Technical Manual

CheckMate™ Mammalian Two-Hybrid System

INSTRUCTIONS FOR USE OF PRODUCT E2440.

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CheckMate™ Mammalian Two-Hybrid System

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1. Description

Two-hybrid systems are extremely powerful ways of detecting protein:protein interactions in vivo. The modular domains found in some transcription factors form the basis for two-hybrid systems. These domains include a DNA-binding domain, which binds to a specific DNA sequence, and a transcriptional activation domain, which interacts with the basal transcriptional machinery (1). A transcriptional activation domain in association with a DNA-binding domain may promote the assembly of RNA polymerase II complexes at the TATA box and increase transcription. In the CheckMate™ Mammalian Two-Hybrid System[a–d], the DNA-binding domain and the transcriptional activation domain, produced by separate plasmids, become closely associated when one protein ("X") fused to a DNA-binding domain interacts with a second protein ("Y") fused to a transcriptional activation domain. In this system, interaction between proteins X and Y results in transcription of the firefly luciferase reporter gene (Figure 1).

Originally developed in yeast (2,3), the two-hybrid system has been adapted for use in mammalian cells (4,5). One major advantage of the CheckMate™ Mammalian Two-Hybrid System over yeast systems is that mammalian protein interactions can be studied in an environment that is more similar to that in vivo. The CheckMate™ System can be used with the Dual-Luciferase® Reporter Assay System (Cat.# E1910), which makes quantitation of luciferase reporter genes a rapid and easy process.

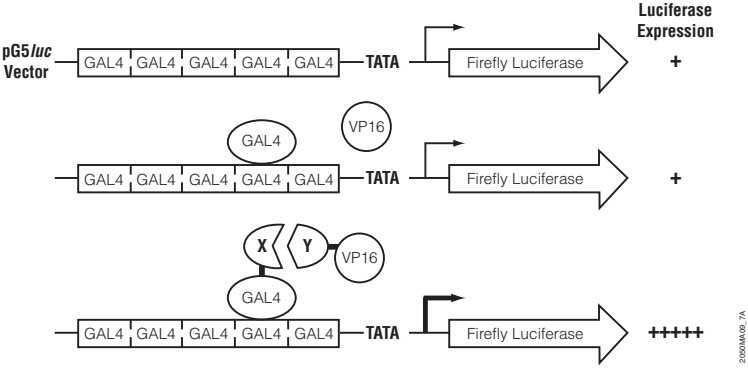

Figure 1. Schematic representation of the CheckMate™ Mammalian Two-Hybrid System. The pG5*luc* Vector contains five GAL4 binding sites upstream of a minimal TATA box, which in turn is upstream of the firefly luciferase gene. In negative controls, the background level of luciferase is measured in the presence of GAL4 (from pBIND) and VP16 (from pACT). Interaction between the two test proteins, expressed as GAL4-X and VP16-Y fusion constructs, results in an increase in luciferase expression over the negative controls.

In the CheckMate™ Mammalian Two-Hybrid System, the pBIND Vector contains the yeast GAL4 DNA-binding domain upstream of a multiple cloning region. The pACT Vector contains the herpes simplex virus VP16 activation domain upstream of a multiple cloning region. In addition, the pBIND Vector expresses the *Renilla reniformis* luciferase under the control of the SV40 promoter, which allows the user to normalize for differences in transfection efficiency. Two genes encoding two potentially interactive proteins of interest are cloned into the pBIND and pACT Vectors to generate fusion proteins with the DNA-binding domain of GAL4 and the activation domain of VP16, respectively. The pG5*luc* Vector contains five GAL4 binding sites upstream of a minimal TATA box, which in turn is upstream of the firefly luciferase gene (*luc*+). The pGAL4 and pVP16 fusion constructs are transfected along with the pG5*luc* Vector into mammalian cells. Two to three days after transfection, the cells are lysed, and the amount of *Renilla* luciferase and firefly luciferase are quantitated using the Dual-Luciferase® Reporter Assay System. Interaction

between the two test proteins, expressed as GAL4 and VP16 fusion constructs, results in an increase in firefly luciferase expression as compared to the negative controls.

The CheckMate™ Mammalian Two-Hybrid System provides two positive control vectors that encode and express two proteins known to interact in vivo. The pBIND-Id and pACT-MyoD Control Vectors encode GAL4:Id and VP16:MyoD fusion proteins, respectively.

Applications of the CheckMate™ Mammalian Two-Hybrid System include confirming suspected interactions between two proteins, identifying residues/domains involved in protein:protein interactions and identifying small molecules that affect protein:protein interactions. When identifying residues/domains involved in an interaction, the GeneEditor™ in vitro Site-Directed Mutagenesis System (Cat.# Q9280) is a useful tool for making site-directed mutants.

2. Product Components and Storage Conditions

Product	Cat.#
CheckMate™ Mammalian Two-Hybrid System	E2440

Each system includes:

- 20 µg pBIND Vector
- 20 µg pACT Vector
- 20 µg pBIND-Id Control Vector
- 20 µg pACT-MyoD Control Vector
- 2 × 20 µg pG5*luc* Vector

Storage Conditions: Store the vectors at –20 °C.

3. General Considerations

3.A. pACT Vector

The pACT Vector is a high-copy plasmid in which the human cytomegalovirus (CMV) immediate early promoter drives expression of the herpes virus VP16 activation domain (amino acids 411–456). A chimeric intron is located 5′ of the gene segment, and a multiple cloning region is located 3′ of the gene segment for insertion of cDNA clones of interest (Figures 2 and 3). The presence of this chimeric intron, in context with the CMV promoter and polyadenylation signal, can result in increased protein expression of cDNA genes linked to these elements (6). The stop codons and SV40 late polyadenylation region, demonstrated to be efficient for mRNA production (7), are at the 3′ end of the fusion gene. The fusion gene region is flanked by T7 and T3 RNA polymerase promoters for the synthesis of sense and antisense RNA products. The T7 promoter allows the construct to be translated using the TnT® T7 Quick Coupled Transcription/Translation System (Cat.# L1170). Also located on this vector is the neomycin phosphotransferase gene (from Tn5) driven by the SV40 early promoter and followed by a synthetic polyadenylation cassette. This gene confers resistance to the neomycin analog, G418 (Geneticin®; 8). The plasmid backbone contains an f1 origin of replication for the production of single-stranded DNA (ssDNA) and the β-lactamase (Amp^r) gene for selection of the vector DNA in *E. coli*.

