Protocol

Performing Yeast One-Hybrid Library Screens

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Yeast one-hybrid (Y1H) assays are used to identify which transcription factor (TF) "prey" molecules can bind a DNA fragment of interest that is used as "bait". Y1H assays involve introducing plasmids that encode TFs into a yeast "bait strain" in which the DNA fragment of interest is integrated upstream of one or more reporters, and activation of these reporters indicates that a TF–DNA interaction has occurred. These plasmids express each TF as a hybrid protein (hence the "one-hybrid" name) fused to the activation domain (AD) of the yeast TF Gal4. The AD moiety activates reporter expression even if the TF to which it is fused typically functions as a repressor. Here, we describe how to perform a Y1H screen of a library of cDNA fragments cloned into a pPC86 plasmid expressing the protein encoded by the cDNA as an AD fusion. The method assumes availability of either commercially available libraries or libraries generated in house using mRNA extracted from a tissue of interest. We also assume that users have access to a yeast bait strain that possesses the DNA fragment of interest integrated upstream of two different reporters—*HIS3*, an auxotrophic marker, and *LacZ*, a colorimetric marker that changes colorless X-gal into a blue compound. Briefly, the screen involves transforming the AD-cDNA library into the yeast bait strain, identifying colonies that show activation of both reporters, retesting the interaction in a freshly grown bait strain, and sequencing the cDNA insert to identify the interacting TF.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

AD-cDNA library DNA (1 µg/µL in TE, pH 7.0)
Agarose gel [1% (w/v) in 0.5× TBE buffer]
Bovine serum albumin (BSA; 1 mg/mL in water)
LiAc (10×; 1 M in water)
pPC86 plasmid (Clontech)
Primers:
- AD primer (5′-CGCGTTTGGAATCACTACAGGG-3′)
- TERM primer (5′-GGAGACTTGACCAAACCTCTGGCG-3′)

Restriction enzymes SalI and BglII and associated 10× buffers

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Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot088955

Salmon sperm DNA (ssDNA; 10 mg/mL; Life Technologies)

This boiled DNA acts as a carrier and increases transformation efficiency.

Sc-Ura-His-Trp plates (150 mm) ± 3AT (without tryptophan) <R>

The concentration of 3AT in the plates should be that at which growth of each strain is minimal, as determined by the HIS3 *autoactivation test (see Protocol:* ***Generating Bait Strains for Yeast One-Hybrid Assays*** *[Fuxman Bass et al. 2015a]).*

TBE buffer <R>
TE (10×; 100 mM Tris–HCl [pH 8.0] and 10 mM EDTA)
YAPD plates <R>
Water (MilliQ, sterile)

Equipment

Agarose gel electrophoresis equipment
Glass beads (sterile)
Incubators (set at 30°C and 37°C)
Nitrocellulose filters (45 µm, 137 mm; Fisher Scientific WP4HY13750)
PCR plates (96-well)
Replica-plating apparatus (Cora Styles #4006 for 150-mm plates)
Thermal cycler
Toothpicks (sterile) and/or sterile disposable plastic loops
Tubes (sterile; 1.5-mL and 15-mL)
Tube shaker
Velvets (sterile, 22-cm × 22-cm pieces of velveteen)

METHOD

Transformation of the AD–Prey Library into the Y1H Bait Strain

1. For each bait strain to be assayed, perform the following high-efficiency yeast transformations, following the procedure described elsewhere (see Reece-Hoyes and Walhout 2012 or follow Steps 3–17 of Protocol: **Generating Bait Strains for Yeast One-Hybrid Assays** [Fuxman Bass et al. 2015a]).
    i. Add 3 µg of AD-cDNA library into ten 1.5-mL tubes, each containing 200 µL of TE–LiAc–ss DNA bait yeast suspension.
    ii. Add 50 ng of empty pPC86 plasmid (i.e., possessing no cDNA insert) into 30 µL of TE–LiAc–ss DNA bait yeast suspension in a 1.5-mL tube.

