Chapter 7

Yeast Two-Hybrid Liquid Screening

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Abstract

Yeast two-hybrid (YTH) method consists of a genetic trap that selects for "prey" cDNA products within a library that interact with a "bait" protein of interest. Here, we provide a protocol for YTH screening using a liquid medium screening method, which improves the sensitivity of this technique and streamlines the laborious classic screening in solid medium plates. The method uses a simple series of dilutions with established yeast strains transformed with diverse baits and complex cDNA libraries. This allows for prompt detection of positive clones revealed by liquid growth, due to activation of HIS3 reporter gene. Activation of a second reporter gene and reconstruction of the YTH interaction is highly reproducible using this system. This approach can either be performed using culture flasks or deep-well 96-well plates and the number of interactions obtained is similar, when compared to the classic method. In addition, the liquid screening method is faster and more economical for YTH screening and has the added benefit of automation if 96-well plates are used.

Key words Liquid screening, Yeast two-hybrid, Protein–protein interaction, cDNA libraries, Bait protein

1 Introduction

The Yeast Two Hybrid system (YTH), developed by Fields and Song [1] was the first molecular genetic screen developed to detect protein–protein interactions [2]. This technique allows researchers to explore the possible roles of a protein in the cell metabolism, by revealing the different interactions it may form and therefore, the molecular complexes it may be involved in. Over the past two decades, this method has been constantly tested and improved in many ways [3–5] in search of better performance and a solution to eliminate false-positive interactions.

Traditionally, YTH positives are selected for the activation of the HIS3 gene on solid medium plates (SD-W-L-H) supplemented with 3-Aminotriazole (3-AT), an inhibitor of the HIS3 gene product. This approach usually results in a substantial number of false-positive interactions being identified along with the true positives. Previous work has reported that using liquid medium as a complementary

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selection of *HIS3* activators shows better efficiency when compared to *lacZ* assays [6]. For the trihybrid system [7], a liquid medium approach proved capable of distinguishing true interactions from false positives by analyzing the yeast growth in the culture during a fixed interval of 24 h [8].

We have used the yeast strains Y190 and KGY37 to develop an effective method of YTH screening using liquid medium. This adaptation improves the selection of two-hybrid interactions reducing the number of false positives selected.

A liquid medium screen can be performed using one of two strategies. The first is to grow the transformants in ten separate flasks of liquid medium that select for activation of the reporter gene. The second is to divide the transformants into 96-deep well plates and select for activation of the reporter gene.

This article will instruct how to perform a yeast two-hybrid screen using each of the liquid screening methodologies. We have used this method numerous times, each time with excellent results. This article will only outline the procedures used in the screen itself. A yeast two-hybrid screen is a complicated undertaking with many different parts. An article documenting each step of a YTH screen can be found here [9].

It is known that some proteins can have a toxic effect on yeast [10]. In this case, smaller fragments of the protein of interest can be used in an attempt to minimize this harmful interference [3]. In the method described here, a method for the monitoring of yeast growth is also described.

This method replaces YTH screening on solid medium. We find the selection in liquid medium to be more stringent producing fewer false positives and marginal positives. We believe that this liquid screening method is more economical and more efficient for YTH screens than the classical method.

2 Materials

2.1 Yeast Culture Media

2.1.1 YPAD (Yeast Extract–Peptone–Adenine–Dextrose) Medium

YPAD medium is used for general yeast growth. The 2×YPAD medium reduces the doubling time of yeast strains and increases transformation efficiency.

Component	YPAD agar	2×YPAD broth
Difco Bacto Yeast extract	8 g	16 g
Difco Bacto Peptone	16 g	32 g
Glucose	16 g	32 g
Adenine hemisulfate	80 mg	80 mg

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Component	YPAD agar	2×YPAD broth
Difco Bacto agar[a]	12 g	–
Distilled/deionized water	800 mL	800 mL

[a]Agar is included for plates and excluded for liquid. Volumes of 800 mL are easier to handle than 1,000 mL and can be made up and autoclaved in 1.0-L bottles or other suitable containers. The powdered ingredients are dissolved in the water and sterilized by autoclaving for 15 min. Medium for plates should be allowed to equilibrate to 60 °C in a water bath before it is poured into petri dishes. This volume makes about 30 plates. YPAD and 2×YPAD liquid media are dissolved, dispensed in aliquots, and autoclaved.

