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Mammalian MATCHMAKER Two-Hybrid Assay Kit User Manual

PT3002-1 (PR17119)

Published 18 September 2001

Catalog #: K1602-1

See List of Components for storage conditions

FOR RESEARCH USE ONLY

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I. Introduction

The Mammalian MATCHMAKER Two-Hybrid Assay Kit provides a complete set of vectors for performing two-hybrid assays in mammalian cells. Two-hybrid assays are based on the fact that many eukaryotic transcriptional activators consist of two physically and functionally separable domains: a DNA-binding domain (DNA-BD) that specifically binds to a promoter or other *cis*-regulatory element, and an activation domain (AD) that directs RNA polymerase II to transcribe the gene downstream of the DNA-binding site. While these domains may be part of the same protein (as in the case of the native yeast GAL4 protein), they can also function as two separate proteins—as long as the AD is tethered to a DNA-BD bound to the promoter. In two-hybrid assays, that tether is the interaction between two additional proteins (X and Y) that are expressed as protein fusions to the AD and DNA-BD peptides, respectively.

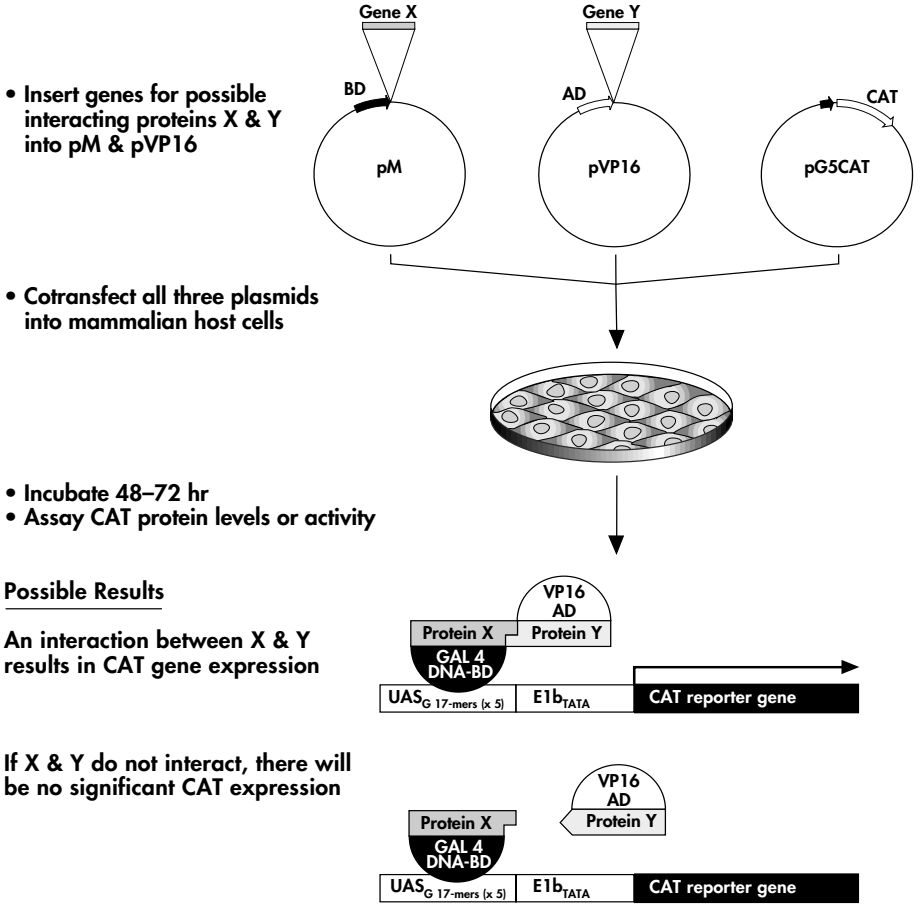

Figure 1. Using the Mammalian MATCHMAKER Two-Hybrid Assay Kit.

