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

Instruction Manual

Catalog #219000 (PathDetect c-Jun *trans*-Reporting System)
#219005 (PathDetect Elk1 *trans*-Reporting System)
#219010 (PathDetect CREB *trans*-Reporting System)
#219015 (PathDetect CHOP *trans*-Reporting System)
#219026 (pFA-ATF2 Plasmid)
#219031 (pFA-cFos Plasmid)
#219036 (pFA-CMV Plasmid)
#219001 (pFR-CAT Plasmid)
#219002 (pFR-βGal Plasmid)
#219004 (pFR-SEAP Plasmid)

Revision C.0

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

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MATERIALS PROVIDED

Catalog #219000 (PathDetect c-Jun *trans*-Reporting System)

Component	Concentration	Quantity
pFR-Luc plasmid (reporter plasmid)	1 mg/ml	50 µg
pFA2-cJun plasmid (fusion *trans*-activator plasmid)	25 ng/µl	5 µg
pFC2-dbd plasmid (negative control)	25 ng/µl	2 µg
pFC-MEKK plasmid (positive control)	25 ng/µl	5 µg

Catalog #219005 (PathDetect Elk1 *trans*-Reporting System)

Component	Concentration	Quantity
pFR-Luc plasmid (reporter plasmid)	1 mg/ml	50 µg
pFA2-Elk1 plasmid (fusion *trans*-activator plasmid)	25 ng/µl	5 µg
pFC2-dbd plasmid (negative control)	25 ng/µl	2 µg
pFC-MEK1 plasmid (positive control)	25 ng/µl	5 µg

Catalog #219010 (PathDetect CREB *trans*-Reporting System)

Component	Concentration	Quantity
pFR-Luc plasmid (reporter plasmid)	1 mg/ml	50 µg
pFA2-CREB plasmid (fusion *trans*-activator plasmid)	25 ng/µl	5 µg
pFC2-dbd plasmid (negative control)	25 ng/µl	2 µg
pFC-PKA plasmid (positive control)	25 ng/µl	5 µg

Catalog #219015 (PathDetect CHOP *trans*-Reporting System)

Component	Concentration	Quantity
pFR-Luc plasmid (reporter plasmid)	1 mg/ml	50 µg
pFA-CHOP plasmid (fusion *trans*-activator plasmid)	25 ng/µl	5 µg
pFC2-dbd plasmid (negative control)	25 ng/µl	2 µg
pFC-MEK3 plasmid (positive control)	25 ng/µl	5 µg

Catalog #219036 (pFA-CMV Plasmid)

Component	Concentration	Quantity
pFA-CMV plasmid (fusion *trans*-activator plasmid)	1 mg/ml	20 µg
XL1-Blue MRF′ cells (host strain)	—	500 µl

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PathDetect *trans*-Reporter Plasmids

Component	Concentration	Quantity	Catalog #
pFR-CAT plasmid	1 mg/ml	50 µg	219001
pFR-βGal plasmid	1 mg/ml	50 µg	219002
pFR-SEAP plasmid	1 mg/ml	50 µg	219004
pFR-Luc plasmid	1 mg/ml	50 µg	219050

PathDetect *trans*-Reporting Activator Plasmids

Component	Type of Plasmid	Concentration	Quantity	Catalog #
pFA2-cJun plasmid	activator plasmid for c-Jun reporting system	1 mg/ml	10 µg	219053
pFA2-Elk1 plasmid	activator plasmid for Elk1 reporting system	1 mg/ml	10 µg	219062
pFA2-CREB plasmid	activator plasmid for CREB reporting system	1 mg/ml	10 µg	219068
pFA-CHOP plasmid	fusion *trans*-activator plasmid for CHOP reporting system	1 mg/ml	10 µg	219054
pFA-ATF2 plasmid	fusion *trans*-activator plasmid	1 mg/ml	10 µg	219026
pFA-cFos plasmid	fusion *trans*-activator plasmid	1 mg/ml	10 µg	219031

PathDetect *trans*-Reporting Control Plasmids

Component	Type of Control	Concentration	Quantity	Catalog #
pFC-MEKK plasmid	positive control plasmid for c-Jun reporting system	1 mg/ml	10 µg	219059
pFC-MEK1 plasmid	positive control plasmid for Elk1 reporting system	1 mg/ml	10 µg	219065
pFC-PKA plasmid	positive control plasmid for CREB reporting system	1 mg/ml	10 µg	219071
pFC2-dbd plasmid	negative control plasmid for all PathDetect *trans*-systems	1 mg/ml	10 µg	219056
pFC-MEK3	positive control plasmid	1 mg/ml	10 µg	219086
pMUT-Elk1	negative control plasmid for pFA2-Elk1	1 mg/ml	10 µg	219040
pMUT-cJun	negative control plasmid for pFA2-cJun	1 mg/ml	50 µg	219041
pMUT-CREB	negative control plasmid for pFA2-CREB	1 mg/ml	50 µg	219042

STORAGE CONDITIONS

All Components: –20°C

ADDITIONAL MATERIALS REQUIRED

Mammalian cells (e.g., HeLa, CHO, CV-1, and NIH3T3)
Cell culture medium [e.g., Dulbecco's minimum essential medium (DMEM)]
DMEM containing 10% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin and streptomycin
Luciferase assay kit or alternate assay for the Alternate Reporter Plasmids
14-ml BD Falcon® polypropylene round-bottom tubes (BD Biosciences Catalog #352059)
Calcium- and magnesium-free PBS
Tissue culture dishes (6-wells)
Transfection reagents
Luminometer
LB-tetracycline agar plates[§]
LB-tetracycline liquid medium[§]
LB-kanamycin agar plates[§]

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

INTRODUCTION

The PathDetect in vivo signal transduction pathway *trans*-reporting systems are designed for specific, rapid, and convenient assessment of the in vivo activation of transcription activators and upstream signal transduction pathways.[1, 2] The systems are useful for identifying whether a newly cloned gene is involved in a signal transduction pathway, and if so, at which step of the pathway. These reporting systems can also be used to study the in vivo effects of growth factors, drug candidates, and extracellular stimuli on PathDetect signal transduction pathways. 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.