2.1.2 SC (Synthetic Complete) Selection Medium

SC selection medium is used to select for yeast plasmids or reporter gene activity. Specific amino-acid mixtures (*see* Subheading 2.1.3) are used for each type of medium needed.

Ingredient	SC selection medium
Difco Yeast Nitrogen Base w/o amino acids	5.4 g
Amino-acid mix	1.6 g
Glucose	16.0 g
Difco Bacto agar*	12.0 g
Distilled/deionized water	800.0 mL

*Omit the agar to make liquid SC selection medium.

Dissolve the ingredients in water and adjust the pH to 5.6 with 1.0 N NaOH. Sterilize by autoclaving for 15 min. When plates are required allow the medium to cool to 60 °C before pouring. The plates should be stored in the dark at room temperature for 1 or 2 days to dry and then stored in sealed bags in the dark at 4 °C. Liquid medium should also be stored in the dark at 4 °C.

A sterile 10.0 M solution of 3 aminotriazole (3-AT, Sigma) is made by dissolving 84.08 g in 100 mL of water and sterilizing by filtration. There are two concentrations of 3-AT that are used in liquid medium depending on the strain that is being employed; 0.5 mM and 2.5 mM (KGY37 and Y190, respectively). Solid medium requires higher concentrations of 3-AT; 2.0 mM and 50 mM for KGY37 and Y190, respectively.

2.1.3 Amino-acid Mix

Mix the following ingredients [11] in a plastic container by shaking thoroughly with 2 or 3 glass marbles. The compounds omitted in specific SC selection media are in bold type. The types of medium used for this screen are as follows: SC minus Trp (SC-W), SC minus Trp, Leu (SC-W-L), and SC minus Trp, Leu, His (SC-W-L-H). Each type of medium can be prepared by omitting the listed ingredient from the mix.

Adenine SO_4	**0.5 g**	Methionine	2.0 g
Arginine	2.0 g	Phenylalanine	2.0 g
Aspartic acid	2.0 g	Serine	2.0 g
Glutamic acid	2.0 g	Threonine	2.0 g
Histidine HCl	**2.0 g**	**Tryptophan**	**2.0 g**
Inositol	2.0 g	Tyrosine	2.0 g
Isoleucine	2.0 g	**Uracil**	**2.0 g**
Leucine	**4.0 g**	Valine	2.0 g
Lysine HCl	2.0 g	*p*-Aminobenzoic acid	0.2 g

2.2 Yeast Transformation Solutions

All solutions for yeast transformation can be found in this volume [12].

2.3 Liquid Screening Protocol

1. The liquid screening is done in 96-well deep-well polypropylene plates.
2. The plates are sealed with SealPlate® adhesive sealing film.
3. Liquid cultures were grown in 250-mL culture flasks with baffles.

2.4 ß-Gal Activity Assay

1. Z Buffer: $NaH_2PO_4 \cdot H_2O$ 13.79 g/L, KCl 0.75 g/L, $MgSO_4 \cdot 7H_2O$ 0.246 g/L. Titrate with 10 *N* NaOH to pH 7.0.
2. Z buffer/β-ME: This should be made fresh by adding 270 mL of b-mercaptoethanol (β-ME)/100 mL of Z buffer.
3. X-Gal: 20 mg/mL, dissolve 1.0 g of X-Gal in 50 mL of *N*,*N*-dimethylformamide and store at −20 °C.
4. Z buffer/β-ME/X-Gal: This should be made fresh by adding 270 mL of β-ME and 1.67 mL of X-Gal solution to 100 mL of Z buffer.

