

## A Field-Proven Yeast Two-Hybrid Protocol Used to Identify Coronavirus–Host Protein–Protein Interactions

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### Abstract

Over the last 2 decades, yeast two-hybrid became an invaluable technique to decipher protein–protein interaction networks. In the field of virology, it has proven instrumental to identify virus–host interactions that are involved in viral embezzlement of cellular functions and inhibition of immune mechanisms. Here, we present a yeast two-hybrid protocol that has been used in our laboratory since 2006 to search for cellular partners of more than 300 viral proteins. Our aim was to develop a robust and straightforward pipeline, which minimizes false-positive interactions with a decent coverage of target cDNA libraries, and only requires a minimum of equipment. We also discuss reasons that motivated our technical choices and compromises that had to be made. This protocol has been used to screen most non-structural proteins of murine hepatitis virus (MHV), a member of *betacoronavirus* genus, against a mouse brain cDNA library. Typical results were obtained and are presented in this report.

**Key words** Murine hepatitis virus, Host–pathogen interactions, Yeast two-hybrid, Interactomics, Proteomics

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## 1 Introduction

The yeast two-hybrid system (Y2H) was first developed in 1989 by Fields and Song [1], and quickly became a popular technology to detect protein–protein interactions [2]. Although multiple flavors of this system have been developed, it is in essence a complementation assay based on the reconstitution of a functional transcription factor mediated by protein interaction. Indeed, many transcription factors such as Gal4 exhibit a modular organization with a DNA binding domain (DB) that can be separated from the transactivation domain (AD) [3, 4]. In the Y2H system, a first protein of interest is fused to DB and a second one is fused to AD. When co-expressed in yeast, these two hybrid proteins called “bait” and

“prey”, respectively, reconstitute a functional transcription factor if they physically interact. This activates a set of reporter genes, including selectable markers. For example, interaction-dependent reporter gene *HIS3* enables yeast growth on a synthetic culture medium depleted of histidine (–His). This provides a system to co-express a bait of interest with a whole set of prey proteins, and then select for *HIS3* positive yeast colonies that express interacting protein pairs.

Collections of prey proteins can be expressed in yeast from cDNA libraries cloned in frame with the AD sequence (AD-cDNA library). However, such libraries often have a complexity in the range of five million independent clones for complex organisms like human, which adds to the strong enrichment bias for house-keeping proteins like actin. This implies that when performing an Y2H screen, several times more yeast transformants must be obtained to cover all possible bait–prey combinations and probe underrepresented preys. This can be difficult to achieve by standard yeast transformation and requires large amounts of the AD-cDNA plasmid preparation. This limitation is partially overcome by taking advantage of haploid Mat-a and Mat- $\alpha$  yeast to mate and thus form diploid cells [5]. Haploid yeast of opposite mating type are pre-transformed with the DB plasmid and the AD-cDNA library, and then mated to obtain a large number of diploids co-expressing bait and prey proteins. In this experimental setting, up to 50 million diploids that represent ten times the original complexity of the AD-cDNA library can be easily generated. Besides the technical benefit of yeast mating, it is now possible to generate normalized libraries by pooling thousands of prey plasmids originating from large collections of full-length ORFs like the human ORFeome [6–8]. Since prey plasmids are represented at equimolar concentrations in such libraries, their full coverage by yeast transformation or yeast mating is by far easier to reach. However, only full-length prey proteins are expressed from such normalized libraries and because isolated protein domains often better interact in the Y2H system, this can be a source of undetected interactions (false negatives; see below). This is in contrast with AD-cDNA libraries that usually encode full-length proteins but also protein fragments generated by random priming and premature arrest of reverse-transcription when building the library from cellular mRNAs.

Although multiple protein–protein interaction assays have been developed in the last decade [9], the Y2H system is often preferred because it does not require protein purification steps, which can be technically challenging, and is amenable to high-throughput settings [10–12]. As a matter of fact, Y2H system takes advantage of yeast genetic power to dissect complex problems and perform high-throughput genetic screens by opposition to biochemical screens. Most importantly, this assay essentially provides information on binary protein–protein interactions in contrast to

