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Use of Rolling-Circle Amplification for Large-Scale Yeast Two-Hybrid Analyses

Xiaodong Ding, Yan Zhang, and Wen-Yuan Song

Summary

Detection of protein–protein interactions on a large-scale has become a major focus of functional genomics after the completion of genome sequencing. The information generated from these studies not only assembles proteins into signaling networks, but also reveals potential functions of uncharacterized proteins when their interacting partners have known functions. We have developed a rolling circle amplification-based yeast two-hybrid scheme that allows one to test reproducibility and specificity of the interactions on a large scale. Using this scheme, technical false-positives from yeast two-hybrid analyses can be efficiently minimized.

Key Words: Protein–protein interactions; yeast two-hybrid; rolling-circle amplification; high throughput; plasmids.

1. Introduction

Proteins often exist by interacting with other proteins to fulfill their physiological role. Such interactions are critical for the stabilization and subcellular localization of many proteins. The protein–protein interactions also regulate enzymatic activities and provide the connectivity and specificity of signaling networks. Yeast two-hybrid analysis is a genetic method of choice for detecting pair-wise protein–protein interactions in a cellular setting *(1–3)*. Instead of using complex technologies to purify protein complexes and identify interacting partners, yeast two-hybrid analysis manipulates plasmids in yeast to test for interactions of the proteins (also called bait and prey) produced by the plasmids. It has been estimated that more than half of the protein interactions reported in the literature were originally identified by yeast two-hybrid analyses *(4)*. The viability of this method relies on its low costs, simplicity in manipula-

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tion, and sensitivity in detection (both stable and transient interactions can be detected by yeast two-hybrid).

Like any technique used for the study of protein–protein interactions, the current yeast two-hybrid procedures have their limitations. A large number of false-positives have been observed in a variety of yeast two-hybrid screenings, particularly high-throughput analyses. These include the artificial interactions resulting from the activation of the yeast two-hybrid reporters in the absence of interacting proteins. This group of false-positive interactions has been categorized as technical false-positives *(5)*. It has been estimated that as many as 40% of the interactions, obtained from the initial library screen, cannot be confirmed by retransforming the identified prey into fresh yeast cells that contains the original bait *(5)*. However, examination of the reproducibility of the interactions is often not performed in many high-throughput yeast two-hybrid analyses because plasmid isolation from yeast cultures and subsequent propagation in *Escherichia coli* are extremely labor-intensive and time-consuming when carried out on a large scale.

Rolling-circle amplification (RCA), used by bacteria to replicate circular plasmids or viruses in nature *(6)*, has been developed as a powerful tool to amplify plasmid DNA in vitro *(7,8)*. Because of its proofreading and high processive activities, the Phi29 DNA polymerase used in RCA can efficiently amplify plasmids with a broad size range at a high fidelity *(9–12)*. The simplicity, robustness, and contamination-resistant features make RCA particularly useful for high-throughput assays. We adapted RCA to simplify yeast two-hybrid procedures *(12–14)*. The entire bait and prey plasmids can be equally amplified from single-yeast colonies or isolated plasmids in a 96-well format. The amplified, linear concatemeric DNA is sufficient and suitable for a variety of molecular analyses including restriction digestion, DNA sequencing, yeast transformation, and even bacterial transformation *(13,14)*. When retransformed into yeast, the bait plasmid can be excluded from the prey using distinct counterselection methods, which allows for the examination of specificity and reproducibility of the interactions. By using the RCA-based yeast two-hybrid scheme, the interactors found in the initial library screen can be verified in subsequent one-on-one-based assays.

2. Materials

2.1. Yeast Strains

1. CG1945 (*MAT***a** *ura3-52 his3-200 lys2-801 trp1-901 ade2-101 leu2-3,112 gal4-542 gal80-538 LYS2*::*GAL1-HIS3 cyh*^{r}*2 URA3*::[*GAL4* 17-mers]$_3$-*CYC1-lacZ*).
2. Y187 (*MAT*α*, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4*Δ*, gal80*Δ*, met, URA3:: GAL1*$_{UAS}$ *- GAL1*$_{TATA}$ *- lacZ MEL1*).

3. MaV203 (*MATα leu2-3,112 trp1-901 his3200Δ ade2-101 cyh2^r can1^r gal4Δ gal80Δ GAL1::lacZ HIS3$_{UASGAL1}$::HIS3@LYS2 SPAL10$_{UASGAL1}$::URA3*).

