Complete Control Inducible Mammalian Expression System

Instruction Manual

Catalog #217460 (pERV3 Vector), #217461 (pEGSH Vector), and #217468 (Complete Control Vector Kit)

Revision B.0

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217468-12

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Complete Control Inducible Mammalian Expression System

Contents

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MATERIALS PROVIDED

Materials provided	Concentration	Quantity		
		Catalog #217460	Catalog #217461	Catalog #217468
pERV3 receptor vector	1 µg/µl	100 µg	—	100 µg
pEGSH expression vector	1 µg/µl	—	20 µg	20 µg
pEGSH-Luc vector (positive control)	1 µg/µl	—	20 µg	20 µg
pEGSH sequencing primer (lyophilized)[a]	—	—	2.5 µg	2.5 µg
T3 20-mer sequencing primer (lyophilized)[b]	—	—	2.5 µg	2.5 µg
XL1-Blue host strain (glycerol stock)[c]	—	—	—	500 µl
Ponasterone A (lyophilized)[d]	—	—	—	1 mg

[a] pEGSH forward sequencing primer: 5′-CTCTGAATACTTTCAAAAGTTAC-3′.

[b] T3 promoter sequencing primer: 5′-AATTAACCCTCACTAAAGGG-3′.

[c] Genotype: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q} *ZΔM15 Tn*10 (Tet^{r})].

[d] See *Preparation of Media and Reagents*.

STORAGE CONDITIONS

XL1-Blue Host Strain: –80°C
Lyophilized Sequencing Primers: Room Temperature
All Other Materials: –20°C

ADDITIONAL MATERIALS REQUIRED

1× Phosphate-buffered saline (PBS) solution[§]
LB agar[§]
LB liquid medium[§]
Ampicillin
Cesium chloride
G418 sulfate or Geneticin® antibiotic
Hygromycin
Luciferase assay kit
Luminometer
Mammalian transfection solutions
Restriction enzymes
Plasmid DNA isolation solutions
T4 DNA ligase
10× Ligase buffer[§]
TE buffer[§]
10 mM rATP
100% (v/v) Ethanol

[§] See *Preparation of Media and Reagents.*

Revision B.0

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INTRODUCTION

System Overview

The Complete Control inducible mammalian expression system is a gene transfer system that allows precise control of gene expression in a wide variety of mammalian cell types. Development of the Complete Control system is based upon the finding that the insect hormone ecdysone or its analog ponasterone A (ponA) can activate transcription in mammalian cells harboring both the gene for the *Drosophila melanogaster* ecdysone receptor and a promoter containing a binding site for the ecdysone receptor.[1]

The Complete Control system has several advantages over other inducible systems. PonA has no known measurable effect on mammalian physiology. PonA has a short in vivo half-life, and its lipophilic nature allows it to efficiently penetrate all tissues, including the brain. The result is rapid and potent induction of gene expression and rapid clearance. A 1000-fold induction of a reporter gene, with negligible basal expression, has been obtained with the Complete Control system.[2]

System Design

The ecdysone receptor (EcR) is a member of the retinoid-X-receptor (RXR) family of nuclear receptors and is composed of three domains: an N-terminal activation domain (AD), a central DNA-binding domain (DBD), and a C-terminal ligand-binding and dimerization domain (LBD). In insect cells, EcR and the nuclear receptor ultraspiracle (USP) form a promoter-bound heterodimer, which regulates transcription (see Figure 1). In the absence of ecdysone, the receptor heterodimer binds to corepressors and tightly represses transcription.[3] When ecdysone binds to the EcR LBD, the corepressors are released, coactivators are recruited to the complex, and transcriptional activation is enabled.

In mammalian cells harboring the EcR gene, EcR heterodimerizes with RXR, the mammalian homologue of USP. The EcR–RXR heterodimer binds to multiple copies of the ecdysone-responsive element (EcRE), and in the absence of ponA, represses transcription of an expression cassette. When ponA binds to the receptor, the receptor complex activates transcription of a reporter gene or a gene of interest. To avoid pleiotropic interactions with endogenous pathways in mammalian host cells, both the EcRE recognition sequence and the EcR protein were modified.

