Promega

Technical Manual

CheckMate™/Flexi® Vector Mammalian Two-Hybrid System

INSTRUCTIONS FOR USE OF PRODUCT C9360.

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Promega

CheckMate™/Flexi® Vector Mammalian Two-Hybrid System

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1. Description

Protein interactions are an integral aspect of functional proteomic studies, and the CheckMate™/Flexi® Vector Mammalian Two-Hybrid System[a–g] provides a means to confirm, validate and study suspected interactions between two proteins or domains. This system can also be used to generate stable cell lines for cell-based assays to identify modulators of a specific protein:protein interaction. The original CheckMate™ Mammalian Two-Hybrid System (Cat.# E2440) has been used to study proteins involved in processes including transcriptional regulation (1,2), signal transduction (3,4), developmental biology (5), pathology (6) and viral protein interactions and functions (7,8).

Developed particularly for mammalian proteins of interest, the CheckMate™/Flexi® Vector System can allow protein expression and post-translational modifications in an environment mimicking the native cell milieu. The CheckMate™/Flexi® Vector System is patterned on the yeast two-hybrid system (9–12) with one protein of interest ("X") fused to a DNA-binding domain and the other protein ("Y") fused to a transcriptional activation domain. Association of both domains, driven by the interaction of proteins "X" and "Y", results in binding to the promoter region and transcriptional activation of a firefly luciferase reporter gene (Figure 1). Assay of firefly luciferase activity is sensitive, rapid and easy.

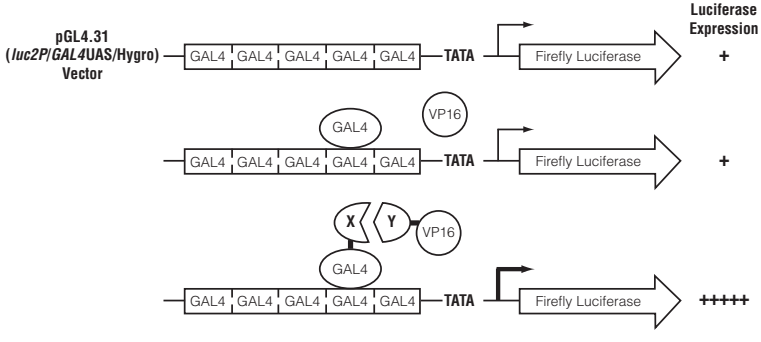

Figure 1. Schematic representation of the CheckMate™/Flexi® Vector Mammalian Two-Hybrid System. The pGL4.31[*luc2P/GAL4*UAS/Hygro] Vector contains five GAL4 binding sites upstream of a minimal TATA box, which in turn is upstream of the firefly luciferase gene. In negative controls, the background level of luciferase is measured in the presence of GAL4 DNA-binding domain (from pBIND) and VP16 activation domain (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.

The CheckMate™/Flexi® Vector System relies upon three plasmids that are co-transfected into mammalian cells. The pFN10A (ACT) Flexi® Vector contains a herpes simplex virus VP16 transcriptional activation domain upstream of the cloning site, and the pFN11A (BIND) Flexi® Vector contains the yeast GAL4 DNA-binding domain upstream of the cloning site. The pFN11A (BIND) Flexi® Vector also expresses the *Renilla reniformis* luciferase under the control of the SV40 promoter, allowing normalization for differences in transfection efficiency. The third vector, pGL4.31[*luc2P/GAL4*UAS/Hygro], contains five GAL4 binding sites upstream of a minimal TATA box, which is upstream of a firefly luciferase gene that acts as a reporter for interactions between proteins "X" and "Y".

The CheckMate™/Flexi® Vector System differs from the original CheckMate™ System in the properties of the three supplied plasmids. The vectors, pFN10A (ACT) and pFN11A (BIND), are compatible with the Flexi® Vector System, which allows directional cloning and a rapid, efficient and high-fidelity transfer of protein-coding sequences between a variety of other Flexi® Vectors. Both of the Flexi® Vectors supplied in the CheckMate™/Flexi® Vector System incorporate two rare-cutting restriction enzyme sites, SgfI and PmeI, flanking the protein-coding sequences, and also contain the lethal barnase gene for positive selection during the cloning step. For additional information on Flexi® Vectors, see the *Flexi® Vector Systems Technical Manual* #TM254 available at: **www.promega.com/tbs**. The reporter gene in the CheckMate™/Flexi® Vector System is supplied on the pGL4.31[*luc2P*/*GAL4*UAS/Hygro] Vector, which contains a firefly luciferase gene (*luc2P*) that has been engineered for rapid response characteristics and a gene for hygromycin resistance to allow generation and maintenance of stably transformed cells. To learn more about the pGL4 series of vectors, see the *pGL4 Luciferase Reporter Vectors Technical Manual* #TM259 available at: **www.promega.com/tbs/**

Positive control vectors provided in the CheckMate™/Flexi® Vector System encode and express two proteins known to interact in vivo (13–16). The pACT-MyoD and pBIND-Id Control Vectors encode VP16-MyoD and GAL4-Id fusion proteins, respectively. The provided vectors pACT and pBIND, which lack fusion proteins, may be used as negative controls.

