PathDetect in Vivo Signal Transduction Pathway *cis*-Reporting Systems

Instruction Manual

Catalog #219073 (AP-1 *cis*-Reporting System)
#219075 (CRE *cis*-Reporting System)
#219077 (NF-κB *cis*-Reporting System)
#219079 (SRE *cis*-Reporting System)
#219081 (SRF *cis*-Reporting System)
#219083 (p53 *cis*-Reporting System)
#219092 (ISRE *cis*-Reporting System)
#219093 (GAS *cis*-Reporting System)
#219094 (NFAT *cis*-Reporting System)
#219095 (TARE *cis*-Reporting System)
#240111 (C/EBP *cis*-Reporting System)
#240113 (DR1 *cis*-Reporting System)
#240115 (DR3 *cis*-Reporting System)
#240119 (DR5 *cis*-Reporting System)
#240129 (Egr-1 *cis*-Reporting System)
#240131 (LILRE *cis*-Reporting System)
#240133 (GRE *cis*-Reporting System)
#240135 (DR4 *cis*-Reporting System)
#240049 (pAP-1-hrGFP Plasmid)
#240050 (pCRE-hrGFP Plasmid)
#240051 (pNF-κB-hrGFP Plasmid)
#240053 (pNFAT-hrGFP Plasmid)
#219087 (pLuc-MCS Plasmid)

Revision D

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PathDetect in Vivo Signal Transduction Pathway *cis*-Reporting Systems

MATERIALS PROVIDED

PathDetect AP-1 *cis*-Reporting System (Catalog #219073)

Component	Concentration	Quantity
pAP-1-Luc plasmid	1 µg/µl	50 µg
pFC-MEKK positive control plasmid	25 ng/µl	5 µg

PathDetect CRE *cis*-Reporting System (Catalog #219075)

Component	Concentration	Quantity
pCRE-Luc plasmid	1 µg/µl	50 µg
pFC-PKA positive control plasmid	25 ng/µl	5 µg

PathDetect NF-κB *cis*-Reporting System (Catalog #219077)

Component	Concentration	Quantity
pNF-κB-Luc plasmid	1 µg/µl	50 µg
pFC-MEKK positive control plasmid	25 ng/µl	5 µg

PathDetect SRE *cis*-Reporting System (Catalog #219079)

Component	Concentration	Quantity
pSRE-Luc plasmid	1 µg/µl	50 µg
pFC-MEKK positive control plasmid	25 ng/µl	5 µg

PathDetect SRF *cis*-Reporting System (Catalog #219081)

Component	Concentration	Quantity
pSRF-Luc plasmid	1 µg/µl	50 µg
pFC-PKA positive control plasmid	25 ng/µl	5 µg

PathDetect p53 *cis*-Reporting System (Catalog #219083)

Component	Concentration	Quantity
p53-Luc plasmid	1 µg/µl	50 µg
pFC-p53 positive control plasmid	25 ng/µl	5 µg

PathDetect ISRE *cis*-Reporting System (Catalog #219092)

Component	Concentration	Quantity
pISRE-Luc plasmid	1 µg/µl	50 µg
pCIS-CK negative control plasmid	1 µg/µl	50 µg

PathDetect GAS *cis*-Reporting System (Catalog #219093)

Component	Concentration	Quantity
pGAS-Luc plasmid	1 µg/µl	50 µg
pCIS-CK negative control plasmid	1 µg/µl	50 µg

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PathDetect NFAT *cis*-Reporting System (Catalog #219094)

Component	Concentration	Quantity
pNFAT-Luc plasmid	1 µg/µl	50 µg
pCIS-CK negative control plasmid	1 µg/µl	50 µg

PathDetect TARE *cis*-Reporting System (Catalog #219095)

Component	Concentration	Quantity
pTARE-Luc plasmid	1 µg/µl	50 µg
pCIS-CK negative control plasmid	1 µg/µl	50 µg

PathDetect C/EBP *cis*-Reporting System (Catalog #240111)

Component	Concentration	Quantity
pC/EBP-Luc plasmid	1 µg/µl	50 µg
pCIS-CK negative control plasmid	1 µg/µl	50 µg

PathDetect DR1 *cis*-Reporting System (Catalog #240113)

Component	Concentration	Quantity
pDR1-Luc plasmid	1 µg/µl	50 µg
pCIS-CK negative control plasmid	1 µg/µl	50 µg

PathDetect DR3 *cis*-Reporting System (Catalog #240115)

Component	Concentration	Quantity
pDR3-Luc plasmid	1 µg/µl	50 µg
pCIS-CK negative control plasmid	1 µg/µl	50 µg

PathDetect DR5 *cis*-Reporting System (Catalog #240119)

Component	Concentration	Quantity
pDR5-Luc plasmid	1 µg/µl	50 µg
pCIS-CK negative control plasmid	1 µg/µl	50 µg

PathDetect Egr-1 *cis*-Reporting System (Catalog #240129)

Component	Concentration	Quantity
pEgr-1-Luc plasmid	1 µg/µl	50 µg
pCIS-CK negative control plasmid	1 µg/µl	50 µg

PathDetect LILRE *cis*-Reporting System (Catalog #240131)

Component	Concentration	Quantity
pLILRE-Luc plasmid	1 µg/µl	50 µg
pCIS-CK negative control plasmid	1 µg/µl	50 µg

PathDetect GRE *cis*-Reporting System (Catalog #240133)

Component	Concentration	Quantity
pGRE-Luc plasmid	1 µg/µl	50 µg
pCIS-CK negative control plasmid	1 µg/µl	50 µg

PathDetect DR4 *cis*-Reporting System (Catalog #240135)

Component	Concentration	Quantity
pDR4-Luc plasmid	1 µg/µl	50 µg
pCIS-CK negative control plasmid	1 µg/µl	50 µg

