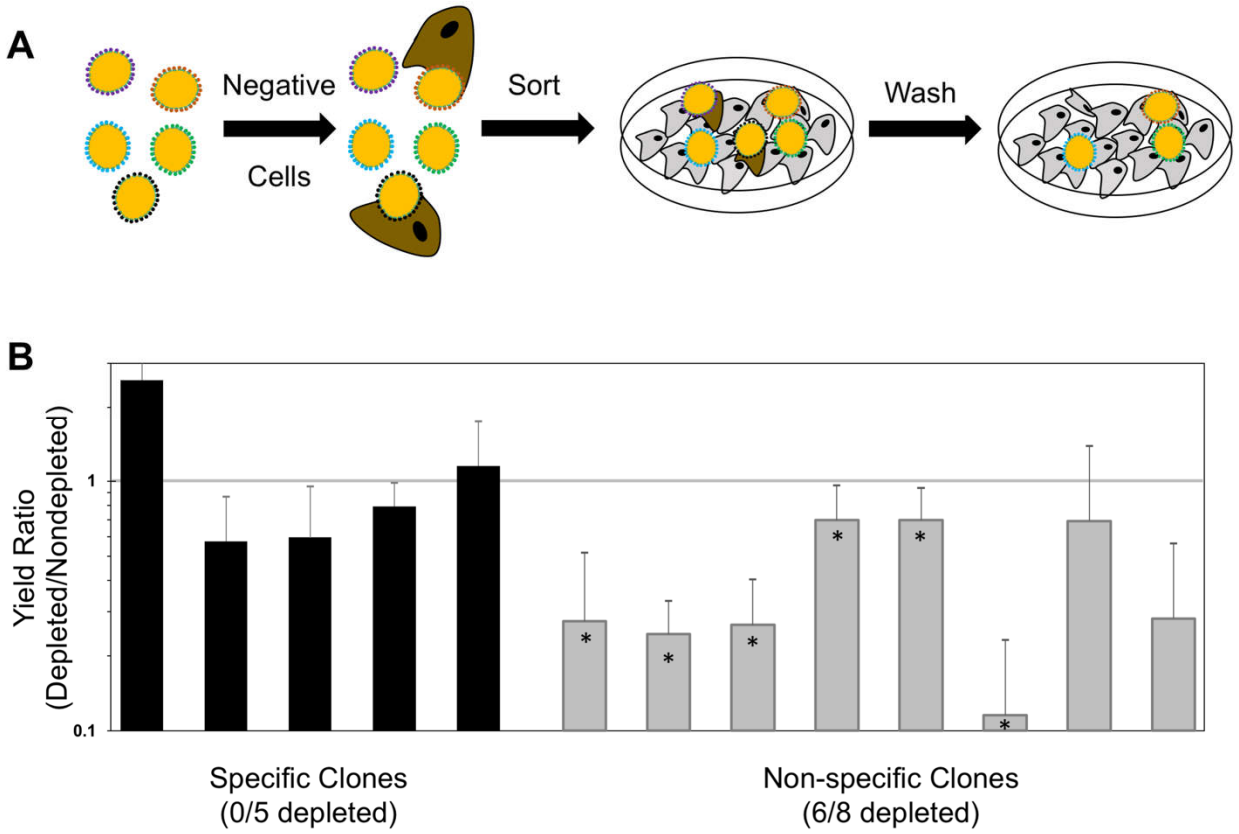

Figure 5. *Cell panning with depletion by mammalian cell pre-blocking.* Yeast expressing a ligand library are incubated with disadhered target-negative mammalian cells. This mixture is then panned against target-positive mammalian cells, resulting in depletion of non-target specific binding yeast and enrichment of target specific interactions (A). Previous experiments panning a panel of yeast-displayed affibody domains on target-expressing cells as per 3.1 (nondepleted) or 3.2 (depleted) using target-negative cells as a depletion agent inform expectations. The yields of specific ligands (black) are not significantly different between the two procedures. In contrast, the yield of nonspecific ligands (grey) is significantly decreased ($p<0.05$) in 6 of 8 clones using target-negative cells as a depletion agent (B).