I. Introduction *continued*

In the Mammalian MATCHMAKER Two-Hybrid Assay (Figure 1), the pM cloning vector is used to generate fusions of some protein X to the GAL4 DNA-BD. Similarly, pVP16 is used to construct fusions of some protein Y to an AD derived from the VP16 protein of herpes simplex virus. pG5CAT is a reporter vector which contains the CAT gene downstream of five consensus GAL4 binding sites and the minimal promoter of the adenovirus E1b gene (Figure 1). The minimal E1b promoter will not express significant levels of the CAT gene, so background is low in the absence of activation from the GAL4 sites. Once you have constructed your two hybrid plasmids, all three plasmids are cotransfected into a suitable mammalian host cell line. 48–72 hours later, the interaction between proteins X and Y is assayed by measuring CAT gene expression.

Once an interaction between two proteins has been detected in mammalian cells, the Mammalian Two-Hybrid Assay is a powerful tool for functionally analyzing that interaction using deletional or site-directed mutagenesis (Table I). The Mammalian MATCHMAKER Two-Hybrid Assay is also useful for confirming the relevance of protein-protein interactions identified via yeast two-hybrid screens using yeast-based two-hybrid systems such as CLONTECH's MATCHMAKER Two-Hybrid Systems and cDNA and Genomic Libraries. Such confirmation eliminates the possibility of a false positive that is an artifact of working in yeast cells. For more information on yeast two-hybrid technology, see Fields & Sternglanz (1994), Bartel *et al.* (1993) or the MATCHMAKER Two-Hybrid System 3 User Manual (PT3247-1) available at **www.clontech.com**.

TABLE I. EXAMPLES OF PROTEIN-PROTEIN INTERACTIONS CHARACTERIZED WITH MAMMALIAN TWO-HYBRID ASSAYS

GAL4 DNA-BD Hybrid	VP16 AD Hybrid	Cell Type	Reference
E2F-1	Rb	Saos-2	Fagan, R., *et al.* (1994) *Cell* **78**:799–811
E2F-1	Adenovirus E4 protein	Saos-2	Fagan, R., *et al.* (1994) *Cell* **78**:799–811
FOS	JUN	CHO	Dang, C. V., *et al.* (1991) *Mol. Cell. Biol.* **11**:954–962
LCK	CD4	CHO	Fearon, E. R., *et al.* (1992) *Proc. Natl. Acad. Sci. USA* **89**:7958–7962
R6-K	R6-K	CV1	Vasavada, H. A., *et al.* (1991) *Proc. Natl. Acad. Sci. USA* **88:**10686–10690
TAL1	E47	Jurkat	Hsu, H., *et al.* (1994) *Proc. Natl. Acad. Sci. USA* **91**: 3181–3185
VSV protein N	VSV protein PI	CHO	Takacs, A. M., *et al.* (1993) *Proc. Natl. Acad. Sci. USA* **90**:10375–10379

II. List of Components

Store all components at –20°C. Refer to Appendix B for maps and detailed descriptions of the cloning and reporter vectors.

- 20 µl **pM** GAL4 DNA-binding domain cloning vector (0.5 µg/µl)
  3.5-kb DNA-BD cloning vector used to express a fusion of a test protein with the GAL4 DNA-BD
- 20 µl **pVP16** activation-domain cloning vector (0.5 µg/µl)
  3.3-kb AD cloning vector used to express a fusion of a test protein with VP16 AD, a herpes virus protein that acts as a transcriptional activator in mammalian cells
- 20 µl **pG5CAT** mammalian reporter vector (0.5 µg/µl)
  4.5-kb CAT reporter plasmid for cotransfection into mammalian cells with recombinant plasmids derived from pM and pVP16
- 20 µl **pM3-VP16** positive control vector (0.5 µg/µl)
  4.4-kb positive control plasmid that expresses a fusion of the GAL4 DNA-BD to the VP16 AD
- 20 µl **pM-53** positive control vector (0.5 µg/µl)
  4.6-kb positive control plasmid that expresses a fusion of the GAL4 DNA-BD to the mouse p53 protein
- 20 µl **pVP16-T** positive control vector (0.5 µg/µl)
  5.3-kb positive control plasmid that expresses a fusion of the VP16 AD to the SV40 large T-antigen, which is known to interact with p53
- 20 µl **pVP16-CP** negative control vector (0.5 µg/µl)
  4.5-kb negative control plasmid that expresses a fusion of the VP16 AD to a viral coat protein, which does not interact with p53