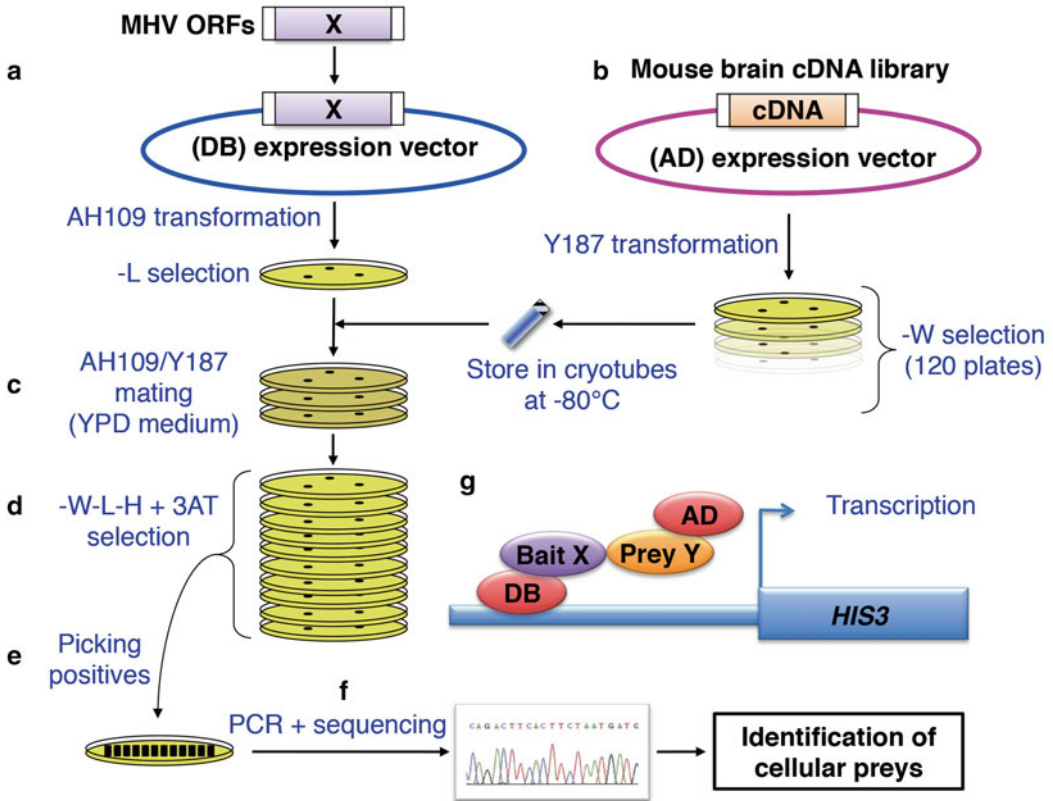
protein complex analysis by mass spectrometry, which does not distinguish direct from indirect partners. Nevertheless, technical limitations of the Y2H system must be considered when performing a screen. Contrary to common thought, Y2H screens generate high-quality datasets with relatively low rates of false-positive interactions when properly performed. In particular, this implies to properly evaluate for each bait construct the level of self-transactivation in yeast, and titrate down this activity at an appropriate stringency with a selective medium containing a competitive inhibitor of HIS3 gene product when performing the screen. Another important point is the elimination of satellite prey plasmids that often contaminate positive yeast colonies, and this is achieved by their serial passage on selective medium [13]. Finally, it is established that filtering out Y2H interactions supported by only one or two positive yeast colonies is essential to enrich datasets for high-quality interactions [7]. When following these recommendations, about 80 % of the interactions identified by Y2H properly retest in another experimental systems [7, 14, 15]. Although this validates Y2H data at a biophysical level, this does not imply that identified interactions are biologically relevant and participate to a specific biological process. This should be kept in mind since functional validation can be a daunting task, and represents the true bottleneck for such interaction-mapping approaches.

As a matter of fact, undetected interactions or false negatives are more problematic. The sensitivity of this assay has been estimated to 20–30 %, meaning that Y2H detects at best a quarter of the interactions from a positive control set [16]. Misfolding, mislocalization, poor expression levels, or the lack of appropriate post-translational modifications of bait and prey proteins that are both tagged and forced to enter the yeast nucleus can explain the high false-negative rate of this assay. In addition, and even if the two hybrid proteins properly interact, steric constraints often prevent the formation of a functional transcription factor to drive reporter gene expression. To some extent, this can be circumvented by using isolated protein domains, different Y2H systems, and by swapping DB and AD tags to both extremities of bait and prey proteins [17, 18]. Beyond sensitivity of the assay, the usual incompleteness of prey libraries that are often missing several binding partners of the bait, and the multiplicity of isoforms, are the main source of false negatives. This is why screening several AD-cDNA libraries from different tissues can be advantageous to cover as much as possible the complexity of the proteome [19, 20]. Finally and as already discussed above, it can be difficult to fully probe the complexity of some libraries despite the use of yeast mating protocols and the production of millions of diploid yeast [14].

In the last decade, Y2H has been extensively used to map virus–host interactions. The main objective for different research groups was to address the lack of information in literature, and

obtain proteome-scale pictures of virus infection networks [21, 22]. So far, SARS-coronavirus (SARS-CoV) is the only member of Coronaviridae family for which interactions with host factors were investigated systematically by Y2H [23]. This led to identify 132 SARS–host interactions in the high-confidence dataset (which was only partially disclosed), and an extensive mining of literature also retrieved an additional list of 27 SARS-CoV–host interactions. In particular, this report identified nsp1 interaction with several members of immunophilin and calcipressin families, which led to demonstrate SARS sensitivity to cyclosporin A. More recently, another Y2H screen performed with the C-terminal domain of the spike glycoprotein (S) identified Ezrin as a binding partner and a restriction factor of SARS-CoV [24].

