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A recombination based method to rapidly assess specificity of two-hybrid clones in yeast

R. Petermann, B. M. Mossier, D. N. T. Aryee and H. Kovar*

Children's Cancer Research Institute, St. Anna Kinderspital, Kinderspitalgasse 6, A-1090 Vienna, Austria

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ABSTRACT

The yeast two-hybrid system is frequently used to identify protein–protein interactions. Confirming the specificity of candidate clones requires separation and isolation of yeast plasmids, propagation in bacteria and testing combinations of DNA-binding and activation domain hybrids in yeast. In order to simplify this procedure, we developed a rapid method based on PCR amplification of library insert DNAs and *in vivo* cloning into the activation domain hybrid vector. Reporter gene activity is assayed in parallel for combinations with different DNA-binding domain hybrids. Further characterization of inserts does not require plasmid isolation and intermediate hosts.

The yeast two-hybrid system is a powerful tool to screen DNA libraries for proteins that interact with a specific protein of interest. It is based on the functional reconstitution of a transcriptional activator. The target protein (bait) fused to a DNA-binding domain (DBD) and library encoded proteins (prey) are fused to the activation domain (AD) (1,2). Interaction between bait and prey hybrids reconstitutes transcriptional activation function and stimulates reporter gene expression.

In the course of such a screen, artifactually positive yeast clones may be isolated. In order to eliminate false positives additional genetic assays have to be performed that involve coexpression of different combinations of AD- and DBD-hybrids (3). To this end, AD- and DBD-hybrid vectors have to be separated. This can be achieved alternatively by generation of segregant yeast strains or by isolation of plasmids from yeast and propagation in *Escherichia coli*. Standard protocols for separation, isolation and analysis of yeast plasmids are time consuming and are prone to undesired plasmid alterations.

To circumvent plasmid segregation/isolation and intermediate hosts we developed a method based on PCR mediated *in vivo* cloning, a powerful technique to construct recombinant plasmids in yeast (4,5). Library insert DNAs of GAL4 AD-hybrid vectors are amplified from transformants (6) by PCR using oligonucleotides complementary to flanking plasmid sequences, generating vector sequence tags at both 5′ and 3′ ends of the product. Sequence analysis of the PCR product can be performed using an internal oligonucleotide homologous to the upstream vector sequence tag. An indicator strain is then transformed with PCR products, linearized AD-hybrid vector and alternative DBD-hybrid controls as recommended by Bartel *et al*. (3). Homology between linear GAL4 AD vector and plasmid sequences in the PCR product mediates efficient conversion of both linear DNA molecules into circular, recombinant plasmids (Fig. 1). Coexpression of bait and control prey hybrids allows to assay the specificity of interaction between target and prey hybrids in yeast.

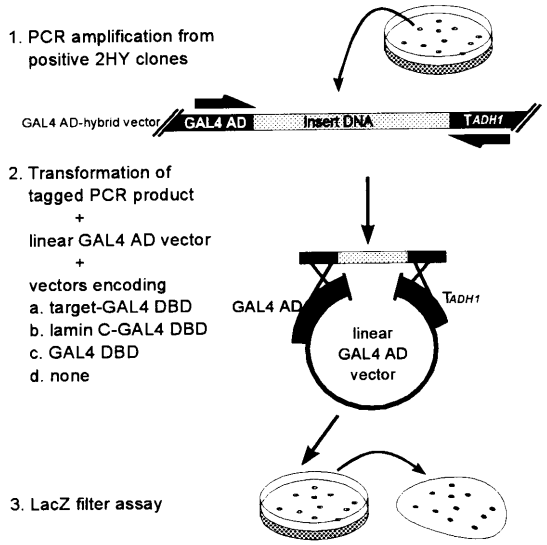

Figure 1. Schematic diagram of recombination based characterization of two hybrid clones in yeast. 1. Insert of the GAL4-AD hybrid vector is amplified from whole yeast cells using vector specific primers. 2. PCR products and linearized GAL4-AD vector are transformed into indicator cells. Recombination between linear vector and homologous sequences in the PCR product (black bars) generates circular recombinant GAL4 AD-hybrid vectors. Coexpression of GAL4 AD-library hybrids and control GAL4 DBD-hybrids allows to determine the specificity of interaction in yeast. 3. Replica plating onto nitrocellulose membranes and β-galactosidase assay.

However, self-ligation or integration of the linearized AD vector in the yeast genome as well as errors introduced during PCR amplification may give rise to artifactually negative colonies. Only 3–6% of false negatives were obtained in total in an assay using two proteins known to interact in the two hybrid system, p53 and SV40 large T antigene (7,8). Since >270 colonies per plate were typically assayed for β-galactosidase activity this error rate does not influence the results obtained by this method (Fig. 2).

