LacSwitch II Inducible Mammalian Expression System

INSTRUCTION MANUAL

Catalog #217450

Revision B

For In Vitro Use Only

217450-12

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LacSwitch II Inducible Mammalian Expression System

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LacSwitch II Inducible Mammalian Expression System

MATERIALS PROVIDED

Materials provided	Quantity	Storage conditions
pCMVLacI repressor vector	100 µg (1 mg/ml)	–20°C
pOPRSVI/MCS operator vector	20 µg (1 mg/ml)	–20°C
pOPI3CAT operator vector	20 µg (1 mg/ml)	–20°C
XL1-Blue MR strain[a]	1.5-ml tube (500 µl)	–20° or –80°C[b]
IPTG[c]	1 g	–20°C

[a] XL1-Blue MR strain genotype: *Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1supE44 thi-1 recA1 gyrA96 relA1lac*

[b] The XL1-Blue MR strain[5] is shipped as a glycerol stock. Do not allow the contents of the vial to thaw. The vials can be stored at –20° or –80°C. Most strains remain viable longer if stored at –80°C. Use LB broth for both plates and glycerol stocks.

[c] The IPTG is provided as a powder. Prepare a 1 M solution of IPTG before beginning the protocol (see *Preparation of Media and Reagents*).

STORAGE CONDITIONS

Bacterial Glycerol Stock: –20° or –80°C
All Other Components: –20°C

ADDITIONAL MATERIALS REQUIRED

G418 sulfate or gentamicin
Hygromycin
Not I enzyme or alternative cloning enzyme
T4 DNA ligase
Cesium chloride
Plasmid DNA isolation solutions
Mammalian transfection solutions
Tissue culture media and supplies
1× PBS solution (see *Preparation of Media and Reagents*)

NOTICE TO PURCHASER

The use of the CMV Promoter is covered under U.S. Patent Nos. 5,168,062 and 5,385,839 owned by the University of Iowa Research Foundation and licensed FOR RESEARCH USE ONLY. For further information, please contact UIRF at 319-335-4546.

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Revision B

INTRODUCTION

The ability to reversibly turn genes off and on is a powerful tool in the investigation of various genetic functions. In the *Escherichia coli* lactose (*lac*) operon, the Lac repressor binds as a homotetramer to the *lac* operator, blocking transcription of the *lac*Z gene. Physiological or synthetic inducers, such as allolactose or isopropyl β-D-thiogalactopyranoside (IPTG), respectively, bind to the Lac repressor, causing a conformational change and effectively decreasing the affinity of the repressor for the operator. When the repressor is removed from the operator, transcription from the *lac* operon resumes.[1]

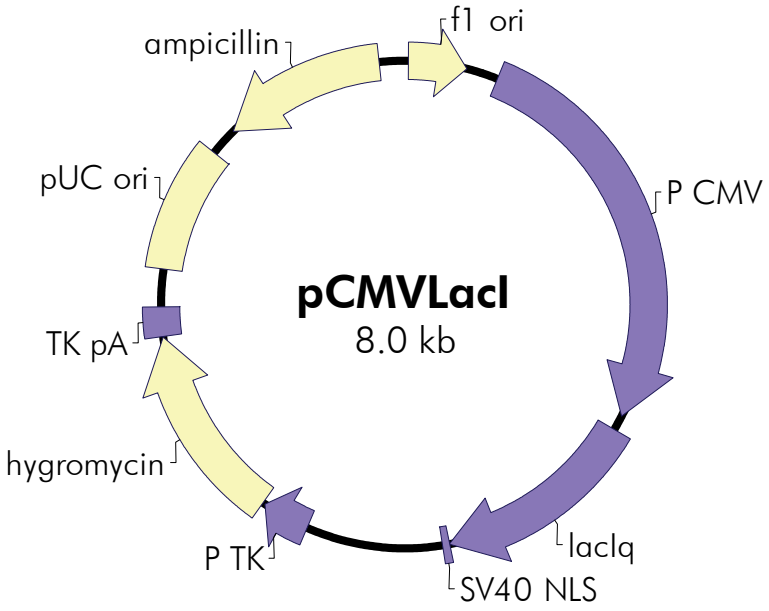
The Stratagene LacSwitch II inducible mammalian expression system*[2, 3] utilizes an improved vector system in which several elements of the *lac* operon have been modified for use in eukaryotic cells for the control of gene expression. This method for inducible expression of exogenous genes in eukaryotic cells consists of a eukaryotic Lac-repressor-expressing vector, pCMVLacI (Figure 1), and two eukaryotic *lac*-operator-containing vectors, pOPRSVI/MCS (Figure 2), and pOPI3CAT (Figure 3), into which the gene of interest is inserted by cloning. These vectors are transfected into a cultured cell line in which expression of the inserted gene is repressed until an inducer is added to the media. Upon induction, expression of the inserted gene resumes.

