Chapter 4

A Stringent Yeast Two-Hybrid Matrix Screening Approach for Protein–Protein Interaction Discovery

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Abstract

The yeast two-hybrid (Y2H) system is currently one of the most important techniques for protein–protein interaction (PPI) discovery. Here, we describe a stringent three-step Y2H matrix interaction approach that is suitable for systematic PPI screening on a proteome scale. We start with the identification and elimination of autoactivating strains that would lead to false-positive signals and prevent the identification of interactions. Nonautoactivating strains are used for the primary PPI screen that is carried out in quadruplicate with arrayed preys. Interacting pairs of baits and preys are identified in a pairwise retest step. Only PPI pairs that pass the retest step are regarded as potentially biologically relevant interactions and are considered for further analysis.

Key words: Protein–protein interactions, Interactome mapping, Yeast-two hybrid, Large-scale screen, Network biology, STUB1/Chip, FKBP6/FKBP36, PRKACA/PKC alpha

1. Introduction

The yeast two-hybrid (Y2H) system (1) is a widely used tool for the discovery of protein–protein interactions (PPIs). Hundreds of laboratories successfully used the system to find novel proteins that prove to be important for the biological system under study. In the last 10 years, the Y2H system has been developed as a tool enabling the systematic, large-scale analysis of protein–protein interactions (2–15). At present, it is one of the most powerful methods for the generation of proteome-wide, binary protein–protein interaction maps (16–22) and will play a crucial role in whole-organism interactome mapping (23–27).

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Here, we describe our version of the Y2H system, which should be useful for experimentalists searching for protein–protein interactions as well as researchers who use the protein–protein interaction data generated in our lab for further computational and experimental analysis.

In contrast to Y2H library screens, matrix screens use ordered arrays of hybrid proteins, so-called baits and preys that are subcloned individually and characterized. The major advantage of array screens is that all protein pairs are tested with equal probabilities and interaction screens can be repeated. This allows quality-controlled, systematic generation of protein–protein interaction data on a large scale.

We describe a three-step matrix approach. First, after preparation of yeast strains in array format, we identify and eliminate autoactivating bait strains that would compromise the whole screen. Nonautoactivating strains are then used for the primary PPI screen and interacting pairs of baits and preys are identified in a third step using fresh yeast, a pairwise retest. Only PPI pairs that pass the third step are regarded as potentially biologically relevant interactions and are considered for further analysis.

1.1. Parameters for a Stringent Y2H System

The stringency of a Y2H screening experiment critically depends on three parameters. These parameters are of great importance for obtaining high quality interaction data in Y2H analyses; however, they can be addressed differently as exemplified by other versions of the Y2H system used in several other laboratories (28–31).

First, it is important to express bait and prey fusion proteins at very low levels. It is the rule rather than the exception that bait and prey proteins cannot be seen via western blotting of total yeast lysates even though the protein pair generates a genuine positive Y2H signal. In our system, low protein expression levels are achieved by using very weak promoters.

Second, reporter genes integrated in the genome of the Y2H yeast strains should not allow any activity in the absence of interacting baits and preys even after long incubation times. In particular for large-scale PPI experiments a background-free setup is essential as every single bait–prey combination has to be unambiguously classified as positive or negative. As the strength of the Y2H signal depends on the protein expression level, stability, localization and other parameters which are variable between different proteins, colony size is not a reliable measure for interaction detection and shows only weak correlation with affinity (32). Weak Y2H signals, which may result from high affinity interactions and give a strong signal in other interaction assays, can only be detected efficiently in a background-free assay. In our system, the *his3* reporter is the most stringent readout fully suppressing growth in the absence of an interaction.

Third, to detect interactions with a weak Y2H signal, the experiments have to be performed in several replicas using fresh yeast strains. Statistical analysis of the data helps identifying those interactions and largely improves data quality. Although established Y2H PPIs are highly reproducible, in large-scale screens only a subset of interactions is detected in every experiment. The sampling sensitivity of matrix screening approaches has recently been determined from repeated screening experiments (27). In our system, after four rounds of primary screen and retest approximately 42, 26, 18, and 14% of PPIs seen in the 1st, 2nd, 3rd, and 4th repeat of screening are new, respectively. Approximately 46% of the detected interactions are found once, 44% two times and 10% of the PPIs are detected more than two times. Thus, the number of interactions found only once decreases in successive screens and interactions that have been identified multiple times become the majority. Therefore, statistical data analysis can efficiently identify interactions, also those that give relatively weak Y2H signals, in a large-scale screen, even at low repeat numbers.

1.2. Performance of the Y2H System

Recent studies demonstrated that large-scale Y2H screens can result in high precision data. A study examining large-scale PPI data from yeast (22) has shown that data from Y2H and affinity purification coupled to mass spectrometry are of similar quality when the benchmarking sets used for quality measurements account for the different and complementary nature of the interactions. Quality estimates were also provided for large-scale *Caenorhabditis elegans* (33) and human PPI data (27) using empirical measurements with standard interaction sets and independent PPI detection methods. Venkatesan et al. measured the precision of two large single screen/single retest human Y2H datasets (18, 19) retesting random samples of 200 interactions from each with MAPPIT (34). These screens, each of which examined more than 25×10^6 protein pairs for interaction, showed an average precision of ~80% in independent experiments.

However, the sensitivity of the individual Y2H systems is 5–20%, only (24, 27). This holds true for other PPI-detection methods as well. Importantly, when a set of true interaction from the literature is being examined with different PPI methods, each method detects its own subset (24, 27). In the overlap we find exactly the number of interactions that is statistically expected for independent measures. This means that sensitivity can be increased by combining different PPI detection methods (35–37). Notably, this does also hold true for different versions of the Y2H system. Provided that different Y2H systems are producing high-precision data, parallel use of several Y2H setups will simply increase sensitivity and yield more complete interaction maps (24, 26, 30).

Clone selection is decisive for whether interactions are found with Y2H. Since a high fraction of baits is either autoactive and

cannot be used in a Y2H experiment in the first place or simply does not yield interactions, the use of several different clones covering each protein increases the chance of finding PPIs substantially. Sometimes even a second full-length ORF for a protein that is inserted differently in the Y2H vector interacts while the first does not. To increase the chance of finding PPI partners, for large proteins in particular, it is advisable to screen with well characterized domains or protein fragments that ideally have been shown to be functional in other systems. In the end, the Y2H system is an extremely powerful PPI discovery tool. As such, it provides a wealth of high quality information for further experimental and computational analysis.