III. Additional Materials Required

For mammalian cell culture and harvesting

- **Cell culture medium** (e.g., Dulbecco's modified Eagle medium [DMEM] or another appropriate growth medium for mammalian cells in culture)
- **Fetal bovine serum, newborn calf serum, or equivalent** (to supplement the growth medium)
- **Phosphate buffered saline (PBS) (pH 7.4)**

Component	Final Conc.	Amount for 2 L
Na_2HPO_4	58 mM	16.5 g
NaH_2PO_4	17 mM	4.1 g
NaCl	68 mM	8.0 g

Dissolve the above components in 1.8 L of deionized H_2O. Adjust to pH 7.4 with 0.1N NaOH. Add deionized H_2O to final volume of 2 L. Store at room temperature.

- **1X Trypsin/EDTA** (LTI #25300-054)

For Mammalian Cell Transfection

Reagents will depend on which transfection method you use. We generally use calcium-phosphate-mediated transfection for Mammalian MATCHMAKER Two-Hybrid experiments and recommend using the CalPhos™ Mammalian Transfection Kit (#K2051-1). For liposome-mediated transfection, we recommend CLONfectin Transfection Reagent (#8020-1). See Appendix A for recipes and protocols. Protocols for other methods can be found in Freshney (1993) and Ausubel *et al.* (1994).

For CAT Assay

Reagents will vary depending on which CAT assay you choose. Although radioactive CAT assays are common, there are several nonradioactive alternatives that are easy to perform and provide quantitative data. At CLONTECH, we generally measure CAT protein directly in Mammalian MATCHMAKER Two-Hybrid experiments using the CAT ELISA Kit from Boehringer Mannheim (#1 363 727). Nonradioactive enzymatic CAT assays such as the FAST CAT® (Deoxy) Chloramphenicol Acetyltransferase Assay Kits from Molecular Probes, Inc. (phone #: 1-503-344-3007) are also available. Protocols for other CAT assays can be found in Ausubel *et al.* (1994).

IV. Mammalian Two-Hybrid Assay Protocols

PLEASE READ THROUGH THE ENTIRE PROTOCOL BEFORE BEGINNING.

A. Transformation of Plasmids into *E. coli* and Plasmid Isolation

1. Transform each of the plasmids provided in this kit into a suitable *E. coli* host strain (e.g., DH5α) to ensure that you have a renewable source of DNA.
2. You will need to perform large-scale plasmid preparations of any plasmid that will be introduced into mammalian cells (e.g., your hybrid gene constructs derived from pM and pVP16, pG5CAT, and the control plasmids). To insure the purity of the DNA, isolate all plasmids for transfection by banding on CsCl gradient (Sambrook *et al.*, 1989), or by an equivalent method. For optimal results, you may wish to purify twice on CsCI gradients.

B. Construction of Plasmids Expressing Hybrid Proteins

Construct fusion genes using standard molecular biology techniques. A brief outline of the protocol is given below (for more detailed information, see Sambrook *et al.*, 1989). The gene of test protein X is fused to the GAL4 DNA-BD in the pM vector. The gene of test protein Y is fused to the VP16 AD in the pVP16 vector. The orientation and reading frame of both fusions must be correct for hybrid proteins to be expressed. The GAL4 Binding Domain Sequencing Primer (#6474-1) can be used to sequence across the DNA BD/protein X junction in pM-derived clones. The VP16 Sequencing Primer (#6469-1) is designed for sequencing across the VP16 AD/protein Y junction in pVP16-derived clones.

In some cases, the fusion gene can be generated using compatible restriction sites that are present in the test gene and the cloning vector. Many of the sites in pM are in the same reading frame as in the yeast two-hybrid vectors pGBT9, pAS2-1, and pLexA (see map, Appendix B). If no such sites are present, the gene fragment can be generated by PCR with useful restriction sites incorporated into the primers (Scharf, 1990). Often a restriction site at the end of the gene of interest can be changed into a different site or put into a different reading frame by using a PCR primer which incorporates the desired restriction site at the desired place.

