Chapter 22
Mammalian Two-Hybrid Assay for Detecting Protein-Protein Interactions in Vivo

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Abstract The mammalian two-hybrid system is a very powerful tool to investigate protein-protein interactions in terms of functional domains and identify potential binding ligands and partners of a protein. Compared with the yeast two-hybrid system, the mammalian two-hybrid system provides the milieu for the bona fide posttranslational modifications and localizations of most eukaryotic proteins and, therefore, should be a better choice to study proteins of mammalian origin. This chapter depicts the detailed experimental procedures adapted by various laboratories. Researchers with experience in molecular biology could modify the procedures according to their own needs, that is, the choice of restriction sites in the cloning process. The reference list could be of use to researchers who wish to understand more of the system and explore its wider applications.

Keywords mammalian two hybrid, secreted alkaline phosphatase (SEAP), protein-protein interaction, PCR, cloning

1 Introduction

Protein-protein interaction is one of the most important mechanisms utilized by mammalian cells to regulate a variety of cellular and molecular activities, such as transcription, translation, signal transduction and enzyme reaction [1, 2]. Proteins of microorganisms also interfere with host cell proliferation and functions by interacting with intracellular proteins [3]. There are various methods to characterize protein-protein interactions including coimmunoprecipitation, mass spectrometry [4], chemical cross-linking [5], and the two-hybrid system [6, 7], which has been used widely in recent years because of its high-throughput screening capability and powerful ability of isolating unknown binding partners of a protein. The first two-hybrid system was yeast based and was introduced by Fields and Song in 1989 [8]. It is composed of two GAL-based transcription activation domains of S*accharomyces cerevisiae*, one is the DNA binding

domain (DBD) domain, while the other is the transcription activation domain (AD). To perform the two-hybrid analysis, an X gene is cloned into a two-hybrid vector fusing to DBD, with a Y gene cloned into another two-hybrid vector fused to the AD. If the X protein interacts with Y protein, the DBD and AD domains come into close proximity and form an active transcription activation complex (Fig. 22.1). The complex binds to and activates its cognate promoter sequence, which is linked to a reporter gene(s), such as *Lac* Z, or an essential nutrient [8]. These gene products generate distinctive phenotypes that can be easily recognized by a change of color of yeast colonies or a gained ability to grow in nutrient-deficient media.

A lot of modifications have been made to improve the sensitivity and specificity of the two-hybrid system: the addition of more reporter genes and genetic alterations of the DBD and AD domains, such as the *LexA* system [9, 10]. However, the yeast-based two-hybrid assay has its limitations. One major issue is that posttranslational modifications such as glycosylation in yeast can be quite different from that in mammalian cells. In addition, the intracellular milieu that supports the protein-protein interaction in yeast is different from that of mammalian cells [11, 12]. Therefore, when considering the study of interactions of proteins derived from mammalian cells, the yeast system may not be able to provide the optimal intracellular environment.