2. Materials

2.1. Labware

1. 96-Well MTPs with lid, PP, sterile, flat bottom (Greiner Bio-One GmbH).
2. 384-Well MTPs with lid, PP, sterile, flat bottom (Greiner Bio-One GmbH).
3. Omnitrays (Nunc GmbH & Co. KG).
4. Agar-plates (low profile bioassay dishes 241 × 241 × 20; Nunc GmbH & Co. KG).
5. 96-Well PCR plate (Costar).
6. 96-Well deepwell plates (2,000 µl/well; Eppendorf).
7. Pin tools with 96 and 384 pins. The steel pins are cylindrical with a diameter of 1.3 mm and the edge of the flat top that is touching the agar is beveled 45°at 0.2 mm. Sterilize by heating the pins until they glow red. Let them cool in a sterile environment (see Note 1).
8. Plastic tape for sealing PCR plates and MTPs (Costar or Thermo Scientific).
9. Sterile breathable sealing films (Aeraseal).

2.2. Solutions

1. Ampicillin stock (100 mg/ml): Dissolve 100 mg ampicillin sodium salt in 1 ml water. Store at –20°C. Dilute to a final concentration of 100 µg/ml (see Note 2).
2. Tetracycline stock (12.5 mg/ml): Dissolve 12.5 mg tetracycline hydrochloride in 1 ml 50% ethanol. Store at –20°C until use. Dilute to a final concentration of 20 µg/ml.
3. Tris/EDTA buffer pH 7.5 (10× TE): 100 mM Tris–HCl, pH 7.5, 10 mM EDTA, pH 8. Autoclave. Store at room temperature.

2.3. Media Stocks

1. LB medium: 10 g Bacto tryptone, 5 g yeast extract, 10 g NaCl, adjust to a final volume of 1 l with water and autoclave. Store at room temperature. Supplement with the appropriate antibiotics and mix before use.
2. LB agar: 10 g Bacto tryptone, 5 g yeast extract, 10 g NaCl, 20 g agar, adjust to a final volume of 1 l with water and autoclave. Store at room temperature. For agar plates, heat until the agar is dissolved, cool to 60°C, supplement with the appropriate antibiotics, stir and pour into sterile Petri dishes. Store plates at 4°C.
3. 1.25× YPD liquid medium: 5 g Yeast extract, 10 g peptone. Fill up to 400 ml with water and autoclave. Store at room temperature.
4. 1.25× YPD agar: 5 g Yeast extract, 10 g peptone, 10 g agar. Fill up to 400 ml with water and autoclave. Store at room temperature.
5. 2.5× Yeast medium (NB): 6.7 g Yeast nitrogen base. Fill up to 400 ml with water and autoclave. Store at room temperature.
6. 1.25× Yeast liquid medium (NB): 3.35 g Yeast nitrogen base. Fill up to 400 ml with water and autoclave. Store at room temperature.
7. 20× Glucose stock solution: 200 g Glucose monohydrate. Fill up to 500 ml with water and autoclave. Heating up helps dissolving the glucose before autoclaving. Store at room temperature.
8. 100× Amino acid/nucleoside stock solutions: Dissolve 4 g of leucine (L) in 400 ml water and autoclave. For histidine (H), adenine (A), uracil (U), and tryptophan (T) dissolve 0.8 g of the corresponding amino acid/nucleoside in 400 ml water and autoclave. Store at room temperature.
9. 2.5× Agar: For 500 ml of selective medium, autoclave 10 g agar in 200 ml water, store at room temperature.
10. 1.25× Yeast storage medium (NBG): 3.35 g Yeast nitrogen base, 250 ml glycerol (99%), and 29.44 g betain. Adjust to 400 ml with water and autoclave. Store at room temperature.
11. Sterile water (see Note 2).

2.4. Yeast Media Preparation

In this section, we describe the preparation of different media from the stock solutions. The media are named after the missing and required amino acids/nucleosides. Amino acids/nucleosides are abbreviated with a single letter as followed: “H” for histidine, “A” for adenine, “U” for uracil, “L” for leucine, and “T” for tryptophan. Anabolites omitted are marked by a minus sign and separated by a slash from the amino acids/nucleosides which are added

to the media. The order on both sides of the slash is always HAULT.

1. Liquid medium: Add 25 ml 20× glucose stock solution and 5 ml of each required 100× amino acid/nucleoside stock solution to 400 ml 1.25× NB or 1.25× NBG and adjust to a final volume of 500 ml with sterile water. Pipette 100–120 µl into each well of 96-well MTPs and 35–45 µl into each well of 384-well MTPs.
2. Solid medium: Add 200 ml of 2.5× NB, 25 ml 20× glucose stock solution, and 5 ml of each required 100× amino acid/nucleoside stock solution to 200 ml 2.5× agar. Adjust to a final volume of 500 ml with sterile water. Heat up in a microwave until everything is dissolved. Cool the medium to 60°C and pour 200 ml into each agar plate under a sterile hood (see Note 3).
3. YPD liquid medium: Add 25 ml 20× glucose stock solution and 5 ml 100× adenine stock solution (optional) to 400 ml 1.25× YPD and adjust to a final volume of 500 ml with sterile water.
4. YPD solid medium: Add 25 ml 20× glucose stock solution and 5 ml 100× adenine stock solution (optional) to 400 ml 1.25× YPD agar, fill up to 500 ml with sterile water and heat in a microwave until dissolved. Cool to 60°C and pour 200 ml into each agar plate under a sterile hood.

2.5. Y2H Vectors

We use Gateway-compatible Y2H destination vectors: the DNA binding domain (BD)-containing pBTM116-D9 (baits) is a derivate of pBTM116 (Clontech); the activation domain (AD)-containing vector pACT4-DM (preys) is based on pACT2 (Clontech). As an alternative prey vector, pGAD426-D3 is used, which originates from pGAD426 (Clontech). The vectors contain a bacterial origin of replication and selectable antibiotic markers, the β-lactamase gene Amp^R (pACT4-DM, pGAD426-D3) or the tetracycline-resistance gene Tet^R (pBTM116-D9), respectively. All three yeast expression vectors are 2 µm vectors and contain a truncated ADH1 promoter and an ADH1 terminator.

Bait open reading frames (ORFs) are subcloned into pBTM116-D9 which contains an N-terminal LexA DNA-binding domain and a *TRP1* selection marker enabling growth selection of yeast transformants. The preys are generated by inserting ORFs into pACT4-DM or pGAD426-D3, respectively; both carry the *LEU2* marker gene and contain an N-terminal GAL4 transcription activation domain. An advantage of the N-terminal BD- and AD-fusions is that ORFs can be used irrespective of whether they contain a stop codon at the end. Only a few C-terminal amino acids are added to the open ORFs because all vectors contain a C-terminal stop codon after the attB2 Gateway recombination site.