2.2. Yeast Media

1. Yeast peptone dextrose (YPD; 1.0 L): To 950 mL of H_2O, add 10 g of bacto-yeast extract, 20 g of bacto-peptone, 18 g of agar (for plate only). Adjust pH to 5.8, autoclave, and cool to approx 55°C. Then add dextrose (glucose) to 2% (50 mL of a sterile 40% stock solution).
2. Yeast complete medium (YCM; 1.0 L): To 950 mL of H_2O, add 10 g of bacto-yeast extract, 10 g of bacto-peptone, 18 g of agar (for plate only). Adjust pH to 3.5 (liquid) or 4.5 (agar plate), autoclave, and cool to approx 55°C. Then add dextrose (glucose) to 2% (50 mL of a sterile 40% stock solution).
3. Synthetic dropout (SD; 1.0 L): To 950 mL of H_2O, add 6.7 g of Difco yeast nitrogen base without amino acids (Difco; cat. no. 0919-15-3), 20 g of agar (for plate only) and one of the following amino acid supplements:
   a. SD/-Trp: 0.74 g of -Trp DO supplement (BD Biosciences; cat. no. 630413).
   b. SD/-Leu: 0.7 g of -Leu DO supplement (BD Biosciences; cat. no. 630414);
   c. SD/-Trp-Leu: 0.64 g of -Trp-Leu DO supplement (BD Biosciences; cat. no. 630417).
   d. SD/-Trp-Leu-His: 0.62 g of -Trp-Leu-His DO supplement (BD Biosciences; cat. no. 630419).
   e. SD/-Leu-Ura-His: 0.65 g of -Leu-Ura-His DO supplement (BD Biosciences; cat. no. 8614-1).

   Adjust pH to 5.8, autoclave, and cool to approx 55°C, and then add dextrose (glucose) to 2% (50 mL of a sterile 40% stock solution).
4. SD/-Leu-Ura+Trp(L): To 950 mL of H_2O, add 6.7 g of Difco yeast nitrogen base without amino acids, 0.65 g of -Leu-Ura-Trp DO supplement (BD Biosciences cat. no: 630426), 0.1 mg of tryptophan (Sigma, cat. no. T-0254), and 20 g of agar (for plate only). Adjust pH to 5.8, autoclave, and cool to approx 55°C, and then add dextrose (glucose) to 2% (50 mL of a sterile 40% stock solution).

2.3. Solutions

1. All the solutions are prepared using double-distilled H_2O.
2. 40% Dextrose, autoclaved or filter-sterilized (avoid prolonged or repeated autoclaving).
3. 1 *M* 3-Amino-1,2,4-triazole (3-AT; Sigma, cat. no. A-8056), filter-sterilized.
4. 1 mg/mL Cycloheximide (Sigma, cat. no. C-6255), filter-sterilized.
5. 0.5 g/mL FAA (2-amino-5-fluorobenzoic acid; Fluka, cat. no. 07973) in absolute ethanol.
6. 10 mg/mL Herring testes carrier DNA (single-stranded DNA [ssDNA]; Sigma, cat. no. D-1626).
7. 50% Polyethylene glycol (PEG) 4000 (average mol. wt. = 3350; Sigma, cat. no: P-3640), filter-sterilized.

8. 10X TE buffer: 0.1 *M* Tris-HCl, 10 m*M* ethylene diamine tetraacetic aicd, pH 7.5. Autoclave. 1X TE buffer (TE solution) is prepared by diluting the 10X stock.
9. 10X LiAc: 1 *M* lithium acetate (Sigma, cat. no: L-6883). Adjust to pH 7.5 with dilute acetic acid and autoclave.
10. PEG/LiAc solution: Mix 8 mL of 50% PEG 4000, 1 mL of 10X TE, and 1 mL of 10X LiAc. Prepare just before use.
11. Dimethyl sulfoxide (DMSO; Sigma, cat. no. D-8779).
12. 1 *M* Sorbitol (Fisher, cat. no. BP439-500).
13. 50% Glycerol (Fisher, cat. no. G153-1).

2.4. Equipment and Supplies

1. SmartSpec3000 (Bio-Rad).
2. Centrifuges 5417C and 5810R (Eppendorf).
3. Isotemp 110 waterboth (Fisher).
4. Bioassay dishes (NUNC).
5. Analytical funnels (Fisher).
6. PVC vacuum manifolds (Fisher).
7. HydroTech vacuum pump (Bio-Rad).
8. 47-mm Water membrane (pore size = 0.45 μm; Fisher).
9 96 Deepwell plates (Fisher).
10. 96-Well amplification plates with chimney (NUNC).
11. 96-Hydra microdispenser (Robbins Scientific Corporation).
12. 96-Pin replicators (V&P Scientific).
13. Single-well Omnitrays (NUNC).
14. PTC-200 Peltier Thermo Cycler (Bio-Rad).
15. TempliPhi™500 Amplification kit (Amersham Biosciences).

3. Methods

3.1. Transformation of Bait Constructs Into Yeast (Small-Scale Yeast Transformation)

1. We have constructed a pair of gateway compatible bait vectors (pXDGATcy86 and pXDGATU86) (**Fig. 1**) for RCA-based yeast two-hybrid analysis (*see* **Note 1**). To transform bait constructs into CG1945, streak a small portion of the frozen yeast stock of CG1945 onto a freshly prepared YPD agar plate. Incubate at 30°C for 4 to 5 d.