1. Purify the gene fragment, whether generated by PCR or cut out of a plasmid, by any standard method (Sambrook *et al.*, 1989).
2. Digest pM (or pVP16) with the appropriate restriction enzyme(s), treat with phosphatase, and purify.
3. Ligate pM (or pVP16) and the insert encoding protein X (or protein Y).
4. Transform ligation mixtures into *E. coli* to maintain a working stock of the recombinant plasmid.
5. Identify insert-containing plasmid by restriction analysis.
6. Check orientation and reading frame by sequencing fusion junctions.

IV. Mammalian Two-Hybrid Assay Protocols *continued*

C. Guidelines for Transfection of Mammalian Cells

Plasmids may be cotransfected into mammalian cells by any standard transfection method. At CLONTECH, we typically use calcium phosphate (Chen & Okayama, 1988) and recommend the CalPhos Mammalian Transfection Kit (#K2051-1) for mammalian transfections. A protocol is provided in Appendix A. For liposome-mediated transfection, we recommend CLONfectin Transfection Reagent (#8020-1). DEAE-dextran (Rosenthal, 1987) also works well in our hands. Useful general references on cell culture techniques are:

- *Culture of Animal Cells,* Third Edition, ed. by R. I. Freshney (1993, Wiley-Liss; available from CLONTECH Press, #V2128-1)
- *Current Protocols in Molecular Biology*, ed. by F. M. Ausubel *et al.* (1994, Greene Publishing Associates and Wiley & Sons)

The efficiency of transfection for different cell lines may vary by several orders of magnitude. A method that works well for one host cell line may be inferior for another. Therefore, when working with a cell line for the first time, you may want to compare the efficiencies of several transfection protocols. This can be done by cotransfecting the pG5CAT and pM-VP16 plasmids and assaying for CAT activity. Alternatively, you can transfect the host cell line with one of CLONTECH's Great EscAPe™ SEAP reporter vectors (#K2040-1), and assaying for reporter gene activity.

After a method of transfection is chosen, it may be necessary to optimize parameters such as cell density, the amount and purity of the DNA, media conditions, and transfection time. Once optimized, these parameters should be kept constant to obtain reproducible results. With each method, CAT activity may be detected 48–72 hr after transfection, depending on the host cell line used.

For rapid, qualitative results, we generally obtain reproducible results using single transfections. For quantitative data, we recommend that you perform duplicate or triplicate transfections and average the results. You may also wish to normalize for transfection efficiency by cotransfecting a constant amount of a second reporter under the control of a constitutive promoter. The values obtained in each sample for the primary reporter (i.e., CAT) are then normalized to the values obtained for the second reporter in the same sample. Useful vectors for this purpose are CLONTECH's pCMVβ (#6177-1), pβgal-Control (#6047-1), or pSEAP-Control (#6043-1).

IV. Mammalian Two-Hybrid Assay Protocols *continued*

Table II describes the basic set-up for Mammalian MATCHMAKER Two-Hybrid experiments, including positive and negative controls.

TABLE II. RECOMMENDED SET-UP FOR MAMMALIAN TWO-HYBRID ASSAYS

Input DNA: Transfection	GAL4 DNA-BD Plasmid (10 µg [a])	VP16 AD Plasmid (10 µg [a])	Reporter Plasmid (2 µg [a])	CAT Protein or CAT Activity
1 (experiment)	pM-insert X	pVP16-insert Y	pG5CAT	to be determined
2 (untransfected control)	none	none	none	– [b]
3 (basal control)	pM	pVP16	pG5CAT	– [c]
4 (X control)	pM-insert X	pVP16	pG5CAT	to be determined [d]
5 (Y control)	pM	pVP16-insert Y	pG5CAT	to be determined [e]
6 (positive control)	pM3-VP16 [f]	(pM3-VP16)	pG5CAT	+++

[a] These amounts of DNA are optimized for calcium-phosphate mediated transfection of HeLa cells.When using other transfection methods, adjust the total amount of DNA accordingly, but maintain the ratio of the different plasmids. The optimal amount of DNA may also vary with different cell types.

[b] This value is the background CAT signal in your cells. For quantitative CAT assays, subtract this value from all other experimental values.