*To whom correspondence should be addressed. Tel: +43 1 40 470; Fax: +43 1 408 72 30; Email: kovar@ccri.univie.ac.at

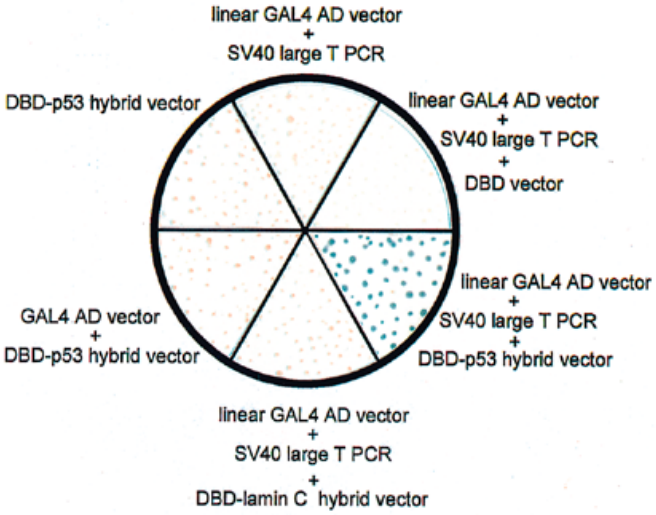

Figure 2. Linear GAL4 AD vector and SV40 large T PCR product recombine in yeast. Expressed GAL4 AD-SV40 large T hybrid specifically interacts with the GAL4 DBD-p53 hybrid and activates the GAL4 dependent *lacZ* reporter gene.

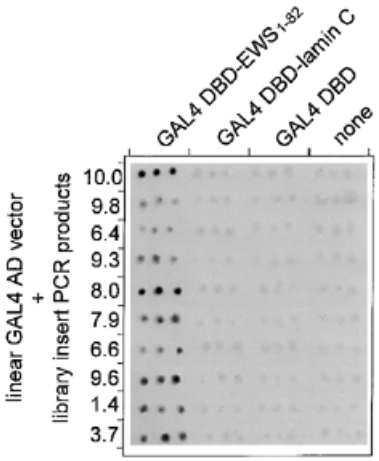

Figure 3. Ten independent, initially positive two-hybrid clones obtained in a screen for EWS-interactive proteins were tested for specificity in yeast according to the above protocol with the following exceptions. The number of PCR cycles was reduced to 30, and to further simplify this procedure, yeast cells already transformed with GAL4 DBD-$EWS_{1–82}$, GAL4 DBD-lamin C and GAL4 DBD expression vectors were used.

This technique does not involve long term liquid culturing, plasmid isolation and bacterial hosts and therefore significantly reduces the time required for characterization of initially positive two-hybrid clones.

The specificity of the recommended procedure has been demonstrated using the interaction between murine p53 and SV40 large T antigen hybrids as a model system using the following brief protocol. Subsequently, we employed the reported method to assay initially positive two-hybrid clones obtained in a screen for proteins that interact with the N-terminus of the human EWS gene product (Fig. 3) (9). For this analysis, yeast cells pretransformed with DBD-hybrid vectors were used.

Amplification of library inserts, vector preparation: a pinch of HF7c colonies (Clontech Laboratories, Palo Alto) harboring a murine p53-GAL4 DBD hybrid vector (pVA3) (7) and an SV40 large T antigen-GAL4 AD hybrid plasmid (pTD1) (1,8) were transferred in a 50 µl PCR reaction mixture and directly used as templates in a reaction containing 0.1 mM dNTPs, 20 pmol AD727+ (5′-CGCGTTTGGAATCACTACAGGGATG) and TER976– (5′-GGTTACATGGCCAAGATTGAAACTTAGAGGAG), 2.0 U DynaZyme^{TM} II DNA polymerase and buffer supplied by the manufacturer (Finnzymes Oy). The reaction was heated at 94°C for 7 min and subjected to 45 rounds of amplification (92°C, 45 s; 62°C, 30 s; 72°C, 90 s); of note, 25–30 cycles were found to yield sufficient product (not shown). The AD-hybrid vector (pGAD3F) (7) was linearized with *Bam*HI, dephosphorylated (alkaline phosphatase, Boehringer Mannheim) and gel purified with Biotrap^{TM} (Schleicher & Shuell).

Yeast transformation, reporter gene assay: strain HF7c was transformed using a modified lithium acetate procedure (10) with 200 ng of PCR product, 50 ng of linear GAL4 AD vector and 400 ng of one of the following controls encoding (i) p53-GAL4 DBD hybrid (pVA3), or (ii) lamin C-GAL4 DBD hybrid (pLAM5′) (1), or (iii) GAL4 DBD (pGBT9) (1), or (iv) alone. We routinely obtained 3×10^3–1×10^4 transformants/µg using uncut pVA3. Cells were spread on selective media and incubated at 30°C for 3 days. Colonies were transferred onto nitrocellulose membranes (Protran BA 85, Schleicher & Schuell) and assayed for β-galactosidase activity (11).

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