Each PathDetect *trans*-reporting system includes a unique fusion *trans*-activator plasmid that expresses a fusion protein (see Figure 2 in *Appendix I*). The fusion protein is a *trans*-acting, pathway-specific transcriptional activator (i.e., a fusion *trans*-activator protein). The fusion *trans*-activator protein consists of the activation domain of either the c-Jun,[3,–6] Elk1,[6–9] CREB,[6, 10] CHOP,[2, 11] ATF2,[12, 13] or c-Fos[12, 13] transcriptional activator fused with the yeast GAL4 DNA binding domain (DBD),[14, 15] residues 1–147. The transcriptional activators c-Jun, Elk1, CREB, and CHOP are phosphorylated and activated by c-Jun N-terminal kinase (JNK), mitogen-activated protein kinase (MAPK), cyclic AMP-dependent kinase (PKA), or p38 MAP kinase, respectively, and their activity reflects the in vivo activation of these kinases and the corresponding signal transduction pathways. The signal transduction pathways leading to the phosphorylation of the transcription activators ATF2 and c-Fos are not characterized.

The fusion *trans*-activator plasmid contains the human cytomegalovirus (CMV) immediate early promoter to drive the constitutive expression of the *trans*-activator protein in a wide variety of eukaryotic cell lines. Selection in bacteria is made possible by the kanamycin-resistance gene, which is under control of the prokaryotic β-lactamase promoter. The neomycin-resistance gene, driven by the SV40 early promoter, provides stable selection with G418 in mammalian cells.

The pFA-CMV *trans*-Activator Plasmid

The pFA-CMV plasmid is a mammalian expression vector that is designed to allow the study of any transcription activator and signal transduction pathway of interest. The pFA-CMV plasmid is designed for convenient insertion of the activation domain sequence of any transcription activator. The fusion *trans*-activator protein expressed by the pFA-CMV plasmid consists of the activation domain of the transcription activator of interest fused with the DNA binding domain of the yeast GAL4.

The pFR-Luc Reporter Plasmid

The pFR-Luc reporter plasmid contains a synthetic promoter with five tandem repeats of the yeast GAL4 binding sites that control expression of the *Photinus pyralis* (American firefly) luciferase gene (see Figure 3 in *Appendix I*). The DNA binding domain of the fusion *trans*-activator protein binds to the reporter plasmid at the GAL4 binding sites. When a fusion *trans*-activator plasmid, reporter plasmid, and an uncharacterized gene are cotransfected into mammalian cells, direct or indirect phosphorylation of the transcription activation domain of the fusion *trans*-activator protein by the uncharacterized gene product will activate transcription of the luciferase gene from the reporter plasmid (Figure 1). Expression (or activity) levels of luciferase reflect the activation status of the signaling events.

Alternate Reporter Proteins

We have constructed a series of plasmids that enable the use of the PathDetect system with reporter proteins other than luciferase. These alternate reporter proteins may offer advantages for certain laboratories or experiments. These reporter proteins include chloramphenicol acetyltransferase (CAT), β-galactosidase, and the secreted alkaline phosphatase (SEAP). The plasmids have the same backbone as the pFR-Luc plasmid and have been validated to function with the PathDetect reporting systems (see Figure 3 in *Appendix I*). For protocols outlining the use of these alternate reporter proteins see *Alternate Reporter Plasmids.*

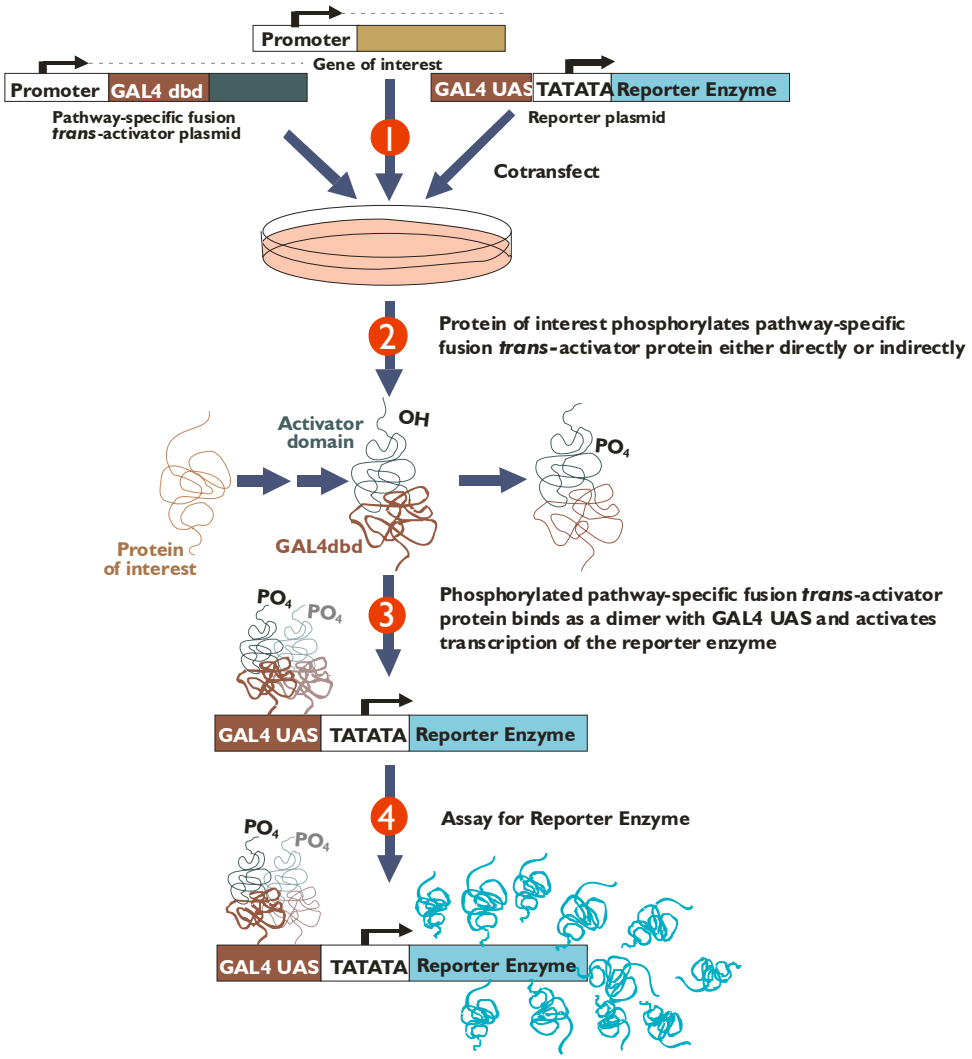


Figure 1 The PathDetect in vivo signal transduction pathway *trans*-reporting system.

