Protocol

Generating Bait Strains for Yeast One-Hybrid Assays

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Yeast one-hybrid (Y1H) assays are used to identify which transcription factor (TF) "preys" can bind a DNA fragment of interest that is used as the "bait." Undertaking Y1H assays requires the generation of a yeast "bait strain" for each DNA fragment of interest that features the DNA bait coupled to a reporter(s). Plasmids encoding TFs fused to the Gal4 activation domain (AD) are then introduced into the bait strain, and activation of the reporter(s) indicates that a TF–DNA interaction has occurred. Here, we present a protocol for the first part of the strategy—the generation of a bait strain for Y1H assays. We assume that the DNA bait has already been cloned into two different reporter constructs: One places the fragment of interest upstream of *HIS3*, an auxotrophic growth marker, whereas the other places the DNA bait upstream of *LacZ*, a colorimetric marker that changes colorless X-gal into a blue compound. Briefly, generation of the bait strain involves using homologous recombination to integrate the two reporters into the genome of the yeast strain, screening individual integrants for background reporter expression (i.e., expression in the absence of a TF), and using polymerase chain reaction (PCR) and sequencing to confirm the DNA bait identity from both integrated reporter cassettes.

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

Agarose gel (1%, w/v, in 0.5× TBE buffer]
Bovine serum albumin (BSA; 1 mg/mL in distilled water)
DNA bait cloned into either pHisi-1 and pLacZi (Clontech) or pMW#2 and pMW#3 (Addgene)

The reporter constructs pHisi-1 and pLacZi from Clontech allow restriction-endonuclease-based cloning, whereas pMW#2 and pMW#3 are from the Addgene repository and allow Gateway recombination-based cloning.

DNA molecular mass markers
Glycerol solution (15%, v/v, in sterile water)
LiAc (10×; 1 M in water)
Nitrocellulose filters (45 µm, 137 mm; Fisher Scientific WP4HY13750)
PEG 3350 (40% w/v, sterile; Fisher Scientific)
Primers

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HISRV primer (anneals both pHisi-1 and pMW#2; 5′-GCTTTCTGCTCTGTCATCTTTG-3′)
LACFW primer (anneals both pLacZi and pMW#3; 5′-GTTCGGAGATTACCGAATCAA-3′)
LACRV primer (anneals both pLacZi and pMW#3; 5′-ATCTGCCAGTTTGAGGGGAC-3′)
pHisi-1FW primer (5′-AACAAATAGGGGTTCCGC-3′)
pMW#2FW primer (5′- AGCTATGACGTCGCATGCAC-3′)

Restriction enzymes (various, see Step 1) and associated 10× buffers

The suitable restriction enzymes are AflII, ApaI, BseR1, NcoI, NsiI, StuI, and XhoI.

Salmon sperm DNA (ssDNA; 10 mg/mL; Life Technologies)
Sc-Ura-His plates (150 mm) ± 3AT (see Step 19) <R>
TBE buffer <R>
TE (10×; 100 mM Tris–HCl [pH 8.0] and 10 mM EDTA)
Water (MilliQ, sterile)
YAPD liquid medium <R>

YAPD medium is yeast extract peptone dextrose (YEPD) medium with extra adenine, which is added because the yeast strain used has mutations in the adenine synthesis pathway.

YAPD plates <R>
Y1HaS2 yeast strain (genotype: *MATa, ura3-52, his3-Δ1, ade2-101, ade5, lys2-801, leu2-3,112, trp1-901, tyr1-501, gal4Δ, gal80Δ, ade5::hisG*)

Equipment

Agarose gel electrophoresis equipment
Camera (optional; see Step 20)
Conical tubes (50-mL, sterile)
Freezer (−80°C)
Glass beads (sterile)
Replica-plating apparatus (Cora Styles #4006 for 150-mm plates)
Shaking incubator (30°C)
Toothpicks and/or disposable plastic loops (sterile)
Tubes (1.5-mL, sterile)
Velvets (220 × 220-mm pieces of velveteen [100% cotton velveteen without rayon])
Water baths (37°C and 42°C)