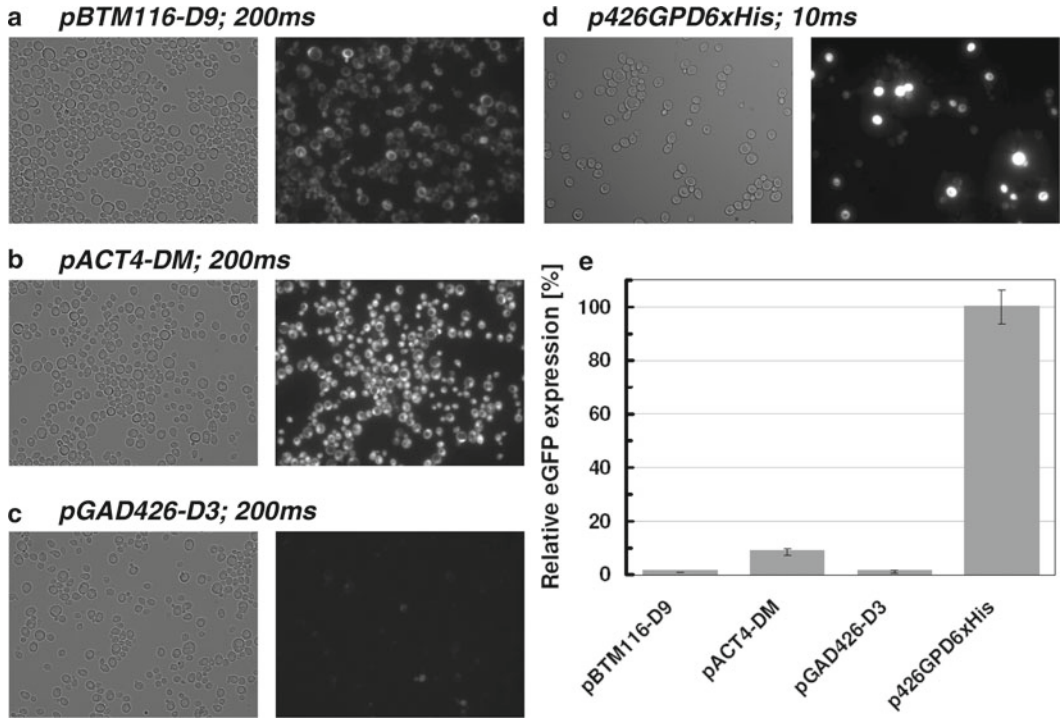

Fig. 1. Semiquantitative comparison of promoter activity of Y2H plasmids in *Saccharomyces cerevisiae*. Y2H MAT*a* strain was transformed with eGFP inserted in the Y2H pBTM116-D9 bait (**a**), pACT4-DM (**b**) and pGAD426-D3 (**c**) prey vectors. For comparison, eGFP expression was also assessed from a Gateway compatible p426GPD6xHis vector (**d**, 2 μ, ura auxotrophic marker). In this vector, eGFP is constitutively expressed from a glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter, while in the Y2H vectors a truncated ADH1 promoter is used. Yeast strains were grown on selective media and then replicated (solid–liquid–solid) on YPD. After 30 h, bright light and fluorescence pictures of eGFP-expressing yeast cells were taken for 200 ms (Y2H plasmids) and 10 ms (PGPD6xHis). (**e**) Comparison of eGFP expression after normalization to cell density measured at 600 nm. Relative fluorescence was measured in an MTP reader (Biomek DTX 800/880).

Relative protein expression levels have been assessed with eGFP, demonstrating that the truncated ADH1 promoter on our vectors drives very low levels of gene expression (Fig. 1). Although the pACT4-DM and pGAD426-D3 contain the same truncated ADH1 promoter, they differ in protein expression levels.

2.6. Gateway-Cloning

1. Destination vector (150 ng/μl).
2. Entry clone (obtained via 96-well *Escherichia coli* mini-prep, see Subheading 3.3).
3. pENTR-gus for positive controls (Invitrogen).
4. LR Clonase Enzyme Mix II (Invitrogen).
5. Proteinase K Solution (Invitrogen).

2.7. Transformation of Competent *E. coli*

1. pUC19 DNA for positive controls (Invitrogen).
2. Chemically competent DH10B *E. coli* cells.

3. SOC Medium: for 100 ml SOC medium supplement 99 ml SOB medium with 1 ml 20× glucose stock solution. SOB medium (1 l): 20 g tryptone, 5 g yeast extract, 0.5 g NaCl. Add water to a final volume of 1 l and autoclave. Add 10 ml of filter-sterilized 1 M $MgCl_2$ and 10 ml of filter-sterilized 1 M $MgSO_4$.
4. LB Plates containing 100 µg/ml ampicillin or 20 µg/ml tetracycline.

2.8. Ninety Six-Well *E. coli* Mini-prep

1. 50% Glycerol: 581 ml 86% glycerol (Merck), bring up to 1 l with water and autoclave. Store at room temperature.
2. Buffer P1: 50 mM Tris, pH 8, 10 mM EDTA, pH 8. Autoclave, store at 4°C after addition of 50 mg/l RNAse A (f.c.).
3. Buffer P2: 200 mM NaOH, 1% SDS (w/v). Store at room temperature.
4. Buffer P3: 300 ml 5 M potassium acetate pH 5.5, 57.5 ml glacial acetic acid, 145.5 ml water. Store at room temperature.
5. Isopropanol (p.a.) (Merck).
6. 70% (v/v) ethanol: 700 ml absolute ethanol (Merck), bring up to 1 l with water.

2.9. Yeast Strains

We use L40ccu (MAT*a*) and L40ccα (MATα) (7), both of which have a *LacZ* ((*lexAop*)$_8$-GAL1TATA-*lacZ*) and a *HIS3* ((*lexAop*)$_4$-HIS3TATA-*HIS3*) reporter, additionally L40ccu has a *URA3* reporter ((*lexAop*)$_8$-GAL1TATA-*URA3*). L40ccu and L40ccα are auxotroph for leucine (*leu2-3,112*) and tryptophan (*trp1-901*), the *ADE2* gene is deleted in L40ccα (see Note 4). While choosing strains, keep in mind that the two strains, typically with the same genetic background, have to be of different mating type. Furthermore, the strains have to carry reporter genes with promoters compatible with the binding domain of your bait vector.

2.10. Yeast Transformation

1. YPDA liquid medium.
2. MAT*a* and MATα yeast strains to be transformed, e.g., L40ccα for preys cloned into pACT4-DM or pGAD426-D3, and L40ccu for baits in pBTM116-D9.
3. 1 M LiOAc, autoclave and store at room temperature.
4. 2 M Sorbitol, autoclave and store at room temperature.
5. 60% PEG-3350, autoclave and store at room temperature.
6. Expression vectors, e.g., pBTM116-D9 as the BD- and pACT4-DM or pGAD426-D3 as the AD-containing vectors, respectively.
7. Empty prey vector for the autoactivation test.

8. Carrier DNA: Dissolve 5 mg/ml salmon sperm DNA (Sigma) in 1× TE, heat up to 95°C for 5 min, put on ice, aliquot and store at –20°C.
9. Selective agar plates (-L/HAUT for preys and -AT/HUL for baits, see Subheading 2.4).

