The CheckMate™ Mammalian Two-Hybrid System

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Two-hybrid systems provide powerful methods for detecting protein:protein interactions in vivo. *The two-hybrid system, originally developed in yeast, has been adapted for use in mammalian cells. Promega recently introduced the CheckMate™ Mammalian Two-Hybrid System, which allows protein:protein interactions to be studied in the cell line of choice. In this article, the CheckMate™ Mammalian Two-Hybrid System is demonstrated in HeLa, NIH3T3 and CHO mammalian cell lines, as well as used to confirm* in vivo *association of the cloned Raf-1 kinase and oncogenic Ras proteins.*

INTRODUCTION

Interactions between proteins play critical roles in cellular regulatory events, such as transcription, signal transduction pathways and enzyme-mediated metabolic effects. Identification, verification, characterization and manipulation of discrete protein:protein interactions within eukaryotic cells can be quite difficult and time-consuming. The introduction by Fields and co-workers of an *in vivo* yeast genetic screening method provided a means to accelerate the rate of progress in the identification of novel protein:protein interactions (1,2). The method, known as the yeast two-hybrid system, is a clever adaptation based upon the structural nature of certain eukaryotic transcription factors. Some transcription regulatory factors, such as yeast GAL4, which was first used in the yeast two-hybrid system, possess separate functional portions of the protein, or domains, that bind DNA and activate transcription (1,3-5). These protein domains can be expressed individually from recombinant vectors, and functional transcriptional activation that is specified by the DNA binding moiety can be reconstituted. The DNA binding domain and the transcriptional activation domain, which are produced by separate plasmids, are closely associated when one protein ("X") fused to a GAL4 DNA binding domain interacts with a second protein ("Y") fused to a GAL4 transcriptional activation domain. The physical interaction between proteins X and Y brings the two functional regions of the GAL4 protein in close proximity and enables activation of gene expression when placed in the proximity of a target promoter. Usually, one protein is known (X or Y), and the system is used to find a protein that interacts with the known protein. Newer applications of the two-hybrid system include assessing levels of protein:protein interactions.

The CheckMate™ Mammalian Two-Hybrid System is a modification of the original yeast system (6,7). In the mammalian-based system, reconstituted transcription factor activity comes from two different protein domains that are expressed from two separate vectors. The DNA binding domain of GAL4 protein and the activation domain of herpes simplex virus type 1 VP16 protein provide functional transcriptional activation from RNA polymerase II basal promoters with upstream GAL4 binding sites (8). In the CheckMate™ System, five GAL4 binding sites are positioned upstream of the firefly luciferase gene (*luc*+)[b,c], providing a sensitive and quantitative reporter system for functional assessment of reconstituted GAL4:VP16 activity (Figure 1). Mammalian cells are cotransfected with the firefly luciferase reporter vector, pG5*luc*, along with vectors that express the GAL4 DNA binding domain and VP16 activation domain. In the absence of interacting fusion partners to GAL4 and VP16, the level of transcription from the GAL4 element promoter is low, and consequently the luciferase expression is low. Cotransfection of mammalian cells with vectors coding for GAL4-X and VP16-Y, in which "X" and "Y" are interacting protein domains, results in close physical association of GAL4 and VP16 domains. The GAL4 promoters are functionally activated with concomitant increases in the luciferase reporter activity (Figure 1).