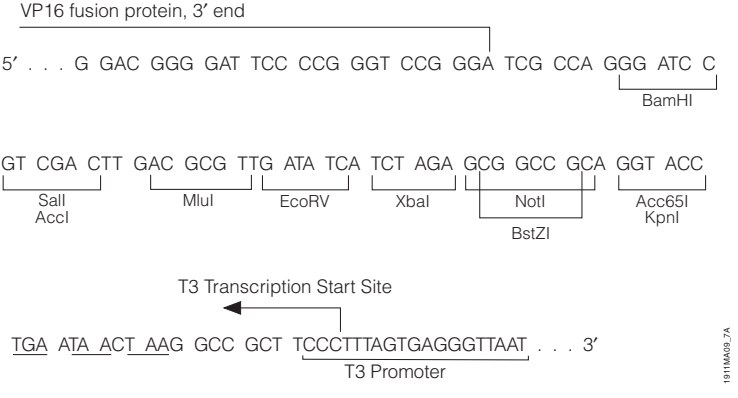

Figure 2. pACT Vector multiple cloning region. Bases listed in triplets correspond to codons in the open reading frame of the VP16 fusion protein. Stop codons (underlined) in three different reading frames are located downstream of the multiple cloning region and before the T3 promoter sequences.

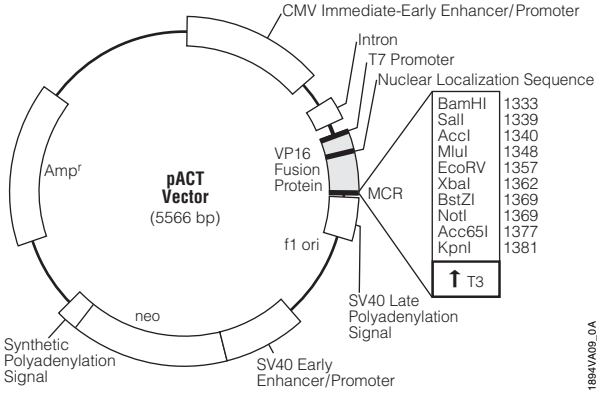

Figure 3. pACT Vector circle map and sequence reference points.

pACT Vector sequence reference points:

CMV immediate-early enhancer	1–659
CMV immediate-early promoter	669–750
chimeric intron	890–1022
T7 EEV sequencing primer binding site	1053–1074
T7 RNA polymerase promoter (–17 to +2)	1067–1085
GAL4 1–11 amino acids	1116–1148
VP16 fusion protein	1188–1325
multiple cloning region	1333–1382
T3 RNA polymerase promoter (–16 to +3)	1402–1420
SV40 late polyadenylation signal	1429–1650
phage f1 origin of replication	1693–2148
SV40 early enhancer/promoter	2181–2526
SV40 minimum origin of replication	2424–2489
neomycin (neo) phosphotransferase coding region	2571–3365
synthetic polyadenylation signal	3429–3477
β-lactamase (Amp^r) coding region	3874–4734

Use the T7 EEV Promoter Primer (Cat.# Q6700) for sequence analysis of the pACT Vector. Do not use the T7 Promoter Primer (Cat.# Q5021) to sequence the pACT Vector. There is a sequence difference between the primer and the promoter sequences.

3.B. pBIND Vector

The pBIND Vector is a high-copy plasmid in which the CMV immediate early promoter drives expression of a portion of the yeast GAL4 gene (amino acids 1–147) containing a DNA-binding domain. As with the pACT Vector, the DNA-binding domain sequence is flanked by a chimeric intron, a multiple cloning region, stop codons and an SV40 late polyadenylation region (Figures 4 and 5). The fusion gene region is flanked by T7 and T3 RNA polymerase promoters for the purpose of synthesizing sense and antisense RNA products, respectively. The *Renilla* luciferase gene on this vector is preceded by the SV40 early promoter and a growth hormone intron. Introns can increase protein expression through mRNA stability and nuclear to cytoplasmic transport effects (9–12). A synthetic polyadenylation sequence resides 3′ of the *Renilla* luciferase gene. The plasmid backbone contains an f1 origin of replication for the production of ssDNA and the β-lactamase gene (Amp^r) for selection in *E. coli*.

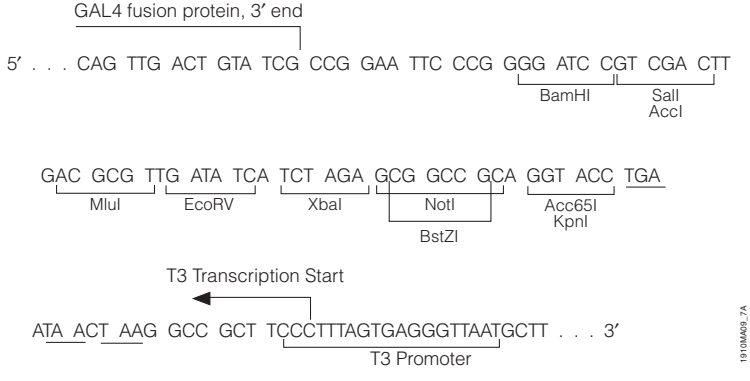

Figure 4. pBIND Vector multiple cloning region. Bases listed in triplets correspond to codons in the open reading frame of the GAL4 fusion protein. Stop codons (underlined) in three different reading frames are located downstream of the multiple cloning region and before the T3 promoter sequences.