The EcRE sequence was modified to create a synthetic recognition site that does not bind any endogenous transcription factors. The wild-type EcRE sequence consists of two inverted repeat sequences separated by a single nucleotide: AGTGCA N TGCACT. The EcRE sequence was changed to AGTGCA N_1 TGTTCT (and renamed E/GRE). Recognition of the synthetic E/GRE recognition sequence by either a steroid receptor or a wild-type RXR heterodimer receptor is extremely unlikely. The E/GRE recognition sequence has imperfect inverted half sites separated by one nucleotide. A steroid receptor recognizes perfect inverted repeat sequences. A wild-type RXR heterodimer requires single nucleotide separation of the inverted repeats, and the majority bind to direct repeats rather than inverted repeats (EcRE is an exception).

The EcR protein was modified to create a synthetic ecdysone-binding receptor that does not transactivate any host genes. Three amino acids in the EcR DBD were mutated to change its DNA-binding specificity to that of the glucocorticoid receptor (GR), which recognizes the half-site AGAACA.[1] Like all steroid receptors and unlike RXR receptors, the GR protein homodimerizes and recognizes two inverted repeat sequences separated by three nucleotides. The GR–EcR fusion protein (GEcR) retains the ability to dimerize with RXR and activate, with ponA-dependence, reporter genes that contain the synthetic E/GRE recognition sequence.

The GEcR receptor was further modified by replacing the EcR AD with the more potent VP16 AD. The result of all the modifications is the synthetic ecdysone-binding receptor VgEcR. VgEcR is a fusion of the ligand-binding and dimerization domain of the *D. melanogaster* ecdysone receptor, the DNA-binding domain of the glucocorticoid receptor, and the transcription activation domain of herpes simplex virus (HSV) VP16.