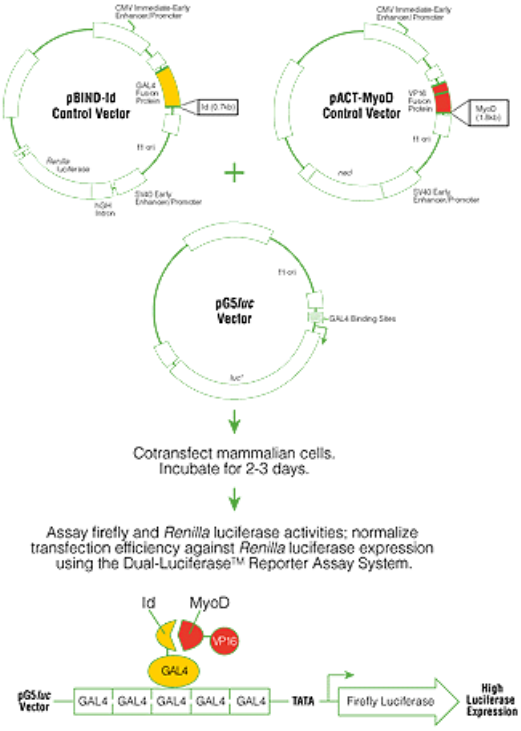

Figure 1. Schematic representation of a positive control reaction in the CheckMate™ Mammalian Two-Hybrid System for protein:protein interactions. For a positive control reaction, the pBIND-Id and the pACT-MyoD Control Vectors are cotransfected into mammalian cells along with the pG5*luc* Vector. The pG5*luc* Vector contains five GAL4 binding sites upstream of a minimal TATA box that is upstream of the firefly luciferase gene. The cells are incubated for 2-3 days, lysed and then assayed for firefly and *Renilla* luciferase using the Dual-Luciferase™ Reporter Assay System**(a). In positive control experiments, interaction between the two proteins, Id and MyoD, in the GAL4-Id and VP16-MyoD fusion proteins, results in high levels of luciferase expression from the pG5*luc* Vector.

***Patents Pending.*

(a)*U.S. Pat. Nos. 5,283,179, 5,641,641 and 5,650,289 have been issued to Promega Corporation for a firefly luciferase assay method, which affords greater light output with improved kinetics as compared to the conventional assay.*

(b)*U.S. Pat. No. 5,670,356 has been issued to Promega Corporation for a modified luciferase technology.*

(c)*The method of recombinant expression of* Coleoptera *luciferase is covered by U.S. Pat. No. 5,583,024 assigned to the Regents of the University of California.*

The strength of the mammalian two-hybrid system is the ability to confirm putative protein:protein interactions *in vivo* that were initially identified by the yeast two-hybrid system or by *in vitro* biochemical methods. A two-hybrid system for mammalian cells allows characterization of mammalian protein:protein interactions within a cellular environment that more closely mimics the native protein environment. Differences exist between yeast and mammalian cells in patterns of post-translational modifications, such as glycosylation, phosphorylation and acylation (9), as well as in the intracellular localization of proteins. These types of protein modifications, as well as other unique factors or modulators present in mammalian cells, may influence the ability of protein domains to interact (10). Using the CheckMate™ System with mammalian cells, one can potentially eliminate false positive results obtained from initial screening with the yeast two-hybrid system and confirm true positive results in a more native context.

Features of the CheckMate™ Mammalian Two-Hybrid System

Mammalian two-hybrid systems have been used to characterize several heteromeric protein:protein interactions, as well as homodimer protein interactions. Examples include interactions between Jun and Fos (4), MyoD and Id (11), Myc and Max (12), retinoid X receptor and thyroid hormone receptor (13), Sp1 and steroidogenic factor-1 (14), and CREB-binding protein and NF-kappaB p65 subunit (15). The CheckMate™ System offers several advantages and options compared to earlier systems used for mammalian two-hybrid assays.