The LacSwitch II inducible mammalian expression system is a versatile and proven alternative to other systems. The system uses a nontoxic, fast-acting inducer, IPTG, which permits induction in 4–8 hours, partly due to the rapid transportation of IPTG into eukaryotic cells.[4] The LacSwitch II expression system exhibits low basal expression of a luciferase reporter gene (~10–20 molecules/cell) when in the repressed state. This repression level is partially dependent on the half-life of the inserted gene.

The establishment of a highly regulated expression system in eukaryotic cells, maintained in a repressed state until induced by an exogenous stimulus, is an invaluable tool for the study of cell cycle, oncogenicity, cytogenicity, and the mechanisms of gene regulation.

* U.S. Patent No. 4,833,080 and Patent Pending.

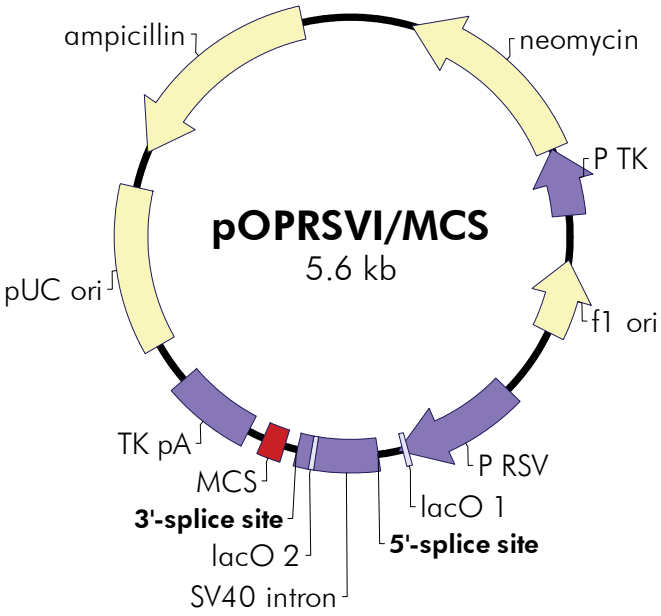
The pCMVLacI Vector



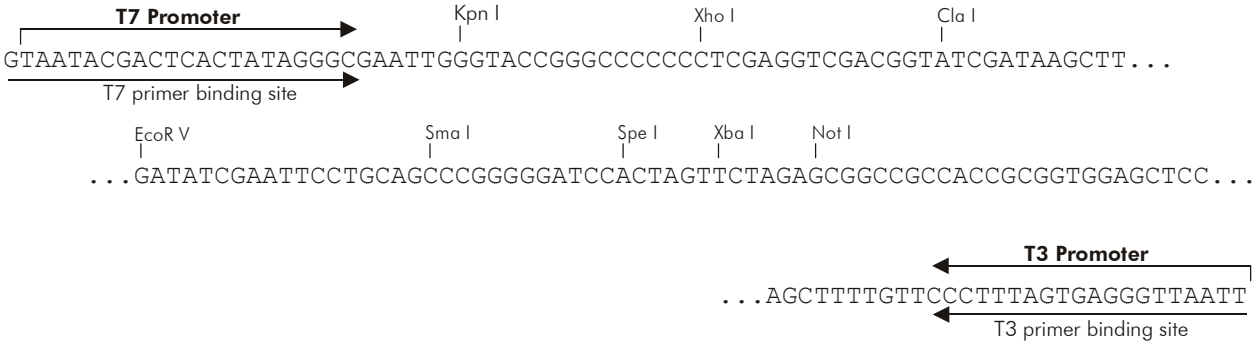
Feature	Nucleotide Position
f1 origin of ss-DNA replication	21–327
CMV promoter	496–2604
lacI^{q} repressor ORF	2685–3764
SV40 nuclear localization signal (NLS)	3765–3800
HSV-thymidine kinsase (TK) promoter (the **HSV-TK promoter/hygromycin ORF/TK polyA signal** fragment may be excised using Hind III (4322) and Nsi I (6127); these sites are not unique in the vector)	4525–4775
hygromycin resistance ORF	4789–5826
HSV-thymidine kinase (TK) polyA signal	5809–5984
pUC origin of replication	6185–6852
ampicillin resistance (*bla*) ORF	7003–7860