The pFN10A (ACT) Flexi® Vector and pFN11A (BIND) Flexi® Vector **should not** be used directly as negative control vectors because they both contain the barnase gene, which is toxic when expressed in cells.

2. Product Components and Storage Conditions

Product	Cat. #
CheckMate™/Flexi® Vector Mammalian Two-Hybrid System	C9360

Includes:

- 5µg pFN10A (ACT) Flexi® Vector
- 5µg pFN11A (BIND) Flexi® Vector
- 20µg pGL4.31[*luc2P*/*GAL4*UAS/Hygro] Vector
- 20µg pBIND Vector
- 20µg pACT Vector
- 20µg pBIND-Id Control Vector
- 20µg pACT-MyoD Control Vector

Product	Cat. #
CheckMate™ Positive Control Vectors	C9370

Includes:

- 20µg pBIND-Id Control Vector
- 20µg pACT-MyoD Control Vector

Product	Cat. #
CheckMate™ Negative Control Vectors	C9380

Includes:

- 20µg pBIND Vector
- 20µg pACT Vector

Items Available Separately

Product	Size	Cat. #
pFN10A (ACT) Flexi® Vector	20µg	C9331
pFN11A (BIND) Flexi® Vector	20µg	C9341
pGL4.31[*luc2P*/*GAL4*UAS/Hygro] Vector	20µg	C9351

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

3. General Considerations

The protein-coding regions of interest should be cloned into the pFN10A (ACT) and pFN11A (BIND) Flexi® Vectors. The SgfI and PmeI restriction sites in these vectors are designed for in-frame protein fusions with the VP16 activation domain and GAL4 DNA-binding domain, respectively. The protein-coding region can be obtained by transfer from other Flexi® Vectors or by capture of amplification products generated with SgfI and PmeI primers. For a complete description of the Flexi® Vector Systems, please refer to Technical Manual #TM254.

Certain protein-coding regions appear to perform differently depending upon whether they are fused to the VP16 activation domain or the GAL4 DNA-binding domain (13), and the interaction between the pairs may be vector-dependent. Because of this phenomenon, we advise cloning the protein-coding regions of interest into both the pFN10A (ACT) and pFN11A (BIND) Flexi® Vectors and testing for possible fusion protein interactions.

Transcriptional activation domains for a protein of interest can be tested by fusion with the pFN11A (BIND) Flexi® Vector. Co-transfection of this construct together with the reporter pGL4.31[*luc2P/GAL4*UAS/Hygro] Vector would be expected to give higher levels of luciferase activity compared to co-transfection of the reporter vector with the pBIND Vector alone, or the pFN11A (BIND) Flexi® Vector with a protein-coding region that is not a transcriptional activator.

3.A. pFN10A (ACT) Flexi® Vector

The pFN10A (ACT) Flexi® Vector contains several specialized features. The vector is designed to express a functional fusion protein comprising the herpes simplex virus VP16 activation domain (amino acids 411–456), nuclear localization sequences, a linker segment and an in-frame protein-coding sequence flanked by SgfI and PmeI sites at the 5´ and 3´ ends, respectively (Figure 2). Fusion protein expression is under control of the human cytomegalovirus (CMV) immediate early promoter. The protein-coding sequence can be captured directly into the ampicillin-resistant pFN10A (ACT) Flexi® Vector from an amplification reaction or transferred from another Flexi® Vector, preferably encoding kanamycin resistance, via the compatible SgfI/PmeI sites. The transcribed RNA sequence for the fusion protein contains a chimeric intron 5´ of the protein coding region (17,18) and polyadenylation region 3´ of the coding region (19). A T7 RNA polymerase promoter embedded upsteam of the VP16 activation domain allows the construct to be transcribed and translated in vitro [e.g., using the TNT® T7 Quick Coupled Transcription/Translation System (Cat. #L1170)]. The pFN10A (ACT) Flexi® Vector also contains the neomycin phosphotransferase gene driven by the SV40 early promoter. Neomycin phosphotransferase confers resistance to the antibiotic G418 (Geneticin®) to transfected cells (20). Once the protein coding sequence of interest is cloned into the pFN10A (ACT) Flexi Vector, the resulting plasmid can be propagated in *E. coli* under ampicillin selection.

