# Chapter 6

# Virus–Human Cell Interactomes

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## Abstract

Using global approaches and high-throughput technologies in virology brings a new vision of the infections physiology and allows the identification of cellular factors, mandatory for viral life cycle, that could be targeted by original therapeutic agents. It opens perspectives for the treatment of viral infections by acting on cellular pathways that the virus must use for its own replication. Combining these new molecules with classical antiviral drugs and immunomodulators diversifies and enlarges the antiviral arsenal and contributes to fight drug resistance.

Our laboratory and others are constructing virus–human interactomes to propose a comprehensive analysis of viral infection at the cellular level. Studying these infection maps, where the viral infection can be visualized as perturbation of the human protein–protein interaction network, and identifying the biological functions that are impaired by these perturbations may lead to discovery of new therapeutic targets. These virus–human interaction maps are constructed in a stringent yeast two-hybrid system by screening human cDNA libraries with viral proteins as bait and integrating interactions mined from literature and public databases.


**Key words:** Yeast two-hybrid screen, Mating, Yeast two-hybrid array, Protein–protein interaction

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## 1. Introduction

The rapidly growing knowledge of protein–protein interaction networks (interactomes) for model organisms and human provides a network-based model to understand molecular and cellular biology. Recently, virus–host relationships also began to be studied at the proteome level by identifying interactions between viral and host-cell proteins (1–5), as reviewed in ref. 6. These virus–host interactomes compose a repertoire of interactions between viral and human proteins that can be analyzed in a network approach. By this mean, viral infections can be viewed as the expression of new constraints imposed by the virus on the cellular interactome.

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Study of topological and functional properties that are lost, deregulated, or that emerged in the "infection network" leads to the identification of cellular functions that are mandatory for the virus life cycle. By developing therapeutic agents that act directly on these cellular factors, new ways for treatment of viral infections may emerge.

There are several methods to detect protein–protein interactions. The yeast two-hybrid system is widely used because it requires the manipulation of DNA exclusively (allowing standardization and automation) and because of its efficacy. However, the two-hybrid system is not amenable to detect all protein–protein interactions, since it is based on the nuclear localization of a transcriptional reporter system. Additional approaches need to be used to complement and improve coverage of protein–protein interaction maps generated by yeast two hybrid.

The yeast two hybrid is based on the observation that a transcription factor consists of two separate functional domains: a DNA-binding (DB) domain and a transactivation domain (AD). These two domains are separated and each is fused to a protein of interest (a bait X and a prey Y). Physical interaction between X and Y reconstitutes a transcription factor that binds to responsive elements upstream of a reporter gene and, thus, can activate its transcription (7). In the screening procedure we developed, we use DB and AD from the yeast transcription factor Gal4, *HIS3* as reporter gene, and the mating protocol, in which pretransformed haploid years cells form diploids that carry both bait and prey vectors (8). [AU1]

Here, we describe yeast two-hybrid screens, DNA isolation protocols, and analysis strategies (Fig. 1) that were recently developed to generate the HCV infection map (4). For this interactome approach, 27 HCV ORFs (encoding full-length proteins or domains) were used as baits to screen 2 human cDNA libraries and generate a virus–host cell interaction map composed of 314 virus–host protein interactions. We added interactions retrieved from literature, constructing an HCV–human interaction network composed of 481 interactions involving 11 viral proteins and 421 cellular proteins. For this purpose, we developed VirHostNet, a database gathering virus–host protein interactions from literature and public databases, allowing a comprehensive analysis of virus–human protein interactions through a network view (9).

## 2. Materials

### 2.1. Plasmids

1. pGBKT7-gw: pGBKT7 is commercialized by Clontech (10). It is a bait vector, encoding Gal4DB domain (aa1-147) under the control of *ADH1* promoter (see Note 1). A Gateway® cassette was introduced downstream and in frame with Gal4DB

