Chapter 1

A High-Throughput Yeast Two-Hybrid Protocol to Determine Virus-Host Protein Interactions

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Abstract

The yeast two-hybrid (Y2H) system is a powerful method to identify and analyze binary protein interactions. In the field of virology, the Y2H system has significantly increased our knowledge of structure and function of viral proteins by systematically assessing intraviral protein interactions. Several comprehensive approaches to determine virus-host interactions have provided insight into viral strategies to manipulate the host for efficient replication and to escape host-derived countermeasures. To expand our knowledge of intraviral and virus-host protein interactions, we here present a Y2H protocol that is well suited for high-throughput screening. Yeast mating followed by liquid handling in a 96-well format as well as fluorescent readout of the reporter system provides a highly standardized and fully automated screening situation. The protocol can either be applied to screen complex host cDNA libraries or protein pairs arrayed for cross-testing. The ease of use, the cost-effectiveness as well as the robotic handling allows for extensive and multiple rounds of screening providing high coverage of protein-protein interactions. Thus, this protocol represents an improved "deep" screening method for high-throughput Y2H assays.

Key words Yeast two-hybrid, Y2H, High-throughput, Liquid handling, Automation, Yeast mating protocol, Virus-host interactions, Protein-protein interactions, Herpesvirus

1 Introduction

Soon after its development in 1989 [1], the yeast two-hybrid (Y2H) system turned into the most valuable high-throughput method to determine binary protein interactions. In the Gal4-based Y2H system, two proteins to be tested for interaction are fused to either the Gal4 DNA binding domain (bait) or the Gal4 activation domain (prey), and co-expressed in budding yeast cells. In case of interaction of the candidate proteins, the Gal4 transcriptional activator is reconstituted to form a functional entity able to activate one or several reporter genes present in the screening strain.

Over the years, the Gal4-Y2H system has been widely used to determine protein-protein interactions (PPI) in screening situations of varying complexity (for review *see* ref. 2). Initially, individual proteins were tested for interaction by transforming yeast

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reporter cells with both one bait and prey plasmid. A more open approach followed the same principle though by exposing a defined bait protein to a whole collection of potential preys which were provided as a cDNA prey library co-expressed with the bait protein. More recently, the yeast mating protocol that takes advantage of the natural ability of haploid yeast cells to mate in order to form diploids provided a much more efficient way to bring bait and prey plasmids together. In contrast to the *yeast transformation protocol* which is difficult to control and unlikely to result in a screening system covering all potential combinations, the *yeast mating protocol* allows for efficient cross-combination of a large number of pre-transformed bait and prey pairs providing an important prerequisite for high-throughput Y2H screening. Depending on the preys available, Y2H screening can be performed against a complex mixture of preys provided as cDNA libraries [3], alternatively, individual bait and prey pairs can be cross-tested for interaction using array-based mating systems.

Naturally—like all assays—the Y2H system is challenged by limitations. The use of the yeast organism as such could potentially disable the interaction of proteins that require particular species-specific modifications. But in general the environment seems to be sufficiently natural for the analysis of other species as has been demonstrated [4]. Similarly, the nuclear reporter system disables modifications provided by membrane compartments and integral membrane proteins need to be trimmed of their membrane anchors to access the nucleus. And finally, transcriptional activators are not suited to be tested in the Y2H system due to the use of a transcriptional reporter system.

Sceptics perceive the Y2H system as being prone to false-positive interactions. Large-scale analysis, however, revealed that the Y2H system is comparable in specificity to other assays detecting binary protein interactions [4–6]. Clearly however, the screening protocol is instrumental in providing sufficient sensitivity: (1) To ensure a "deep" screening situation, all proteins potentially interacting have to be exposed to each other in pairwise combinations. High-quality complex cDNA libraries enhance the screening success [3]. Alternatively, large ORF collections like the Mammalian Gene Collection (http://mgc.nci.nih.gov/) have been generated based on whole genome sequencing and subsequent recombinational cloning of all genes of a particular organism. Collections of this size and complexity can be screened either in batch procedures or by cross-testing of all potential protein interactions using arrays. (2) Classically, bait and prey proteins are tagged at their amino-terminal end with the bait fused to the Gal4 DNA binding domain and the prey fused to the Gal4 transcriptional activator. Fusion of tags to the carboxy-terminal end of a bait or a prey as well as testing of a given protein both as bait and prey fusion, allows for more refined screening [7, 8].