PREPROTOCOL CONSIDERATIONS

Choosing a Cell Line

The PathDetect signal transduction pathway *trans*-reporting systems may be used for various mammalian cell lines, provided the cells contain the protein kinases that activate the fusion *trans*-activator protein. Cell lines vary in signaling proteins and other properties. The endogenous protein kinases and transcription activator activities in the cell line used will determine the background and hence the sensitivity of the assay. Although these systems have been found to work in various cell lines including HeLa, CHO, CV-1, and NIH3T3, a given reporting system might work better in one cell line under defined conditions. Parameters for use of the PathDetect system must be determined experimentally for each cell type.

Choosing a Transfection Method

As with all transfection assays, the sensitivity of an assay using a PathDetect signal transduction pathway reporting system is greatly 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. Because the luciferase assay is very sensitive, various transfection methods, such as calcium phosphate precipitation and lipid-mediated transfection, may be used.

Tissue Cultureware

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.

DESIGNING THE EXPERIMENT

Studying the Effects of a Gene Product

The c-Jun, Elk1, CREB, and CHOP reporting systems include positive controls that are known upstream activators and are intended to ensure the quality of the components and to test if the reporting system works in the specific cell line chosen. The signal transduction pathways leading to the phosphorylation of the transcription activators ATF2 and c-Fos are not characterized; therefore, providing positive controls is not possible. The experimental plasmid without the gene of interest that may lead to activation of the fusion *trans*-activator protein should be used as a negative control to ensure that the effect observed is not caused by the introduction of viral promoters (e.g., CMV, RSV, or SV40) or other proteins expressed from the plasmid. Depending on the purpose of the experiment, other controls such as a nonactivatable mutant of the fusion *trans*-activator protein might be required. Typical initial experiments for using the PathDetect *trans*-reporting system to study the effects of a gene product are outlined in Table I. As all assays are to be run in triplicate, eight samples will utilize one 24-well tissue culture dish. Amounts listed in Table I below are for a 6-well tissue culture dish. Sample numbers are indicated in Column A.

TABLE I

Sample Experiment to Study the Effects of a Gene Product

A	B	C	D	E	F	G	H
#	Reporter plasmid	Fusion *trans*-activator plasmid[a]	pFC2-dbd (negative control for pFA plasmid)	Positive control	Experimental plasmid with gene of interest	Experimental plasmid without insert	Carrier Plasmid DNA
1[b]	1.0 µg (1 µl)	50 ng (2 µl)	—	—	—	50 ng	950 ng
2[c]	1.0 µg (1 µl)	50 ng (2 µl)	—	—	—	100 ng	900 ng
3[d]	1.0 µg (1 µl)	50 ng (2 µl)	—	—	—	1000 ng	—
4[e]	1.0 µg (1 µl)	50 ng (2 µl)	—	—	50 ng	—	950 ng
5[f]	1.0 µg (1 µl)	50 ng (2 µl)	—	—	100 ng	—	900 ng
6[g]	1.0 µg (1 µl)	50 ng (2 µl)	—	—	1000 ng	—	—
7[h]	1.0 µg (1 µl)	50 ng (2 µl)	—	50 ng (2 µl)	—	—	950 ng
8[i]	1.0 µg (1 µl)	—	50 ng (2 µl)	—	100 ng	—	900 ng

[a] This quantity may need to be optimized, usually within the range of 1–100 ng.

[b] Sample 1 lacks the gene of interest and, therefore, controls for sample 4.

[c] Sample 2 lacks the gene of interest and, therefore, controls for sample 5.

[d] Sample 3 lacks the gene of interest and, therefore, controls for sample 6.

[e] Sample 4 measures the effect of the gene product on the signal transduction pathway involved.

[f] Sample 5 measures the effect of the gene product on the signal transduction pathway involved.

[g] Sample 6 measures the effect of the gene product on the signal transduction pathway involved.

[h] Sample 7 measures the efficacy of the assay for the cell line chosen.

[i] Sample 8 does not contain an activation domain and should show results similar to samples 1–3.

Studying the Effects of Extracellular Stimuli

The PathDetect reporting systems may also be used to study the effects of extracellular stimuli, such as growth factors or drug candidates, on corresponding signal transduction pathways (see Table II). Cells are cotransfected with the fusion *trans*-activator plasmid and reporter plasmid and then treated with the stimulus of interest. Luciferase expression from the reporter plasmid indicates the activation of the fusion *trans*-activator protein and, therefore, the presence of the endogenous protein kinase (e.g., MAPK, JNK, PKA, p38 kinase or an uncharacterized upstream activator). Typical initial experiments for using the PathDetect *trans*-reporting system to study the effects of extracellular stimuli are outlined in Table II. As all assays are to be run in triplicate, eight samples will utilize one 24-well tissue culture dish. Amounts listed in Table II below are for a 6-well tissue culture dish.

TABLE II

Sample Experiment to Study the Effects of Extracellular Stimuli

#	Reporter plasmid	Fusion *trans*-activator plasmid[a]	pFC2-dbd (negative control)	Positive control	Extracellular stimuli
1[b]	1.0 µg (1 µl)	—	50 ng (2 µl)	—	Serum (10%)
2[c]	1.0 µg (1 µl)	50 ng (2 µl)	—	—	Serum (10%)
3[d]	1.0 µg (1 µl)	—	50 ng (2 µl)	—	EGF (100 ng/ml)
4[e]	1.0 µg (1 µl)	50 ng (2 µl)	—	—	EGF (100 ng/ml)
5[f]	1.0 µg (1 µl)	—	50 ng (2 µl)	—	Medium
6[g]	1.0 µg (1 µl)	50 ng (2 µl)	—	—	Medium
7[h]	1.0 µg (1 µl)	50 ng (2 µl)	—	50 ng (2 µl)	—
8[i]	1.0 µg (1 µl)	—	50 ng (2 µl)	—	—

[a] This quantity may need to be optimized, usually within the range of 1–100 ng.

[b] Sample 1 lacks the fusion *trans*-activator protein and, therefore, controls for sample 2.

[c] Sample 2 measures the effect of fetal bovine serum on kinase activation.

[d] Sample 3 lacks the fusion *trans*-activator protein and, therefore, controls for sample 4.

