



**Promega**

# Technical Manual

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## **Regulated Mammalian Expression System**

INSTRUCTIONS FOR USE OF PRODUCT C9470.



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# Regulated Mammalian Expression System

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 Please visit the web site to verify that you are using the most current version of this Technical Manual. Please contact Promega Technical Services if you have questions on use of this system. E-mail: [techserv@promega.com](mailto:techserv@promega.com)

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## 1. Description

The Regulated Mammalian Expression System<sup>(a-e)</sup> is designed to allow tight control of gene expression in mammalian cells. The system is based on a novel on/off switch that relies on the rapid and sensitive modulation of a chimeric transactivator protein by coumerin-related compounds (1). Nanomolar concentrations of the antibiotic coumermycin (i.e., coumermycin A1) promote homodimerization of a chimeric transactivator resulting in activation and binding to  $\lambda$  operator sequences located upstream of a minimal promoter that drives transcription of the coding sequence of interest. The levels of protein expression can be regulated by adjusting the coumermycin concentration. The expression can be rapidly switched off by adding novobiocin, which acts as an antagonist by dissociating the dimerized transactivator protein, inhibiting expression.

The Regulated Mammalian Expression System features high levels of induction of gene expression in stable cell lines with low levels of basal activity. This is achieved by combining a transactivator and a positive regulatory feedback loop to express the transactivator with reduced intrinsic dimerization activity, thus assuring low steady-state expression levels of the transactivator.

The Regulated Mammalian Expression System is well adapted for in vivo regulation of gene expression. Both modulator compounds, coumermycin and novobiocin, have excellent pharmacokinetic properties, display next to no toxicity and do not bind to endogenous targets in mammalian cells.

In addition, the Regulated Mammalian Expression System is compatible with other Flexi® Vectors for cloning and transfer between vectors.

### Advantages of the Regulated Mammalian Expression System

- High level of controlled induction combined with low basal protein expression
- Dose-response induction of protein expression
- Rapid and sensitive on/off switches for protein expression
- Auto-amplification of transactivator expression
- Excellent pharmacokinetic properties of modulator compounds
- Flexi® Vector compatibility

## 2. Product Components and Storage Conditions

Product	Size	Cat.#
Regulated Mammalian Expression System	1 system	C9470

Includes:

- 20µg pF12A RM Flexi® Vector
- 20µg pF12K RM Flexi® Vector
- 20µg pReg neo Vector

**Storage Conditions:** Store at -20°C. See expiration date on product label.

### Available Separately

Product	Size	Cat.#
pF12A RM Flexi® Vector	20µg	C9431
pF12K RM Flexi® Vector	20µg	C9441
pReg neo Vector	20µg	C9421
Coumermycin A1	5mg	C9451
Novobiocin Sodium Salt	1g	C9461

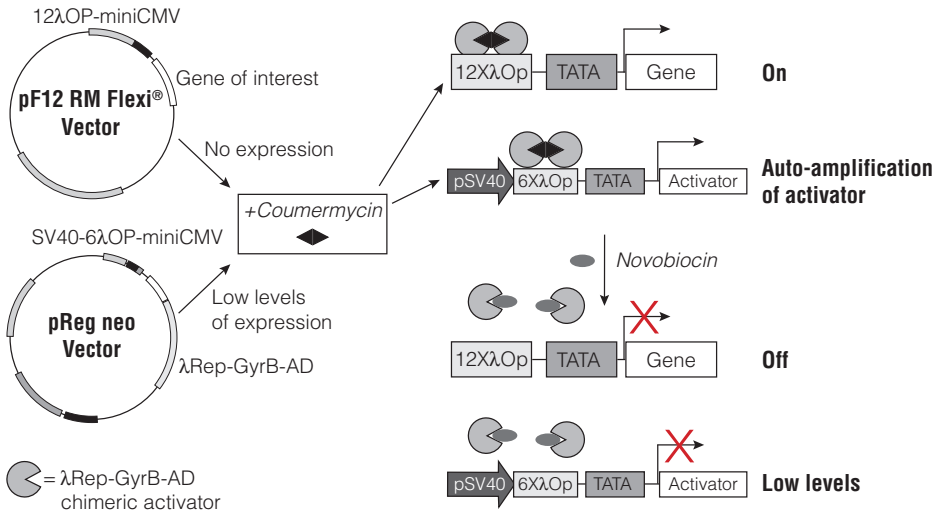
### 3. System Overview

The Regulated Mammalian Expression System relies upon two specially designed plasmids that are cotransfected into mammalian cells, along with the modulator compounds coumermycin and novobiocin that are added directly to cell culture medium. The protein coding region of interest is cloned into a specialized Flexi® Vector, either the pF12A RM Flexi® Vector or the pF12K RM Flexi® Vector, both of which were designed for Regulated Mammalian (RM) protein expression. The RM Flexi® Vector with the protein-coding region of interest is co-transfected into mammalian cells together with the pReg neo Vector. The pReg neo Vector is designed to express a chimeric transactivator protein that interacts with the promoter region in the pF12A RM or pF12K RM Flexi® Vector in response to coumermycin and novobiocin.