Fig. 1. Schematic drawing of the pXDGATcy86 **(A)** and pXDGATU86 **(B)** vectors for initial yeast two-hybrid screening and subsequent verification of candidate interactors. The gateway conversation cassette (*att*R1-C[R]-ccdB-*att*R2) inserted between *Sal*I (2) and *Eco*RI (1724) is from Invitrogen. The recombination sites *att*R1 and *att*R2 in this cassette are underlined. T-ADH, yeast alcohol *dehydrogenase* gene transcription terminator; TRP1, *phosphoribosylanthranilate isomerase* gene; ARS4/CEN6, for replication and low copy-number maintenance in yeast; Amp (R), ampicillin resistance; Cm(R), chloramphenicol resistance; ColE1 ori, for replication in *E. coli*;

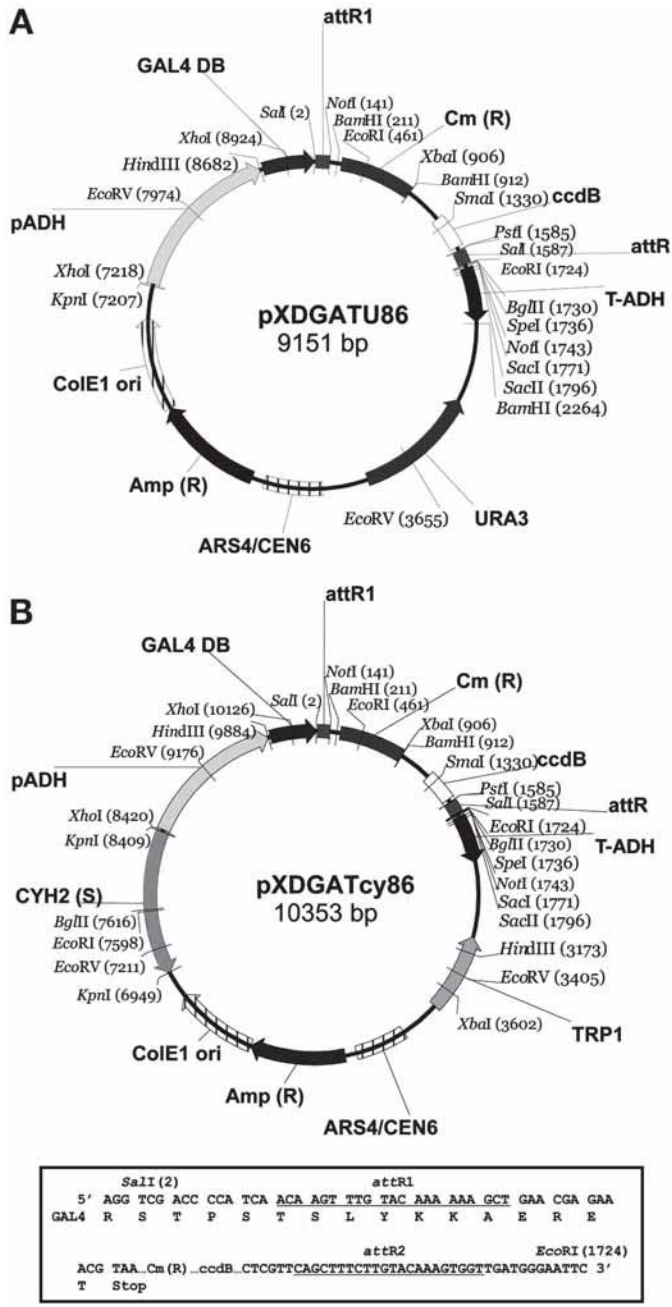

Fig. 1. (*continued from opposite page*) CYR2 (S), cyclohexamide sensitivity; pADH, yeast alcohol *dehydrogenase* gene promoter; GAL4 DB, GAL4 DNA binding domain; URA3, orotidine *5′-phosphatedecarboxylase* gene.

2. Pick two to three colonies (3 mm in diameter) and transfer into 1 mL of freshly prepared YPD liquid medium in a 1.5-mL microfuge tube. Vortex to completely disperse the cells and then transfer into 50 mL of YPD.
3. Incubate at 30°C for approx 18 h with shaking at 250 rpm. Dilute the culture to 300 mL of YPD to make $OD_{600} = 0.2$. Continue to incubate until $OD_{600} = 0.8$.
4. Harvest the cells by centrifugation at 1700*g* for 5 min at room temperature. Wash the cells by resuspending the pellet in 50 mL of H_2O and recentrifugation at 1700*g* for 5 min at room temperature. Discard the supernatant.
5. Resuspend the cell pellet in 1.5 mL of freshly prepared 1X LiAc/TE buffer.
6. Add 10 µL of denatured ssDNA (10 mg/mL), 0.1 µg of pXDGATcy86 or pXDGATU86 derived bait construct, and 0.1 mL of yeast competent cells prepared previously to a 1.5-mL microfuge tube and mix well.
7. Add 0.6 mL of PEG/LiAc solution into the cell mixture and vortex for at least 1 min.
8. Incubate at 30°C for 30 min with shaking at 200 rpm.
9. Add 70 µL of DMSO to a final concentration of 10% and mix gently by inversion.
10. Heat shock for 15 min in a 42°C water bath. Chill the cells on ice.
11. Pellet the cells by centrifugation at 6800*g* for 10 s. Discard the supernatant. Resuspend the cell pellet into 100 µL of TE.
12. Plate the cells on SD/-Trp medium for pXDGATcy86 or SD/-Ura medium for pXDGATU86.
13. Incubate at 30°C for 4 to 5 d.
14. Collect the cells to make glycerol stocks. Store at –80°C for future use.
15. Streak the transformants onto SD/-Trp and SD/-Trp-His media, respectively, to test autoactivation of the bait constructs (*see* **Note 2**).

