Preface

Genetic technology is the buzzword of the new millennium. Via the media, we are informed day by day about new genes, disease-causing mutations, cloned animals, "Frankenstein" food, and further advances in the Human Genome Project. However, in spite of the constant media attention, most people remain confused about the importance and application of these new discoveries. It seems that only the high priests of this new technology, armed with PhDs in molecular biology, understand the meaning, importance, and consequences of these new breakthroughs. But should it be like this? After all, molecular biology is only a tool, though one that enables us to study the secrets of life, death, and disease development.

Vision Research Protocols has been written for those scientists, optometrists, and ophthalmologists who are interested in eye research, but have not been trained in molecular biology. It covers molecular biological techniques from the basics to the most sophisticated recent technologies. In each case, the techniques described have been adapted to the special requirements of eye research. The first four chapters discuss crucial molecular biological methodologies that create a basis for the more complex methodologies presented later. The last chapter discusses the impact of these new technologies on everyday clinical services.

The first four chapters follow the basic dogma of molecular biology. They describe the extraction of DNA and detection of mRNA and protein expression in vitro and in vivo. In addition, Chapter 2 gives a description of the basics of tissue culture technology, which has significantly advanced biological research since the 1970s.

In a philosophical sense, diseases can be considered as disturbances in the normal balance within a complex organism. In molecular terms, certain genes become up or down regulated, or turned on and off out of sequence. In this respect, the new molecular biological techniques can have a significant impact both in the development of animal models and on new treatments for diseases. Chapters 5, 6, and 12 discuss technologies suitable for the down regulation of gene expression. Chapters 7–10 are devoted to the new gene therapy technologies. Following a very enthusiastic start in the early 1990s,

gene therapy proved to be a difficult technology to apply, and further improvements are required before it can be considered ready for clinical application. However, even in its present form, it is particularly suitable for the development of animal models and for testing new therapies in transgenic and knockout animal models. *Vision Research Protocols* contains a detailed description of the three most popular viral delivery vehicles: recombinant adeno, adeno-associated, and retroviruses. In addition, delivery methods for the most popular animal models are also described.

In summary, I recommend *Vision Research Protocols* to all medical and nonmedical scientists who are thinking of using these new technologies in their research, or those clinicians who would like to find out the current state of progress in this important field of molecular medicine.

I would like to sincerely thank all the contributors, who are leading scientists in their fields with real hands-on experience in the different techniques. They were wonderful to work with, and I would specifically thank them for providing their invaluable comments in the troubleshooting sections of each chapter. I also would like to thank Ms. Louise Kemp, who has been superb in organizing the contributions of experts from three continents.

P. Elizabeth Rakoczy

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Transient Transfection of Human Retinoblastoma Cells

Application to the Analysis of the Regulatory Regions of Photoreceptor-Specific Genes

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1. Introduction

Retinoblastoma (Rb) is an intraocular tumor usually diagnosed in children under four years of age ***(1)***. The tumor rises when both alleles of the Rb tumor suppressor gene become inactivated in a retinal precursor cell during development ***(2,3)***. The first retinoblastoma cell line to be established in culture, Y-79 ***(4)***, has been shown to originate from neuroectodermal cells that express both neuronal and glial cell markers ***(3)***. Both Y-79 cells and Rb tumor cells produce mRNAs encoding several proteins unique to the photoreceptors ***(5)***, including different subunits of cone- and rod-specific cGMP-phosphodiesterases ***(6)***. Therefore, cultured Y-79 cells, which have a human retinal origin, could be particularly useful for studying the regulatory mechanisms of photoreceptor-specific gene expression ***(7)***.

In order to introduce DNA into eukaryotic cells several methods have been developed. The most commonly used techniques are calcium phosphate-mediated transfection, electroporation, and

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lipofection. Briefly, calcium phosphate-mediated transfection takes advantage of the formation of DNA-calcium phosphate precipitates which enhance the introduction of foreign DNA into the cells ***(8)***. It is a convenient and inexpensive technique, although the efficiency of DNA uptake may vary significantly between different cell types. During electroporation, a high-voltage electric pulse of a brief duration is applied to the cells to generate transient and reversible "electropores" in the plasma membrane. The optimal conditions for efficient electroporation which is not harmful to the cells must be determined empirically for each cell type ***(9)***. Lipofection ***(10)*** is often useful for cell types that transfect inefficiently by other methods. However, the selection of an efficient, convenient, and cost-effective transfection technique depends, to a large extent, on the particular cell line to be transfected.