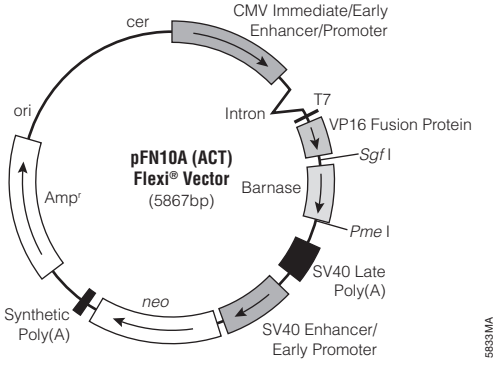

Figure 2. pFN10A (ACT) Flexi® Vector circle map and sequence reference points.

pFN10A (ACT) Flexi® Vector Sequence Reference Points:

CMV immediate/early enhancer/promoter	1–742
Chimeric intron	857–989
T7 RNA polymerase promoter (–17 to +3)	1033–1052
VP16 fusion protein	1083–1292
IgA linker	1299–1340
SgfI site	1332–1339
Barnase coding region	1363–1698
PmeI site	1700–1706
SV40 late polyadenylation signal	1859–2080
SV40 enhancer/early promoter	2179–2597
Neomycin phosphotransferase coding region	2642–3436
Synthetic polyadenylation signal	3500–3548
β-lactamase (Amp^r) coding region	3809–4669
ColE1-derived plasmid origin of replication	4824–4860
cer region (site for *E. coli* XerCD recombinase)	5531–5816

Note: The IgA linker encodes the polypeptide AIPSTPPTPSPAIA.

3.B. pFN11A (BIND) Flexi® Vector

The pFN11A (BIND) Flexi® Vector is designed to functionally express a fusion protein comprised of a DNA-binding domain of the yeast GAL4 gene (amino acids 1–147), a linker segment and an in-frame protein-coding sequence flanked by SgfI and PmeI sites at the 5´ and 3´ ends, respectively, under the control of the human cytomegalovirus (CMV) immediate early promoter (Figure 3). As with the pFN10A (ACT) Flexi® Vector, this ampicillin-resistant vector can be used as an acceptor vector for protein-coding sequences obtained directly by PCR amplification or transferred from another Flexi® Vector, preferably encoding kanamycin resistance, via the compatible SgfI/PmeI sites. In the pFN11A (BIND) Flexi® Vector, the *Renilla* luciferase gene is preceded by the SV40 early promoter and a growth hormone intron (hGH) for high expression levels (17,18). The *Renilla* luciferase gene may be used to normalize for transfection differences between samples within an experiment. A T7 RNA polymerase promoter is included upstream of the GAL4 DNA binding domain and allows the construct to be transcribed and translated in vitro using the TnT® T7 Quick Coupled Transcription/Translation System (Cat. #L1170).

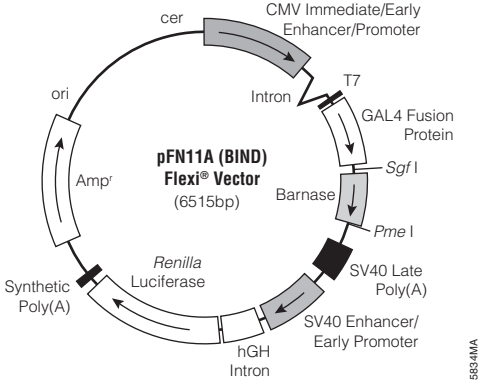

Figure 3. pFN11A (BIND) Flexi® Vector circle map and sequence reference points.

pFN11A (BIND) Flexi® Vector Sequence Reference Points:

CMV immediate/early enhancer/promoter	1–742
Chimeric intron	857–989
T7 RNA polymerase promoter (–17 to +3)	1033–1052
GAL4 fusion protein	1083–1520
IgA linker	1521–1553
SgfI site	1554-1561
Barnase coding region	1585–1920
*Pme*I site	1922–1929
SV40 late polyadenylation signal	2081–2302
SV40 enhancer/early promoter	2401–2819
hGH intron	2871–3130
Renilla luciferase coding region	3155–4090
Synthetic polyadenylation signal	4148–4194
β-lactamase (Amp^r) coding region	4457–5317
ColE1-derived plasmid origin of replication	5472–5508
cer region (site for *E. coli* XerCD recombinase)	6179–6464

Note: The IgA linker encodes the polypeptide AIPSTPPTPSPAIA.

3.C. pGL4.31[*luc2P/GAL4*UAS/Hygro] Vector

The pGL4.31[*luc2P/GAL4*UAS/Hygro] Vector is designed for transcriptional activation of the firefly luciferase reporter gene by association of the GAL4 DNA-binding and VP16 activation domains bound upstream of the luciferase gene. This reporter vector contains five consensus binding sequences, or Upstream Activating Sequences (UAS), for the GAL4 DNA-binding domain (*GAL4*UAS) upstream of a minimal adenoviral promoter. The *luc2P* reporter gene is a synthetic firefly luciferase sequence engineered for increased mammalian expression by optimizing the codons used and removing the consensus sequences for transcription factor binding sites. In addition, the *luc2P* gene incorporates a protein degradation sequence, PEST, from the C-terminal region of mouse ornithine decarboxylase (21) to give a destabilized reporter protein with a faster response that may be better suited to monitor rapid processes. The pGL4.31 Vector codes for hygromycin resistance, which allows long-term selection of stably transfected cells (22), and ampicillin resistance for propagation in *E. coli*.

