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Construction of yeast surface-displayed cDNA libraries

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Abstract

Using yeast display, heterologous protein fragments can be efficiently displayed at high copy levels on the *Saccharomyces cerevisiae* cell wall. Yeast display can be used to screen large expressed protein libraries for proteins or protein fragments with specific binding properties. Recently, yeast surface displayed cDNA libraries have been constructed and used to identify proteins that bind to various target molecules such as peptides, small molecules, and antibodies. Because yeast protein expression pathways are similar to those found in mammalian cells, human protein fragments displayed on the yeast cell wall are likely to be properly folded and functional. Coupled with fluorescence-activated cell sorting (FACS), yeast surface-displayed cDNA libraries potentially allow the selection of protein fragments or domains with affinity for any soluble molecule that can be fluorescently detected. In this report, we describe protocols for the construction and validation of yeast surface displayed cDNA libraries using pre-existing yeast two-hybrid cDNA libraries as a starting point.

Keywords

Yeast surface; cDNA; display; library

1. Introduction

Various techniques have been developed to screen large expressed protein libraries for proteins or protein fragments with specific binding properties, including the yeast two-hybrid system and phage display (1–9). However, these systems have limitations. Because it relies on the internal coexpression of “bait” and “prey” fusion proteins, the two-hybrid system cannot be used to identify proteins that bind to externally synthesized or modified proteins or compounds. Phage display is limited by potential expression bias against eukaryotic proteins expressed in a prokaryotic host and the low number of fusion proteins displayed on each phage particle (2, 6). To address these limitations, yeast surface-displayed libraries of human cDNA fragments can be utilized. Heterologous protein fragments can be efficiently displayed at high copy levels on the *Saccharomyces cerevisiae* cell wall (10), and yeast surface display technology has been successfully used to affinity mature human antibody fragments and map antibody-binding epitopes (11,12). Because yeast protein expression pathways are similar to those found in mammalian cells, human protein fragments displayed on the yeast cell wall are likely to be properly folded and functional. Yeast surface displayed human cDNA libraries have been successfully used to screen for proteins that bind to various target molecules (13–16). Coupled with fluorescence-activated cell sorting (FACS), yeast surface-displayed cDNA libraries potentially allow the selection of protein fragments or domains with affinity for any soluble molecule that can be

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fluorescently detected. In this report, we describe protocols for the construction and validation of yeast surface displayed cDNA libraries using pre-existing yeast two-hybrid cDNA libraries as a starting point.

2. Materials

2.1 Generation of frameshift variants of pYD1

1. pYD1 (*see* Note 1)
2. BamHI restriction enzyme (New England BioLabs)
3. 10x React 3 (supplied with restriction enzymes)
4. Klenow fragment (New England BioLabs)
5. 10x React 2 (supplied with klenow fragment)
6. Mung bean nuclease (New England BioLabs)
7. 10x mung bean nuclease buffer (supplied with mung bean nuclease)
8. Spin prep PCR isolation kit (Qiagen)
9. Elution buffer EB (supplied with Qiagen spin prep kit)
10. T4 DNA ligase (New England BioLabs)
11. 10x ligation buffer (supplied with T4 DNA ligase)
12. 1,000x ampicillin: 1 g ampicillin, add ddH₂O to 10 mL, sterilize by filtration and aliquot, store aliquots at −20°C.
13. 150 mm 2xYT + ampicillin plates: 16 g tryptone, 10 g yeast extract, 5 g NaCl, 17 g agar, bring volume to 1 L with ddH₂O, adjust pH to 7.0, sterilize by autoclaving, let cool until not too hot to touch and add 1 mL 1,000x ampicillin, pour plates and allow to cool at RT, store plates at 4°C until ready to use.
14. Gap5 primer (5'-TTAAGCTTCTGCAGGCTAGTG -3')

2.2 Library construction

1. Random-primed, size-selected cDNA libraries in pYesTrp (Invitrogen)
2. EcoRI restriction enzyme (New England BioLabs)
3. 10x React 3 (supplied with restriction enzymes)
4. Shrimp alkaline phosphatase (SAP) (Fermentas)
5. Spin prep gel isolation kits (Qiagen)
6. T4 DNA ligase (New England BioLabs)
7. 10x ligation buffer (supplied with T4 DNA ligase)
8. Ultracel YM-30 microconcentrator (Millipore)
9. 10G Supreme electrocompetent cells (Lucigen)
10. Transformation recovery medium (supplied with 10G Supreme cells)

¹The pYD1 yeast display vector and the EBY100 yeast strain were commercially available from Invitrogen at the time of our library construction work. Please check with the vendor for currently availability.

