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CHAPTER

MAMMALIAN TWO-HYBRID SYSTEM

Chapter Two: Mammalian Two-hybrid System

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Mammalian two-hybrid system overview

The mammalian two-hybrid system is similar to the yeast two-hybrid system in that both are based on the fact that eukaryotic transcription factors are comprised of two distinct physical and functional domains: a DNA binding domain (DBD) and an activation domain (AD). The DBD recognizes a specific DNA sequence, in this case a promoter/enhancer element. The AD coordinates the assembly of the elements required for transcription allowing RNA polymerase II to transcribe a specific reporter gene downstream of the DBD.

The mammalian two-hybrid system relies upon three plasmids that are co-transfected into mammalian cells. Each plasmid has unique features. One plasmid contains a transcriptional activation domain upstream of coding sequences for the prey protein (AD-Y). The second vector contains a DBD upstream of coding sequences of the bait protein (DBD-X). The third vector contains five DNA binding sites upstream of a minimal TATA box, which is upstream of a specific reporter gene.

Interaction between proteins “X” and “Y” result in association of the DBD with the transcriptional activation domain. When the complex binds to the DNA binding sites on a specially designed reporter vector, transcriptional activation of the reporter gene occurs (expression of the two domains individually will not lead to activation of the reporter gene) (Figure 2).



Mammalian two-hybrid system formats

The mammalian two-hybrid system allows characterization of mammalian protein:protein interactions within a cellular environment that mimics native conditions. Yeast and mammalian cells differ in patterns of post-translational modification, such as glycosylation, phosphorylation and acylation, as well as in the intracellular localization of proteins. These types of protein modifications, as well as other unique factors or modulators present in mammalian cells, may influence the ability of protein domains to interact.

Another advantage of the mammalian two-hybrid system is that the assay is less time-consuming than the yeast two-hybrid system. Instead of waiting 3–4 days for yeast colonies to grow to a reasonable size for a blue-color

assay, typical reporter assays in the mammalian system can be performed within 48 hours of transfection.

The most common format of the mammalian two-hybrid system consists of one vector containing the DBD of the GAL4 protein, another vector containing the AD of the herpes simplex virus VP16, and a third vector containing 4–5 GAL4 binding sites upstream of a specific reporter gene.

The primary difference between the various systems is the reporter gene used for detection of positive interactions. The three most commonly used reporter genes are luciferase, β -galactosidase and secreted alkaline phosphatase (SEAP).