The chimeric transactivator expressed from the pReg neo Vector is composed of three parts: a  $\lambda$  repressor DNA binding domain ( $\lambda$ Rep), a bacterial gyrase B subunit domain (GyrB) and a NF- $\kappa$ B p65 transcriptional activation domain (AD). Activation of transcription is based on coumermycin-dependent binding to the bacterial GyrB domain and subsequent dimerization of the chimeric transactivator protein. One molecule of coumermycin dimerizes two subunits of the GyrB domain (2,3), allowing the  $\lambda$  repressor domain to bind  $\lambda$  operator sequences (4). Binding to the operator sequences brings the NF- $\kappa$ B p65 transcriptional activation domain of the chimeric transactivator protein into close proximity with the minimal promoter and increases transcription of the sequences of interest (Figure 1).

Additionally, the pReg neo Vector encodes a neomycin phosphotransferase gene that allows stable cell selection and generation with the antibiotic G-418.

The regulatory compounds coumermycin and novobiocin are added directly to cell culture medium. Novobiocin acts as an antagonist to coumermycin by competing with coumermycin for binding to the GyrB subunit of the chimeric transactivator protein. Novobiocin binds GyrB in a one-to-one ratio and interferes with dimerization of the transactivator protein by coumermycin (2,3).



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**Figure 1. Schematic representation of the coumerin-regulated system.**

In the Regulated Mammalian Expression System, the gene of interest is cloned into the pF12A or pF12K RM Flexi® Vector behind a minimal CMV promoter. Basal levels of transcription are low until binding of the homodimerized chimeric transactivator (λRep-GyrB) with associated AD occurs in close proximity and upstream of the promoter. The degree of induction and level of protein expression may be controlled by varying the concentration of coumermycin (0.5–10nM) added to the cell culture medium. The induction process is reversed by adding novobiocin (1–10μM).

## 4. General Considerations

### 4.A. Transient Versus Stable Transfectants

The performance of the Regulated Mammalian Expression System will depend upon the cell type and whether the transactivator gene is transiently or stably expressed. Transient transfection of both the pReg neo Vector and pF12 RM Flexi<sup>®</sup> Vector containing the protein-coding region of interest introduces multiple episomal copies of the vectors. Depending upon the particular cell type and associated repertoire of transcription factors as well as transfection efficacy, transcription of both the transgene and chimeric transactivator gene may be high even in the absence of coumermycin. This is due to background transcription from the promoters. Background and induced levels of protein expression will also be affected by the ratio of the transfected pReg neo Vector to the pF12 RM Flexi<sup>®</sup> Vector with the protein-coding region of interest.

Stable integration of the pReg neo Vector into the chromosome will result in lower basal expression levels of the transgene. This is an important consideration when toxic proteins are being studied. Enhanced performance of the system in stable transfected cells depends on the plasmid integration sites and requires screening to select the best clones exhibiting desired characteristics. Depending upon the sites of integration, chromatin effects can act to minimize and repress transcription and expression of the chimeric transactivator protein. Higher induction:background ratios may be obtained with such a stable cell line, compared to transient transfectants, due to lower basal expression levels of the transgene.

### 4.B. pF12A RM Flexi<sup>®</sup> Vector and pF12K RM Flexi<sup>®</sup> Vector

These Flexi<sup>®</sup> Vectors incorporate several specialized features designed to be compatible with both the Regulated Mammalian Expression System as well as with the Flexi<sup>®</sup> Vector Cloning System. Both vectors are designed for the capture of protein-coding region sequences intended for controlled protein expression. Controlled expression is made possible by the promoter region containing 12 tandem repeats of a consensus  $\lambda$  operator sequence, followed by a minimal CMV promoter. This provides low basal transcriptional activity within the cell. Upon induction with coumermycin, the chimeric transactivator dimer binds to the  $\lambda$  operator sequences resulting in high levels of transcription.

These expression vectors are part of the Flexi<sup>®</sup> Vector System, which uses two rare-cutting restriction enzymes, SgfI and PmeI, in a simple yet powerful directional cloning method for protein-coding sequences. These systems provide a rapid, efficient and high-fidelity way to transfer these sequences between a variety of Flexi<sup>®</sup> Vectors. The Flexi<sup>®</sup> Vectors contain various expression or peptide tag options to enable expression of native or fusion proteins to study protein structure and function as well as protein:protein interactions. All Flexi<sup>®</sup> Vectors carry the lethal barnase gene, which is replaced by the DNA fragment of interest and acts as a positive selection for successful