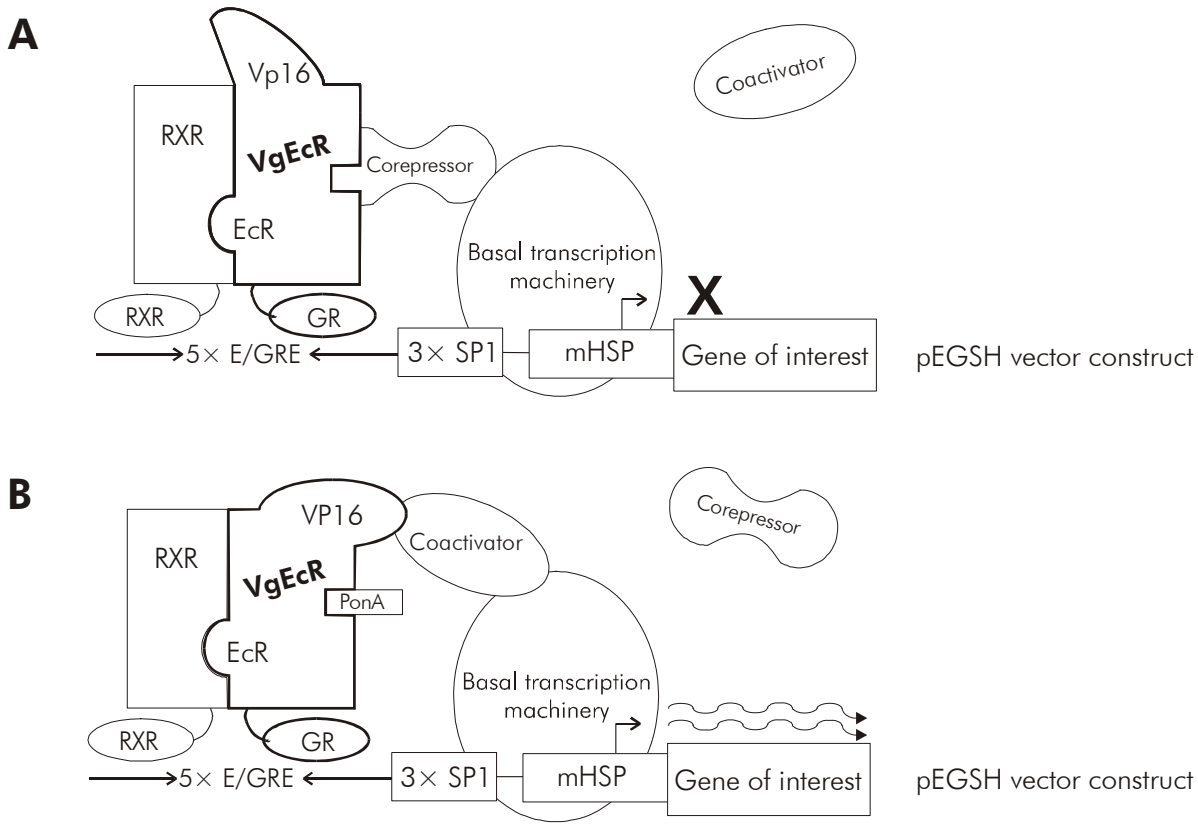


FIGURE 1 Regulation of transcription in the Complete Control system. The synthetic receptor VgEcR is a fusion of the ligand-binding and dimerization domain of the Drosophila ecdysone receptor (EcR), the DNA-binding domain of the glucocorticoid receptor (GR), and the transcriptional activation domain of HSV VP16. (A) VgEcR and RXR bind as a heterodimer to five copies of the E/GRE recognition sequence (5× E/GRE), which are located upstream of a minimal promoter composed of three SP1 binding sites (3× SP1) and the minimal heat shock promoter (mHSP). The E/GRE recognition sequence consists of inverted half-site recognition elements for the RXR and the GR DNA-binding domains (which are separated by one nucleotide). In the absence of ponA (the inducer), the promoter is tightly repressed by corepressors. (B) When ponA binds to VgEcR, the corepressors are released, coactivators are recruited, and the complex becomes transcriptionally active.

Vectors

Two vectors are required to express the gene of interest: the pERV3 receptor vector and the pEGSH expression vector.* The pERV3 vector contains an expression cassette from which the VgEcR and RXR proteins are constitutively expressed. The pEGSH vector contains the ponA-inducible expression cassette and a multiple cloning site (MCS) for inserting the gene of interest. The pEGSH-Luc vector, containing the luciferase reporter gene, is included for use as a positive control.

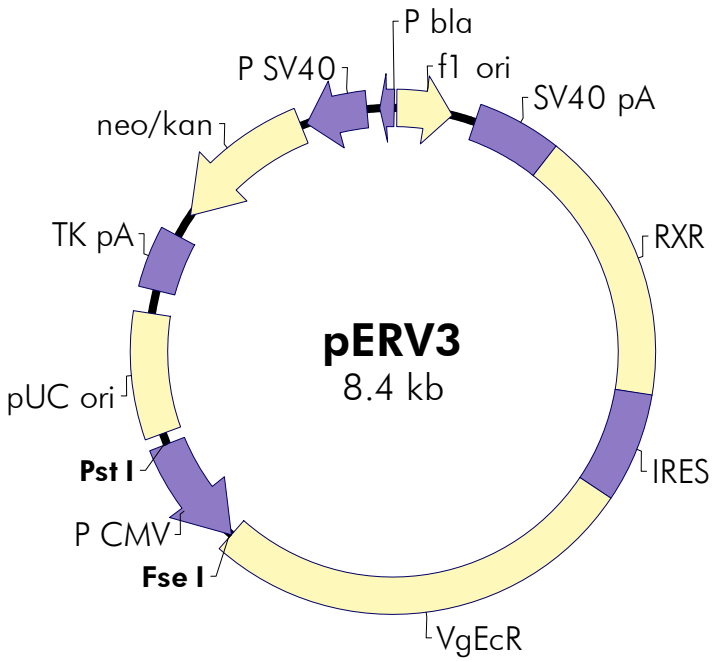
The pERV3 vector (see Figure 2) is designed so that the genes for both VgEcR and RXR are expressed from a dicistronic message transcribed from the CMV promoter. This mode of expression was achieved by placing the internal ribosome entry site (IRES), which is from the encephalomyocarditis virus, between the VgEcR and RXR open reading frames (ORF). This construction allows efficient, high-level internal (cap-independent) initiation of translation of ORFs positioned downstream in an appropriate context. The pERV3 vector also contains a neomycin-resistance gene so that mammalian cell transfectants can be selected with the antibiotic G418.

The pEGSH vector (see Figure 3) contains a ponA-inducible expression cassette that includes five copies of the E/GRE recognition sequence. The ponA-inducible expression cassette is located immediately upstream of a minimal promoter that consists of three SP1 sites (3× SP1) and the *D. melanogaster* minimal heat shock promoter 27 (mHSP). The MCS contains restriction sites positioned for directional insertion of sequences derived from the most widely used cDNA vectors. The pEGSH vector allows seamless insertion of the gene of interest by direct *Eam* 1104 I seamless cloning.[4] Expression of the gene of interest can be monitored either at the protein level by α-FLAG® immunodetection[5] or by RNA detection using T3 antisense RNA probes.