[e] Sample 4 measures the effect of EGF on kinase activation.

[f] Sample 5 controls for the extracellular stimulus and the fusion *trans*-activator protein and therefore controls for sample 6.

[g] Sample 6 controls for the extracellular stimulus.

[h] Sample 7 measures the efficacy of the assay for the cell line chosen.

[i] Sample 8 does not contain an activation domain and should show results similar to samples 1–5.

CLONING PROTOCOL FOR THE pFA-CMV PLASMID

The pFA-CMV plasmid is designed for the convenient insertion of the activation domain sequence of any transcription activator to express a fusion *trans*-activator protein consisting of the transcription activator activation domain of interest and the DNA binding domain of the yeast GAL4 protein. This plasmid features a multiple cloning site (MCS) with 10 unique, conveniently arranged cloning sites for insertion of the DNA sequence of a transcription activator activation domain of interest. Expression of the fusion *trans*-activator protein is driven by the human cytomegalovirus (CMV) promoter, a strong promoter that allows high-level constitutive expression in a variety of mammalian cell lines. The plasmid has a neomycin-resistance gene for selection of stable cell lines that express the fusion *trans*-activator protein of interest. See *Appendix I: Plasmid Information* for a map of the pFA-CMV plasmid.

Host Strain and Genotype

XL1-Blue MRF´ Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F´ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^{r})]

For the appropriate medium, please refer to the following table:

Bacterial strain	Agar plate for bacterial streak	Medium for bacterial glycerol stock
XL1-Blue MRF´	LB–tetracycline[a,b]	LB–tetracycline[a,b]

[a] 12.5 µg/ml.

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

On arrival, prepare the following from the glycerol stock:

Note *Upon receipt, store the host strains immediately at–80°C. It is also best to avoid repeated thawing of the host strain in order to maintain extended viability.*

1. Revive the stored cells by scraping off splinters of solid ice with a sterile wire loop.

2. Streak the splinters onto an LB-tetracycline agar plate.

3. Restreak the cells fresh each week.

Preparation of a –80°C Bacterial Glycerol Stock

1. In a sterile 50-ml conical tube, inoculate 10 ml of the LB-tetracycline liquid medium with one or two colonies from the plate. Grow the cells to late log phase.

2. Add 4.5 ml of a sterile glycerol–liquid medium solution (5 ml of glycerol + 5 ml of liquid medium) to the bacterial culture from step 1. Mix well.

3. Aliquot into sterile centrifuge tubes (1 ml/tube).

This preparation may be stored at –80°C for more than 2 years.

Preparing the pFA-CMV Plasmid

- The gene of interest can be inserted in any of the 10 restriction sites in the MCS (see Figure 4 in *Appendix I*); however, ensure that the proper coding sequence of the insert is in the same reading frame as the sequence of the GAL4 DNA binding domain gene.

- Dephosphorylate the digested pFA-CMV plasmid with CIAP prior to ligation with 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 removing the desired plasmid band through electroelution, leaving behind the small fragment that appears between the two restriction enzyme sites.

- 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).

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 ratio is calculated using the following equation:

$$X\ \mu g \text{ of insert} = \frac{(\text{Number of base pairs of insert})\,(0.1\ \mu g \text{ of pFA - CMV vector})}{4576 \text{ bp of pFA - CMV vector}}$$

where *X* is the quantity of insert (in micrograms) required for a 1:1 insert-to-vector molar ratio. Multiply *X* by 2 to get the quantity of insert required for a 2:1 ratio.

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

	Control			Experimental	
Ligation reaction components	**1**[a]	**2**[b]	**3**[c]	**4**[d]	**5**[d]
Prepared pFA-CMV plasmid (0.1 µg/µl)	1.0 µl	1.0 µl	0.0 µl	1.0 µl	1.0 µl
Prepared insert (0.1 µg/µl)	0.0 µl	0.0 µl	1.0 µl	*Y* µl	*Y* 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	*Z* µl	*Z* µl

[a] This control tests for the effectiveness of the digestion and the CIAP treatment. Expect a low number of transformant colonies 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 transformant colonies if the digestion is complete.

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

[d] These experimental ligation reactions vary the insert-to-vector ratio. Expect a majority of the transformant colonies 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-kanamycin agar plates. Refer to reference 16 for a transformation protocol.

Note *The XL1-Blue MRF´ cells supplied with the pFA-CMV plasmid are not competent cells. Refer to Hanahan (1983) for a protocol for producing competent cells.*

Verifying the Presence and Reading Frame of the Insert

Individual colonies can be examined to determine the plasmids with inserts and the insert size directly by PCR (see *Appendix II: Polymerase Chain Reaction Amplification of DNA from Individual Colonies*) or by restriction analysis. The junction between the GAL4 DNA binding domain and the activation domain of the transcription activator should be sequenced to confirm that the two domains are in the same reading frame. **The expected size of the PCR product is 208 bp plus the size of the insert**. Additional information can be obtained by further restriction analysis of the PCR products.

Sequences of Primers for Use in PCR Amplification and Sequencing

Plasmid	Primer	Binds to nucleotide (nt)	Nucleotide sequence (5´ to 3´)
pFA-CMV plasmid	Forward	1012–1030	AGCATAGAATAAGTGCGAC
	Reverse	1220–1200	GACTGTGAATCTAAAATACAC

CELL CULTURE AND TRANSFECTION

Note *The DNA used for transfections must be of high purity (i.e., double cesium chloride banded). Ensure that the plasmid DNA has an $OD_{260/280}$ of ~1.8–2.0 and is endotoxin free. The plasmids supplied with this kit are of high quality and are ready for 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 cell lines.*

1. Thaw and seed frozen cell stocks in growth medium (see *Preparation of Media and Reagents*) in 50-ml or 250-ml tissue culture flasks.

2. Split the cells once confluence is reached.

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

Preparing the Cells

1. Seed 3×10^5 cells in 2 ml of complete medium in each well of a 6-well tissue culture dish.

2. Incubate the cells at 37°C in a 5% CO_2 incubator for 24 hours.

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.

1. **If studying the effects of a gene product,** perform step 1a. **If studying the effects of extracellular stimuli**, perform step 1b.