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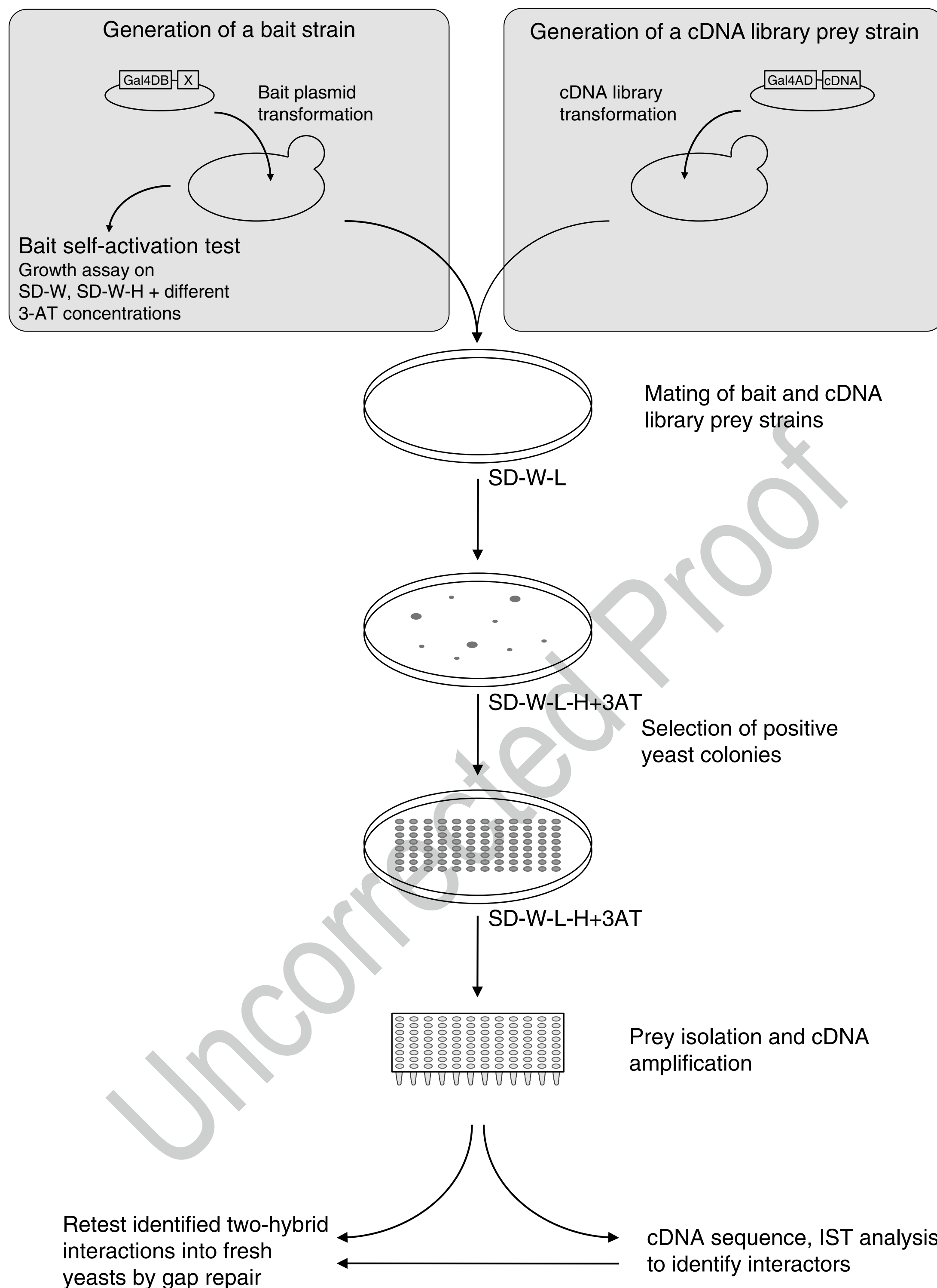


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Fig. 1. Flowchart of the two-hybrid screening using the mating strategy. See text for details.

to render it a destination vector (kindly provided by Dr. Yves Jacob, Institut Pasteur (11), Fig. 2a, see Notes 2 and 3). 74 75

2. pACT2-gw: pACT2 is commercialized by Clontech (12). It is a prey vector, encoding Gal4AD domain (aa 768–881) under the control of *ADH1* promoter (see Note 4). A gateway® cassette 76 77 78

[AU2]

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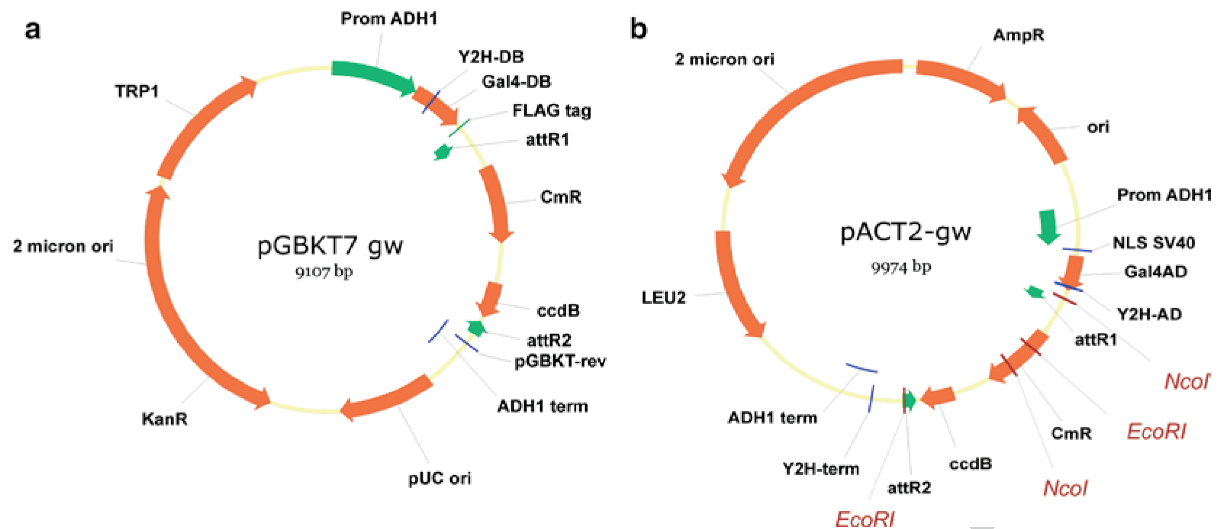


Fig. 2. Bait and prey plasmid maps used for yeast two hybrid. (**a**) pGBKT7-gw, bait vector: it encodes Gal4DB–bait protein of interest. See text for details. (**b**) pACT2-gw, prey vector: it encodes Gal4AD–prey protein. See text for details. Human cDNA libraries are cloned in pACT2-gw using the Invitrogen CloneMiner technique (see Note 5). In red are represented restriction sites used for gap repair.

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79 was introduced downstream and in frame with Gal4AD to
80 render it a destination vector (kindly provided by Dr. Yves
81 Jacob, Institut Pasteur (11), Fig. 2b). We introduced human
82 cDNA libraries into pACT2-gw (see Note 5).