METHOD

Linearization of the Y1H Reporter Constructs

The protocol begins with a DNA bait that has already been cloned into the HIS3 *(pHisi-1 or pMW#2) and* LacZ *(pLacZi or pMW#3) reporter constructs.*

1. Set up the following digests with restriction enzymes in 1.5-mL tubes and incubate in a water bath for 3 h at 37°C.

Tube 1:

pMW#2 (or pHisi-1) construct including DNA bait	1 to 4 µg
Bovine serum albumin (1 mg/mL)	2.5 µL
10× Restriction buffer	2.5 µL
AflII, XhoI, NsiI, or BseR1 (select one)	2 µL (20 units)
Water	to 25 µL

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Tube 2:

pMW#3 (or pLacZi) construct including DNA bait	1 to 4 µg
Bovine serum albumin (1 mg/mL)	2.5 µL
10× Restriction buffer	2.5 µL
NcoI, ApaI, or StuI (select one)	2 µL (20 units)
Water	to 25 µL

The restriction enzyme of choice must not cut within the DNA-bait sequence. These digests linearize the Y1H reporter constructs such that regions of homology with the yeast genome occur at both ends. When these linear constructs are transformed into the Y1HaS2 host yeast strain, the pMW#2 (or pHisi-1) construct is integrated into the mutant HIS3 *locus (*his3-Δ1*), whereas the pMW#3 (or pLacZi) construct is integrated into the mutant* URA3 *locus (*ura3-52*).*

2. Using agarose gel electrophoresis equipment, verify linearization of the constructs by running 1–2 µL of the restriction digest reaction mixture on a 1% (w/v) agarose gel in 0.5× TBE buffer (pH 8.3) next to an equal amount of the undigested constructs as well as DNA molecular mass markers.

High-Efficiency Transformation to Generate Integrated Colonies of Y1HaS2

3. Using a sterile toothpick or loop, thickly spread the Y1HaS2 strain onto a YAPD plate. Incubate overnight at 30°C.
4. Using a sterile toothpick or loop, resuspend the Y1HaS2 strain into 80 mL of liquid YAPD to an OD_{600} of 0.15–0.20.
5. Incubate the culture in a shaking incubator (30°C, 200 rpm) until the OD_{600} reaches 0.4–0.6.

   This usually takes ~4–6 h.
6. Pellet the cells by centrifugation (700*g*) in conical 50-mL tubes for 5 min at room temperature.
7. Discard the liquid from the pellet formed, and wash the cells with 20 mL of sterile water. Resuspend the cells by pipetting or by shaking the tube. Do not vortex.
8. Centrifuge the yeast suspension as in Step 6, discard the supernatant, and resuspend the cells in 5 mL of a freshly made solution of 1× LiAc in 1× TE in sterile water ("TE–LiAc solution") at room temperature. Do not vortex.
9. Centrifuge the suspension as in Step 6, remove the supernatant carefully by aspiration, and then resuspend the cells in 400 µL of TE–LiAc solution by pipetting up and down.
10. Prepare 50 µL of denatured salmon sperm DNA (ssDNA, 10 mg/mL) by boiling for 5 min, and then keep on ice.
11. Add 40 µL denatured ssDNA to the yeast suspension (from Step 9) and mix by pipetting up and down.
12. In a 1.5-mL tube, transform 20 µL of *both* linearized constructs for the same DNA bait (using the digest reaction mixes from Step 1) into the *same* 200 µL of competent Y1HaS2 yeast cell suspension in TE–LiAc–ssDNA.

    Remember to also prepare a negative control in which no linearized vector DNA is added to the yeast.
13. Add 1250 µL of 40% PEG in TE–LiAc solution to the tube and mix by multiple inversions or by pipetting up and down (do not vortex).
14. Incubate all transformation reactions for 30 min at 30°C.
15. Heat-shock the cells for 20 min at 42°C (in a water bath).
16. Centrifuge the tubes for 1 min at room temperature at 700*g* and remove the supernatant by aspiration.