Various reporter genes may be used in transient transfection assays for transcriptional regulation studies. These include chloramphenicol acetyl transferase (CAT) ***(11)***, β-galactosidase ***(12)***, luciferase ***(13)***, and β-globin ***(14)***. In particular, we have used the GeneLight™ reporter vectors pGL2-Basic and pGL2-Control (Promega Corp., Madison, WI) that contain the coding region of the firefly *Photinus pyralis* luciferase gene. Luciferase activity can be easily quantified with low background (there is no endogenous luciferase activity in eukaryotic cells) using a rapid and sensitive assay that allows the analysis of a large number of samples.

Transient transfections have been employed for the study of the regulatory *cis*-elements and their interactions with *trans*-acting nuclear factors that control the level of transcriptional activation of various photoreceptor-specific genes including the β-subunit of cGMP-phosphodiesterase (β-PDE) gene ***(7)***. In order to prepare constructs suitable for transient transfections, various lengths of the 5'-flanking region of the human β-PDE gene were generated by polymerase chain reaction (PCR) using sequence-specific primers; the 3' primers contained a *Bgl*II linker and the 5' primers contained an *Nhe*I linker. PCR products were digested with *Bgl*II and *Nhe*I, and directionally subcloned into the pGL2-Basic vector upstream of the luciferase reporter gene. Inserts were sequenced in both

directions to assure 100% identity with the 5'-flanking region of the human β-*PDE* gene.

This chapter describes the detailed protocols for the propagation of Y-79 retinoblastoma cells in culture, for calcium phosphate-mediated transient transfections of Y-79 cells using luciferase reporter constructs, for the preparation of Y-79 cell extracts and for the determination of the levels of the luciferase gene expression.

2. Materials

2.1. Equipment

1. Luminometer (Monolight 2010; Analytical Luminescence, MD).
2. Standard cell culture equipment (e.g., a cell-culture incubator preset at 37°C with 5% CO_2 in air atmosphere, laminar flow hood, centrifuge, microscope, Coulter counter or hemocytometer).

2.2. Reagents

2.2.1. Cell Culture

1. Y-79 Human retinoblastoma cells obtained from American Type Culture Collection (Rockville, MD, Cat. No. HTB18).
2. Complete growth medium: RPMI-1640 with L-glutamine and without sodium phosphate (Gibco BRL, Grand Island, NY) supplemented with 15% fetal bovine serum (FBS; Gibco BRL).
3. Transfection medium: Dulbecco's modified Eagle's medium (DMEM)/F12 (50/50 mix, Cellgro™, Mediatech, Inc., Herndon, VA) supplemented with 15% FBS.

2.2.2. Transient Transfections of Human Rb Cells Using Calcium Phosphate Precipitation

1. 2× HEPES-buffered saline/Na_2HPO_4, pH 7.0 (HBS/P): 45 m*M* HEPES (tissue-culture grade), 280 m*M* NaCl, and 2.8 m*M* Na_2HPO_4. Adjust pH accurately to 7.0 with NaOH, filter sterilize. This buffer may be stored at 4°C for several months; recheck pH after prolonged storage.
2. Phosphate-buffered saline (PBS), pH 7.4: 140 m*M* NaCl, 2.7 m*M* KCl, 4.5 m*M* Na_2HPO_4, and 1.5 m*M* KH_2PO_4. Adjust pH to 7.4 with HCl, filter sterilize.

3. 2.5 *M* $CaCl_2$ (tissue-culture grade), filter sterilize.
4. 10× TE buffer, pH 7.0: 10 m*M* Tris-HCl and 1 m*M* ethylenediaminetetraacetic acid (EDTA), filter sterilize. This buffer may be stored at 4°C for several months.
5. 0.2 mg/mL poly-D-lysine in PBS, filter sterilize, store at 4°C.
6. 5 μg/mL fibronectin (Sigma Chemical Company, St. Louis, MO) in PBS, filter sterilize, store at 4°C.