This reveals otherwise unrecognized interactions—*false negatives*—due to misfolding of tagged proteins or steric constraints that a particular tag may impose on the Gal4 reporter system. (3) Technical limitations significantly influence the screening output. Therefore, multiple rounds of screening of the same set of proteins will provide large and more reliable data sets. A Y2H mating protocol that allows for automated and standardized robotic screening is thus of utmost importance in replicate screening of large sets of protein pairs.

Post-screening analysis is required to identify interaction pairs of biological relevance. Prey and bait counts reveal the frequency a given prey or bait has been identified in one or several replicate screen(s). Interactions that were identified repeatedly with one particular bait or prey in screens of varying setups are likely to be specific, while preys isolated only once should be considered as uncertain interactors. Preys that are found very often while screening different baits, are probably "sticky," that is to say they may interact with other proteins in a nonphysiological manner and should be excluded from the evaluation as likely-false positives [3], while frequently identified bait proteins may partially self-activate the reporter system. Next, high-confidence data sets are subjected to computational analysis (*see* Friedel, Chapter 8 of this series). Database searches are performed to generate virus-host protein interactomes which are then connected to other high-throughput screens such as RNAi screens (*see* Griffiths, Chapter 5 of this series). Altogether these data are likely to provide insights into so far unknown viral strategies and to reveal viral or host factors that may serve as targets for antiviral therapy.

Meanwhile several methods have been developed that are suited to validate high confidence PPIs identified by Y2H analysis (for review *see* ref. 2). These include *methods for affinity-isolation* of co-expressed bait and prey proteins, e.g., the luminescence-based mammalian interactome (LUMIER) pull-down assay [9], and *protein fragment complementation assays*, e.g., the bimolecular fluorescence complementation assay (BiFC; *see* Becker and von Einem, Chapter 3 of this series). While most of these methods are of limited use in high-throughput applications either because the method is rather time-consuming, costly or requires extensive optimization on the level of a single protein pair, the LUMIER assay [9] has been adapted for high-throughput analysis (*see* Blasche and Koegl, Chapter 2 of this series). In brief, this assay is based on co-expression of two proteins in mammalian cells: while the bait protein is tagged by the protein A- or Flag-tag and used to immobilize the complex, the co-isolated interacting prey protein is detected via the enzymatic activity of its luciferase fusion partner. Most importantly, like the Y2H system, the LUMIER assay primarily detects binary interactions, provides high confirmation rates and thus represents the best method of choice to validate Y2H interactions.

Whole species Y2H interactomes have been generated for yeast [10, 11], humans [12, 13], *D. melanogaster* [14], *C. elegans* [15], and various pathogens (for review *see* ref. 2, 16; Friedel, Chapter 8 of this series). The analyses of intraviral interactions are of particular importance in complex viruses like Vaccinia virus [17], herpesviruses [7, 18–23], papillomaviruses [24], and SARS coronavirus [25]. Moreover, the identification of virus-host protein interactions is instrumental in understanding viral strategies to manipulate the host for efficient replication. Several very recent insights into the virus-host interplay come from high-throughput Y2H studies involving HCV [21], Influenza virus [26], SARS coronavirus [27], the gammaherpesviruses EBV [22], and MHV68 [23] as well as a collection of tumor viruses [24].

Current efforts combine Y2H screening with affinity-purification of larger protein complexes [24]. Since these two strategies are rather complementary than confirmatory, they are well suited to increase the coverage of interactomes. Individual proteins are fused to ProtA-, FLAG-, or GFP-tag to serve as baits for the isolation of protein complexes the composition of which is subsequently analyzed by mass spectrometry (*see* Rowles et al., Chapter 4 of this series). While functional tagging in the chromosomal context is still not feasible for higher eukaryotic proteins, the BAC technology which is available for numerous virus systems allows for genomic tagging of viral genes and thus their use in affinity purification of virus-host protein complexes. This way, PPIs of a particular viral protein can be investigated at different time-points of viral infection and upon infection of various cell systems. Most importantly, in case of viral genes with relevance for the infection cycle, the functional integrity of the viral fusion product can easily be assessed. A powerful combination of the Y2H system and affinity-isolation of protein complexes has recently been presented for four tumor virus families (the papilloma viruses, the polyoma viruses, the herpesviruses, and the adenoviruses) providing high stringency virus-host interactomes [24].

In order to generate a PPI data set of high coverage and high confidence, a high-throughput Y2H screening system is necessary. Here we present a Gal4-Y2H protocol that is well suited for high-throughput screening of intraviral as well as virus-host protein interactions. This method not only allows for simple and cost-effective screening. In particular, it can be fully automated and performed using a liquid handling robot and thus enables screening of extensive bait and prey combinations as well as multiple rounds of library screenings, thereby overcoming major drawbacks of the Y2H system. Thus, this protocol represents an improved "deep" screening method for high-throughput robotic Y2H assays.