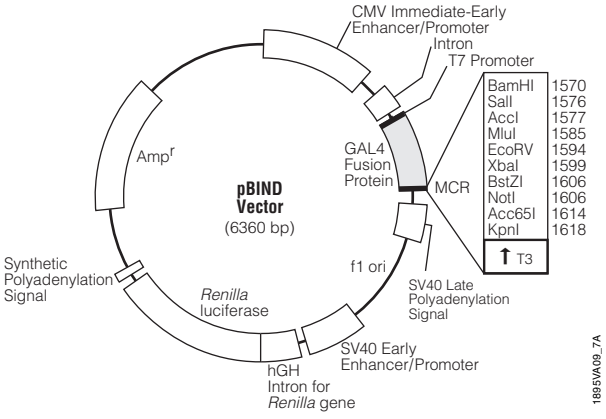

Figure 5. pBIND Vector circle map and sequence reference points.

pBIND Vector sequence reference points:

CMV immediate-early enhancer	1–659
CMV immediate-early promoter	669–750
chimeric intron	890–1022
T7 EEV sequencing primer binding site	1053–1074
T7 RNA polymerase promoter (–17 to +2)	1067–1085
GAL4 fusion protein	1116–1556
multiple cloning region (MCR)	1570–1619
T3 RNA polymerase promoter (–16 to +3)	1639–1657
SV40 late polyadenylation signal	1666–1887
phage f1 origin of replication	1982–2437
SV40 early enhancer/promoter	2527–2872
SV40 minimum origin of replication	2770–2835
hGH intron for *Renilla* gene	2924–3183
Renilla luciferase gene coding region	3208–4143
synthetic polyadenylation signal	4201–4249
β-lactamase (Amp^r) coding region	4668–5528

Use the T7 EEV Promoter Primer (Cat.# Q6700) for sequence analysis of the pBIND Vector. Do not use the T7 Promoter Primer (Cat.# Q5021) to sequence the pBIND Vector. There is a sequence difference between the primer and the promoter sequences.

3.C. Control Vectors

The CheckMate™ Mammalian Two-Hybrid System provides positive control vectors that encode and express two proteins known to interact in vivo (13). MyoD is expressed in skeletal muscle and is a myogenic regulatory protein (14,15). The Id protein acts as a negative regulator of myogenic differentiation (16). MyoD and Id are members of the helix-loop-helix family of nuclear proteins. The pBIND-Id Control Vector contains a segment of murine Id cDNA (amino acids 29–148; 13,16) cloned in-frame with the GAL4 DNA-binding domain of the pBIND Vector (Figure 6). The pACT-MyoD Control Vector contains a segment of murine MyoD cDNA (amino acids 1–318; 13,15) cloned in-frame with the VP16 activation domain of the pACT Vector (Figure 6).

Both positive control vectors are provided as high-quality DNA preparations qualified for transfection experiments.

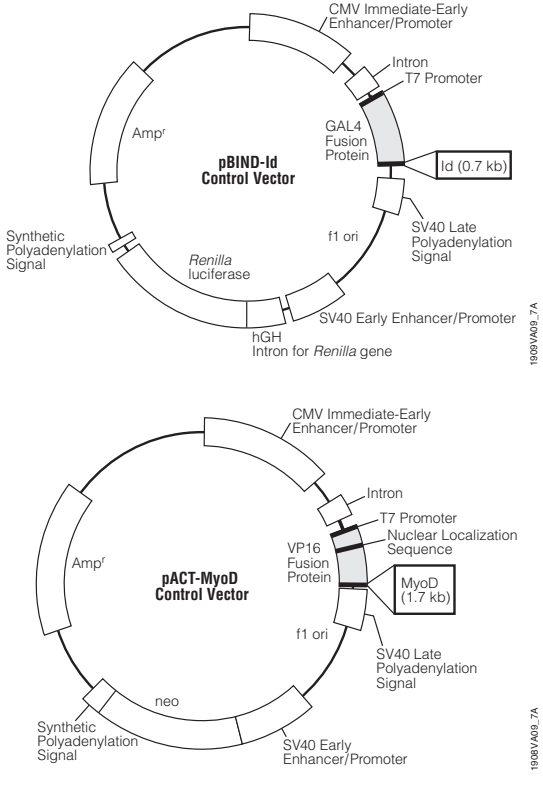

Figure 6. pBIND-Id and pACT-MyoD Control Vector circle maps. The protein product of the Id cDNA sequence in the pBIND-Id Vector is known to interact with the protein product of the MyoD cDNA sequence in the pACT-MyoD Vector.

3.D. Assessing Protein:Protein Interaction

The cDNA sequences encoding the polypeptides of interest should be subcloned into pACT and pBIND Vectors to generate fusion proteins with the DNA-binding domain of GAL4 and the activation domain of VP16, respectively. The pGAL4 and pVP16 fusion constructs are transfected with the pG5*luc* Vector into mammalian cells. The pG5*luc* Vector contains five GAL4 binding sites upstream of a minimal TATA box, which in turn is upstream of the firefly luciferase gene (*luc*+; Figure 7). Association of the DNA-binding domain and the transcriptional activation domain results in transcriptional activation of the firefly luciferase reporter gene.

The insert in each vector must be in the correct orientation and reading frame. See Figure 2 for the sequence following the 3´ end of the VP16 fragment, the multiple cloning region and the T3 promoter for pACT Vector; see Figure 4 for the sequence following the 3´ end of the GAL4 fragment, the multiple cloning region and the T3 promoter for pBIND Vector. For a complete description of various techniques used in subcloning, please refer to the *Subcloning Notebook* (17). All vectors in the CheckMate™ Mammalian Two-Hybrid System are ampicillin-resistant and are compatible with *E. coli* strains such as JM109. We strongly recommend sequencing the 5´ junction between the insert and the vector to ensure that the insert is subcloned properly. The T3 Promoter Primer (Cat.# Q5741) or T7 EEV Promoter Primer (Cat.# Q6700) can be used for sequence verification.

Certain inserts appear to have vector "directionality" (or preference), in which the interaction between a pair of proteins is vector-dependent (13). Protein:protein interactions may appear stronger given a particular vector context for the inserts. Because of this phenomenon, we advise subcloning each cDNA of interest into both the pACT and the pBIND Vectors and testing the two possible fusion protein interactions.