17. Resuspend each transformation in 700 µL of sterile water and use sterile glass beads to spread on a 150-mm Sc-Ura-His plate. Incubate the plates for 3–5 d at 30°C until individual colonies appear.

    The Y1H reporter constructs are integrative (YIp) vectors that cannot be replicated within yeast, so any colonies that grow at this stage must be integrants. The minimal promoter in pHisi-1 or pMW#2 drives enough HIS3 *expression to enable growth in the absence of histidine. The number of colonies obtained usually varies between 10 and 100. The negative control should give rise to no colonies.*

    See Troubleshooting.

Test DNA Bait Strain Autoactivity

Autoactivity is the level of reporter expression in the absence of binding to the AD–prey. Autoactivation is likely to be due to an endogenous yeast TF binding the DNA bait (Deplancke et al. 2004). Different integrant strains arising from the same transformation can show varying levels of autoactivity, which can be because differing numbers of reporter cassettes have been integrated into each strain (and even at each locus within each strain). It is important to test several independent integrants to select the one with the lowest autoactivity for both reporters, so that interactions are easily detected in subsequent Y1H assays.

18. Using a sterile toothpick or loop, transfer six to 24 yeast colonies from the Sc-Ura-His plate in Step 17 to a new 150-mm Sc-Ura-His plate in "96-spot format." Incubate the plates for 1–2 d at 30°C.

    If yeast strains that show low, medium, and high HIS3 and *lacZ reporter expression levels are available, add these to the Sc-His-Ura plate.*

19. Using sterile velvets and the replica-plating apparatus, replica-plate the yeast from Step 18 onto a fresh 150-mm Sc-Ura-His plate (without 3AT), a set of 150-mm Sc-Ura-His+3AT plates (containing 10, 20, 40, 60, or 80 mM 3AT), and a 150-mm YAPD plate onto which a nitrocellulose (NC) filter has been placed (so that the yeast will grow on the filter). Using sterile velvets and the replica-plating apparatus, replica-clean only the 3AT-containing selective medium plates until no yeast are visible (usually three cleans are needed). Incubate all of the plates at 30°C.

    For replica-plating, a sterile velvet is placed onto the replica-plating block and locked into place using the collar. The yeast plate is placed yeast side down onto the velvet and evenly pressed to transfer the yeast from the plate to the velvet. Then, the yeast plate is removed, and a new plate is placed medium side down onto the velvet and pressed evenly to transfer the yeast from the velvet to the new plate. In this way, multiple (up to five) transfers can be performed from one velvet. To replica-clean the 3AT-containing plates, press the plate (yeast side down) onto a series of sterile velvets (used only once) to remove excess yeast that can interfere with the yeast growth assay.

    The 3-aminotriazole (3AT) acts as a competitive inhibitor of the His3p enzyme.

20. After 1 d at 30°C, use the nitrocellulose filter–YAPD plate from Step 19 in a β-galactosidase colorimetric assay (see Protocol: **Colony Lift Colorimetric Assay for β-Galactosidase Activity** [Fuxman Bass et al. 2015a]). Record how much blue compound was generated by each integrant strain by taking a photograph with a camera and/or noting the color in a qualitative manner (e.g., white, light blue, dark blue, very dark blue).

21. After 5–10 d at 30°C, inspect the Sc-Ura-His+3AT plates from Step 19 and record how much 3AT was required to inhibit the growth of each integrant strain. This information will help determine which yeast bait strain(s) to screen in Y1H assays, as explained in Step 22.

Confirming the Identity of the Integrated DNA Bait

22. Choose up to four integrant strains showing the lowest autoactivity for *both* reporters. For each integrant, verify that the correct DNA bait is upstream of *both* reporters by amplification of the inserts, as described elsewhere (see Protocol: **Zymolase Treatment and Polymerase Chain Reaction Amplification from Genomic and Plasmid Templates from Yeast** (Fuxman Bass et al. 2015b), using the appropriate primers, followed by DNA sequencing of the PCR products.