Since 2005, a technological platform is up and running in our laboratory at Institut Pasteur, and dedicated to virus–host interaction mapping for a large panel of RNA viruses. Our Y2H protocol is a combination of tools and techniques greatly inspired by previous reports from Yves Jacob [25] and Marc Vidal’s group [10]. Here, we detail our Y2H protocol (Fig. 1), and present results obtained with both structural and non-structural proteins of Mouse Hepatitis Virus (MHV, strain A59). MHV is a *Coronaviridae* from *betacoronavirus* genus—like SARS-CoV—that encodes for 8 structural proteins and 16 non-structural proteins (nsps), which are poorly characterized at the functional level. In this screen we focused on those viral proteins that are known or suspected to be involved in RNA replication and transcription; nsp1–16 (with the exception of the very small nsp11) and the nucleocapsid protein N. The very large nsp3 was divided into three parts of approximately similar size (nsp3a, 3b, and 3c). In total, 15 MHV full-length proteins or isolated domains (including nsp3a and 3b) were used as bait to screen a mouse brain cDNA library. Because nsp3c, nsp6, and nsp12 segments could not be cloned in the Y2H vector, corresponding screens were not pursued. In total, 1,410 positive yeast colonies were recovered, and potential interactors identified by PCR analysis and sequencing. High-quality sequences were obtained for 1,096 positive yeast colonies, and retrieved interactions were filtered using statistical criteria to generate a high-quality dataset. In total, 39 novel interactions were identified with no precedent in literature (Table 1). Interestingly, nsp2 was found to interact with three MARK proteins (Microtubule Affinity-Regulating Kinase), suggesting a role in microtubule assembly, and this echoes Pfefferle et al. report that showed ORF9b and nsp13 of SARS-CoV binding with MARK2 and 3 [23]. In addition, nsp7 was found to bind the small glutamine-rich tetratricopeptide repeat-containing protein alpha (SGTA), like ORF7a from SARS-CoV [26].



**Fig. 1** Summary of the yeast two-hybrid screening pipeline. Cloning and transformation of the viral bait construct in AH109 yeast strain is shown in the *upper left* (A). Cloning and transformation of the prey AD-cDNA library in Y187 yeast is shown in the *upper right* (B). *Lower part* of the figure is showing successively yeast mating (C), growth on selective medium (D), picking of positives (E), amplification and sequencing of interacting preys (F). A schematic of *HIS3* reporter gene transactivation by DB-X bait interaction with AD-Y prey is also presented (G)

## 2 Materials

### 2.1 Yeast Strains, Plasmids, and Media

1. Yeast strain AH109 (*MAT $\alpha$* , *trp1-901*, *leu2-3, 112*, *ura3-52*, *his3-200*, *gal4 $\Delta$* , *gal80 $\Delta$* , *LYS2::GAL1<sub>UAS</sub>-GAL1<sub>TATA</sub>-HIS3*, *GAL2<sub>UAS</sub>-GAL2<sub>TATA</sub>-ADE2*, *URA3::MEL1<sub>UAS</sub>-MEL1<sub>TATA</sub>-lacZ*) (Clontech).
2. Yeast strain Y187 (*MAT $\alpha$* , *ura3-52*, *his3-200*, *ade2-101*, *trp1-901*, *leu2-3, 112*, *gal4 $\Delta$* , *met-*, *gal80 $\Delta$* , *URA3::GAL1<sub>UAS</sub>-GAL1<sub>TATA</sub>-lacZ*) (Clontech). The two haploid strains AH109 and Y187 are of opposite mating types, which enables library screens by mating.
3. pDEST32 (Life Technologies) or pPC97-GW (provided by Dr. Vidal) yeast two-hybrid vectors, containing sequence corresponding to viral protein of interest in frame with Gal4DB.
4. Mouse brain cDNA library cloned into yeast two-hybrid vector pPC86 (Life Technologies), or similar.

**Table 1**  
**Matrix of MHV–host protein–protein interactions identified by Y2H**

Ensembl gene IDs	Gene name	N	nsp1	nsp2	nsp3a	nsp3b	nsp4	nsp5	nsp7	nsp8	nsp9	nsp10	nsp13	nsp14	nsp15	nsp16
29392	Rilp1															63
64181	Rab3ip															33
46204	Pnma2															22
5417	Mpr1p												14			17
20776	Fbf1												20			10
32267	Usp28															3
71650	Ganab													20		
3573	Homer3												77			
19856	Fam184a												6			
38170	Pde4dip												4			
34602	Mon2												3			
42156	Dzip1												3			
42766	Trim46												3			
28249	Sdcbp											175				
39197	Adk										12					
68329	Htra2									6						
20375	Rufy1									4						
25855	Prkar1b								43							
4937	Sgta							150								
32405	Pias1							23								
976	Heatr6							3								
37259	Dzank1						35									
46818	Ddit4l						18									
52539	Magi3						17									
34354	Mtmr3						6									
4931	Apba3						5									
55128	Cgrrf1						5									
29312	Klh18						4									
8348	Ubc				5											
31788	Kifc3				5											
23353	Centg3				4											
64351	mt-Co1				3											
28367	Txn1			28												
24969	Mark2		29													
26620	Mark1		26													
7411	Mark3		16													
37443	Ccdc21		6													
<b>Number of Interactions supported by &lt;3 positive yeast colonies</b>		10	0	2	52	31	2	6	1	3	0	2	2	18	5	19

First and second columns correspond, respectively, to Ensembl gene IDs and canonical gene names for interacting cellular proteins. Columns 3–16 provide, for indicated MHV proteins, numbers of positive yeast colonies obtained for each cellular protein. Last row corresponds to numbers of interactions supported by less than three positive yeast colonies, which were filtered out as explained in Subheading 3.8, step 6

