# 24 Identification of Protein Single-chain Antibody Interactions In Vivo Using Two-hybrid Protocols

Antje Pörtner-Taliana, Karen F. Froning, Isolde Kusser, and Marijane Russell

*Invitrogen Corporation, Carlsbad, California 92008*



## INTRODUCTION

As naturally occurring protein–protein interactions become increasingly well defined, it is of interest to develop a means of regulating the interactions of specific proteins in order to achieve a desirable biological or clinical effect. One means to do this is to develop a targeted agent that can bind a protein of interest, and alter its biological activity. Naturally occurring antibodies have long been known to bind to a great diversity of target proteins with high affinity. Antibody engineering efforts have built on the great wealth of structural knowledge concerning antibody–antigen recognition, to build enhanced reagents that can work not only in the extracellular milieu within which antibodies normally function, but also for specific intracellular applications. The goal of this chapter is to describe issues related to the use of modified antibodies as intracellular protein-targeted agents and to provide a protocol using a modified dual-bait two-hybrid system to generate such agents.

*Protein–Protein Interactions: A* Molecular Cloning *Manual*, © 2002 by Cold Spring Harbor Laboratory Press, Chapter 24.

Single-chain antibodies (sFvs) are genetically engineered antibodies that consist of the variable domain of a heavy chain at the amino terminus joined to the variable domain of a light chain by a flexible peptide linker. They are generated by PCR and preserve the affinity of the parent antibody. Neutralizing sFvs against antigens of interest enable researchers to study protein function within the cell (Cattaneo and Biocca 1997; Rondon and Marasco 1997). Richardson et al. (1995) expressed a sFv against the α-subunit of the high-affinity human interleukin-2 receptor and achieved a phenotypic knockout of the receptor. Cochet et al. (1998) showed that expression of a Ras-specific sFv was able to inhibit Ras signaling pathways in *Xenopus laevis* oocytes and NIH-3T3 fibroblasts and specifically promoted apoptosis in human cells. Such neutralizing antibodies can be used to study protein function within cells and have potential in gene therapy.

sFvs are usually generated by PCR from hybridoma cell lines that express monoclonal antibodies (mAbs) with known target specificity (Nicholls et al. 1993), or they are selected by phage display from sFv libraries isolated from spleen cells or lymphocytes (Coloma et al. 1991, 1992; Hoogenboom et al. 1991; Marks et al. 1991). Neither method guarantees the isolation of sFvs that function in antigen recognition in vivo. The formation of disulfide bonds in the reducing environment of the cytoplasm and nucleus (Hwang et al. 1992) is hindered (Martineau et al. 1998), resulting in low expression levels and a limited half-life of the antibodies. Therefore, not all mAbs can be converted into sFvs and maintain their function within the cell. However, some sFvs do not require this bond for antigen recognition (Proba et al. 1998). In search of a protocol to identify intracellular binders, several groups have applied the yeast two-hybrid technology (Chapter 7) to evaluate sFv–protein interaction. This work has proven that the two-hybrid system is useful to predict whether or not a sFv will be able to recognize its target protein in vivo (Visintin et al. 1999; De Jaeger et al. 2000; Pörtner-Taliana et al. 2000). All groups cloned a known sFv or sFv libraries into the prey vector and expressed them as fusions to an activation domain (AD). Visintin et al. (1999) analyzed several sFvs with known antigen specificity. They studied a human immunodeficiency virus (HIV) integrase sFv that was derived from a mAb and exhibits neutralizing function when expressed in human cells. The sFv was able to bind to its antigen in a yeast two-hybrid system also. Next, they investigated the interaction of different sFvs isolated by phage display against the antigens Syk (a tyrosine kinase) or p21-ras using the yeast two-hybrid system. From a set of different antibodies none or only a few sFvs were able to target their antigen in a yeast two-hybrid in vivo assay. Therefore, the isolation of sFvs from phage display libraries is an inadequate criterion for their subsequent use as interacting intracellular antibodies.

The application of the yeast two-hybrid technology was also suitable for identifying intracellular binders from a set of in vitro binding sFvs. De Jaeger et al. (2000) present data of dihydroflavonol-4-reductase interacting with specific sFvs isolated by phage display. Their results correlated with previous expression analysis of the same sFvs in plant cytosol (De Jaeger et al. 1999). In a model screen, Visintin et al. (1999) isolated a sFv with known target specificity that was diluted with DNA from a library encoding nonrelevant sFv-VP16 fusion proteins. Pörtner-Taliana et al. (2000) were able to isolate transcription-factor-specific sFvs from a library using the yeast two-hybrid system and showed that the sFv isolated using a full-length bait was able to recognize this protein in mammalian cells.

In the last decade, yeast two-hybrid systems have been used to isolate interactors of many proteins of interest (see Chapter 7). However, screens with some baits result in the isolation of false positives, whereas in other cases, no interactor can be isolated. When the technology is applied to study sFv–protein interactions, the same problems may occur. Additionally, some researchers may want to screen sFv libraries with truncated bait proteins. The sFv isolated with truncated baits are sometimes not able to bind the native protein in mammalian cells (Pörtner-Taliana et al. 2000). This could be due to the inaccessibility of the fragment in the native protein. In the last years, effort has been spent on improving the yeast two-hybrid system, especially to reduce the number of false positives. The dual-bait interaction trap system (Serebriiskii et al. 1999) is a versatile expansion of the interaction trap system originally developed by Gyuris et al. (1993). A second bait plasmid was added coding for a different DNA-binding domain (DBD) that binds upstream of an additional set of unique reporter genes. The system allows testing for cross-reactivities with

an unrelated or related bait protein in the same cell and for library screens with two baits at the same time. This approach has been used to test sFv–antigen interactions. Initial tests showed that the B42 AD was not strong enough to show interaction of sFvs that were isolated in a yeast two-hybrid screen that used VP16 AD. Therefore, VP16 AD was used for additional screens. This modified system was able to distinguish between two different sFv–protein interactions and can be applied for library screens (unpublished results).

Another approach to testing sFv–protein interaction in vivo is a mammalian two-hybrid system. Although mammalian systems do not offer easy library screens, this approach has the advantage that specific sFvs are tested in an environment in which they are most likely used to study protein function. A thorough titering of bait-to-prey ratio is required when characterizing interactions (Pörtner-Taliana et al. 2000). Two groups have used the mammalian two-hybrid approach and compared their results with the yeast two-hybrid approach (Visintin et al. 1999; Pörtner-Taliana et al. 2000). In contrast to the yeast two-hybrid system, Visintin et al. (1999) were only able to verify one sFv–antigen interaction with a mammalian two-hybrid system. They believe this is due to a low sensitivity of their reporter construct. Pörtner-Taliana et al. (2000) used a mammalian two-hybrid bait and prey fusion compatible with the yeast system and a sensitive luciferase reporter. The reporter plasmid contains a splice intron in the polyadenylation signal that increases reporter transcription. They showed that the sFv isolated in a yeast two-hybrid screen was also capable of recognizing the antigen in mammalian cells.

## The Yeast Two-hybrid System to Test for sFv–Antigen Interactions In Vivo

Two different yeast two-hybrid systems have been used to study sFv–protein interactions. De Jaeger et al. (2000) describe studies using a Gal4-based yeast two-hybrid system using a Gal4 AD-fused sFv and a Gal4 DBD-fused bait. The laboratories of Cattaneo (Visintin et al. 1999) and Invitrogen (Pörtner-Taliana et al. 2000) used the same modified LexA-based yeast two-hybrid system originally developed by Vojtek et al. (1993), shown in Figure 1. The protein of interest is expressed as a LexA DBD fusion. The LexA DBD binds to the lexA-operator (LexA-op) upstream of the reporter. The prey proteins are sFvs fused to the VP16 AD. If a sFv binds to the bait protein, the transcription of the two reporter genes *His3* and *lacZ* is induced. The bait and prey vectors of the system are shown in Figure 2. The bait vector pBTM116 contains a *TRP1* gene for selection in yeast and the *LexA* DBD with a downstream polylinker to generate LexA-bait fusion proteins. The prey plasmid pVP16* (Pörtner-Taliana et al. 2000) is derived from the vector pVP16 (Vojtek et al. 1993). The vector pVP16 contains a *LEU2* selectable marker and was modified by inserting an ATG, followed by a nuclear localization signal and recognition sequences for *Sfi*I and *Not*I to allow cloning of sFvs (Pörtner-Taliana et al. 2000).