PathDetect *cis*-Reporting Plasmids

Component	Concentration	Quantity	Catalog #
pAP-1-Luc plasmid	1 µg/µl	50 µg	219074
pCRE-Luc plasmid	1 µg/µl	50 µg	219076
pNF-κB-Luc plasmid	1 µg/µl	50 µg	219078
pSRE-Luc plasmid	1 µg/µl	50 µg	219080
pSRF-Luc plasmid	1 µg/µl	50 µg	219082
p53-Luc plasmid	1 µg/µl	50 µg	219085
pISRE-Luc plasmid	1 µg/µl	50 µg	219089
pGAS-Luc plasmid	1 µg/µl	50 µg	219091
pNFAT-Luc plasmid	1 µg/µl	50 µg	219088
pTARE-Luc plasmid	1 µg/µl	50 µg	240039
pC/EBP-Luc plasmid	1 µg/µl	50 µg	240112
pDR1-Luc plasmid	1 µg/µl	50 µg	240114
pDR3-Luc plasmid	1 µg/µl	50 µg	240116
pDR5-Luc plasmid	1 µg/µl	50 µg	240120
pEgr-1-Luc plasmid	1 µg/µl	50 µg	240130
pLILRE-Luc plasmid	1 µg/µl	50 µg	240132
pGRE-Luc plasmid	1 µg/µl	50 µg	240134
pDR4-Luc plasmid	1 µg/µl	50 µg	240136
pAP-1-hrGFP plasmid	1 µg/µl	50 µg	240049
pCRE-hrGFP plasmid	1 µg/µl	50 µg	240050
pNF-κB-hrGFP plasmid	1 µg/µl	50 µg	240051
pNFAT-hrGFP plasmid	1 µg/µl	50 µg	240053
pLuc-MCS plasmid	1 µg/µl	50 µg	219087

PathDetect *cis*-Reporting Control Plasmids

Component	Type of Control	Concentration	Quantity	Catalog #
pFC-p53 plasmid	positive control plasmid recommended for use with p53-Luc plasmid	1 µg/µl	10 µg	219084
pFC-MEKK plasmid	positive control plasmid recommended for use with pNF-κB-Luc, pNF-κB-hrGFP, pAP-1-Luc, pAP-1-hrGFP, and pSRE-Luc plasmids	1 µg/µl	10 µg	219059
pFC-PKA plasmid	positive control plasmid recommended for use with pCRE-Luc, pCRE-hrGFP, and pSRF-Luc plasmids	1 µg/µl	10 µg	219071
pCIS-CK plasmid	negative control plasmid recommended for use with all *cis*-reporting plasmids	1 µg/µl	50 µg	219090

STORAGE CONDITIONS

All Components: –20°C

ADDITIONAL MATERIALS REQUIRED

Mammalian cells (e.g., HeLa, 293, CHO, CV-1, and NIH3T3)
Cell culture medium [e.g., Dulbecco's minimum essential medium (DMEM)]
Complete medium [DMEM containing 10% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin and streptomycin]
Luciferase assay kit
5-ml BD Falcon® polystyrene round bottom tubes (BD Biosciences catalog #352054)
Calcium- and magnesium-free PBS
Tissue culture dishes
Transfection reagents
Luminometer
Extracellular stimuli (e.g., interferon, ionomycin, etc.)

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INTRODUCTION

The Agilent PathDetect in Vivo Signal Transduction Pathway *cis*-Reporting Systems are designed for simple, rapid, and convenient assessment of the in vivo activation of signal transduction pathways. We have created a series of inducible reporter plasmids that contain the luciferase reporter gene driven by a basic promoter element (TATA box) plus a defined inducible *cis*-enhancer element (see Figure 2 in *Appendix*). These plasmids are particularly suited for the in vivo readouts of signal transduction pathways since these enhancers are convergent points of many signal transduction pathways. These systems are useful in studying the in vivo effects of a new gene, growth factor, or drug candidate on the signaling pathway. Some of the reporters may also be used for cloning novel signal transduction genes, and may be useful for identifying drug candidates in a high-throughput format.

cis-Reporting Systems

The PathDetect in vivo signal transduction pathway *cis*-reporting systems include a reporter plasmid and a positive or negative control plasmid. Expression of the *Photinus pyralis* (firefly) luciferase gene in the reporter plasmid is controlled by a synthetic promoter that contains direct repeats of the transcription recognition sequences for the tumor suppressor protein p53,[1,2] the activator protein 1 (AP-1),[1–3] the CCAAT-enhancer binding protein (C/EBP), the consensus sites for cyclic AMP response element (CRE),[1–3] the retinoid X receptor response element (DR1/RXRE),[4] the vitamin D receptor response element (DR3/VDR),[4] the active thyroid hormone receptor response element (DR4/TR), the retinoic acid receptor response element (DR5/RARE),[4,5] the early growth response factor 1 (Egr-1),[6] the interferon γ-activated sequence (GAS),[1,2] the glucocorticoid response element (GRE),[7] the interferon-stimulated response element (ISRE),[1,2] the LPS IL-1β response element (LILRE),[8,9] the nuclear factor of activated T cells (NFAT),[1,2,10] the binding sites for nuclear factor κB (NF-κB),[1–3] the serum response element (SRE),[1–3] the serum response factor (SRF),[1–3] or the TGF-β/Activin response element (TARE) (see *Appendix: Plasmid Information*).

In addition to the luciferase reporter format, the CRE, AP-1, NF-kB and NFAT *cis*-reporting plasmids are available with an hrGFP reporter (humanized green fluorescent protein). With the hrGFP reporter, one can detect the signal without disrupting the cells, an advantage that is particularly suitable for functional cloning and potentially for high-throughput screening.

When a reporter plasmid and an uncharacterized gene are cotransfected into mammalian cells, the activation of endogenous protein kinases initiated by the cotransfected gene product will result in the activation of corresponding *trans*-activators which in turn stimulate reporter expression (Figure 1). The effects of extracellular stimuli can also be studied with these systems.

Multiple Cloning Site Plasmid

Instead of repeats of a *cis*-acting DNA element, pLuc-MCS contains a multiple cloning site upstream from the luciferase gene and the TATA box (see Figure 3 in *Appendix*). Therefore, pLuc-MCS allows the insertion of any *cis*-acting DNA element if a specific enhancer other than those described above is desired.

Positive and Negative Control Plasmids

The pCIS-CK negative control plasmid contains the luciferase reporter gene and does not contain any *cis*-acting DNA elements, thus serving as a negative control for all PathDetect *cis*-reporting plasmids. The negative control is included with each of the following PathDetect *cis*-reporting systems: C/EBP, DR1, DR3, DR4, DR5, Egr-1, GAS, GRE, ISRE, LILRE, NFAT, and TARE. For all other systems, the pCIS-CK negative control plasmid is available separately.

Each positive control plasmid expresses a kinase from the constitutive CMV promoter that is ultimately responsible for activating transcription of the reporter from the enhancer element–TATA box region. A positive control plasmid containing an appropriate kinase for a given pathway is provided with each of the following *cis*-reporting systems: AP-1, CRE, NF-κB, p53, SRE, and SRF.