[c] This control (or a control using just pM and pG5CAT) provides the basal expression level of CAT protein or CAT activity in your experiments. To determine the fold-activation caused by an interaction between protein X and protein Y, divide your experimental CAT values by this number. For example, if you used the Boehringer Mannheim CAT ELISA assay and obtained an absorbance of 1.5 with your experimental example and an absorbance of 0.05 with this negative control, you would conclude that the interaction between protein X and protein Y caused a 30-fold activation of CAT expression. This sort of analysis is particularly useful when comparing multiple protein interactions (e.g., the interactions between protein X and a series of protein Y mutants.)

[d] Critical control to determine whether or not your protein X functions autonomously as a transcriptional activator. The level of CAT should be similar to the background value obtained in transfection 3.

[e] Critical control to determine whether or not your protein Y functions autonomously as a DNA-BD or binds directly to the DNA-BD encoded by pM. The level of CAT should be similar to the background value obtained in transfection 3.

[f] Use 20 µg when transfecting *only* a DNA-BD or AD vector with pG5CAT. The pM3-VP16 positive control plasmid encodes a fusion of the GAL4 DNA-BD and the VP16 AD and therefore gives very

IV. Mammalian Two-Hybrid Assay Protocols *continued*

D. Positive Control Experiment

Perform the positive control experiment described in Table III to confirm that the mammalian two-hybrid assay works in your hands and in your cells, and to optimize your transfection and CAT assay protocols. This experiment compares the interaction between the p53 protein and two other proteins: the SV40 large T-antigen, which is known to interact with p53; and a polyoma virus coat protein (CP), which does not interact with p53. All the plasmids in Table III are included in this kit.

TABLE III. POSITIVE CONTROL MAMMALIAN TWO-HYBRID EXPERIMENT

Input DNA: Transfection	GAL4 DNA-BD Plasmid (10 µg [a])	VP16 AD Plasmid (10 µg [a])	Reporter Plasmid (2 µg [a])	CAT Protein or CAT Activity
1A (positive control)	pM-53	pVP16-T	pG5CAT	+++
1B (negative control)	pM-53	pVP16-CP	pG5CAT	–
2 (untransfected control)	none	none	none	– [b]
3 (basal control)	pM	pVP16	pG5CAT	– [c]
4 (negative control)	pM-53	pVP16	pG5CAT	– [d]
5 (negative control)	pM	pVP16-T	pG5CAT	– [e]
6 (positive control)	pM3-VP16 [f]	(pM3-VP16)	pG5CAT	+++

[a–f] See Table II (previous page).

E. Recommendations for Performing CAT Assays

There are many alternatives for performing CAT assays. At CLONTECH, we generally measure CAT protein directly in Mammalian MATCHMAKER Two-Hybrid experiments using the CAT ELISA Kit from Boehringer Mannheim (#1 363 727). CAT activity can be measured nonisotopically with the FAST CAT® kit from Molecular Probes, Inc. (phone #: 1-503-344-3007). Protocols for other CAT assays can be found in Ausubel *et al.* (1994).

IV. Mammalian Two-Hybrid Assay Protocols *continued*

F. Further Experiments

1. Verification of Hybrid Protein Expression [Optional]

   Expression of the GAL4 DNA-BD/Protein X fusion in mammalian cells can be verified by preparing Western blots using soluble protein extracts from the cells. Probe the blots with CLONTECH's GAL4 DNA-BD-specific monoclonal antibody (#5399-1) using standard Western blotting procedures (Harlow & Lane, 1988; Sambrook *et al.,* 1989).

2. Mapping Protein Structure

   Once an interaction between two proteins has been detected in mammalian cells, the Mammalian Two-Hybid Assay provides a powerful tool for functionally dissecting that interaction using deletional or site-directed mutagenesis. CLONTECH's Transformer™ Site-Directed Mutagenesis Kit (#K600-1) is useful for generating mutant proteins that can then be tested in the Mammalian Two-Hybrid Assay:

3. Confirmation in Yeast MATCHMAKER Two-Hybrid System

   Interactions that are detected in the Mammalian Two-Hybrid Assay may also be detectable in a yeast-based two-hybrid assay such as CLONTECH's MATCHMAKER Two-Hybrid System 3 (#K1612-1). Much less DNA is required for each two-hybrid experiment in yeast. Furthermore, yeast two-hybrid assays are much less expensive, since they do not require tissue-culture facilities and supplies for the growth and transfection of mammalian cells. For more information on yeast two-hybrid technology, see the references by Fields & Sternglanz (1994) and Bartel *et al.* (1993) or the MATCHMAKER Two-Hybrid System 3 in User Manual (PT3247-1) available at **www.clontech.com**.