### 2.2. Yeast Strains

83 The yeast strains used in our lab are from Clontech. AH109 is
84 *MATa* and Y187 *MATα* (13, 14). The bait vectors are transformed
85 in AH109 and the cDNA libraries (encoded in prey vector, pACT2-
86 gw) are transformed in Y187. By simplicity, AH109 is called the
87 yeast bait strain and Y187 the prey strain.

88 Genotypes (see Note 6):

89 AH109: *MATa, trp1-901, leu2-3 112, ura3-52, his3-200,*
90 *gal4∅, gal80∅, LYS2::UAS*$_{GAL1}$*- TATA*$_{GAL1}$*- HIS3, UAS*$_{GAL2}$*-*
91 *TATA*$_{GAL2}$*- ADE2, URA3::UAS*$_{MEL1}$*- TATA*$_{MEL1}$*- lacZ.*

92 Y187: MATα, trp1-901, leu2-3 112, ura3-52, his3-200, ade2-
93 101, met–, gal4∅, gal80∅, URA3::UAS$_{GAL1}$- TATA$_{GAL1}$- lacZ.

### 2.3. Yeast Media

94 1. Rich medium – YPAD: Allowing propagation of AH109 and
95 Y187, also used for the mating step during the yeast two-
96 hybrid screen. YPAD (1% yeast extract, 2% peptone, 2% dex-
97 trose, 0.004% adenine) is dissolved in deionized water and
98 autoclaved for 20 min at 120°C. Solid media are made by add-
99 ing 2% agar.

100 2. Selective medium – SD: Used for selection of yeast transfor-
101 mants and detection of DB-X and AD-Y interactions. SD (8 g/l
102 SD mix, 2% dextrose) is dissolved in deionized water and auto-
103 claved for 20 min at 120°C. This medium does not contain

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histidine, leucine, and tryptophan. Add 4 ml of stock solution of each required amino acids after autoclave; see ref. 5. 104 105

3. SD mix powder for 20 l: 34 g YNB (w/o amino acids and $(NH_4)_2SO_4$), 26 g amino acids mix, 100 g $(NH_4)_2SO_4$. Mix well to homogenize the powder. 106 107 108
4. Amino acids mix: Mix 6 g of each amino acid: alanine, arginine, asparagine, cysteine, glutamate, glutamine, glycine, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine. Add 6 g of adenine sulfate. Mix well to homogenize. 109 110 111 112 113
5. Stock solutions of histidine: 100 mM, leucine: 100 mM and tryptophan: 40 mM. Autoclave all, but tryptophan solution (heat labile): filter sterilize using 0.22-μm filter. Histidine and leucine solution is stored at RT. Tryptophan solution should be stored in the dark at 4°C. 114 115 116 117 118
6. 3-AT: 2 M stock solution. Filter sterilize using 0.22-μm filter. Store in the dark at 4°C. 119 120

#### *2.4. Transformation Solutions*

1. LiAc/TE: 100 mM LiAc, 10 mM Tris, pH 7.5, 1 mM EDTA from sterile stock solutions (1 M LiAc and 10× TE (100 mM Tris, pH 7.5, 10 mM EDTA); see Note 7). 121 122 123
2. LiAc/TE/PEG: 100 mM LiAc, 10 mM Tris, pH 7.5, 1 mM EDTA from sterile stock solutions (as above) and 40% PEG from a sterile 50% solution (see Note 8). 124 125 126
3. ssDNA: Deoxyribonucleic acid, single stranded from salmon testes (Sigma D-9156). This DNA is single-stranded denaturated at a concentration of 10 mg/ml. 127 128 129

#### *2.5. Primers*

Y2H-AD: 5′-CGATGATGAAGATACCCCACCAAA. 130
Y2H-Term: 5′-ACCAAACCTCTGGCGAAGAA. 131
Y2H-DB: 5′-GCACATCTGACAGAAGTGGA. 132
pGKBT7-rev: 5′-GAAATTCGCCCGGAATTAGC. 133

## 3. Methods 134

#### *3.1. Yeast Two-Hybrid Library Screening Using the Mating Strategy*

A bait strain is generated and self-activation is tested. Then, a cDNA library is transformed in a prey yeast strain, which allows interaction screening by mating with the bait strain. Selection of positive interactors leads to their identification by sequence analysis. These positive interactors are then retested into fresh yeasts by gap repair (schemed in Fig. 1). 135 136 137 138 139 140

A prerequisite step is to transform AH109 yeast strain with the bait vector (i.e., pGBKT7 bait of interest). The bait-encoding sequence, already cloned into a pDONR, is transferred into 141 142 143

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pGBKT7-gw by an LR reaction (Invitrogen, Gateway® recombination system). This highly efficient cloning method eliminates the need for gene-specific manipulations, such as restriction enzymes. The bait is tested for basal self-activation of *HIS3* reporter gene (see below) and toxicity in yeast. If the bait is not toxic for yeast and if it does not self-activate the expression of the reporter gene, it can be further used in a two-hybrid screen.