2.11. Beta-Galactosidase Assay

1. Diploid yeast containing putatively interacting bait–prey combinations.
2. Liquid nitrogen.
3. Sterile nylon membranes (MagnaCharge from MSI).
4. Z-buffer: 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$.
5. 1 M Dithiothreitol (DTT).
6. Whatman 3MM Chromatography paper.
7. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), 20 mg/ml in dimethylformamide.
8. Agar plates containing-HAULT medium.

3. Methods

We describe a three-step matrix screening protocol (Fig. 2), including a description of basic tools (see Subheadings 2.5 and 2.9) and yeast handling procedures (Subheading 3.4), the generation of the hybrid constructs (Subheadings 3.1–3.3), the transformation of yeast strains in 96-well format (Subheading 3.5), assaying for autoactivation (Subheading 3.6), screening of a prey matrix for primary interaction partners (Subheading 3.7) and the final identification of interacting protein pairs by retesting (Subheadings 3.8 and 3.9).

3.1. Gateway-Cloning

This method is used for the parallel site-specific recombination of ORFs from entry into Y2H destination vectors in 96-well format. Gateway-compatible entry plasmids carrying ORFs can be obtained from various sources and distributors (38–40).

1. Prepare a master mix of 0.5 µl destination vector (150 ng/µl, see Note 5 for 2-in-1-LR Clonase reaction), 2 µl TE-buffer and 1 µl LR Clonase Enzyme Mix II per reaction, mix and transfer 3.5 µl master mix per well into a PCR plate.
2. Add 1.5 µl entry vector per well (DNA obtained with the 96-well mini-prep can be used here; protocol described in Subheading 3.3). Include a negative control (i.e. only master mix and water or elution buffer instead of the entry vector)

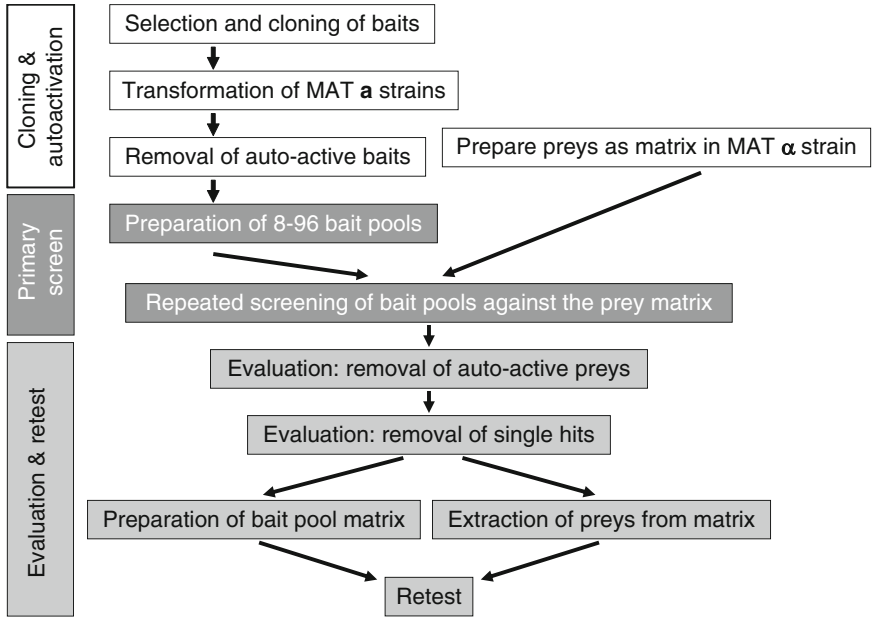

Fig. 2. Schematic depiction of the experimental flow. First, baits proteins are selected for cloning and MAT*a* strains are transformed with the bait plasmids and tested for autoactivity. After removal of autoactives, baits are pooled and screened against a previously prepared prey matrix. The resulting interacting pairs are filtered and tested again in an independent experiment using fresh yeast. *Shading* indicates the three parts of the protocol.

and a positive control (i.e. a well-tried entry vector or the pENTR-gus control vector provided by Invitrogen).

3. Seal the PCR plate with plastic tape, mix and spin down.
4. Incubate the plates at 25°C for 3–18 h.
5. Add 0.5 µl of Proteinase K solution to each reaction.
6. Seal the 96-well plate with plastic tape, vortex for 20 s and spin down.
7. Incubate the plates for 10 min at 37°C.
8. Use reaction directly for transformation or store at –20°C.

3.2. Transformation of Competent E. coli with LR Reaction Mixtures

This protocol is suitable for 96-well format transformations and yields colonies for 95–100% of the LR reactions if chemically competent DH10B are used (41). For missing clones, we use electroporation to obtain colonies, which is successful most of the time because of the higher transformation efficiency.

1. Prepare 200 µl SOC-Medium per well in a deepwell plate and warm up to 37°C.
2. Thaw the required amount of competent bacteria (30 µl per reaction) on ice and pipette into a PCR plate.
3. Add 2.5 µl (per well) of the Proteinase K-treated LR reaction prepared above (also include a positive control of 10 pg pUC19 DNA (Invitrogen) for transformation efficiency).

4. Seal the PCR plate with plastic tape, vortex softly and incubate on ice for 30 min.
5. Incubate at 42°C for 90 s.
6. Incubate on ice for 5 min.
7. Add 70 µl prewarmed SOC-Medium per well from the first column of the deepwell plate to the first column of the PCR plate and directly transfer the whole content of the wells back into the deepwell plate.
8. Repeat step 7 for the remaining 11 columns. Shake the deepwell plate at 37°C for 1 h.
9. Pipette a total of 50 µl from each well on selective LB plates by making rows of drops of 5 µl on an agar plate. Use LB plates containing 20 µg/ml tetracycline for pBTM116-D9 or 100 µg/ml ampicillin for pACT4-DM.
10. Incubate the LB plates for 16–20 h at 37°C.
11. Use colonies directly for the inoculation of LB-Medium to obtain DNA via 96-well mini-prep.
12. If you do not obtain clones in the first place repeat transformation of 1–2 µl of the remaining LR reaction using an electroporation protocol.

3.3. Ninety Six-Well E. coli Mini-prep

We developed a protocol that produces DNA of sufficient quantity (10–20 µg of plasmid DNA) and quality for LR-reactions, sequencing and yeast transformation. We use a pipetting robot to add buffers and to take off the supernatant but a pipette can also be used.

1. Pipette 1,000 µl LB medium containing the appropriate antibiotic into each well of a deepwell plate and inoculate with a single *E. coli* colony.
2. Seal the deepwell plate with breathable sealing film and grow for 15–18 h in a shaker at 37°C.
3. Transfer 50 µl of culture into a 96-well MTP that is filled with 50 µl 50% glycerol, mix and store at –80°C.
4. Spin the deepwell plate at 4°C, 1,258 × *g* for 30 min; pour off the supernatant and dry by tapping on a paper towel a couple of times.
5. Add 300 µl Buffer P1 per well (ensure that RNAse A has been added), seal the plates with plastic tape (see Note 6) and vortex vigorously for 2–3 min; make sure the pellet is completely dissolved.
6. Add 300 µl Buffer P2 per well, seal the plates with plastic tape, mix thoroughly by inverting the plate 3–4 times, and incubate for 5 min at room temperature.
7. Add 300 µl Buffer P3 per well, seal the plates with plastic tape, mix thoroughly by inverting the plate 3–4 times.