To study sFv–protein interactions in a two-hybrid context, the bait plasmid containing the protein of interest cloned in frame with the LexA DBD, and the prey plasmid coding for the sFv–VP16 fusion, are transformed into the yeast strain L40 (*MATa his3Δ200 trp1-901 leu2-3112 ade2 LYS2::[4lexAop-HIS3]*) (Vojtek et al. 1993). The strain has integrated *His3* and *lacZ* reporter genes. Both reporters have eight or nine *lexA* operators upstream. The transformants are plated and patched on YC-HLUW plates. Leu and Trp are selectable markers for the plasmids. An in vivo interaction is detected by growth on plates lacking histidine, and by qualitative β-galactosidase assay. The interaction is truly positive if both reporter genes are expressed and the following control transformations show no reporter activity: (1) empty bait plasmid with sFv prey plasmid—the sFv should not be able to interact with the LexA DBD; (2) bait plasmid with control protein (e.g., laminin) with sFv prey plasmid—a negative result will verify that the sFv interacts specifically with the protein of interest and not unspecifically with any protein; (3) bait plasmid with protein of interest and empty prey plasmid—the expression bait fusion and the VP16 AD should not result in the activation of the reporter genes; (4) bait plasmid with protein of interest and no prey plasmid (add Leu [L] to medium)—the result of this test will show whether or not the LexA

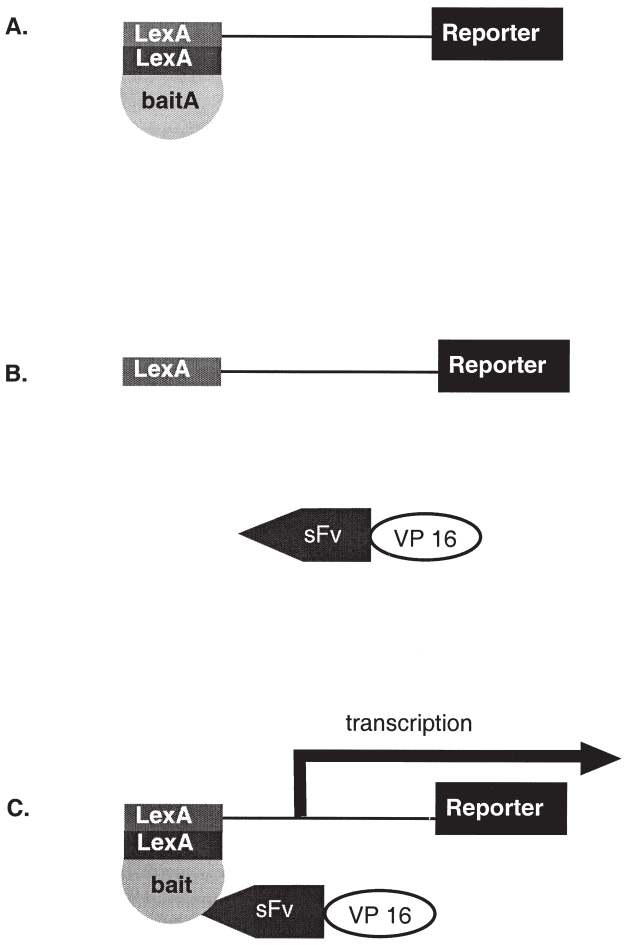


**FIGURE 1.** Modified yeast two-hybrid system to study sFv–protein interaction. The protein of interest (bait) is expressed as fusion to the LexA DNA-binding domain (DBD) that binds to the *lexA* op upstream of a reporter gene (*A*). The sFv is fused to the activation domain (AD) VP16 and cannot activate transcription on its own (*B*). If an sFv binds to the bait protein, the transcription factor function is reconstituted and the reporter gene is expressed (*C*).

bait fusion activates the transcription of the reporter genes alone; (5) sFv prey plasmid with no bait plasmid (add Trp [W] to medium)—a negative result will exclude the possibility that the sFv will interact unspecifically with DNA upstream of the reporter; (6) prey plasmid with nonbinding control sFv and bait plasmid containing protein of interest—the sFv should not bind to the protein of interest, and therefore, cells should not grow on medium lacking His (H) and show no β-gal activity.

If available, a positive control transformation can be performed, using a sFv/protein pair, which is known to interact in a yeast two-hybrid format. Alternatively, an expression plasmid containing a LexA–VP16 fusion could be used to obtain reporter readouts. Reporter gene expression in transformants containing a LexA–VP16 fusion is usually stronger than that obtained from experiments testing interaction of a LexA protein fusion with a sFv prey VP16 fusion.

## sFv–Library Screen Applying the Yeast Two-hybrid Approach

To isolate novel sFvs that bind to a protein of interest in vivo, a bait strain is first constructed. The protein of interest is cloned in frame with the LexA DBD into the bait plasmid pBTM116. The

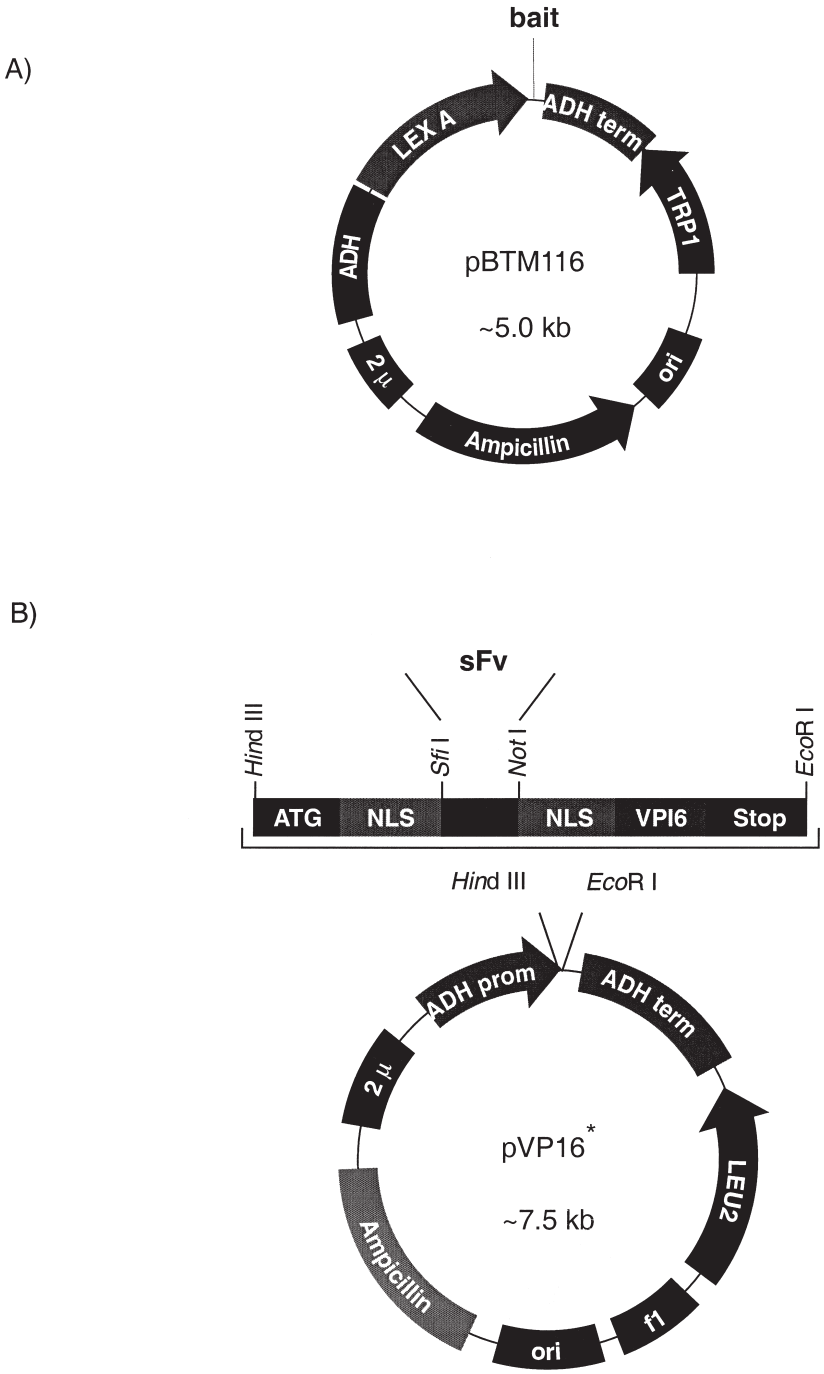


**FIGURE 2.** Yeast two-hybrid bait (*A*) and modified prey vector (*B*).

plasmid is transformed into the L40 yeast strain and streaked on YC-UW plates to select transformants. Expression of the bait fusion protein is verified by western blot analysis. The fusion proteins can be detected with bait-specific antibodies or with commercially available LexA antibodies. The bait fusion by itself should not be able to activate the reporter genes alone. Therefore, the bait strain is patched on a YC-HUW plate. The cells should not be able to grow on plates lacking His (H).