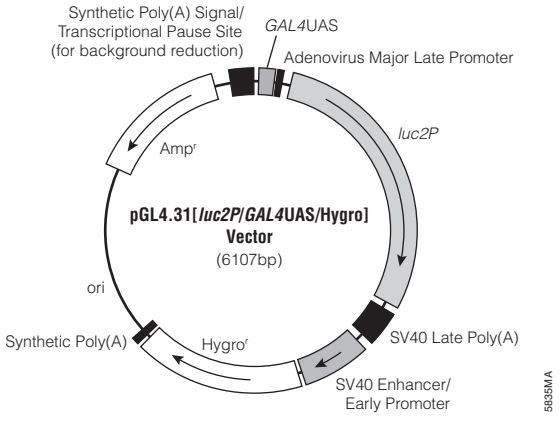

Figure 4. pGL4.31[*luc2P/GAL4*UAS/Hygro] Vector circle map and sequence reference points.

pGL4.31[*luc2P/GAL4*UAS/Hygro] Vector Sequence Reference Points:

*GAL4*UAS binding sites	31–133
Adenovirus major late promoter	145–185
luc2P firefly luciferase reporter	238–2013
SV40 late polyadenylation signal	2053–2274
SV40 enhancer/early promoter	2322–2740
Synthetic hygromycin resistance (Hygro^r) coding region	2765–3802
Synthetic polyadenylation signal	3826–3874
ColE1-derived plasmid origin of replication	4198–4234
Synthetic β-lactamase (Amp^r) coding region	4989–5849
Synthetic poly(A) signal/transcriptional pause site	5954–6107

4. Protocols

4.A. Cloning into the Flexi® Vectors

To clone your coding regions of interest, you can either transfer the region from a Flexi® Vector or capture a PCR amplimer. For detailed protocols on protein-coding region amplification or transfer, please refer to the *Flexi® Vector Systems Technical Manual* #TM254.

Transfer from a Flexi® Vector

1. Clone the coding regions of interest into a Flexi® Vector using the Flexi® System, Entry/Transfer (Cat.# C8640).

   Protein-coding regions can be cloned into Flexi® Vectors, and these inserts can be easily transferred to other Flexi® Vectors following digestion with SgfI and PmeI. Insert orientation and reading frame are maintained, eliminating the need to resequence inserts after each transfer. Vectors that encode no tags or N-terminal fusion tags can serve as acceptors and donors of protein-coding regions. Vectors that encode a C-terminal fusion tag can only serve as acceptors.

2. Transfer the coding regions into the pFN10A (ACT) and pFN11A (BIND) Flexi® Vectors as described in Section 5 of the *Flexi® Vector Systems Technical Manual* #TM254.

   The pFN10A (ACT) and pFN11A (BIND) Flexi® Vectors can serve as acceptors of protein-coding regions from other native-expressing or N-terminal fusion Flexi® Vectors, preferably vectors that encode kanamycin resistance. Since the pFN10A (ACT) and pFN11A (BIND) Flexi® Vectors both contain ampicillin resistance, transfer from a kanamycin-type Flexi® Vector allows selection against the donor plasmid. pFN10A (ACT) and pFN11A (BIND) Flexi® Vectors containing protein-coding inserts can serve as donors to other Flexi® Vectors. Transfer of protein-coding regions between donor and acceptor Flexi® Vectors is accomplished by a 15–30 minute restriction digestion reaction followed by heat-inactivation, a 1-hour ligation reaction and bacterial transformation.

PCR Amplify and Capture Insert

1. Clone the protein-coding regions of interest into the pFN10A (ACT) and pFN11A (BIND) Flexi® Vectors using PCR amplimers.

   To facilitate cloning, PCR primers used to amplify the protein-coding region must append an SgfI site to the amino-terminus and a PmeI site to the carboxy-terminus of the product. Transfer of the protein-coding regions into N-terminal fusion vectors results in translational readthrough of the SgfI site, which encodes the peptide sequence Ala-Ile-Ala. The PmeI site is placed at the carboxy terminus, appending a single valine residue to the last amino acid of the protein-coding region. The valine codon, GTT, is immediately followed by an ochre stop codon, TAA. Primer design guidelines are provided in Technical Manual #TM254 and at: **www.promega.com/techserv/tools/flexivector/**

4.B. Vector Purification, Transfection and Experimental Design

1. Purify the CheckMate™/Flexi® Vector fusion constructs.

   Following successful cloning or transfer of protein-coding regions into the Flexi® Vectors, the plasmids should be purified by methods that provide DNA that is relatively free of protein, RNA and chemical contamination. For a listing of various plasmid purification systems available from Promega, such as the PureYield™ Plasmid Midiprep System (Cat.# A2492), visit the DNA Purification chapter of the online *Protocol and Applications Guide* at: **www.promega.com/paguide/**. The pGL4.31[*luc2P/GAL4*UAS/Hygro] Vector is provided ready for transfection.