*3.1.1. Bait Plasmid Transformation*

1. Patch AH109 on a YPAD plate and incubate overnight at 30°C.
2. Inoculate 5 ml of YPAD with this fresh yeast culture and incubate overnight at 30°C under agitation (see Note 9).
3. Inoculate the yeast strain at $O.D._{600\ nm}$ 0.2 in 5 ml YPAD (5 ml of culture is needed for each plasmid to be transformed, inoculate a minimum of 20 ml). Incubate at 30°C under agitation until it reaches an O.D. of 0.6.
4. Transfer in a 50-ml conical tube and centrifuge for 5 min at 3,000 × *g* at RT. Discard the supernatant. Wash the pellet with an equal amount of sterile water. Discard the supernatant.
5. Resuspend yeast cells with 1 ml LiAc/TE solution (for each transformation to be done) and transfer in 1.5-ml tubes. Centrifuge for 30 s at 6,000 × *g* at RT and discard the supernatant.
6. Resuspend each pellet in 100 µl LiAc/TE.
7. Add to each pellet 2 µl of ssDNA and 1 µg of DNA bait vector to be transformed.
8. Mix by pipetting two times and incubate for 10 min at RT.
9. Add 230 µl LiAc/TE/PEG, and mix by inverting two times the tube. Incubate for 30 min at 30°C.
10. Add 43 µl DMSO and heat shock for 5 min at 42°C (mix by inversion from time to time).
11. Pellet the cells (30 s at 6,000 × *g*), discard the supernatant, and resuspend in 150 µl water.
12. Plate yeast cells on a 10-cm SD-W plate using sterile glass beads, and incubate at 30°C for 2–3 days (see Note 10).

*3.1.2. Bait Basal Self-Activation*

Bait strains should be examined for the bait self-activation prior to two-hybrid analysis. Self-activation is defined as a detectable DB-X-dependent reporter gene activation in the absence of any AD-Y prey protein, allowing the bait strain to grow on medium lacking histidine. Weak to intermediate self-activator DB-X can be used in two-hybrid experiments by titrating the basal yeast growth by using 3-AT, whereas strong self-activators must be discarded. Self-activation is examined on plates containing different concentrations of 3-AT (see Note 11).

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1. Prepare selective plates lacking tryptophan and histidine and containing 0, 2.5, 5, 10, 15, 20, and 30 mM 3-AT. 187 188
2. Inoculate each bait strain in 5 ml SD-W and incubate overnight at 30°C under agitation. 189 190
3. Dilute each bait strain to a 0.D. ≈ 0.2 in SD-W and drop 5 μl of each bait strain on SD-W, SD-W-H, SD-W-H + increasing 3-AT concentration. Let the drops dry, and then incubate the plates for 2–5 days at 30°C. Leave the SD-W plate at day 1 and check others regularly. 191 192 193 194 195
4. Score the 3-AT plates. For bait strains that confer growth on SD-W-H + 30 mM 3-AT, one can test higher concentrations of 3-AT (up to 100 mM). Above this concentration, bait should be discarded for yeast two-hybrid screen: it is not able to discriminate between self-activation and positive interactions with an AD-Y partner during a screen. For bait strains that show a lower level of self-activation, i.e., no growth at 30 mM but at lower 3-AT concentrations, two-hybrid readouts are performed albeit at a slightly higher concentration of 3-AT. For bait strains that do not show any self-activation, e.g., that do not grow even on SD-W-H, we used to screen them on medium containing 5 mM 3-AT to select only positive interactions. 196 197 198 199 200 201 202 203 204 205 206 207

*3.1.3. Transformation of the cDNA Library*

1. Inoculate 50 ml of YPAD with a patch of freshly grown Y187 prey strain, and incubate overnight at 30°C under agitation. 208 209
2. Inoculate the yeast strain at O.D. 0.2 in 200 ml YPAD. Incubate at 30°C under agitation until it reaches an O.D. of 0.6. 210 211
3. Transfer in 50-ml conical tubes and centrifuge for 5 min at 3,000 × *g* at RT. Discard the supernatant. Wash the pellet with an equal amount of sterile water. Discard the supernatant. Wash the yeast cells in 50 ml LiAc/TE solution. Discard the supernatant. 212 213 214 215 216
4. Resuspend the yeasts in 10 ml LiAc/TE and transfer in six 15-ml conical tubes. Centrifuge for 5 min at 3,000 × *g* at RT and discard the supernatant. 217 218 219
5. Resuspend each pellet in 100 μl LiAc/TE. 220
6. Add sequentially in each tube 160 μl ssDNA, 40 μg of pACT2-cDNA library, 200 μl DMSO, and 10 ml LiAc/TE/PEG. 221 222
7. Mix by inverting the tubes 5–6 times, and incubate for 30 min at 30°C. 223 224
8. Heat shock for 15 min at 42°C (in a water bath, mix by inversion every 5 min). 225 226
9. Pellet the yeasts (5 min at 3,000 g), discard the supernatant, and resuspend the cells in 10 ml sterile water (for each 15-ml tube). 227 228
10. Plate 300 μl on large SD-L plates (200 plates). Incubate at 30°C for 4 days (see Note 12). 229 230

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231 232 233 *3.1.4. Harvesting and Pooling the Transformed Library*

The transformed library is pooled in slurry, then aliquoted, and frozen. Each aliquot is a representative of the set of primary transformants and is used in subsequent mating.

234 235 1. Prepare 400 ml YPAD containing 20% glycerol, and autoclave it (see Note 13).

236 237 238 2. Pour 2 ml YPAD + glycerol per plate and scrap the yeast colonies using a spreader (see Notes 14 and 15). Collect the yeast slurry from each plate and pool in 50-ml conical tubes.

239 240 3. Resuspend the cells and aliquot by 1.8 ml in 2-ml sterile tubes, and freeze at −80°C.