3.D. Assessing Protein:Protein Interaction (continued)

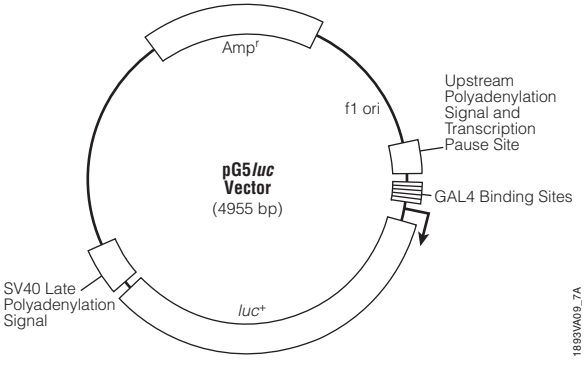

Figure 7. pG5*luc* Vector circle map and sequence reference points.

pG5*luc* Vector sequence reference points:

five GAL4 binding sites	18–120
major late promoter of adenovirus	132–172
predicted transcriptional start site	168
luciferase (*luc*+) gene	225–1877
SV40 late polyadenylation signal	1909–2130
β-lactamase (Amp^r) coding region	4077–3217
phage f1 origin of replication	4209–4664
upstream polyadenylation signal and transcriptional pause site	4795–4948

4. Protocols

4.A. Subcloning into pACT and pBIND Vectors

Introductory comments on subcloning, isolating and purifying fragments of DNA may be found in the *Subcloning Notebook* (17) or in any basic molecular biology text (e.g., *Current Protocols in Molecular Biology*, reference 18; *Molecular Cloning: A Laboratory Manual*, reference 19).

4.B. Purification of Vector and Transfection

Following the successful subcloning of the cDNAs into the pACT and pBIND Vectors, the resulting plasmids should be purified such that the DNA is free of protein, RNA and chemical contamination. Before completing any experiments with the CheckMate™ System, optimize the transfection method for the cell type being transfected. Many DNA delivery agents exist for transfecting mammalian cells. Transfection of DNA into mammalian cells may be mediated

by cationic lipids, calcium phosphate, DEAE-dextran or electroporation. Transfection systems based on cationic lipids (TransFast™ Transfection Reagent, Transfectam® Reagent, Tfx™-20 and Tfx™-50 Reagents, and calcium phosphate (ProFection® Mammalian Transfection System) are available from Promega. **Note:** See Related Products, Section 6, for a complete listing of the transfection reagents available from Promega.

The most efficient transfection method is highly dependent upon the cell type. When optimizing a transfection method for a particular cell type, use a reporter gene such as the firefly luciferase gene whose activity is easily and rapidly assayed. The pGL3-Control Vector (Cat.# E1741) expresses the firefly luciferase gene from the SV40 early promoter. For additional information about transfection reagents and cell lines, visit:
www.promega.com/transfectionasst/

Table 1 presents the recommended combinations of vectors to properly control an experiment when using the CheckMate™ System to determine the extent to which two proteins interact in a two-hybrid assay. Figure 8 presents representative data from typical control transfection experiments.

Table 1. Recommended Experimental Design to Determine the Magnitude of Interaction Between Two Proteins.

Transfection	pACT Vector	pBIND Vector	pG5*luc* Vector
1	pACT Vector	pBIND Vector	pG5*luc* Vector
2	pACT-MyoD Control Vector	pBIND-Id Control Vector	pG5*luc* Vector
3	–	–	–
4	pACT Vector	pBIND-**X** Vector	pG5*luc* Vector
5	pACT-**Y** Vector	pBIND Vector	pG5*luc* Vector
6	pACT-**Y** Vector	pBIND-**X** Vector	pG5*luc* Vector

Notes:

1. The background level of firefly luciferase expression from the pG5*luc* Vector is determined in the presence of the pACT and pBIND Vectors (Transfection #1).
2. The positive control reaction (Transfection #2) is expected to provide firefly luciferase levels well above the background levels measured in Transfection #1. Adequate vector amounts are provided for approximately 20 positive control transfection reactions for cells cultured in 60 mm dishes.
3. The nontransfected control (Transfection #3) is used to determine background activity for the firefly luciferase assay, which can be subtracted from each transfection reaction.
4. *Renilla* luciferase activity expressed from the pBIND Control Vector or pBIND-X Vector can be used to normalize for differences in transfection efficiencies.

4.B. Purification of Vector and Transfection (continued)

5. It is critical that the amount of pG5*luc* Vector is identical in all transfection reactions for accurate comparisons between samples. Dispensing a larger volume of the pG5*luc* Vector to the transfection mixtures generally correlates with higher reproducibility between transfection reactions within an experiment. For example, adding a 25 µl volume containing 1 µg of pG5*luc* DNA to each transfection mixture will provide less variability between mixtures compared to adding a 1 µl volume of pG5*luc* DNA to each transfection mixture. The pG5*luc* Vector can be diluted in buffer or media, depending upon the transfection method used.
6. Transfection #4 tests for interaction of the GAL4-X fusion protein with the VP16 activation domain and transcriptional activation activity of the X protein. Transfection #5 tests for interaction of the VP16-Y fusion protein with the GAL4 binding domain and GAL4 DNA binding activity of the Y protein.

The amount of vector DNA to use will depend upon the method of transfection. However, we recommend a 1:1 molar ratio of the pACT:pBIND Vector constructs. We have varied the amount of the pG5*luc* Vector in experiments with the positive control vectors and have found that the signal-to-noise ratio of firefly luciferase expression does not differ significantly. We routinely use a molar ratio of 1:1:1 for pACT:pBIND:pG5*luc* Vector in the CheckMate™ Mammalian Two-Hybrid System. To maintain a constant mass of DNA for each transfection reaction within an experiment, add plasmid DNA such as pGEM®-3Zf(+) Vector (Cat.# P2271).