8. Centrifuge for 1 h at 3,220 × *g* at room temperature.
9. Transfer 750 µl of the supernatant from step 8 to a new deepwell plate and add 530 µl isopropanol to each well.
10. Seal the plates with plastic tape, mix thoroughly by inverting the plate 3–4 times.
11. Spin the deepwell plate at 3,220 × *g* for 45 min at room temperature; pour off the supernatant and dry by tapping on a paper towel a couple of times.
12. Add 1,000 µl 70% ethanol per well, seal the plates with plastic tape, and mix by inverting the plate 3–4 times.
13. Spin the deepwell plate at 3,220 × *g* for 30 min at 4°C; pour off the supernatant and dry by tapping on a paper towel a couple of times.
14. Allow for the pellet to dry. This might take up to 30 min.
15. Add 100 µl sterile water per well and dissolve the pellet by incubating over night at 4°C or by shaking for 1 h.
16. Control the success of the mini-prep in a BsrGI restriction analysis (see Note 7).

3.4. Yeast Handling

For Y2H experiments, yeast needs to be grown in and thus be transferred to different liquid and solid media. The yeast can be transferred from liquid to solid medium, solid to liquid, liquid to liquid and solid via liquid to solid. Instead of a solid–solid transfer, the solid–liquid–solid step is used to get a better selection of the yeast, because it prevents the yeast from clumping together. Correct growth and storage of yeast is important for successful screening. The yeast gets inoculated and streaked out in different formats as described below:

1. Liquid–solid: Streak (inoculation loop), drop (pipette) or stamp (pin tool) liquid yeast culture on agar plates. Take care that the liquid culture is mixed well. To grow the yeast on agar, streak it out, wrap the agar plate in foil and incubate it for 2–7 days at 30°C (see Note 8).
2. Solid–liquid: Scrape off yeast colonies from agar plates and transfer to liquid medium with an inoculation loop or with a pin tool. It is important to vortex the medium to separate the yeast clumps and get a uniform suspension of cells. Using a pin tool, scrape off yeast from the agar and transfer it to MTPs containing the liquid medium, repeat at least once and mix well. The liquid cultures obtained in the MTPs are quite dense and do not grow to much higher densities when incubated.
3. Solid–liquid–solid: Scrape off yeast colonies from agar plates like described above and transfer the resuspended yeast culture to solid medium with a pin tool, pipette or inoculation loop.

If using a pin tool stamp the yeast culture at least two times on the agar, wrap in foil and incubate at 30°C for 2–7 days.

4. Liquid–liquid: It is possible to inoculate liquid media with liquid yeast culture. To grow yeast in MTPs, inoculate each well with 5 µl of yeast culture, mix, wrap MTPs in foil and incubate at 30°C for 18–20 h. In contrast to 96-well MTPs, 384-well MTPs are incubated without shaking. To grow yeast in deepwell plates pipette 10 µl of yeast culture per well into deepwell plates containing 1 ml medium per well. Seal the deepwell plate with breathable sealing film and grow for 18–20 h in a shaker at 30°C.
5. Yeast storage: Yeast colonies can be stored for at least 1 month on foil-wrapped agar at 4°C. For longer storage scrape off yeast from agar, transfer to NBG medium (see solid–liquid replication), incubate for 18–20 h, mix and freeze at −80°C. Frozen yeast can be thawed two times only and needs to be replicated on agar (liquid–solid) for an extra generation before starting a Y2H experiment.

3.5. Ninety-Six-Well Yeast Transformation

With this 96-well format yeast transformation protocol, haploid MAT*a* and MATα yeast strains are transformed with the bait and prey plasmids respectively. Selection of the transformed yeast cells requires -AT/HUL and -L/HAUT media because in our system the bait vector pBTM116-D9 has a tryptophan and the prey vectors (pACT4-DM/pGAD429-D3) have a leucine selection marker (see Subheading 2.5). It is important that the MATα strain is also transformed with a prey vector without insert because this strain is needed for the autoactivation test. Colonies obtained from the transformation have to be solid–liquid–solid replicated at least once (see Subheading 3.4) before they are used in an autoactivation or mating experiment. We generally create four biological replicas (quadruplicates) and keep them separated throughout the autoactivation test, the primary screen and the retest.

The following protocol describes the transformation of eight 96-well plates in parallel:

1. Inoculate 12.5 ml of YPDA liquid medium with yeast strains freshly grown on YPD agar, vortex and grow for 15–18 h at 30°C with shaking.
2. Prepare PCR plates filled with plasmid DNA: pipette 5 µl plasmid DNA into each well (DNA from the 96-well mini-prep described in Subheading 3.3. can be used here), spin down, add 5 µl carrier DNA to each well, mix and spin down. Include one negative control (i.e. only carrier DNA) and a positive control (i.e. a well-tried vector preparation).
3. Inoculate 250 ml of YPDA to an OD_{600} of 0.10–0.15 with the over-night culture and grow at 30°C with shaking until an OD_{600} of 0.6–0.8 is reached.

4. Freshly prepare Mix 1 and Mix 2. Mix 1 (10 ml for eight plates): 1 ml 1 M LiOAc, 0.5 ml 10× TE, 5 ml 2 M sorbitol, 3.5 ml sterile water. Mix thoroughly. Mix 2 (60 ml for 8 plates): 6 ml 1 M LiOAc, 6 ml 10× TE, 40 ml 60% PEG-3350, 8 ml sterile water. Mix thoroughly.
5. Once the culture reaches the desired OD, split and transfer to five 50 ml screw-cap centrifuge tubes and centrifuge at 805 × *g* for 5 min.
6. Remove the supernatant and resuspend each pellet in 20 ml sterile 1× TE. Centrifuge at 805 × *g* for 5 min, remove the supernatant.
7. Resuspend each pellet in 2,000 µl Mix 1 and pool them (total volume of 10 ml) and incubate at room temperature for 10–60 min.
8. Pipette 11 µl of the yeast Mix 1 into each well of the PCR plate containing plasmid and carrier DNA.
9. Seal the plate with plastic tape and mix. Do not spin the plate.
10. Pipette 58 µl of Mix 2 into each well of the PCR plate.
11. Seal the plate with plastic tape and mix for 1 min.
12. Incubate the plates at 30°C for 30 min.
13. Add 8 µl DMSO to each well.
14. Seal the plate with plastic tape and mix for 1 min.
15. Incubate the plates at 42°C for 7 min in a thermocycler.
16. Create four biological replicas by transferring the cells to four selective agar plates. Transfer baits to -AT/HUL and preys to -L/HAUT to select for transformed yeast. Typically we use a pin tool to spot the cells five times on the same position of selective agar plates (e.g., Petri dishes or Omnitrays). Alternatively a pipette or a pipetting robot can be used to transfer 5 µl to selective agar plates. Allow the spots to dry on the plates (to speed up the process dry the open plates under laminar air flow).
17. Incubate at 30°C for 3 days.
18. Scrape off transformed yeast from the agar using a pin tool, transfer to 96-well MTPs containing NBG, mix well and stamp three times on selective agar (solid–liquid–solid). Incubate the MTP at 30°C for 12 h, mix and store at −80°C.
19. Incubate the agar plates at 30°C for 48–72 h.
20. This freshly grown yeast can be used for yeast two-hybrid experiments.