FIGURE 1 Map of the eukaryotic Lac-repressor-expressing vector, pCMVLacI. Expression of the Lac repressor protein is driven by the CMV promoter. The protein is targeted to the nucleus by the SV40 nuclear localization sequence (NLS). Hygromycin is used for selection in mammalian cells. The complete nucleotide sequence and list of restriction sites is available from *www.stratagene.com* or from the GenBank® database (Accession #U64448).

The pOPRSVI/MCS Vector



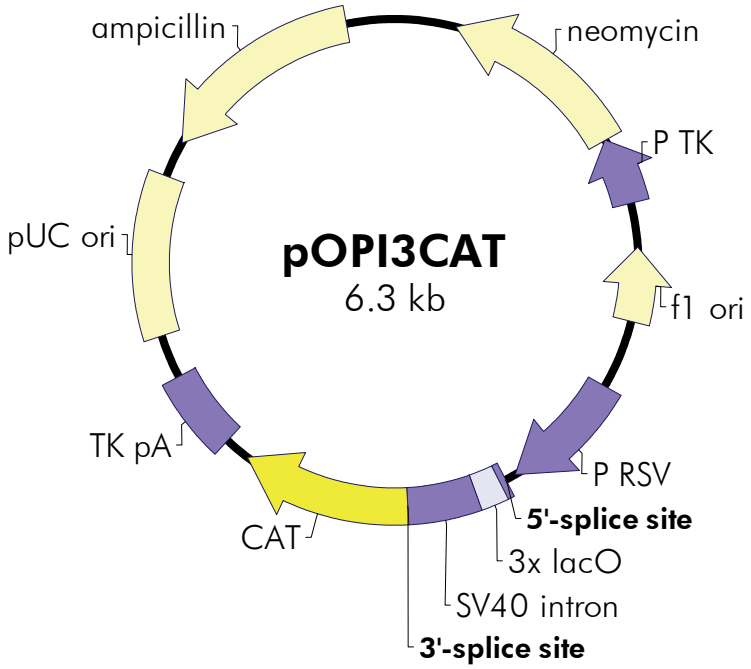
pOPRSVI/MCS Multiple Cloning Site Region (sequence shown 3071–3237)



Feature	Nucleotide Position
neomycin resistance ORF	256–1044
HSV-thymidine kinsase (TK) promoter	1045–1324
f1 origin of ss-DNA replication	1509–1815
RSV promoter (digesting the promoter in this vector may interfere with the operator)	2106–2626
lac operator 1	2588–2608
SV40 intron (5´-splice site 2712; 3´-splice site 3044)	2712–3044
lac operator 2	2967–2987
multiple cloning site	3099–3190
HSV-thymidine kinase (TK) polyA signal	3259–3598
pUC origin of replication	3772–4439
ampicillin resistance (*bla*) ORF	4590–5447

FIGURE 2 Map of the pOPRSVI/MCS vector. The Rous sarcoma virus (RSV) promoter drives expression of the gene of interest inserted into the MCS. Ideal operator sequences for Lac repressor binding are present in the RSV promoter and in the intron. G418 resistance is provided by the neomycin gene. The complete nucleotide sequence and list of restriction sites is available from *www.stratagene.com* or from the GenBank® database (Accession #U64449).