We have successfully transfected NIH/3T3, CHO, 293, BHK-2 and HeLa mammalian cell lines with the positive control vectors. We recommend testing a specific cell line with positive and negative control transfection reactions before initiating protein:protein interaction experiments with that cell line. Replication of the pACT and pBIND Vectors and their recombinant vectors is expected in COS cells or other cells that express the SV40 large T antigen. Use the Dual-Luciferase® Reporter Assay System to quantitate *Renilla* luciferase and firefly luciferase activities.

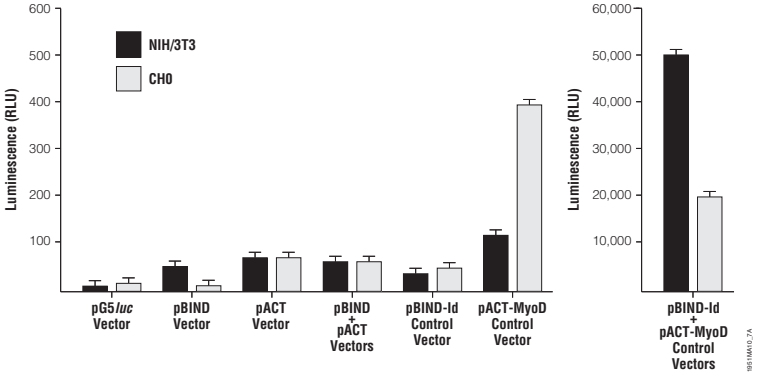

Figure 8. Sample data for representative control transfection experiments using the CheckMate™ Mammalian Two-Hybrid System in two mammalian cell lines. Data were recorded as relative light units (RLU) using a Turner luminometer following calcium phosphate transfection.

5. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**

Symptoms	Causes and Comments
Low *Renilla* luciferase activity in all samples	Low transfection efficiency. Re-optimize transfection conditions for your cell line with a reporter vector such as pGL3-Control Vector (Cat.# E1741).
	Check that cell cultures are not contaminated with *Mycoplasma*.
High variability in firefly luciferase activity between replicates	Unequal distribution of pG5*luc* DNA in transfection mixtures. Prepare a "master mix" of diluted pG5*luc* Vector in buffer or medium for more reproducible aliquots.
High luciferase activity from controls but low luciferase activity after co-transfection with recombinant vectors of interest (i.e., false-negative result)	Recombinant plasmid DNA impure. Purify plasmid DNA using methods that provide transfection-quality DNA. Co-transfect with a reporter DNA to determine if there are contaminants that interfere with transfection in the DNA preparation.
	One of the recombinant fusion proteins is labile. Assay for luciferase activity in cell extracts at earlier time points.

5. Troubleshooting (continued)

Symptoms	Causes and Comments
High luciferase activity from controls but low luciferase activity after co-transfection with recombinant vectors of interest (i.e., false-negative result; continued)	Expression of a test protein is vector-dependent. Subclone the "X" and "Y" test proteins into the other's respective pACT Vector or pBIND Vector.
	Weak interaction between proteins cloned into the pACT and pBIND Vectors: • Additional macromolecules may be required for interaction if the X and Y proteins are part of a multicomponent complex. • Endogenous cellular proteins may compete for interactions with the X or Y proteins. • The X and Y proteins may have relatively low affinities.

6. Related Products

Product	Size	Cat.#
Dual-Luciferase® Reporter Assay System	100 assays	E1910
Dual-Luciferase® Reporter 1000 Assay System	1,000 assays	E1980
Dual-Glo™ Luciferase Assay System	10 ml	E2920
	100 ml	E2940
	10 × 100 ml	E2980
T7 EEV Promoter Primer	2 µg	Q6700
TransFast™ Transfection Reagent	1.2 mg	E2431
Transfectam® Reagent for the Transfection of Eukaryotic Cells	1 mg	E1231
	0.5 mg	E1232
Tfx™-20 Reagent	4.8 mg (3 × 1.6 mg)	E2391
Tfx™-50 Reagent	2.1 mg (3 × 0.7 mg)	E1811

7. References

1. Sadowski, I. *et al.* (1988) GAL4-VP16 is an unusually potent transcriptional activator. *Nature* **335,** 563–4.
2. Fields, S. and Song, O. (1989) A novel genetic system to detect protein-protein interactions. *Nature* **340,** 245–6.
3. Chien, C. *et al.* (1991) The two-hybrid system: A method to identify and clone genes for proteins that interact with a protein of interest. *Proc. Natl. Acad. Sci. USA* **88**, 9578–82.
4. Dang, C.V. *et al.* (1991) Intracellular leucine zipper interactions suggest c-Myc hetero-oligomerization. *Mol. Cell. Biol.* **11**, 954–62.
5. Fearon, E.R. *et al.* (1992) Karyoplasmic interaction selection strategy: A general strategy to detect protein-protein interactions in mammalian cells. *Proc. Natl. Acad. Sci. USA* **89**, 7958–62.
6. Brondyk, W. (1995) The pCI-neo Mammalian Expression Vector. *Promega Notes* **51**, 10–4.
7. Carswell, S. and Alwine, J.C. (1989) Efficiency of utilization of the simian virus 40 late polyadenylation site: Effects of upstream sequences. *Mol. Cell. Biol.* **9**, 4248–58.
8. Southern, P.J. and Berg, P. (1982) Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J. Mol. Appl. Genet.* **1**, 327–41.
9. Gross, M.K., Kainz, M.S. and Merrill, G.F. (1987) Introns are inconsequential to efficient formation of cellular thymidine kinase mRNA in mouse L cells. *Mol. Cell. Biol.* **7**, 4576–81.
10. Buchman, A.R. and Berg, P. (1988) Comparison of intron-dependent and intron-independent gene expression. *Mol. Cell. Biol.* **8**, 4395–405.
11. Evans, M.J. and Scarpulla, R.C. (1989) Introns in the 3´-untranslated region can inhibit chimeric CAT and beta-galactosidase gene expression. *Gene* **84**, 135–42.
12. Huang, M.T.F. and Gorman, C.M. (1990) Intervening sequences increase efficiency of RNA 3´ processing and accumulation of cytoplasmic RNA. *Nucleic Acids Res.* **18**, 937–47.
13. Finkel, T. *et al.* (1993) Detection and modulation in vivo of helix-loop-helix protein-protein interactions. *J. Biol. Chem.* **268**, 5–8.
14. Weintraub, H. *et al.* (1991) The myoD gene family: Nodal point during specification of the muscle cell lineage. *Science* **251**, 761–6.
15. Davis, R.L., Weintraub, H. and Lassar, A.B. (1987) Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* **51**, 987–1000.
16. Benezra, R. *et al.* (1990) The protein Id: A negative regulator of helix-loop-helix DNA binding proteins. *Cell* **61**, 49–59.
17. *Subcloning Notebook* (2004) Promega Corporation. (**www.promega.com/guides/subcloning_guide/**)
18. Ausubel, F.M. *et al.*, eds. (1993) In: *Current Protocols in Molecular Biology*, Greene Publishing Associates and John Wiley and Sons, New York, NY.
19. Sambrook, J., Fritsch, E.F. and Maniatis, T. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd edition, Cold Spring Laboratory, Cold Spring Harbor, NY.