2.2.3. Preparation of Cell Lysate and Measurement of Luciferase Activity

1. Luciferase assay mixture (LAM): 20 m*M* Tricine, 1 m*M* $MgCO_3$, 2.7 m*M* $MgSO_4$, 0.1 m*M* EDTA, 30 m*M* dithiothreitol (DTT), 0.3 m*M* coenzyme A, 0.5 m*M* luciferin, and 0.5 m*M* ATP; adjust pH to 7.8 with 1 *M* HCl. Store 1 mL aliquots at –20°C in complete darkness (may be wrapped in aluminum foil).
2. Prepare the cell lysis buffer by adding 1 vol of 5× Reporter Lysis Buffer (Promega) to 4 vol of ddH_2O. Vortex.

2.2.4. Determination of Galactosidase Activity and Quantification of Transcription Levels

1. β-galactosidase assay buffer, pH to 7.0 (buffer Z): 60 m*M* Na_2HPO_4, 40 m*M* NaH_2PO_4, 10 m*M* KCl, 1 m*M* $MgSO_4$, and 50 m*M* β-mercaptoethanol ***(15)***. Do not autoclave. This buffer is stable at 4°C.
2. 4 mg/mL o-nitrophenyl-β-D-galactopyranoside (ONPG) in buffer Z, store in 1-mL aliquots at –20°C.
3. 1 *M* Na_2CO_3 in ddH_2O.

3. Methods

In the identification of specific nucleotides involved in protein-DNA interactions, it is important to ensure that mutational analysis does not disrupt the spatial relations between potential regulatory elements. In this regard, site-specific nucleotide substitutions are preferred to sequence deletions or additions as they preserve the overall length and the internal structure of the tested DNA region.

There are thee main criteria for selecting Y-79 human Rb cells as the cell line in which to study the transcriptional regulation mecha-

nisms of photoreceptor-specific genes in general and the β-PDE gene in particular:

1. Y-79 Rb cells express a number of photoreceptor-specific genes ***(5,6)*** that indicates that they possess the regulatory factors necessary for their transcription. For example, the fact that Y-79 Rb cells produce readily detectable levels of the β-subunit of cGMP-phosphodiesterase (β-PDE) indicates that these cells have the appropriate transcription machinery for the expression of the gene encoding this protein.
2. A convenient method for the introduction of DNA into Y-79 Rb cells by transient transfections has been established (*see* **Subheading 3.3.**) that allows for sufficiently high transfection efficiency.
3. Promoters of many photoreceptor-specific genes, e.g., the β-PDE gene, as well as the SV40 early promoter are capable of directing high levels of expression of the luciferase reporter gene in Y-79 retinoblastoma cells ***(7)***.

3.1. Propagation of Y-79 Human Rb Cells in Culture

1. Rapidly thaw in a water bath preheated to 37°C the frozen stock of Y-79 human Rb cells. Rinse the exterior of the cryovial with 70% ethanol and dry it.
2. Transfer the cells to a 175-cm^2 flask containing 25 mL of the complete growth medium.
3. Incubate the cells at 37°C in 5% CO_2 and 95% air for 24 h (*see* **Note 1**).
4. Replace the growth medium as follows:
   a. Gently pellet the cells by centrifugation at 125*g* for 2 min at room temperature.
   b. Carefully aspirate the culture medium.
   c. Resuspend the cells in 35 mL of fresh complete growth medium.
5. Allow the cells to grow undisturbed in the humidified incubator at 37°C in 5% CO_2 and 95% air for 7–10 d or until the medium becomes slightly yellow. When this occurs, change the medium again.
6. Allow the cells to grow in 35 mL of the complete growth medium for another 10–14 d or until the medium turns light yellow. Clusters of Y-79 cells in suspension can now be seen with the naked eye. As the population of cells increases, they may be transferred into two or more flasks, and the growth medium needs to be changed more frequently. Thus, daily observation of the cells is recommended.

3.2. Isolation of Plasmid DNA Suitable for High-Efficiency Transient Transfections

For efficient and consistent transfection results in Y-79 Rb cells, it is very important to prepare highly purified plasmid DNA (*see* **Note 2**) (**Fig. 1**).