The pEGSH-Luc vector is included for use as a positive control reporter vector to confirm expression of the VgEcR and RXR receptors in cell lines stably transfected with the pERV3 vector. The pEGSH-Luc vector is derived from the pEGSH vector and has the luciferase reporter gene inserted between the *Kpn* I and *Sal* I sites of the MCS. The pEGSH-Luc vector does not contain the FLAG epitope sequence.

* The pEGSH expression vector is manufactured under license from Sigma-Aldrich, Co. using FLAG technology.

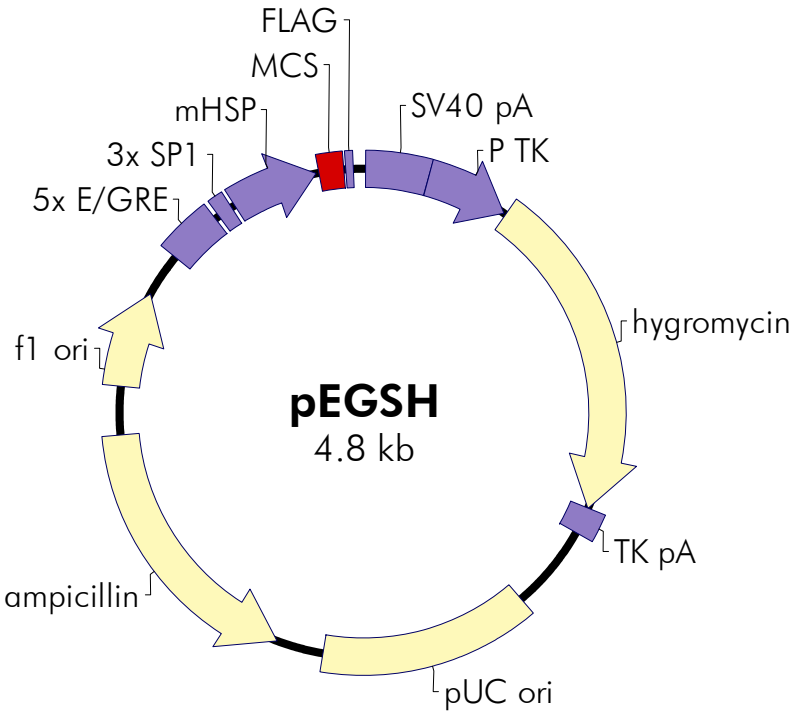
The pERV3 Vector



Feature	Position
f1 origin of ss-DNA replication	24–330
SV40 polyA signal	463–901
retinoid-X-receptor ORF	912–2216
internal ribosome entry site (IRES)	2332–2902
synthetic VP16-glucocorticoid/ecdysone receptor (VgEcR) ORF	2912–5179
Fse I	5186
CMV promoter	5201–5789
Pst I	5801
pUC origin of replication	5868–6535
HSV-thymidine kinase (TK) polyA signal	6664–7119
neomycin/kanamycin resistance ORF	7123–7930
SV40 promoter	7949–8287
bla promoter	8310–8431

FIGURE 2 Map of the pERV3 vector. The pERV3 vector contains an expression cassette from which a dicistronic message encoding the VgEcR and RXR receptors is transcribed from the CMV promoter. It is possible to replace the CMV promoter with a promoter of interest using *Pst*I and *Fse*I. The RXR protein expressed in this system is essentially wild-type. The internal ribosome entry site (IRES), which is derived from the encephalomyocarditis virus (EMCV) 5'UTR, allows efficient internal initiation of translation of the RXR receptor. The plasmid contains the neomycin-resistance gene downstream of both the SV40 early promoter and the β-lactamase promoter to allow selection of mammalian cell transfectants (with G418) and *Escherichia coli* transformants (with kanamycin).

The pEGSH Vector



pEGSH Multiple Cloning Site Region
(sequence shown 4670–4820)

mHSP leader

Eam1104 I, Mun I, Kpn I, BamH I, EcoR V, Cla I

```
GA ACT CAG ACA CAG CAG AAG AGC AAT TGG TAC CGG ATC CGA TAT CGA...
```

Not I, Xho I, Spe I, Sac I, Sal I, Xba I, Eam1104 I

```
...TGC GGC CGC TCG AGA CTA GTG AGC TCG TCG ACT CTA GAC TCT TCT...
                         STOP              STOP
```