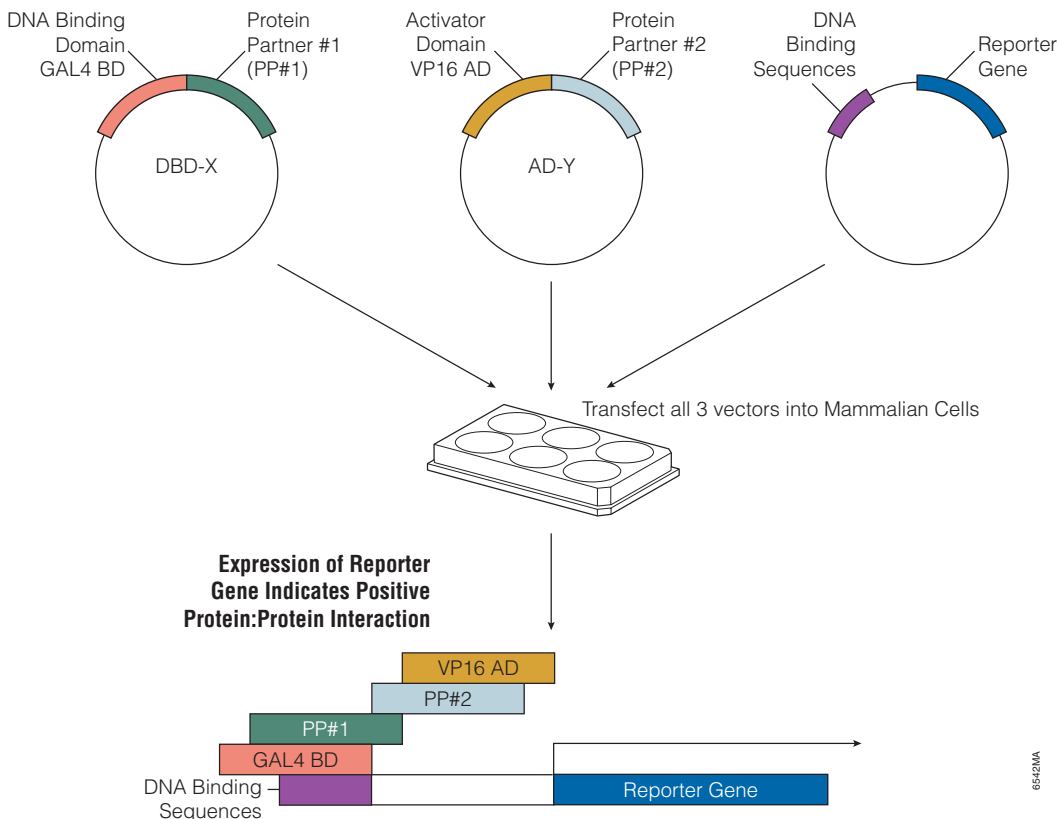


Figure 2. Principle of the mammalian two-hybrid system. The protein coding sequence for the bait protein is cloned into a vector that contains the DNA binding sequence (DBD-X (bait) fusion). The protein coding sequence for the prey protein is cloned into a vector that contains the sequences for transcriptional activation (AD-Y (prey) fusion). The vectors also must contain the necessary elements for growth and protein expression in mammalian cells. The recombinant vectors are then transfected along with a third plasmid containing the appropriate DNA binding sites upstream of a reporter gene. Only if proteins X and Y physically interact are the DBD and AD brought together to reconstitute a functionally active factor that binds to upstream sequences and activate expression of the reporter gene.

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Using luciferase as the reporter gene

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Luciferase

Firefly luciferase is a monomeric enzyme of 61kDa that catalyzes a two-step oxidation reaction to yield light, usually in the green to yellow region, typically 550–570nm. Upon mixing with substrates, firefly luciferase produces an initial burst of light that is measured by a luminometer. The assay for firefly luciferase is easily quantitated and is linear over at least seven orders of magnitude.

SEAP

SEAP is a protein that is secreted from cells and thus can be assayed using a small aliquot of cell culture media. The enzyme can withstand temperatures as high as 65°C. Therefore, endogenous alkaline phosphatase activity can be eliminated by pretreatment of the samples at 65°C. SEAP activity can be measured using chemiluminescence or fluorescence detection methods.

 β -Galactosidase

Bacterial β -galactosidase consists of four identical 116kDa subunits, and is extremely stable and resistant to proteolytic degradation. The most common method used to detect

β -galactosidase activity is based on the ability of the enzyme to hydrolyze ONPG to free o-nitrophenol. The substrate o-nitrophenol is yellow in aqueous solutions and absorbs light at 420nm. Fluorescent, chemiluminescent and colorimetric detection methods are available. The fluorescent and chemiluminescent methods are 20- to 1,000-fold more sensitive than the standard colorimetric method.

When to use the mammalian two-hybrid system

In conjunction with other techniques, the mammalian two-hybrid system is used to characterize protein:protein interactions in a true mammalian environment. Due to the format, this technique is not usually used to screen large numbers of prey proteins.

Primary reagent requirements for the mammalian two-hybrid system

- Mammalian expression vector containing DNA binding domain (e.g., GAL4) upstream of coding sequences for the bait protein
- Mammalian expression vector containing activation transcriptional domain (e.g., VP16) and coding sequences for the prey protein
- Appropriate mammalian cell line
- Cell culture media
- Transfection reagent
- Reagents for detection of reporter gene output (e.g., luminometer, fluorometer)

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