1. Late in the afternoon, transform DH5α™ competent cells (Gibco-BRL) with a desired plasmid, e.g., a pGL2-based construct or the pSV-β-Galactosidase control plasmid (Promega). Select the transformed colonies by growing the cells overnight in an appropriate medium supplemented with antibiotic, e.g., LB medium supplemented with ampicillin.
2. The following morning inoculate 2 mL of LB/ampicillin media with one small colony selected from the plate (large overgrown colonies may not grow well). Grow the bacterial culture in a 14-mL Falcon® tube in a shaker incubator at 250 rpm at 37°C until the OD_{595} is about 0.5 (in the late afternoon).
3. Inoculate the entire volume (2 mL) into a 2-L flask containing 250 mL of LB/ampicillin and incubate in a shaker incubator at 250 rpm at 37°C until the OD_{595} is about 1.0 (usually by the next morning).
4. Harvest the cells by centrifuging at 6000*g* for 15 min at 4°C. Decant the supernatant. At this stage, the cells can either be frozen or processed further for plasmid isolation using the Endofree Maxi kit (Qiagen, Valencia, CA).

The concentration of the purified DNA is calculated based on the OD_{260} measurements, the plasmid is digested with a restriction endonuclease and both digested and undigested samples are resolved on a 1% agarose gel to ensure the quality of the plasmid preparation.

3.3. Transient Transfections of Human Rb Cells Using Calcium Phosphate Precipitation

1. Initiate Y-79 Rb cell cultures at least 2 wk before transfection. Cells maintained in culture for a long period of time may not produce optimal results, therefore, we prefer to use cell cultures less than 1.5 mo old. Propagate the cells in suspension as described above. The day before plating the cells for transfection, change the complete growth medium.

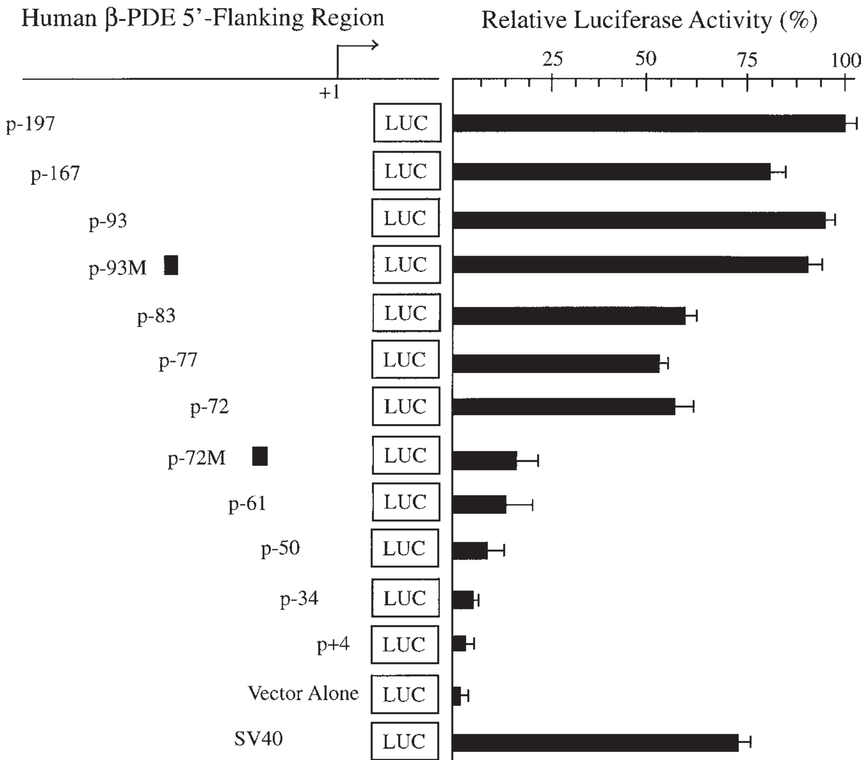

Fig. 1. Relative luciferase activity in transient transfections of Y-79 human Rb cells using pGL2-based constructs containing unidirectional nested deletions of the –197 to +4 bp of the 5'-flanking region of the human PDE6B gene. Plasmid nomenclature (p-197 to p+4) refers to the 5'-most nucleotide in the subcloned fragment. Constructs p-93M and p-72M contain site-specific nucleotide substitutions *(7)*. Plasmids were cotransfected with pSV-β-Galactosidase control vector. Luciferase activity was normalized to the corresponding β-galactosidase activity for each sample and expressed as percent activity of the construct p-197. Values represent the average of at least three transfections and standard deviation bars are shown.