2. Transfect the purified CheckMate™/Flexi® Vector constructs into your cell line.

   Transfection optimization with your cell type of interest is important to successfully assay protein:protein interactions. The optimization process is easier using a reporter gene and assay system, such as the firefly luciferase reporter pGL4.13 [*luc2/SV40*] Vector (Cat.# E6681) and the Bright-Glo™ Luciferase Assay System (Cat.# E2610). Standard transfection methods to use include cationic lipid [e.g., TransFast™ Transfection Reagent (Cat. #E2431)], calcium phosphate [e.g., ProFection® Mammalian Transfection System—Calcium Phosphate (Cat.# E1200)], DEAE-dextran [e.g., ProFection® Mammalian Transfection System—DEAE-Dextran (Cat.# E1210)], electroporation and nucleofection protocols.

3. Measure the bioluminescent signal from the mammalian two-hybrid experiment.

   Identification of protein:protein interactions with the CheckMate™/Flexi® Vector System requires co-transfection of three plasmids: the pFN10A (ACT) and pFN11A (BIND) Flexi® Vectors containing the protein-coding regions of interest and the pGL4.31[*luc2P/GAL4*UAS/Hygro] Vector. Determine the optimal amount of plasmid DNA needed for transfection with your system. This can be done using a control reporter vector. Using this optimized quantity as a starting point, transfect similar amounts of each plasmid in the co-transfection mixture with the total equal to the optimal amount of plasmid DNA.

(!) Do **not** transfect pFN10A (ACT) or pFN11A (BIND) Flexi® Vectors directly for baseline or negative control comparisons. These vectors contain the barnase gene, which is toxic in mammalian cells. Instead, use the pACT and pBIND Vectors as negative controls. These vectors contain identical transcriptional activation and DNA-binding domains, respectively, to the corresponding Flexi® Vectors. Alternatively, protein-coding regions known not to interact can be cloned into the pFN10A (ACT) and pFN11A (BIND) Flexi® Vectors and used as negative controls. The pACT-MyoD and pBIND-Id Control vectors can be used together as positive controls when co-transfected with the reporter vector. The MyoD and Id proteins are known to interact and provide a positive readout signal in many cell types tested (23), and can be used to troubleshoot the CheckMate™/Flexi® Vector System.

4.B. Vector Purification, Transfection and Experimental Design (continued)

Table 1 presents recommended combinations of vectors to properly control experiments using the CheckMate™/Flexi® Vector System. "X" and "Y" represent protein-coding regions to test for protein:protein interactions.

Table 1. Experimental Design for Testing Protein:Protein Interactions.

Sample	ACT Vector	BIND Vector	pGL4.31 (*luc2P/GAL4*UAS/Hygro) Vector
1	pFN10A (ACT)-**Y**	pFN11A (BIND)-**X**	+
2	pACT Vector	pFN11A (BIND)-**X**	+
3	pFN10A (ACT)-**Y**	pBIND Vector	+
4	pACT Vector	pBIND Vector	+
5	pACT-MyoD Control	pBIND-Id Control	+

Sample 1 is used to test for protein interactions between the X and Y protein-coding regions.

Sample 2 is used to test for the background level of reporter activity when one of the protein-coding regions is fused to the GAL4 DNA-binding domain. This also tests for transcriptional activation domain activity of protein **X** when fused to the GAL4 DNA-binding domain.

Sample 3 is used to test for the background levels of reporter activity when one of the protein-coding regions is fused to the activation domain.

Sample 4 generally provides the lowest level of background activity of luciferase activity from the reporter plasmid.

Sample 5 provides a positive control with interacting proteins fused to the activation and GAL4 DNA-binding domains.

Notes:

1. Samples 2 or 3 may provide the appropriate luciferase background level to use as the baseline for protein-protein interaction studies.
2. *Renilla* luciferase activity expressed from the BIND-type vectors can be used to normalize transfection efficiency.

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 *Renill*a luciferase activity in all samples	Low transfection efficiency. Re-optimize transfection conditions for your cell line with a reporter vector such as pGL4.13[*luc*2/SV40] Vector (Cat.# E6681).
	Check that cell cultures are not contaminated with *Mycoplasma*.
High variability in firefly luciferase activity between replicates	Unequal distribution of pGL4.31[*luc2P/GAL4*UAS/Hygro] Vector in transfection mixtures. Prepare a "master mix" of diluted pGL4.31[*luc2P/GAL4*UAS/Hygro] 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 or toxic. Assay for luciferase activity in cell extracts at earlier time points.
	Expression of a test protein is vector-dependent. Subclone the "X" and "Y" test proteins into the other's respective pFN10A (ACT) Flexi® Vector or pFN11A (BIND) Flexi® Vector.
	Weak interaction between proteins cloned into the ACT and BIND 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. References

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7. Related Products

Flexi® Vector Systems

Product	Size	Cat#
Flexi® System, Entry/Transfer	5 entry and 20 transfer reactions	C8640
Flexi® System, Transfer	100 transfer reactions	C8820
Carboxy Flexi® System, Transfer	50 transfer reactions	C9320
10X Flexi® Enzyme Blend (SgfI and PmeI)	25µl	R1851

Mammalian Two-Hybrid System

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

Luciferase Assay Systems

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	10ml*	E2920
EnduRen™ Live Cell Substrate	0.34mg*	E6481
ViviRen™ Live Cell Substrate	0.37mg*	E6491

*Larger sizes available.