FLAG tag

```
   G   S   G   D   Y   K   D   D   D   D   K
...GGT TCT GGC GAC TAT AAG GAT GAC GAT GAC AAG TAA TAG CCC TTT AGT GAG GGT TAA TT
                                              STOP STOP
```

pEGSH Vector Features

Feature	Position
SV40 polyA signal	8–208
HSV-thymidine kinase (TK) promoter	210–472
hygromycin resistance ORF	481–1518
HSV-thymidine kinase (TK) polyA signal	1519–1603
pUC origin	1877–2544
ampicillin resistance (*bla*) ORF	2695–3552
f1 origin of ss-DNA replication	3708–4014
5× ecdysone/glococorticoid responsive elements	4155–4325
3× Sp1 binding sites	4340–4387
minimal heat shock promoter (mHSP)	4401–4676
pEGSH forward sequencing primer binding site	4637–4659
multiple cloning site	4686–4764
FLAG tag	4771–4794
T3 promoter primer binding site	4820–4801

FIGURE 3 Map of the pEGSH vector. The pEGSH vector contains a ponA-inducible expression cassette composed of a ponA-inducible promoter and the MCS, which is located downstream from the promoter. The MCS contains 11 unique restriction sites flanked by a pair of inverted *Eam* 1104 I sites so that inserts may be cloned seamlessly between the mHsp 5′ untranslated leader (5′UTR) and the coding sequence for the 8 amino acid FLAG epitope. Seamless cloning may be achieved by direct ligation to the *Eam* 1104 I sites. The restriction site *Mun* I has a 5′ overhang that is compatible with *Eco*R I, thus *Eco*R I–*Xho*I inserts derived from pBluescript-based cDNA vectors may be inserted between the *Mun* I and *Xho* I sites in pEGSH. The T3 promoter is positioned both for convenient sequencing of the 3′ junction of inserted DNA and for the production of antisense RNA probes to monitor expression levels. There are two termination codons internal to the MCS, both of which are out of frame with the FLAG epitope sequence and both of which are underlined.

PREPARING THE HOST CELLS

Note *Immediately upon arrival, the vials should be stored at –20 or –80°C. Most strains remain viable longer if stored at –80°C. Avoid repeated freeze–thaw cycles of the host strain to maintain extended viability.*

Streaking the Host Cells

1. Scrape a few splinters of solid ice from the stored cells with a sterile wire loop.

2. Streak the splinters onto an LB agar plate (see *Preparation of Media and Reagents*).

3. Incubate the plates overnight at 37°C.

4. Store the plates at 4°C for up to 1 week, then restreak the colonies onto a fresh plate.

Preparing –80°C Bacterial Glycerol Stocks

1. In a sterile 50-ml conical tube, inoculate 10 ml of LB liquid medium (See *Preparation of Media and Reagents*) with one or two bacterial colonies from step 4 of *Streaking the Host Cells*. Incubate the cells at 37°C with vigorous agitation until the cells reach late log phase (OD_{600} = ~1.0).

2. Add 4.5 ml of a sterile glycerol–LB liquid medium solution (1:1) to the cells and mix well.

3. Aliquot the glycerol stock into sterile microcentrifuge tubes (1 ml/tube).

   The glycerol stocks can be stored at –20°C for 1–2 years or at –80°C for more than 2 years.

Note *These XL1-Blue cells may be made competent for later use in transformation.*[6]

OVERVIEW OF THE COMPLETE CONTROL INDUCIBLE MAMMALIAN EXPRESSION SYSTEM

Establishing the ponA-inducible expression system in cultured cells involves the following sequence of procedures:

- Digest the pEGSH vector with the desired restriction enzyme(s) and ligate the insert.
- Transform competent cells with the pEGSH construct.
- Purify the pEGSH construct and determine whether the expression construct can be induced in the cultured cell line by transient cotransfection of the pERV3 and pEGSH vectors.
- Stably transfect cultured cells with the pERV3 vector.
- Isolate G418-resistant stable clones and expand the colonies.
- Examine receptor expression by transient transfection of the pEGSH-Luc vector into G418-resistant clones.
- Stably transfect the receptor-expressing cell line with the pEGSH construct.
- Isolate hygromycin- and G418-resistant stable clones and expand the colonies.
- Induce gene expression with ponasterone A.
- Perform the assay for gene expression.