3.2. Transformation of a Complementary DNA Library Into Yeast (Library-Scale Yeast Transformation)

1. Streak a small portion of the frozen yeast stock of Y187 onto a freshly-prepared YPD agar plate. Incubate at 30°C for 4 to 5 d.
2. Pick two to three colonies (3 mm in diameter) and inoculate into 1 mL of freshly prepared YPD liquid medium in a 1.5 mL microfuge tube. Vortex to completely disperse the cells and then transfer into 50 mL of YPD.
3. Incubate at 30°C for approx 18 h with shaking at 250 rpm. Dilute the culture to 1 L of YPD to make $OD_{600} = 0.2$. Continue to incubate until $OD_{600} = 0.8$.
4. Harvest the cells by centrifugation at 1700*g* for 10 min at room temperature. Wash the cells by resuspending the pellet in 500 mL of H_2O and recentrifugation at 1700*g* for 10 min at room temperature. Discard the supernatant.
5. Resuspend the cell pellet in 8 mL of freshly prepared 1X LiAc/TE buffer.
6. Add 2 mL of denatured ssDNA (10 mg/mL), 400 µg of library DNA (*see* **Note 3**), and 8 mL of yeast competent cells prepared previously into a 50-mL tube and mix well.
7. Combine the aformentioned mixture with 20 mL of 50% PEG in a flask (250 mL) and vortex for at least 1 min.
8. Incubate at 30°C for 30 min with shaking at 200 rpm.

9. Add DMSO to a final concentration of 10% and mix gently by inversion.
10. Heat shock for 15 min in a 42°C water bath. Chill the cells on ice.
11. Pellet the cells by centrifugation at 2700*g* for 10 min. Discard the supernatant. Resuspend the cell pellet into 15 mL of TE solution.
12. Spread the cells onto SD/-Leu medium in about 40 Bioassay dishes (22×22 cm^2). To determine the transformation efficiency, spread 0.2 µL, 0.5 µL, and 1 µL onto the same medium in 100-mm Petri dishes. Incubate at 30°C for 3 d. More than 10 million total transformants are expected for subsequent library screenings.
13. Harvest the cells with 200 mL of 1 *M* sorbitol and briefly disperse the cell suspension at setting 5 for 10 s with an ultrasonic cell disruptor (Microsonix).
14. Mix the cell suspension with an equal volume of 50% glycerol and aliquot 1.2 mL of cells into 1.5-mL tubes.
15. Spread 100 µL, 200 µL, and 500 µL of the diluted cells (10^5- to 10^6-fold) onto SD/-Leu plates. Incubate at 30°C for 3 to 4 d.
16. Wrap the freezer boxes containing the aliquoted cells with five layers of paper towels and store at –80°C.
17. Thaw a tube of frozen cells and determine the viable cells after the freeze/thaw cycle as described in **step 15**.
18. Viability = (cell number after frozen × unit/vol)/(cell number before frozen × unit/vol)%. The expected viability is 40 to 45%.

3.3. Screening of a Complementary DNA Library

1. To screen the complementary DNA (cDNA) library, streak the CG1945 cells carrying the pXDGATcy86 derived bait constructs (stored at –80°C) onto freshly prepared SD/-Trp medium and incubate at 30°C for 4 to 5 d (*see* **Note 4**).
2. Pick two to three (2 mm in diameter) colonies and inoculate into 2 to 3 mL of SD/-Trp liquid medium and shake at 30°C (250 rpm) for approx 18 h.
3. Dilute the culture to $OD_{600} = 0.2$ in 20 mL of SD/-Trp medium and continue to shake for 4 to 5 h until $OD_{600} = 0.8$.
4. Thaw α-mating type cells containing the cDNA library (stored at –80°C as described above) at room temperature for 10 to 15 min.
5. Mix 1.6×10^8 (approx 8 mL) cells containing the bait construct with the thawed cDNA library cells (7×10^7 viable cells) to make the 2.5:1 (bait:library) cell ratio.
6. Centrifuge at 1700*g* for 2 mins and discard the supernatant.
7. Resuspend the cells in 2.3 mL of YCM (pH 3.5) to make a cell density of 10^8 cells/mL.
8. Shake at 220 rpm for 105 min at 30°C.
9. Dilute the cells 100-fold by adding H_2O and vortex at maximum speed for 1 min to disperse the cells.
10. Harvest the cells onto a 47-mm water membrane (pore size = 0.45 µm) using vacuum filtration (**Fig. 2**; *see* **Note 5**).
11. Transfer the membrane (cell side up) onto YCM medium (pH 4.5) and incubate for 4.5 h at 30°C (*see* **Note 6**).
12. The zygotes can be observed under a microscope (pick cells with a tip, resuspend into 100 µL of H_2O, and spread onto a glass slide).