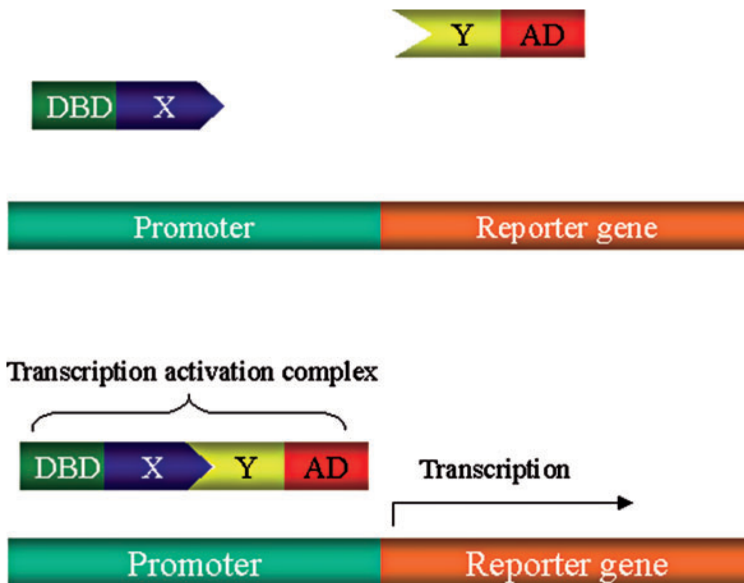

Fig. 22.1 Principles of the-two hybrid system: DBD and AD are DNA binding and transcriptional activation domains, X and Y are the bait and prey genes

To alleviate the pitfalls of the yeast two-hybrid system, the mammalian two-hybrid system was introduced.

The design of the mammalian two-hybrid system is similar to that of the yeast two-hybrid system, which is composed of two vectors carrying DNA binding domain and DNA activation domain, respectively, in two mammalian expression vectors. Two genes of interest (X and Y) are cloned into the vectors and fused with DBD and DA, correspondingly. The interaction of protein X to protein Y brings the DBD and AD to a close proximity and forms the transcriptional activation complex, which binds to the promoter of a reporter gene encoded in a reporter plasmid and starts the gene transcription. The reporter genes can be secreted alkaline phosphatase (SEAP), chloramphenicol acetyl transferaseor (CAT), or luciferase [13–15]. GAL4 DNA binding domain and herpes virus DNA transcription activator VP16 are commonly used in mammalian two-hybrid systems [16]. This chapter depicts the general procedure adopted by various laboratories.

2 Materials

2.1 PCR and Cloning of Genes of Interest into Mammalian Two-Hybrid Vectors

1. High fidelity DNA polymerase (i.e., iProof high fidelity DNA polymerase, BioRad).
2. dNTPs at 25 m*M* concentration (New England Biolabs).
3. PCR primers (cloning sites are added to the 5′ ends of the primers):

   5′ GTACGAATTCATGTCTGATAATGGACCC 3′.
   5′ GTACGGATCCTTATGCCTGAGTTGAATCAG 3′.
4. PCR template (cDNA reversely transcribed from SARS-CoV genome).
5. Thermal cycler, such as Master Cycler EP (Eppenforf).
6. Thin-wall PCR tubes or 96-well plates.
7. Ampicillin (Sigma-Aldrich).
8. PCR Kleen Spin Columns (BioRad).
9. Restriction enzymes: *Eco* RI and *Bam* HI (New England Biolabs).
10. Mammalian two-hybrid system with pM and pVP16 vectors (Clontech).
11. T4 DNA ligase (New England Biolabs).
12. Taq polymerase for colony screening assay (i.e., HotStar PCR Master Mix Kit, Qiagen).
13. Water bath at 37 °C.
14. Chemical or electro competent cells (Invitrogen).
15. Water bath at 42 °C.
16. Qiaprep Spin Miniprep Kit (Qiagen).

17. 10X TBE buffer: 108 g of Tris-base, 55 g of boric acid, 9.3 g of Na-EDTA, and add ddH_2O to 1 L.
18. Agarose (Invitrogen).
19. LB (Luria Broth) per liter: bacto-tryptone 10 g, bacto-yeast extract 5 g, NaCl 10 g, pH 7.0; ddH_2O to make 1 L.
20. Preparation of LB agarose plates: add 15 g of agarose to 1 L of LB media and autoclave for 20 min, cool to 45 °C, and dispense approximately 25 mL into per 100-mm plate.
21. SOC medium per liter: add 2% bacto-tryptone, 0.5% yeast extract, 10 m*M* NaCl, 2.5 m*M* KCl, 10 m*M* $MgCl_2$, 10 m*M* $MgSO_4$, 20 m*M* glucose, pH 7.0, and bring the final volume to 1 L with ddH_2O.
22. Shaking incubator (Thermo Electron Corporation).
23. 20% glycerol solution (v/v).