The prey vector pVP16* allows an easy transfer of most existing sFv libraries with *Sfi*I and *Not*I restriction enzymes. sFv libraries are derived from mRNA isolated from human and murine spleen cells or peripheral blood lymphocytes (Marks et al. 1991; Coloma et al. 1992), and the mRNA is used as a template for synthesis of a single-stranded cDNA. The variable light-chain ($V_L$) and heavy-chain ($V_H$) regions are amplified by PCR. The PCR primers are designed such that the immunoglobulin variable regions of heavy and light chain can be directly amplified without prior knowledge of their sequence (Coloma et al. 1991). The linking of $V_H$ and $V_L$ can occur in two ways. The 3′ primer of the $V_H$ region and the 5′ $V_L$ primer contain additional sequences that overlap in a recombinant PCR, and the overlapping sequence codes for the linker peptide. A second approach is to purify the $V_L$ and $V_H$ fragments and to reamplify them with the same set of primers containing restriction sites for cloning into an expression vector. The heavy chain is cloned 5′ of the peptide linker sequence, and the light chain 3′ of the linker sequence. The libraries used for screens with the yeast two-hybrid systems were cloned from phagemid vectors using *Sfi*I and *Not*I restriction sites.

Successful library screens with this system have been performed using the large-scale lithium acetate transformation protocol from Schiestl and Gietz (1989), Hill et al. (1991), Gietz et. (1992), and Mount et al. (1996). Experiments with other two-hybrid systems have shown that small-scale transformation is also sufficient to isolate interactors (Golemis et al. 1996). To measure primary transformation efficiency, different dilutions are prepared and plated on medium lacking Leu and Trp (YC-LUW) to select transformants that contain bait and prey plasmid. The residual transformation is plated on medium lacking additionally His (YC-HLUW). The *His* gene is under control of lexA-op and can only be transcribed when the bait protein interacts with a sFv antibody. His-positive clones generally show up after 2–3 days. The clones are patched on duplicate plates (YC-HLUW). After 1–2 days, β-gal activity is measured using a filter lift (Breeden and Nasmyth 1985) or overlay assay (Gleeson et al. 1998; Golemis and Serebriiskii 1998).

For further characterization, the sFv prey plasmid DNA has to be isolated from double-positive clones and transferred into *E. coli*. Both bait and prey plasmids contain ampicillin as a selectable marker in bacteria. Therefore, the plasmid DNA needs to be analyzed by restriction digest for the presence of sFv-pVP16* plasmid with *Sfi*I and *Not*I. The specificity of the isolated sFv is tested as described above for the interaction of characterized sFvs with their antigens (a–f). Only yeast transformations containing the bait plasmid with the protein of interest and the isolated sFv should grow on plates lacking His (H) and show β-gal activity.

## A Dual-bait Yeast Two-hybrid System That Can Distinguish between Two Different sFv–Protein Interactions in a Single Step

The original yeast two-hybrid system has been modified and improved in multiple ways. The interaction trap system developed by Gyuris et al. (1993), for example, was expanded by a second bait vector (Serebriiskii et al. 1999). This vector codes for a second bait-DBD (c*I*) fusion binding upstream of a unique second set of reporters. The system allows testing for cross-reactivity of a prey protein with another bait in the same cell. This second bait can be any other protein of interest, a mutant, or a protein related to bait 1. To study sFv-binding sites, two different truncated mutants could serve as bait proteins. Additionally, the dual-bait system allows a library screen with two baits at the same time and therefore saves time and reagents.

A modified version of the interaction trap dual-bait system is shown in Figure 3 (unpublished results). Within a yeast cell, the two different bait proteins can be expressed: one as a LexA DBD fusion and the other as a c*I* DBD fusion. Each fusion has two unique reporter genes to identify interaction. The LexA DBD binds upstream of a *LEU2* and *LACZ* reporter, the c*I* DBD upstream of a *LYS2* and *GUS* (glucuronidase) reporter. Experiments have shown that the B42 AD is not suitable to examine sFv–protein interaction, but that an amino-terminal VP16–sFv fusion results in a stronger activation of reporter genes. Therefore, the sFvs are expressed as carboxy-terminal

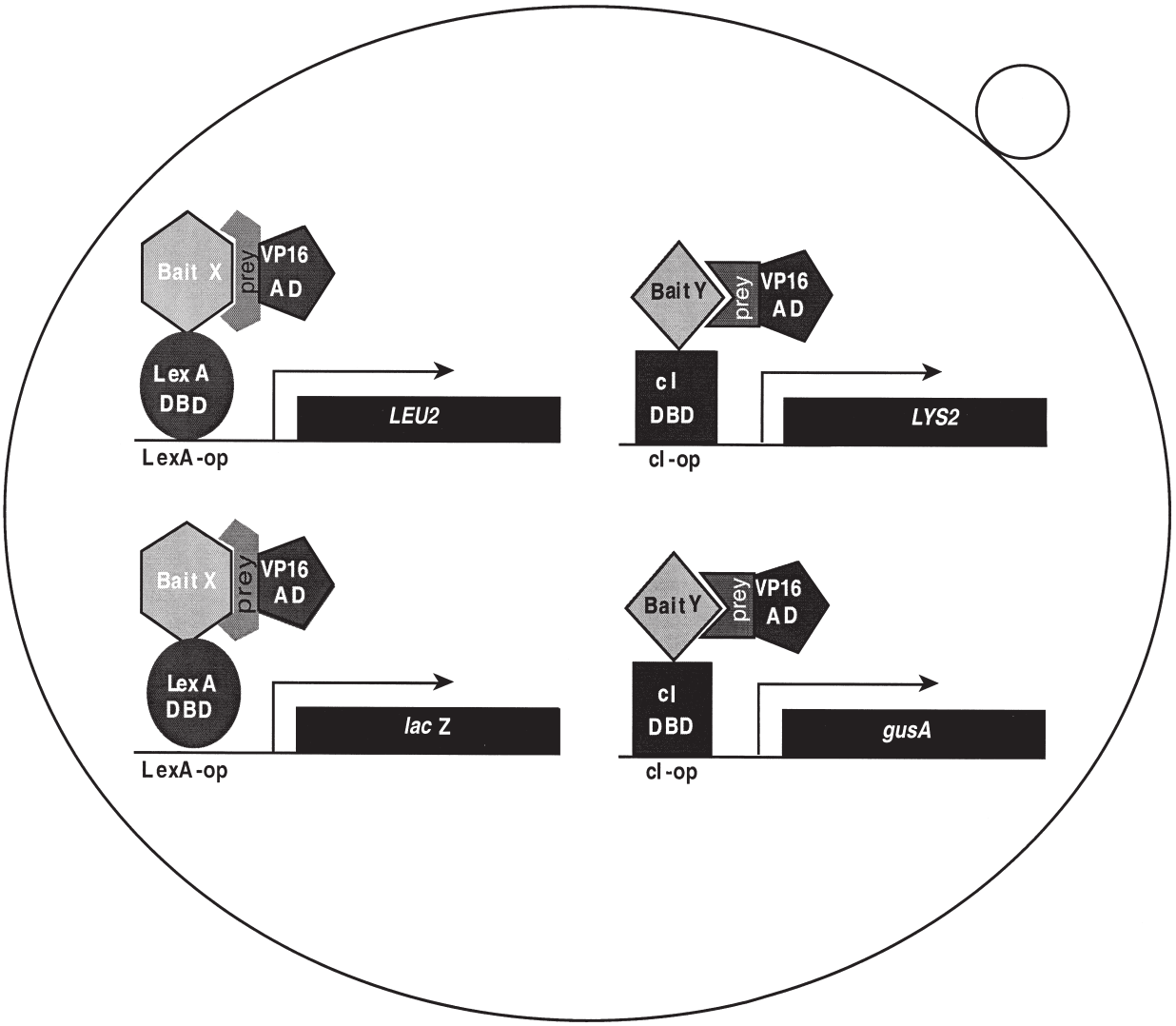


**FIGURE 3.** Modified dual-bait yeast two-hybrid system to study sFv–protein interaction. A VP16 activation domain-fused sFv interacts with a LexA-fused bait to drive transcription of lexA-op-responsive *LEU2* and *LacZ* reporters. Another sFv interacts with bait 2 fused to the c*I* DBD and activates transcription of cI-op-responsive *LYS2* and *GUS* reporters. This system can be exploited to screen sFv libraries with two baits simultaneously. It also allows testing for cross-reactivity of a sFv with a second bait in the same cell.