The primers for pLacZi and pMW#3 (LACFW and LACRV) will add ~640 nucleotides to the original bait sequence, pHisi-1 (pHisi-1FW and HISRV) ~240 nucleotides, and pMW#2 (pMW#2FW and HISRV) ~210 nucleotides. Proceed to Step 23 while waiting for the sequencing results.

Preparing Glycerol Stocks of Yeast Integrants

23. Using a sterile toothpick, transfer the integrants selected in Step 22 from the Sc-Ura-His plate replicated in Step 19 to a new Sc-Ura-His plate and incubate the plate overnight at 30°C.

    Only one integrant strain per DNA bait is needed for library screens, but it is useful to have at least one backup strain.

24. Using a sterile toothpick, transfer a match-head-sized amount of this freshly grown yeast from Step 23 into 200 µL of sterile 15% (v/v) glycerol solution in 1.5-mL tubes. Vortex the yeast–glycerol solution for 5 sec and store the resulting yeast suspension in a freezer at −80°C.

    The frozen yeast stocks can be stored, for up to 2 yr, and then revived by transferring some (~5 µL) of the frozen stock to a YAPD plate and allowing growth for at least 2 d at 30°C. Remember to discard any frozen stocks of yeast that proved incorrect via sequencing in Step 22.

TROUBLESHOOTING

Problem (Step 17): Transformation fails to generate yeast colonies.

Solution: Assuming that the medium was made correctly (e.g., glucose was not forgotten), the next-most-common issue is that one of the reporters failed to integrate. Check this by transforming only the *HIS3* construct and plating on Sc-His, or only the *LacZ* construct and plating on Sc-Ura. It might be necessary to digest the refractory construct with a different enzyme (Step 1), generate a new or more-concentrated miniprep, or even remake the construct. Integration efficiency is also dependent on the "health" of the yeast, so only use Y1HaS2 revived from frozen stocks <7 d previously, and do not mix harshly at any step.

DISCUSSION

Optimal integrant strains generate a little blue compound in the colorimetric assay (driven by the minimal promoter upstream of *LacZ*), and have some growth on 10 mM 3AT plates, but minimal or no growth on 20 mM 3AT plates. If no blue compound at all is observed, or no growth even on 10 mM 3AT plates is observed, this might indicate a problem with either reporter construct, and such integrants should ideally not be selected. The lowest concentration of 3AT that totally prevents growth should be used for library screens (i.e., integrants that grow on 20 mM 3AT but not on 40 mM 3AT should be screened at 40 mM) (see Protocol: **Performing Yeast One-Hybrid Library Screens** [Fuxman Bass et al. 2015c]). Integrants that grow strongly on 80 mM 3AT cannot be used in Y1H library screens because few (if any) protein–DNA interactions can activate the *HIS3* reporter enough to overcome this high background, and because higher concentrations of 3AT are toxic to yeast. However, integrants with low *HIS3* autoactivity and high *LacZ* autoactivity can be screened because interactions are detected by growth assay using the *HIS3* reporter first, and the *LacZ* activity in these "HIS-positive" yeast might be higher than background when observed closely. However, results obtained with highly autoactive baits should be judged with caution. Note that, for some DNA baits (10%–20%), the autoactivity levels for all integrants will be too high, making Y1H screens impossible (Deplancke et al. 2004; Reece-Hoyes et al. 2013). For these highly autoactive DNA baits, it might be desirable to use smaller fragments of the DNA bait that confer lower autoactivity.

RECIPES

Sc-Ura-His 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
Tryptophan (40 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 plates containing 3AT, add 10–80 mL of 3AT together with the leucine and the tryptophan 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 Liquid Medium

Reagent	Quantity (for 1 L)
Peptone	20 g
Yeast extract	10 g
Adenine hemisulfate dehydrate	0.16 g
Glucose (40%, w/v, in water; sterile)	50 mL

Dissolve powders in 950 mL of water in a 1-L bottle. Autoclave for 40 min at 15 psi on liquid cycle. Add the glucose, mix well, and store for up to 6 mo at room temperature.

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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 at room temperature for up to 6 mo.

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