#### 4.B. pF12A RM Flexi® Vector and pF12K RM Flexi® Vector (continued)

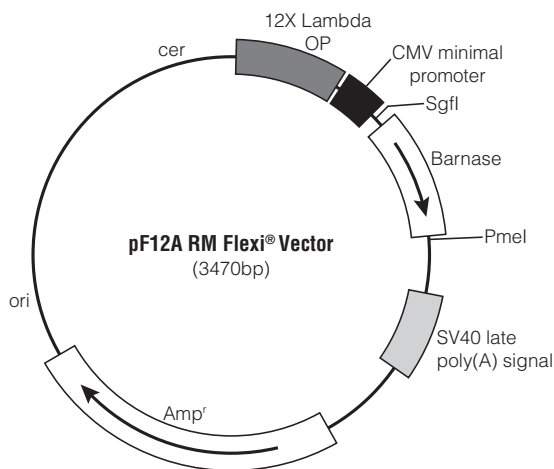
ligation of the insert. The pF12A RM Flexi® Vector and pF12K RM Flexi® Vector have identical properties, with the exception that the “A” vector provides ampicillin resistance and the “K” vector provides kanamycin resistance in *E. coli*. These resistance features are important and decrease the number of recipient clones to screen when considering protein-coding region transfer from one type of Flexi® Vector to another.



**Do not** use the pF12 RM Flexi® Vectors without an insert as a negative control for transfection. The barnase gene decreases the viability of the transfected cells.

The SgfI and PmeI sites in these vectors provide the common foundation for this cloning and transfer system. The SgfI site is most proximal to the promoter and is located one base upstream of the start codon for the protein coding region. The PmeI site contains the stop codon and defines the beginning of the 3' untranslated region of the transcript. SgfI has the fewest restriction sites in the protein-coding regions of human cDNA sequences, and PmeI has the next fewest. This enzyme pair also cuts infrequently in the open reading frames of many organisms (5). Most (>98%) of the known human open reading frames are not affected by the use of these restriction enzymes for directional cloning. However, we recommend scanning your protein-coding region for SgfI and PmeI sites. The presence of SgfI or PmeI sites within the protein-coding region will interfere with cloning of the full-length protein-coding region. If your protein-coding region contains these sites, consider cloning a portion of the protein-coding region or using RecA protein to protect the SgfI or PmeI sites within the protein-coding region from digestion (6). Alternatively, PCR-based, site-directed mutagenesis methods (7,8) can be used to mutate restriction enzyme sites without changing the amino acid sequence of a protein-coding region. The desired protein-coding region can be amplified by PCR to append SgfI and PmeI sites to the protein-coding region before being cloned into the Flexi® Vectors (see the *Flexi® Vector System Technical Manual #TM254* for a detailed protocol).

**Note:** Promega provides a tool that scans your sequence for SgfI and PmeI sites to ensure these sites are absent and to help with primer design. This tool is available at: [www.promega.com/techserv/tools/flexivector/](http://www.promega.com/techserv/tools/flexivector/)



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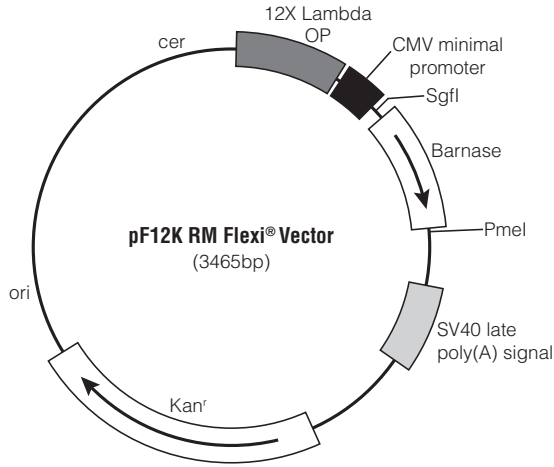
**Figure 2. Circle map of pF12A RM Flexi® Vector with features.**

pF12A RM Flexi® Vector Sequence Reference Points:

12X $\lambda$ operator	16–309
Minimal CMV promoter	322–434
SgfI site	446–453
Barnase coding region	477–812
PmeI site	814–821
SV40 late polyadenylation signal	973–1194
$\beta$ -lactamase (Amp <sup>r</sup> ) coding region	1455–2315
ColE1-derived replication of origin	2470–2506
cer site (site for <i>E. coli</i> XerCD recombinase)	3177–3462

Vector sequences are available in the GenBank® database (GenBank®/EMBL Accession Number EF030520) and at: [www.promega.com/vectors/](http://www.promega.com/vectors/)





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**Figure 3. Circle map of pF12K RM Flexi® Vector with features.**

pF12K RM Flexi® Vector Sequence Reference Points:

12X $\lambda$ operator	16–309
Minimal CMV promoter	322–434
SgfI site	446–453
Barnase coding region	477–812
PmeI site	814–821
SV40 late polyadenylation signal	973–1194
Kanamycin resistance gene	1502–2296
ColE1-derived origin of replication	2465–2501
cer site (site for <i>E. coli</i> XerCD recombinase)	3172–3457