241 242 243 244 245 4. Determine the transformed library titer by plating several dilutions ($10^6$, $10^7$, and $10^8$) of a thawed aliquot on SD-L plates. Count the CFU for each dilution and determine the number of yeast cells/milliliter of the transformed library. It should be around $10^9$ cells/ml.

246 247 248 *3.1.5. Two-Hybrid Screening by Mating Bait and Prey Yeast Strains*

249 250 251 252 253 As bait and prey strains are ready, the next step is to mate both strains. For this purpose, the bait strain is mixed with a thawed aliquot of the prey strain. The cells are plated on rich media for 4–5 h to induce mating of cells of opposed mating type and forming diploids (see Note 16). The diploids cells, along with haploids (unmated yeasts), are collected and plated on interaction selection medium (SD-W-L-H + 3 AT). On this medium, only diploids, where an interaction between bait and prey takes place, are able to grow.

254 255 256 1. Inoculate 50 ml of SD-W with a patch of freshly grown bait strain, and incubate overnight at 30°C under agitation (see Note 17).

257 258 259 2. Thaw an aliquot of the prey strain, and take $60 \times 10^7$ cells that are incubated in 20 ml YPAD for 10 min at 30°C (in a 50-ml conical tube).

260 261 262 263 3. Measure the O.D. of the bait culture (a $O.D._{600\ nm}$ of 1 corresponds to $10^7$ cells/ml), and collect the equivalent of $72 \times 10^7$ cells. Add this volume to the prey culture, and mix by inverting.

264 265 266 4. Centrifuge at 3,000 × *g* for 5 min at RT. Discard the supernatant and resuspend the yeasts in 1.1 ml YPAD (the yeast pellet corresponds approximatively to 400 µl).

267 268 5. Plate on three large YPAD plates (500 µl per plate), and incubate at 30°C for 4–5 h.

269 270 271 272 273 6. Scrap the yeasts using a spreader with 2 ml SD-W-L-H per plate. Collect the slurry and plate on ten selective medium (SD-W-L-H + appropriate concentration of 3-AT, according to the made basal self-activation test), thus 500 µl per plate (see Note 18).

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7. Incubate for 6–10 days at 30°C. From day 6, inspect the plates daily and mark the location of colonies using a given color and each day mark the arising colonies with different colors. At day 10, collect the colonies (following the order of appearance, generally the first appeared are the biggest) using toothpicks and patch them in a 96-well format on a selective medium plate. Incubate for 48 h at 30°C and replica plate twice on selective medium (see Note 19).

*3.1.6. Prey Isolation and cDNA Identification*

AD prey-encoding sequences can be retrieved by PCR amplification directly from yeast colonies. For this purpose, we use primers that hybridize in Gal4-AD sequence and in *ADH1* Term (Y2H-AD and Y2H-Term, respectively; see Subheading 2). We perform yeast colony PCR directly on yeast colonies arrayed on a plate in a 96-well format.

1. Replica patch the yeasts, and incubate the plate 1 day at 30°C (see Note 20). [AU3]
2. Prepare on ice a PCR mix for a plate (≈ 100 PCR): 1,000 μl GoTaq (Promega) 5× buffer, 300 μl $MgCl_2$ (supplied with GoTaq), 20 μl dNTP's (solution stock 20 mM), 15 μl Y2H-AD primer 100 μM, 15 μl Y2H-Term primer 100 μM, 3,130 μl distilled water, and 20 μl GoTaq (5 U/μl).
3. Transfer 45-μl aliquots of the PCR mix into each well of a 96-well PCR plate.
4. Using a sterilized pin-tool replicator, pick and resuspend 96 yeasts into 50 μl of water (dispensed in a 96-well PCR plate) and incubate at 100°C for 5 min.
5. Add 5 μl of boiled yeasts to the PCR mix and run the PCR with the following program: (a) 94°C, 5 min; (b) 94°C, 30 s; (c) 54°C, 45 s; (d) 72°C, 5 min (see Note 21); (e) 72°C, 3 min by repeating steps b–d 34 more times.
6. Load 5 μl on a 1% agarose gel (see Note 22). If there is an amplification band, we proceed for its sequencing.

***3.2. Yeast Two-Hybrid Array Screening***

In a two-hybrid array experiment or also called "two-hybrid matrix," pairs of interactions are examined individually by mating bait and prey yeast strains. After generation of diploids, yeasts representing single potential interaction pairs are dropped onto selective media and examined for two-hybrid phenotype. Such matrix experiments are limited by the number of pairwise combinations that can be reasonably tested (from a few hundreds when working manually to a few thousands when having a multichannel pipetting robotic platform). Since the baits' and preys' identity is known throughout the experiment, no sequencing step is needed to identify them, making this method rapid and cheap. But it requires that all bait and prey protein-encoding sequences are already cloned.

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318 Another advantage of the array screen is its parallel character, 319 allowing to compare baits' as well as preys' global responses. If 320 different preys have different affinities to a given bait, it will be 321 indicated by different colony growth on selective plates.

322 *3.2.1. Retesting Identified* 323 *Two-Hybrid Interactions*

An array strategy can be used for the confirmation of interactions 324 identified during a screen. In this case, preys are retested against 325 the bait that trapped them during the two-hybrid screen. This array 326 protocol is specific because prey plasmids are reconstituted by "gap 327 repair" into a yeast strain already containing the bait plasmid, and 328 the two-hybrid interaction phenotype scoring is conducted into a 329 haploid strain. The gap repair is based on the homologous recom- 330 bination that occurs in vivo in yeast and allows the reconstitution 331 of the prey plasmid due to the identity between the PCR fragment 332 and the pACT2-gw at the priming sites (15). The yeast strain is 333 transformed by linearized prey plasmid and AD prey sequences obtained by PCR.