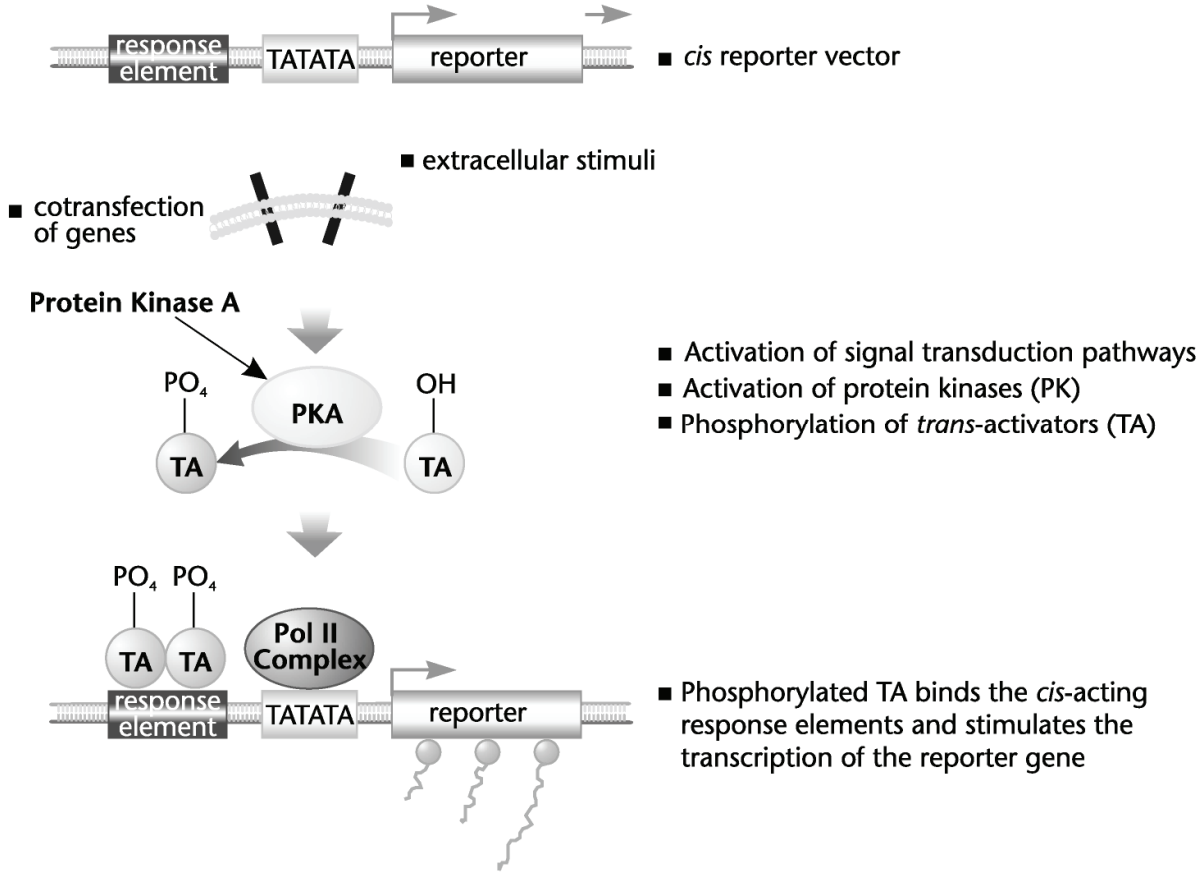


FIGURE 1 Extracellular signals trigger the activation of a series of intracellular signaling molecules such as protein kinases and phosphatases. Many of these signal transduction pathways converge at transcription factors that bind to specific enhancer elements found in the promoters of various genes and modulate the transcription of these genes. The activation of a signal transduction pathway can therefore be monitored by the expression level of the luciferase reporter gene controlled by a promoter containing these enhancer elements. The PathDetect reporter vector can be used to monitor the activation of signal transduction pathways converging at a specific response element. The PathDetect vectors contain the firefly luciferase gene or the hrGFP gene controlled by synthetic promoters containing a basic TATA element and various *cis*-acting elements. In vivo, the promoters contain transcription factors including *trans*-acting activators. The activation of endogenous PKA or other protein kinases initiated by extracellular stimuli or the product of a cotransfected gene will result in the activation of corresponding *trans*-activators, which in turn stimulate reporter expression.

PREPROTOCOL CONSIDERATIONS

Choosing a Cell Line

The PathDetect signal transduction pathway *cis*-reporting systems may be used for various mammalian cell lines. Cell lines vary in both types and levels of signaling proteins and other properties. The endogenous protein kinases and transcriptional activator activities in the cell line used will determine the background and hence the sensitivity of the assay. **Cell culture and transfection protocols described in this manual apply to adherent cell lines. It should be noted that certain cell lines (e.g. Jurkat), are grown in suspension and consequently may require alternative cell culture and transfection methods.** Optimal conditions for each cell line used with this system will need to be determined experimentally.

Choosing a Transfection Method

The sensitivity of the assay is influenced by the transfection efficiency. A high transfection efficiency generally provides a more sensitive assay that requires a smaller volume of sample. Transfection conditions should be optimized before performing the assays with a reporter plasmid. Sufficient plasmid is included for several optimization experiments. Because the luciferase assay is very sensitive, various transfection methods, such as calcium phosphate precipitation and lipid-mediated transfection, may be used. As with all transfection methods, the quality of plasmid DNA is critical. The plasmids provided in this kit have been double cesium-chloride banded.

Normalizing the Transfection Efficiency

Overexpression of some gene products may affect transfection efficiency or cause overall changes in the growth properties of mammalian cells. Normalizing the transfection efficiency is often necessary to interpret results.[11]

DESIGNING THE EXPERIMENT

Typical initial experiments for the *cis*-reporting systems are outlined in Tables I–III. All assays are to be run in triplicate (for example, eight samples will utilize four 6-well tissue culture dishes).

Studying the Effects of a Gene Product

When a reporter plasmid and an uncharacterized gene are cotransfected into mammalian cells, the activation of endogenous protein kinases initiated by the cotransfected gene product will result in the activation of corresponding *trans*-activators which in turn stimulate reporter expression. A reporter plasmid-only transfection should be used as a negative control to measure the background luciferase expression in the cell line and ensure that the effect observed in the experimental reaction is not caused by endogenous proteins. The parental plasmid used to clone the gene of interest should be used as a negative control (without insert) to ensure that the effect observed is not caused by other proteins expressed from the plasmid. The pCIS-CK control plasmid does not contain any *cis*-acting DNA elements and can be used as a negative control for all PathDetect *cis*-reporting plasmids. Background luciferase activity from the pCIS-CK control plasmid indicates that the effects seen in the experimental samples are valid. Each available positive control plasmid expresses a kinase from the constitutive CMV promoter resulting in the activation of corresponding *trans*-activators which in turn stimulate reporter expression from the reporter plasmid. Depending on the purpose of the experiment, other controls such as nonfunctional mutants of the enhancer elements might be required.