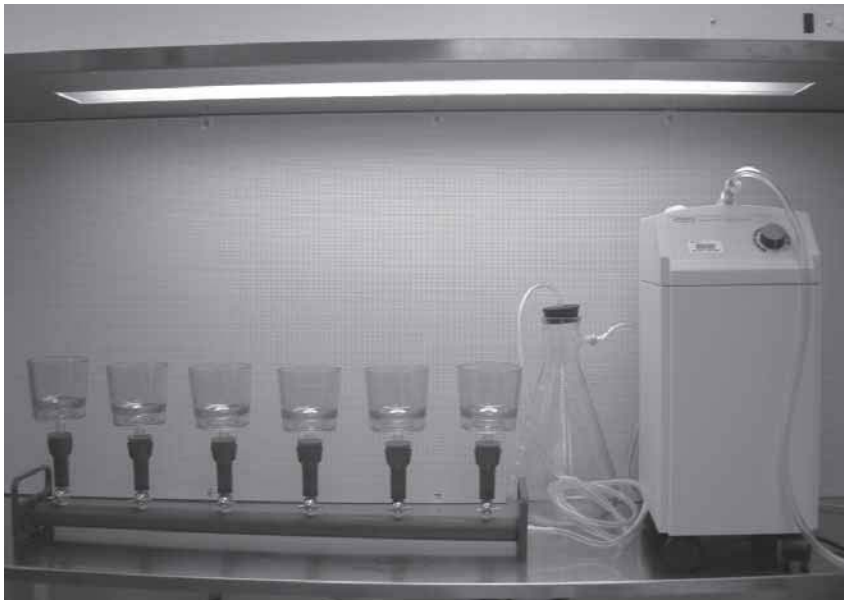

Fig. 2. Six-channel filtration system. Yeast cells in liquid medium are collected on the 47-mm water membranes contained in the funnels by the vacuum drawn from a pump (right).

13. Transfer the membrane into 10 mL of 1 *M* sorbitol solution and vortex vigorously for 1 min to wash the cells off the membrane.
14. Pellet the cells by centrifugation at 1700*g* for 2 to 3 min.
15. Resuspend the cells into 2 mL of TE solution by vortexing for 1 min.
16. Spread the cells onto SD/-Trp-Leu-His + 2 m*M* 3-AT in four Bioassay dishes (*see* **Note 7**).
17. Spread 0.1 µL and 0.2 µL of the cell suspension in **step 15** onto selective medium (SD/-Leu, SD/-Trp, and SD/-Trp-Leu) to determine the mating efficiency: Mating efficiency = Total number of colonies on SD/-Trp-Leu/the sum of total number of colonies on SD/-Trp and SD/-Leu.
18. Incubate at 30°C for 6 to 10 d.
19. Pick the colonies growing on the SD/-Trp-Leu-His + 2 m*M* 3-AT medium and inoculate into 1 mL of the SD/-Trp-Leu-His medium contained in a 96-deepwell plate (master plate) with a glass bead in each well. Incubate at 30°C with shaking at 250 rpm for 2 to 3 d.
20. Transfer 200 µL of cells from each well, of the master plate, into two fresh 96-well microplates with chimney, respectively, by using the 96-Hydra microdispenser. Pellet the cells by centrifugation at 1700*g* for 2 min and discard the supernatant. Add 600 µL of 50% glycerol into each well of the master plate. Store the three plates at –80°C.