2 Materials

If not indicated otherwise, all media and stock solutions are prepared using deionized water, sterilized by sterile filtration (pore size 0.2 μm), and stored at 4 °C. To avoid bacterial contamination, all media are supplemented with penicillin/streptomycin prior to use. Yeast cells are cultivated in a shaking incubator at 30 °C and 200 rpm unless otherwise stated.

2.1 Yeast Strains and Vectors

1. *Saccharomyces cerevisiae* strains Y187 (MATα, *ura3-52*, *his3-200*, *ade2-101*, *trp1-901*, *leu2-3*, *112*, *gal4Δ*, *met-*, *gal80Δ*, *MEL1*, *URA3*::$GAL1_{UAS}$-$GAL1_{TATA}$-*lacZ*) and AH109 (MATa, *trp1-901*, *leu2-3*, *112*, *ura3-52*, *his3-200*, *gal4Δ*, *gal80Δ*, *LYS2*::$GAL1_{UAS}$-$GAL1_{TATA}$-*HIS3*, *GAL2UAS-GAL2TATA-ADE2*, *URA3*::$MEL1_{UAS}$-$MEL1_{TATA}$-*lacZ*, *MEL1*) of opposite mating type are used throughout all yeast two-hybrid analyses.
2. Y2H vectors pGADT7 (prey) and pGBKT7 (bait) containing the genes of interest are applied.
3. *S. cerevisiae* strain Y187 is transformed with the prey plasmid pGADT7, while *S. cerevisiae* strain AH109 is holding the bait vector pGBKT7 (*see* **Note 1**).
4. For library-based Y2H screening pretransformed cDNA libraries can be purchased, usually consisting of a tissue-specific cDNA library cloned into prey vector pACT2 or pGADT7-Rec and transformed into the yeast strain Y187.

2.2 Media and Stock Solutions

1. Prepare 10× concentrated stock solution of SD medium (a synthetic minimal medium) according to the manufacturer's protocol (*see* **Note 2**).
2. Prepare 10× dropout supplements according to the manufacturer's protocol. Dropout supplements (DO) needed are DO-Leucine (-L), DO-Tryptophane (-W), DO-Leucine/Tryptophane (-L/W), and DO-Leucine/Tryptophane/Histidine (-L/W/H).
3. Purchase or prepare 100× concentrated sterile penicillin/streptomycin (Pen/Strep) solution.
4. SD-L: Dilute 100 mL 10× SD medium, 100 mL 10× DO-L, and 5 mL 100× Pen/Strep in 795 mL autoclaved water under aseptic conditions.
5. SD-W: Dilute 100 mL 10× SD medium, 100 mL 10× DO-W, and 5 mL 100× Pen/Strep in 795 mL autoclaved water under aseptic conditions.
6. SD-L/W: Dilute 100 mL 10× SD medium, 100 mL 10× DO-L/W, and 5 mL 100× Pen/Strep in 795 mL autoclaved water under aseptic conditions.

7. SD-L/W/H: Dilute 100 mL 10× SD medium, 100 mL 10× DO-L/W/H, and 5 mL 100× Pen/Strep in 795 mL autoclaved water under aseptic conditions.
8. 2× YPD medium: Weigh 40 g peptone, 20 g yeast extract and 40 g glucose into a beaker and add water to a volume of 1 L (*see* **Note 3**).
9. Prepare 100× Adenine solution by weighing 0.2 g Adenine into 100 mL water.
10. YPDA-medium: Dilute 500 mL 2× YPD medium, 5 mL 100× Adenine solution, and 5 mL 100× Pen/Strep in 490 mL autoclaved water under aseptic conditions.
11. Prepare a 0.15 M 4-Methylumbelliferyl-α-D-galactopyranoside (4-MUx) solution with sterile DMSO (dimethyl sulfoxide) and store at −20 °C.
12. Prepare a 1 M solution of 3-Amino-1,2,4-triazole (3-AT) and store at 4 °C.
13. Agar plates: Prepare a 2× Agar solution in 500 mL bottles by adding 15 g Agar to 250 mL deionized water and autoclave. Prepare 250 mL of 2× medium from the respective SD medium stock with sterile water. Bring both solutions to a temperature of 55 °C, mix, add 5 mL 100× Pen/Strep solution and spread onto Petri dishes under aseptic conditions.
14. YPD medium (20 % PEG): Weigh 10 g peptone, 5 g yeast extract, 10 g glucose, and 20 g Polyethylenglycol (PEG 6000) into a beaker and add water to a volume of 500 mL.
15. 0.25 % SDS aqueous solution.