Systems Providing a Positive Control Plasmid

The positive control plasmids included in the PathDetect AP-1, CRE, NF-κB, SRE, SRF, and p53 reporting systems ensure the quality of the components and transfection method and test whether the reporting system works in the specific cell line chosen. The pCIS-CK negative control plasmid can be used as a negative control and is available separately. See Table I for typical experimental and control sample setup.

TABLE I

Sample Experiment to Study the Effects of a Gene Product[a]

#	Reporter plasmid	Positive control plasmid	pCIS-CK negative control plasmid[b]	Experimental vector with gene of interest	Experimental vector without insert	Carrier DNA
1[c]	1.0 μg (1 μl)	—	—	—	—	1.0 μg
2[d]	1.0 μg (1 μl)	—	—	—	50 ng	950 ng
3[d]	1.0 μg (1 μl)	—	—	—	100 ng	900 ng
4[d]	1.0 μg (1 μl)	—	—	—	1000 ng	—
5[e]	1.0 μg (1 μl)	—	—	50 ng	—	950 ng
6[e]	1.0 μg (1 μl)	—	—	100 ng	—	900 ng
7[e]	1.0 μg (1 μl)	—	—	1000 ng	—	—
8[f]	—	—	1.0 μg (1 μl)	50 ng	—	950 ng
9[f]	—	—	1.0 μg (1 μl)	100 ng	—	900 ng
10[f]	—	—	1.0 μg (1 μl)	1000 ng	—	—
11[g]	1.0 μg (1 μl)	50 ng (2 μl)	—	—	—	950 ng

[a] The protocols given are based on 6-well tissue culture dishes with a well diameter of ~35 mm and a surface area of ~9.4 cm^2. When dishes with smaller wells are used, decrease the number of cells per well and the volume of reagents according to the surface area of the wells.

[b] The pCIS-CK negative control plasmid is not included with all *cis*-reporting systems and is available separately.

[c] Sample 1 measures the background luciferase expression in the cell line.

[d] Samples 2–4 lack the gene of interest and, therefore, control for samples 5–7, respectively.

[e] Samples 5–7 measure the effect of the gene product on the signal transduction pathway involved.

[f] Samples 8–10 lack *cis*-acting elements. Background luciferase activity in these samples indicates that the effects seen in samples 5–7 are valid.

[g] Sample 11 measures the efficacy of the assay for the cell line chosen.

Systems Providing the pCIS-CK Negative Control Plasmid

A negative control plasmid is included with each of the following PathDetect *cis*-reporting systems: C/EBP, DR1, DR3, DR4, DR5, Egr-1, GAS, GRE, ISRE, LILRE, NFAT, and TARE. See Table II for typical experimental and control sample setup.

TABLE II

Sample Experiment to Study the Effects of a Gene Product[a]

#	Reporter plasmid	pCIS-CK negative control plasmid	Experimental vector with gene of interest	Experimental vector without insert	Carrier DNA
1[b]	1.0 μg (1 μl)	—	—	—	1 μg
2[c]	1.0 μg (1 μl)	—	—	50 ng	950 ng
3[c]	1.0 μg (1 μl)	—	—	100 ng	900 ng
4[c]	1.0 μg (1 μl)	—	—	1000 ng	—
5[d]	1.0 μg (1 μl)	—	50 ng	—	950 ng
6[d]	1.0 μg (1 μl)	—	100 ng	—	900 ng
7[d]	1.0 μg (1 μl)	—	1000 ng	—	—
8[e]	—	1.0 μg (1 μl)	50 ng	—	950 ng
9[e]	—	1.0 μg (1 μl)	100 ng	—	900 ng
10[e]	—	1.0 μg (1 μl)	1000 ng	—	—

[a] The protocols given are based on 6-well tissue culture dishes with a well diameter of ~35 mm and a surface area of ~9.4 cm^2. When dishes with smaller wells are used, decrease the number of cells per well and the volume of reagents according to the surface area of the wells.

[b] Sample 1 measures the background luciferase expression in the cell line.

[c] Samples 2–4 lack the gene of interest and, therefore, control for samples 5–7, respectively.

[d] Samples 5–7 measure the effect of the gene product on the signal transduction pathway involved.

[e] Samples 8–10 lack *cis*-acting elements. Background luciferase activity in these samples indicates that the effects seen in samples 5–7 are valid.

Studying the Effects of Extracellular Stimuli

PathDetect *cis*-reporting systems may also be used to study the effects of extracellular stimuli (e.g. growth factors or drug candidates) on corresponding signal transduction pathways. Cells are transfected with the *cis*-reporter plasmid and treated with the stimulus of interest. Luciferase or hrGFP expression from the reporter plasmid indicates the activation of corresponding transcriptional activators and the signal transduction pathways acting through the enhancer elements. See Table III for typical experimental and control sample setup. Many *cis*-reporting plasmids have been tested with various extracellular stimuli (see Table IV for reaction conditions).

TABLE III

Sample Experiment to Study the Effects of Extracellular Stimuli[a]

#	Reporter plasmid	Positive control plasmid[b]	pCIS-CK negative control plasmid[c]	Extracellular stimuli
1[d]	1.0 µg (1 µl)	—	—	e.g., Interferon, Ionomycin, etc.
2[e]	1.0 µg (1 µl)	—	—	—
3[f]	1.0 µg (1 µl)	10–200 ng	—	—
4[g]	—	—	1.0 µg (1 µl)	e.g., Interferon, Ionomycin, etc.

[a] The protocols given are based on 6-well tissue culture dishes with a well diameter of ~35 mm and a surface area of ~9.4 cm^2. When dishes with smaller wells are used, decrease the number of cells per well and the volume of reagents according to the surface area of the wells.

[b] A positive control plasmid is not included with all *cis*-reporting systems.

[c] The pCIS-CK negative control plasmid is not included with all *cis*-reporting systems and is available separately.

[d] Sample 1 measures the effect of the extracellular stimulus.

[e] Sample 2 measures the background luciferase expression in the cell line.

[f] Sample 3 indicates whether the reporting system works in the chosen cell line.

[g] Sample 4 determines whether the effect seen in Sample 1 is specific.