VP16 fusions. Compared to the first yeast two-hybrid system described above, the expression of the prey fusion is inducible from a *Gal1* promoter that guarantees a higher representation of the library in the yeast.

The dual-bait expression plasmids are shown in Figure 4. The bait 1 plasmid pHybLex/Zeo contains a LexA DBD upstream of a multiple cloning site (MCS) and a Zeocin marker for selection in yeast and bacteria. The bait 2 plasmid contains a c*I* DBD of the bacteriophage λ, a MCS downstream, a kanamycin gene for selection in bacteria, and a *HIS3* gene for selection in yeast. The prey plasmid pYesTrp was modified by replacing the B42 AD with VP16 and introducing an *Sfi*I and *Not*I downstream to clone sFvs easily. It contains a TRP1 marker for selection in yeast and an ampicillin marker for selection in bacteria. The dual-reporter plasmid pLacGus codes for the lexA-op-responsive *lacZ* and cI-op-responsive *GUS* reporter genes. The *Saccharomyces cerevisiae*

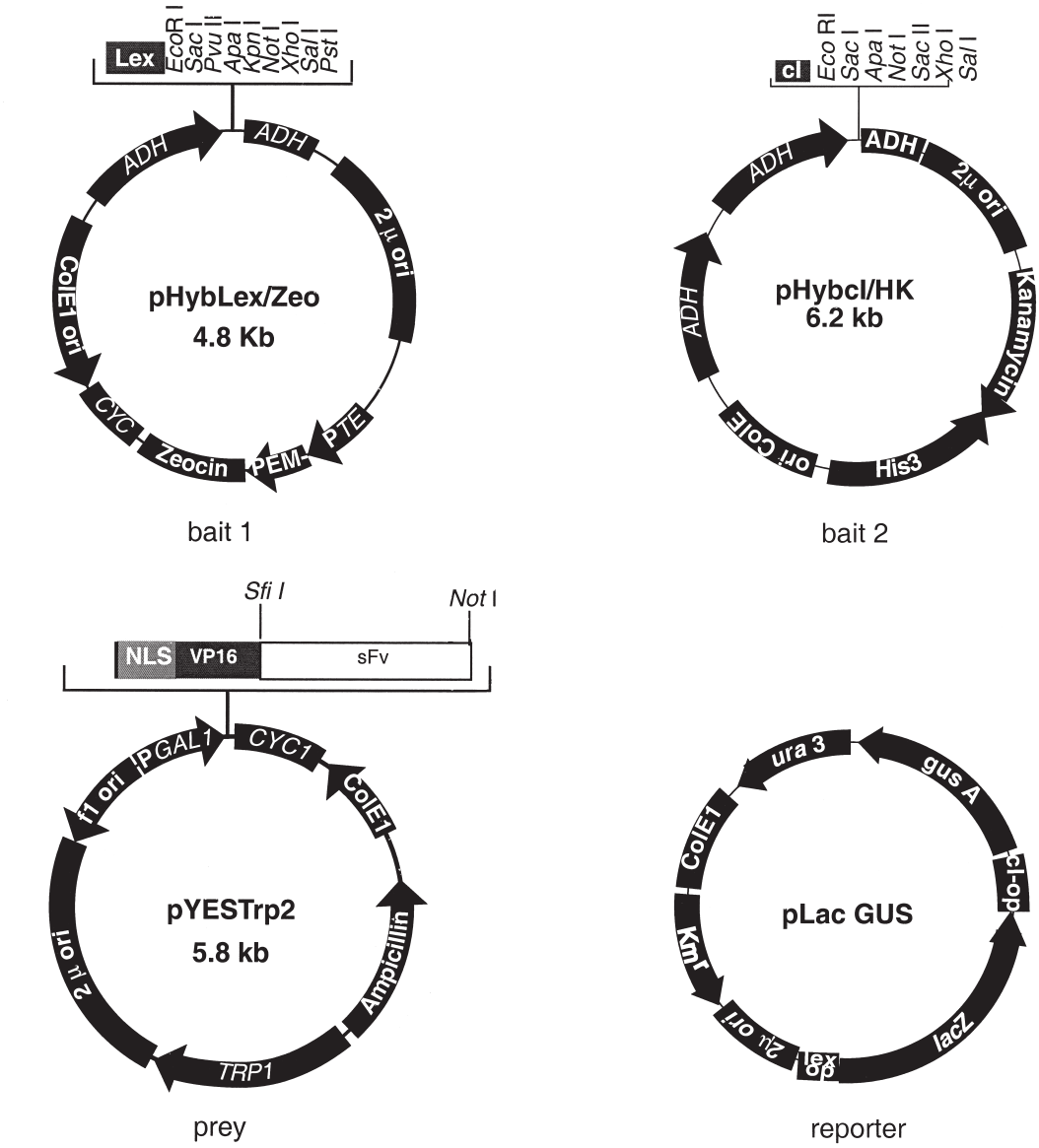


**FIGURE 4.** Dual-bait two-hybrid bait 1, bait 2, modified prey, and dual reporter plasmids.

strain SKY 48 (*Matα ura3 trp1 his3 6lexAop-LEU2, 3cIop-LYS2*) has integrated lexA-op-responsive *LEU2* and cI-op-responsive *LYS2* reporters (Serebriiskii et al. 1999).

The system can be applied for testing sFv–protein interaction in vivo. After transformation, the cells are first plated on glucose/Zeocin plates, which lack the amino acids His, Trp, and Ura, to select for transformants containing the plasmids bait 1, bait 2, prey, and dual reporter. The prey protein expression is controlled by a galactose-inducible promoter and induced by transferring the cells on galactose medium. The medium lacks additionally Leu (L) or Lys (K) to obtain the first reporter readout for both baits. Positive clones are patched on duplicate –L or –K plates. After a maximum of 2 days' incubation, overlay or filter lift assays are performed to detect β-gal or GUS activity, respectively. Controls are required as described for the single-bait two-hybrid system. The bait fusion proteins as well as the VP16–sFv fusion should not be able to activate reporter transcription on their own. The cross-reactivity of the sFvs with cI and LexA needs to be examined, also.

For library screens, a dual-bait strain has to be created transforming competent SKY48 cells containing the reporter plasmid pLacGUS with the two bait plasmids. Bait protein expression can

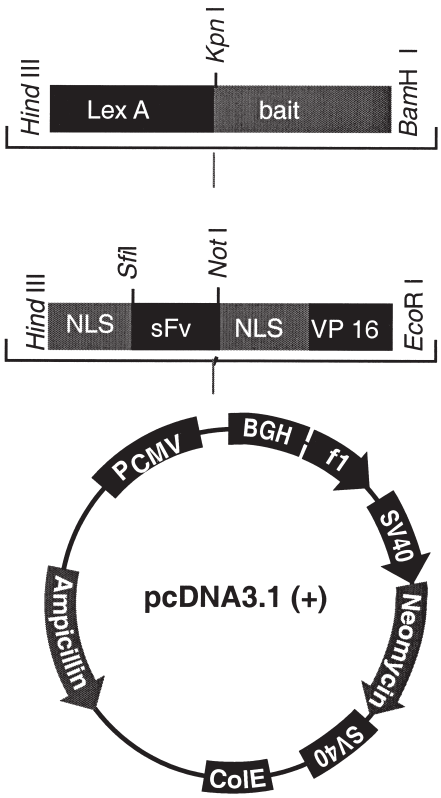


**FIGURE 5.** Mammalian two-hybrid bait and prey constructs.

be detected by western blot with bait-specific or LexA- and cI-specific antibodies. At the same time, it should be tested whether or not the bait fusion proteins can activate reporter transcription on their own. Either the large-scale or small-scale library transformation can then be applied. Transformation efficiency is measured by plating a sequence of 1:10 dilutions of the transformation on YC-UWH + Zeo plates. The transformants are harvested and prey protein expression is induced by incubating the cells in galactose-containing liquid medium. Different volumes are plated on galactose plates containing Zeocin, lacking UWH, and L or K. $Leu^+$ and $Lys^+$ colonies are tested for β-gal or GUS activity, respectively.

The recovery of prey plasmid DNA from double-positive clones is simplified due to the different bacterial selection markers on prey, bait, and dual reporter plasmid (cf. Golemis and Serebriiskii 1998).