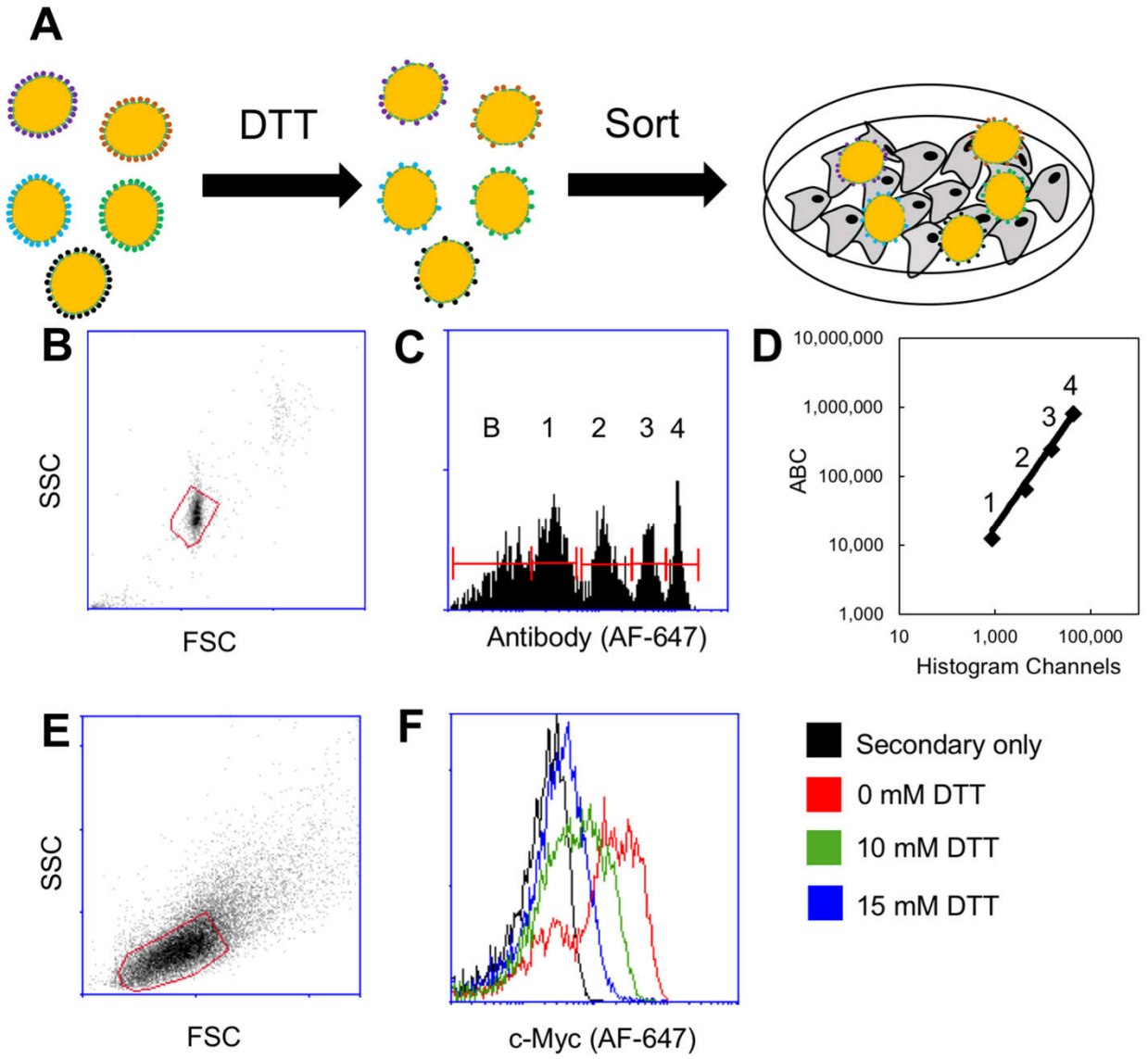

Figure 6. *Titratable avidity reduction*. Yeast expressing a ligand library are treated with DTT to decrease the number of ligands per cell. The avidity-reduced library is sorted by cell panning on target-expressing mammalian cell monolayers (A). In order to quantify ligand expression on yeast cells, a fluorescence calibration curve is generated by Quantum Simply Cellular beads. Gating on the most abundant population in FSC vs SSC yields singlet beads (B). The beads show five fluorescence peaks, corresponding to four different quantities of antibody binding sites and negative control beads (C). Entering the median fluorescences into the manufacturer's spreadsheet yields a calibration curve for converting sample fluorescence to the number of antibody binding sites (D). Yeast fluorescence is quantified first by gating on the most abundant population in FSC vs SSC, yielding singlets (E). Examples of fluorescence displayed by secondary-only control yeast (black), untreated yeast (red), and yeast treated with increasing levels of DTT (green and blue) are shown (F).