TABLE IV
Guidelines for Determining the Concentrations of Extracellular Stimuli

Reporter plasmid	Plasmid concentration	Extracellular stimulus	Concentration
pC/EBP-Luc, pDR1-Luc, pDR5-Luc, or pEgr-1-Luc	0.1 μg/well[a]	Ionomycin PMA	0.5 μg/ml 50 ng/ml
pDR3-Luc	0.16 μg/well[a]	9-cis-retinoic acid 1-alpha-25-dihydroxy Vitamin D3	1 μM 0.1 μM
pDR4-Luc	0.16 μg/well[b]	9-cis-retinoic acid 3,3′,5-triiodo-L-thyronine	1 μM 1 μM
pGAS-Luc	0.25 μg/well[c]	Interferon γ	20–1000 U/ml
pGRE-Luc	0.15 μg /well[a]	Dexamethasone	100 μM
pISRE-Luc	0.25 μg/well[c]	Interferon α Interferon β	200–2000 U/ml 200–2000 U/ml
pLILRE-Luc	4 μg/well[d]	LPS	5 μg/ml
pNFAT-Luc or pNFAT-hrGFP	0.25 μg/well[c]	Ionomycin PMA	1 μg/ml 60 ng/ml
pTARE-Luc	0.25 μg/well[c]	TGF-β	50 pM

[a] Each well of a 96-well plate contains ~1.6×10^4 adherent cells (e.g. HeLa or 293) or ~2×10^5 suspension cells (e.g. Jurkat).

[b] Each well of a 96-well plate contains ~3×10^4 adherent cells (e.g. Caco-2).

[c] Each well of a 24-well plate contains ~8×10^4 adherent cells (e.g. HeLa or 293) or ~1×10^6 suspension cells (e.g. Jurkat).

[d] Each well of a 6-well plate contains ~1×10^6 suspension cells (e.g. Jurkat).

CELL CULTURE AND TRANSFECTION

Growing the Cells

Note *The following protocol is designed for adherent cell lines such as HeLa and NIH3T3. Optimization of media and culture conditions may be required for other adherent cell lines. Suspension cell lines will require alternative cell culture and transfection protocols.*

1. Thaw and seed frozen cell stocks in complete medium in 50-ml or 250-ml tissue culture flasks.

2. Split the cells when they just become confluent.

3. Subculture the cells at an initial density of ~1×10^5–2×10^5 cells/ml every 3–4 days.

Preparing the Cells for Transfection

1. Seed 3×10^5 cells in 2 ml of complete medium in each well of a 6-well tissue culture dish. (Perform transfection within 24 hours of seeding.)

2. Incubate the cells at 37°C in a CO_2 incubator for 24 hours.

PREPARING THE DNA MIXTURES FOR TRANSFECTION

Studying the Effects of a Gene Product

Combine the plasmids to be cotransfected in sterile 5-ml BD Falcon polystyrene round bottom tubes as indicated in Tables I and II. As each assay is run in triplicate, the amount of plasmid DNA in each tube should be sufficient for three transfections. See Tables I and II for the appropriate amounts. For example, to prepare sample #2 **in triplicate** as indicated in Table I, combine the following components and then proceed to *Transfecting the Cells*:

3 µl (3 µg) of reporter plasmid
150 ng of experimental vector without an insert
2.9 µg of unrelated plasmid DNA

Studying the Effects of Extracellular Stimuli

Combine the plasmids to be cotransfected in sterile 5-ml BD Falcon polystyrene round bottom tubes as indicated in Table III. As each assay is run in triplicate, the amount of plasmid DNA in each tube should be sufficient for three transfections. See Table III for appropriate amounts. For example, to prepare sample #1 **in triplicate** as indicated in Table III, add 3 µl (3 µg) of reporter plasmid to a microcentrifuge tube and then proceed to *Transfecting the Cells*.

TRANSFECTING THE CELLS

A number of transfection methods, including calcium phosphate precipitation and lipid-mediated transfection, may be used. Transfection efficiencies vary between cell lines and according to experimental conditions. Transfection procedures should be optimized for the cell line chosen.

If studying the effects of a gene product, perform step a. **If studying the effects of extracellular stimuli**, perform step b.

a. Replace the medium with fresh DMEM containing 0.5% FBS 18–24 hours after the beginning of transfection. After incubating an additional 18–24 hours, proceed to *Extracting the Luciferase*.

Note *Due to the possible induction of pathways by unknown factors in the serum, low serum concentrations are used; however, the use of 10% serum may also yield satisfactory results.*

b. Replace the media with fresh media containing the appropriate extracellular stimuli (e.g., EGF) 18–24 hours after the beginning of transfection. After incubating an additional 5–7 hours, proceed to *Extracting the Luciferase*.

EXTRACTING THE LUCIFERASE

1. Remove media from the cells and carefully wash the cells twice with 2 ml of 1× PBS buffer.[§]

2. Remove as much PBS from the wells as possible with a Pasteur pipet. Add 400 µl of 1× cell lysis buffer[§] to the wells and swirl the dishes gently to ensure uniform coverage.

3. Incubate the dishes for 15 minutes at room temperature. Swirl the dishes gently midway through the incubation.

4. Assay for luciferase activity directly from the wells within 2 hours.

5. To store for later analysis, transfer the solutions from each well into a separate microcentrifuge tube. Spin the samples in a microcentrifuge at full speed for 2 minutes. Store the supernatant at –80°C. (More than one freeze–thaw cycle of the extract will result in the loss of luciferase activity.)

Note *If this passive lysis method does not yield satisfactory results, refer to the active lysis method in* Troubleshooting.

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

PERFORMING THE LUCIFERASE ACTIVITY ASSAY

1. Mix 5–20 µl of cell extract equilibrated to room temperature with 100 µl of room temperature 1× luciferase assay reagent (see *Preparation of Media and Reagents*) in a 5-ml BD Falcon polystyrene round bottom tube.

2. Measure the light emitted from the reaction with a luminometer using an integration time of 10–30 seconds.

3. Luciferase activity may be expressed in relative light units (RLU) as detected by the luminometer from the sample. The activity may also be expressed as RLU/well, RLU/number of cells, or RLU/mg of total cellular protein.

USING THE HRGFP *CIS*-REPORTER PLASMIDS

The hrGFP protein may be visualized by fluorescence microscopy, or analyzed semi-quantitatively by flow cytometry. Because visualization of the induction of the hrGFP reporters requires no cellular disruption, the reporters are particularly amenable for functional screening of cDNA expression libraries for signal transduction genes involved in specific pathways. In addition, they may prove useful for the production of cell-based assays for high-throughput drug screening. In most cases, mammalian host cell lines transfected with plasmids should show expression of hrGFP 24–72 hours after transfection. The hrGFP excitation/emission peaks are at 500 and 506 nm, respectively, and hrGFP can be visualized using standard FITC filters.

Specifications for hrGFP and EGFP Excitation and Emission Spectra

GFP Form[a]	Excitation/Emission Spectra Maxima (nm)
hrGFP	500/506
EGFP	488/509[b]

[a] Both forms of GFP compared in this table have been codon-optimized for maximum expression in human cells.