2.3 Equipment and Consumables

1. Shaking and non-shaking incubator at 30 °C.
2. For high-throughput analyses the use of a robotic liquid-handling platform is recommended. If neither is at hand, it is also possible to complete the Y2H screening with a manually supported 96-well pipetting system (2–200 μL), e.g., the Liquidator96, Mettler Toledo.
3. A fluorescence microplate reader (excitation spectrum 365 nm, emission spectrum 448 nm) preferably equipped with an automatic stacker.
4. 96-well microtiter plates (MTPs) with both F- and U-bottom shape.
5. A centrifuge for microplates.
6. Polystyrene tubes of 50 mL volume.
7. Appropriate sterile pipet tips and reservoirs.
8. Self-adhesive foil for MTPs.
9. Laboratory plastic foil.

10. Sterile Petri dishes.
11. 96-well PCR plates.
12. Taq DNA polymerase kit of your choice, preferably equipped with loading dye.
13. PCR cycler.
14. PCR DNA primer to amplify prey vector inserts and accordingly a sequencing primer.
15. 0.8 % agarose gel.

3 Methods

3.1 Array-Based Yeast Two-Hybrid Screening

This procedure allows cross-testing of single proteins of interest with a defined set of possible interaction partners that are arranged as array (Fig. 1). Hence, any potential interaction pair is tested individually. The protocol displayed here is performed in 96-well MTPs and liquid medium. For convenience, the assay is described using a single bait protein to test against a prey library, although naturally it can also be carried out in the opposite setting.

1. Inoculate SD-W medium with a single yeast colony transformed with a bait vector (e.g., strain AH109) and incubate in a 50 mL tube over night at 200 rpm and 30 °C (*see* **Note 4**). To control for auto-activation of reporter gene activity potentially induced by the prey collection, also prepare a culture with yeast cells carrying an empty bait vector.
2. In an MTP (F-bottom), inoculate 150 μL SD-L medium with your choice of preys transformed into a yeast strain of opposing mating type (e.g., Y187) arranged in the preferred matrix, cover plate with adhesive foil, and incubate over night at 30 °C (*see* **Note 5**). It is strongly recommended to occupy three wells with controls: (1) blank control containing only medium but no yeast, (2) negative control containing yeast transformed with prey vector lacking an insert, and (3) as positive control a bait-prey pair proven to give a positive result (*see* **Note 6**).
3. On the next day the yeast mating is performed in an MTP equipped with U-bottom. For this purpose, 25 μL of each bait and prey culture is added to 100 μL YPDA and the MTP is gently centrifuged (1 min, 180 × *g*) to collect the yeast cells at the bottom of the wells. Incubate over night.
4. To select for diploid yeasts, transfer 15 μL of mating plate to a fresh MTP (F-bottom) with 150 μL SD-L/W medium and incubate for 2 days. Add the positive control to the designated well at this step.
5. To monitor the reporter gene activity, the diploid yeasts are then divided onto five different MTPs (F-bottom). 10 μL each