The pOPI3CAT Vector



Feature	Nucleotide Position
neomycin resistance ORF	256–1044
HSV-thymidine kinsase (TK) promoter	1045–1324
f1 origin of ss-DNA replication	1509–1815
RSV promoter (can be excised using BstX I and Bgl II)	2106–2626
SV40 intron (5´-splice site 2672; 3´-splice site 3090)	2672–2687
lac operator 1	2699–2719
lac operator 2	2742–2762
lac operator 3	2785–2805
chloramphenicol acetyl transferase (CAT) ORF	3091–3792
HSV-thymidine kinase (TK) polyA signal	3901–4231
pUC origin of replication	4414–5081
ampicillin resistance (*bla*) ORF	5232–6089

FIGURE 3 The pOPI3CAT vector shown in the figure above contains the Rous sarcoma virus (RSV) promoter, intron sequences from SV40 small t intron and VP1 intron, two *Not* I sites for insertion of a reporter gene, and ideal operator sequences for Lac repressor binding inserted at various positions. It also contains the CAT gene for added vector stability. pOPI3CAT allows for insertion of various promoters into the *Bst*X I and *Bgl* II restriction sites. The complete nucleotide sequence and list of restriction sites is available from *www.stratagene.com* or from the GenBank® database (Accession #U42373).

PREPARATION OF HOST CELLS

Note *The host strain may thaw during shipment. The vials should be stored immediately upon arrival 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 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.[§]
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 media with one or two bacterial colonies from step 4 of *Streaking Host Cells*. Incubate the cells at 37°C with vigorous agitation until the cells reach late log phase (OD_{600} = ~1.0–2.0).
2. Add 4.5 ml of a sterile glycerol–liquid media solution (1:1) to the cells and mix well.
3. Aliquot the glycerol stock into sterile microcentrifuge tubes (1 ml/tube).

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

Note *These Lac$^-$ cells may be made competent for later use in transformation.*

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

PROTOCOL

Insertion of the Gene of Interest

Two operator-containing expression vectors, pOPRSVI/MCS and pOPI3CAT, are included in the LacSwitch II inducible mammalian expression system.

Note *The operator positioning in pOPRSVI consistently produces stable clones with high induction levels (20- to 50-fold), while pOPI3 has yielded the highest-inducing clone (95-fold). pOPI3 also has unique restriction sites for the removal of the RSV promoter. Perform the transfection with only one of the vectors. The choice of vector depends on individual experimental requirements.*

1. Digest the pOPRSVI/MCS operator vector with the desired restriction enzyme(s), and/or digest the pOPI3CAT operator vector with *Not* I.

   Note *Digesting the pOPI3CAT vector with* Not *I will remove a portion of the CAT open reading frame.*

2. Gel-isolate the ~5647-bp pOPRSVI/MCS fragment and/or gel-isolate the 5513-bp pOPI3 fragment.

   Note *The gene of interest to be inserted into the pOPRSVI-digested and/or the digested pOPI3 vectors should have compatible restriction site ends. To achieve* Not *I-compatible restriction site ends, the gene may have to be isolated by either* Not *I digestion, gene amplification via polymerase chain reaction (PCR) using primers with a* Not *I sequence, or digestion with a compatible enzyme (e.g.,* Xma *III).* Not *I requires more than ten bases flanking the recognition sequence for efficient digestion.*

3. Ligate the gene of interest into the operator vectors using T4 DNA ligase (see *Appendix I: Vector Ligation*).

4. Transform the resulting ligation into Lac^- competent cells which may increase the stability of the operator-containing vectors. (XL1-Blue MR supercompetent cells achieve $>1 \times 10^9$ colonies/µg of pUC18 DNA; Stratagene Catalog #200229.) Small-scale DNA preps of the resulting transformants can be screened for the gene of interest by restriction digestion. Inserts in pOPRSVI/MCS can also be screened by sequencing with T3 or T7 promoter sequencing primers.

   Notes *To ensure that deletion of operator sequences has not occurred in the selected transformants, digest with* Hin*d III to release the SV40 intron fragment. Digestion of pOPRSVI (one intron operator) with* Hin*d III releases a 381-bp fragment, and digestion of pOPI3 (three intron operators) with* Hin*d III releases a 467-bp fragment.*

Prepare cesium-chloride-purified DNA from positive transformants (pOPRSVI + gene or pOPI3 + gene) before transfection into cultured cells.[5] Aliquot the DNA preps into several tubes and avoid repeated freeze-thaw cycles.

Replacement of the RSV Promoter (Optional)

The pOPI3CAT vector contains three ideal *lac* operator sequences in the SV40 intron region and none in the promoter region. For this reason, the RSV promoter can be replaced with another promoter of interest.

1. Digest pOPI3CAT with *Bgl* II, followed by *Bst*X I.

2. Gel-isolate the 5723-bp fragment (or larger fragment if the gene of interest has been inserted) away from the 566-bp promoter fragment.