V. References

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VI. Related Products

Product	Cat. #
• CalPhos™ Mammalian Transfection Kit	K2051-1
• Transformer™ Site-Directed Mutagenesis Kit	K1600-1
• GAL4 AD Monoclonal Antibody	5398-1
• GAL4 DNA-BD Monoclonal Antibody	5399-1
• MATCHMAKER AD LD-Insert Screening Amplimer Set	9103-1
• MATCHMAKER One-Hybrid System	K1603-1
• MATCHMAKER Two-Hybrid System 3	K1612-1
• MATCHMAKER Two-Hybrid System	K1605-1
• MATCHMAKER cDNA and Genomic Libraries	many
• YEASTMAKER™ Yeast Transformation Kit	K1606-1

Appendix A: Calcium Phosphate Transfection Protocol

A. Materials Required

We recommend using the CalPhos Mammalian Transfection Kit (#K2051-1) for mammalian transfection. Follow the protocol in the User Manual provided.

The recipes and calcium phosphate transfection protocol below are adapted from *Current Protocols in Molecular Biology*, Supplement 14, Section 9.1.3.

- **2X BES-buffered solution (BBS, pH 6.95)**

Component	Final Conc.
N, N-bis (2-hydroxyethyl)-2-aminoethane-sulfonic acid (BES; CALBIOCHEM)	50 mM
NaCl	280 mM
Na_2HPO_4	1.5 mM

It is critical that the pH of this solution be between pH 6.95 and 6.98. We recommend that you check each new batch of 2X BES buffer against a reference stock prepared (and tested) earlier.

Filter sterilize through a 0.45-µm nitrocellulose filter (Nalgene).

Store in aliquots at –20°C (can be frozen and thawed repeatedly).

- **2.5 mM $CaCl_2$**

Add 183.7 g of $CaCl_2$ dihydride (Sigma; tissue culture grade) to 500 ml of H_2O.

Filter sterilize through a 0.45-µm nitrocellulose filter (Nalgene).

Store at –20°C in 10-ml aliquots (can be frozen and thawed repeatedly).

B. Protocol for Calcium Phosphate Transfection

All plasmids should be CsCl-banded and diluted to a concentration of 1.0 µg/µl. Store the DNA solution at 4°C. For initial experiments, we recommend that each transfection be performed with a total of 22 µg of DNA (as shown in Tables II and III). However, the optimal concentration of DNA may vary with different cell types.

Day 1

1. For each transfection, seed 5×10^5 exponentially growing cells in a 10-cm tissue culture plate in 10 ml of complete medium (with serum). There should be $< 10^6$ cells/plate (~30% confluency) just prior to transfection. This provides enough surface area on the plate for at least two more doublings.

Appendix A: Calcium Phosphate Transfection...*continued*

Day 2

2. Two hours before transfection, replace the medium with fresh medium (with serum) to stimulate cell growth.
3. For each transfection, combine the DNAs in a sterile 1.5-ml microcentrifuge tube.
4. Bring the total volume to 450 µl by adding TE or deionized H_2O.
5. Add 50 µl of 2.5 M $CaCl_2$ and vortex thoroughly. (The final concentration of $CaCl_2$ is 0.25 M.)
6. Add 500 µl of 2X BBS, mix well, and incubate at room temperature for 10–20 min to allow a precipitate to form.
7. Add the calcium phosphate-DNA solution dropwise to the medium while swirling the plate.
8. Incubate overnight in a 37°C, 3% CO_2 incubator.

   Note: The level of carbon dioxide is critical. We recommend using a Fyrite gas analyzer to measure percent CO_2 prior to incubation.