Transfection Reagents

Product	Size	Cat#
TransFast™ Transfection Reagent	1.2mg	E2431
Tfx™-20 Reagent	4.8mg (3 × 1.6mg)	E2391
Tfx™-50 Reagent	2.1mg (3 × 0.7mg)	E1811
ProFection® Mammalian Transfection System—Calcium Phosphate	40 reactions	E1200

8. Appendix

8.A. pFN10A (ACT) Flexi® 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 **DQ487211**) and on the Internet at: **www.promega.com/vectors/**

Table 2. Restriction Enzymes That Cut the pFN10A (ACT) Flexi® Vector 1–5 Times.

Enzyme	# of Sites	Location	Enzyme	# of Sites	Location
Acc65 I	2	1721, 2227	**BstZI**	2	1847, 2677
AccI	2	1309, 1743	**ClaI**	1	3552
AccIII	1	5531	**Csp45I**	2	3453, 4672
AflIII	4	1259, 1380, 4767, 5744	**CspI**	1	3287
			DraI	1	1704
AgeI	1	4699	DrdI	4	810, 2614, 2798, 4875
Alw44I	2	3925, 5081			
AlwNI	2	3611, 5183	EarI	4	1120, 3115, 3325, 3798
AvaI	3	1281, 1555, 1725			
AvaII	5	1285, 1666, 3287, 4233, 4455	**EclHKI**	1	4597
			EcoRI	1	1709
AvrII	1	2577	FspI	3	2181, 2873, 4374
BalI	3	11, 65, 2853	**HaeII**	2	2774, 5015
BamHI	2	1730, 3565	**HincII**	4	670, 1310, 1744, 1990
BanII	2	1719, 3136			
BbeI	1	2774	**HindIII**	2	749, 2593
BbsI	2	929, 1088	**HpaI**	1	1990
BglII	1	5863	KasI	1	2770
BlpI	1	1817	**KpnI**	2	1725, 2231
BsaAI	3	494, 3075, 3659	**MluI**	1	5744
BsaBI	3	1250, 3564, 5862	**NaeI**	1	3273
BsaI	2	883, 4531	**NarI**	1	2771
BsaMI	2	1911, 2004	**NcoI**	5	514, 1340, 2188, 2484, 3203
BspHI	2	3757, 5487			
BspMI	5	845, 1755, 2658, 3039, 3489	**NdeI**	1	388
			NgoMIV	1	3271
BsrGI	1	97	**NheI**	1	1053
BssHII	1	3168	**NotI**	1	1847
BssSI	3	3363, 3928, 4940	**NsiI**	2	2327, 2399
Bst98I	3	821, 1018, 2625	**PmeI**	1	1704
BstXI	1	3492	**PstI**	3	831, 1752, 2824

8.A. pFN10A (ACT) Flexi® Vector Restriction Sites (continued)

Table 2. Restriction Enzymes That Cut the pFN10A (ACT) Flexi® Vector 1–5 Times (continued).

Enzyme	# of Sites	Location	Enzyme	# of Sites	Location
PvuI	1	1337	**SnaBI**	2	494, 3659
PvuII	2	2253, 2877	**SpeI**	1	153
SalI	2	1308, 1742	**SspI**	4	6, 53, 2101, 3792
ScaI	2	4116, 5828	**StuI**	1	2576
SfiI	1	2530	**Tth111I**	1	2889
SgfI	1	1337	**VspI**	3	161, 1033, 4422
SinI	5	1285, 1666, 3287, 4233, 4455	**XbaI**	1	1736
			XmaI	3	1281, 1555, 1725
SmaI	3	1283, 1557, 1727	**XmnI**	1	3997

Table 3. Restriction Enzymes That Do Not Cut the pFN10A (ACT) Flexi® Vector.

AccB7I	**Bsu36I**	**EcoRV**	PmlI	SgrAI
ApaI	DraIII	FseI	PpuMI	SwaI
AscI	**Eco47III**	**I-PpoI**	PshAI	XcmI
BclI	**EcoICRI**	**NruI**	**SacI**	**XhoI**
BstEII	EcoNI	PacI	**SacII**	

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

8.B. pFN11A (BIND) Flexi® 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 **DQ487212**) and on the Internet at: **www.promega.com/vectors/**

Table 4. Restriction Enzymes That Cut the pFN11A (BIND) Flexi® Vector 1–5 Times.