334 All interactors found (after identification of interaction 335 sequence tag (IST) by BLAST, see below) are retested against the 336 bait protein as well as the empty bait vector using this array screen- 337 ing method. Recombinant plasmids are selected onto SD-W-L 338 plates.

339 DNA preparation

340 1. Linearize pACT2-gw using *NcoI* and *EcoRI* (plasmid purifica- 341 tion is not necessary); 2.5 μg of vector are necessary for 96 gap 342 repairs.
343 2. Per reaction, transform ≈ 25 ng linearized pACT2-gw and 344 3–5 μl PCR product in a 96-well plate format (see Notes 23 345 and 24).

346 Transformation

347 1. Inoculate 5 ml SD-W with fresh bait strain and incubate over- 348 night at 30°C under agitation.
349 2. Inoculate the yeast strain at O.D. 0.2 in 200 ml SD-W. Incubate 350 at 30°C under agitation until it reaches an O.D. of 0.6.
351 3. Transfer in 50-ml conical tubes and centrifuge for 5 min at 352 3,000 × *g* at RT. Discard the supernatant. Wash the pellet with 353 an equal amount of sterile water. Discard the supernatant.
354 4. Wash the yeast cells in 50 ml LiAc/TE solution and pool in a 355 single 50-ml conical tube. Discard the supernatant.
356 5. Wash the yeasts in 10 ml LiAc/TE solution. Discard the 357 supernatant.
358 6. Resuspend the pellet in 2.5 ml LiAc/TE. Add 300 μl ssDNA. 359 Transfer 30 μl of the suspension in each well of a 96-well PCR 360 plate containing DNAs (linearized pACT2 and PCR product).

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7. Add 100 μl of LiAc/TE/PEG solution in each well and resuspend carefully. 361 362
8. Incubate for 30 min at 30°C. 363
9. Heat shock for 15 min at 42°C. 364
10. Centrifuge the 96-well plate for 5 min at 1,500 × *g* at RT. Remove the supernatant using a multichannel pipette. Add 110 μl of water to each well and directly remove 100 μl (see Note 25). 365 366 367 368
11. Resuspend the yeasts in the remaining volume and spot the cells on an SD-W-L plate. Incubate overnight at 30°C. 369 370
12. Replicate softly the plate, and incubate for 2 days at 30°C (see Note 26). 371 372

    Compare the number of transformants with and without PCR products (linearized pACT2-gw without PCR product). The gap repair is considered successful when at least a minimum fivefold induction is observed. 373 374 375 376

Two-hybrid interaction phenotype scoring 377

1. From the master plate containing all the yeast transformants, inoculate a 96-well deep-well plate (each well containing 1.2 ml SD-W-L) using a pin-tool replicator. Incubate overnight at 30°C under agitation. 378 379 380 381
2. Dilute five times the yeast cultures and drop 5 μl using a multichannel pipette on large plates in a 96-well format: SD-W-L, SD-W-L-H, SD-W-L-H containing increasing amounts of 3-AT (classically: 2.5, 5, 10 mM 3-AT) (see Note 27). 382 383 384 385
3. Let the drops dry, and then incubate the plates for 2–5 days at 30°C. Leave the SD-W-L plate at day 1 and check others regularly. 386 387 388
4. Score the 3-AT plates and compare to controls (see Note 28). 389

#### 3.2.2. Array Screening

Arrays can also be used as a general tool to screen a defined subset of proteins as a family of proteins or the members of a given cellular pathway. A prerequisite step is to clone the protein-encoding sequences to be tested. In our lab, this procedure has been used to screen whole-viral proteomes against a specific cellular pathway (for example, autophagy or innate immunity pathway that are both functionally related to viruses). 390 391 392 393 394 395 396

Generation of a prey strains collection. 397

Clone a set of preys into the pACT2-gw from entry clones using the Gateway® LR recombination and transform the Y187 yeast strain by these prey plasmids. In our particular case, preys are cellular proteins and baits viral proteins. Following the number of transformation, one can use the simple transformation protocol, as described in Subheading 3.1. (Bait plasmid transformation) or the 96-well transformation protocol described in Subheading 3.2. 398 399 400 401 402 403 404

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405 (Preparing a prey array using gap repair.) In this case, yeasts are
406 transformed by prey plasmids without the need of a gap repair.

407 Dispose the prey yeasts collection in a 96-array format on SD-L
408 plates. Include per plate at least a yeast strain, including empty prey
409 vector.

410 Generation of a bait strains collection.

411 By the same way, clone a set of baits into the pGBKT7-gw
412 from entry clones using the Gateway® LR recombination and
413 transform the AH109 yeast strain by these bait plasmids. In our
414 case, the baits are viral proteins and many of them are already
415 cloned in pDONR vectors (see viral ORFeome database, http://
416 www.viralorfeome.com (16)).

417 Dispose the bait yeasts collection in a 96-array format on SD-W
418 plates. Include at least a yeast strain, including empty bait vector.