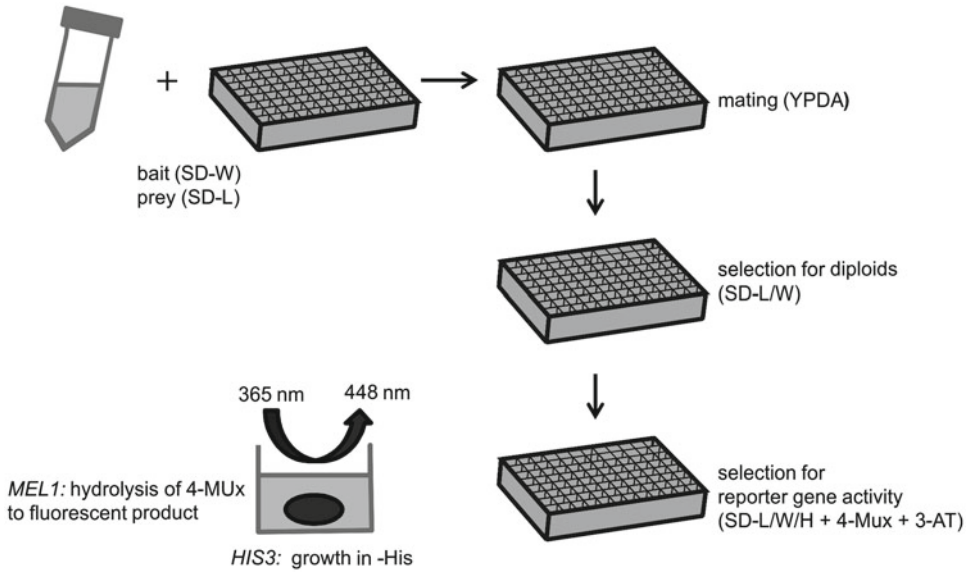

Fig. 1 The array-based yeast two-hybrid (Y2H) screening allows the cross-testing of a single protein against a defined set of possible interaction partners. The haploid yeast strain carrying the bait vector is propagated under selective conditions in synthetic minimal medium lacking Tryptophane (SD-W), while yeast cells of opposing mating type transformed with the various prey vectors are grown arrayed in 96-well microtiter plates (MTP) in SD medium lacking Leucine (SD-L). The combination of the bait yeast strain with the multiple prey strains in complete medium (YPDA) over night leads to mating, resulting in diploid cells carrying both bait and prey vectors, which are selected by growth in SD-L/W medium. The reporter gene activity is examined in SD medium lacking additionally Histidine (SD-L/W/H) and supplemented with 4-Methylumbelliferyl-α-D-galactopyranoside (4-MUx) and 3-Amino-1,2,4-triazole (3-AT) in a fluorescence plate reader (excitation 365 nm, emission 448 nm). The interaction of the bait protein fused to Gal4 DNA binding domain with the prey protein fused to Gal4 activation domain leads to the reconstitution of Gal4 transcription factor and subsequently expression of *HIS3* and *MEL1* reporter genes. Expression of the reporter genes allows growth in a Histidine-free environment as well as the hydrolysis of 4-MUx to fluorescent methylumbelliferone, whose fluorescent activity is a measure of protein-protein interaction. Usually 3-AT is added in increasing concentration as competitive inhibitor of the leaky expression of the *HIS3* reporter gene

are transferred to 150 μL SD-L/W/H medium containing 50 μM 4-Methylumbelliferyl-α-D-galactopyranoside (4-MUx) and increasing concentrations of 3-Amino-1,2,4-triazole (3-AT) (typically 0, 1, 2.5, and 5 mM) as competitive inhibitor of the leaky expression of the *HIS3* reporter gene. Also, 10 μL are used to inoculate 150 μL SD-L/W medium for growth control. All MTPs are incubated for 3 days.

6. The MTP with SD-L/W is controlled by eye for yeast growth. If there is no detection of growth in some wells, the corresponding wells of the SD-L/W/H MTPs cannot be analyzed.
7. The SD-L/W/H MTPs are then analyzed for reporter gene activity in a fluorescence plate reader (excitation 365 nm,

emission 448 nm). While the gene product of *HIS3* permits growth in Histidine-free medium, the α-Galactosidase encoded by *MEL1* catalyzes hydrolysis of 4-MuX to fluorescent methylumbelliferone.

8. The resulting data is analyzed by comparing the relative fluorescence intensity of each bait/prey-pair to the negative controls (empty vectors). For this, the relative fluorescence units (RFU) for all 3-AT concentrations of each protein pair are plotted on a bar diagram and compared to the values produced by expression of each bait or prey with the respective empty vector (*see* **Note 7**). The values of the positive control help to ease the interpretation of results. Cutoff values for positive interactions are usually defined individually for each bait, as RFU values tend to take a broad range in between the individual tested proteins.
9. Inclusion of several fragments of one possible prey to your matrix as well as repeating the procedure with the opposite vector combination (test your protein of interest as prey against a bait matrix) will help to raise the confidence in your resulting data.

3.2 Library-Based Yeast Two-Hybrid Screening

This protocol describes the yeast two-hybrid screening of individual bait proteins against a prey library consisting of cDNA usually derived from a certain tissue type cloned into a prey vector and pretransformed in yeasts (Fig. 2). This allows testing of single proteins of interest for interaction with a broad spectrum of possible cellular interaction partners. The library-based Y2H screening is performed in 96-well MTPs and liquid medium in analogy to the previously described matrix-based assay (*see* Subheading 3.1).

1. As a first step, the optimal 3-AT concentration for each individual bait should be identified in a prescreen.
2. Inoculate 5 mL SD-W medium with a single yeast colony transformed with a bait vector (e.g., strain AH109) and incubate in a 50 mL tube over night at 200 rpm and 30 °C.
3. Measure OD_{600} of the overnight culture and of the prey library (e.g., strain Y187) you want to apply. Inoculate 15 mL SD-W medium to an OD_{600} of 0.1 using the overnight bait culture and accordingly inoculate 15 mL SD-L with the prey library (*see* **Note 8**). Incubate at 30 °C and 160 rpm until OD_{600} of 0.9–1.0 is reached, which is usually about 16 h later.
4. To monitor the mating efficiency, prepare 10^{-1} to 10^{-4} dilutions of both cultures with sterile water and streak out on agar plates with the respective medium for viability count. Incubate at 30 °C for 3 days. Count the colonies on the plate with the appropriate dilution and calculate the colony forming units (cfu) per mL of culture.