2.2 DNA Purification for Mammalian Cell Transfection

1. Endofree Plasmid Maxi Kit (QIAGEN).
2. 70% ethanol v/v (for the QIAGEN Maxi Plasmid Kit).
3. 99% isopropanol (for the QIAGEN Maxi Pladmid Kit).
4. 50-mL tissue culture conical tubes.
5. Ultracentrifuge tubes.
6. TE buffer: 10 m*M* Tris-HCl; 1 m*M* EDTA, pH 8.0.

2.3 Tissue Culture

1. Growth medium: Dulbecco's Modified Eagle Medium (Invitrogen).
2. Fetal calf serum (Invitrogen).
3. 100X penicillin/streptomycin (Invitrogen).
4. Vero E6 cells (ATCC).
5. 0.5% trypsin-EDTA (Invitrogen).
6. Phosphate buffered saline: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, add double distilled H_2O to 1L. pH 7.4.
7. 24-well tissue culture plates (Nalgene Nunc).
8. Water bath set at 37 °C.
9. Inverted microscope (Zeiss).
10. Hemacytometer (VWR).
11. Tissue culture incubator (Thermo Electron Corporation).

2.4 Transfection and Mammalian Two-Hybrid Analysis

1. Transfection reagent: Effectene (QIAGEN).
2. pM and pVP-based constructs (Clontech, Palo Alto, CA).

3. pG5SEAP reporter vector (included in the mammalian two-hybrid kit).
4. SEAP (secreted alkaline phosphatase) Chemiluminescent Assay Kit (Clontech).
5. 96-well chemiluminescence reader (Tecan) or single-well chemiluminescence reader (Turner Designs).
6. 96-well PCR plates.

3 Methods

3.1 PCR Amplification of Genes of Interest and Preparation of Mammalian Two- Hybrid Vectors

3.1.1 PCR Reaction

1. Add 10 µL of iProof HF buffer, 1 µL of dNTP, two primers at 500 n*M* each, 0.5 U of DNA polymerase, a PCR template for the genes of interest, and ddH_2O to 50 µL.
2. PCR conditions: after 30 s of the initial denaturation at 98 °C, run 25–35 cycles of the following steps—denaturation at 98 °C for 10 s, annealing at 45–72 °C for 30 s, and extension at 72 °C (15–20 s per kb).
3. Clean PCR products with PCR Kleen columns, according to the manufacturer's protocol and elute DNA into a TE buffer.
4. Subject the PCR product to 1.2% agarose gel for electrophoresis, then isolate the band corresponding to the size of the desired gene. Purify the DNA from the gel piece using the Qiagen DNA Gel Purification Kit (optional step).

3.1.2 Vector Preparation

1. Inoculate two single colonies of *E. coli* top 10 strain containing pM and pVP16 vectors into 5 mL of LB respectively, add ampicillin to 100 µg/mL and grow in a shaking incubator at 37 °C and shake at 250 rpm overnight.
2. Harvest the cells by centrifugation at 10,000 *g* for 1 min.
3. Isolate the constructs using the Qiaprep Spin Miniprep Kit (Qiagen).

3.2 Cloning PCR Genes of Interest into of pM, pVP16 Vectors

3.2.1 Restriction Digestion

In designing the primers, *Eco*RI and *Bam*H I sites were added to the 5′ ends of the two PCR primers not present in the genes to be cloned. Cloning at *Eco*RI site fuses the gene of interest in frame with both DBD and AD domains.

1. Digest pM and pVP vectors (150 ng each) with 3 U of *Eco*RI and *Bam*HI, respectively, in 2 µL *Eco*RI buffer supplied by the manufacturer in 20 µL reaction mixture. Incubate at 37 °C for 1 h.
2. Digest PCR products flanked with *Eco*RI and *Bam*HI sites on the 5′ and 3′ ends respectively, using conditions just described.
3. Clean the digested vectors and PCR products with PCR Kleen columns.