Testing of putative interacting protein domains using the CheckMate™ System requires cotransfection of three vectors into eukaryotic cells. The DNA binding domain of a yeast GAL4 gene product (amino acids 1-147) is provided in the pBIND Vector (Figure 2A). This

GAL4 domain also contains an endogenous nuclear localization sequence (16) followed by a multiple cloning site (MCS) for the insertion of the cDNA clone of interest. The activation domain of herpes simplex virus type 1 VP16 (amino acids 411-456) and an added nuclear localization sequence (5,17) are supplied in the pACT Vector (Figure 2B). A multiple cloning site at the 3´-end of the coding domain provides a convenient location to clone the other cDNA of interest. The restriction enzyme sites in these two vectors are identical, making insertion of the cDNAs in both vectors more convenient. The vectors are designed to provide high level expression of the recombinant GAL4 and VP16 proteins. The human cytomegalovirus[(d)] (CMV) immediate-early promoter, which is an active promoter in mammalian cells (18), drives expression of both the GAL4 and VP16 genes. The cDNA regions are preceded by a chimeric intron and followed by the late SV40 polyadenylation signal sequence, which serve to provide efficient processing and optimized steady-state levels of the transcribed RNA (19-23).

[(d)]*The CMV Vector technology is the subject of U.S. Pat. No. 5,168,062 assigned to the University of Iowa Research Foundation.*

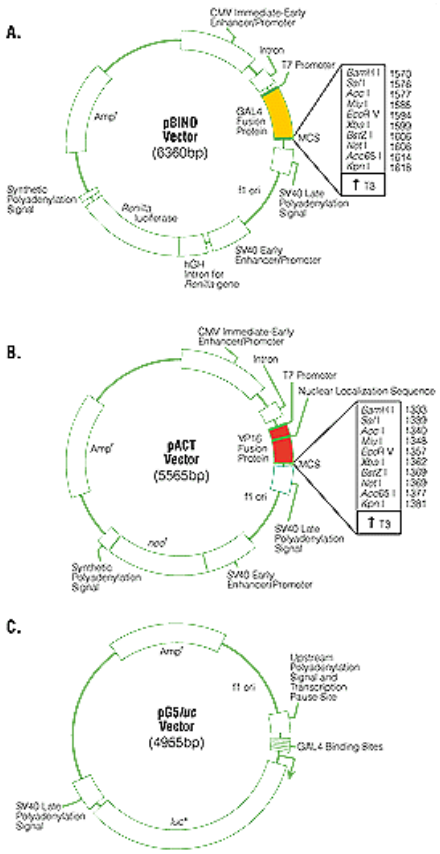

Figure 2. Circle maps of the pBIND Vector (Panel A), pACT Vector (Panel B) and pG5*luc* Vector (Panel C).

The GAL4 and VP16 chimeric proteins expressed in the transfected cells are tested for functional protein:protein interactions in combination with the reporter vector, pG5*luc*, which encodes the firefly luciferase gene (Figure 2C). Luciferase expression provides the means to report transcriptional activity from the GAL4:VP16 protein interaction. The assay for firefly luciferase is easily quantitated, is linear over at least seven orders of magnitude with a luminometer (24) and is more sensitive than the assay for chloramphenicol acetyltransferase, the reporter gene in other mammalian two-hybrid systems (6,25). The use of a luciferase reporter enzyme may also make this system easy to use in a high throughput format. The pG5*luc* Reporter Vector contains five DNA elements in tandem that bind GAL4. These five DNA elements are located 5´ of the basal TATA promoter from the adenovirus major late promoter, which has been demonstrated to have very low basal activity in this type of application (26). A polyadenylation signal and transcriptional pause site upstream of the GAL4 sites are present to reduce nonspecific luciferase expression due to transcription from the plasmid backbone of the vector.

Additional features designed into our vectors allow normalization of transfection efficiencies and establishment of stable cell lines. The pBIND Vector includes the *Renilla* luciferase[(e)] gene driven by the SV40 early promoter and enhancer. Insertion of the human growth hormone intron 5´ of the gene results in increased expression of the *Renilla* luciferase in the cell lines tested (data not shown). Cells transfected with identical levels of pBIND and its recombinant versions will express *Renilla* luciferase, and the levels of *Renilla* luciferase activity reflect the relative levels of transfection efficiency between dishes of cells within a transfection experiment. The Dual-Luciferase™ Reporter Assay System (Cat.# E1910), used in combination with a luminometer, is a convenient and simple method to assay both *Renilla* luciferase and firefly luciferase enzyme activities from the same cell lysate sample. Stable cell lines can also be obtained using the CheckMate™ System due to the presence of the neomycin phosphotransferase (*neo*) gene in the pACT Vector. Cells

expressing the *neo* gene can be selected in the presence of G418, or Geneticin® (27). However, continued passage and propagation of selected stable cells should include careful analysis for the functional presence of the other two cotransfected plasmids that do not have selection marker genes.