    To prepare 2 mL of competent bait yeast cells, the volumes described in Protocol: ***Generating Bait Strains for Yeast One-Hybrid Assays*** *(Fuxman Bass et al. 2015a) should be scaled accordingly (e.g., start with a 400 mL culture of the bait yeast strain). The amount of library DNA used in the transformation will depend on the complexity of the library. The amount listed here is for a* Caenorhabditis elegans *cDNA library of* 4×10^7 *clones, representing approximately 16,000 of the approximately 20,000 genes, in the pPC86 backbone. pPC86 features a* TRP1 *expression cassette that enables the Y1H bait strain to grow in the absence of tryptophan. Remember to also prepare a negative control that has no plasmid added to the yeast, which should yield no growth.*

2. After the heat-shock stage in the high-efficiency yeast transformation protocol (i.e., Step 15 in Protocol: **Generating Bait Strains for Yeast One-Hybrid Assays** [Fuxman Bass et al. 2015a]), resuspend each aliquot of the cDNA library transformations in 500 µL of sterile water and combine in a 15-mL tube. Resuspend the empty plasmid control yeast in 30 µL of sterile water.
3. To determine the transformation efficiency, dilute 5 µL of the library transformation mix from Step 2 in 495 µL of sterile water in a 1.5-mL tube to create a 1/100 dilution. Take 50 µL from the

1/100 dilution and add 450 µL of sterile water to create a 1/1000 dilution. Use sterile glass beads to spread each dilution onto a 150-mm Sc-Ura-His-Trp plate.

An effective library screen needs to have generated at least 1 million clones. For libraries of higher complexity (i.e., more genes and/or isoforms), a higher number of clones might need to be screened. If the yield is low, then the screen might need to be repeated until the required number of colonies is attained.

4. Using sterile glass beads, plate the remainder of the library transformation across ten 150-mm Sc-Ura-His-Trp+3AT plates.
5. Pipette 5 µL of the empty pPC86 control yeast suspension as a single spot onto a Sc-Ura-His-Trp plate.

   There is no need to spread for single colonies. The yeast transformed with empty vector will be used in Step 8 as a "negative control" yeast to show reporter expression levels when no interaction is occurring.

6. Use incubator to maintain all plates from Steps 3 to 5 at 30°C.
7. After 3 d, check for yeast colonies, which should be obvious on the plates from Step 3 and Step 5. If colonies appear on the negative control plate (with no plasmid) prepared in Step 1, the screen cannot be used as there has been a contamination in one of the reagents. Calculate the transformation efficiency using data from the plates from Step 3. Store the plate from Step 5 at room temperature until needed in Step 8.
8. Continue to incubate the plates from Step 4 at 30°C for up to 14 d. Check the plates regularly and, using sterile toothpicks or loops, transfer "HIS-positive" colonies as they become visible on 150-mm Sc-Ura-His-Trp plates in "96-spot format." Also transfer some of the empty pPC86 control yeast from Step 5 to these plates. Incubate the plates for 1–2 d at 30°C.

   The number and size of colonies obtained varies greatly and depends on the bait used. It is important to select faster- and slower-growing colonies where feasible because different TFs drive different levels of reporter activation, resulting in different yeast phenotypes.

   If positive-control yeast strains that express known levels of reporters are available, they should also be transferred to these plates. The number of colonies picked per bait is limited only by how many grow, but we usually do not exceed 96 (which can be plated on a single 150-mm plate and is convenient for 96-well polymerase chain reaction [PCR]). Some bait strain transformations might not yield any "HIS-positive" colonies despite good transformation efficiency. Such baits can be screened again, perhaps using medium with lower concentrations of 3AT or using a different cDNA library (e.g., obtained with mRNA from another tissue or source). However, it is also possible that some bait strains might yield many hundreds of colonies. Such baits might be interacting with many partners or they interact with a highly abundant protein that is overrepresented in the library (this latter issue is particularly relevant for non-normalized cDNA libraries). Baits with many interactors might need to be screened again using medium with higher concentrations of 3AT.

Identification of "Double-Positive" Yeast

9. Using sterile velvets and replica-plating apparatus, replica-plate the "HIS-positive" (and empty pPC86 control) yeast onto a fresh 150-mm Sc-Ura-His-Trp plate, a 150-mm Sc-Ura-His-Trp plate containing the 3AT concentration used for screening, and a 150-mm YAPD plate onto which a nitrocellulose filter has been placed (so that the yeast will grow on the filter).
10. Using sterile velvets and the replica-plating apparatus, replica-clean the plate containing 3AT (usually three cleanings are needed) immediately after replica-plating and incubate for 5–10 d at 30°C.

    This plate will confirm the "HIS-positive" result.