[b] The emission spectrum for EGFP also shows a shoulder at 540 nm.

Note *Filter sets compatible with the detection of hrGFP and EGFP are sold by Omega Optical, Inc. (Phone: 802 254 2690, or see* www.omegafilters.com*):*

Exciter filter: *XF1073*

Emitter filter: *XF3084*

Beam splitter: *XF2010*

Microscope cube set with the exciter filter, emitter filter and beam splitter: *XF100-2*

CLONING PROTOCOL FOR THE PLUC-MCS PLASMID

The pLuc-MCS plasmid is designed for the convenient insertion of enhancer elements upstream of the TATA box and the firefly luciferase gene. This plasmid features a multiple cloning site (MCS) with 6 unique cloning sites for insertion of double stranded oligonucleotides. It is constructed by replacing the 5× GAL4 binding sites in the pFR-Luc plasmid of the PathDetect Trans-Reporting Systems with the MCS. See *Appendix: Plasmid Information* for a map of the pLuc-MCS plasmid.

Design and Synthesis of Oligonucleotides to Construct Enhancer Elements of Choice

Enhancer sequences can be constructed by annealing two synthetic, complementary oligonucleotides that contain the sequence for the enhancer element of interest. For example, in order to create a CREB-responsive enhancer element, two oligonucleotides should be synthesized.

1. 5´ TCGA GCC TGACGTCAGAG 3´

2. 5´ TCGA CTCTGACGTCAGGC 3´

The underlined sequence represents the cAMP-response element and surrounding sequence is derived from the promoter of the somatostatin gene. When annealed together, the two oligonucleotides will form a double stranded segment of DNA with a 5´ single stranded overhang at both ends. These overhangs are complementary to the sticky ends created when double stranded DNA (for example pLuc-MCS) is digested with *Sal* I. Of course other restriction sites in the multicloning site (MCS) can be used for cloning the enhancer element of choice. The oligonucleotides should be designed so that when they are annealed, the overhangs are complementary to the sticky ends to be used in the MCS. Clones with various copies of the response element inserted can be selected and tested for function.

It is usually desirable for the cloned enhancer element to be present in multiple copies. If the enhancer element of interest has a known number of optimal repeats, *cis*-reporter plasmids can be made by designing longer oligonucleotides with defined copies of the enhancer element (e.g. 3× CRE). An advantage of this approach is that the annealed, double stranded enhancer DNA can be inserted into pLuc-MCS digested with two different restriction enzymes (directional cloning), which is more efficient. In this case, the overhangs at the 5´-ends of the two oligonucleotides should be designed to be complementary to the sticky ends created in the double-digested pLuc-MCS.

Preparing the pLuc-MCS Plasmid

- The enhancer element of interest can be inserted in any of the 6 restriction sites in the MCS. Following restriction digestion, we suggest dephosphorylation of the pLuc-MCS plasmid with CIAP prior to ligation. If the pLuc-MCS plasmid is digested with two restriction enzymes, the background can be further reduced by agarose gel purification of the digested plasmid band.

- After purification and ethanol precipitation of the DNA, resuspend in a volume of TE buffer (see *Preparation of Media and Reagents*) that will allow the concentration of the plasmid DNA to be the same as the concentration of the insert DNA (~0.1 µg/µl).

Preparing the Insert

- Determine the concentration of the oligonucleotide by measuring the absorption at 260 nm.

- Mix the two complementary oligonucleotides in equal molar amounts in a low salt buffer (e.g. 10 mM Tris-HCl, pH 7.5, containing 50 mM NaCl).

- Heat the mixture at 95-100°C for 5 min and let it cool gradually to room temperature over a period of ~30-60 min.

Ligating the Insert

For ligation, the ideal insert-to-vector molar ratio of DNA is variable; however, a reasonable starting point is a 2:1 ratio. The ligation reactions outlined below allow the setup of two different insert-to-vector ratios. Generally, as the amount of double stranded oligonucleotide in the ligation reaction is increased, the percentage of clones containing multiple copies of the repeat will also increase.

1. Prepare three control and two experimental 10-µl ligation reactions by adding the following components to separate sterile 1.5-ml microcentrifuge tubes:

Ligation reaction components	Control			Experimental	
	1[a]	2[b]	3[c]	4[d]	5[d]
Prepared pLuc-MCS digested plasmid (0.1 µg/µl)	1.0 µl	1.0 µl	0.0 µl	1.0 µl	1.0 µl
Double stranded oligo (1 nmol/µl)	0.0 µl	0.0 µl	1.0 µl	1 µl	3 µl
rATP [10 mM (pH 7.0)]	1.0 µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl
Ligase buffer (10×)[e]	1.0 µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl
T4 DNA ligase (4 U/µl)	0.5 µl	0.0 µl	0.5 µl	0.5 µl	0.5 µl
Double-distilled (ddH_2O) to 10 µl	6.5 µl	7.0 µl	6.5 µl	5.5 µl	3.5 µl

[a] This control tests for the effectiveness of the digestion and the CIAP treatment. Expect a low number of transformants if the digestion and CIAP treatment are effective.

[b] This control indicates whether the plasmid is cleaved completely or whether residual uncut plasmid remains. Expect an absence of transformants if the digestion is complete.

[c] This control verifies that the insert is not contaminated with the original plasmid. Expect an absence of transformants if the insert is pure.

[d] These experimental ligation reactions vary the insert-to-vector ratio. Expect a majority of the transformants to represent recombinants.

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

2. Incubate the reactions for 2 hours at ~22°C or overnight at 4°C. For blunt-end ligation, reduce the rATP to 5 mM and incubate the reactions overnight at 12–14°C.

Transformation

Transform competent bacteria with 1–2 µl of the ligation reaction, and plate the transformed bacteria on LB-ampicillin agar plates. Refer to reference 12 for a transformation protocol. Agilent competent cells with transformation efficiencies $\geq 5 \times 10^9$ cfu/µg are also available separately.

Verifying the Presence of Insert

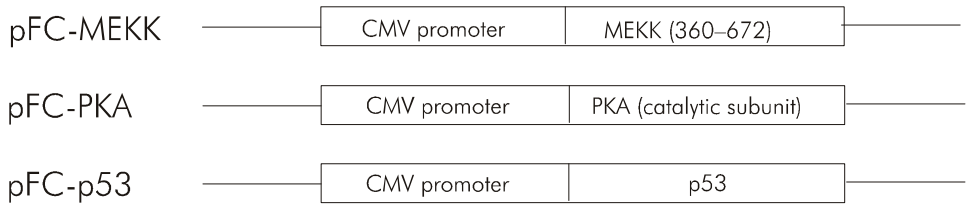
Depending on the ligation efficiency, various numbers of single colonies should be picked from the plate and grown in 1.5 ml LB-ampicillin broth. Prepare plasmid DNA from the liquid culture and sequence with the Luc-B1 primer (GCT CTC CAG CGG TTC CAT C). It is complementary to nucleotides 2837–2855 in pLuc-MCS.