3.2.2 Ligation

1. Add 100 ng of digested pM or pVP16 vector, 500 ng of digested PCR product, 2 µL of T4 Ligase buffer, 1 U of T4 ligase, and add ddH$_2$O to 20 µL.
2. Incubate at 16 °C for overnight.

3.2.3 Transformation

1. Thaw and aliquot 50 mL top 10 chemical competent cells into an Eppendorf tube (1×10^8 cells/mL), and chill on ice for 30 min.
2. Add 10–50 ng of the previously ligated products (see Sect. 3.2.2) into the previous *E coli* cells.
3. Immediately transfer the Eppendorf tube to a 42 °C water bath and incubate for 45 s.
4. Add 1 mL of SOC buffer and incubate in a shaking incubator at 37 °C for 30 min. Transfer the transformed *E coli* cells to a LB-ampicillin plate and incubate at 37 °C for 1 h. Turn the plate upside down and incubate at the same condition overnight.

3.2.4 Screening for Clones That Contain the Gene of Interest

There are many ways to screen for colonies that contain the gene of interest. In this protocol, we introduce a PCR-based screening, which is fast, simple, specific, and economical.

1. Perform PCR reaction with Taq polymerase. For each screening, prepare a master PCR-mix sufficient for 5–10 PCR reactions (48 µL/per PCR). The master mix is prepared by adding appropriate PCR buffer, dNTPs, polymerase, primers used for the previous PCR amplifications of the genes of interest or other primers that can amplify the gene, and ddH$_2$O.
2. Aliquot this PCR mix into 5–-10 PCR tubes.
3. Pick a single colony and dip into the PCR mix, then dip the residual of the colony onto a LB-ampicillin master plate with numbers labeled on the bottom. The master plate is incubated at 37 °C overnight.

4. Perform PCR cycling using following program: 1 cycle of 95 °C for 10 min; 40–45 cycles of 95 °C for 1 min, 45–72 °C for 30 s, and 72 °C at 1 kb/min.
5. Subject PCR products to electrophoresis with 1.2% TBE agarose gel prepared in 1X TBE buffer. Samples with a band corresponding to the desired size are the potential positive clones.
6. Inoculate the positive colonies from the master LB-ampicillin plate into 5 mL of culture, incubate at 37 °C overnight, then perform DNA Maxiprep as described next (see Sect. 3.3).
7. Sequence the selected plasmids to verify the correct DNA sequence.
8. The positive *E coli* clone should be stored in LB-ampicillin broth containing 20% glycerol at −80 °C.

3.3 Large-Scale, High-Purity DNA Purification

1. Inoculate from the master plates just described containing pM and pVP16 constructs selected from the previous screenings into two culture tubes with 5 mL LB-ampicillin and incubate overnight at 37 °C.
2. Transfer these two 5 ml culture into two culture flasks containing 100 mL of LB plus ampicillin (100 μg/mL) and incubate until A_{600}=1.0–1.1 (approximately 1 × 10^9cell/mL).
3. Harvest the cells by centrifugation at 4 °C for 10 min. at 5,000 *g*.
4. Isolate the pM and pVP16-based constructs using a Qiagen Endofree Plasmid Maxi Kit, following the manufacturer's protocol.

3.4 Tissue Culture and Transfection

3.4.1 Culturing and Preparing Vero E6 Cells

1. Supplement DMEM medium with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin.
2. Maintain the Vero E6 cells (African green monkey kidney cells) in culture flasks inside a humidified incubator at 37 °C supplied with 5% CO_2.
3. To set up cell culture plates: Wash the cells with PBS, then treat the cells with 0.5% trypsin-EDTA before incubation at 37 °C for 5–10 min. Add 5 mL of the supplemented DMEM to the culture flask, transfer the supernatant into a screw-top conical tube, and spin at 1,500 *g* for 5 min. Aspirate out the supernatant and add fresh medium to make the final concentration at 150,000 cells/mL.
4. Aliquot 0.5 mL of these cells into each well of a 24-well plate (75,000 cells/well), then incubate at 37 °C overnight in a humidified chamber supplemented with 5% CO_2.