2.5 Isolation of Plasmid DNA from Yeast

1. Yeast Lysis buffer: 20 mM Tris–HCl pH 8.0, 10 mM EDTA, 100 mM NaCl, 1 % (w/v) SDS, 2 % (v/v) Triton X-100. Make 100 mL by adding 2 mL of 1.0 M Tris pH 8.0, 2 mL of 0.5 M EDTA pH 8.0, 2 mL of 5.0 M NaCl, 5 mL of 20 % (w/v) SDS, and 2 mL of Triton X-100 to 87 mL of sterile distilled water.
2. Acid-Washed Glass beads, 425–600 μm.

2.6 Bacterial Media

1. LB (Luria–Bertani) medium + ampicillin (600 mL): tryptone 6 g, yeast extract 3 g, NaCl 6 g, distilled water 600 mL. Titrate to pH 7.0 with 10 *N* NaOH. For plates: add 10 g Difco Bacto Agar to 600 mL volume in each flask prior to sterilization. When cooled to 60 °C add 300 μL of a 100 mg/mL stock of ampicillin, mix, and pour plates.

2. SOB medium (1.0 L): tryptone 20 g, yeast extract 5 g, NaCl 0.5 g, KCl 0.15 g, distilled water 990 mL. Dissolve ingredients and titrate to pH 7.0 with 10 N NaOH and autoclave. Add 10 mL of 2.0 M solution (1.0 M $MgSO_4$ and 1.0 M $MgCl_2$). To make SOC medium add 0.5 mL of 2.0 M sterile glucose to 49.5 mL of SOB medium.

3 Methods

3.1 Yeast Strain

There are a number of yeast strains currently available for YTH screening [9]. The strains KGY37 and Y190 were tested and validated for the liquid screen protocol. KGY37 yields higher transformation efficiency and can therefore be better for screening complex libraries. The addition of 3-aminotriazole is common while performing YTH assays to suppress the "leaky" expression of the reporter gene HIS3. In the liquid medium, lower concentrations of 3-AT are required for this purpose, and each of these strains requires a different concentration (0.5 mM for KGY37 and 2.5 mM for Y190).

Yeast strain	Genotype	Reporter genes	Plasmid selection	References
Y190	*MAT*a, *ade2-101*, *gal4*Δ, *gal80*Δ, *his3*Δ*-200*, *leu2-3,112 trp1*Δ*-901*, *ura3-52*, *URA3::GAL1-lacZ*, *lys2::GAL1-HIS3*, *cyhrs*	*lacZ HIS3 MEL1*	*TRP1 LEU2 LYS2*	[13]
KGY37	*MAT*a, *ade2-101*, *gal4*Δ, *gal80*Δ, *his3*Δ*-200*, *leu2*Δ*-inv pUC18*, *trp1*Δ*-901*, *ura3*Δ*-inv::GAL1-lacZ*, *lys2*Δ*-inv::GAL1-HIS3*	*lacZ HIS3*	*TRP1 LEU2 URA3 LYS2*	[14]

3.2 Yeast Transformation

Yeast transformation is carried out using the protocols listed in this volume [12]. Each screen can be performed using either a sequential or co-transformation approach. If your bait construct has little or no effect on yeast growth or survival, it is best to use the sequential approach. Bait plasmid construction is not covered here but can be reviewed in other publications [9]. Bait plasmids are transformed into the selected yeast strain using either the Quick and Easy or high-efficiency protocols found elsewhere this volume [12].

Each bait plasmid–strain combination should be tested for reporter gene auto-activation as well as bait fusion protein expression

as outlined in [9]. Your YTH screen can proceed after testing for Library transformation efficiency again outlined in [9]. The library screening protocol found in [12] in this publication can then be used to transform your cDNA activation domain library into your yeast strain containing the bait plasmid. This protocol can be scaled up from 30× to 120× to generate the number of transformants to cover the complexity of your cDNA library.