APPENDIX: PLASMID INFORMATION

Antibiotic Resistance of the PathDetect *cis*-Reporting System Plasmids

Plasmid	Prokaryotic Selection	Eukaryotic Selection
***cis*-Reporter Plasmids**		
p53-Luc	ampicillin	none
pAP-1-hrGFP	ampicillin	hygromycin
pAP-1-Luc	ampicillin	none
pC/EBP-Luc	ampicillin	none
pCRE-hrGFP	ampicillin	hygromycin
pCRE-Luc	ampicillin	none
pDR1-Luc	ampicillin	none
pDR3-Luc	ampicillin	none
pDR4-Luc	ampicillin	none
pDR5-Luc	ampicillin	none
pEgr-1-Luc	ampicillin	none
pGAS-Luc	ampicillin	none
pGRE-Luc	ampicillin	none
pISRE-Luc	ampicillin	none
pLILRE-Luc	ampicillin	none
pLuc-MCS	ampicillin	none
pNFAT-hrGFP	ampicillin	hygromycin
pNFAT-Luc	ampicillin	none
pNF-kB-hrGFP	ampicillin	hygromycin
pNF-kB-Luc	ampicillin	none
pSRE-Luc	ampicillin	none
pSRF-Luc	ampicillin	none
pTARE-Luc	ampicillin	none
Control Plasmids		
pFC-MEKK	ampicillin	none
pFC-PKA	ampicillin	none
pFC-p53	ampicillin	none
pCIS-CK	ampicillin	none

Configuration of the PathDetect *cis*-Reporting System Positive Control Plasmids



The *cis*-Reporter Plasmids

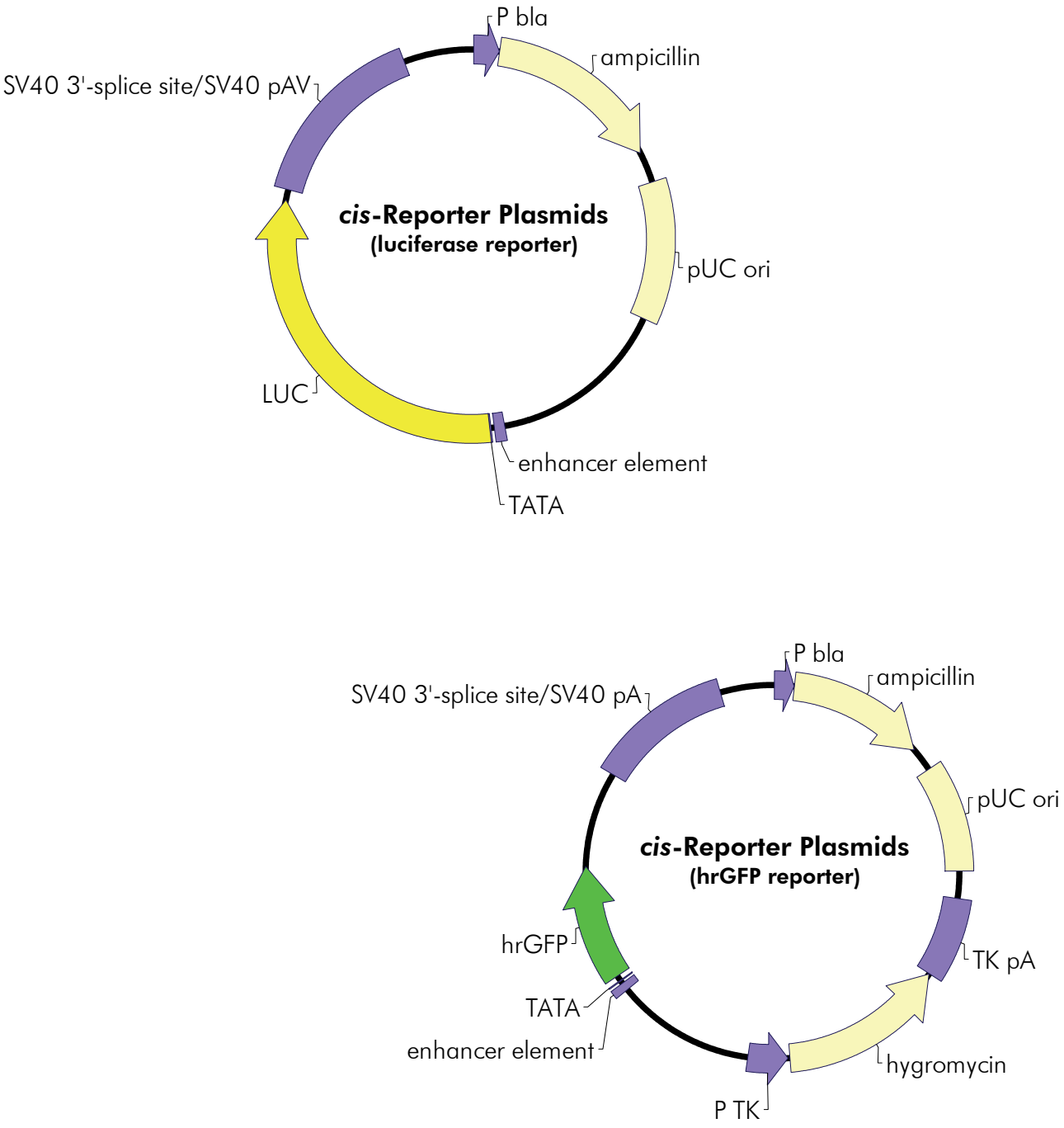
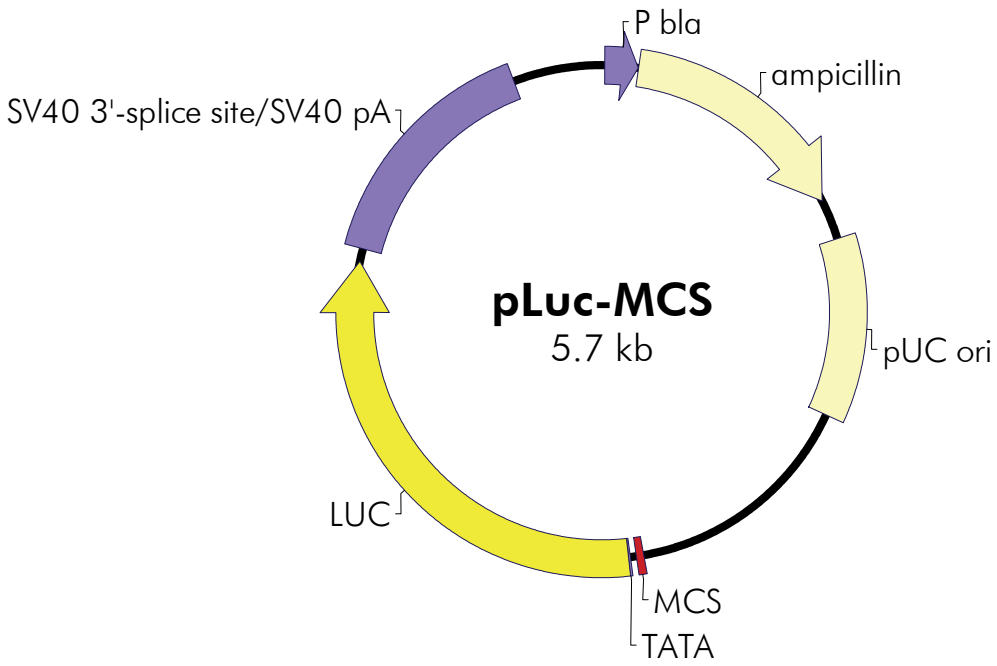