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

        Note *Due to the possible induction of pathways by unknown serum-borne factors, low serum concentrations are used; however, the use of 10% serum has also yielded satisfactory results in some cases.*

    b. Replace the medium with fresh medium 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 *Luciferase Activity Assay.*

LUCIFERASE ACTIVITY ASSAY

Preparing the Cell Lysates

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

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

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. Store at –80°C. **Each freeze–thaw cycle results in a significant loss of luciferase activity (as much as 50%).**

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

Performing the Luciferase Activity Assay

1. Mix 5–20 µl of cell extract equilibrated to room temperature with 100 µl of room temperature luciferase assay reagent[§] in a 14-ml BD Falcon round-bottom polypropylene 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.

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

ALTERNATE REPORTER PLASMIDS

Firefly luciferase assays offer many advantages over other reporter enzyme assays. These advantages include high sensitivity, broad linear range (4 orders of magnitude), minimal endogenous luciferase activity, relative economy, and nonradioactivity. Other reporter enzymes, however, may offer advantages for certain laboratories or experiments. For example, chloramphenicol acetyltransferase (CAT) may be preferred by laboratories that routinely perform CAT assays. β-Galactosidase or secreted alkaline phosphatase (SEAP), on the other hand, will be better choices for a laboratory that does not have access to a luminometer. For these reasons, we offer a series of plasmids: pFR-CAT, pFR-βGal, and pFR-SEAP, that encode CAT, β-galactosidase, or SEAP, respectively. These reporter plasmids express the reporter enzyme under the control of GAL4 binding element as does the pFR-Luc plasmid. The plasmids have the same basic structure as the pFR-Luc plasmid and have been validated to function with the PathDetect reporting systems. The following sections outline how these reporter plasmids may be used with the PathDetect systems and the assay that was used to validate them. (Please refer to reference 17 for an overview and alternative assay protocols of various reporter enzymes.)

Using the pFR-CAT Plasmid with PathDetect Systems

Background

CAT catalyzes the transfer of acetyl groups from acetyl-coenzyme A to chloramphenicol. The level of background CAT activity in mammalian cells is minimal, as it is a prokaryotic enzyme. The CAT protein is very stable, with a half-life in mammalian cells of ~50 hour as compared with the ~5 hour half-life of luciferase. Various formats for CAT activity assay are available, including isotopic and nonisotopic scintillation and thin-layer chromatography (see reference 17). The following protocol employs a liquid scintillation counting (LSC) method using ^{14}C chloramphenicol.

Materials Required

pFR-CAT plasmid and other PathDetect system plasmids
^{14}C Chloramphenicol 25 µCi (DuPont Co./New England Nuclear, Catalog #NEC408A)
n-Butyryl Coenzyme A (Sigma Catalog #B1508)
Xylenes (Aldrich Chemical Company, Inc. Catalog #24-764-2)
Scintillation cocktail
PBS or TBS buffer (see *Preparation of Media and Reagents*)

Protocol

1. Perform transfections with the pFR-CAT plasmid as described for the pFR-Luc plasmid in *Cell Culture and Transfection*. Replace the pFR-Luc plasmid with the same amount of pFR-CAT plasmid.

2. Once the cells are ready to be harvested, rinse twice with PBS or TBS.

3. Scrape the cells into a small volume of wash buffer (1.0 ml of PBS or TBS). Pellet the cells in a microcentrifuge at 10,000 × *g* for 1 minute at 4°C.

4. Discard the supernatant. Resuspend the cell pellet in 100 µl of ice-cold 0.25 M Tris-HCl, pH 7.5.

5. Freeze the cells for 5 minutes in dry ice–ethanol. Transfer to a 37°C water bath and thaw for 5 minutes. Repeat this freeze–thaw process twice (for a total of three freeze/thaw cycles).

6. Chill cell lysates on ice, microcentrifuge at maximum speed for 5 minutes at 4°C. Remove and save the supernatant for CAT assay.

7. Prepare the following reaction mixture in a 1.5-ml microcentrifuge tube:

Cell lysate	50.5 µl
^{14}C-chloramphenicol (0.05 mCi/ml)	1.5 µl
n-Butyryl Coenzyme A	2.5 µl
Distilled water	8.0 µl
Final volume	62.5 µl

8. Incubate the reaction mixture at 37°C for 3–5 hours.

9. Briefly spin the tubes in a microcentrifuge.

10. For LSC assays, terminate the reactions by adding 150 µl of mixed xylenes to each tube. Vortex for 30 seconds. Spin at maximum speed in a microcentrifuge for 3 minutes. Transfer the entire volume of the upper phase to a new tube. Add 100 µl of fresh 0.25 M Tris-HCl, pH 8.0. Vortex for 30 seconds. Spin for 3 minutes at maximum speed. Carefully remove the upper xylene phase and transfer to a scintillation vial. Add an appropriate amount of scintillation fluid and count the samples in a liquid scintillation counter.

Using the pFR-βGal Plasmid with PathDetect Systems

Background

The β-galactosidase enzyme catalyzes hydrolysis of β–galactosides including lactose and the galactoside analog o-nitrophenyl-β–D-galacto-pyranoside (ONPG). The β–galactosidase gene functions well as a reporter gene because its protein product is extremely stable, resistant to proteolytic degradation in cellular lysates, and enzymatic activity is easily assayed. Several formats are available to assay the activity of β–galactosidase, including colorimetric, fluorescent, and chemiluminescent methods. β–Galactosidase activity can be assayed using a spectrophotometer or microplate reader. The following protocol uses ONPG as the enzymatic substrate and is the most convenient and inexpensive method available.

Materials Required

pFR-βGal plasmid and other PathDetect system plasmids

Protocol

1. Perform transfections and cell treatments with the pFR-βGal plasmid as described for the pFR-Luc plasmid in *Cell Culture and Transfection*. Replace pFR-Luc plasmid with the same amount of pFR-βGal plasmid.

2. Once the cells are ready to be harvested, rinse the cells twice gently with PBS. Be careful not to dislodge any of the cells. Remove as much PBS as possible from the wells.

3. Add 100 µl of lysis buffer to the wells, and gently scrape down the cells or pipet cells off the plate. Transfer the suspension into a 1.5-ml microcentrifuge tube.

4. Spin the tubes at 12,000 × *g* for 5 minutes in a microcentrifuge to pellet the cellular debris. Transfer the supernatant to a fresh microcentrifuge tube for the β–galactosidase assay.