1. Grow the yeast strain for transformation as described in [12].
2. Transform your "prey" plasmid library or "bait" plasmid and "prey" library together into the competent yeast cells to give good coverage as described in [12].

3.3 Liquid Screening

1. Mix the transformed cells to the appropriate volume in liquid SD-W-L-H medium containing 3-AT (0.5 mM or 2.5 mM for KGY37 and Y190, respectively). Ampicillin (100 μg/mL) can be optionally added to this medium (*see* **Note 1**). The volume of medium required corresponds to the scale of transformation performed, 1 mL for each transformation (e.g., for 30, 60, or 120× use a final volume of 300, 600, and 1,200 mL, respectively). Ensure that the final volume includes the 50 mL used to resuspend the transformed yeast cells ([12] Subheading 3.2.3). Plate 2, 20 and 200 μL of this cell suspension to duplicate plates of SC-W-L medium to estimate the number of total transformants in this solution.
2. Distribute 60 mL into each 250-mL flask (Flask method) or 1.5 mL into each well of the 96-well deep-well plates (96-well method) and seal with SealPlate® sterile tape (*see* **Note 2**).
3. Incubate the flasks or 96-well plates at 30 °C on a rotary shaker at 200 rpm for 72 h. In some cases the bait protein can affect yeast growth, which affects the timing of this incubation. To adapt the first incubation and dilution timing (*see* **Note 3**).
4. Prepare fresh SD-W-L-H + 3-AT + Amp medium and using fresh sterile flasks or 96-well plates dilute the culture in each culture flask 1 in 10. When using 96-well plates distribute 1.25 mL of this medium into each well of new sterile 96-well plates. Using a multichannel pipette replicate each 96-well plate by removing 150 μL and dispensing into the wells of a new plate. Seal each plate with SealPlate® membrane (*see* **Note 4**).
5. Incubate at 30 °C for 24 h in a rotary shaker at 200 rpm.
6. Prepare fresh SD-W-L-H + 3-AT + Amp medium using fresh sterile flasks. Dilute the culture in each culture flask 1 in 100. For 96-well plates distribute 1.5 mL of this medium into each well of new sterile 96-well plates. Using a multichannel pipette replicate each 96-well plate by removing 15 μL and

dispensing into the wells of a new plate. Seal each plate with SealPlate® membrane.

7. Incubate at 30 °C for another 24 h in a rotary shaker. Check each culture for growth in each well or flask (*see* **Notes 5** and **6**).

3.4 Identification of YTH-Positive Clones

In general, strongly activating clones will cause turbidity that is noticeable by eye. However, each flask culture should be have a sample removed and the OD at 600 nm determined. Any culture giving an OD_{600} higher than 0.1 should be analyzed further. Alternatively, the cell concentration of each flask culture can be determined with a haemocytometer and those with a cell concentration above 10^7 cells/mL should be analyzed further. If your YTH screen was done in deep-well plates, transfer 400 µL of the final culture into ELISA plates and analyze with a plate reader at 600 nm. Continue your analysis with those wells containing cultures with OD_{600} higher than 0.1.

Those flasks or wells from the 96-well plate containing growth as indicated from the OD_{600} analysis should be plated onto SC-W-L-H + 3-AT solid medium for isolation of colonies (2.5 mM for KGY37 and 50 mM for Y190). Plate approximately 500–1,000 cells onto a plate of SC-W-L-H + 3-AT and incubate at 30 °C for ~2–3 days or until colonies appear.

3.5 Analysis and Validation

Validation of YTH positives consists of a number of steps, the first being testing for activation of the second reporter gene. Colonies from plates corresponding to flasks or single wells usually represent clones from a single transformant that have populated the culture. The frequency of this event is rare. In a 10-flask screen (600× transformation scale up) up to ½ of the flasks can present with some form of growth. In these cases the flask could contain numerous YTH positives with the faster-growing ones dominating the culture. Using a 96-well-format screen (4 deep-well plates, 600× transformation scale up) we have seen up to 30 wells that present with some form of growth.