## A Mammalian Two-hybrid System to Evaluate sFv–Protein Interaction In Vivo

The mammalian two-hybrid system can serve as an alternative to test sFv–antigen interaction in vivo, but it cannot be applied for library screens (Visintin et al. 1999; Pörtner-Taliana et al. 2000). The system has the advantage that the sFv–protein interaction is directly assessed within a mammalian cell. Both the LexA DBD bait and the sFv–VP16 fusions are cloned into mammalian expression vector pcDNA3.1 (Fig. 5) (Pörtner-Taliana et al. 2000). Thorough titering of bait/prey ratios is required to ensure detection of interaction. A good strategy is to start with a low-bait plasmid concentration and titer the sFv–VP16 expression plasmid. The reporter readout will reach an optimum at a certain sFv–VP16 plasmid concentration and decrease with higher prey concentrations. In control experiments, it should be tested whether or not the bait fusion or the sFv–VP16 fusion is able to activate reporter transcription on its own.

In addition, the system requires a reporter with high sensitivity (Visintin et al. 1999). A luciferase reporter plasmid applied in the study of Pörtner-Taliana et al. (2000) was created by cloning 8 lex-op sequences and the minimal thymidine kinase (TK) promoter upstream of the luciferase gene in pGL-Basic (Promega). A splice intron in the polyadenylation signal increases reporter transcription. Sensitive luciferase detection assays facilitate the detection of sFv–protein interaction.

To normalize for transfection efficiency, a plasmid with a different reporter gene under control of a constitutive promoter should be cotransfected. This reporter will be a good internal control for nonspecific effects of the VP16 AD. The system described was successfully applied in CHO and COS cells with sFvs selected in yeast two-hybrid screens (Pörtner-Taliana et al. 2000). Optimal results were obtained with 25 ng of bait DNA and 250 ng of sFv–VP16 plasmid using 2 μg of reporter plasmid. Plasmid was added to a final DNA concentration of 4 μg in a 35-mm TC plate.

## OUTLINE OF PROCEDURE

This chapter describes three related two-hybrid approaches to test for sFv–protein interactions in vivo. Studies with the first yeast two-hybrid system showed that the method is a valuable tool to predict whether or not a specific sFv recognizes its antigen in an in vivo environment. The second method represents an expansion of the traditional yeast two-hybrid system with a second bait and a second set of reporters. It has the advantage that cross-reactivities of sFvs with a second bait can be studied in one yeast cell, and sFv–library screens can be performed with two baits at the same time. In contrast to the first system, the prey protein expression is inducible. Therefore, a two-step selection protocol should be chosen for library screens. The first step selects for transformants containing bait, prey, and reporter plasmids. This guarantees that most of the genes in the library are represented in the population of transformants. Prey protein expression is induced by transferring the transformants to galactose-containing medium, and interaction can be assessed by examining reporter expression. The two-step selection is more time-consuming and requires more material, but it increases the probability of isolating sFvs that interact with the protein of interest. The third method describes a mammalian two-hybrid approach which has the advantage that sFv–antigen interaction can be studied in a mammalian host cell in which a neutralizing sFv is most likely used.

Protocol 1

# Yeast Two-hybrid Test for sFv–Protein Interaction

The two-hybrid approach described here can be used to predict whether or not a specific sFv recognizes its antigen in an in vivo environment. The protocol uses a second bait and a second set of reporters so that the cross-reactivation of sFv can be studied in one yeast cell; in addition, sFv–library screens can be performed with two baits at the same time. This protocol is related in concept and materials to Chapter 7 and should be cross-checked to this chapter.

## MATERIALS

**CAUTION:** See Appendix for appropriate handling of materials marked with <!>.

### Buffers and Solutions

Cracking buffer
- 8 M urea
- 5% SDS <!>
- 40 mM Tris-HCl (pH 6.8)
- 0.1 mM EDTA
- 1% β-mercaptoethanol <!>
- 0.4 mg/ml bromophenol blue <!>

Store at 4°C or –20°C.

Dimethylsulfoxide (DMSO) <!>

Glycerol solution
- 25 mM Tris-HCl (pH 8.0)
- 0.1 M magnesium sulfate ($MgSO_4$)<!>
- 65% (v/v) glycerol

Autoclave the solution and store at room temperature.

1x LiAc
- 100 mM lithium acetate (pH 7.5)

10x LiAc
- 1 M lithium acetate (pH 7.5)

Adjust pH to 7.5 using diluted glacial acetic acid <!>, filter-sterilize, and store at room temperature.

1x LiAc/1x TE
- 100 mM lithium acetate (pH 7.0)
- 10 mM Tris (pH 7.5)
- 1 mM EDTA

Mix together 10 ml of 10x LiAc and 10 ml of 10x TE. Add deionized $H_2O$ to 100 ml, filter-sterilize, and store at room temperature.

1x LiAc/0.5x TE
- 100 mM lithium acetate (pH 7.5)
- 5 mM Tris (pH 7.5)
- 0.5 mM EDTA

Filter-sterilize and store at room temperature.

1x LiAc/40% PEG-3350/1x TE
100 mM lithium acetate (pH 7.5)
40% (w/v) PEG 3350 <!>
10 mM Tris-HCl (pH 7.5)

Mix together 20 ml of 10x LiAc, 20 ml of 10x TE, and 80 g of PEG 3350 <!>. Add deionized $H_2O$ to 200 ml and dissolve the PEG. The solution may have to be heated. Autoclave (15 psi) for 20 minutes at 121°C and store at room temperature.

1x TE
10 mM Tris (pH 7.5)
1 mM EDTA

Filter-sterilize.

10x TE
100 mM Tris (pH 7.5)
10 mM EDTA

Filter-sterilize.

X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactose)
25 mg or 50 mg/ml X-Gal /ml in *N,N*-dimethylformamide (DMF)<!>

X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid)
25 mg of X-Gluc /ml in DMF<!>

YC medium and plates (YC is minimal defined medium for yeast)

1. Dissolve 0.12% w/v Yeast nitrogen base (without either amino acids or ammonium sulfate), 0.5% w/v ammonium sulfate, <!>, 1% w/v succinic acid, 0.6% w/v NaOH <!>, 2% w/v glucose, 0.01% w/v (adenine, arginine, cysteine, leucine [L], lysine [K], threonine, tryptophan [W], uracil [U]), and 0.005% w/v (aspartic acid, histidine [H], isoleucine, methionine, phenylalanine, proline, serine, tyrosine, valine).

   The amino acids with the one-letter code are those that need to be omitted to make selective plates, depending on the genotype of the host, plasmid markers, and reporters.
2. Add 2% w/v agar after dissolving the reagents above, if you are making plates.
3. Autoclave at 15 psi for 20 minutes at 121°C.
4. Cool to 50°C and add 10% v/v of filter-sterilized 20% w/v glucose.

   Add Zeocin (Zeo) at this point (if needed) to a final concentration of 200 μg/ml. For plates that contain galactose and raffinose, add 10% v/v 20% w/v galactose and 5% v/v 20% w/v raffinose instead of glucose.
5. Pour plates and allow to harden. Invert the plates and store at +4°C. Plates are stable for 6 months unless they contain Zeo. Plates containing Zeo are stable for about 1 month.

Yeast extract peptone dextrose medium

1. Dissolve 1% w/v yeast extract, 2% w/v peptone.
2. Add 2% w/v agar, if making plates.
3. Autoclave for 20 minutes on liquid cycle.
4. Add 2% w/v dextrose (D-glucose).
5. If desired, cool the solution to <50°C and add 200 μg/ml Zeo. Store medium at room temperature. Store medium containing Zeo at 4°C protected from exposure to light. The shelf life is ~1–2 months.

Yeast lysis buffer
50 mM Tris-HCl (pH 8.0)
2.5 M lithium chloride (LiCl)<!>
4% (v/v) Triton X-100
62.5 mM EDTA

Adjust the pH if necessary with NaOH or HCl and bring the volume to 100 ml. Store at room temperature.