5. Nonselective medium (YPD) agar plates: 1 % yeast extract w/v, 2 % Bacto-Peptone w/v, 100 mg/l adenine hemisulfate, 2 % glucose w/v, 2 % agar w/v (*see Note 1*). Store at 4 °C.
6. Liquid YPD medium: 1 % yeast extract w/v, 2 % Bacto-Peptone w/v, 100 mg/l adenine hemisulfate, 2 % glucose w/v (*see Note 1*). Store at room temperature.
7. Amino acid powder: 10 g L-alanine, 10 g L-arginine, 10 g L-aspartic acid, 10 g L-asparagine, 10 g L-cysteine, 10 g L-glutamic acid, 10 g L-glutamine, 10 g L-glycine, 10 g L-isoleucine, 10 g L-lysine, 10 g L-methionine, 10 g L-phenylalanine, 10 g L-proline, 10 g L-serine, 10 g L-threonine, 10 g L-tyrosine, 10 g L-valine, 10 g of adenine hemisulfate. Mix and grind carefully in a mortar. Store at room temperature.
8. 100 mM L-leucine drop-out solution sterilized through 0.22 µm filter. Store at room temperature.
9. 40 mM L-tryptophan drop-out solution sterilized through 0.22 µm filter. Store at 4 °C in the dark.
10. 20 mM uracil drop-out solution sterilized through 0.22 µm filter. Store at room temperature.
11. 100 mM L-histidine drop-out solution sterilized through 0.22 µm filter. Store at room temperature.
12. 3-aminotriazole powder (3-AT).
13. Petri dishes.

## **2.2 Yeast Transformation Reagents**

1. TE/LiAc solution: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM lithium acetate (*see Note 2*).
2. TE/LiAc/PEG solution: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM lithium acetate, 35.2 % polyethyleneglycol (PEG 3350) (*see Note 2*).
3. 10 mg/ml salmon sperm DNA denatured by boiling in water for 10 min and chilled on ice.
4. Glycerol.
5. Cryotubes.
6. Glass beads, autoclaved prior to use.

## **2.3 PCR Amplification of AD-cDNA from Positive Yeast Colonies**

1. Zymolyase 20T.
2. La Taq PCR kit (Takara) or similar (*see Note 3*).
3. pPC86-For (5'-GACGGACCAAACCTGCGTATA-3') and pPC86-Rev (5'-ACCAAACCTCTGGCGAAGAA-3') primers.
4. 96-well E-gel (Life Technologies) or 1 % agarose gel.



### 3 Methods

For convenience, yeast cultures are manipulated in a biosafety level-2 cabinet to avoid contaminations. However, yeast cultures can be manipulated on a regular bench, even without a flame, depending on air quality in laboratory spaces.

#### 3.1 Production of Selective Medium Agar Plates

1. Make up base medium as follows: 0.29 % amino acid powder w/v, 0.38 % yeast nitrogen base (without amino acid and ammonium sulfate) w/v, 1.12 % ammonium sulfate w/v. Adjust pH to 5.9 with NaOH.
2. Make a stock of 4 % agar.
3. Autoclave both solutions (*see Note 1*).
4. Supplement base medium with 40 % glucose solution to a final concentration of 4 %.
5. Mix the base medium and agar at a 1:1 ratio.
6. Add 8 ml/L of the appropriate amino acid drop-out solutions and 3-AT whenever required (*see Note 4*). For example, uracil, histidine, and tryptophan should be added to obtain some synthetic medium lacking leucine (-L medium).
7. Pour in 15 cm petri plates and dry for 3–4 days. Store at 4 °C.

#### 3.2 Establishment of cDNA Library into Yeast Culture

A mouse brain cDNA library cloned in yeast two-hybrid vector pPC86 is first established into yeast strain Y187 (*see Note 5*).

1. Inoculate 500 ml of nonselective YPD medium at 0.007 optical density (OD) at 600 nm with a fresh yeast culture.
2. Grow overnight in a shaker at 30 °C.
3. Determine the OD at 600 nm. Harvest culture when OD is in a 0.4–0.5 range.
4. Centrifuge cells in 10×50 ml tubes at 750×g for 5 min. Discard supernatant and resuspend each yeast pellet in 40 ml water.
5. Centrifuge cells at 750×g for 5 min. Discard supernatant, and resuspend each yeast pellet in 40 ml TE/LiAc.
6. Centrifuge cells at 750×g for 5 min. Discard supernatant and pool the ten yeast pellets in 5 ml TE/LiAc.
7. Add 500 µl heat-denatured carrier DNA and 150 µg cDNA library to competent yeast, and mix carefully.
8. Dispense 250 µl of this preparation in twenty 2 ml tubes, and then add 1.6 ml TE/LiAc/PEG.
9. Mix by gently vortexing the tube and incubate for 45 min at 30 °C.
10. Heat-shock for 20 min at 42 °C.



11. Centrifuge at  $750\times g$  for 5 min. Discard supernatant and fill tube with water without resuspending the pellet.
12. Centrifuge at  $750\times g$  for 5 min. Discard supernatant and resuspend the pellet in 1 ml water. Pool the 25 transformation reactions in a single 50 ml tube.
13. Spread 200  $\mu$ l of yeast using glass beads onto one hundred twenty-five 15 cm petri dishes with -W agar.
14. Make 1/100 and 1/1,000 dilutions in water and plate on two 15-cm petri dishes with -W agar to determine the transformation efficiency.
15. Grow cells for 3 days at 30 °C. Calculate the total number of yeast transformants (*see Note 6*).
16. Add 5 ml YPD medium to each plate and scrape cells into medium using for example a Pasteur pipette bent using a flame. Pool into a 2-l flask.
17. Add glycerol to obtain a 20 % (w/v) solution, and determine the final OD. Calculate the volume  $V$  of yeast suspension required to perform one two-hybrid screen considering that  $V=60/\text{OD}$ .
18. Aliquot in cryotubes with the volume required for one yeast two-hybrid screen. Store at -80 °C.