3.6. Autoactivation Test

It is important to remove autoactive bait strains before the matrix screen, because autoactivating baits will always grow after mating and mask any interaction signal of other baits in a pool.

Autoactivation is operationally defined as detectable bait-dependent reporter gene activation in the presence of any prey vector, even without insert. To detect autoactivity, the bait strains are mated with a prey strain carrying the prey plasmid without insert. Baits growing on -HAULT medium are autoactive and should not be used in a pooled matrix screen (see Note 9). Test all four replicas for autoactivation.

1. Prepare bait strains on selective agar in 96-well format and incubate for 3–4 days.
2. One day before mating inoculate freshly grown prey strain carrying the plasmid without insert in liquid -L/HAUT medium (solid–liquid), vortex and grow 18–20 h at 30°C in a shaker. Prepare 20 ml for each bait plate to be tested.
3. Mating: Pipette the prey strain into 96-well MTPs (100 µl per well). Transfer each of the baits from the agar into the MTPs, which contain the prey strain without insert and stamp the bait and prey strain mixture directly onto YPD agar. Incubate for 36–44 h at 30°C.
4. To select for diploid yeast cells take off the yeast from the YPD agar, resuspend in -ALT/HU MTPs and transfer to -ALT/HU agar (solid–liquid–solid). This is important because autoactivation can only be reliably assayed in diploid strains (see Note 10).
5. After four nights of incubation at 30°C transfer the yeast from -ALT/HU agar to -HAULT agar (solid–liquid–solid via -ALT/HU MTPs) to select for the growth reporter gene activity. Take pictures of the -ALT/HU agar plates. Incubate the -HAULT agar plates for 5–7 days at 30°C.
6. Take pictures of the -HAULT agar plates.

Remove all bait strains which do not grow on -ALT/HU agar plates as well as those growing on -HAULT agar plates. Usually, autoactive baits grow in all replicas, but occasionally single autoactive spots are detected and must not be used in further experiments (see Note 11).

3.7. Screening Bait Pools Against a Prey Matrix

Yeast strains expressing BD-fusion proteins are screened for primary protein–protein interactions with every strain in the prey matrix. Independent of the actual pool size we repeat each screen four times with distinct bait replicas. For large screens, when many prey MTPs have to be assayed, we create bait pools that contain between 8 and 96 different bait strains. Very efficient pooling strategies have been reported that can increase specificity and sensitivity of large screens without an additional deconvolution step (42, 43). However, we use a retest (Subheading 3.8) that shows which baits in a pool are interacting with the prey that was positive in the primary screen. Importantly, bait strains are grown separately and the baits belonging

to one pool are combined directly before mating. Each prey is mated with each pool of baits and primary protein–protein interactions are identified after transfer to selective medium. For large prey matrixes automated screening is recommended. Prey vector controls are not required as protein–protein interactions are very rare events (27, 44) and thus a large majority of preys will not give any growth signal.

1. Three to four days before mating, prepare prey strains on selective agar in 384-well format and bait strains on selective agar in 96-well format (see Subheading 3.4).
2. Day 1: Prepare bait strains in liquid culture: Transfer (solid–liquid) the baits to -AT/HUL MTPs. Liquid–liquid inoculate -AT/HUL medium in flasks or deepwell plates with the bait strains (see Note 12) and grow them for 18–22 h at 30°C in a shaker to early stationary phase (OD_{600} = 1.5–3). Inoculate at least 20-ml liquid medium per prey MTP to be screened. Grow each bait strain separately to avoid growth competition.
3. Day 2: Mating: Make sure that the baits are grown to early stationary phase and that all the yeast is completely suspended before pooling. Combine all bait strains belonging to the same pool in one beaker and mix thoroughly, keeping replicas separated. Pipette the bait strains into 384-well MTPs (40 µl per well). Scrape off prey strains from the agar using a pin tool, transfer to the 384-well MTPs containing the corresponding bait pool cultures, mix well and stamp on YPD agar. This way in each position one prey strain is mated with all baits in one pool. Incubate for 36–44 h at 30°C to allow mating. In essence the mating step is a solid–liquid–solid replication step of the prey matrix, from -L/HAUT agar to YPD agar, except that the MTPs do not contain fresh medium but rather bait pools.
4. Day 4: Interaction selection: Transfer the colonies from YPD agar to 384-well MTPs containing -ALT/HU medium, then to -HAULT agar (solid–liquid–solid). Incubate the agar plates at 30°C for 5–7 days.
5. Mating control: Control the diploid recovery by taking some of the -ALT/HU 384-well MTPs with the yeast mixtures from step 4 and stamping onto -ALT/HU agar (liquid–solid). Grow for 3–4 days at 30°C.
6. Day 8: Take pictures of mating control plates. The diploid recovery should be close to 100% (see Note 13).
7. Day 11: Take pictures of the -HAULT agar plates.

3.8. Retesting

After the primary screen, a retest is necessary to verify and deconvolute the results. High confidence in the final interaction set is guaranteed by using fresh yeast with a low generation number and small

culture volumes keeping the probability of acquiring mutation or recombination events very low (29).

In principle, all bait pool–prey combination that result a growth signal in the primary screen should be retested. However, there is a trade-off between the absolute number of interactions recovered and the retest success rate. We present simple rules designed to optimize this. We exclude autoactive/"sticky" preys and bait pool–prey combination that grow only once out of four times from the retest.

As in the autoactivation test, the bait and prey handling is opposite to the screen. The baits are prepared in matrix format on agar while the preys are grown in flasks. This way, each yeast spot corresponds to one bait–prey combination only. Also like in the autoactivation test, there is a diploid selection step between the mating on YPD and the interaction-specific selection.