Enzyme	# of Sites	Location
Acc65I	2	1943, 2449
AccB7I	1	3003
AccI	2	1531, 1965
AccIII	1	6179
AflIII	4	1602, 3338, 5415, 6392
AgeI	1	5347
Alw44I	2	4573, 5729
AlwNI	3	3064, 4259, 5831
AvaI	3	1301, 1777, 1947
AvrII	1	2799
BalI	3	11, 65, 3007
BamHI	2	1952, 4213
BanII	1	1941
BbsI	4	929, 1088, 1313, 3996
BbuI	4	1115, 1980, 2547, 2619
BclI	2	3440, 3649
BglII	1	6511
BlpI	1	2039
BsaAI	3	494, 3888, 4307
BsaBI	2	4212, 6510
BsaI	3	883, 1226, 5179
BsaMI	2	2133, 2226
BspHI	3	3724, 4405, 6135
BspMI	2	845, 1977
BsrGI	3	97, 1374, 3854
BssSI	3	3814, 4576, 5588
Bst98I	3	821, 1018, 2847
BstZI	1	2069
Bsu36I	2	2909, 2955
ClaI	1	4200
Csp45I	2	3162, 5320
DraI	1	1926
DraIII	1	2903
DrdI	3	810, 2836, 5523
EarI	3	1478, 3326, 4446
EclHKI	1	5245
EcoNI	1	3068
EcoRI	2	1931, 2857
FspI	2	2403, 5022
HaeII	2	2879, 5663
HindIII	2	749, 2815
HpaI	2	1361, 2212
KpnI	2	1947, 2453
MluI	1	6392
NcoI	4	514, 1562, 2410, 2706
NdeI	1	388
NheI	1	1053
NotI	1	2069
NsiI	2	2549, 2621
PmeI	1	1926
PstI	2	831, 1974
PvuI	1	1559
PvuII	1	2475
SalI	2	1530, 1964
ScaI	2	4764, 6476
SfiI	1	2752
SgfI	1	1559
SmaI	2	1779, 1949
SnaBI	2	494, 4307
SpeI	1	153

8.B. pFN11A (BIND) Flexi® Vector Restriction Sites (continued)

Table 4. Restriction Enzymes That Cut the pFN11A (BIND) Flexi® Vector 1–5 Times (continued).

Enzyme	# of Sites	Location	Enzyme	# of Sites	Location
SphI	4	1115, 1980, 2547, 2619	**XbaI**	1	1958
			XcmI	1	3805
SspI	4	6, 53, 2323, 4440	**XhoI**	1	1301
StuI	1	2798	**XmaI**	2	1777, 1947
VspI	4	161, 1033, 3256, 5070	**XmnI**	2	3690, 4645

Table 5. Restriction Enzymes That Do Not Cut the pFN11A (BIND) Flexi® Vector.

ApaI	**CspI**	KasI	PmlI	SwaI
AscI	**Eco47III**	**NaeI**	PpuMI	**Tth111 I**
BbeI	**EcoICRI**	**NarI**	PshAI	
BssHII	**EcoRV**	**NgoMIV**	**SacI**	
BstEII	FseI	**NruI**	**SacII**	
BstXI	**I-PpoI**	PacI	SgrAI	

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

8.C. pGL4.31[*luc2P/GAL4*UAS/Hygro] 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 **DQ487213**) and on the Internet at: **www.promega.com/vectors/**

Table 6. Restriction Enzymes That Cut the pGL4.31[*luc2P/GAL4*UAS/Hygro] Vector 1–5 Times.

Enzyme	# of Sites	Location
Acc65I	1	15
AccI	4	3041, 3241, 3892, 5572
AccIII	1	116
AflIII	2	2950, 4141
AgeI	2	1370, 3812
Alw26I	1	5905
Alw44I	1	4455
AlwNI	1	4557
ApaI	2	269, 3178
AvaII	5	297, 1543, 3415, 3715, 5201
AvrII	1	2720
BalI	4	1220, 1745, 3453, 5297
BamHI	1	2286
BanII	3	269, 3178, 3632
BbeI	1	1190
BbsI	4	141, 297, 3328, 3970
BbuI	2	2468, 2540
BglI	1	9
BglII	1	187
BlpI	1	1193
BsaAI	1	2951
BsaBI	2	686, 2285
BsaMI	2	2105, 2198
BspHI	3	822, 1428, 4861
BspMI	1	6078
BsrGI	1	729
BssHII	1	3776
BssSI	3	1166, 3597, 4314
BstEII	1	4988
BstXI	1	4985
BstZI	4	2037, 2041, 3389, 4961
Bsu36I	1	5419
ClaI	2	2279, 6006
Csp45I	1	3877
DraI	4	2245, 3801, 4900, 4919
DraIII	1	1385
DrdI	4	1640, 2027, 3726, 4249
EarI	5	1547, 2314, 2854, 4025, 5281
EclHKI	2	3272, 5063
Eco47III	4	332, 443, 2972, 4017
EcoRI	2	112, 1888
FseI	1	2043
FspI	3	1061, 2324, 3567
HincII	3	1444, 2184, 3893
HindIII	1	204
HpaI	2	1444, 2184
Hsp92I	4	1187, 1635, 1830, 5600
KasI	1	1186
KpnI	1	19
NarI	1	1187
NcoI	3	237, 2331, 2627
NheI	1	140
NotI	1	4961
NsiI	2	2470, 2542

8.C. pGL4.31[*luc2P/GAL4*UAS/Hygro] Vector Restriction Sites (continued)

Table 6. Restriction Enzymes That Cut the pGL4.31[*luc2P/GAL4*UAS/Hygro] Vector 1–5 Times (continued).