419 Screening the prey array with different baits.

420 When both prey and bait arrays are consolidated, the goal is to
421 mate each bait against each prey strain.

422 Mating

423 1. Incubate the yeast prey array overnight in SD-L using a 96-well
424 deep-well plate (each well containing 1.2 ml SD-L) inoculated
425 by a pin-tool replicator under agitation at 30°C.
426 2. Grow each yeast bait colony overnight in 2 ml SD-W under
427 agitation at 30°C (see Note 29).
428 3. Dilute five times the bait culture in a sterile reservoir and fill
429 each well of a 96-well microtiter plate with 25 μl of the yeast
430 bait suspension.
431 4. Transfer 5 μl of the prey array to the bait microtiter, mix the
432 yeast suspension, and drop 5 μl of each mix on a large YPAD
433 plate. Let the drops dry and then incubate overnight at 30°C.
434 The bait and prey strains conjugate on this medium, forming
435 diploids.
436 5. Using a velvet, replica plate the mated yeasts on a large SD-W-L
437 plate. Incubate for 2 days at 30°C. This step selects diploids
438 and ensures that all colonies have mated properly.

439 Screening the prey array

440 1. Inoculate the diploid colonies in a 96-well deep-well plate
441 filled of SD-W-L (1.2 ml per well) using a pin-tool replicator,
442 and incubate overnight at 30°C under agitation.
443 2. Dilute five times the cultures (in a 96-well microtiter plate) and
444 drop 5 μl on selective plates: SD-W-L, SD-W-L-H, SD-W-
445 L-H+increasing amounts of 3-AT (2.5, 5, and 10 mM 3-AT).
446 3. Let the drops dry, and then incubate the plates for 2–6 days at
447 30°C. Leave the SD-W-L plate at day 1 and check others
448 regularly.

449 Score the 3-AT plate and compare to the controls.

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**3.3. IST Analysis**

The two-hybrid screens generate a large amount of ISTs obtained by sequencing all AD cDNA isolated from yeast colonies (17). To establish a standardized system to analyze these ISTs, we developed pISTil, a bioinformatics pipeline combined to a user-friendly Web interface (see Note 30) (18).

The pISTil system is highly flexible and allows (a) systematic and fast assignation of ISTs to a unique protein accession number; (b) annotation of "in-frame" or not-in-frame ISTs; (c) manual checking and visualization of annotated ISTs through a user-friendly Web interface; and (d) export of ppi in multiple formats, such as MIMIx standard format (19).

IST chromatogram files, in Applied Biosystems INC (ABI) or Standard Chromatogram Format (SCF) formats, are filtered by Phred-pregap4 software (20) to extract nucleic sequences and their associated quality values. The resulting nucleic sequence of each IST is then translated into three frames and aligned against a protein sequence database (as defined in the configuration file by users, typically Ensembl or NCBI databases) by using BLASTX alignment software (21). Only alignment information for the best hit is subsequently retained. In addition, identification of Gal4AD on ISTs allows the true delineation of in-frame and not-in-frame ISTs. Thus, this filter can discard putative not-in-frame ISTs that may lead to false-positive interaction. All information generated at each step of the IST pipeline is stored into the pISTil database, such as sequence quality, percent identity, e-value, alignment position, frame, and protein sequence database source. Other data supplied by users, such as bait protein used for the screen (GenBank accession number) or description of cDNA libraries (host organism, tissue origin, cell type), can be integrated to the pISTil database. A demonstration of the Web site capabilities is available at http://www.pbildb1.univ-lyon1.fr/pistil.

# 4. Notes

1. The *ADH1* promoter of pGBKT7 is a truncated form (700 bp), leading to relatively high expression level of Gal4DB–bait fusion protein (22). This plasmid harbors a *TRP1* yeast marker, allowing its selection on medium lacking tryptophan, and the *KanR* marker, for its selection in bacteria. It possesses the 2 μ yeast replication origin (multicopy plasmid).
2. We are using the Gateway® recombinational cloning system (Invitrogen, (23, 24)) in order to clone viral ORFs and to construct viral ORFeomes (see our LIMS, viralORFeome at http://www.viralorfeome.com (16)). Using these viral ORFs entry clones, we can recombine them by an LR reaction into pGBKT7-gw.

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494 3. We swapped the original c-myc epitope for a FLAG epitope
495 between Gal4DB and the Gateway® cassette.

496 4. The *ADH1* promoter of pACT2 is a truncated form (410 bp),
497 leading to medium expression level of Gal4AD–prey fusion
498 protein (25). It harbors the *LEU2* yeast marker, allowing trans-
499 formed yeasts to grow on medium lacking leucin, and the
500 *AmpR* marker, for its selection in bacteria. It also possesses the
501 2 μ yeast replication origin (multicopy plasmid).

502 5. cDNA are obtained from mRNA extracted from tissues or cells
503 by using a polyA primer fused to *attB2* sequence and a linker
504 containing *attB1* sequence. Then, they are cloned into a
505 pDONR vector by a BP recombination (Invitrogen, Gateway®
506 CloneMiner cDNA library construction kit, ref: 18249–029).
507 Next, the whole cDNA library is swapped into the pACT2-gw
508 destination vector using an LR recombination. Using the
509 Gateway® recombination system to clone cDNA avoids the use
510 of restriction enzymes, and thus avoids cutting cDNA into
511 fragments.

512 6. The genes encoding Gal4 and Gal80 (its inhibitor) are deleted
513 in both yeast strains. AH109 possesses three reporter genes:
514 *HIS3*, *ADE2*, and *LacZ* under control of different promoters
515 (UAS*GAL TATAGAL*), whereas Y187 possesses one reporter
516 gene, *LacZ*. We decided to only analyze the transactivation
517 phenotype of the *HIS3* reporter gene, which encodes imida-
518 zole glycerolphosphate dehydratase or, more simply, His3
519 enzyme. A competitive inhibitor has been described: 3-amin-
520 ino 1,2,4 triazole (3-AT) that can be used to inhibit low levels
521 of *HIS3* expression, and thus to suppress background growth
522 (26, 27). It can also be used when baits show basal self-activa-
523 tion and titrate His3 that is produced by this basal activation.