Vector sequences are available in the GenBank® database (GenBank®/EMBL Accession Number EF030521) and at: [www.promega.com/vectors/](http://www.promega.com/vectors/)

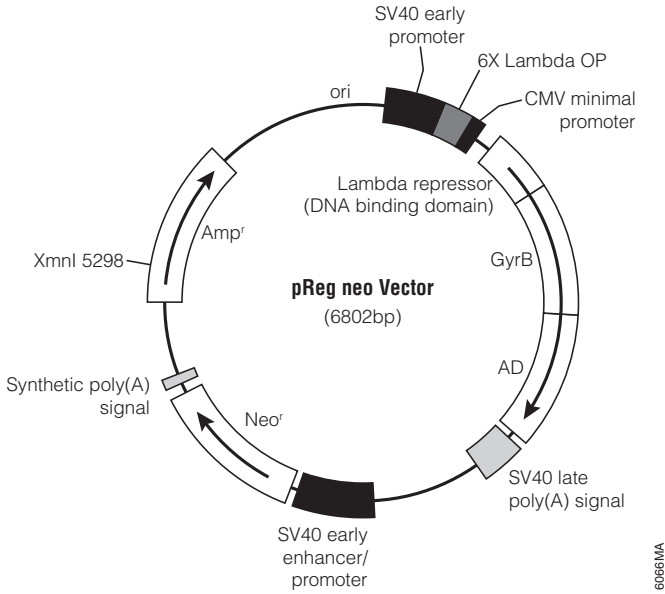
#### 4.C. pReg neo Vector

The pReg neo Vector expresses a chimeric transactivator protein with a positive feedback regulatory system. The chimeric transactivator comprises three different functional protein domains. The amino terminal region incorporates the DNA-binding domain of the bacteriophage  $\lambda$  repressor, which binds as a dimer to  $\lambda$  operator sequences. In addition, the repressor domain has been mutated to minimize intrinsic self-dimerization (1). This domain is fused downstream to the amino-terminal 24K subdomain of bacterial DNA gyrase B (amino acids 2-220), which upon binding to coumermycin results in protein dimerization. At the C-terminus is the p65 NF- $\kappa$ B activation domain (AD), which enhances transcription when bound to promoter regions.

Another notable feature of the pReg neo Vector is the specially designed hybrid promoter that controls expression of the chimeric transactivator and provides an autoamplification loop. The promoter comprises an SV40 early promoter followed by 6 tandem repeats of the  $\lambda$  operator sequence and the TATA box from the CMV promoter (9) for transcription initiation. Low levels of transcriptional activity occur from the SV40 promoter in the non-induced state and provide a basal level of the chimeric transactivator available for binding to coumermycin. This avoids overexpression of a strong activator that can result in transcriptional “squenching” by binding to endogenous transcription factors (10,11).

The addition of coumermycin promotes homodimerization of the transactivator, allowing for more efficient binding to the  $\lambda$  operator sequences and positioning the p65 NF- $\kappa$ B activation domain in the promoter region that increases polymerase II-dependent transcription.

The pReg neo Vector also encodes the neomycin phosphotransferase gene driven by the SV40 early enhancer/promoter to allow selection with G-418 for stable cell transfectants.



**Figure 4. Circle map of pReg neo Vector with features.**

pReg neo Vector Sequence Reference Points:

SV40 early promoter	141-433
6X $\lambda$ operator	444-587
Minimal CMV promoter	600-659
Rep-GyrB-AD	770-2443
Lambda repressor (DNA binding domain)	770-1085
GyrB (amino terminal domain)	1086-1758
p65 Activation Domain (AD)	1759-2443
SV40 late polyadenylation signal	2497-2718
SV40 early enhancer/promoter region	3330-3748
Neomycin phosphotransferase (Neor <sup>r</sup> ) coding region	3793-4587
Synthetic polyadenylation signal	4651-4699
$\beta$ -lactamase (Amp <sup>r</sup> ) coding region	5110-5970
ColE1-derived plasmid origin of replication	6695-6732

Vector sequences are available in the GenBank<sup>®</sup> database (GenBank<sup>®</sup>/EMBL Accession Number EF030522) and at: [www.promega.com/vectors/](http://www.promega.com/vectors/)

#### 4.D. Modulator Compounds: Coumermycin and Novobiocin

The Regulated Mammalian Expression System is controlled by a combination of the coumerin antibiotics, coumermycin and novobiocin. Coumermycin is produced in *Streptomyces* and consists of two coumerin-substituted rings joined by a linker (12). Novobiocin is based on a single coumerin-substituted ring, similar to the monomeric version of coumermycin.