Day 3

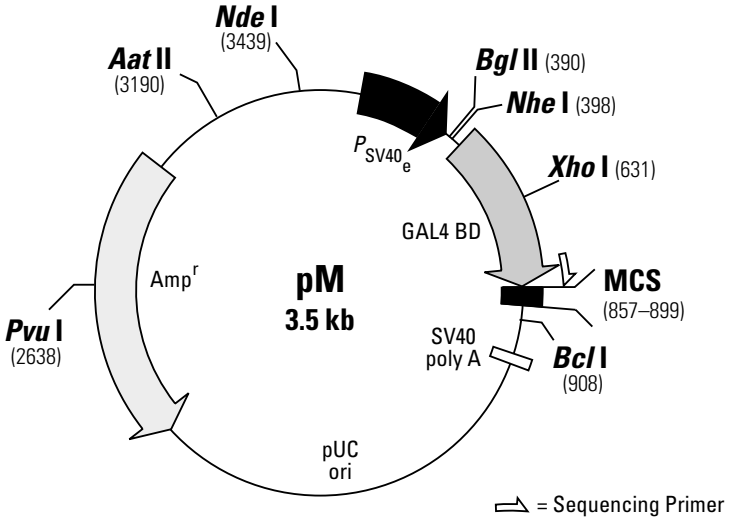
9. Carefully wash the cells twice with 5 ml of PBS, then add 10 ml of complete medium (with serum).
10. Incubate the cells for an additional 24–48 hr.

Harvesting and lysing

The protocol for harvesting and lysing cells will depend on the particular CAT assay and cell type being used. If you are using a commercial kit, please consult the manufacturer's recommendations. Protocols for several other CAT assays can be found in *Current Protocols in Molecular Biology* by Ausubel *et al.* (1994).

Troubleshooting note: The amounts of DNA in Tables II and III have been optimized for calcium phosphate transfection of HeLa cells; however, the optimal amount of total DNA may vary with different cell types. (The ratio of input DNAs should stay the same.) To determine the optimum amount of plasmid, transfect three plates of cells with 10, 20, and 30 µg of plasmid DNA and incubate overnight. The next day examine the plates with a microscope at 100X. A coarse, clumpy precipitate will form at DNA concentrations that are too low, a fine (almost invisible) precipitate will form at concentrations that are higher than optimal, and an even, granular precipitate will form with optimal DNA concentrations.

Appendix B: Plasmid Maps & Multiple Cloning Sites



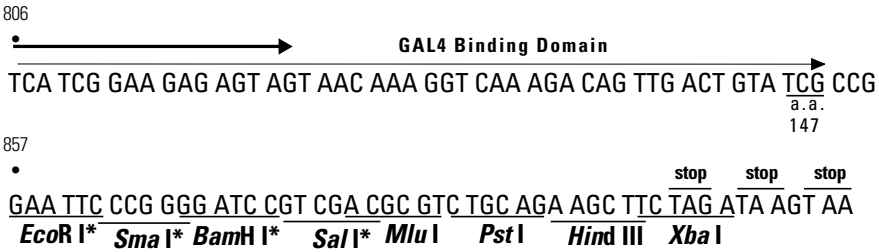
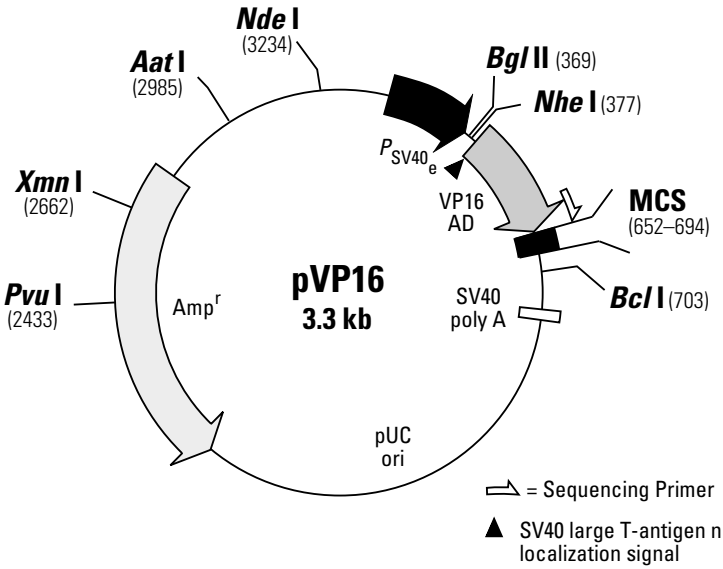