11. 1 mm electroporation cuvettes (Molecular BioProducts)
12. Electroporators (Eppendorf 2510, Bio-Rad Gene Pulser II)
13. 2xYT: 16 g tryptone, 10 g yeast extract, 5 g NaCl, bring volume to 1 L with ddH₂O, adjust pH to 7.0, sterilize by autoclaving.
14. Maxiprep kit (Qiagen)

2.3 Transformation of library into yeast

1. EBY100 *Saccharomyces cerevisiae* strain (see Note 1)
2. YPD: 10 g of yeast extract, 20 g of bacteriological peptone, 20 g dextrose, bring volume to 1 L with ddH₂O, autoclave to sterilize.
3. 10x SD-CAA for making plates: 70 g yeast nitrogen base w/o amino acids, 50 g bacto casamino acids, 100 g dextrose, bring volume to 500 mL with ddH₂O, filter sterilize.
4. 50% PEG-3350: 250 g PEG-3350, bring volume to 500 mL with ddH₂O, filter sterilize.
5. 1M Lithium acetate: 33 g lithium acetate, bring volume to 500 mL with ddH₂O, filter sterilize.
6. SD-CAA plates: 5.4 g Na₂HPO₂, 7.4 g NaH₂PO₄, 17 g agar, bring volume to 900 mL with ddH₂O and autoclave, let cool until not too hot to touch and add 100 mL 10X SD-CAA, pour plates of desired size (100 mm or 150 mm) and allow to cool at RT, store plates at 4°C until ready to use.
7. 2x SR-CAA yeast growth media: 20 g raffinose, 14 g yeast nitrogen base, 10 g bacto casamino acids, 5.4 g Na₂HPO₄, 7.4 g NaH₂PO₄, bring volume to 1 L with ddH₂O, filter sterilize.

2.4 Test library induction

1. 20% Galactose: 100 g galactose, bring volume to 500 mL with ddH₂O, filter sterilize.
2. Mouse anti-Xpress (Invitrogen)
3. Mouse anti-V5 (Invitrogen)
4. Goat anti-mouse-CY5 (Jackson ImmunoResearch)

3. Methods

The methods below are divided into four categories: 3.1) Generation of frameshift variants of pYD1 vector. 3.2) Library construction. 3.3) Transformation of library into yeast. 3.4) Test library induction. The construction of yeast display libraries and the generation of frameshifted variants of the original pYD1 yeast display vector have been described previously (13,14). The pYD1 frameshift variants theoretically allow for the in-frame expression of a larger number of cDNA inserts. The cDNA in the protocol we describe comes from pre-made Invitrogen cDNA libraries designed for yeast two-hybrid experiments. We chose libraries designed for two-hybrid experiments because they have been random-primed and size-selected (0.3 – 1.5kb) and will thus display domain-sized protein fragments that may be more efficiently expressed and folded than full-length proteins. The choice of cDNA source material will depend on the final application for the library. A wide variety of pre-made cDNA libraries are available and it should be possible to adapt the presented

cloning scheme to use other cDNA sources. Other protocols for the *de novo* generation of cDNA libraries have been described and can also be adapted for the generation of yeast surface-displayed cDNA libraries based on the protocols we describe.

3.1 Generation of pYD1(+1) and pYD1(−1) frameshift variants of the pYD1 vector

1. Set up the following digestion:
 - 3 µl 10x React 3
 - 10 µg pYD1 plasmid
 - 2 µL BamHI
 - Bring volume to 30 µL with ddH₂O
 - Incubate at 37°C for 3 h.
2. Run digestion on 0.75% agarose gel, cut out linearized band with a clean razor blade, and isolate DNA using Qiagen gel isolation kit following manufacturer's protocols. Elute in 50 µL EB buffer.
3. Set up the following reactions:

<u>Klenow fill in for pYD1(+1)</u> 3 µL 10x React 2 2 µL 0.5 mM dNTP mix 20 µL Linearized pYD1 (≈2 µg) 2 µL Klenow fragment (5U/ul) 4 µL ddH ₂ O Incubate at RT for 30 min	<u>Mung bean chew back for pYD1(−1)</u> 3 µL 10x Mung bean reaction buffer 20 µL Linearized pYD1 (≈2 µg) 1 µL Mung bean nuclease (10U/µL) 6 µL ddH ₂ O Incubate at 30°C for 30 min
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4. Purify Klenow and mung bean reactions using the Qiagen PCR purification kit according to manufacturer's directions. Elute in 50 µL buffer EB.
5. Set up ligations to re-circularize:
 - 2 µL 10x ligation buffer
 - 10 µL Klenow filled in pYD1 *or* mung bean chewed back pYD1
 - 7 µL ddH₂O
 - 1 µL T4 DNA ligase (400U/µL)
 - Incubate ligations at RT for 2 h
6. Transform ligations into bacteria using any standard method, plate on 2xYT-ampicillin plates, and incubate overnight at 37°C.
7. Prepare minipreps from colonies using any standard method compatible with DNA sequencing and analyze by sequencing with the Gap5 primer to identify the correct clones.
8. Prepare large scale DNA preps of pYD1(+1) and pYD1(−1) for future library cloning.