PROTOCOL FOR THE COMPLETE CONTROL INDUCIBLE MAMMALIAN EXPRESSION SYSTEM

Digesting the pEGSH Vector and Ligating the Insert

The pEGSH vector contains 11 unique restriction enzymes in the MCS for insertion of the gene of interest (see Figure 3). See reference 6 for protocols covering basic DNA manipulations. The gene of interest to be inserted into the pEGSH vector should contain a Kozak translation initiation sequence.[7] If the gene does not contain a stop codon, insert the gene in reading frame with one of the stop codons found in the *Xba* I (TCTAGA) or *Spe* I (ACTAGT) sites in the MCS or in reading frame with a stop codon located downstream of the sequence of the FLAG epitope.

Dephosphorylate the digested vector with calf intestinal alkaline phosphatase (CIAP) prior to ligation with the insert DNA. If the vector is digested with more than one restriction enzyme, the small fragment between the two restriction sites (which appears as background) can be removed by electrophoresing the DNA on an agarose gel and recovering the desired vector by electroelution.

After purification and ethanol precipitation of the DNA, resuspend the DNA in TE buffer (see *Preparation of Media and Reagents*). The concentration of the vector DNA should be the same as the concentration of the insert DNA (~0.1 µg/µl).

For ligation, the ideal insert-to-vector ratio is variable; however, a reasonable starting ratio is 2:1 (measured in available picomole ends). The insert-to-vector ratio is calculated as follows:

$$\text{picomole ends / microgram of DNA} = \frac{2 \times 10^6}{\text{number of base pairs} \times 660}$$

Digestion and Ligation Protocol

1. Digest the pEGSH vector with the desired restriction enzyme(s).

2. Prepare the experimental and control samples for ligation by combining the components specified in the following table in microcentrifuge tubes:

Samples (1 and 2) and controls (3–5)	Experimental samples		Controls		
	Insert:vector (1)	Insert:vector (2)	CIAP (3)	Background (4)	Insert purity (5)
Prepared vector (0.1 µg/µl)	1 µl	1 µl	1 µl	1 µl	0 µl
Prepared insert (0.1 µg/µl)	X µl	X µl	0 µl	0 µl	1 µl
10 mM rATP (pH 7.0)	1 µl	1 µl	1 µl	1 µl	1 µl
10× Ligase buffer[a]	1 µl	1 µl	1 µl	1 µl	1 µl
T4 DNA ligase	0.5 µl	0.5 µl	0.5 µl	0 µl	0.5 µl
ddH_2O to 10 µl	X µl	X µl	6.5 µl	7.0 µl	6.5 µl
Expected results	Many colonies	Many colonies	Few colonies	No colonies	No colonies

[a] See *Preparation of Media and Reagents*.

3. Incubate the tubes overnight at 4°C. When performing a blunt end ligation, incubate the tubes overnight at 12–14°C.

Transforming Competent Cells with the pEGSH Construct

1. Transform competent cells with 1–2 µl of the ligation mixture.

2. Plate the transformation on LB–ampicillin agar plates (see *Preparation of Media and Reagents*).

Small-scale DNA preps of the transformants can be screened for the gene of interest by restriction digestion or PCR. The provided pEGSH forward sequencing primer and T3 promoter sequencing primer can be used to sequence the 5′ and 3′ junctions of the inserted DNA, respectively (see Table I).

TABLE I

Primer Sequences and Positions in the pEGSH Vector

Sequencing primer	Sequence	Position (bp)
pEGSH forward sequencing primer	5′-CTCTGAATACTTTCAAAAGTTAC-3′	4637–4659
T3 promoter sequencing primer	5′-AATTAACCCTCACTAAAGGG-3′	4820–4801

Determining Whether the Expression Construct Can Be Induced in the Cultured Cell Line

Note *Prepare DNA from positive transformants using cesium chloride-banding (or a comparable protocol) before transfection into cultured cells.*

The cultured cell line chosen for expression of the gene of interest can be transiently cotransfected with the pERV3 receptor vector and the pEGSH construct (containing the gene of interest) as a quick method to determine whether or not the expression construct can be induced in the cell line. The preferred method of transfection depends on the chosen cultured cell line. See *www.genomics.agilent.com* for information on our calcium phosphate- and liposome-mediated transfection kits. Transfect the cells at least in duplicate to enable a comparison of uninduced and induced expression levels.