3.4.2 Transfection

1. Sample arrangement: the following six samples are recommended for each mammalian two-hybrid analysis (see Table 22.1). All samples should be three to four repeat sets.
2. For each transfection: add 150 µL EB buffer, 8 µL enhancer, 1 µg DNA (400 ng pM-X, 400 ng pVP16-Y, and 200 ng pG5SEAP), followed by incubation for 5 min at room temperature. Afterward, add 15 µL effectene (Qiagen) and incubate at room temperature for 10 min.
3. Add 40 µL of this transfection mix dropwise into each well of a 24-well plate, three or four repeats of each sample is needed to generate standard deviation when SEAP assay is performed later.
4. Gently shake the culture plate to mix, then incubate in a tissue culture chamber supplemented with 5% CO_2 at 37 °C for 48 h.

3.5 SEAP Analysis (Chemiluminescence)

3.5.1 SEAP Reaction

1. Aliquot 100 µL of the precedinjg cell culture supernatants from each well, transfer to an Eppdorf tube, and spin for 1 min at 10,000 *g*.
2. Add 15 µL of 1X dilution buffer from the GreatEscape SEAP Chemiluminescence Kit into each well of a conical bottom 96-well plate.
3. Add 40 µL of the cell culture supernatant into each well of the 96-well plate.
4. Seal the wells with adhesive aluminum foil pads, and incubate at 65 °C for 30 min.
5. Cool the plate on ice for 3 min and equilibrate the plate to room temperature.
6. Add 60 µL of assay buffer to each well and incubate for 5 min at room temperature.
7. Mix 1:20 ratio of chemiluminescent enhancer and CSPD SEAP substrate.
8. Add 60 mL of the mix to each well and incubate the reaction mixture for 15–30 min at room temperature.

Table 22.1 Sample arrangement of mammalian two-hybrid assays

Sample number	pM	pVP16	pG5SEAP vector
1	pM-X	pVP16-Y	pG5SEAP vector
2	pM	PVP16	pG5SEAP vector
3	pM	pVP16-Y	pG5SEAP vector
4	pM-X	PVP16	pG5SEAP vector
5	pM-p53	pVP-(SV40)T	pG5SEAP vector
6	Blank	Blank	Blank
7	Blank	Blank	pG5SEAP vector

Note: In the array of transfection for mammalian two-hybrid analysis, the pM, pVP, and pG5SEAP plasmid constructs are added in 2:2:1 ratio. Each combination should be transfected in triplicates or quadruplicates to obtain the optimal level of standard deviation for SEAP analysis.