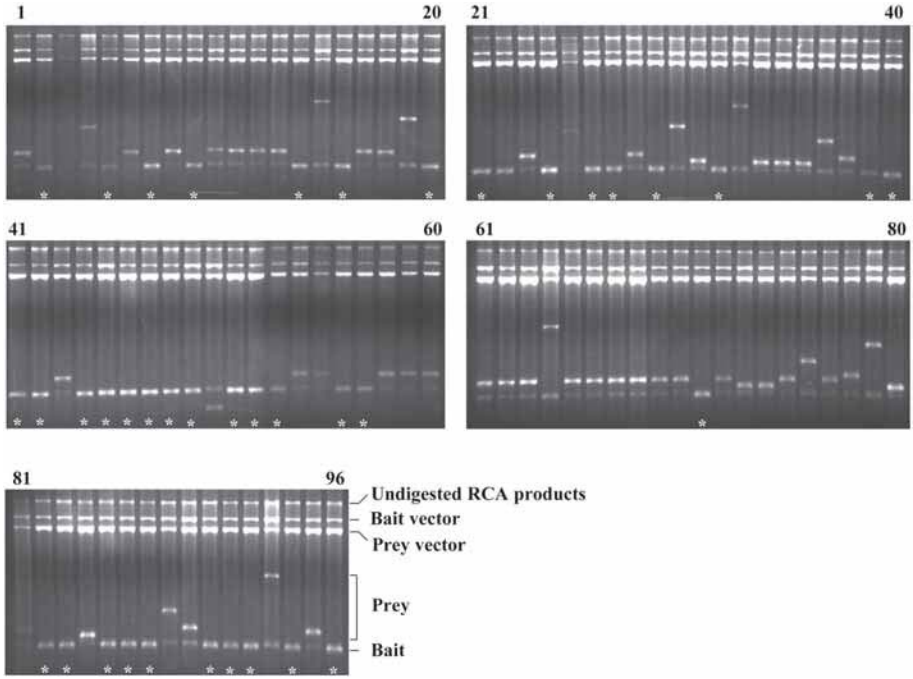

Fig. 3. High-throughput amplification of both bait and prey from the yeast cells using rolling-circle amplification. Cell lysates from single-yeast colonies were used as templates for the amplification. The amplified DNA was digested with *Sal*I and *Not*I. The samples were resolved by agarose gel electrophoresis. The prey samples containing inserts identical in size to the bait are indicated by asterisks.

3.4. Amplification of Bait-and-Prey Plasmids by RCA

1. To prepare the templates for RCA, thaw the cells contained in one microplate as described in **Subheading 3.3.**, **step 20** at room temperature. Resuspend the cells into 50 µL of TE solution containing 0.5 µL of zymolase (5 U/µL). Mix well by gently vortexing and incubate at 30°C for 1 h (*see* **Note 8**).
2. Transfer 5 µL of 10X diluted cell lysate into to a new regular 96-well microplate. Heat the plate on a thermocycler at 96°C for 3 min. Chill on ice/water bath for 10 min.
3. RCA is performed using the TempliPhi100 Amplification kit (Amersham Biosciences). Add 0.2 µL of the premixed enzyme mixture (Phi29 DNA polymerase) and 5 µL of reaction buffer (Amersham Biosciences) into each well of the above plate. Briefly vortex and centrifuge.
4. Incubate at 30°C for 20 to 30 h.
5. The amplified DNA can be analyzed by restriction digestion followed by gel electrophoresis (**Fig. 3**). Alternatively, a pipettor tip can be used to check the viscosity, as the RCA amplified products are concatemeric DNA.

6. Add 15 µL of H_2O to dilute the amplified DNA. Transfer 10 µL into two new plates, respectively. One plate is subjected to DNA sequencing, whereas the other one is used for yeast retransformation as described herein.

3.5. Retransformation of RCA DNA Into Yeast

1. Streak the MaV203 strain carrying the pXDGATU86 derived verification bait onto a freshly prepared SD/-Ura +5 µg/mL cycloheximide medium. Incubate at 30°C for 6 to 7 d.
2. Pick two to three (2 mm in diameter) colonies and inoculate into 5 mL of SD/-Ura medium and incubate at 30°C for approx 18 h with shaking (250 rpm).
3. Dilute 1 mL of the cell culture into 50 mL of SD/-Ura (for transformation of 96 samples).
4. Incubate at 30°C for 6 to 8 h until $OD_{600} = 0.8$.
5. Harvest the cells by centrifugation at 1700*g* for 5 min at room temperature. Wash the cells by resuspending into 50 mL of H_2O and recentrifuge at 1700*g* for 5 min at room temperature.
6. Resuspend the cells into 1.0 mL of 1X LiAc/TE buffer.
7. Add 100 µL of denatured ssDNA (10 mg/mL) to the cell suspension and mix well. Aliquot 10 µL of the cell mixture into each well of the 96-well plate containing the RCA amplified DNA in **Subheading 3.4.6.**
8. Incubate at room temperature for 15 min.
9. Add 50 µL of PEG/LiAc solution containing 10% DMSO to each well. Gently and thoroughly mix.
10. Incubate at 30°C for 30 min and heat shock in a 42°C water bath for 30 min.
11. Transfer 10 µL of the cells using a 96-pin replicator (V&P Scientific, INC) onto a SD/-Leu-Ura+10 µg/mL cycloheximide medium.
12. Dry the plate in a clean hood for 10 to 20 min and incubate at 30°C for 6 to 7 d.
13. Replicate the colonies growing from the above plate onto SD/-Leu-Ura+Trp(L) + 0.5 g/L FAA medium (*see* **Note 9**).
14. Incubate at 30°C for 2 to 3 d.
15. Replicate the cells on SD/-Leu-Ura medium and incubate at 30°C for 3 d.
16. Replicate the colonies growing on the above plate onto SD/-Leu-Ura-His + 40 m*M* 3-AT medium and incubate at 30°C for 6 to 7 d (**Fig. 4**; *see* **Note 10**).