1. Collect all primary PPIs in a relational database (SQL): For each yeast colony on the -HAULT agar plates, determine the matrix position, corresponding to the prey strain, as well as the bait pool. Make sure that agar numbers, plate numbers, row and column denominators are collected in separate fields and that each colony has a separate entry (see Note 14). All combinations of prey positions and bait pools collected constitute the primary retest list.
2. Removal of autoactive/"sticky" preys: For each prey position, determine the number of colonies and the number of bait pools that produce colonies (see Note 15). Remove any bait pool–prey combination with prey positions that score higher than 50% in both categories from the retest list (see Note 16).
3. Remove singletons: Remove all bait pool–prey combinations that produce yeast spots in only one out of four replica screens from the retest list.
4. Prepare prey cultures: Prepare an agar plate with freshly grown preys from the retest list by pipetting 5 µl of liquid culture on -L/HAUT agar and incubating for 24–36 h (see Note 17). Determine the number of bait pools for each prey. For retesting, prey spots are resuspended directly in 20–100 ml -L/HAUT liquid medium (20 ml per bait pool) and grown 18–22 h at 30°C. For preys with more than five bait pools, prepare 3 ml precultures 1 day in advance and inoculate cultures with 1% of preculture.
5. Prepare bait pool agars: For each bait pool, a 384-well MTP is prepared by combining the four 96-well MTPs containing the four replicas in the following manner. Replica A and B are put into the first (A1 of the 96-A replica in 384-A1) and fourth (A1 of the 96-B replica in 384-B2) quadrant, respectively. For replica C and D the 96-well MTPs are turned by 180° and put

into the second (H12 of the 96-C replica in 384-A2) and third (H12 of the 96-D replica in 384-B1) quadrant, respectively (see Note 18). One of these MTPs is sufficient for stamping 120 copies on agar (see Note 19).

6. Stamp (liquid–solid) the 384-well bait pool MTPs on -AT/HUL agar once for each associated prey on the retest list and incubate at 30°C for 60–72 h.
7. Mating: Transfer prey cultures from flasks to 384-well MTPs (40 µl/well). Scrape off bait colonies from the agar using a pin tool, transfer to the 384-well MTPs containing the corresponding prey solutions (one MTP per bait pool–prey combination), mix well, and stamp on YPD agar (solid–liquid–solid).
8. Recover diploids: After 38–44 h of incubation at 30°C, take off the yeast spots, resuspend in -ALT/HU MTPs, and stamp on -ALT/HU agar (solid–liquid–solid).
9. Interaction selection: After four nights of incubation at 30°C, take off the yeast spots, resuspend in -ALT/HU MTPs, and stamp on -HAULT agar (solid–liquid–solid). For assaying β-galactosidase activity (see Subheading 3.9) save the MTPs at this point.
10. After 5–7 days, take pictures of the agar plates.

Count the number of colonies for each interaction. Expect MTPs with more than one interaction at this point (see Note 20). Verified interactions show up as characteristic patterns, i.e., four yeast colonies that appear as two "anticorrelated" diagonal pairs of yeast colonies (Fig. 3). The Y2H interactions can be further validated applying other PPI detection methods (see Note 21). However, the Y2H PPI information as such is very useful for network analyses (45–48) and most promising starting points for functional studies (7, 12, 49, 50).

3.9. Beta-Galactosidase Assay

In our Y2H system the activity of the third reporter, the *E. coli LacZ* gene, is not tested via growth but in an enzymatic assay. The gene's product, beta-galactosidase, is a protein that cleaves 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) into galactose and 5-bromo-4-chloro-3-hydroxyindole, which oxidizes to 5,5′-dibromo-4,4′-dichloro indigo, resulting in a blue stain. The enzymatic assay is used in addition to growth reporter selection to further qualify Y2H interactions. The protocol for this assay is detailed here.

1. Place a nylon-membrane on a -HAULT agar by lifting two opposing corners with two pairs of forceps. Place the other two corners on an agar plate, then carefully let go of the first two corners. Remove any air bubbles that you may have created in the process. Prepare one agar for every six MTPs to be tested.

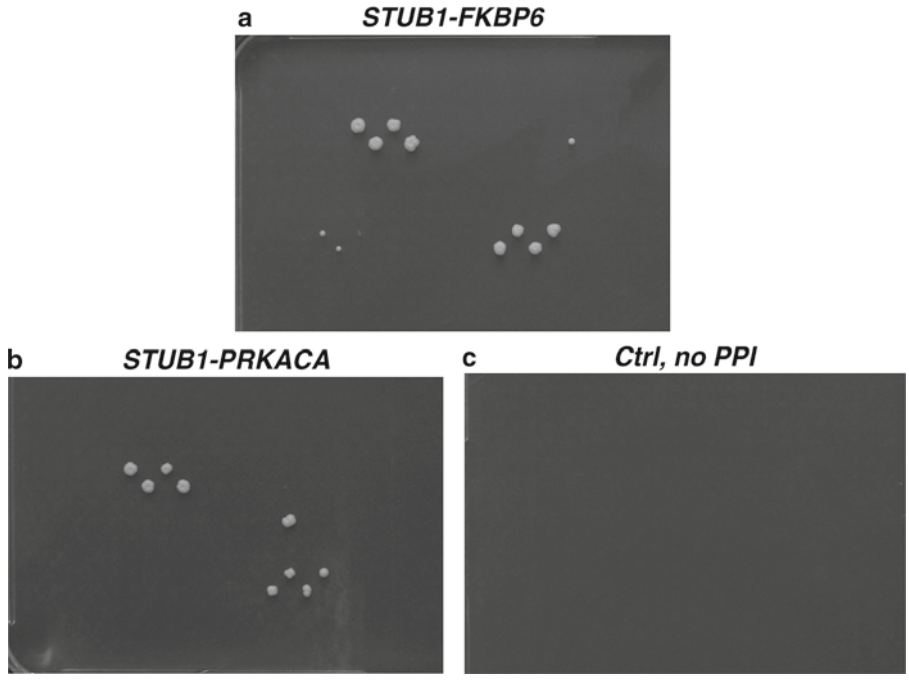

Fig. 3. Characteristic growth patterns indicate interactions in a retest experiment. The Y2H interactions between STUB1/Chip and FKBP6/FKBP36 (**a**) and STUB1/Chip and PRKACA/PKC alpha (**b**) are shown together with an experiment with a prey that does not interact with any of the baits (**c**). In this retest experiment, a 384 MTP that contains 96 baits in four replicas is tested against preys that were positive in the primary screen. Two full-length bait clones of STUB1/Chip (NP_005852, aa 1–303) are in position E7, F8, K18, L17 and E9, F10, K16, L15, respectively. Both constructs grow on selective media (–HAULT) when tested with FKBP6/FKBP36 (NP_003593, aa 1–322) or PRKACA/PKC alpha (NP_002721, aa 1–335) preys for interaction resulting a characteristic pattern of yeast colonies. STUB1/Chip is an E3 ubiquitin-protein ligase and HSPA8/HSC70 cochaperone for which quite a few protein interaction partners have been reported, e.g., ref. 51. STUB1/Chip function has been linked to neurodegenerative diseases (52). FKBP6/FKBP36, a peptidyl-prolyl *cis/trans* isomerase, is associated with Clathrin and Hsp72 in spermatocytes (53). FKBP6 deletions were shown to be associated with Williams–Beuren syndrome. The second potential STUB1/Chip interaction partner reported here, Protein Kinase C alpha (PRKACA/PKC alpha), is a major player in several well studied signaling cascades (54, 55).