3.2 DNA digestion, ligation, and transformation

1. Set up digests of the pYD1, pYD1(+1), and pYD1(−1) yeast display vectors and the cDNA library (*see* Note 2) as follows:

3 μ L 10x React 3	5 μ L 10x React 3
10 μ g pYD1 plasmid	20 μ g cDNA library plasmid
2 μ L EcoRI	2 μ L EcoRI
Bring volume to 30 μ L with ddH ₂ O	Bring volume to 50 μ L with ddH ₂ O
Incubate at 37°C for 3 h.	

For simplification, the pYD1, pYD1(+1), and pYD1(−1) digests can be pooled.

- To dephosphorylate the ends of the linearized pYD1 vectors, add 6 μ L SAP (1U/ μ L) to the pooled vector digests and incubate at 37°C for 1 h. Heat inactivate the SAP at 65°C for 15 min.
- Run the vector and library digests on a 1% agarose gel. Using clean razor blades, cut the linearized vector and the desired size range of cDNA inserts (0.3–1.5 kb). Isolate the DNA from the gel slices using the Qiagen gel isolation kit following the manufacturer's protocol. Verify the quality of the isolated DNA fragments by running a fraction on an agarose gel. Determine the concentration of the purified fragments using a spectrophotometer.
- Set up ligations as follows (*see* Note 3):

<u>Control ligation</u>	<u>Library ligation</u>
2 μ L 10x ligation buffer	2 μ L 10x ligation buffer
700 ng linearized vector	700 ng linearized vector
1 μ L T4 DNA ligase	300 ng cDNA insert
Bring volume to 20 μ L with ddH ₂ O	1 μ L T4 DNA ligase
	Bring volume to 20 μ L with ddH ₂ O
Incubate ligations for 16 h at 16°C.	

- Desalt ligations with a YM-30 microconcentrator according to manufacturer's instructions. Spin at least 20 volumes (400 μ L) of ddH₂O through the filter unit. The retentate volume should be approximately 50 μ L.
- Prewarm transformation recovery medium at 37°C. Place electroporation cuvettes on ice. Thaw 10G supreme cells completely on ice (10–20 min) and aliquot 25 μ L of cells to pre-chilled 1.5 mL eppendorf tubes on ice. Add 1–3 μ L of each of the ligations to the tubes with cells and stir gently without pipeting up and down.
- Transfer 25 μ L of the cell/ligation mixture to the chilled electroporation cuvettes and tap the cuvette a few times to distribute the cells across the bottom of the chamber. Electroporate using the following conditions (*see* Note 4):
 - 10 μ F
 - 600 Ohms
 - 1800 Volts

²We used random primed, size-selected (0.3–1.5 kb; average size, 0.75 kb) human cDNA libraries from Invitrogen as the source material for the libraries we have made so far. Other pre-made cDNA libraries can be used by modifying the cloning scheme.

³This is a standard ligation condition with 1 μ g DNA in 20 μ L with a 3:1 insert to vector molar ratio, but could be optimized for each individual case.

⁴These are the conditions suggested by Lucigen and they have worked well for us. We have generated similar results with Bio-Rad (Gene Pulser II) and Eppendorf (2510) electroporators.

8. Immediately add approximately 1 mL of the pre-warmed recovery medium to the cuvette, resuspend the cells, and transfer to 17 mm culture tubes.
9. Incubate tubes at 37°C with shaking at 250rpm for 1 h. Dry 2xYT-ampicillin plates at 37°C while transformation are recovering.
10. Plate 1/10,000 of each transformation on a 2xYT-ampicillin plate for titering and plate the remaining transformation cultures on three more plates at approximately 350 µL/plate. Incubate the plates overnight at 37°C.
11. Calculate the number of total colonies for the control and library transformations using the titer plates. The library transformation should have at least 100 times as many colonies as the control (*see* Note 5).
12. Prepare minipreps from at least 20 colonies from the library titer plate and sequence using the Gap5 primer to verify the quality and diversity of the library. The colonies can also be analyzed using colony PCR (Fig 1)(*see* Note 6).
13. Repeat the transformation of the library ligation until the desired library size is achieved (*see* Note 7).
14. Add 3 mL 2xYT+ampicillin to each of the library transformation plates and resuspend cells with a flame-sterilized spreader. Collect cells from all the plates in one 50 mL conical using a pipetman.
15. Prepare several bacterial freezer stocks of the library by mixing 0.5 mL 50% glycerol and 0.5 mL of the collected transformants in a cryotube and storing at -80 °C.
16. Prepare a Qiagen maxiprep of the remaining library transformants according to manufacturer's protocols. Determine the concentration of the library DNA prep using a spectrophotometer.