11. After 1–2 d incubation of the nitrocellulose filter and YAPD plate at 30°C, perform a colony-lift colorimetric assay (see Protocol: **Colony-Lift Colorimetric Assay for β-Galactosidase Activity** [Fuxman Bass et al. 2015b]). Combining the results observed in this step and in Step 10, identify which colonies are "double-positive" (i.e., show induction of both reporters above the level observed in the negative control yeast).

    For baits that display moderate to high autoactivity, it might be necessary to compare potential positive yeast with the negative control very often and early in the readout period to see increased reporter expression.

 Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot088955

INTERACTION RETESTING BY GAP-REPAIR

It is necessary to retest every interaction identified in Step 11 because some of the "double-positive" yeast phenotypes are not actually due to the AD–prey interacting with the bait. The easiest method to confirm many interactions is to use gap-repair. Gap-repair involves using PCR to amplify the cDNA from the pPC86 clone in each positive yeast colony, and then cotransforming each amplicon with linear pPC86 vector into fresh bait strain yeast. Homologous recombination within the yeast reconstitutes the vector and insert into a new construct, and reporter activation within the resulting transformed yeast can then be reassayed. Without gap repair, every interaction would need to be confirmed by transforming the AD–prey construct into fresh yeast, and, although extracting the plasmid from the double-positive yeast for such a transformation is technically possible, it is challenging to do this for many interactions simultaneously.

12. Using sterile toothpicks or loops (or by replica-plating), transfer the double-positive yeast identified in Step 11 to fresh Sc-Ura-His-Trp plates and incubate overnight at 30°C.

13. Use yeast colony PCR from these freshly grown yeast with the AD primer and TERM primer to amplify the insert from all interacting library clones (see Protocol: **Zymolase-Treatment and Polynerase Chain Reaction Amplification from Genomic and Plasmid Templates from Yeast** [Fuxman Bass et al. 2015c]). Store the PCR products at −20°C.

    The PCR extension time should reflect the average insert size. Use in later steps only PCR reactions that amplify a single band. Yeast lysates that generate multiple bands likely contain multiple AD–prey clones, and will not give clean sequence data to identify the interactor.

14. In a sterile 1.5-mL microcentrifuge tube, set up the following restriction enzyme digest:

pPC86 plasmid	10 µg
Bovine serum albumin (1 mg/mL)	4 µL
10× Restriction buffer	4 µL
SalI	2 µL (20 units)
BglII	2 µL (20 units)
Water (molecular biology grade)	to 40 µL

    Mix the digest with a tube shaker and incubate at 37°C overnight. With agarose gel electrophoresis equipment, verify the digest by running 2 µL of the restriction digest on a 1% (w/v) agarose gel in 0.5× TBE buffer next to an equal amount of the undigested plasmid as well as DNA molecular mass markers.

    This digest excises a small (~20-bp) fragment from the multicloning site of the vector, resulting in a linear fragment with noncompatible ends. If the library of choice is not pPC86-based then restriction enzymes should be chosen for the appropriate vector that results in the same type of linear fragment. There is no need to purify the linear plasmid backbone from the digest mix.

15. For each bait yeast strain, perform a high-efficiency yeast transformation, as described elsewhere (see Reece-Hoyes and Walhout 2012 or follow Steps 3–17 of Protocol: **Generating Bait Strains for Yeast One-Hybrid Assays** [Fuxman Bass et al. 2015a]), that introduces 40 ng of linear pPC86 and 5 µL of prey PCR product (from Step 13 of this protocol) into 20 µL of TE–LiAc–ss DNA yeast suspension. Three negative controls without PCR product should be included: no DNA, 40 ng of linear pPC86 alone, and 40 ng of uncut pPC86.

    This transformation can be performed in a 96-well PCR plate (200 µL well volume), with incubations in a thermal cycler. At either end of these PCR products is ~100 bp of sequence matching the vector sequence on either side of the pPC86 multicloning site. These sequences will facilitate homologous recombination.

16. Resuspend all transformations in 20 µL of sterile water by pipetting up and down and use 5 µL of each resuspension to generate a single spot (in 96-spot format) onto Sc-Ura-His-Trp plates. If positive-control yeast strains that express known levels of reporters are available, also transfer them to these plates. Incubate the plates for 2 d at 30°C.

    The number of transformants should be an order of magnitude higher in gap-repair samples and uncut pPC86 controls compared with the linear pPC86-alone control. No transformants should be present in the no-DNA control.