[e]*The cDNA encoding luciferase from* Renilla reniformis *is covered by U.S. Pat. No. 5,292,658 assigned to the University of Georgia Research Foundation, Inc., and sublicensed from SeaLite Sciences, Inc., Norcross, GA. The pRL family of* Renilla *luciferase cDNA vectors is for research use only.*

Once confirmation of protein:protein interactions is obtained with this vector system, further experiments can be performed to further define the domains and amino acids involved in the interactions. Promega's GeneEditor™ *in vitro* Site-Directed Mutagenesis System** (Cat.# Q9280) for making site-directed mutants and the Erase-a-Base® Systems for deletion analysis are useful tools, and they are fully compatible with the pBIND and pACT Vectors of the CheckMate™ System.

POSITIVE CONTROL MYOD:ID INTERACTING PROTEIN DOMAINS

The MyoD and Id proteins are members of the helix-loop-helix family of nuclear proteins and have been demonstrated to interact *in vitro* (28). MyoD is a regulator of myogenic differentiation that is expressed in skeletal muscle cells (29,30), and Id acts as a negative regulator of myogenic differentiation (28). The murine MyoD sequence corresponding to amino acids 1-318 was cloned into the pACT Vector, and the murine Id sequence corresponding to amino acids 29-148 was cloned into the pBIND Vector (11) (Figure 1). These recombinant vectors are included as control vectors in the CheckMate™ Mammalian Two-Hybrid System.

HeLa, NIH3T3 and CHO cells were co-transfected with three plasmid DNAs: pG5*luc* Vector in combination with pBIND and pACT Vectors, or with the respective Control Vectors. Cells were plated in 24 well plates and transfected with the aid of Tfx™-20[f] or TransFast™ cationic liposome reagents. As shown in Figure 3, cells transfected with both pBIND-Id and pACT-MyoD Control Vectors expressed approximately 50-700 times more firefly luciferase activity (depending upon the cell type) than cells transfected with pACT and pBIND Vector combinations. In addition, the level of firefly luciferase activity was low in cells transfected with vector combinations that did not reconstitute transcriptionally functional GAL4:VP16 (i.e., pBIND and pACT-MyoD; pBIND-Id and pACT). (*Renilla* luciferase expression was not used to normalize for transfection efficiency in these experiments because we previously observed anomalous levels of *Renilla* luciferase activity with the pACT-MyoD Vector following cotransfection.) As shown in Figure 3, the pBIND-Id and pACT-MyoD Control Vectors, in combination with pG5*luc*, provide excellent positive control vectors to verify functionality of the system with different cell types.

[f]*The cationic lipid component of the Tfx™ Reagents is covered by U.S. Pat. No. 5,527,928 assigned to The Regents of the University of California and pending foreign patents.*