3. Ligate the isolated plasmid to the promoter of interest containing *Bst*X I (5´)- and *Bgl* II (3´)-compatible restriction-site ends (see *Appendix I: Vector Ligation*).

4. Transform the ligation reaction into competent cells and screen the resulting transformants for the insertion of the promoter fragment through restriction mapping. Prepare cesium chloride-purified DNA from the resulting construction.[5]

Transient Transfection (Optional)

1. Both the pCMVLacI repressor and operator constructs with the gene of interest can be transiently transfected simultaneously into cultured cells. The preferred method of transfection depends on the cultured cell line chosen for expression of the gene of interest. Transfect in duplicate to enable a comparison of repressed expression to induced expression.

   Note *If using a calcium phosphate transfection protocol, incubate the cells with the DNA precipitate for 12–24 hours. After incubation, wash the cells with PBS and then add fresh media. Expression of the inserted gene can be induced by addition of 1–5 mM IPTG (see* Preparation of Media and Reagents*) to the media 4–12 hours before harvesting the cells.*

Stable Transfection

Option 1: Sequential Transfection

1. Stably transfect cultured cells with the pCMVLacI-repressor construct. Select transfectants by adding the antibiotic hygromycin to the media (150–400 µg/ml, depending on the cell line used). Expanded clones can be isolated and examined for expression of the Lac repressor by any of the following methods:

   - Western hybridization using a polyclonal antiserum to Lac repressor to detect the 38,000-dalton monomer
   - Indirect immunofluorescence, allowing detection of fluorescence-labeled Lac repressor protein by microscopy[6]
   - RNA isolation and Northern hybridization, which detects a transcript between 1.5 and 2.0 kb and a transcript between 3.5 and 4.5 kb.

2. Cell lines from step 1 that are positive for expression of the Lac repressor can now be stably transfected with the pOPRSVI operator vector containing the gene of interest or with the pOPI3 operator vector containing the gene of interest. Hygromycin in the media maintains selective pressure for the pCMVLacI repressor construction. Addition of 200–800 µg/ml of G418 sulfate or gentamicin to the media allows selection for clones containing the *lac* operator vectors. Expanded cell lines can then be induced with IPTG to allow expression of the gene of interest.

   Note *Expression levels may be insertion-site dependent. Isolation of at least 15 clonal cell lines in recommended.*[2]

Option 2: Cotransfection

The pCMVLacI-repressor plasmid plus either the pOPRSVI vector with the gene of interest or the pOPI3 vector with the gene of interest can be stably cotransfected into a cultured cell line. Clones positive for both vectors can be selected by the addition of hygromycin and either G418 sulfate or gentamicin to the media.

Notes *As a control, we recommend performing stable or transient transfection of only the operator vector containing the gene of interest. Expression of the gene of interest in this transfectant confirms that the inserted gene is functional with the promoter and cell line chosen in the absence of repressor.*

Induction

After selection of clones positive for both the pCMVLacI and operator vectors, expression of the gene of interest can be controlled by the addition of IPTG (see *Preparation of Media and Reagents*) to the media.

1. Split each sample of stably transfected cultured cells into two 100-mm culture dishes at a density that allows them to reach confluence after the induction period (the density will vary with the type of cells used).

2. Add IPTG (MW 238.31) to a final concentration of 1–5 mM (diluted from a 1M stock) to one plate of each pair of plates. The plate without IPTG is the repressed sample plate.

3. Incubate the cells at 37°C for 4–12 hours. Optimal induction time for either operator construction will be gene-dependent.

After washing the cells with PBS, harvest the cells using a rubber policeman and examine the cells for expression of the desired gene product in the induced plates versus the repressed sample plates. Use a protein quantitative assay to determine total protein levels in each harvested sample.

Note *The use of expression vectors involves many variables including the gene of interest, promoter (if altered), cultured cell line, stable vs. transient transfection, and expression assay method. Parameters must be optimized for each system.*

APPENDIX I: VECTOR LIGATION

Dephosphorylate the digested vector with calf intestinal alkaline phosphatase (CIAP) prior to ligating to the insert DNA. If more than one restriction enzyme is used, the background can be reduced further by electrophoresing the DNA on an agarose gel and recovering the desired vector band through electroelution, removing the small fragment that appears between the two restriction enzyme sites.