Several properties of these antibiotics make them excellent compounds for use in mammalian systems. Novobiocin and coumermycin have been tested in animals with minimal toxicity. Both coumermycin and novobiocin bind bacterial GyrB with high affinity ( $K_d = 3-5 \times 10^{-8}M$ ) (13). In this system, coumermycin effectively induces gene expression at concentrations between 0.5 and 10nM. Concentrations above 500nM may decrease protein expression due to excess coumermycin causing dissociation of the homodimers. The concentration of novobiocin required to completely block coumermycin-induced gene expression and dissociate the protein homodimers is approximately 1,000-fold higher than for coumermycin, although the binding affinities for both antibiotics is similar.

Rapid on-off characteristics for protein expression control are related to the relatively short half-lives of the compounds. The half-lives in animals in serum are reported as 5.5 to 6 hours (14,15). In addition, these compounds are specific for the bacterial form of GyrB, and no known targets exist for high-affinity binding in mammalian cells.



#### Recommended Use of Reagents with this System

A stock solution of coumermycin at a final concentration of 5mM may be prepared in DMSO and stored in aliquots at  $-20^{\circ}C$ . Before use, the stock solution may be diluted in full media or PBS. We recommend preparing novobiocin in media or PBS to a final concentration of 1mM just prior to use. Do not store 1 $\mu$ M Novobiocin solution. To block coumermycin-induced gene expression, add novobiocin (1–10 $\mu$ M final concentration) directly to the cell culture medium. There is no need to replace the cell culture medium prior to addition.

## 5. Protocols

### 5.A. Cloning into the Flexi<sup>®</sup> Vectors

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

#### Transfer from a Flexi<sup>®</sup> Vector

1. Clone the coding regions of interest into a Flexi<sup>®</sup> Vector using the Flexi<sup>®</sup> System, Entry/Transfer (Cat.# C8640). Protein-coding regions can be cloned into Flexi<sup>®</sup> Vectors, and these inserts can be easily transferred to other Flexi<sup>®</sup> 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 donors of protein-coding regions. Vectors that encode a C-terminal fusion tag can not serve as donors.
2. Transfer the coding regions into the pF12A RM or pF12K RM Flexi<sup>®</sup> Vectors as described in Section 5 of the *Flexi<sup>®</sup> Vector Systems Technical Manual #TM254*. The pF12A RM and pF12K RM Flexi<sup>®</sup> Vectors can serve as acceptors of protein-coding regions from other native-expressing or N-terminal fusion Flexi<sup>®</sup> Vectors. Transfer protein coding regions between the pF12A RM Flexi<sup>®</sup> Vector and donor Flexi<sup>®</sup> Vector with kanamycin resistance or between the pF12K RM Flexi<sup>®</sup> Vector and Flexi<sup>®</sup> Vector donor plasmids with ampicillin resistance. pF12A RM and pF12K RM Flexi<sup>®</sup> Vectors containing protein-coding inserts can serve as donors to other Flexi<sup>®</sup> Vectors. Transfer of protein-coding regions between donor and acceptor Flexi<sup>®</sup> Vectors is accomplished by a 15- to 30-minute restriction digestion reaction followed by heat-inactivation, a 1-hour ligation reaction and bacterial transformation.

#### PCR Amplify and Capture Insert

Clone the protein-coding regions of interest into the pF12A RM and pF12K RM Flexi<sup>®</sup> Vectors using PCR amplicons. 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. The SgfI site is placed one base upstream of the start codon. This allows de novo initiation at the native translation start site. 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 a stop codon, TAA. Primer design guidelines are provided in *Flexi<sup>®</sup> Vector Systems Technical Manual #TM254* and at: [www.promega.com/techserv/tools/flexivector/](http://www.promega.com/techserv/tools/flexivector/)

## 5.B. Optimization Experiments and Experimental Designs for Transient Transfections

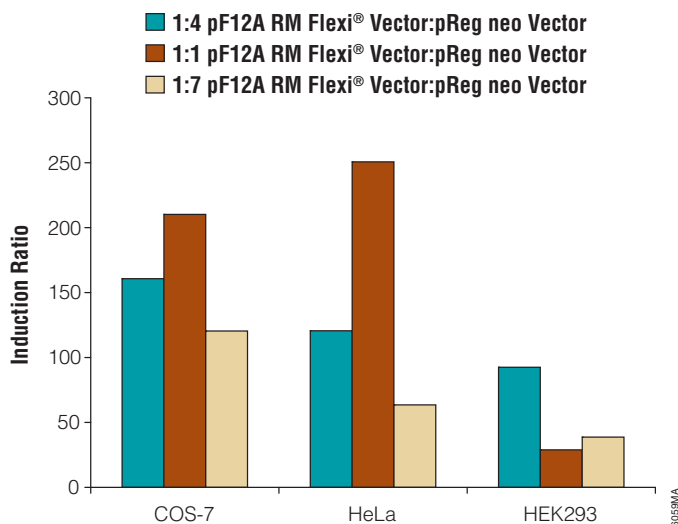
We recommend optimizing the system for lowest basal and highest induction values using a transient transfection assay. For optimization experiments we suggest using a pF12 RM Flexi® Vector with a suitable reporter gene. Section 5.A. describes cloning into Flexi® Vectors.