Notes *Transfect cells with several different amounts of DNA in order to determine the optimal concentration. The optimal amount of DNA will vary between cell lines and transfection methods. Follow the manufacturer's transfection protocol. Expression of the inserted gene can be induced by adding 1–10 μM ponA (diluted from a 1 mM stock, see* Preparation of Media and Reagents*) to the medium 4–20 hours before harvesting the cells. Parameters will need to be optimized for each particular system.*

Results from transient assays should be used to ascertain whether the gene of interest can be induced in the cell line of choice and to determine the maximal expression levels that can be expected in stable cells or transgenic animals. Uninduced pEGSH background expression will be considerably higher in transient transfections than stable transfections. This background is due to the high copy number of the plasmid and accessibility of the free nuclear DNA to core transcription factors. In stably transfected cells, however, the pEGSH vector is integrated into the host chromosome and is naturally repressed.

Stably Transfecting Cultured Cells with the pERV3 Vector

Perform a stable transfection of cultured mammalian cells with the pERV3 receptor vector using an appropriate method for the chosen cell line. We recommend calcium phosphate-based transfections for stable transfection. Calcium phosphate-based transfections require 5–15 μg of DNA/100-mm culture plate.

Use of Antibiotics for Cell Selection

Not all mammalian cell lines are equally sensitive to the antibiotics G418 and hygromycin. The minimal lethal concentration can range from 100 µg/ml to 1 mg/ml. The antibiotic concentration to be used for selection must be determined for **each** cell line **before** beginning the experiment.

Consult the available literature on the sensitivity of cell lines to G418 and hygromycin. If no information about the sensitivity of a particular cell line is available, a simple way to determine sensitivity is to grow cultures in a multiwell plate with a range of antibiotic concentrations between the individual wells. The optimal concentration is the lowest one that kills all of the cells within 10–14 days. (Rapidly dividing cells may be killed more readily since the antibiotic appears to act mainly on dividing cells.)

In some cases, it may be possible to reduce the concentration of the antibiotic after initial selection and still maintain selective pressure for the marker gene. For example, NIH3T3 cells are generally selected in 400 µg/ml G418, but selective pressure for the neomycin-resistance (Neo^r) gene can be maintained in 250 µg/ml.

Isolating G418-Resistant Stable Clones and Expanding the Colonies

1. Select for transfected cells by adding G418 (100 µg/ml–1 mg/ml, depending on the cell line) to the medium.

2. Isolate individual clones and expand the resulting colonies.

Examining Receptor Expression

Examine the expanded clones for expression of the VgEcR and RXR receptors by transiently transfecting the G418-resistant clones with the pEGSH-Luc vector and inducing luciferase transcription with ponA.

Note *Examine at least 20 individual clones, as the site of integration of the plasmid into the chromosome will affect how well the receptors are expressed. For most cell types, relatively small quantities of pEGSH-Luc (10–100 ng/10^5 cells plated) should give induction ratios of ≥20-fold and reach ≥10^5 RLUs when fully induced in these assays.*

Alternatively, prescreened stable cell lines that express the VgEcR and RXR receptors at optimal levels are available separately (see www.genomics.agilent.com for more information).

Stably Transfecting the Receptor-Expressing Cell Line with the pEGSH Construct

Once the luciferase assay confirms that the VgEcR and RXR receptors are being expressed, the cell line from *Examination of Receptor Expression* can be stably transfected with the pEGSH vector containing the gene of interest. Use an appropriate method of stable transfection for the chosen cell line.

Isolating Hygromycin- and G418-Resistant Stable Clones and Expanding the Colonies

1. Apply selective pressure to the cells by adding hygromycin to the medium (100 µg/ml–1 mg/ml, depending on the cell line). G418 in the medium maintains selective pressure for the pERV3 vector.

2. Isolate individual clones and expand the resulting colonies.

   Note *Isolate at least 20 individual clones, as expression is insertion-site dependent.*

Inducing Gene Expression with Ponasterone A

Once expression of the pERV3 vector and the pEGSH construct containing the gene of interest has been confirmed, expression of the gene of interest can be induced by adding ponA to the medium.

1. Split each sample of stably transfected cells into at least two 35–100-mm culture dishes at a density that will allow the cells to reach 70–80% confluence by the end of the induction period (seeding density will depend on the chosen cell type).

2. Add ponA to a final concentration of 1–10 µM to one of the plates. Add an equivalent volume of solvent (ethanol) to the uninduced sample plates.

3. Incubate the cells at 37°C in a CO_2 incubator for 4–20 hours. The optimal induction time and ponA concentration will depend on the chosen cell type and gene of interest.