4. Notes

1. The pXDGATcy86 vector, derived from the pPC86 and pPC97 plasmids ***(12)***, is designated for initial library screen. The plamid contains all the features of a bait vector including the sequences coding for the GAL4 DNA binding domain (GAL4-DB) followed by a gateway cassette, the *TRP1* marker for selecting the presence of this plasmid in yeast cells, and the $CYH2^S$ marker. The gateway cassette facilitates the rapid cloning of a gene of interest into this vector using the gateway reactions, whereas the *TRP1* and $CYH2^S$ markers allow for the elimination of pXDGATcy86 derived constructs by the FAA (2-amino-5-fluorobenzoic

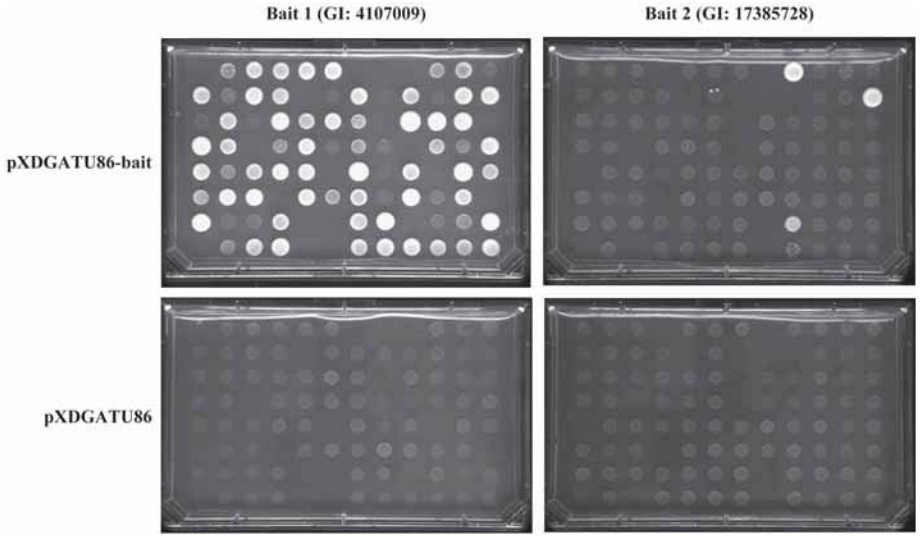

Fig. 4. Verification of candidate interactors by yeast retransformation. Yeast cells, containing the constructs indicated on the left and top, were transformed with candidate interactors and grown on the selective medium (SD/-Leu-Trp-His+ 40 m*M* 3-AT). Colonies capable of growing on the selective medium indicate activation of the reporter gene *His3*.

acid) and/or the cycloheximide counterselection in the RCA products after yeast re-transformation ***(12,15)***.

The pXDGATU86 bait vector is developed for the verification of the identified interactions. Unlike pXDGATcy86, this vector does not contain the above counterselection markers. The *URA3* gene can be used for selecting the presence of this plasmid in yeast cells. Before library screening, the gene of interest is in-frame fused with the GAL4-DB domain in the two bait vectors, respectively. We transform the pXDGATcy86 derived bait constructs into the yeast strain CG1945 for two-hybrid library screening. The **a**-mating type strain (e.g., CG1945) can be mated with an α-mating type strain (e.g., Y187) carrying a cDNA library. In addition, the wild-type CG1945 is resistant to cycloheximide, therefore can be subjected to cycloheximide counterselection when transformed with the pXDGATcy86 derived bait.

To transform a bait vector into CG1945, a number of procedures, for example, Walhout et al. ***(16)***, are suitable. The protocol described here is modified from the user manual of the MATCHMAKER GAL4 Two-Hybrid system (Clontech).

2. Certain gene products can activate the transcription of reporter genes in a prey-independent manner. These bait constructs are not suitable for yeast two-hybrid screening. To test the autoactivation capability of bait, the frozen cells carrying

the pXDGATcy86 derived construct are streaked onto SD/-Trp and SD/-Trp-His media, respectively. If a construct supports the growth of yeast cells these two types of media after incubation at 30°C for 5 to 6 d, the bait autoactivates the *HIS3* reporter.

3. The cDNA libraries are constructed using the HybriZAP-2.1 Two-Hybrid system (Stratagene) by following the manufacture's instructions. The pAD-GAL4-2.1 vector, containing the *LEU2* marker, is compatible with the pXDGATcy86 and pXDGATU86 vectors. Other cDNA libraries constructed using a similar vector may also be compatible to the bait vectors.
4. The screening procedure is modified from Soellick and Uhrig ***(17)***. With the standard laboratory equipment and our multichannel filtration system, we can conduct 15 to 20 screenings simultaneously.
5. We have assembled a six-channel filtration system for rapid harvest of yeast cells onto membranes for mating. The cells, resuspended in 250 mL of H_2O, are vortexed at maximum speed, poured into a funnel containing a 47-mm water membrane (pore size = 0.45 μm) and collected onto the membrane after a vacuum is drawn by a pump. Six samples can be processed simultaneously. To further increase the throughput, additional six-channel filtration apparatus can be added to the system.
6. When transferring the 47-mm water membrane containing the yeast cells onto the solid YCM (pH 4.5) medium for mating, do not allow any bubbles to occur between the membrane and the medium.
7. Do not apply too many cells onto the selection medium (about 3–4 million diploid zygotes/Bioassay dish). The screening stringencies, adjusted by the concentration of 3-AT in the media, vary greatly with different yeast strains.
8. Both cell lysates and isolated plasmids can be used as templates to amplify bait and prey plasmids. Compared with cell lysates, higher yields and reproducibility of amplification can be achieved by using the isolated plasmids. We use the Yeast Plasmid Miniprep kit (Zymo Research, cat. no. D2001) to isolate plasmid DNA from yeast as the template for RCA. In brief, add 30 μL of buffer 1 containing 0.2 μL of zymolase into each well by a 12-channel pipettor. Mix well and incubate at 30°C for 1 h. Add 30 μL of buffer 2 to lyse the cells and add 30 μL of buffer 3. Mix well and centrifuge at 15,000*g* for 10 min. Transfer 100 μL of the supernatant into a new plate and mix with an equal volume of 2-propanol. Centrifuge at 15,000*g* for 20 min. Discard the supernatant.
9. For FAA counterselection, the concentration of tryptophan in the medium must be lowered to 0.1 mg/L. To avoid the carrying over of a clump of yeast cells, do not transfer too many cells onto counterselection medium. The reason for this is to ensure that all of the cells come into contact with the counterselection medium, thus the bait carrying yeast cells can be eliminated.
10. We consider the interactions that cannot be confirmed by yeast retransformation as false-positives. After analyses of more than 60 rice kinases, we found that the false-positive rate varies significantly depending on the baits. **Figure 4** shows the verification results from two rice kinases. For the first kinase, most of the