YPD ± Zeocin

Z buffer
60 mM sodium hydrogen phosphate ($Na_2HPO_4$) <!>
40 mM sodium hydrogen diphosphate ($NaH_2PO_4$)<!>
10 mM potassium chloride (KCl) <!>
1 mM magnesium sulfate ($MgSO_4$) (pH 7.0)<!>
Zeocin (Zeo) concentration and selective medium

### Bacteria and Yeast

*Escherichia coli:* 25 μg/ml in low-salt LB medium
Efficient selection requires that the concentration of NaCl be no more than 5 g/liter (<90 mM).
*Saccharomyces cerevisiae* (SKY48/pLacGUS): 200 μg/ml in YPD or other selective medium

### Plasmids and Yeast Strains

Plasmids and strains for modified yeast two-hybrid system developed originally by Vojtek et al. (1993)
pVP16*
pBTM116
L40 yeast strain (*MATα his3D200 trp1-901 leu2-3112 ade2 LYS::9lexAop-HIS3)*
Plasmids and strains for the modified dual-bait yeast two-hybrid system
pHybLex/Zeo
pHybcI/HK
pYesTrp/VP16
SKY 48 (*MATα trp1 ura3 his3 6lexop-LEU2 3cIop-LYS2*) pretransformed with pLacGus

## METHOD

### Small-scale Yeast Transformation (Schiestl and Gietz 1989; Hill et al. 1991; Gietz et al. 1992; Mount et al. 1996)

This protocol can be used to test whether or not sFvs isolated from libraries by phage display or mAbs are able to target their antigen in vivo. It is also applicable to create bait strains for a library screen and to control for true positive interactions after sFvs are isolated from a yeast two-hybrid library screen.

Bait strains are constructed by transforming the bait plasmid(s) containing the gene of interest in frame with the DNA-binding domain into the appropriate bait strain. These bait strains should be used to test whether or not the baits are able to activate reporter transcription on their own; the expression of the bait fusion protein should be tested by western blotting.

1. Inoculate 10 ml of YPD with a colony of SKY48/pLacGUS and shake overnight at 30°C.
2. Determine the $OD_{600}$ of your overnight culture. Dilute culture to an $OD_{600}$ of 0.4 in 50 ml of YPD and grow an additional 2–4 hours.
3. Pellet the cells at 2500 rpm and resuspend the pellet in 40 ml of 1x TE.
4. Pellet the cells at 2500 rpm and resuspend pellet in 2 ml of 1x LiAc and 0.5x TE.
5. Incubate the cells at room temperature for 10 minutes.
6. For each transformation, mix together 1 μg of plasmid DNA and 100 μg of denatured sheared salmon sperm DNA with 100 μl of the yeast suspension from step 5.

7. Add 700 µl of 1x LiAc, 40% PEG-3350, 1x TE and mix well.
8. Incubate solution for 30 minutes at 30°C.
9. Add 88 µl of DMSO, mix well, and heat shock for 7 minutes at 42°C.
10. Centrifuge in a microcentrifuge for 10 seconds and remove supernatant.
11. Resuspend the cell pellet in 1 ml of 1x TE and re-pellet.
12. Resuspend the pellet in 50–100 µl of 1x TE and plate on an appropriate selective plate.

## Expression of Bait Fusion Proteins (Golemis et al. 1996; Golemis and Serebriiskii 1998)

This protocol describes how to prepare cell lysates from your L40 bait strain ($Trp^+$ and $Ura^+$) or $His^+$, Zeo-resistant SKY48 pLacGUS bait strain for western blot analysis. Test several transformants in case of heterogeneity in LexA and cI fusion expression levels. Run lysates of untransformed L40 or SKY48/pLacGUS as negative controls.

1. Inoculate 10 ml of YC-UW (L40) or YC-UH Zeo200 (SKY48 pLacGUS) with a single colony of the bait strain. Inoculate 10 ml of YC-U with L40 or SKY48/pLacGUS as a negative control. Grow overnight, with shaking, at 30°C.
2. Streak a sample of each culture onto a fresh plate. After confirmation of bait expression, return to this plate and use it as a subsequent source of the bait strain.
3. Pellet the cells in step 1 by centrifuging at 2500 rpm for 5 minutes at room temperature. Decant the medium.
4. Transfer the cell pellets to a –80°C freezer for 10 minutes.
5. Thaw the cell pellet in 100 µl of prewarmed (60°C) cracking buffer and resuspend by pipetting the cell pellet in the buffer.
6. Transfer the cell suspension to a 1.5-ml microcentrifuge tube containing 100 µl of glass beads.
7. Incubate the solution for 10 minutes at 70°C.
8. Vortex the solution for 1 minute.
9. Centrifuge at 14,000 rpm for 5 minutes at room temperature and transfer the supernatant to a new tube.
10. Add SDS-PAGE sample buffer and boil sample for 5 minutes. Use 30–50 µl for immunoblot analysis. Detect LexA and cI fusions using antibodies to the proteins of interest or LexA and cI antibodies (available from Invitrogen).

The calculated molecular weights of the LexA and cI protein expressed from pHybLex/Zeo and pHybcI/HK, respectively, are listed below. The calculated molecular weight of each protein includes additional amino acids encoded by the multiple cloning sites. The table also lists the observed migration of each protein on an SDS polyacrylamide gel.

| Protein | Calculated molecular mass | Observed molecular mass |
|---|---|---|
| LexA | 26 kD | 32 kD |
| cI | 29 kD | 36 kD |

## Library Transformation

SFv libraries are isolated from spleen cells or peripheral blood lymphocytes. The mRNA is isolated and the cDNA is generated. The amplifications of the $V_H$ and $V_L$ chains are performed with primers that do not require the knowledge of the variable domain sequences. The $V_L$ chain is

linked to the carboxyl terminus of the $V_H$ chain by using overlapping PCR. The overlapping sequences at the $V_H$ 3′ and $V_L$ 5′ ends are designed to code for a $([Gly]_4Ser)_3$ linker (Marks 1991), or the purified chains are reamplified with a set of primers containing overhangs with restriction sites to clone them into an expression vector (mostly phagemids). The $V_H$ chain is cloned amino-terminally of the linker coding sequence, and the $V_L$ chain carboxy-terminally of that linker sequence (Finnern et al. 1997). Existing libraries can be easily cloned into the modified prey vectors of both systems using *Sfi*I and *Not*I restriction enzymes.

The large-scale protocol was applied for the single-bait system using an L40 bait strain (Schiestl and Gietz 1989; Hill et al. 1991; Gietz et al. 1992; Mount et al. 1996). The dual-bait yeast two-hybrid screen was performed using the small-scale protocol (Golemis et al. 1996; Golemis and Serebriiskii 1998). The procedures are generally interchangeable. The user should consider that the two-step selection is recommended for the dual-bait system, and the large-scale protocol should be appropriately adapted. The selection of transformants will change accordingly.

### *Large-scale Library Transformation for Single-bait Two-hybrid System into the L40 Bait Strain*

1. Grow 5 ml of an overnight culture of the L40 bait strain in selective yeast medium lacking W and U.
2. Inoculate an aliquot of the overnight culture into 100 ml of the same medium and grow overnight at 30°C with constant shaking.
3. On the following day, dilute the culture to a final $OD_{600}$ of 0.3 in 1 liter of YPAD (YPD with 40 μg/ml adenine). Grow for 3 hours at 30°C with constant shaking.
4. Pellet at room temperature (RT) by centrifugation at 2500 rpm for 10 minutes in a fixed-angle rotor for a medium-speed centrifuge.
5. Wash in 500 ml of 1x TE, resuspend in 20 ml of 100 mM LiAc and 0.5x TE, and transfer to a sterile 1-liter flask.
6. Add a mixture of 1 ml of 10 mg/ml denatured salmon sperm DNA, 500 μg library DNA, and 140 ml of 100 mM LiAc, 40% PEG-3350, 1x TE to the cell suspension. Incubate for 30 minutes at 30°C.
7. Swirl the mixture with 17.6 ml of DMSO, heat shock for 6 minutes at 42°C, and immediately dilute with 400 ml of YPA (YPAD without dextrose). Cool rapidly to room temperature in a water bath.
8. Pellet the cells at 2500 rpm for 10 minutes. Wash with 500 ml of YPA, resuspend in 1 liter of YPAD, and incubate for 1 hour at 30°C with constant shaking.
9. Centrifuge the suspension at 2500 rpm for 10 minutes, wash the pellet, and incubate with 1 liter of selective medium lacking W, U, and L for 16 hours with shaking.
10. Repeat the centrifugation, wash with YPA, and incubate for 1 hour with 1 liter of YPAD.
11. Plate aliquots from the 1-liter suspension on selective yeast medium lacking U, W, and L to measure primary transformation efficiency.
12. Pellet and wash the cells twice with selective medium lacking W, U, L, and H, and resuspend the final pellet in 10 ml of this selective medium. Plate aliquots of 5 μl, 10 μl, 25 μl, and 50 μl on 40 selective plates (10 plates for each aliquot) lacking W, L, U, and H. Incubate 2–3 days until colonies appear.
13. Patch plate $His^+$ colonies onto duplicate plates lacking W, U, L, and H, and test one plate after 2 days for β-gal activity.