2. Prepare diploid yeast containing bait and prey plasmids for each interaction to be tested like before. If you saved the -ALT/HU MTPs from the retest (step 10), these can be used.
3. Stamp the diploid yeast onto the -HAULT agars with membranes (liquid–solid, if using MTPs from retest). Use a solid–liquid–solid step if fresh diploids are grown on -ALT/HU agar (see Note 22).
4. After 5–7 days, remove the membranes from the agar plates with forceps, freeze them in liquid nitrogen, let thaw at room temperature, freeze and thaw again (see Note 23).
5. Add 400 µl 1 M DTT and 624 µl 20 mg/ml X-Gal solution to 40 ml Z-buffer for each membrane.

6. Place two sheets of Whatman 3MM Chromatography paper in an empty agar dish and soak with Z-buffer.
7. Carefully place the membrane on the Whatman paper (again with forceps) and remove air bubbles.
8. Incubate the membrane for 3 h at 37°C or until blue staining can be seen (see Note 24).
9. Dry the membranes and take pictures. Blue staining signifies interactions.

4. Notes

1. The pins can also be sterilized by moving through a 70% ethanol bath equipped with a brush, moving into an ultrasonic bath for 10 s, moving through a second 70% ethanol bath with a brush and drying with hot air (300–400°C). This option is relevant when using a robot for automated stamping. On a related note, the tips of a pipetting robot can be sterilized by subsequently pipetting the maximal volume 70% ethanol, bleach (~1% free chlorine), 70% ethanol, and sterile water three times each. When working with robots, be sure to include sterility controls in every experiment.
2. In this publication, water refers to deionized water with a specific resistance of at least 18 MΩ.
3. It is possible to prepare plates with an agarclave. For 10 l selective agar, autoclave (15 min, 121°C) 67 g yeast nitrogen base and 300 g agar in 9.2–9.5 l water (depending on the required amino acids/nucleosides), cool the medium to 60°C, add 500 ml 20× glucose stock solution and 100 ml of each required amino acid/nucleoside stock solution. Note that you need 1.5 times the amount of agar when autoclaving agar and nitrogen base together. YPD agar plates can be prepared with the agarclave, too. For 10 l YPD agar, autoclave 100 g yeast extract, 200 g peptone and 200 g agar in 9.5 l water. Cool to 60°C and add 500 ml 20× glucose. Pour 200 ml into each agar plate under a sterile hood.
4. In contrast to L40ccu, L40ccα needs adenine-supplemented medium. If the adenine concentration is low the yeast turns red. This can be avoided by adding adenine to the YPD agar or doubling the amount of adenine to -L/HAUT plates.
5. It is possible to shuttle one ORF into two destination vectors in a single 2-in-1-LR Clonase reaction provided that the selection markers are different (e.g., ampicillin and tetracycline resistance in pACT4-DM and pBTM116-D9, respectively). Just add

0.25 µl of each destination vector instead of 0.5 µl of one destination vector. After the transformation of *E. coli*, plate one half of the SOC medium on LB agar containing ampicillin and the other half on LB-Agar containing tetracycline. Checking for cotransformation is very important because *E. coli* cotransformants containing both pACT4 and pBTM116 plasmids can lead to yeast cotransformed with both vectors. This results in growth on -HAULT agar if the protein in question is able to form homodimers, i.e., the protein will appear autoactive although it may not be. To check for cotransformation, plate 5 µl of the overnight culture on LB-Agar with ampicillin and on LB-Agar with tetracycline. If the overnight culture grows on both plates, dilute the plasmid-DNA 1:1,000 and transform again or pick a different colony.

6. Care should be taken to seal the plates properly in all steps because vigorous vortexing or inversion might cause cross-contamination.
7. Normally the 96-well *E. coli* mini-prep protocol yields 10-20 µg plasmid-DNA. BsrGI restriction analysis is necessary because free nucleotides are copurified making the measurement of the OD at 260 nm meaningless. To determine the DNA concentration, compare to known amounts of DNA on the same gel.
8. The yeast growth rate depends on the medium. Grow bait and prey strains for 3 days on -AT/HUL or -L/HAUT, respectively. To allow mating, grow yeast on YPD agar for 30–48 h. Select diploid strains by growing on -ALT/HU medium for 3–4 days. Select for protein–protein interactions by growing yeast on -HAULT medium for 5–7 days.
9. We do not test for prey autoactivity because autoactive preys occur in less than 1% of all cases and preys are not pooled. Autoactive preys are not removed from prey arrays as they are useful as mating controls and allow identification of prey plates from large collections at first glance once you gathered some experience.
10. Some degree of autoactivation can also be observed testing haploid bait strains for reporter activity, but only baits that do not autoactivate in a diploid context can be used for a pooled screen.
11. If three out of four replicas are autoactive, consider removing the fourth copy from the pooled approach and screening the bait separately.
12. Depending on the number of baits use flasks or deepwell plates. We use flasks for pools of 8 baits and deepwell plates for pools of 96 baits.
13. We differentiate diploid recovery and mating efficiency. Mating efficiency is defined as the number of diploids produced by an

equal mixture of MAT*a* and MATα haploid strains divided by half the number of haploid cells before mating and can be determined for individual mating reactions. The diploid recovery is defined as the fraction of positions of a MTP that grow on -ALT/HU agar after mating.

14. Since the growth rate of yeast colonies is only weakly correlated with the probability of successful retesting, be sure to count colonies regardless of size, but do not count very faint spots.
15. If you have information about primary hits from a previous screen with the same prey matrix, this information should be added to the current screens. Also, if all the baits are functionally related, the rules for removing preys should be relaxed.
16. This number is chosen to yield a success rate of about 80% in retesting. By adjusting the number of preys removed, the absolute number of interactions recovered and the success rate can be traded off.
17. Alternatively, preys can be stamped in 96-well format with a pin tool (liquid–solid). In that case, incubate the agar plate for 2–3 days.
18. Removal of autoactive baits is not necessary at this point.
19. Propagation of bait pool plate in liquid 384-well MTP format is not recommended.
20. At this point, it is important to exclude autoactive baits from the analysis. Autoactive preys can be recognized by stochastic distribution of a high number of colonies.
21. Keep in mind that protein–protein interaction assays are orthogonal. This means that while interactions validated by other methods do have a higher likelihood of being true, a large number of true interactions will inadvertently be lost.
22. The β-galactosidase activity can also be assayed from diploid strains gown on -ALT/HU, and the results can be compared to independent growth reporter readouts. However, we use this assay on top of the growth reporter readout as the most stringent way of assaying PPIs.
23. Before thawing the membranes the second time, they can be stored at −80°C for weeks.
24. Since the leuco form of the indigo reaction product is soluble, unspecific staining will occur once the DTT is oxidized, so make sure to stop the assay before this point.

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