### **3.3 Yeast Transformation with Bait Constructs Expressing Viral Proteins Fused to Ga4-DB**

1. In a 100 ml flask, inoculate 50 ml of nonselective YPD medium with a patch of fresh AH109 yeast cells scooped from a YPD plate stored at room temperature (few days old at most).
2. Grow overnight in a shaker at 30 °C.
3. Determine the OD at 600 nm. Take the appropriate volume of yeast culture considering that 1 ml at 5 OD is sufficient to perform ten transformations.
4. Centrifuge cells at  $750\times g$  for 5 min. Discard supernatant and resuspend yeast in  $>500\ \mu$ l water per transformation.
5. Centrifuge cells at  $750\times g$  for 5 min. Discard supernatant, and resuspend yeast in  $>100\ \mu$ l TE/LiAc solution per transformation.
6. Centrifuge cells at  $750\times g$  for 5 min. Discard supernatant and resuspend the pellet in 20  $\mu$ l of TE/LiAc per transformation.
7. Add 2  $\mu$ l heat-denatured carrier DNA and 50–250 ng Gal4-DB plasmid.
8. Add 120  $\mu$ l TE/LiAc/PEG and mix by gently vortexing the tube.
9. Incubate for 45 min at 30 °C.
10. Heat-shock for 15 min at 42 °C.
11. Centrifuge at  $750\times g$  for 5 min. Discard supernatant, and resuspend the pellet in 20  $\mu$ l water.

12. Spot 10  $\mu$ l on a petri dish with –L agar.
13. Grow cells for 3 days at 30 °C.

**3.4 Testing  
the Transactivation  
of HIS3 Reporter Gene  
by Gal4-DB Bait  
Constructs**

Before performing a screen, determine basal transactivation of *HIS3* reporter gene for each viral bait protein fused to Gal-DB (*see Note 7*).

1. With a loop, take a small patch of transformed yeast from Subheading 3.3, step 13.
2. Dilute in 1 ml water, and spot 10  $\mu$ l on petri dishes with –L and –L–H agar. After evaporation of water, the remaining yeast layer should be almost transparent and barely visible (if not, increase yeast dilution and repeat).
3. Incubate cells for 5 days at 30 °C.
4. Read plates. If no growth is observed on –L–H agar when compared to –L plate, then perform the screen on –L–W–H plates.
5. If yeast growth is observed on –L–H plates, repeat the same experiment by plating yeast on –L–H agar supplemented with increasing concentrations of 3-AT (*see Note 7*).
6. Determine a minimal 3-AT concentration sufficient to block yeast growth. Then, prepare –L–W–H plates containing the ad hoc concentration of 3-AT to perform the screen.

**3.5 Yeast Mating  
and Selection  
of Positive Yeast  
Colonies**

1. In a 100 ml flask, inoculate 50 ml YPD medium with a patch of fresh AH109 yeast expressing the bait protein of interest (*see Note 8*).
2. Grow overnight in a shaker at 30 °C.
3. Determine OD at 600 nm. Expected value should be in a 2–6 range. Calculate the volume  $V$  of AH109 yeast culture required to perform the screen considering that  $V=72/\text{OD}$  (*see Note 9*).
4. Thaw one vial containing the cDNA library in Y187 yeast and transfer into a 50 ml tube containing 10 ml fresh YPD medium.
5. Incubate 10 min in a shaker at 30 °C.
6. Add AH109 yeast with the bait protein to the tube containing Y187 yeast with the cDNA library. Mix by inverting.
7. Centrifuge at  $750 \times g$  for 5 min. Discard supernatant and resuspend the pellet in 1.5 ml of YPD medium.
8. Spread 500  $\mu$ l onto three YPD plates using beads.
9. Incubate for 4.5 h at 30 °C to allow yeast mating (*see Note 10*).
10. Add 8 ml of water to each YPD plate and resuspend yeast with a scraper (made, for example, from a Pasteur pipette bent by heating in a flame).
11. Repeat the same procedure to wash the three YPD plates at least once more and pool yeast suspensions into one 50 ml tube.