Figure 2. pM map and multiple cloning site (MCS). pM is used to generate a fusion of the GAL4 DNA-BD (amino acids 1–147) and a protein of interest. The hybrid protein is targeted to the cell's nucleus by the GAL4 nuclear localization sequence (Silver *et al.,* 1984). Genes encoding test proteins should be cloned, *in the correct orientation and reading frame,* into one of the unique restriction sites in the MCS region at the 3' end of the GAL4 DNA-BD. Restriction sites marked with an (*) are in the same reading frame as pGBT9 and pAS2-1. Sites marked with an (*) and *Pst* I are in the same reading frame as pLexA. Transcription is initiated from the constitutive SV40 early promoter (P); transcription is terminated at the SV40 poly A transcription termination signal.

Appendix B: Plasmid Maps & MCS *continued*



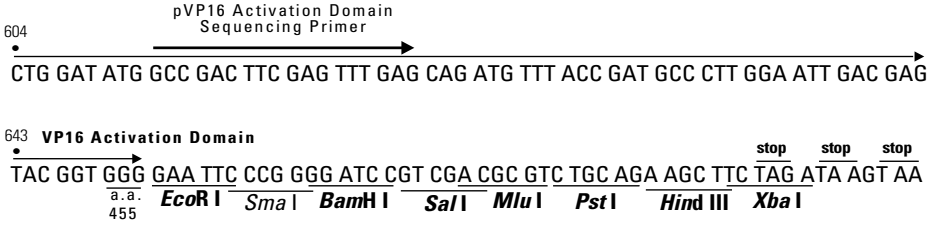


Figure 3. pVP16 map and multiple cloning site (MCS). Unique sites are in bold. pVP16 is used to generate a fusion of the VP16 AD (amino acids 411–455) and a protein of interest. The hybrid protein is targeted to the cell's nucleus by the SV40 nuclear localization sequence. Genes encoding test proteins should be cloned, *in the correct orientation and reading frame*, into one of the unique restriction sites in the MCS region at the 3' end of the VP16 AD. Transcription is initiated from the constitutive SV40 early promoter (P); transcription is terminated at the SV40 poly A transcription termination signal.

Appendix B: Plasmid Maps & MCS *continued*

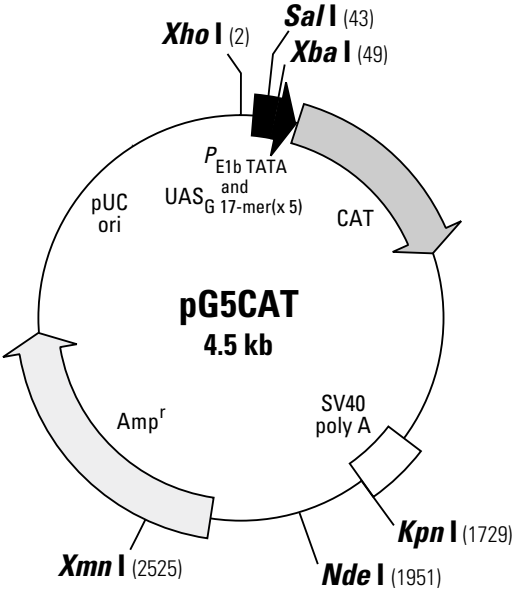


Figure 4. pG5CAT reporter vector map. pG5CAT contains five consensus GAL4 binding sites ($UAS_{G\ 17\text{-}mer\ (x\ 5)}$) and an adenovirus E1b minimal promoter upstream of the chloramphenicol acetyltransferase (CAT) gene. As seen in Figure 1 in the Introduction, the DNA-BD portion of a hybrid protein expressed from a pM-derived plasmid localizes to the GAL4 binding sites in pG5CAT. If the hybrid test protein X interacts with test protein Y (expressed as a hybrid protein from a pVP16-derived plasmid), the CAT gene will be transcribed.

Notes