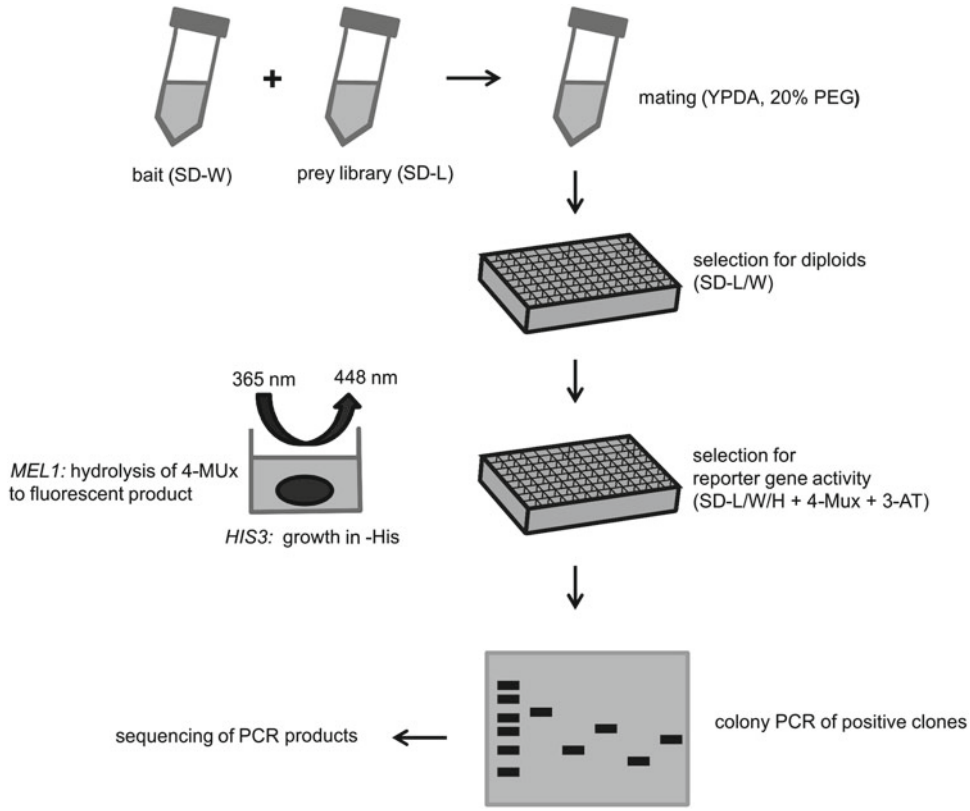

Fig. 2 The library-based yeast two-hybrid (Y2H) screening is applied to test an individual bait protein for interaction against a complex prey library, usually consisting of cDNA of a certain tissue type and pretransformed in yeasts. Haploid yeast strains of opposing mating type carrying either bait or prey vectors are propagated under selective conditions in synthetic minimal medium lacking Tryptophane (SD-W) or Leucine (SD-L), respectively. The mating process to gain diploid cells containing both bait and prey vector is performed in complete medium (YPDA + 20 % Polyethyenglycol) under gentle shaking. Afterwards, the yeast culture is spread on 96-well microtiter plates (MTP) and incubated under selective conditions (SD-L/W). The reporter gene activity is then examined in SD medium lacking additionally Histidine (SD-L/W/H) and supplemented with 4-Methylumbelliferyl-α-D-galactopyranoside (4-MUx) and 3-Amino-1,2,4-triazole (3-AT) in a fluorescence plate reader (excitation 365 nm, emission 448 nm). The interaction of the bait protein fused to the Gal4 DNA binding domain with a prey protein fused to the Gal4 activation domain leads to reconstitution of the Gal4 transcription factor and subsequently expression of *HIS3* and *MEL1* reporter genes. The reporter genes allow growth in a Histidine-free environment as well as the hydrolysis of 4-MUx to fluorescent Methylumbelliferone, whose fluorescent activity is a measure of protein-protein interaction. The optimal 3-AT concentration as a competitive inhibitor to leaky reporter gene *HIS3* expression was defined in a previously performed prescreen. To identify the prey genes that resulted in a positive outcome, a colony PCR of the respective diploid cells is carried out. The agarose gel electrophoresis reveals those hits, where only one prey gene is present. These PCR products then undergo sequencing analysis and their gene ID is identified using an alignment tool like NCBI's BLAST (http://blast.ncbi.nlm.nih.gov/)