3.3 Transformation of library into yeast

1. Inoculate a 5 mL YPD culture with EBY100 and grow overnight with shaking at 30 °C and 200rpm.
2. Determine concentration of the overnight yeast culture by measuring the OD₆₀₀ of a 1:20 dilution using a spectrophotometer (1 OD₆₀₀ = 2 × 10⁷/mL).
3. Use the overnight culture to inoculate a 50 mL YPD culture at 0.5 OD₆₀₀ and incubate at 30°C, 200 rpm for 4–5 h (at least two cell divisions).
4. Harvest cells by centrifugation at 300 g for 5 min, wash in 25 mL sterile ddH₂O, and resuspend in 1 mL sterile ddH₂O. Transfer to a 1.5 mL eppendorf tube and wash again with 1 mL sterile ddH₂O and resuspend in 1 mL sterile ddH₂O.
5. Add 100 µL EBY100 cell suspension to ten 1.5 mL eppendorf tubes, spin at top speed for 30 sec, and remove supernatant.
6. To each tube of cells add the following in this order:

⁵If there are too many colonies on the vector control transformation plate, the vector digestion, dephosphorylation, and purification should be repeated.

⁶Analyze the sequence to verify that the inserts are cDNA and diverse. Check to ensure that all three frameshift variants of pYD1 are present. Colony PCR on a larger number of colonies can be carried out to check the size distribution and percentage of empty vector (should be very low), but sequencing is the best method of quality control.

⁷Transformation plates can be sealed with parafilm and stored at 4°C until enough colonies have been accumulated over the course of several days. Then they can be recovered simultaneously and pooled.

240 μ L 50% PEG-3350

36 μ L 1.0 M lithium acetate

5 μ g plasmid DNA

Bring volume to 360 μ L with sterile ddH₂O

Vortex transformation mix vigorously and incubate tubes at 42°C in a water bath for 40 min.

7. Spin transformation tubes at top speed for 30 sec and remove transformation mix. Add 200 μ L sterile ddH₂O to each tube and resuspend by vortexing. Pool transformants by transferring to a 15 mL conical (approximately 2 mL total volume).
8. Pre-warm 150 mm SD-CAA plates at 30°C. To titer the transformation, add 10 μ L of pooled transformants to 1 mL of sterile ddH₂O, mix and plate 20 μ L or 2 μ L (1/10,000 or 1/100,000) by pipetting into a 100 μ L puddle of sterile water on the SD-CAA plates. Gently spread the cells onto the plate using a sterilized spreader. Plate the rest of the transformed cells onto SD-CAA plates at a density of 200 μ L/plate and once again spread gently. If necessary, allow fluid to be taken up by plates prior to incubation (*see* Note 8). Incubate the plates inverted at 30°C for 3–4 days until colonies grow.
9. Count the transformants on the titer plates. If larger library size is desired, more transformations can be done (*see* Note 9).
10. Add 3 mL SR-CAA to each library transformation plate and collect cells by scraping with a spreader. If there is any minor contaminating bacteria or fungus on the plates, it should be excised with a flame-sterilized scalpel prior to resuspending the cells. Pool transformants in a 50 mL conical using a pipetman. To prepare freezer stocks, add 1/2 volume of sterile 50% glycerol to the transformants (i.e. add 10 mL 50% glycerol to 20 mL cells), pipet 1 mL aliquots into cryotubes, and store at –80°C.
11. To test the freezer stocks, pre-warm and dry six 150 mm SD-CAA plates in a 30°C incubator. Thaw a 1 mL aliquot of yeast surface-displayed cDNA library at RT. To titer the library aliquot, dilute 5 μ L of the thawed library into 50 mL sterile H₂O, mix well, and then add 10 μ L of the dilution to a 100 μ L puddle of sterile H₂O on a 150 mm SD-CAA plate and gently spread. At the same time, plate the remaining library aliquot on the remaining five 150 mm SD-CAA plates (200 μ L/plate). Incubate plates upside down at 30°C for 2–3 days. A solid lawn of cells should grow. Count colonies on the titer plate and multiply by 10⁶ to obtain the total CFU for the aliquot. This number should be > 10X the estimated diversity of the original library (*see* Note 10). The recovered library can be kept for several weeks on the plates if necessary by sealing the plate edges with parafilm and storing at 4°C.