Enzyme	# of Sites	Location	Enzyme	# of Sites	Location
PmeI	1	3801	**SpeI**	1	5892
PshAI	2	3145, 3956	**SphI**	2	2468, 2540
PstI	1	4985	**SspI**	2	5867, 5923
PvuI	2	93, 5433	**StuI**	1	2719
PvuII	2	313, 2396	**XbaI**	4	26, 184, 2024, 3806
SacII	1	5457			
SalI	1	3891	**XhoI**	1	69
SfiI	1	9	**XmnI**	1	2803
SgrAI	1	302			
SinI	5	297, 1543, 3415, 3715, 5201			

Table 7. Restriction Enzymes That Do Not Cut the pGL4.31[*luc2P/GAL4*UAS/Hygro] Vector.

AatII	**CspI**	**MluI**	PpuMI	SwaI
AccB7I	**EcoICRI**	**NdeI**	**SacI**	**Tth111 I**
AscI	EcoNI	**NruI**	**SgfI**	**VspI**
BsaI	**EcoRV**	PacI	**SmaI**	XcmI
Bst98I	**I-PpoI**	PmlI	**SnaBI**	**XmaI**

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

8.D. Control Vector Maps

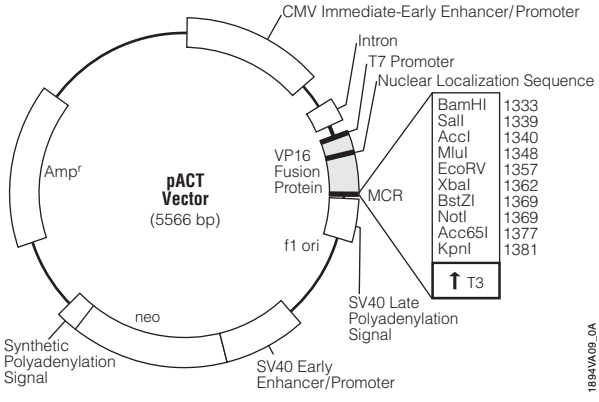

Figure 5. pACT Vector circle map and sequence reference points.

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

Vector sequences are available in the GenBank® database (GenBank®/EMBL Accession Number **AF264723**) and on the Internet at: **www.promega.com/vectors/**

8.D. Control Vector Maps (continued)

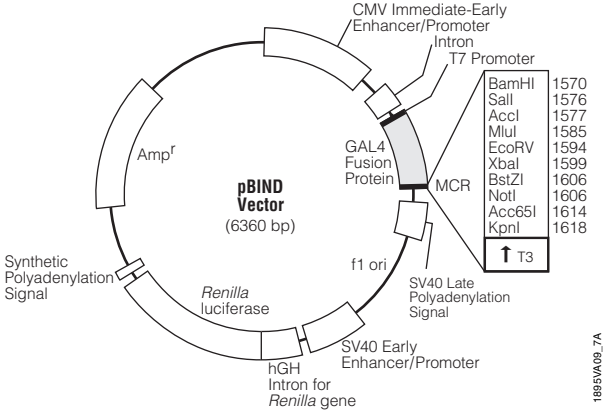

Figure 6. pBIND Vector circle map and sequence reference points.

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

Vector sequences are available in the GenBank® database (GenBank®/EMBL Accession Number **AF264722**) and on the Internet at: **www.promega.com/vectors/**

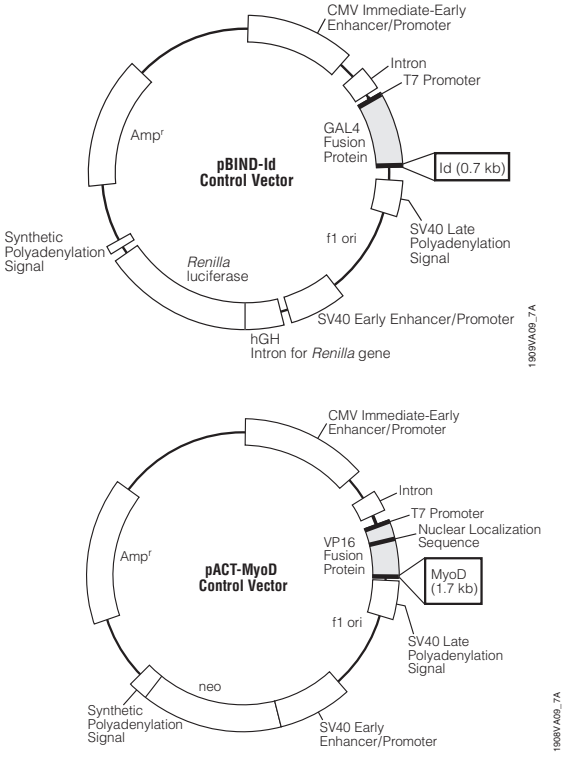

Figure 7. pBIND-Id and pACT-MyoD Control Vector circle maps. The protein product of the Id cDNA sequence in the pBIND-Id Vector is known to interact with the protein product of the MyoD cDNA sequence in the pACT-MyoD Vector.

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