5. The mating process is performed in 50 mL tubes. A similar number of yeast cells of both bait and prey culture should be applied. Calculate the culture volume needed to represent an OD_{600} of 12 in 1 mL ($12/OD_{600}$), mix both volumes in a tube and centrifuge for 2 min at 860 × *g*. Discard supernatant and resuspend the cell pellet in 25 mL YPD medium (20 % PEG). Incubate at 30 °C with gentle agitation (100 rpm) for exactly 3 h (*see* **Note 9**).
6. After mating, centrifuge cells at 860 × *g* for 3 min. Resuspend the cell pellet in 19 mL SD-L/W and strictly avoid vortexing (*see* **Note 10**). Prepare 10^{-1} to 10^{-4} dilutions for viability count and streak out on SD-L/W agar plates. Incubate for 3 days, then count the colonies to calculate the colony forming units (cfu) per mL.
7. Centrifuge cells again and resuspend the cell pellet in 1 mL SD-L/W/H.
8. Prepare SD-L/W/H medium containing 50 μM 4-MuX sufficient for seven MTPs with 200 μL medium per well (134.4 mL) plus the dead volume needed for the reservoir. Add the mated yeasts.
9. Now the mated yeasts are divided onto seven MTPs (F-bottom) with increasing 3-AT concentration (0, 0.5, 1, 2.5, 5, 10, 50 mM). First, transfer 200 μL each to an MTP without any 3-AT (0 mM plate). Then add the appropriate volume of 1 M 3-AT to the remaining yeast culture to obtain a 0.5 mM concentration in the remaining volume and fill the next MTP. Repeat this pipetting scheme until all seven plates contain cells in SD-L/W/H medium with 50 μM 4-MuX and the respective 3-AT concentration (*see* **Note 11**).
10. Stack the MTPs, cover the topmost with self-adhesive foil and wrap the stack in plastic foil. Incubate for 6 days at 30 °C.
11. To monitor the mating efficiency, divide the cfu/mL of diploids by cfu/mL of the limiting partner (the strain which yielded the fewest viable cells). Multiply with 100 to obtain the mating efficiency in percent. The mating efficiency should lie between 2 and 5 %.
12. The MTPs are analyzed after 6 days of incubation for reporter gene activity in a fluorescence plate reader (excitation 365 nm, emission 448 nm).
13. Plot the resulting RFU values in a line chart so that each plate is represented by a line. Choose the 3-AT concentration that results in a reasonable number of positive results to perform the Y2H screening (*see* **Note 12**).
14. To perform the actual Y2H screen repeat **steps 1–7**.
15. Prepare SD-L/W/H medium containing 50 μM 4-MuX and the optimal 3-AT concentration for the individual bait evaluated

by prescreen sufficient for ten MTPs with 200 μL medium per well (192 mL) plus the dead volume needed for the reservoir. Add the mated yeasts. Transfer 200 μL of mated yeasts to each well of the ten MTPs.

16. Repeat **steps 10–12**.
17. Plot the resulting RFU values in a line chart so that each plate is represented by a line. Define a reasonable cutoff value and identify the position of positive hits.
18. Unite the positive colonies on one or several MTPs by transferring 10 μL of each well with positive results to a fresh MTP containing 150 μL SD-L/W/H medium (hitpicking plate). Incubate for 3 days.
19. The identity of positive preys is analyzed by colony PCR of positive hits and subsequent sequencing of PCR products. Prior to performing the colony PCR, 5 μL of each well of the hitpicking plate is transferred to 20 μL of 0.25 % SDS solution in 96-well PCR plates and boiled for 5 min at 95 °C in a thermal cycler in order to break down the yeast cell walls. Spin down shortly (180 × *g*, 1 min).
20. Now a colony PCR is performed to amplify the cDNA inserts of positive prey vectors with DNA primers specific for prey vector sequences upstream and downstream of an insert sequence. Apply 5 μL of boiled yeast suspension in SDS solution as template. The primer sequence naturally depends on the prey vector applied. Use any Taq Polymerase Kit, preferably equipped with a reaction buffer already containing gel loading dye. We performed a nested PCR with two pairs of short primers partially overlapping to amplify the insert sequence from a pACT2 prey vector (*see* **Note 13**).
21. Load an aliquot of PCR reaction onto an 0.8 % agarose gel (e.g., 20 μL from a total volume of 50 μL). Those PCR reactions that exhibit a single DNA band in the gel are then sent for DNA sequencing to identify the respective cDNA insert (*see* **Note 14**).
22. Perform a blast search to determine the identity of the positive and sequenced prey cDNAs (http://blast.ncbi.nlm.nih.gov/).
23. To evaluate your results choose the following approach. High-confidence interaction partners are those preys, which lead to specific and reproducible results and encode for naturally occurring proteins. This means that preys should be found more than once with your specific bait. Preys that are found very often while screening different baits, are probably sticky preys and should be excluded from evaluation as likely-false positives, as well as noncoding 3′-UTR (untranslated regions) sequences as true false positives. Preys that were isolated only once should be considered as uncertain interactors.