5. Add 35 µl of cell lysate to each well of a 96-well microtiter dish.

6. For each well, prepare 160 µl of buffer A/β–mercaptoethanol mixture.[§]

7. Add 125 µl of buffer A/β–mercaptoethanol mixture to each well for a final volume of 160 µl. Incubate the microtiter dish for 5 minutes at 37°C.

8. Add 50 µl of ONPG substrate[§] to each well and cover the dish. Incubate the dish at 37°C until mixture turns bright yellow.

9. Terminate the reaction by adding 90 µl of stop solution[§] to each well and scan the dish in a microtiter dish reader set at 420 nm.

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

Using the pFR-SEAP Plasmid with PathDetect Systems

Human placental secreted alkaline phosphatase (SEAP) is released from producer cells following synthesis. This feature allows the activity of SEAP to be continuously monitored without disturbing the cells. This also reduces background alkaline phosphatase activity and facilitates the automation of the sampling and assay processes. There are several commercially available systems that may be employed to assay SEAP activity.

Materials Required

pFR-SEAP plasmid and other PathDetect system plasmids

Protocol

1. Perform transfections and cell treatments with the pFR-SEAP plasmid as described in *Cell Culture and Transfection*. Replace the pFR-Luc plasmid with the same amount of pFR-SEAP plasmid.

2. Follow the manufacturer's recommendations for assessment of SEAP activity.

TROUBLESHOOTING

Observation	Suggestions
The background luciferase activity is too low to calculate	The mammalian transcription activators are binding to the GAL4 UAS inefficiently causing the expression of the luciferase gene to be low. Increase the concentration of cell lysate used in the assay
	Use more pFR-Luc plasmid for transfection
	Plot and compare the absolute luciferase activity rather than the activation fold increase
Results vary among triplicate samples	Variations are occurring in pipetting, growth conditions, extraction efficiency of luciferase, etc.. Use the same DNA–transfection reagent mixture for the three wells
	Take care when washing the cells to avoid removing the cells from the wells
The activity increase of the luciferase over the background is low	The results indicate the gene or stimulus of interest is not involved in these signaling pathways
	The fusion *trans*-activator protein is not expressed. Confirm expression of the fusion *trans*-activator protein by running a western blot of the cell lysate (the GAL4-dbd protein is ~18 kDa)
	Excess pFA plasmid is used. Use only 10–50 ng of the pFA plasmid in the experiment
	The cell line used has low MAPK, JNK, PKA, or p38 kinase activity. Run a positive control to ensure the cell line used is appropriate for the assay
All samples exhibit very low or no luciferase activity	Passive cell lysis is not effective. 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
	Transfection is not successful. Optimize the transfection procedure with a reporter plasmid such as pCMV-βGAL

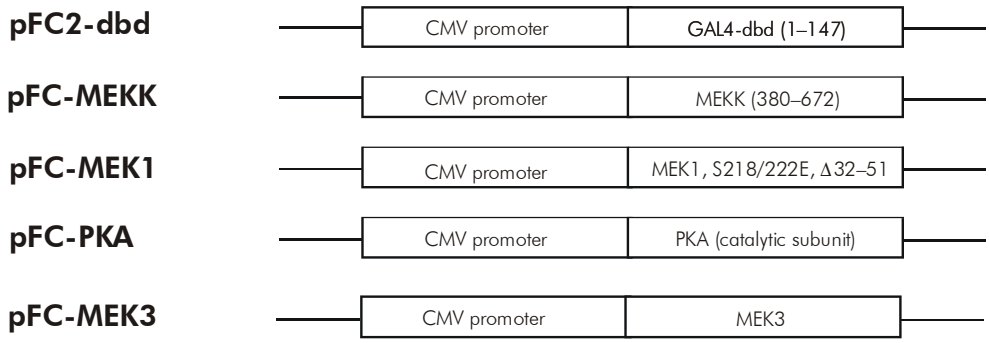
APPENDIX I: PLASMID INFORMATION

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

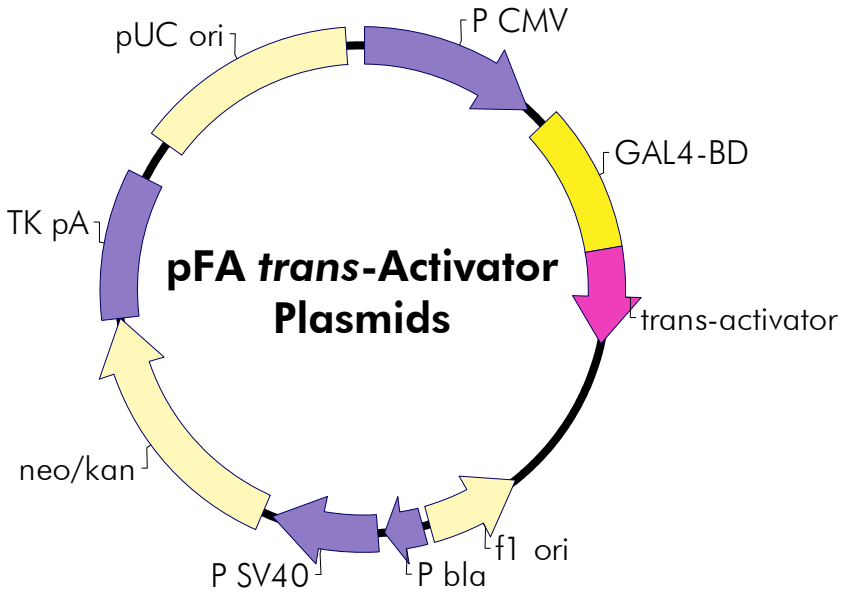
Plasmid	Prokaryotic selection	Eukaryotic selection
pFR *trans*-reporter plasmids		
pFR-Luc plasmid	Ampicillin	None
pFR-CAT plasmid	Ampicillin	None
pFR-βGal plasmid	Ampicillin	None
pFR-SEAP plasmid	Ampicillin	None
Control plasmids		
pFC-MEKK control plasmid	Ampicillin	None
pFC-MEK1 control plasmid	Ampicillin	None
pFC-PKA control plasmid	Ampicillin	None
pFC-MEK3 control plasmid	Ampicillin	None
pFC2-dbd control plasmid	Kanamycin	G418
pFA *trans*-activator plasmids		
pFA2-cJun plasmid	Kanamycin	G418
pFA2-Elk1 plasmid	Kanamycin	G418
pFA2-CREB plasmid	Kanamycin	G418
pFA-CHOP	Kanamycin	G418
pFA-ATF2 plasmid	Kanamycin	G418
pFA-cFos plasmid	Kanamycin	G418
pFA-CMV plasmid	Kanamycin	G418