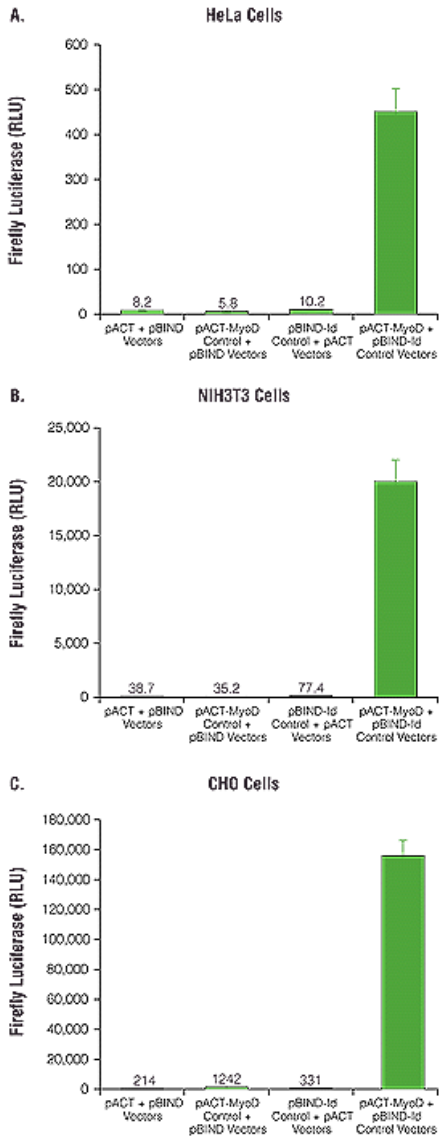

Figure 3. Transfection of HeLa, NIH3T3 and CHO cells with combinations of pACT, pBIND and Control Vectors plus pG5*luc* Vector. The day prior to transfection, cells were seeded in 24 well tissue culture plates at a density of approximately 5 x 10^4 cells per well. Cells were transfected using the optimal DNA:lipid complex, which had been previously determined. For HeLa cells (Panel A), a total of 0.25µg plasmid DNA was added per well using Promega's Tfx™-20 Transfection Reagent (Cat.# E2391) in a 2:1 lipid:DNA ratio per instructions (for information, request Promega's *Tfx™-10, Tfx™-20 and Tfx™-50 Reagents for the Transfection of Eukaryotic Cells Technical Bulletin* #TB216). For NIH3T3 cells (Panel B), a total of 1µg of plasmid DNA was added per well using Promega's TransFast™ Transfection Reagent (Cat.# E2431) in a 1:1 lipid:DNA ratio per instructions (for information, request Promega's *TransFast™ Transfection Reagent Technical Bulletin* #TB260). For CHO cells (Panel C), the same protocol was followed as for the NIH3T3 cells, except 0.5µg total DNA was used. Briefly, the firefly luciferase plasmid pG5*luc* and two control plasmid vectors (pACT Vector, pBIND Vector or the corresponding pBIND-Id and pACT-MyoD Vectors) were mixed in 1:1:1 ratios in serum free medium. The recommended amount of transfection reagent was added to the DNA/medium mixture, vortexed briefly and allowed to incubate at room temperature for 10 minutes. The growth medium was removed from the cells; the transfection mixture was added; and the cells were incubated at 37°C. After a one-hour incubation, 1ml of complete medium was added to the cells and incubation continued for 48 hours when luciferase expression was determined. Luciferase data were generated using a Luminoskan (Labsystems) 96 well plate reading luminometer. Results are represented as the mean relative light units per well ± the standard deviation of six replicates.

IN VIVO TESTING OF RAF-1:RAS INTERACTIONS WITH THE CHECKMATE™ SYSTEM

Ras, the gene product of a proto-oncogene, and Raf-1, a serine/threonine protein kinase, are regulatory proteins involved in cell signaling pathways in mammalian cells that control activation of mitogen-activated protein kinases, differentiation and development (31-33). Interacting protein domains of Ras and Raf-1 have been identified by several methods, including *in vitro* binding assays (34-37), *in vivo* Ras inhibition assays (34,38), and the yeast two-hybrid system (39-41). The amino terminal region of Raf-1, including a cysteine-rich region and intact zinc finger, was reported to be required for direct physical interaction of Ras with Raf-1 (34,37,38,42).