In transient transfections, performance of the Regulated Mammalian Expression System will primarily depend on basal activity of the minimal CMV promoter upstream of the transgene for the protein of interest, as well as on SV40 hybrid promoter activity driving expression of the transactivator protein. Higher levels of the transactivator protein, even in the absence of coumermycin, may transiently homodimerize, leading to increased expression of the transgene. Basal promoter activities will vary in different cell types and with transfection efficiencies. Therefore, an optimization strategy may involve changing the ratio between the pReg neo and pF12 RM Flexi® Vectors for transfection, as well as the total amount of pF12 RM Flexi® Vector and pReg neo Vector DNA used for transfection (Table 1; Figure 5). Carrier DNA may be added to maintain an optimal amount of DNA for transfection purposes and may include a reporter gene for monitoring transfection efficiency, such as the pGL3-Control Vector (Cat.# E1741) or phRL-SV40 Vector (Cat.# E6261) that encodes *Renilla* luciferase.

**Table 1. Optimization of Plasmid DNA Ratios and Amounts for Transfection.** The DNA amounts in this table are based on a total of 1µg, which may be suitable for liposome-mediated transfections of a single well in a 24-well plate. Ratios are based on the amount of pF12 RM Flexi® Vector DNA with the cloned insert of interest to pReg neo Vector DNA. “Total Expression System DNA” refers to the combination of pF12 RM Flexi® Vector plus pReg neo Vector. “10%” refers to the total amount of Expression System DNA compared to the previous column. Carrier DNA amounts are included to maintain a total of 1µg DNA. Carrier DNA may include a plasmid with a reporter gene, such as luciferase, for transfection normalization. Proportionate changes to these amounts would need to be made for different recommended transfection reagents or methods, as well as for changes in the well size.

	1:1 Ratio	1:4 Ratio	1:4 Ratio (10%)	1:7 Ratio
<b>pF12 RM Flexi® Vector</b>	0.25µg	0.1µg	0.01µg	0.1µg
<b>pReg neo Vector</b>	0.25µg	0.4µg	0.04µg	0.7µg
<b>Total Expression System DNA</b>	0.5µg	0.5µg	0.05µg	0.8µg
<b>Carrier DNA</b>	0.5µg	0.5µg	0.95µg	0.2µg

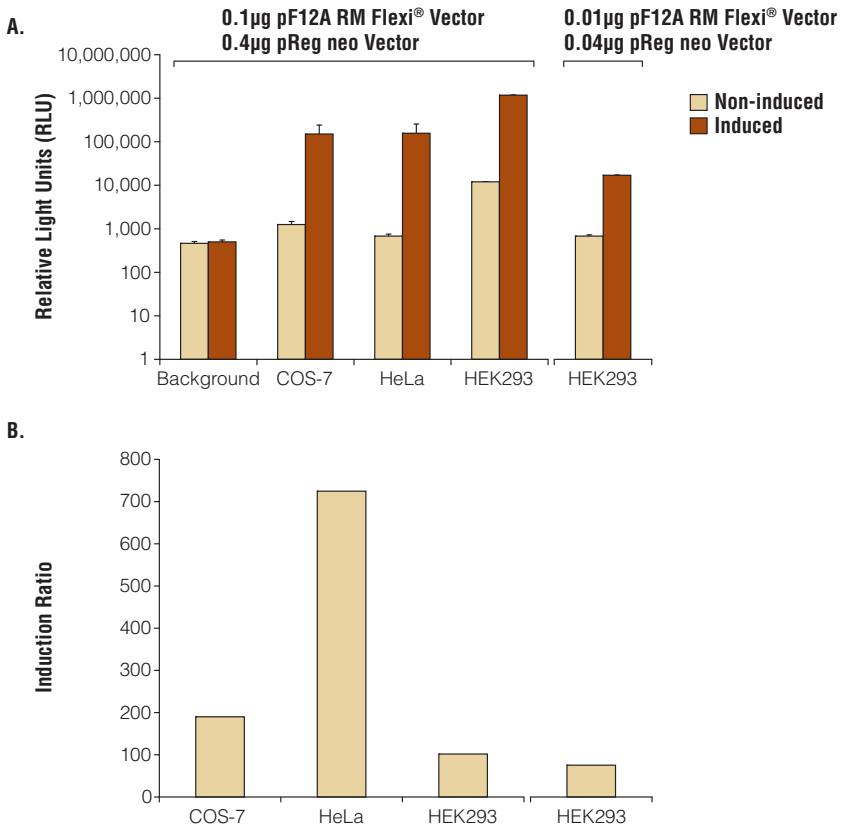
## 5.B. Optimization Experiments and Experimental Designs (continued)