### *Small-Scale Library Transformation for Dual-bait Screen (SKY48/pLacGUS bait strain)*

In the following example, interactors are being identified in parallel for a LexA-fused and a cI-fused bait. See also Chapter 7.

1. Inoculate 20 ml of YC-UH Zeo200 with SKY48/pLacGUS containing the baits expressed in plasmids pHybLex/Zeo and pHybcI/HK. Grow overnight at 30°C.
2. The next day, dilute the culture into 300 ml of YC-UH Zeo200 to 2 x 10E6 cells/ml ($OD_{600}$ = ~0.10). Incubate at 30°C until the culture reaches 2 x 10E7 cells/ml ($OD_{600}$ = 1).
3. Centrifuge for 5 minutes at 1000–1500*g* in a low-speed centrifuge at room temperature to harvest cells. Resuspend in 30 ml of sterile $H_2O$ and transfer to a 50-ml conical tube.
4. Centrifuge for 5 minutes at 1000–1500*g*. Decant the supernatant and resuspend the cells in 1.5 ml of 1x LiAc, 1x TE.
5. Add 1 µg of library DNA and 50 µg of high-quality sheared salmon sperm carrier DNA to each of 30 sterile 1.5-ml microcentrifuge tubes. Add 50 µl of the resuspended yeast solution from step 4 to each tube. The total volume of library and salmon sperm DNA added should be <20 µl and preferably <10 µl.
6. Add 300 µl of sterile 1x LiAC, 40% PEG-3350, 1x TE to each tube, and invert to mix thoroughly. Incubate for 30 minutes at 30°C.
7. Add DMSO to 10% (~40 µl per tube) and invert to mix. Heat-shock for 10 minutes in a 42°C heating block.
8. Take 28 of the 30 tubes from step 7 and plate the complete contents of one tube onto a 150-mm YC-UHW Zeo200 plate and incubate for 1–2 days at 30°C.
9. For the two remaining tubes, plate 360 µl from each tube onto separate 150-mm YC-UHW Zeo200 plates. Use the remaining 40 µl from each tube to make a series of 1:10 dilutions in sterile $H_2O$. Plate dilutions on 100-mm YC-UHW Zeo200 plates. Incubate all plates for 2–3 days at 30°C until colonies appear.

   The dilution series gives an idea of the transformation efficiency and allows an accurate estimate of transformants obtained.

### *Collect Primary Transformants*

Conventional replica plating does not work well in the selection process because so many cells are transferred to new plates that very high background levels inevitably occur. Instead, the procedure described below creates a slurry in which cells derived from $>10^6$ primary transformants are homogeneously dispersed. A precalculated number of these cells are plated for each primary transformant.

10. Cool all of the 150-mm plates containing transformants from step 8 for several hours at 4°C to harden agar.
11. Wearing gloves and using a sterile cell scraper, gently scrape yeast cells off the plate. Be careful not to damage the agar. Pool the cells from the 30 plates into one or two sterile 50-ml conical tubes.

    This is the step where contamination is most likely to occur. Be careful, and if possible use a sterile hood.
12. Wash cells by resuspending the transferred cells into an equal volume of sterile TE buffer or $H_2O$. Centrifuge at 1000–1500*g* for ~5 minutes at room temperature, and discard the supernatant. Repeat the wash.
13. Resuspend the pellet in 1 volume of glycerol solution, mix well, and store up to 1 year in 1-ml aliquots at –80°C.

### *Determine Replating Efficiency*

14. Thaw an aliquot of frozen transformed yeast (step 12, above) and dilute 1:10 with YC-UHW Zeo200 Gal/Raff medium. Shake for 4 hours at 30°C to induce the *GAL1* promoter to express the library.

    Raffinose (Raff) is not required for growth, but it helps the cells to grow faster without diminishing transcription from the GAL1 promoter.

15. Make serial dilutions of the culture using the YC-UHW Zeo200 Gal/Raff medium. Plate on 150-mm YC-UHW Zeo200 Gal/Raff plates and incubate for 2–4 days at 30°C until colonies are visible.

16. Count the colonies and determine the number of colony-forming units (cfu) per aliquot of transformed yeast. If the harvest is done carefully, viability will generally be >90%.

    Some researchers perform this step simultaneously with plating on leucine- or lysine-deficient selective medium.

### *Screening for Interacting Proteins*

Not all transformants will contain interacting proteins. Therefore, plating should be done on leucine- or lysine-deficient medium. It is desirable that for actual selection, each primary colony obtained from the transformation be represented on the selection plate by 3–10 individual yeast cells. This will, in some cases, lead to multiple isolations of the same cDNA; however, because the slurry is not perfectly homogeneous, it will increase the likelihood that all primary transformants are represented by at least one cell on the selective plate.

It is easiest to visually scan for $Leu^+$ or $Lys^+$ colonies using cells plated at ~$10^6$ cfu per 150-mm plate. Plating at higher density can contribute to cross-feeding between yeast, resulting in spurious background growth. Thus, for a transformation in which $3 \times 10^6$ colonies are obtained, plate ~$1 \times 10^7$ cells on a total of 10 selective plates.

17. Thaw the appropriate quantity of transformed yeast based on the plating efficiency (calculated on previous page), dilute 1:10 with YC-UHW Zeo200 Gal/Raff medium, and incubate as in step 14.

18. Centrifuge at 1000–1500*g* for 5 minutes at room temperature and resuspend the pellet in 1 ml of YC-UHW Zeo200 Gal/Raff medium.

19. Plate 50 µl on each of 10 YC-UHWL Zeo200 Gal/Raff plates and 10 YC-UHWK Zeo200 Gal/Raff plates. Incubate for 2–3 days at 30°C until colonies appear. Carefully pick appropriate $Leu^+$ or $Lys^+$ colonies and patch on new YC-UHWL Zeo200 Gal/Raff or YC-UHWK Zeo200 Gal/Raff master plates. Incubate for 2 days at 30°C until colonies appear, and perform LacZ and GUS assays to obtain secondary reporter readouts. Note, true interactors should have a positive phenotype only on Gal/Raff plates, not on Glu plates.

### β-*Galactosidase Filter Assay*

The filter assay is applicable for a qualitative GUS assay also, but generally requires a lower substrate concentration than β-galactosidase (~20%).

1. Lay dry nitrocellulose filter onto the yeast colonies plated on selective medium.

   Remove the filter and float it colony side up in an aluminum foil boat in a thin layer of liquid nitrogen for 30 seconds. Then immerse the filter for 5 seconds in the liquid nitrogen.

2. Remove the filter and place it at room temperature, colony side up, until thawed.

3. Prepare a petri dish for the reaction. Place 1.5 ml of Z buffer containing 15 µl of a 50 mg/ml X-gal in the lid, and lay one #1 Whatman filter circle in the Z buffer, followed by the nitro-

cellulose filter with colonies facing up.

4. Use the bottom of the dish to cover and incubate the dish at 30°C (if longer incubations are required for positive signals to be detected, place the petri dish in a humidified chamber).

   Strong interactions yield detectable color in less than 30 minutes.

   Alternatively, assay β-galactosidase or β-glucuronidase activity with an overlay assay.

### *Overlay Assay (Duttweiler 1996; Golemis and Serebriiskii 1998; Gleeson et al. 1998)*

1. Add 1 g of low-melt agarose (LMA) to 100 ml of 100 mM potassium phosphate <!> buffer (pH 7.0). Dissolve the LMA by heating for 3–5 minutes in the microwave. Do not overheat the agarose because the solution will boil over.
2. Allow the agarose solution to cool to 65°C.
3. Prepare X-Gluc/DMF or X-Gal/DMF solution by adding the following amount of X-Gluc or X-Gal solution to DMF:

   For X-Gluc, add 100 µl of freshly prepared 25 mg/ml X-Gluc to 8 ml of DMF.

   For X-Gal, add 800 µl of freshly prepared 25 mg/ml X-Gal to 8 ml of DMF.
4. Mix the 8 ml of X-Gluc/DMF or X-Gal/DMF solution from step 3 with 12 ml of the dissolved LMA solution to make an X-Gluc/LMA or X-Gal/LMA solution with a final concentration of 40% DMF, 0.6% LMA. The total volume will be 20 ml.
5. Incubate the X-Gluc/LMA or X-Gal/LMA solution for 5 minutes at 65°C.
6. Carefully overlay the patched plates with the following amount of X-Gluc/LMA or X-Gal/LMA solution:

   For 100-mm plates, use 5–6 ml per plate.