Ras is a membrane-bound protein and binds to the amino terminal region of Raf-1 in a GTP-dependent manner (35,40,41). We obtained human clones of the full-length human Raf-1 (pCGN-raf) and c-Ha-ras, containing an oncogenic mutation of glutamine to leucine at amino acid 61 (pZIP-rasH(61L)) (34). The oncogenic c-Ha-ras protein has been shown to exhibit nearly identical affinity as the wild-type Ras protein for Raf-1 by *in vitro* methods (42).

The *ras* sequence representing all but the last three amino acids at the C-terminus was cloned into the *Mlu* I site of the pBIND Vector (pBIND-Ras). The *raf-1* sequence representing amino acids 23-284 of the 648 amino acid protein was cloned into the *Bam*H I site of the pACT Vector (pACT-Raf). NIH3T3 cells were transfected with the pACT and pBIND Vector combinations along with pG5*luc* Vector, and cell lysates were prepared 24, 48 and 72 hours post-transfection. As depicted in Table 1, firefly luciferase levels were approximately 7 to 35 times higher in cells cotransfected with both *ras* and *raf-1* recombinant fusion vectors compared to cells transfected with only one of the two recombinant fusion vectors. The highest levels of firefly and *Renilla* luciferase enzyme activities in this transient transfection were assayed 48 hours post-transfection. From these results we were able to confirm *in vivo* association of the cloned Raf-1 kinase and oncogenic Ras proteins with the CheckMate™ Mammalian Two-Hybrid System.

Table 1. Interaction of pBIND-Ras and pACT-Raf Fusion Proteins Assayed with NIH3T3 Cells 24, 48 and 72 Hours Post-Transfection.

	24 Hours		48 Hours		72 Hours	
Vector Combinations	Firefly	*Renilla*	Firefly	*Renilla*	Firefly	*Renilla*
pBIND-Ras/pACT-Raf	2,580	10,000	5,060	23,000	1,440	3,180
pBIND-Ras/pACT	340	10,400	180	13,500	40	1,060
pBIND/pACT-Raf	180	6,940	160	11,300	20	980

The day prior to transfection, NIH3T3 cells were seeded in 60mm tissue culture plates at a density of approximately 1 x 10^5 cells. Each plate of cells was transfected with a total of 5µg plasmid DNA, using the calcium phosphate procedure (see Promega's ProFection® Mammalian Transfection System Technical Manual *#TM012) with 1µg pBIND Vector, 1µg pACT Vector (or the corresponding Ras and Raf fusions, respectively), 1µg pG5*luc *Vector and 2µg pGEM®-3Zf(+)[g] Vector as carrier. The culture medium was refreshed the following day, and cell lysates were prepared with Passive Lysis Buffer (Cat.# E1914) 24, 48 or 72 hours post-transfection. Firely and* Renilla *luciferase activities were quantified with the Dual-Luciferase™ Reporter Assay System using a Turner Designs Luminometer Model 20e. The results are expressed as the mean relative light units per plate for duplicates.*

[g]U.S. Pat. No. 4,766,072 has been issued to Promega Corporation for transcription vectors having two different bacteriophage RNA polymerase promoter sequences separated by a series of unique restriction sites into which foreign DNA can be inserted.

SUMMARY

The CheckMate™ Mammalian Two-Hybrid System is an *in vivo* genetic system allowing characterization of protein:protein interactions within the context of mammalian cells. This system offers several advantages over earlier vector systems including a firefly luciferase reporter gene, *Renilla* luciferase for normalization of transfection efficiency, strong CMV promoters driving expression of the GAL4 binding domain and VP16 activation domain chimeric proteins, neomycin phosphotransferase gene marker for selection of stable clones, and positive control vectors expressing MyoD and Id fusion proteins.

ACKNOWLEDGEMENTS

We would like to acknowledge the expert advice and assistance of Jennifer Oler, Virginia Goiffon, Patti Aha, Michael Tennier, Carol Lindsay and Jude Stevens during the course of this work.

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TransFast™ Transfection Reagent	E2431
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