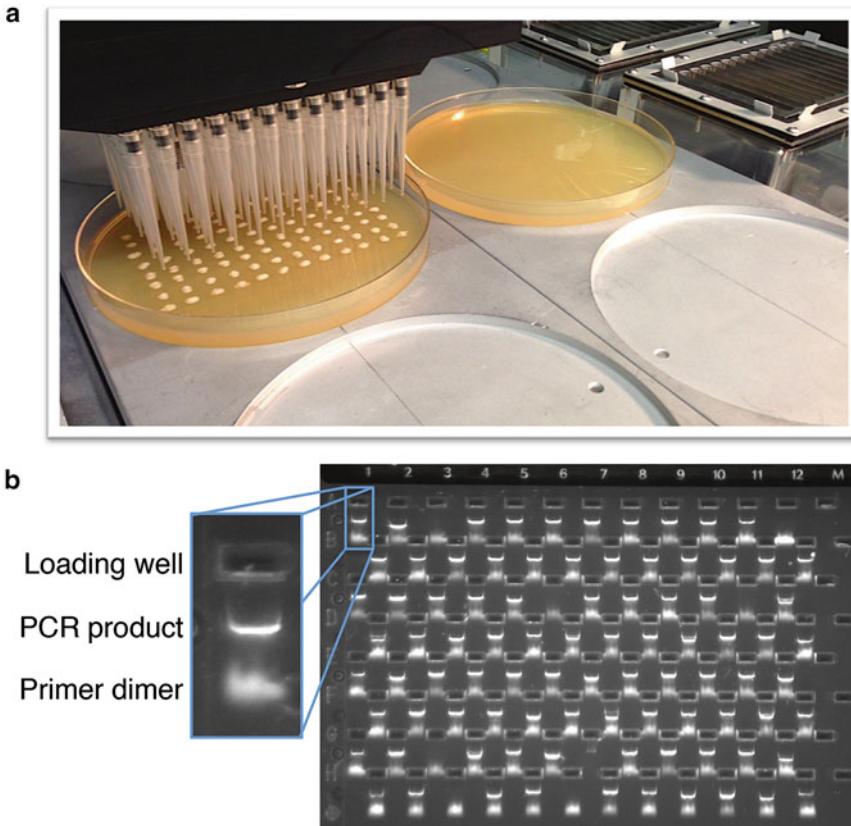
12. Centrifuge at  $750 \times g$  for 5 min. Discard supernatant and resuspend the pellet in 6 ml water.
13. Take 4  $\mu$ l of yeast suspension to prepare a 1/10,000 dilution in water that will be used to determine mating efficiency.
14. Using beads, spread 500  $\mu$ l yeast suspension onto 12 -L-W-H plates containing the ad hoc concentration of 3-AT (determined in Subheading 3.4, step 6). In addition, spread 500  $\mu$ l of the 1/10,000 dilution on a -L-W plate.
15. Incubate for 6 days at 30 °C.
16. In order to determine the efficiency of mating, count yeast colonies on the -L-W plate. Then, multiply by  $10,000 \times 12$  to obtain the total number of diploids generated during the screen (*see* Note 11).

### 3.6 Identification Interacting Partners

1. Cherry-pick positive yeast colonies from the 12 screening plates with a sterile toothpick or tip, and patch them on fresh -L-W-H plates containing the ad hoc concentration of 3-AT (determined in Subheading 3.4, step 6) to maintain selection pressure on *HIS3* reporter gene. It is best to organize positive yeast colonies at the standard 96-well format with the help of a grid paper placed underneath the petri dish.
2. Grow at 30 °C for 3–4 days (*see* Note 12).
3. To eliminate contaminations with satellite AD-cDNA plasmids, which can be present in yeast aside plasmids encoding for *bona fide* interactors, purify positive colonies by replication every 3–4 days on fresh selective medium over 3 weeks. This can be quickly achieved with the extremity of tips mounted on a multichannel or using an automated platform, as displayed in Fig. 2a.

### 3.7 PCR Identification of Positive Colonies

1. Prepare a 2.5 mg/ml solution of zymolyase 20T in water and dispense 50  $\mu$ l per well in a 96-well PCR plate.
2. For each positive colony, take a patch of yeast and resuspend in the zymolyase solution in one well.
3. Incubate for 5 min at 37 °C and then for 5 min at 95 °C to inactivate the enzyme.
4. Perform a PCR using pPC86-For and pPC86-Rev with LaTaq or similar, according to manufacturer's instructions (*see* Notes 3 and 13). An amplification cycle of 94 °C for 1 min then 35 cycles of 98 °C for 10 s and 68 °C for 5 min followed by a single incubation of 72 °C for 10 min should be performed.
5. Analyze PCR products on a 1 % agarose gel, e.g., a 96-well E-Gel (Fig. 2b).
6. Sequence PCR products with pPC86-For primer using standard procedures (*see* Note 13).