3.5.1 LacZ Activation Assay

The colonies on each SC-W-L-H + 3-AT plate containing putative YTH positives should be validated by testing for activation of the lacZ gene. This can be done using the method below.

1. Carefully place a sterile 75 mm circle of Whatman #1 filter paper on top of the colonies or patches growing on selective medium. Ensure that the filter paper makes good contact with the colonies. Mark the orientation of the filter paper relative to the plate using an 18 gauge needle to punch through the filter in an asymmetric pattern.
2. Remove the filter from the plate with sterile forceps after it has fully contacted the colonies and immerse into liquid nitrogen for 10–15 s or freeze at –80 °C for 10 min.

3. Thaw the filter colony side up on a piece of plastic wrap. Repeat the freeze–thaw cycle twice more.
4. Place the filter colony side up, onto a filter paper in an empty petri plate (100 × 15 mm) presoaked with 1.5 mL of Z buffer/β-ME/X-GAL taking care that the filters line up to distribute the solution evenly.
5. Place the lid on each plate and transfer to a plastic bag and incubate at 37 °C.

Strong activation of the *lacZ* gene will give a blue color within 1 h. Filters can be incubated overnight if color does not develop immediately. A faint blue color after overnight incubation is considered minimal *lacZ* activation and is generally not the sign of a strong YTH interaction. Strong activation of the *lacZ* gene indicates a putative YTH positive. These colonies should be archived by freezing in 1 mL of 20 % (v/v) sterile glycerol.

The second step in validation of YTH positive is reconstruction as described in [9]. The prey plasmids can be isolated from these putative positives and used to reconstruct the two hybrid positives with the original bait plasmid. Prey plasmid isolation is described below.

3.5.2 Miniprep Yeast

The prey library plasmid can be isolated from the YTH positives showing strong activation of both reporter genes. This is accomplished by a quick and effective method described in [15] which uses glass beads and phenol–chloroform to extract nucleic acids. These nucleic acid preparations will include both *bait* and *prey* plasmids and can be transformed into an *E. coli* host for recovery.

This protocol, modified from [15], can be used to isolate DNA from yeast cells either grown in liquid culture or harvested from a plate.

1. Inoculate individual YTH positives from SC-W-L-H + 3-AT plates into 2 mL of SC-H or SC-W-L liquid medium and incubate at 30 °C overnight. Alternatively, scrape a 50 µL blob of cells from an SC-W-L-H + 3-AT plate and resuspend in 500 µL of sterile water in a 1.5 mL microcentrifuge tube.
2. Collect the yeast cells from the liquid culture by centrifugation at 13,000 × *g* for 30 s.
3. Remove the supernatant and add 200 µL of Yeast Lysis buffer and gently resuspend the cell pellet using a micropipette tip to avoid the generation of bubbles.
4. Add an approximately 200 µL volume of glass beads and 200 µL of buffer-saturated phenol–chloroform (1:1 v/v).
5. Vortex each sample vigorously for 30 s and then place on ice. Repeat twice, leaving samples for 30 s on ice between treatments.
6. Centrifuge tubes at 13,000 × *g* for 1 min.
7. Remove the aqueous phase (~200 µL) to a fresh tube and precipitate the nucleic acids by adding 20 µL of 3.0 M sodium

acetate (pH 6.0) and 500 µL of 95 % ethanol. Incubate at −20 °C for 30 min and collect the precipitate by centrifugation at 13,000 × *g* for 5 min at 4 °C. Wash the pellet with 100 µL of 70 % ethanol (room temp) and dry the pellet for 5 min at room temperature.

8. Dissolve the pellet in 25 µL of TE buffer and store at −20 °C.

3.5.3 YTH Prey Plasmid Isolation

The most effective method of transforming a yeast DNA extract into *E. coli* is the electroporation method [16]. The protocol listed below gives electroporation conditions that work with *E. coli* strain DH5α in our hands; however, you should determine the conditions for your strain experimentally. Alternatively, the chemical treatment/heat shock method [17] can be used to transform *E. coli*.