8. Appendix

8.A. pACT Vector Restriction Sites

The following restriction enzyme tables were constructed using DNASTAR® sequence analysis software. Please note that we have not verified this information by restriction digestion with each enzyme listed. The location given specifies the 3´ end of the cut DNA (the base to the left of the cut site). For more information on the cut sites of these enzymes, or if you identify a discrepancy, please contact your local Promega Branch or Distributor. In the U.S., contact Promega Technical Services at 800-356-9526. Vector sequences are available in the GenBank® database (GenBank®/EMBL Accession Number **AF264723**) and on the Internet at: **www.promega.com/vectors/**

Table 2. Restriction Enzymes That Cut the pACT Vector Between 1 and 5 Times.

Enzyme	# of Sites	Location
AatII	5	278, 331, 414, 600, 3742
AccI	1	1340
AccIII	1	1670
Acc65I	1	1377
AflII	4	828, 847, 1050, 2553
AflIII	2	1291, 1348
Alw44I	3	3492, 3989, 5235
AlwNI	1	5140
ApaI	2	1178, 1313
ApaLI	4	1317, 3215, 4297, 4519
AvrII	1	2504
BalI	3	10, 64, 2781
BamHI	1	1333
BanII	4	729, 1228, 1852, 3064
BbeI	1	2702
BbsI	2	961, 1120
BbuI	5	1147, 1263, 2253, 2325, 3104
BglII	1	5561
BsaI	2	915, 4595
BsaAI	3	493, 1923, 3003
BsaBI	2	1282, 1661
BsaMI	2	1480, 1573
BsmI	2	1480, 1573
BspHI	3	3716, 3821, 4829
BspMI	5	877, 1365, 2586, 2967, 3417
BsrGI	1	96
BssHII	1	3096
BssSI	4	3291, 3685, 3992, 5376
Bst98I	4	828, 847, 1050, 2553
BstXI	1	3420
BstZI	2	1369, 2605
Cfr10I	4	1818, 3018, 3199, 4576
ClaI	1	1665
CspI	1	3215
Csp45I	1	3381
DraI	4	1620, 4083, 4775, 4794
DraII	1	3681
DraIII	1	1926
DsaI	3	513, 2412, 3131
EagI	2	1369, 2605
EarI	4	1152, 3043, 3253, 3862
EclHKI	1	4661
Eco52I	2	1369, 2605
EcoICRI	2	727, 1226
EcoRI	1	1173
EcoRV	1	1357
EheI	1	2700

Table 2. Restriction Enzymes That Cut the pACT Vector Between 1 and 5 Times (continued).

Enzyme	# of Sites	Location	Enzyme	# of Sites	Location
FspI	2	2801, 4438	RsrII	1	3215
HaeII	4	1768, 1776, 2702, 5309	**SacI**	2	729, 1228
			SalI	1	1339
HincII	3	677, 1341, 1559	**ScaI**	2	1063, 4180
HindII	3	677, 1341, 1559	**SfiI**	1	2458
HindIII	2	756, 2521	**SgfI**	1	664
HpaI	1	1559	**SinI**	4	1317, 3215, 4297, 4519
I-PpoI	1	851			
KasI	1	2698	**SmaI**	2	1180, 1315
KpnI	1	1381	**SnaBI**	1	493
MluI	1	1348	**SpeI**	1	152
NaeI	2	1820, 3201	**SphI**	5	1147, 1263, 2253, 2325, 3104
NarI	1	2699			
NcoI	3	513, 2412, 3131	**SspI**	4	5, 52, 2131, 3856
NdeI	1	387	**StuI**	1	2504
NgoMIV	2	1818, 3199	**StyI**	4	513, 2412, 2505, 3131
NheI	1	1085			
NotI	1	1369	TfiI	5	1308, 1667, 2527, 3184, 3318
NsiI	2	2255, 2327			
Ppu10I	2	2251, 2323	**Tth111I**	1	2817
PshAI	1	1206	**VspI**	2	160, 4486
PspAI	2	1178, 1313	**XbaI**	1	1362
PstI	2	838, 2752	**XmaI**	2	1178, 1313
PvuI	2	664, 4292	**XmnI**	1	4061
PvuII	2	2181, 2805			

Table 3. Restriction Enzymes That Do Not Cut the pACT Vector.

AccB7I	Bpu1102I	Eco81I	PinAI	SrfI
AgeI	Bsp120I	EcoNI	PmeI	Sse8387I
ApaI	Bst1107I	FseI	PmlI	SwaI
AscI	**BstEII**	**NruI**	PpuMI	XcmI
BbrPI	**Bsu36I**	PacI	Psp5II	**XhoI**
BclI	**Eco47III**	PaeR7I	**SacII**	
BlpI	Eco72I	PflMI	SgrAI	

Note: The enzymes listed in boldface type are available from Promega.

8.A. pACT Vector Restriction Sites (continued)

Table 4. Restriction Enzymes That Cut the pACT Vector 6 or More Times.