17. Replica-plate the transformants from Step 16 onto a fresh 150-mm Sc-Ura-His-Trp plate as well as a 150-mm Sc-Ura-His-Trp plate with the 3AT concentration used for screening, and a 150-mm

nitrocellulose-filter-equipped YAPD plate for assays of *LacZ* expression (see Protocol: **Colony Lift Colorimetric Assay for β-Galactosidase Activity** [Fuxman Bass et al. 2015b]).

The first plate is required to maintain the yeast transformants, whereas the other two plates are for retesting the HIS3 *and* LacZ *expression, respectively.*

18. Identify double-positive yeast colonies, as outlined in Steps 10 and 11.

Identification of Prey Identity

19. Determine the identity of the double-positive preys that retest successfully by gap repair by sequencing the PCR products generated in Step 13 using the AD primer.

The majority (>95%) of retrieved proteins will be predicted regulatory TFs containing known DNA-binding domain(s). Interactors that are not obvious TFs might possess a novel DNA-binding domain. If an expected interaction is not observed, bear in mind that interactions that require additional protein partners or post-translational modifications not available in yeast will not be recorded using this assay.

RECIPES

Sc-Ura-His-Trp Plates (150 mm) ± 3AT

Reagent	Quantity (for 2 L)
Drop-out mix synthetic minus histidine, leucine, tryptophan, and uracil, adenine rich (2 g) without yeast nitrogen base (US Biological, D9540-02)	2.6 g
Yeast nitrogen base (YNB) without amino acids and without ammonium sulfate	3.4 g
Ammonium sulfate	10 g
Agar	35 g
Glucose (40%, w/v, in water, sterile)	100 mL
Leucine (100 mM, filter sterilized)	16 mL
3-Amino-1,2,4-triazole (3AT) (2 M, filter sterilized) (optional)	10–80 mL

Dissolve the drop-out mix, the YNB, and the ammonium sulfate in 920 mL of water, and pH to 5.9 with NaOH (5 M). Pour into a 2-L flask and add a stir bar. In a second 2-L flask, add the agar to 950 mL of water (do not add a stir bar as it will cause the agar to boil over in the autoclave). Autoclave for 40 min at 15 psi on liquid cycle. Immediately pour the contents of the first flask, including the stir bar, into the agar-containing flask. Add the glucose, mix well on a stir plate, and cool to 55°C. Add the leucine and the tryptophan. Mix well on a stir plate and pour into 150-mm sterile Petri dishes (~80 mL per dish), dry for 3–5 d at room temperature, wrap in plastic bags, and store for up to 6 mo at room temperature. For Sc-Ura-His-Trp plates containing 3AT, add 10–80 mL of 3AT together with the leucine for a final concentration of 10 to 80 mM 3AT, and store them for up to 1 mo at room temperature.

TBE Buffer

Prepare a 5× stock solution in 1 L of H_2O:
54 g of Tris base
27.5 g of boric acid
20 mL of 0.5 M EDTA (pH 8.0)

The 0.5× working solution is 45 mM Tris-borate/1 mM EDTA. TBE is usually made and stored as a 5× or 10× stock solution. The pH of the concentrated stock buffer should be ~8.3. Dilute the concentrated stock buffer just before use and make the gel solution and the electrophoresis buffer from the same concentrated stock solution. Some investigators prefer to use more concentrated stock solutions of TBE (10× as opposed to 5×). However, 5× stock solution is more stable because the solutes do not precipitate

during storage. Passing the 5× or 10× buffer stocks through a 0.22-µm filter can prevent or delay formation of precipitates.

YAPD Plates

Reagent	Quantity (for 2 L)
Peptone	40 g
Yeast extract	20 g
Adenine hemisulfate dehydrate	0.32 g
Glucose (40%, w/v) in water, sterile	100 mL
Agar	35 g

Dissolve the first three powders in 950 mL of water in a 2-L flask and add a stir bar. In a second 2-L flask, add the agar to 950 mL of water (do not add a stir bar as it will cause the agar to boil over in the autoclave). Autoclave for 40 min at 15 psi on liquid cycle. Immediately pour the contents of the first flask, including the stir bar, into the agar-containing flask. Add the glucose, mix well on a stir plate, and cool to 55°C. Pour into 150-mm sterile Petri dishes (~80 mL per dish), dry for 3–5 d at room temperature, wrap in plastic bags, and store for up to 6 mo at room temperature.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health grants DKO68429 and GM082971 to A.J.M.W. J.I.F.B was partially supported by a postdoctoral fellowship from the Pew Latin American Fellows Program.

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