**Figure 5. Effect of the ratio of pF12A Flexi® and pReg neo Vectors on induction ratio.** Cells were plated in 12-well culture dishes at  $10^6$  cells/well and transfected with a total of  $1\mu\text{g}$  of DNA. Each DNA sample for transfection included  $0.1\mu\text{g}$  of pF12A RM Flexi® Vector with a firefly luciferase coding sequence and 0.4, 0.1 or  $0.7\mu\text{g}$  of pReg neo Vector, plus  $0.2\mu\text{g}$  of phRL-SV40 DNA. Carrier DNA was added to samples such that the final amount of DNA was  $1\mu\text{g}$ . Three hours post-transfection, coumermycin was added ( $5\text{nM}$  final concentration) to induce protein expression (firefly luciferase) and no coumermycin was added to the corresponding control wells to monitor basal activity. Twenty-four hours after coumermycin addition, cells were harvested, lysed and assayed for firefly and *Renilla* luciferase activities with Dual-Glo™ Luciferase Assay System (Cat.# E2980). The firefly luciferase expression was normalized to *Renilla* luciferase as the internal transfection normalization factor. The induction ratio for firefly luciferase was calculated from the firefly/*Renilla* luciferase ratio in coumermycin-induced vs. non-induced control samples.

In our experience, basal activity of the minimal CMV promoter was low in HeLa and COS-7 cells (1.5- to 2-fold above assay background). However, in HEK293 cells under identical transfection conditions, basal activity from the minimal CMV promoter was more than 10-fold higher in comparison. Basal activity in HEK293 cells was reduced to near background levels by lowering the total amount of plasmid DNA transfected by 10-fold. Induction levels ranged from 100- to 1,000-fold, depending on the cell type and experimental design (Figure 6).

For induction, coumermycin is diluted in culture medium and added directly to cells 3-hours post-transfection. Depending upon the cell type, maximal induction occurs using 5 to  $10\text{nM}$  of coumermycin. Titration of coumermycin between  $0.5\text{nM}$  and  $50\text{nM}$  can be tested to determine the dose-response and maximal induction level by coumermycin. Protein expression is monitored 24 to 48 hours following coumermycin addition.



**Figure 6. Effect of the total amount of pF12A Flexi® and pReg neo Vectors used for transfection. Panel A.** COS-7, HeLa and HEK293 cells were plated in 12-well culture dishes at  $10^6$  cells/well and transfected with a total of  $1\mu\text{g}$  of DNA. Each DNA sample for transfection included a 1:4 ratio of the pF12A RM Flexi® Vector with a firefly luciferase coding sequence ( $0.1\mu\text{g}$  or  $0.01\mu\text{g}$  in the 10% sample) to pReg neo Vector ( $0.4\mu\text{g}$  or  $0.04\mu\text{g}$  in the 10% sample) plus  $0.2\mu\text{g}$  of pHRL-SV40 DNA. Carrier DNA was added to samples such that the final amount of DNA was  $1\mu\text{g}$ . Three hours post-transfection, coumermycin was added ( $5\text{nM}$  final concentration) to induce protein expression of firefly luciferase (induced), and no coumermycin (non-induced) was added to the corresponding control wells to monitor basal activity. Twenty-four hours after coumermycin addition, cells were harvested, lysed and assayed for firefly and *Renilla* luciferase activities with Dual-Glo™ Luciferase Assay System (Cat.# E2980). Background was measured using non-transfected cells. The firefly luciferase expression was normalized to *Renilla* luciferase as the internal transfection normalization factor and expressed as “Relative Light Units”. **Panel B.** The fold induction for firefly luciferase was calculated using the ratio of firefly luciferase to *Renilla* luciferase in the coumermycin-induced samples compared to the non-induced control samples.



To suppress coumermycin-induced protein expression of the transgene, novobiocin is added directly to cell culture medium with coumermycin. There is no need to change the culture medium prior to addition. A 10 $\mu$ M concentration, or a concentration of novobiocin that is 1,000-fold in excess of the coumermycin concentration, assures adequate competition for coumermycin binding to the GyrB subunit, dissociation of the transactivator dimers, and subsequent decrease of protein expression. Novobiocin suppresses protein expression best in stable cells rather than in transiently transfected cell models.

### 5.C. Establishing Stable Cell Lines with the pReg neo Vector

Lower basal protein expression with a high induction ratio may be achieved with a stable cell line. Chromosomal integration of the pReg neo Vector generally results in lower levels of the chimeric transactivator and, therefore, of the transgene protein as well, compared to transient transfections. A stable cell line that expresses the chimeric transactivator can be used as the recipient cell type for transfections with various pF12 RM Flexi<sup>®</sup> Vectors expressing many different proteins.