1. Mix a 2 µL aliquot of extracted yeast DNA with a 25 µL aliquot of electrocompetent DH5α, and place carefully into a cold electroporation cuvette. Keep loaded cuvette on ice.
2. Place electroporation cuvette into electroporation device and pulse the DNA bacterial mixture with the following settings; 25 µF, 1.25 kV, with a pulse controller in parallel with the samples set at 400 Ω.
3. Immediately after pulse, add 1 mL of warm SOC medium to the electroporation cuvette and resuspend the cells. Transfer to a sterile tube and incubate at 37 °C for up to 30 min.
4. Plate samples of 25–100 µL onto 2–4 LB + Amp (50 µg/mL) plates and incubate at least 16 h at 37 °C.
5. Inoculate 4–5 Amp^R colonies per putative positive into 2 mL LB + Amp liquid medium and incubate at 37 °C overnight with shaking.
6. Extract the plasmid DNA from these cultures and dissolve plasmid DNA in 50 µL of TE.

3.5.4 Updated Validation and Analysis

The use of yeast colony PCR method [18] would eliminate the need to isolate plasmid DNA from each YTH positive. Each putative YTH positive can be analyzed using colony PCR to amplify the inserts from each prey plasmid. Restriction analysis of amplified DNA using enzymes such as *Hae*III or *Alu*I should allow the characterization and grouping of inserts. This approach should allow for the identification of YTH positives that occur in multiples, which is a hallmark of a true YTH positive.

The advances in high-throughput DNA sequencing [19] have changed the way we approach DNA analysis. Those with access to next-generation sequencing technology could speed analysis by using the products from the colony PCR reactions to generate DNA sequence information quickly. This can help eliminate false positives as well as identify YTH positives.

4 Notes

1. Ampicillin can be added to the SD-W-L-H medium containing 3-AT to reduce the occurrence of bacterial contamination.
2. The deep-well plates can be thought to mimic the growth of colonies in solid medium plates. Each well will contain cells descending from a single transformant. In the flask assay, the large volume is intended to select for stronger activating clones, measured by the ability of the yeast containing such interactions to dominate the culture (Donnard, Ortega, Gietz Unpublished data). However, it should be noted that using a single flask for a screen is not the best approach, as a strong false-positive activating clone will also be able to dominate the culture flask.
3. To estimate the length of the growth incubation before each dilution. Utilizing the original suspension of yeast culture from Subheading 3.2.3 dilute 0.5 mL into a culture tube containing SD-W-L selective medium to a final volume of 10 mL (A). This medium selects for yeast cells carrying both bait and prey plasmids but does not require activation of *HIS3* reporter gene. This culture shows yeast growth under the effect of the bait protein expression. From Tube A make 3 serial 1 in 10 dilutions in SD-W-L (Tube B, C, D). These cultures are incubated parallel to the deep-well plates or flasks in the screen. The titre of these cultures can be determined using a haemocytometer. When the cell titre in tube B is 2×10^7 cells/mL, the first dilution in the liquid screen protocol (1:10) should be performed. At this time dilute Tube B 1:10 (Tube B1) and 1:100 (Tube B2). When tube B1 (or B2 in case of expecting rare interaction events) reaches 2×10^7 cells/mL the second liquid screen dilution can be effected. This monitoring has revealed that in cases of interfering bait proteins, an extra 12–24 h of incubation between the 1:10 and 1:100 dilutions in the screen are necessary.
4. In most cases the diluted culture will be turbid.
5. After completing the liquid screen protocol, if no wells show growth it is still possible to remediate it. By keeping the previous deep-well plates at 4 °C while still moving forward with the dilution protocol, after the final step the initial or 1:10 deep-well plates can be re-incubated and re-diluted following the established protocol. However, be careful of contamination during this entire process; make sure plates are correctly sealed.
6. The deep-well plate approach can be easily adapted for a high-throughput robotic screen, increasing the protocol's efficiency and practicality.

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