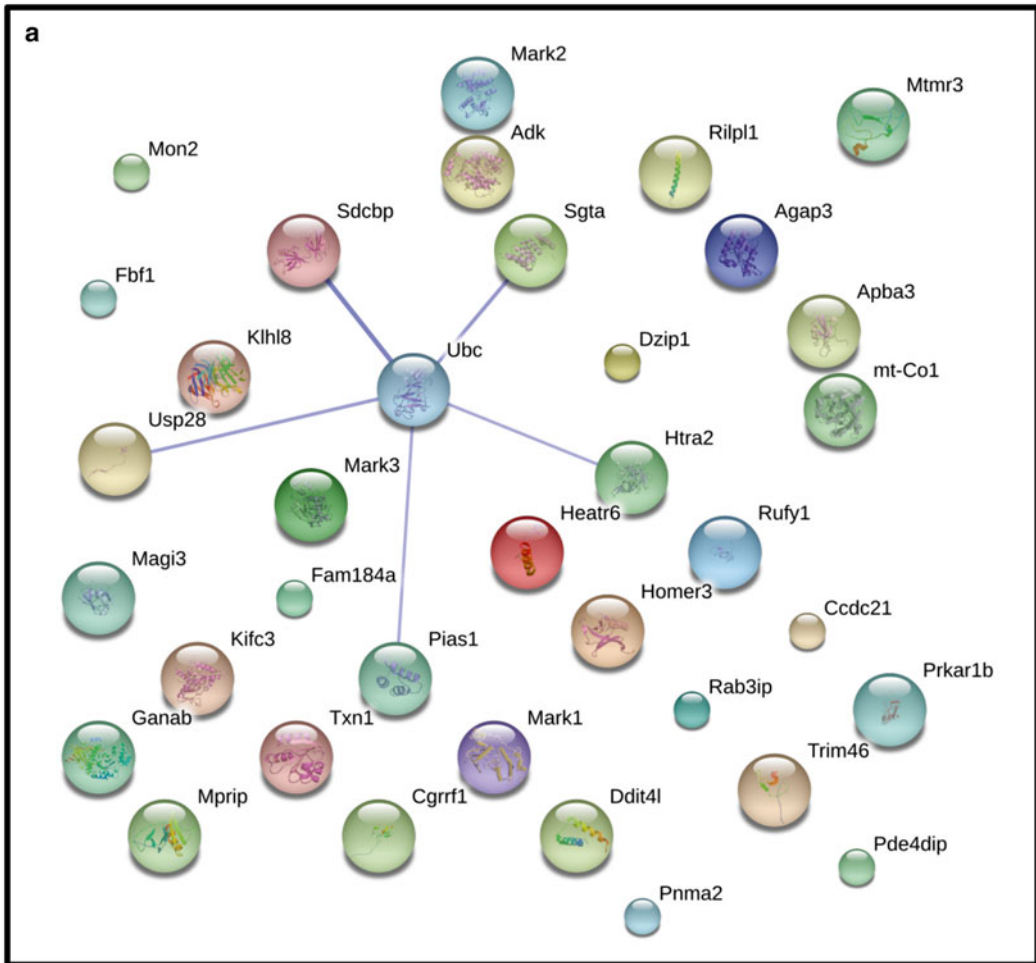
**Fig. 2** Selection and replica plating of positive yeast colonies. **(a)** Automated replica plating of positive yeast colonies arrayed in a 96-well format using a TECAN platform. Notice the customized stand for 15 cm petri dishes that fits into the standard 96-well plate holder. Yeast colonies are replica plated from mother to daughter plates by a 96-Multi Channel Arm (MCA) by simple tip-touching without aspiration or dispense. **(b)** PCR products corresponding to AD-cDNA were amplified from positive yeast colonies and analyzed on a 96-well E-gel

### 3.8 Data Analysis

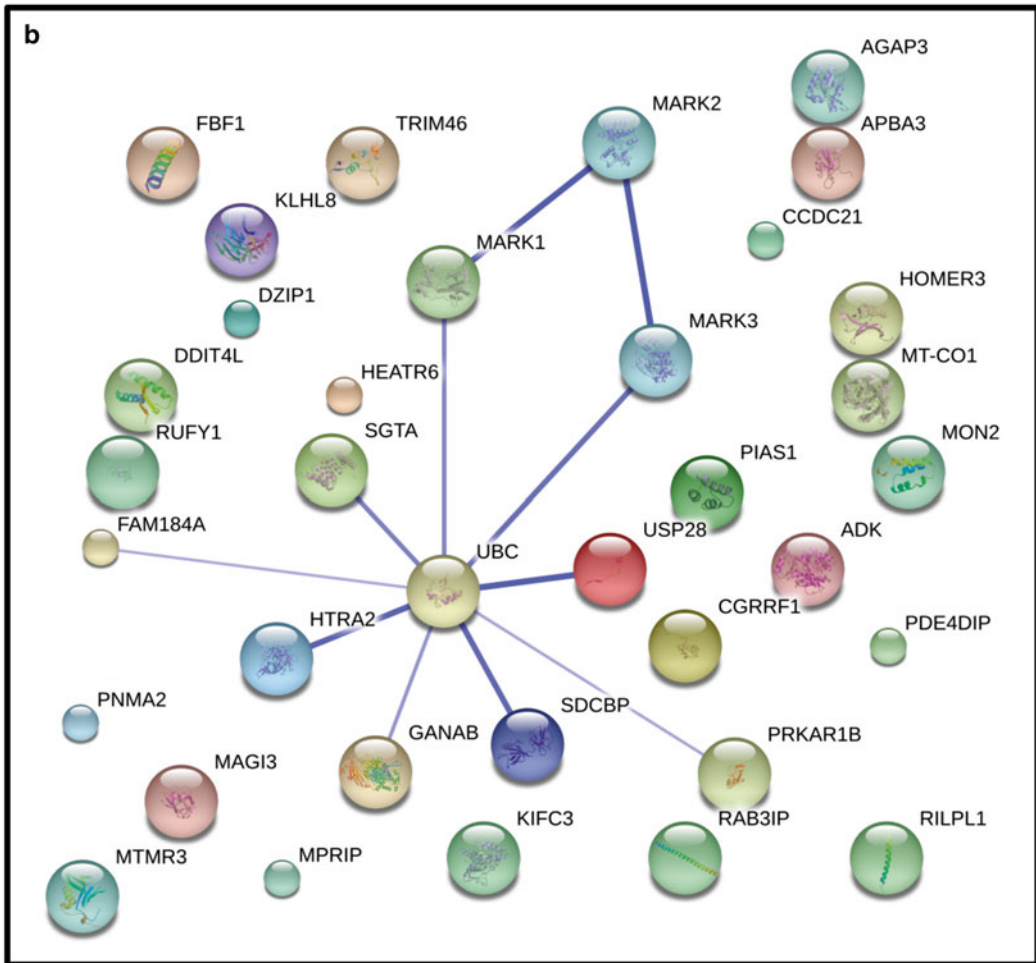
1. Analyze trace files to assign quality scores and generate accurate sequence files.
2. Trim plasmid adaptor sequences.
3. Use BLAST to probe the mouse mRNA and protein sequence databank at EMBL, and determine which host protein corresponds to each prey sequence.
4. Build an Excel spreadsheet with three columns including, for each positive yeast colony, a sequence ID and the corresponding bait and prey protein names.
5. Use the pivot table function of Excel to build an interaction matrix showing the number of positive yeast colonies for each bait-prey combination.
6. Use Data > filter function in Excel to eliminate interactions supported by less than three positive yeast colonies. This is essential

to remove most technical false positives from the final dataset. Table 1 shows an example of the final results obtained for a screen of MHV proteins.