524 7. For better transformation efficiency (for instance, for high-
525 throughput transformation as a library transformation), this
526 solution should be freshly prepared.

527 8. For 100 ml solution, weigh 50 g of polyethylene glycol, MW
528 3350 (Sigma, P-3640), in a 150-ml glass beaker and add 35 ml
529 of distilled water. Stir with a magnetic bar until dissolved (can be
530 heated to accelerate dissolution). Transfer the liquid to a 100-ml
531 graduated cylinder. Rinse the beaker with a small amount of
532 distilled water, add this to the graduated cylinder containing the
533 PEG solution, and bring the volume to exactly 100 ml. Mix well
534 by inversion. Autoclave for 20 min at 120°C.

535 9. Yeast culture and selection are not subjected to antibiotics
536 resistance, as for bacteria. All the experiments should be done
537 under sterile conditions.

538 10. Glass beads are used to plate yeast cells, as well as bacteria. Glass
539 beads, 5-mm diameter, 1 kg (Fisher Scientific, ref: W0130M).

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11. It is useful to have controls for this self-activation test. As negative control, use the bait strain transformed by pGBKT7 empty. As positive control, one can use a bait strain containing a couple of proteins that are known to interact (DB-X and AD-Y). 540 541 542 543
12. Plate $10^4$, $10^5$, and $10^6$ dilutions on SD-L plates to obtain the transformation efficiency. A good transformation efficiency should yield $1–5 \times 10^4$ yeast colonies/μg DNA, thus $1–5 \times 10^4$ colonies per plate. 544 545 546 547
13. We add 10 μg/ml tetracyclin in YPAD in order to avoid microbial contamination of the library. 548 549
14. Spreaders are easily made by heating and bending a 20-cm length of 3-mm-diameter glass rod. Using a Bunsen burner, both ends are flame polished, and then the rod is heated and bent approximately 3 cm from one end to form an angle. Alternatively, one can also make spreaders from Pasteur pipettes. The spreader is sterilized by briefly dipping it in alcohol and passing it through the flame of a Bunsen burner. 550 551 552 553 554 555 556
15. More material is collected if the scrapping is repeated twice but, in that case, pour $2 \times 1$ ml of YPAD + 20% glycerol per plate. 557 558
16. This incubation period is an optimized time for mating and for limiting further growth of diploid cells (28). 559 560
17. We used to preinoculate 5 ml SD-W with a patch of bait strain, incubate it overday, and then dilute this preculture in 50 ml for an overnight culture. 561 562 563
18. At this step, it is important to evaluate the number of diploids that were formed and thus screened. For this purpose, plate different dilutions of the diploids mix ($10^4$, $10^5$, and $10^6$) on SD-W-L plates (unmated yeasts do not grow on this medium). Count the colonies that grow after 2–3 days at 30°C and determine the titer of the mated cells. 564 565 566 567 568 569
19. It is of note that yeast cells can maintain multiple distinct plasmids. When transformed by a cDNA library, the prey strain could be transformed by more than one prey plasmid. Then, when a positive colony is selected during the two-hybrid screen, it could in fact possess more than one prey plasmid, where one giving the positive interaction and the others are simply contaminating. The double round of growth on selection medium maintains positive selection of DB domain bait/AD prey, whereas eventual contaminating prey plasmids are not selected and thus, can be lost (29). 570 571 572 573 574 575 576 577 578 579

[AU4]

20. Fresh cells give rise to the highest level of success in yeast colony PCR. 580 581
21. The elongation time is critical to obtain amplification bands. 582
22. We use precast E-gel 48.1% agarose (Invitrogen), highly convenient for high-throughput DNA manipulations (compatible 583 584

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585 with the utilization of multichannel pipettes). E-gels 96 are
586 also convenient, but the migration distance is less than for
587 E-gel 48.

588 23. Controls should include "no DNA," circular pACT2-gw, and
589 linearized pACT2-gw without PCR product.

590 24. The 96-well transformation protocol is very useful when deal-
591 ing with hundreds to thousands of yeast transformations. It
592 can easily be achieved using a multichannel pipetting robotic
593 platform, on behalf of the centrifugation step. In our lab, we [AU5]
594 use a Tecan® Freedom Evo platform.

595 25. This step allows elimination of most of the transformation mix
596 containing PEG.

597 26. This replica-cleaning step is necessary to dilute out the non-
598 transformed cells present in each spot.

599 27. When dealing with hundreds of tests, we used our multichan-
600 nel pipetting robotic platform to drop yeast cultures onto
601 selective plates, with special labware able to support three large
602 petri dishes.

603 28. Controls should include prey proteins against the empty bait
604 vector and bait proteins against the empty prey vector (circular
605 pACT2-gw, from the "gap repair" transformation). Some prey
606 proteins may show self-activation: transformed yeasts grow on
607 selective reporter media, even those containing empty bait vec-
608 tor. In this case, one should discard these interactions.

609 29. If using dozens of bait strains, incubate them in 96-well deep-
610 well plates.

611 30. pISTil is publicly available for download at http://www.
612 sourceforge.net/projects/pISTil.

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