2. The following day, under sterile conditions coat 60-mm diameter tissue-culture plates with 0.4 mL of poly-D-lysine (spread over the surface with brisk rotational movements), replace the lid and wait for 10 min. Add 0.2 mL of fibronectin solution and spread over the surface, replace the lid, and wait for 30–60 min. Meanwhile, count

the cells and dilute them with complete growth medium to 1.5 × 10^6 cells/mL. Aspirate the remaining fibronectin/poly-D-lysine solution and wash the dishes briefly with 3 mL of serum-free RPMI-1640 followed by aspiration. Plate 3 mL of cell suspension per plate (approx 4.5 × 10^6 cells/plate) and incubate them overnight at 37°C in 5% CO_2 and 95% air. If larger dishes are used, the number of cells plated and the volumes given should be adjusted accordingly.

3. The next morning, gently aspirate the complete growth medium (including any dead cells) and add 4 mL of the transfection medium (*see* **Note 3**). Place the cells back into the humidified incubator for 3 h.
4. In a 14-mL Falcon tube prepare the following transfection mixture that will be used for three plates (transfection of each construct is carried out in triplicate to ensure the reproducibility of the results): 1.35 mL of 10× TE buffer, pH 7.0; 15 µg of pSV-β-Galactosidase vector (5 µg per plate) and 30 µg of the appropriate pGL2 construct (10 µg per plate) in a total volume of 45 µL of TE buffer; 0.15 mL of 2.5 *M* $CaCl_2$. Add 1.5 mL of 2× HBS/P, pH 7.0 and mix thoroughly by pipeting up and down.
5. Add 1.0 mL of transfection mixture to each plate and spread over the cells by grid movements (forward-backward and left-right). Rotational movements are undesirable because the precipitate tends to spread around the periphery of the plate.
6. Place the plates into the humidified incubator overnight (*see* **Note 4**). The following morning, a fine-grained precipitate is readily visualized under the microscope around the cells and attached to the surface of the cells (*see* **Note 5**).
7. Carefully aspirate the medium from the plates to avoid cell loss. Cells may be gently washed with 5 mL of serum-free medium for up to three times. Feed the cells with 5 mL of the complete growth medium, incubate for 24 h, and harvest (*see* **Notes 6** and **7**).

3.4. Preparation of Cell Lysate and Measurement of Luciferase Activity

It is accepted that the level of luciferase activity measured following transient transfection correlates well with the level of the luciferase reporter gene expression.

In order to prepare cell lysates suitable for luciferase activity measurements, the luciferase assay system (Promega) is used according to the manufacturer's instructions with minor modifications:

1. Aspirate the medium from the plates and wash the cells twice with 4 mL of PBS at room temperature (*see* **Note 8**).
2. Add 0.1 mL of cell lysis buffer directly onto the cell monolayer and spread it over the surface by tilting the plate in all directions. Leave the plate horizontal for 15 min at room temperature. The cells will be lysed (if viewed under the microscope, only intact nuclei are visible).
3. Scrape the plate thoroughly with a cell scraper and transfer the cell lysate into a precooled microfuge tube. Keep on ice. Centrifuge for 2 min at 4°C at 16,000*g* to pellet the cell debris, and transfer the supernatant into a clean 1.5-mL tube. Store on ice.
4. Pipet 20 μL of the extract obtained from different plates into appropriately labeled polystyrene luminometer cuvettes. Bring the cuvets and LAM alongside a luminometer and turn it on.
5. Add 0.1 mL of LAM to each cuvet and measure the luciferase activity (*see* **Note 9**).

3.5. Determination of β-Galactosidase Activity and Quantification of Transcription Levels

The luciferase activity measured following transient transfections must be normalized for transfection efficiency and for general effects on transcription using an internal control vector (*see* **Note 10**). Therefore, all cells are routinely cotransfected with a control plasmid containing the bacterial *lacZ* gene driven by the SV40 early promoter, the pSV-β-galactosidase control vector (Promega).

The method of Sambrook et al. is used ***(15)*** with minor modifications.

1. Pipet 40 μL of 4 mg/mL ONPG stock solution into appropriately labeled glass tubes.
2. Add 40 μL of cell extract followed by 120 μL of buffer Z. Mix.
3. Incubate at 37°C until yellow color develops (approx 1.5–3 h).
4. Add 100 μL of 1 *M* Na_2CO_3 to each tube and determine the OD at 420 nm.