4. Wash the cells with 1× PBS (See *Preparation of Media and Reagents*) and harvest them using a rubber policeman. Examine the cells for expression of the desired gene product by comparing the induced plates to the uninduced plates. Use a quantitative protein assay to determine total protein levels in each harvested sample. Normalize the protein levels before comparing expression levels.

   Note *The level of expression can depend on many variables including the gene of interest, cultured cell line, and method of detection. Parameters for each system must be optimized in order to maximize expression levels.*

Performing the Assay for Gene Expression

It is possible to monitor expression of the gene of interest at the level of protein expression or RNA expression. To directly detect the protein of interest, the FLAG epitope can be fused to the C-terminal of the protein of interest, and the expressed protein can be detected by immunoscreening with the M2 α-FLAG antibody. RNA levels can be monitored using antisense RNA probes transcribed from the T3 promoter.

TROUBLESHOOTING

Observation	Suggestion
Transfection efficiencies are low	DNA is not pure. Purify the DNA by cesium chloride banding
	DNA concentration is not optimal. In cotransfections, optimize vector quantities for the chosen cell line
	Antibiotic concentration is not optimal. In stable transfections, perform an antibiotic kill curve on the chosen cell line
Induction of gene expression is low	PonA concentration is not optimal. Optimize the concentration and exposure time of ponA for the chosen cell line
	Integration site of both the pERV3 vector and the pEGSH vector affects expression. Isolate and test at least 20 individual stable clones. Produce pERV3 and pEGSH double-stable cell lines by sequential selection of an optimal pERV3 line. Use this selected line to produce the double-stable line
	Cells have not adequately recovered from transfection. Lengthen the time of recovery from the transfection
	Total protein levels of repressed and induced samples are not normalized. Protein levels should be identical when comparing expression levels
	Expression of the inserted gene is not optimal in the chosen cell line. Confirm that the chosen cell line can support expression of the gene product
	Receptor expression is not compatible with the chosen cell line. Confirm that the CMV promoter is functional in the chosen cell line using a CMV reporter plasmid
	Cell density is too high at the time of induction. Cell density should be ≤80% at the time of induction

PREPARATION OF MEDIA AND REAGENTS

LB Agar (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
20 g of agar
Add deionized H_2O to a final volume of 1 liter
Adjust pH to 7.0 with 5 N NaOH
Autoclave
Pour into petri dishes (~25 ml/100-mm plate)

LB–Ampicillin Agar (per Liter)

1 liter of LB agar, autoclaved
Cool to 55°C
Add 10 ml of 10-mg/ml filter-sterilized ampicillin
Pour into petri dishes (~25 ml/100-mm plate)

10× Ligase Buffer

500 mM Tris-HCl (pH 7.5)
70 mM $MgCl_2$
10 mM dithiothreitol (DTT)

Note *rATP is added separately in the ligation reaction.*

LB Liquid Medium (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
Add dH_2O to a final volume of 1 liter
Adjust to pH 7.0 with 5 N NaOH
Autoclave

1× Phosphate-Buffered Saline (PBS)

137 mM NaCl
2.6 mM KCl
10 mM Na_2HPO_4
1.8 mM KH_2PO_4
Adjust the pH to 7.4 with HCl

1 mM Ponasterone A

Resuspend 1 mg of ponasterone A in 2 ml of 100% (v/v) ethanol
Store at –20°C

TE Buffer

5 mM Tris-HCl (pH 7.5)
0.1 mM EDTA

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ENDNOTES

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MSDS INFORMATION

Material Safety Data Sheets (MSDSs) are provided online at *http://www.genomics.agilent.com*. MSDS documents are not included with product shipments.

Complete Control Inducible Mammalian Expression System

Catalog #271460, #217461, and #217468

QUICK-REFERENCE PROTOCOL

- Digest the pEGSH vector with the desired restriction enzyme(s) and ligate the insert
- Transform competent cells with the pEGSH construct
- Purify the pEGSH construct
- Determine whether the expression construct can be induced in the cultured cell line by transient cotransfection of the pERV3 and pEGSH vectors
- Stably transfect cultured cells with the pERV3 vector
- Isolate G418-resistant stable clones and expand the colonies
- Examine receptor expression by transient transfection of the pEGSH-Luc vector into G418-resistant clones
- Stably transfect the receptor-expressing cell line with the pEGSH construct
- Isolate hygromycin- and G418-resistant stable clones and expand the colonies
- Induce gene expression with ponasterone A
- Perform the assay for gene expression