- The identified proteins may be subjected to further analysis. For example, use the STRING database (Search Tool for the Retrieval of Interacting Genes/Proteins) to determine, for mouse and other organisms, known interaction data between the proteins listed in Table 1 (Fig. 3). Upload protein IDs on the STRING website (<http://string-db.org/>), and follow the instructions.



**Fig. 3** Confidence view of STRING analyses for all interacting host proteins identified in the different screens and listed in Table 1 [29]. Confidence view of STRING analyses of host proteins that were found to interact with the MHV proteins using either *Mus musculus* (a) or *Homo sapiens* (b) as input organism



**Fig. 3** continued

#### 4 Notes

1. When making agar plates, culture medium and agar should be autoclaved separately to avoid chemical interactions when heating. Glucose solution should not be autoclaved, filter sterilize through a 0.22  $\mu\text{m}$  filter and add to medium after autoclaving.
2. Although Tris-EDTA, lithium acetate, and PEG solutions are stored at room temperature, fresh TE-LiAc and TE-LiAc-PEG solutions should be prepared each time before use.
3. Readers should be aware that over a dozen of PCR enzymes tested, only La Taq from Takara gave us satisfactory results. However, other untested PCR enzymes may also be suitable.

4. Synthetic medium lacking leucine (-L) or tryptophan (-W) are used to select yeast transformants for Gal4-DB and Gal4-AD expression plasmids, respectively. Medium lacking both leucine and tryptophan (-L-W) is used to select yeast transformants for both Gal4-DB and Gal4-AD expression plasmids. Finally, synthetic medium lacking leucine, tryptophan and histidine (-L-W-H) is used to select yeast transformants for both Gal4-DB and Gal4-AD expression plasmids and the activation of the two-hybrid reporter gene *HIS3*. Appropriate amount of 3-AT can be added to increase stringency of the screen (*see Note 7*).
5. High transformation efficiency in yeast can be challenging to achieve. The procedure described herein should be tested on a small scale before proceeding to a large-scale transformation.
6. For a good coverage, it is usually accepted that the total number of yeast transformants should correspond to three times the original complexity of the cDNA library. However, this should be considered as a general guideline.
7. A significant fraction of bait proteins transactivate *HIS3* reporter gene in AH109 yeast when expressed in fusion to Gal4-DB. Amino acid stretches with acidic and proline residues in the bait protein are often associated with transactivation [27]. However, transactivation level should be experimentally determined since other poorly defined parameters are also involved. 3-AT, which is a competitive inhibitor of HIS3 enzyme, is used to titrate down yeast growth when the bait protein alone is a transactivator. Concentrations of 5, 10 and 20 mM are usually sufficient, but could be increased up to 200 mM.
8. Bait vectors used herein contain a yeast centromere (CEN) in addition to an autonomously replicating sequence (ARS). Thus, they will be maintained in yeast for several generations when growth is performed on non-selective YPD medium, but yeast could be grown on selective -L medium as well. Situation is different when using plasmids containing a 2 $\mu$  replication origin. In that case, yeast transformants must be permanently maintained on selective medium or the bait plasmid will be quickly lost.
9. A screen is performed by mixing AH109 yeast transformed with the bait-encoding plasmid with Y187 yeast cells transformed with the AD-cDNA library in a final 1.2 ratio. It has been reported that a 2.5 ratio could increase mating efficiency as determined by Soellick et al. [28].
10. Although 4.5 h is sufficient to achieve yeast mating, incubation should not last for too long. After few hours, diploid yeast cells start to divide and this artificially increases numbers of diploids



and positive yeast colonies. To increase mating efficiency, yeast can be resuspended and then spread on YCM (1 % yeast extract, 1 % Bacto-Peptone, 2 % dextrose) at pH 4.5 [28].

11. With this protocol, mating efficiency is usually close to 40–80 million diploids, which represent 8–16 times the original complexity of the mouse cDNA library we used. However, we empirically found that saturation is reached when the number of diploids is superior to 40 times the original complexity of the cDNA library. Thus, the screen should be repeated 3–4 times whenever saturation needs to be reached.
12. The number of positive yeast colonies is highly dependent on the experimental design of the screen and in fact, a significant fraction of Y2H screens generate no positives. In such a situation, screens should be performed with isolated domains of the original bait protein, by swapping DB and AD tags to extremities of bait and prey proteins, and using other prey libraries or alternative Y2H systems [17, 18].
13. PCR on yeast can be challenging when using low-copy plasmids such as pPC86. Besides, the amplification success rate critically depends on the length and nucleoside composition of AD-cDNA sequences corresponding to prey proteins. Thus, PCR success rate is highly variable from one screen to another, and together with poor quality and nonspecific PCR amplification products, could significantly decrease the number of exploitable AD-cDNA sequences. In the MHV screen that is presented in this manuscript, high-quality sequences were obtained for 78 % of positive yeast colonies.

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