AciI	BsrI	**FokI**	**MboI**	**RsaI**
AcyI	**BsrSI**	**HaeIII**	**MboII**	**Sau3AI**
AluI	Bst71I	HgaI	MnlI	Sau96I
Alw26I	**BstOI**	**HhaI**	MseI	ScrFI
AspHI	BstUI	**HinfI**	**MspI**	SfaNI
BanI	**CfoI**	**HpaII**	**MspA1I**	**TaqI**
BbvI	**DdeI**	HphI	**NciI**	**Tru9I**
BglI	**DpnI**	**Hsp92I**	**NdeII**	**XhoII**
BsaOI	DpnII	**Hsp92II**	NlaIII	
BsaHI	DrdI	MaeI	NlaIV	
BsaJI	EaeI	MaeII	NspI	
Bsp1286I	Fnu4HI	MaeIII	PleI	

Note: The enzymes listed in boldface type are available from Promega.

8.B. pBIND Vector Restriction Sites

The following restriction enzyme tables were constructed using DNASTAR® sequence analysis software. Please note that we have not verified this information by restriction digestion with each enzyme listed. The location given specifies the 3´ end of the cut DNA (the base to the left of the cut site). For more information on the cut sites of these enzymes, or if you identify a discrepancy, please contact your local Promega Branch or Distributor. In the U.S., contact Promega Technical Services at 800-356-9526. Vector sequences are available in the GenBank® database (GenBank®/EMBL Accession Number **AF264722**) and on the Internet at: **www.promega.com/vectors/**

Table 5. Restriction Enzymes That Cut the pBIND Vector Between 1 and 5 Times.

Enzyme	# of Sites	Location	Enzyme	# of Sites	Location
AccI	1	1577	**Bst98I**	4	828, 847, 1050, 2899
Acc65I	1	1614			
AccB7I	1	3055	**BstZI**	1	1606
AflII	4	828, 847, 1050, 2899	**Bsu36I**	2	2961, 3007
			Cfr10I	2	2107, 5370
AflIII	2	1585, 3390	**ClaI**	2	1902, 4252
Alw44I	3	4286, 4783, 6029	**Csp45I**	1	3214
AlwNI	2	3116, 5934	**DraI**	4	1857, 4877, 5569, 5588
AvaI	2	1333, 1565			
AvaII	5	3079, 3256, 3978, 5091, 5313	DraII	1	4475
			DraIII	2	2215, 2955
AvrII	1	2851	DrdI	5	817, 2259, 2888, 4372, 6241
BalI	3	10, 64, 3059			
BamHI	1	1570	DsaI	2	513, 2758
BanII	2	729, 2141	EagI	1	1606
BbsI	4	961, 1120, 1345, 4048	EarI	4	1510, 1920, 3378, 4656
BbuI	3	1147, 2599, 2671	**EclHKI**	1	5455
BclI	2	3492, 3701	Eco52I	1	1606
BglII	1	6355	Eco81I	2	2961, 3007
BsaI	3	915, 1258, 5389	**EcoICRI**	1	727
BsaAI	3	493, 2212, 3940	EcoNI	1	3120
BsaBI	2	1898, 4264	**EcoRI**	2	1560, 2909
BsaMI	2	1717, 1810	**EcoRV**	1	1594
BsmI	2	1717, 1810	FspI	2	1961, 5232
BspHI	4	3776, 4510, 4615, 5623	**HaeII**	4	2057, 2065, 2931, 6103
BspMI	2	877, 1602	**HindIII**	2	756, 2867
BsrGI	3	96, 1406, 3906	**HpaI**	2	1393, 1796
BssSI	4	3866, 4479, 4786, 6170	**I-PpoI**	1	851
			KpnI	1	1618

Note: The enzymes listed in boldface type are available from Promega.

8.B. pBIND Vector Restriction Sites (continued)

Table 5. Restriction Enzymes That Cut the pBIND Vector Between 1 and 5 Times (continued).

Enzyme	# of Sites	Location	Enzyme	# of Sites	Location
MluI	1	1585	**SfiI**	1	2804
NaeI	1	2109	**SgfI**	1	664
NcoI	2	513, 2758	**SinI**	5	3079, 3256, 3978, 5091, 5313
NdeI	1	387			
NgoMIV	1	2107	**SmaI**	1	1567
NheI	1	1085	**SnaBI**	1	493
NotI	1	1606	**SpeI**	1	152
NsiI	2	2601, 2673	**SphI**	3	1147, 2599, 2671
PaeR7I	1	1333	**SspI**	4	5, 52, 2420, 4650
PflMI	1	3055	**StuI**	1	2850
Ppu10I	2	2597, 2669	**StyI**	3	513, 2758, 2851
PspAI	1	1565	**VspI**	3	160, 3308, 5280
PstI	1	838	**XbaI**	1	1599
PvuI	3	664, 1942, 5086	XcmI	1	3857
PvuII	1	2527	**XhoI**	1	1333
SacI	1	729	**XmaI**	1	1565
SalI	1	1576	**XmnI**	2	3742, 4855
ScaI	2	1063, 4974			

Table 6. Restriction Enzymes That Do Not Cut the pBIND Vector.

AccIII	Bsp120I	EheI	PmlI	SrfI
AgeI	**BssHII**	FseI	PpuMI	Sse8387I
ApaI	Bst1107I	KasI	PshAI	SwaI
AscI	**BstEII**	**NarI**	Psp5II	**Tth111I**
BbeI	**BstXI**	**NruI**	RsrII	
BbrPI	**CspI**	PacI	**SacII**	
BlpI	**Eco47III**	PinAI	SgrAI	
Bpu1102I	Eco72I	PmeI	SplI	

Table 7. Restriction Enzymes That Cut the pBIND Vector 6 or More Times.