interactions identified from the initial library screening can be confirmed by retransformation. In contrast, the majority of interactors identified from the second kinase failed to be scored as positives after yeast retransformation.

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References

1. Fields, S. and Song, O. (1989) A novel genetic system to detect protein-protein interactions. *Nature* **340,** 245–246.
2. Brent, R., Jr. and Finley, R. L. (1997) Understanding gene and allele function with two-hybrid methods. *Annu. Rev. Genet.* **31,** 663–704.
3. Nodzon, L. and Song, W.-Y. (2004). Yeast two-hybrid technology, in *Encyclopedia of Plant and Crop Science* (Goodman, R. M., ed.), Marcel Dekker, Inc.: New York, pp. 1302–1304.
4. Xenarios, I., Salwinski, L., Duan, X. J., Higney, P., Kim, S. M., and Eisenberg, D. (2002) DIP, the Database of Interacting Proteins: a research tool for studying cellular networks of protein interactions. *Nucleic Acids Res.* **30,** 303–305.
5. Vidalain, P. O., Boxem, M., Ge, H., Li, S., and Vidal, M. (2004) Increasing specificity in high-throughput yeast two-hybrid experiments. *Methods* **32,** 363–370.
6. Kornberg, A. and Baker, T. A. (1992) *DNA Replication*. W. H. Freeman and Company, San Francisco.
7. Fire, A. and Xu, S.-Q. (1995) Rolling replication of short DNA circles. *Proc. Natl. Acad. Sci. USA* **92,** 4641–4645.
8. Dean, F. B., Nelson, J. R., Giesler, T. L., and Lasken, R. S. (2001) Rapid amplification of plasmid and phage DNA using phi29 DNA polymerase and multiple-primed rolling circle amplification. *Genome Res.* **11,** 1095–1099.
9. Blanco, L., Bernad, A., Lazaro, J. M., Martin, G., Garmendia, C., and Salas, M. (1989) Highly efficient DNA synthesis by the phage phi29 DNA polymerase. Symmetrical mode of DNA replication. *J. Biol. Chem.* **264,** 8935–8940.
10. Garmendia, C., Bernad, A., Esteban, J. A., Blanco, L., and Salas, M. (1992) The bacteriophage phi29 DNA polymerase, a proofreading enzyme. *J. Biol. Chem.* **267,** 2594–2599.
11. Esteban, J. A., Salas, M., and Blanco, L. (1993) Fidelity of phi 29 DNA polymerase. Comparison between protein-primed initiation and DNA polymerization. *J. Biol. Chem.* **268,** 2719–2726.
12. Ding, X., Cory, G., and Song, W.-Y. (2004) A high-throughput system to verify candidate interactors from yeast two-hybrid screening using rolling circle amplification. *Anal. Biochem.* **331,** 195–197.

13. Chen, X., Ding, X., and Song, W.-Y. (2003) Isolation of plasmid DNA rescued from single colonies of Agrobacterium tumefaciens by means of rolling circle amplification. *Plant Mol. Biol. Rep.* **21,** 411–415.
14. Ding, X., Snyder, A. K., Shaw, R., Farmerie, W.G., and Song, W.-Y. (2003) Direct retransformation of yeast with plasmid DNA isolated from single yeast colonies using rolling circle amplification. *Biotechniques* **35,** 774–779.
15. Toyn, J. H., Gunyuzlu, P. L., White, W. H., Thompson, L. A., and Hollis, G. F. (2000) A counterselection for the tryptophan pathway in yeast: 5-fluoroanthranilic acid resistance. *Yeast* **16,** 553–560.
16. Walhout, A. J. and Vidal, M. (2001) High-throughput yeast two-hybrid assays for large-scale protein interaction mapping. *Methods* **24,** 297–306.
17. Soellick, T. R. and Uhrig, J. F. (2001) Development of an optimized interaction-mating protocol for large-scale yeast two-hybrid analyses. *Genome Biol.* **2,** research0052.1–0052.7.