4 Notes

1. Naturally, it is also possible to employ yeast strain Y187 as bait and AH109 as prey instead of the combination proposed by us. But we find it more convenient to adapt our protocols to the pretransformed libraries available on the market and to keep this constellation consistent throughout all analyses.
2. The minimal SD medium can be assembled from product systems by different supply companies, which may vary in their respective composition. Make sure your SD medium contains yeast nitrogen base (YNB) without amino acids but with ammonium sulfate. If glucose as a source for carbohydrates is not included by the manufacturer, it has to be added to a final concentration of 2 % (w/v).
3. Alternatively to sterile filtration, YPD medium can also be autoclaved. However, exposing the medium to boiling temperatures will result in caramelizing of the contained glucose. Although we find that this has no negative impact on growth rates of *S. cerevisiae*, some might prefer to add the glucose as a sterile solution after the autoclaving process.
4. Consider the dead volume needed to provide sufficient capacity when pipetting from a reservoir. Add dead volume to the 2.5 mL of yeast culture actually required to perform the assay (e.g., inoculate at least 30 mL for usage of a manual 96-well pipet).
5. Always prepare a glycerol stock of your prey matrix and store at −80 °C to use as inoculum for future analyses. For this purpose, it is also possible to keep the MTP with the prey library at 4 °C for a short period of time (e.g., 2 weeks).
6. Evidently, the positive control should be added only after the mating process on MTP. We found it convenient to keep the positive control in the form of diploid yeasts as glycerol stock at −80 °C and on SD-L/W agar plates stored in the fridge for a short time.
7. It is crucial to include the screening of the empty bait versus the prey collection in every run that is accomplished. Although the auto-activating preys will produce similar results every time, RFU intensities may vary. Slightly auto-activating bait proteins are controlled with the empty prey, which allows to evaluate only those results above a certain 3-AT concentration.
8. It is recommended to measure the OD_{600} of the prey library once and produce aliquots that result in an OD_{600} of 0.1 when 15 mL SD-L medium are inoculated to avoid repeated freezing and thawing.
9. Yeast cells require about 3 h for doubling. If incubation of mated diploids proceeds for more than 3 h, the diploids divide

resulting in overrepresentation of expanded clones obscuring the prey count.

10. Vortexing could dissociate the mating yeast cells as well as early diploids.
11. Keep in mind that the volume is reduced by 19.2 mL after every pipetting step and consider the 3-AT concentration already included for the previous step to calculate the respective volumes of 1 M 3-AT that have to be added after every step.
12. Typically, a low 3-AT concentration produces a lot of high RFU values, which will abate with increasing 3-AT concentration. It needs some experience to choose the optimal concentration. As a rule of thumb, pick a concentration that results in less than ten hits/plate.
13. To amplify cDNA inserts from a pACT2 library, the primer pair used for the first PCR is 5′-cta gag gga tgt tta ata cca cta caa tgg-3′ and 5′-ggt tac atg gcc aag att gaa act tag agg-3′ in a total volume of 25 μL for each PCR reaction. You may need to add 2 % Triton X-100 as detergent. The primer pair applied for the second PCR is 5′-tgt tta ata cca cta caa tgg atg atg-3′ and 5′-cat aaa aga agg caa aac gat g-3′ in a total volume of 50 μL for each PCR reaction. Use 1 μL of the first PCR as template for the second PCR. Ensure that the elongation time is sufficient for insert sizes up to 6 kb or the maximum insert size included in the cDNA prey library according to the manufacturer's protocol. Apply 26 cycles for both PCR reactions.
14. For DNA sequencing of pACT2 cDNA inserts, an aliquot of 20 μL from a total volume of 50 μL PCR reaction was analyzed by applying the pACT2 forward primer (5′-gat gat gaa gat acc cca c-3′).

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