   For 150-mm plates, use 12–15 ml per plate.
7. Let the plates sit at room temperature for 5–10 minutes until the X-Gluc/LMA or X-Gal/LMA solution solidifies. Do not disturb the plates during the solidification process. To prevent exposure to light, keep the plates covered with aluminum foil.
8. Incubate the plates for up to 1 hour at 30°C in the dark, but monitor the color development regularly by eye during this time.

Colonies that are positive for β-glucuronidase or β-galactosidase activity will turn blue. Monitor the color development on the overlay plates carefully within the first hour. We suggest that you check the degree of color development every 15 minutes. This is particularly important for the β-glucuronidase assay because X-Gluc degrades easily, and long incubations can lead to high background that might be interpreted as a false-positive result.

In general, the intensity and length of time that it takes for the color signal to develop should provide an indication of the strength of your positive bait–prey interaction. For a strong positive interaction between your bait and prey, you may see intense blue color develop within 15 minutes. For a weak positive interaction, the blue color may take up to an hour (GUS) or longer (β-gal) to develop.

### *Isolation of pVP16* sFv from His$^+$ β-Gal$^+$ clones*

The prey plasmid DNA from positive yeast clones can be recovered by two alternative methods. The first method was described by Ward (1990). The yeast cells are lysed with lysis buffer and glass beads, and the lysate is extracted with phenol/chloroform. An aliquot of the aqueous phase is transformed into *E. coli* and selected on LB plates with ampicillin. Alternatively, although more

expensive to use, the yield and quality of DNA obtained using a Miniprep kit is generally higher than that isolated with the method detailed above. We have used the S.N.A.P. Invitrogen Miniprep kit; other suppliers are providing specially designed kits to isolate DNA from yeast (e.g., Qiagen).

### *Method developed by Ward (1990)*

1. Grow 5 ml of a yeast overnight culture in selective medium lacking W, L, and H.
2. Pellet and resuspend cells in 300 µl of yeast lysis buffer.
3. Mix the suspension with 150-µl glass beads (0.45–0.50 mm) and 300 µl of phenol/chloroform. Vortex vigorously for 1 minute.
4. Spin the beads and phenol/chloroform, and transfer the aqueous phase to a new tube.
5. Precipitate plasmid DNA in the aqueous phase twice with ethanol, and resuspend in 25 µl of TE.
6. Transform *E. coli* cells with 1–2 µl of DNA and select on LB plates with ampicillin.

### *Isolation of Plasmid DNA from Yeast Using a MiniPrep Kit*

1. Inoculate 5 ml of YC-W with a single positive colony selected as $H^+$ or $K^+$ and incubate overnight at 30°C with shaking. The culture should be in stationary phase ($OD_{600}$ = 1–2) before proceeding further.
2. Pellet the cells in a clinical centrifuge at 2500 rpm for 5 minutes.
3. Resuspend the cell pellet in 1 ml of 1× TE and repellet the cells.
4. Resuspend the cell pellet in 1 ml of 1× TE. Add 1 µl of β-mercaptoethanol and 1.5 µl of zymolyase (3 mg/ml in $H_2O$ [Seikagaku]). Incubate for 1 hour at 30°C.
5. Centrifuge at 1000*g* for 4 minutes at room temperature to pellet the cells gently.
6. Remove the supernatant and resuspend the cell pellet in 150 µl of Resuspension Buffer containing RNase A.
7. Add 150 µl of 1% SDS. Mix gently by inversion. Incubate at 65°C for 10 minutes.
8. Place on ice for 3 minutes.
9. Add 150 µl of ice-cold precipitation salts. Mix by inverting.
10. Centrifuge at 14,000*g* for 10 minutes.
11. Remove supernatant to a new microcentrifuge tube and add 600 µl of binding buffer. Mix by inverting five or six times. Apply the entire solution onto the S.N.A.P. MiniPrep Column/Collection Tube.
12. Centrifuge the S.N.A.P. MiniPrep Column/Collection Tube at 1000–3000*g* for 30 seconds at room temperature. Discard the column flowthrough.
13. Add 900 µl of Wash Buffer. Centrifuge as in step 12.
14. Discard the column flowthrough. Centrifuge the S.N.A.P. MiniPrep Column/Collection Tube at maximum speed for 2 minutes at room temperature to dry the resin.
15. To elute the plasmid DNA, place the S.N.A.P. MiniPrep Column into a new sterile microcentrifuge tube and add 70 µl of 1× TE or sterile $H_2O$ directly to the resin.
16. Incubate for 2 minutes at room temperature.

The prey plasmid DNA can be isolated from ampicillin-resistant colonies. If the first L40-based yeast two-hybrid system was used for the library screen, the plasmid DNA needs to be analyzed by restriction digest because the bait plasmid pBTM116 also contains an ampicillin-resistance gene. The prey plasmid DNA should be retransformed into L40 or SKY48 containing the dual reporter plasmid using the small-scale transformation protocol to confirm whether or not the sFv is interacting specifically with the bait protein. The control transformations should be set up as described under the section The Yeast Two-hybrid System to Test for sFv-Antigen Interactions In Vivo, p. 427.

If true positive clones are identified, the target specificity of the sFv should be investigated by western blotting. For this purpose, the sFv can be cloned into a bacterial expression vector in frame with an expression tag, e.g., myc-tag. The sFv fusion can be expressed in bacteria and the periplasmic preparation can be used to detect the antigen on western blots, detecting the epitope tag with a specific horseradish peroxidase (HRP)-conjugated antibody (Pörtner-Taliana et al. 2000). The in vivo targeting capabilities can also be verified with a mammalian two-hybrid assay (Pörtner-Taliana et al. 2000). If neutralizing sFvs are needed, the inhibiting function of the sFv should be tested with assays specific for the proteins of interest.

17. Centrifuge the S.N.A.P. MiniPrep Column/Collection Tube at maximum speed for 2 minutes at room temperature. The plasmid DNA is now eluted from the column. Remove and discard the column.
18. Transform competent *E. coli* with 10 µl of the DNA suspension and plate out the whole transformation on LB plates containing 50–100 µg/ml ampicillin.

Protocol 2

# Mammalian Transient Transfection

This assay allows the sFv–antigen interaction to be studied in a mammalian host cell. This protocol was adapted from Griffiths et al. (1997) and Pörtner-Taliana et al. (2000).

## MATERIALS

### Buffers and Reagents

β-Galactosidase (RSV)
Growth medium
Lipofectin or Lipofectamine (LTI)
Luciferase assay (Promega)
OptiMEM
Perfect Lipid (pFx-7 for CHO cells; pFx-1 for COS cells) (Invitrogen)
Reporter lysis buffer (Promega)
Tropix β-gal assay (Bedford, Massachusetts)

### Cells

CHO and COS cells

### Plasmids

Lex-op TK pGL Basic luciferase plasmid
PcDNA3.1

### Special Equipment

Incubator
Luminometer (Berthold Lumat LB9501)
Plates (6-well)

## METHOD

1. The day prior to transfection, seed CHO and COS cells with $1 \times 10^5$ cells/well in a 6-well plate.
2. For each well, mix 75 μl of OptiMEM (LTI), 12 μl of Perfect Lipid (pFx-7 for CHO cells or pFx-1 for COS cells) with 1 μg of lex-op TK luciferase plasmid, 250 ng of RSV β-gal, and a low but constant bait plasmid concentration (e.g., 25 ng). The transfection reagents can be replaced by similar transfection reagents such as Lipofectin or Lipofectamine.

3. Add to increasing amounts of sFv-VP16 expression plasmid/well and add empty (e.g., bacterial plasmid DNA) to a final DNA concentration/well of 4 μg. Prepare duplicates or triplicates of every sample.
4. Mix well and incubate for 15 minutes at room temperature.
5. Replace the growth medium with serum-free medium or OptiMEM.
6. Add 75 μl of mixture per well.
7. Incubate for 4 hours in a 37°C incubator.
8. Replace the medium with growth medium.
9. Harvest 44 hours posttransfection by scraping cells into 150 μl of reporter lysis buffer.
10. Use 5–20 μl of sample for the luciferase assay using a kit and 10 μl of sample for the β-gal assay. Obtain readouts for both enzymes with a luminometer.
11. Normalize luciferase readouts to β-gal readouts.

    For the success of the experiment, it is essential to determine the optimal bait/prey ratio. We had good results starting with a low constant bait plasmid concentration and titering the prey plasmid concentration (compare to 2). Under these conditions, the normalized luciferase activity can increase 5–20-fold. This can vary depending on the cell line, bait prey ratio, and the strength of the particular protein–sFv interaction.

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