AatII	**Bsp1286I**	**FokI**	MaeII	NspI
AciI	BsrI	**HaeIII**	MaeIII	PleI
AcyI	**BsrSI**	HgaI	**MboI**	**RsaI**
AluI	Bst71I	**HhaI**	**MboII**	**Sau3AI**
Alw26I	**BstOI**	**HincII**	MnlI	Sau96I
AspHI	BstUI	HindII	MseI	ScrFI
BanI	**CfoI**	**HinfI**	**MspI**	SfaNI
BbvI	**DdeI**	**HpaII**	**MspA1I**	**TaqI**
BglI	**DpnI**	HphI	**NciI**	TfiI
BsaOI	DpnII	**Hsp92I**	**NdeII**	**Tru9I**
BsaHI	EaeI	**Hsp92II**	NlaIII	**XhoII**
BsaJI	Fnu4HI	MaeI	NlaIV	

Note: The enzymes listed in boldface type are available from Promega.

8.C. pG5*luc* Vector Restriction Sites

The following restriction enzyme tables were constructed using DNASTAR® sequence analysis software. Please note that we have not verified this information by restriction digestion with each enzyme listed. The location given specifies the 3´ end of the cut DNA (the base to the left of the cut site). For more information on the cut sites of these enzymes, or if you identify a discrepancy, please contact your local Promega Branch or Distributor. In the U.S., contact Promega Technical Services at 800-356-9526. Vector sequences are available in the GenBank® database (GenBank®/EMBL Accession Number **AF264724**) and on the Internet at: **www.promega.com/vectors/**

Table 8. Restriction Enzymes That Cut the pG5*luc* Vector Between 1 and 5 Times.

Enzyme	# of Sites	Location
AccI	1	2140
AccIII	3	102, 920, 1436
Acc65I	1	1
AcyI	4	232, 258, 1651, 3827
AflIII	2	718, 2397
Alw26I	5	1248, 1480, 1546, 3351, 4127
Alw44I	2	2711, 3957
AlwNI	1	2813
AspHI	4	1690, 2715, 3876, 3961
AvaI	2	55, 1281
AvaII	3	1404, 3428, 3650
BamHI	1	2141
BanII	2	1249, 4368
BbeI	1	261
BbsI	5	127, 235, 1513, 1629, 2226
BbuI	1	888
BclI	1	805
BglI	2	3410, 4678
BglII	1	173
BsaI	1	3351
BsaAI	1	4439
BsaBI	1	2140
BsaHI	4	232, 258, 1651, 3827
BsaMI	3	197, 1960, 2053
BsmI	3	197, 1960, 2053
BspHI	3	808, 3117, 4125
BspMI	3	1614, 1623, 4918

Enzyme	# of Sites	Location
BsrGI	1	715
BssSI	2	2570, 3954
BstZI	3	1892, 1896, 4788
ClaI	3	2134, 4846, 4950
Csp45I	1	394
DraI	4	2100, 3156, 3175, 3867
DraII	1	1404
DraIII	1	4442
DrdI	3	1626, 2505, 4486
DsaI	2	223, 595
EaeI	4	1892, 1896, 3678, 4788
EagI	3	1892, 1896, 4788
EclHKI	1	3290
Eco47III	1	2273
Eco52I	3	1892, 1896, 4788
EcoNI	3	782, 1182, 1842
EcoRI	1	98
EheI	1	259
FseI	1	1898
FspI	2	3512, 4685
HincII	3	1529, 2039, 2149
HindII	3	1529, 2039, 2149
HindIII	1	190
HpaI	1	2039
Hsp92I	4	232, 258, 1651, 3827
KasI	1	257
KpnI	1	5
NaeI	3	1896, 2267, 4336
NarI	1	258

Table 8. Restriction Enzymes That Cut the pG5*luc* Vector Between 1 and 5 Times (continued).

Enzyme	# of Sites	Location	Enzyme	# of Sites	Location
NcoI	1	223	SgrAI	1	1653
NgoMIV	3	1894, 2265, 4334	**SinI**	3	1404, 3428, 3650
NheI	1	126	**SphI**	1	888
NotI	1	4788	**SspI**	3	4094, 4647, 4762
NspI	2	888, 2401	**StyI**	1	223
PaeR7I	1	55	**VspI**	1	3462
PpuMI	1	1404	**XbaI**	3	12, 170, 1879
PshAI	1	2212	XcmI	1	960
Psp5II	1	1404	**XhoI**	1	55
PvuI	3	79, 3660, 4706	**XmnI**	1	3889
SalI	1	2147			

Table 9. Restriction Enzymes That Do Not Cut the pG5*luc* Vector.

AatII	Bsp120I	**EcoRV**	Ppu10I	**SpeI**
AccB7I	**BssHII**	**I-PpoI**	PspAI	SplI
AflII	Bst1107I	**MluI**	**PstI**	SrfI
AgeI	**Bst98I**	**NdeI**	**PvuII**	Sse8387I
ApaI	**BstEII**	**NruI**	RsrII	**StuI**
AscI	**BstXI**	**NsiI**	**SacI**	SwaI
AvrII	**Bsu36I**	PacI	**SacII**	**Tth111I**
BalI	**CspI**	PflMI	**SfiI**	**XmaI**
BbrPI	Eco72I	PinAI	**SgfI**	
BlpI	Eco81I	PmeI	**SmaI**	
Bpu1102I	**EcoICRI**	PmlI	**SnaBI**	

Table 10. Restriction Enzymes That Cut the pG5*luc* Vector 6 or More Times.

AciI	BstUI	HgaI	MnlI	Sau96I
AluI	**CfoI**	**HhaI**	MseI	**ScaI**
BanI	Cfr10I	**HinfI**	**MspI**	ScrFI
BbvI	**DdeI**	**HpaII**	**MspA1I**	SfaNI
BsaOI	**DpnI**	HphI	**NciI**	**TaqI**
BsaJI	DpnII	**Hsp92II**	**NdeII**	TfiI
Bsp1286I	EarI	MaeI	NlaIII	**Tru9I**
BsrI	Fnu4HI	MaeII	NlaIV	**XhoII**
BsrSI	**FokI**	MaeIII	PleI	
Bst71I	**HaeII**	**MboI**	**RsaI**	
BstOI	**HaeIII**	**MboII**	**Sau3AI**	

Note: The enzymes listed in boldface type are available from Promega.

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