The pReg neo Vector encodes the neomycin phosphotransferase gene, providing a means to select for long-term transfectants with the antibiotic G-418 (Cat.# V7983). To generate a stable cell line, we recommend linearization of the pReg neo Vector with XmnI (Cat.# R7271), which digests this plasmid between the Neo resistance cassette and the SV40 promoter. Following transfection with the linearized vector, G-418 is added to the culture 48 hours later. The concentration of G-418 to select and maintain drug resistance depends on the cell type and growth rate. For effective selection, the cells should be subconfluent because confluent cells are very resistant to the antibiotic. It may be necessary to re-seed the cells at a lower density if they are too dense prior to selection. In general, the effective concentration of G-418 for selection is 400–800 $\mu$ g/ml and 200–400 $\mu$ g/ml for maintenance of stable transformants (16). However, it may be necessary to perform a G-418 titration between 100–1,000 $\mu$ g/ml to determine the most effective concentration for a given cell type and growth condition. Death of sensitive cells may take 3 to 10 days.

Resistant clones are isolated by standard methods, propagated and then characterized for performance regarding regulated protein expression. We recommend testing by transfection with a pF12 RM Flexi<sup>®</sup> Vector containing an easy-to-assay reporter gene, such as luciferase. The level of chimeric transactivator protein is generally lower in these cells than in transiently transfected cells, and therefore, it is important to add the appropriate amount of the transgene by titrating the amount of pF12 RM-reporter protein Flexi<sup>®</sup> Vector construct for transfection. Three hours after transfection, cells are treated with and without 5–10nM coumermycin and assayed for the reporter protein, followed by an additional 24–48 hours. Ideal clones exhibit low basal protein expression in the absence of coumermycin and high induction levels in the presence of coumermycin.

## 5.D. Establishing Cell Lines with the pReg neo and pF12 RM Flexi® Vectors

Stable cell lines expressing both the chimeric transactivator and regulated protein of interest can be generated. Two types of approaches may be used. In a one-step approach, both pReg neo and the pF12 RM Flexi® Vector construct plasmids are co-transfected simultaneously. Selection with G-418 and clone screening identifies the clones with stable integration and optimal performance for regulated mammalian protein expression.

In a two-step approach, first a stable cell line with the pReg neo Vector is chosen (as described in Section 5.A), then transfected with a pF12 RM Flexi® Vector construct. In this approach, it is also necessary to co-transfect another plasmid containing another drug selection marker, such as hygromycin or puromycin, and the resultant clones will be resistant to this new marker as well as resistant to G-418. The advantage to this approach is that the cell line containing the pReg neo sequences can be prescreened and characterized for basal and induced properties of regulated protein expression first using transient expression for the protein and also can be used as a recipient for many different proteins expressed with the pF12 RM Flexi® Vectors.

## 6. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: [www.promega.com](http://www.promega.com). E-mail: [techserv@promega.com](mailto:techserv@promega.com)

### For Transient Transfections

Symptoms	Causes and Comments
High background in non-induced state	<p>Nonspecific transcription from minimal CMV promoter.</p> <ul style="list-style-type: none"> <li>• Reduce amount of plasmid for transient transfection.</li> <li>• Optimize ratio between pReg neo and pF12 RM Flexi® Vectors.</li> </ul>
No or low activity after induction	<p>Low transfection efficiency. Use <math>\beta</math>-galactosidase, luciferase, GFP or other fluorescent protein markers (e.g., HaloTag® ligands) to determine transfection efficiency.</p> <p>Insufficient transactivator protein produced. Check the system using reporter genes. If no activity is detected with reporter control, consider replacing SV40 promoter or entire SV40-6X OP-miniCMV cassette with the promoter that is known to function in your cell type of interest.</p> <p>Wrong concentration or degradation of coumermycin. Prepare fresh coumermycin stock.</p>

## 6. Troubleshooting (continued)

### For Stable Cell Line Development

Symptoms	Causes and Comments
No antibiotic-resistant stable cell line clones generated	The vectors do not contain antibiotic selection marker. Check vector sequence. <hr/> Antibiotic concentration is too high.
No detectable activity in stable cell line clones before and after induction	Poor quality or wrong DNA. Check DNA and test in a transient transfection. <hr/> Screen more stable cell line clones. <hr/> SV40 promoter is not functional in your cell line. Replace SV40 promoter or entire SV40-6X OP-miniCMV cassette with the promoter that is known to function in the cell type studied.
Selected stable cell line clones show high activity in non-induced state	The vector has been integrated into a region of chromatin that is highly transcribed. Screen more clones.

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## 8. Related Products

Product		Size	Cat.#
Coumermycin A1		5mg	C9451
Novobiocin Sodium Salt		1g	C9461
Flexi® System Entry/Transfer	5 entry and 20 transfer reactions		C8640
Flexi® System, Transfer	100 transfer reactions		C8820
XmnI		500 units	R7271
TransFast™ Transfection Reagent		1.2mg	E2431

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