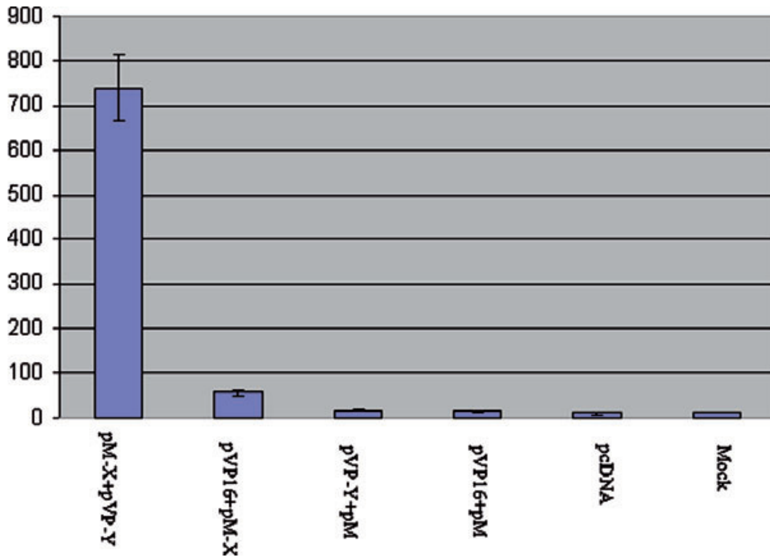

Fig 22.2 SEAP analysis: Chemiluminescence readings of the transfected samples. The depicted constructs were transfected in triplicate, 48 h after transfection, supernatant samples were harvested and subjected to SEAP analysis. The levels of chemiluminescence readings reflect the kinetics of protein-protein interactions

3.5.2 Detection of Chemiluminescence Using Luminometers

1. Detect chemiluminescence using a suitable detector (96-well or a single-tube luminometer can be used for this purpose.
2. Record the readings for phenotype analysis (see Fig. 22.2).

4 Notes

1. The quality of DNA for transfection is very important, we recommend $OD_{260/280}$ to be at least 1.5. The large scale DNA preparation should be aliquoted into several Eppendorf tubes and frozen at –20 °C because frequent freeze-thaw cycles with the same tube may decrease the quality of DNA and affect the analysis.
2. Sometimes the Gal or the VP16 fusion peptides in pM or pVP16 vectors affects the conformation of the targeted protein, it is recommended that pM-X + pVP16-Y, pM-Y, and pVP16-X are analyzed to avoid false negative results.
3. Cells should be 50–70% confluent to allow optimal transcription and translation of the targeted gene.
4. The combination of pM-X + pVP-X is recommended for analysis because many functional forms of proteins are dimers or multimers. In this case, X = Y. The constructs used in this chapter are SARS-CoV nucleocapsid protein in pM and pVP16 vectors (X = Y).

5. The transcription of pM and pVP16 vectors are controlled by a SV40 promoter, which is not as strong as the CMV promoter. Therefore, proteins expressed from these vectors may not be sufficient to perform Western blots or coimmunoprecipitation analyses. Clontech provides another set of expression vectors for the analysis, that is, pCMV-myc and pCM-HA, driven by CMV promoter. Other CMV-based mammalian transcription vectors, such as pcDNA 3.1 can be considered for co-IP or Western blot analysis. Alternatively, there are mammalian two-hybrid vectors carrying CMV promoters, such as pCMX-Gal4-N and pCMX-VP16-N [17].
6. If necessary, other methods should be considered to further confirm the result from the mammalian two-hybrid results. These methods are, but not limited to, GST-pull down assay, fluorescent resonance energy transfer (FRET) or bioluminescence resonance energy transfer (BRET), and mass spectrometry.
7. When pG5SEAP vector alone is used as a negative control, it tends to give high chemiluminescence readings, because no other vector competes for the transfection reagent. When this happens, a "filler" vector (i.e., pcDNA 3.1) that is not related to the mammalian two-hybrid system is recommended (i.e., 800 ng pCDNA 3.1 + 200 ng pG5SEAP).
8. The p53 protein and simian virus large T antigen have very high interaction kinetics. Such positive control included in the mammalian two-hybrid system may yield very high chemiluminescence reading, thereby obscuring the levels of the targeted protein-protein interactions that otherwise would be easily detected as positive reaction. It is recommended that this positive control be used merely to check if the transfection and SEAP assay are successful instead of comparing with the readings from the targeted protein-protein interactions.
9. The chloramphenicol acetyl transferase (CAT) assay is recommended by some manufacturers; however, in some cases, it is not as sensitive as the SEAP assay. The difference of sensitivity level can be more than tenfold.
10. Since mammalian two-hybrid system demonstrates levels of interactions, it can be used for interaction motif mapping. The commonly used mapping methods are sequential deletions or expression of gene fragments. When the sequential deletion method is used, it is recommended to do both amino- and carboxy-terminal sequential deletion so that functional domains can be better defined.

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