The following formula is used to calculate Relative Luciferase Activity (RLA; *see* **Note 10**):

$$\text{RLA} = \text{luciferase activity (light units)} / \beta\text{-galactosidase activity } (\text{OD}_{420}\text{ U}) \times 1000$$

A typical example of relative luciferase activity obtained following transient transfections of Y-79 Rb cells with pGL2-based constructs containing different lengths of the 5'-flanking region of the human *B-PDE* gene and the luciferase reporter gene is shown in **Fig. 1** (*see* **Note 11**).

3.6. Summary

In order to achieve high-efficiency transfection of plasmid DNA into Y-79 Rb cells, sufficiently high levels of expression of the luciferase reporter gene and a good reproducibility of the results, several factors have to be taken into consideration: (1) cell density used for transfections, (2) quality of the plasmid DNA preparation, (3) the length of the incubation of cells with transfection mixture, and (4) the duration of post-transfection incubation.

Briefly, the highest luciferase activity is observed when cells are transfected with plasmid DNA isolated using the Endofree Maxi Prep kit (Qiagen), and when the cells are plated at a density of 3–4.5 million per 60-mm diameter plate. The optimal results are obtained when Y-79 cells are incubated for 16–24 h with calcium phosphate/DNA transfection mixture and harvested 24 h following the removal of the mixture.

4. Notes

1. The medium used for the storage of Y-79 cells in a frozen state contains 95% complete growth medium and 5% dimethyl sulfoxide (DMSO). DMSO is toxic for the growing cells and should be removed within 24 h following thawing of the Y-79 cells by changing the growth medium.
2. We have compared three different plasmid isolation techniques. The Endofree Maxi kit (Qiagen, Valencia, CA), designed to remove bacterial endotoxin, provided the highest levels of luciferase activity, followed by the Maxi Prep kit (Qiagen); the lowest levels were observed using the alkaline lysis/lysozyme isolation technique ***(16)***.
3. The transfection medium differs from the complete growth medium in that it contains DMEM/F12 instead of RPMI-1640 (*see* **Subhead-**

ing 2.). The latter medium has a high Ca^{2+} content which may interfere with calcium phosphate-mediated transfection.

4. We have compared the levels of luciferase activity after Y-79 cells were incubated with the DNA/calcium phosphate transfection mixture for various periods of time (3, 6, 12, 16, and 24 h). The transfection mixture was then replaced with complete growth medium and the cells were harvested the next day. The highest luciferase activity was observed following the 24-h incubation.
5. It is important to examine all the plates for the amount of precipitate formed, which should be comparable, as well as to make sure that the cells remain attached to the plate. If there are plates with significantly less precipitate or with many cells detached, those plates should be disregarded.
6. We have compared the effect of various posttransfection incubation periods on the level of luciferase reporter gene expression. Upon removal of the calcium phosphate mixture, the cells were allowed to grow in fresh complete growth medium for 24 or 56 h. Luciferase activity was measured for both incubation times and found to be the same, but at 56 h many cells were detached from the plate. Therefore, we routinely use the 24-h incubation.
7. Essentially, the same protocol can be used for transient transfections of WERI-Rb-1 human Rb cells.
8. Thoroughly remove all PBS after the final wash by tilting the plate (residual PBS may interfere with the cell lysis buffer and prevent complete lysis of the cells).
9. LAM is thawed at room temperature in the dark. Add LAM to one cuvet at a time, briefly mix by hand-vortex, using one finger tap on the lower portion of the cuvet, insert the cuvet into the luminometer and measure the luciferase activity. It is important to perform these step in a timely fashion consistent for all the cuvets because once LAM is added to the extract, the light-emitting reaction begins. One can perform this sequence to a count of one to four. Alternatively, if the luminometer is equipped with a dispenser, LAM can be injected automatically.
10. Although the luciferase activity measured directly in cell lysates may vary between different transfections or batches of Y-79 cells, the activity of the promoter of interest may still be evaluated provided it is normalized relative to an internal control plasmid, e.g., pSV-β-Galactosidase vector (Promega).

11. For negative controls, the measurements of luciferase activity in LAM alone or in the extracts from mock-transfected cells can be used (between 90 and 150 light units). Luciferase activity measured in Y-79 cells transfected with the pGL2-Control vector can be used as a positive control and the values obtained usually range between 70,000 and 110,000 light units.

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