Figure 2 Circular maps of *cis*-reporter plasmids (luciferase and hrGFP reporters).

Configuration and Sequence of the Enhancer Elements of the *cis*-Reporter Plasmids

***cis*-Reporter Plasmid**	**Enhancer element configuration**	**Enhancer element sequence**
p53-Luc	p53 (14×)	(**TGCCTGGACTTGCCTGG**)$_{14}$
pAP-1-Luc	AP-1 (7×)	(**TGACTAA**)$_7$
pAP-1-hrGFP	AP-1 (6×)	(**TGACTAA**)$_6$
pC/EBP-Luc	C/EBP (3×)	(A**TTGCGCAAT**)$_3$
pCRE-Luc	CRE (4×)	(AGCC**TGACGTCA**GAG)$_4$
pCRE-hrGFP		
pDR1-Luc	DR1 (5×)	(**AGGTCA**N)$_5$
pDR3-Luc	DR3 (4×)	(**AGGTCA**NNN)$_4$
pDR4-Luc	DR4 (2×)	(CAGGA**GGTCA**)$_3$
pDR5-Luc	DR5 (5×)	(**AGGTCA**NNNNN)$_5$
pEgr-1-Luc	Egr-1 (3×)	(**GGGGTGGGG**N)$_3$
pGAS-Luc	GAS (4×)	(AGTTTCATA**TTACTCTAA**ATC)$_4$
pGRE-Luc	GRE (4×)	(**GGTACATTTTGTTCT**)$_4$ (the second repeat is in the antisense orientation)
pISRE-Luc	ISRE (5×)	(T**AGTTTCACTTTC**CC)$_5$
pLILRE-Luc	LILRE (4×)	(**TCACTTCCTGAGAG**)$_4$
pNFAT-Luc	NFAT binding site (4×)	(GGA**GGAAAAACTGTTTCA**TACAGAAGGCGT)$_4$
pNFAT-hrGFP		
pNF-κB-Luc	NF-κB (5×)	(T**GGGGACTTTCC**GC)$_5$
pNF-κB-hrGFP		
pSRE-Luc	SRE (5×)	(AGGATGT**CCATATTAGG**ACATCT)$_5$
pSRF-Luc	SRF (5×)	(GT**CCATATTAGG**AC)$_5$
pTARE-Luc	TARE (3×)	(CATTGTCA**GTCTAGAC**ATACTCCGAGAT**TGTGGATT**GA GA)$_3$
pCIS-CK	—	—
pLuc-MCS	—	—

The pLuc-MCS Plasmid



pLuc-MCS Multiple Cloning Site Region
(sequence shown 2682–2737)

Hind III Srf I Sma I/Xma I Bgl II Xho I Sal I

TATA box

```
AAGCTTGCCCGGGCAGATCTCTCGAGGTCGACAGCGGAGACTCTAGAGGGTATATA
```

Feature	Nucleotide Position
bla promoter	13–134
ampicillin resistance (*bla*) ORF	137–994
pUC origin of replication	1145–1810
multiple cloning site	2682–2713
TATA box	2732–2737
Luciferase ORF	2738–4435
SV40 3´-splice site/SV40 polyA signal	4483–5336

Figure 3 Circular map and features of the pLuc-MCS plasmid.

TROUBLESHOOTING

Observation	Suggestion(s)
The background luciferase activity is too low to calculate	Increase the concentration of cell lysate used in the assay.
	Use more reporter plasmid for the transfection.
	Plot and compare the absolute luciferase activity rather than the fold activation.
Results vary among triplicate samples	Avoid variations in pipetting, growth conditions and extraction efficiency of luciferase. Use the same DNA–transfection reagent mixture for all three wells.
	Avoid removing the cells from the wells when washing the cells.
The activity increase of the luciferase over the background is low	Run a positive control to ensure the cell line used is appropriate for the assay and able to be transfected efficiently.
	Optimize the transfection method.
	The results indicate the gene or stimulus of interest is not involved in these signaling pathways.
	Use a cell line such as SAOS-2 with the p53 *cis*-reporting system to avoid possible endogenous background from other cell lines.
All samples exhibit very low or no luciferase activity	Ensure that the cells are fully lysed. Perform the following active lysis. Scrape all surfaces of the tissue culture dish, pipet the cell lysate to microcentrifuge tube and place on ice. Lyse the cells by brief sonication with the microtip set at the lowest setting or freeze the cells at –80°C for 20 minutes and then thaw in a 37°C water bath and vortex 10–15 seconds. Spin the tubes in a microcentrifuge at high speed for 2 minutes. Use the supernatant for the luciferase activity assay.
	Optimize the transfection procedure with a reporter plasmid such as pCMV-βGAL to ensure successful transfection.

PREPARATION OF MEDIA AND REAGENTS

Cell Lysis Buffer (5×)

40 mM tricine (pH 7.8)
50 mM NaCl
2 mM EDTA
1 mM $MgSO_4$
5 mM DTT
1% Triton® X-100

PBS Buffer (1×)

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

Luciferase Assay Reagent (1×)

40.0 mM tricine (pH 7.8)
0.5 mM ATP
10 mM $MgSO_4$
0.5 mM EDTA
10.0 mM DTT
0.5 mM coenzyme A
0.5 mM luciferin

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.*

TE Buffer (1×)

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.