Configuration of the PathDetect *trans*-Reporting System Control Plasmids

Control Plasmids



The pFA *trans*-Activator Plasmids



Fusion *trans*-Activator Plasmids

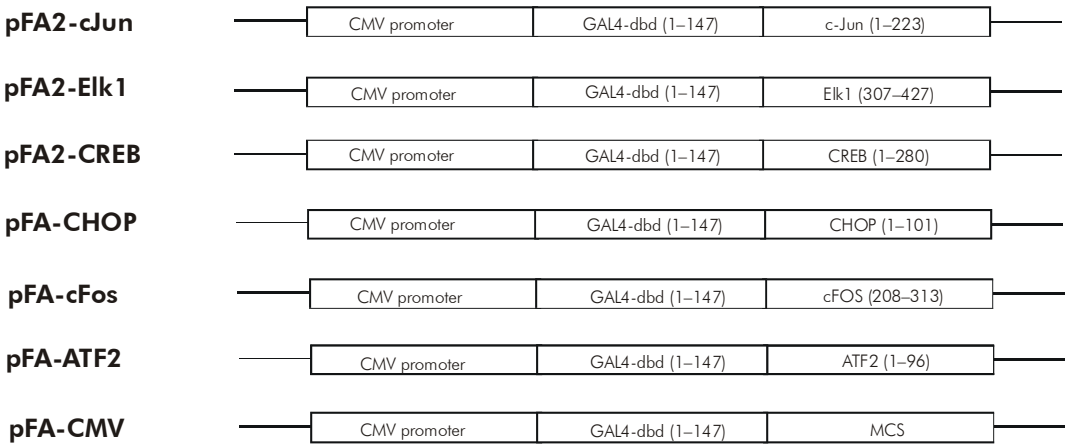
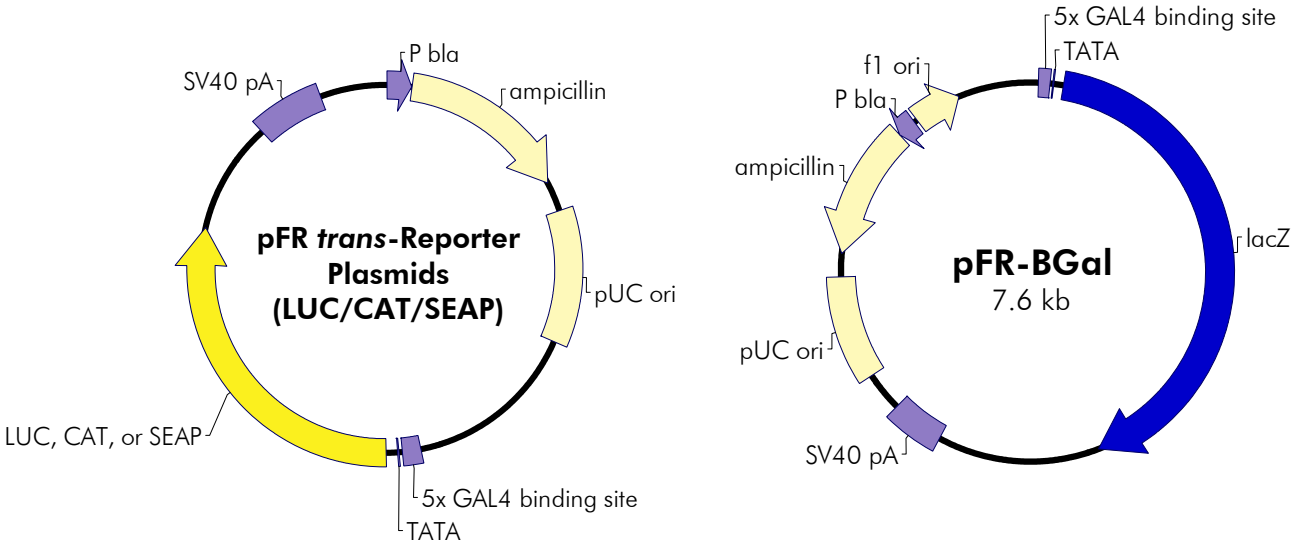


Figure 2 Circular map and configuration of the pFA *trans*-activator plasmids.

The pFR *trans*-Reporter Plasmids



PathDetect *trans*-Reporter Plasmids

Sequence of GAL4 Binding Element in the Reporter Plasmids

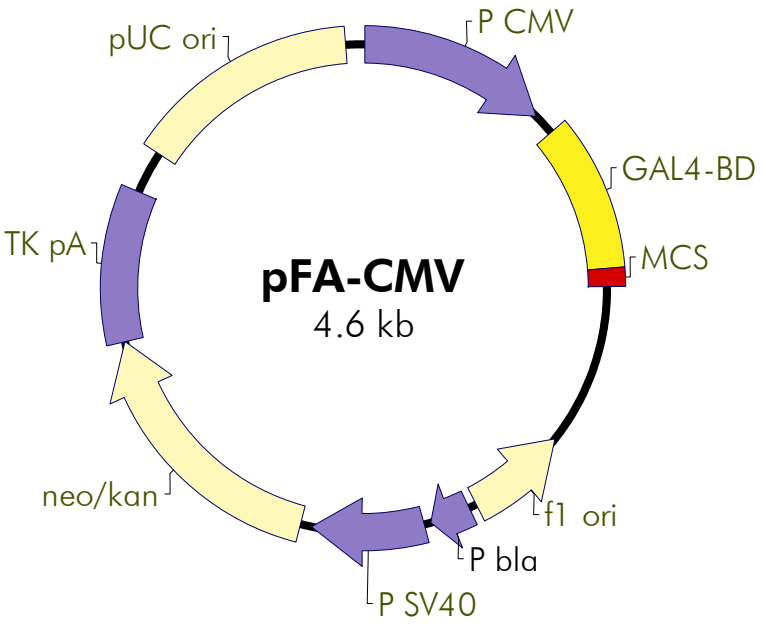
```
AT CTTATCATGTCTGGATC CA AGCTTGCATGCCTGCAG
GT CGGAGTACTGTCCTCCG AG CGGAGTACTGTCCTCCG
AG CGGAGTACTGTCCTCCG AG CGGAGTACTGTCCTCCG
AG CGGAGTACTGTCCTCCG AG CGGAGACTCTAGAGGG
TATAATGGATCCCCGGGT AC CGAGCTCGAATTC...

...CAGCTTGGCATTCCGGTACTGTTGGTAAAATG
```