⁸We found that a longer drying period for the plates allows cells to be more conveniently plated so that cells don't run down the side when plates are inverted.

⁹We usually get > 5 × 10⁷ transformants from ten transformations. Plates can be wrapped in parafilm and stored at 4°C until the desired number of transformants has been collected. Then the transformants can be simultaneously collected and pooled.

¹⁰If the titer is close to the desired titer, more than one aliquot can be thawed for selection experiments. If the titer is too low for some reason, the library should be re-transformed into yeast and aliquots re-frozen.

3.4 Test library induction

1. If there is any minor contaminating bacteria or fungus on the plates, it should be excised with a flame-sterilized scalpel prior to resuspending the cells. Recover library from plates by adding 5 mL 2x SR-CAA to each plate and scraping with a flame-sterilized spreader and then collect the resuspended cells into a 50 mL conical by pipeting. Determine cell number by taking an OD₆₀₀ reading of a 1/50-diluted sample of the resuspended cells ($1 \text{ OD}_{600} \approx 2 \times 10^7$ yeast cells/mL).
2. Using the resuspended library, start a 200 mL 0.5 OD₆₀₀ culture (approximately 2×10^9 cells) in 2x SR-CAA. Grow at 30°C with shaking for 1 h. To induce surface expression of the library, add 22 mL sterile 20% galactose (final 2% galactose) and continue growing at 25°C with shaking for 16 h. The induced library can be stored for several weeks at 4°C.
3. To check induction of the library by FACS, spin down three tubes with 100 µL of the induced library (30 sec maximum speed in a microcentrifuge) and wash twice with 1 mL PBS.
4. Resuspend cells in 500 µL PBS and add 1 µL mouse anti-Xpress antibody to one tube, 1 µL mouse anti-V5 antibody to another tube, and nothing to the control tube. Incubate at RT with rotation for 1 h. Wash cells three times with 1 mL PBS.
5. Resuspend each tube in 500 µL 1:500 goat antimouse-CY5 in PBS and incubate at RT with rotation for 30 min. Wash three times with PBS, resuspend in 500 µL PBS, and place on ice until FACS analysis.
6. Analyze the control anti-Xpress and anti-V5 stained yeast cells by FACS. At least 40% of the population should be Xpress-positive and at least 3% of the population should be V5-positive (Fig 2) (*see* Note 11). The induced library is now ready for selection experiments.

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¹¹There is always a negative population after induction when analyzed by FACS and the maximum induction will vary from experiment to experiment. This is seen even when using pYD1-transformed EBY100 as a positive control. In order for the V5 epitope to be expressed, the cDNA insert must have an open reading frame (ORF) that spans its entire length and is in frame with both the upstream AGA2 coding region and the downstream V5 coding region. Since only one-third of clones with a full length ORF in frame with the AGA2 coding region will also be in frame with the V5 epitope, the actual number of clones with a full length AGA2-fused ORF will be approximately three times the number of V5-positive clones.

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Figure 1. Construction of yeast surface displayed human cDNA library. Size-selected (0.3 – 1.5 kb) human testis cDNAs were cloned into the yeast display vector pYD1 to create a yeast surface display library containing 5×10^7 members. Human protein fragments are expressed as fusions with Aga2p. The cDNA inserts are flanked by epitope-tags for monitoring the level of expression (Xpress, V5). PCR analysis shows that the cDNA inserts range in size from 0.3 kb to 1.5 kb. (This research was originally published in *Mol Cell Proteomics*. Bidlingmaier, S., and Liu, B. (2006) Construction and application of a yeast surface-displayed human cDNA library to identify post-translational modification-dependent protein-protein interactions. *Mol. Cell Proteomics*. 5, 533–540. © the American Society for Biochemistry and Molecular Biology.)

Figure 2.

Library induction is monitored using anti-Xpress and anti-V5 antibodies. The secondary detection reagent was SA-647 and expression is shown on the x-axis in the APC-A channel. In order to express the V5 epitope, the cDNA insert must have an open reading frame (ORF) along its entire length that is in frame with both the upstream *AGA2* coding region and the downstream V5 coding region. Typically, about 3–7% of cells from induced libraries are V5 positive, suggesting that about 10–20% of induced cells express an Aga2p fused ORF that runs the entire length of the insert.