After purification and ethanol precipitation of the DNA, resuspend the DNA in a volume of TE buffer, [5 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA] that will allow the concentration of the vector DNA to be the same as the concentration of the insert DNA (~0.1 µg/µl).

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

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

Ligation of the Vectors

1. Prepare samples 1 and 2 and controls 3–5 in microcentrifuge tubes by combining the components listed in the following table.

	Experimental samples		Controls		
Components	**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 (4 Weiss U/µl)	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*.

2. Incubate the tubes overnight at 4°C. When using blunt ends, incubate overnight at 12–14°C.

3. Transform 1–2 µl of the ligation mixture into Lac^- XL1-Blue MR supercompetent cells. Plate on LB–ampicillin agar plates.[§]

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

TROUBLESHOOTING

Observation	Suggestion
Low ligation efficiencies	Optimize ligation conditions by varying the vector-to-insert ratios (pmole ends)
	Prevent vector self-ligation by treating the vector with calf intestine alkaline phosphatase (CIAP)
Unstable operator vector and insert clones	Transform clones into a *lac* $^{-}$ *E. coli* (e.g., XL1-Blue MR cells) to reduce the effects of repetitive operator sequences being unstable with the inserted DNA
	Harvest cells grown for DNA preparation in the log phase
	Aliquot purified DNA preps of positive clones to prevent repeated freeze-thaw cycles
Low transfection efficiencies	Produce very pure DNA preparations by employing cesium chloride purification
	In double transfections, optimize vector quantities for the cell line used
	In stable transfections, optimize the antibiotic concentration for the cell line used
Low induction values	Optimize the concentration and exposure time of IPTG for the cell line used
	Transfect the Lac-repressor-expressing vector and the two *lac*-operator-containing vectors into cultured cells either both stably or both transiently as the integration site of both the Lac repressor vector and the operator vector will affect expression
	Protein levels should be identical when comparing expression levels; normalize the total protein levels of repressed and induced samples before estimating induction
	Examine at least 15 stable clonal cell lines
Low expression of the inserted gene	Confirm that the chosen cell line can support expression of the gene product
	Check the operator-vector construct for the presence of the operator sequences and correct orientation of the inserted gene, as the integration site of both the Lac repressor vector and the operator will affect expression
	Confirm that the RSV promoter can drive expression of the inserted gene in the chosen cell line

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 Broth (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
Adjust to pH 7.0 with 5 N NaOH
Add deionized H_2O to a final volume of 1 liter
Autoclave

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)

1 M IPTG

238.31 mg IPTG
1 ml PBS
Filter sterilize
Store at –20°C

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*

1× 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

REFERENCES

1. Miller, J. H. and Reznikoff, W. S. (1980). The Operon. Cold Spring Harbor Laboratory Press, Plainview, NY.
2. DuCoeur, L. C., Wyborski, D. L. and Short, J. M. (1992) Strategies 5(3):70-72.
3. Wyborski, D. L., DuCoeur, L. C. and Short, J. M. (1996) Environ Mol Mutagen 28(4):447-58.
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5. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
6. Labow, M. A., Baim, S. B., Shenk, T. and Levine, A. J. (1990) Mol Cell Biol 10(7):3343-56.

ENDNOTES

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MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on the web at *http://www.stratagene.com/MSDS/*. Simply enter the catalog number to retrieve any associated MSDS's in a print-ready format. MSDS documents are not included with product shipments.

STRATAGENE

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LacSwitch II Inducible Mammalian Expression System

Catalog #217450

QUICK-REFERENCE PROTOCOL

- Restriction-digest pOPRSVI/MCS and/or pOPI3CAT operator vectors with *Not* I or desired enzymes
- Ligate gene of interest into operator vectors
- Transform the ligation into Lac$^-$ competent cells (e.g., Stratagene XL1-Blue MR supercompetent cells)
- Replace RSV promoter in pOPI3 vector (optional)
- Prepare cesium chloride-purified DNA from pOPRSVI vector + gene of interest and pOP13 + gene of interest
- Perform stable or transient transfection of pCMVLacI, pOPRSVI + gene of interest and/or pOPI3 + gene of interest into cultured cells
- Induce gene expression by adding IPTG to culture media
- Perform assay for gene expression