5× GAL4 binding element

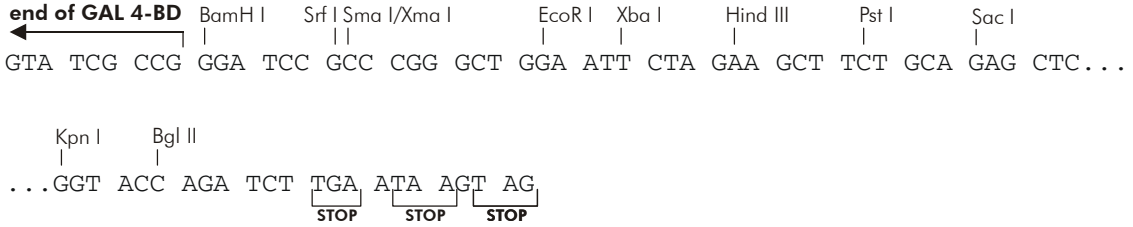
Reporter

Figure 3 Circular maps and configuration of the pFR *trans*-reporting plasmids.

The pFA-CMV Plasmid



pFA-CMV Multiple Cloning Site Region
(sequence shown 1077–1150)



Feature	Nucleotide Position
CMV promoter	7–574
GAL4 DNA-binding domain	642–1085
mutiple cloning site	1086–1139
f1 origin of ss-DNA replication	1630–1936
bla promoter	1961–2082
SV40 promoter	2105–2443
neomycin/kanamycin resistance ORF	2478–3272
HSV-thymidine kinase (TK) polyA signal	3273–3728
pUC origin of replication	3857–4524

Figure 4 Circular map, MCS, and list of features for the pFA-CMV plasmid.

APPENDIX II: POLYMERASE CHAIN REACTION AMPLIFICATION OF DNA FROM INDIVIDUAL COLONIES

The presence and size of a DNA insert in the pFA-CMV plasmid can be determined by PCR amplification of DNA from individual colonies. The sequences of the forward and reverse primers provided below are appropriate for use in PCR amplification and sequencing.

1. Prepare a PCR amplification reaction containing the following components:

   4.0 µl of 10× *Taq* DNA polymerase buffer‡, (see *Preparation of Media and Reagents*)
   0.4 µl of dNTP mix (25 mM each dNTP)
   40.0 ng of forward primer
   40.0 ng of reverse primer
   0.4 µl of 10% (v/v) Tween® 20
   1.0 U of *Taq* DNA polymerase
   dH_2O to a final volume of 40 µl

Plasmid	Primer	Binds to nucleotide (nt)	Nucleotide sequence (5′ to 3′)
pFA-CMV plasmid	Forward	1012–1030	AGCATAGAATAAGTGCGAC
	Reverse	1220–1200	GACTGTGAATCTAAAATACAC

2. Stab the transformed colonies with a sterile toothpick and swirl the colony into reaction tubes. Immediately following inoculation into the reaction mixture, remove the toothpick and streak cells onto antibiotic-containing patch plates for future reference.

3. Gently mix each reaction.

4. Overlay each reaction with 30 µl of mineral oil.

5. Perform PCR using the following cycling parameters:

Number of cycles	Temperature	Length of time
1 cycle	94°C	4 minutes
	50°C	2 minutes
	72°C	2 minutes
30 cycles	94°C	1 minute
	56°C	2 minutes
	72°C	1 minute
1 cycle	72°C	5 minutes

6. Analyze the PCR products for the sizes of the gene inserted into the expression construct using standard 1% (w/v) agarose gel electrophoresis. Because the forward and reverse PCR/sequencing primers are located on both sides of the MCS, **the expected size of the PCR product is 208 bp plus the size of the insert**. Additional information can be obtained by further restriction analysis of the PCR products.

PREPARATION OF MEDIA AND REAGENTS

Buffer A/β-Mercaptoethanol Mixture

Buffer A (pH 7.5)
100 mM NaH_2PO_4
10 mM KCl
1 mM $MgSO_4$
50 mM β-mercaptoethanol

Prepare fresh before each assay

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

Growth Medium

500 ml of Dulbecco's Modified Eagle Medium (DMEM) (high glucose, without L-glutamine, without sodium pyruvate)
5 ml of 200 mM L-glutamine
5 ml of penicillin (5000 U/ml)–streptomycin (5000 µg/ml) mixture
50 ml of fetal bovine serum, heat inactivated

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
Add deionized H_2O to a final volume of 1 liter
Adjust to pH 7.0 with 5 N NaOH
Autoclave

LB–Kanamycin Agar (per Liter)

Prepare 1 liter of LB agar
Autoclave
Cool to 55°C
Add 50 mg of filter-sterilized kanamycin
Pour into petri dishes (~25 ml/100-mm plate)

LB–Tetracycline Agar (per Liter)

Prepare 1 liter of LB agar
Autoclave
Cool to 55°C
Add 12.5 mg of filter-sterilized tetracycline
Pour into petri dishes (~25 ml/100-mm plate)
Store plates in a dark, cool place or cover plates with foil if left out at room temperature for extended time periods as tetracycline is light-sensitive

LB–Tetracycline Broth (per Liter)

Prepare 1 liter of LB broth
Autoclave
Cool to 55°C
Add 12.5 mg of filter-sterilized tetracycline
Store broth in a dark, cool place as tetracycline is light-sensitive

Ligase Buffer (10×) 500 mM Tris-HCl (pH 7.5) 70 mM $MgCl_2$ 10 mM dithiothreitol (DTT) ***Note*** *rATP is added separately in the ligation reaction*	**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
o-Nitrophenyl-β-D-Galactopyranoside (ONPG) Substrate 4 mg/ml ONPG in 100 mM NaH_2PO_4 Adjust the pH to 7.5	**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
Stop Solution 1M Na_2CO_3	
TE Buffer 10 mM Tris-HCl (pH 7.5) 1 mM EDTA	**TBS Buffer** 20 mM Tris-HCl (pH 7.5) 150 mM NaCl

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ENDNOTES

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