PathDetect Reporter Cell Lines

INSTRUCTION MANUAL

Catalog #800050 (PathDetect HLR Cell Line)
#800055 (HLR-Elk1 Cell Line)
#800060 (HLR-CHOP Cell Line)
#800065 (HLR-CREB Cell Line)

Revision A
BN #800050-12

For In Vitro Use Only

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MATERIALS PROVIDED

Catalog #800050	Component	Concentration	Quantity
	HLR Cell Line	—	1×10^6 viable cells
	pFA2-CREB fusion *trans*-activator plasmid	25 ng/µl	5 µg
	pFC-PKA plasmid (positive control)	25 ng/µl	5 µg
	pFC-CMV negative control plasmid	25 ng/µl	5 µg
Catalog #800055	**Component**	**Concentration**	**Quantity**
	HLR-Elk1 *trans*-Reporting Cell Line	—	1×10^6 viable cells
	pFC-CMV negative control plasmid	25 ng/µl	5 µg
	pFC-MEK1 plasmid (positive control)	25 ng/µl	5 µg
Catalog #800060	**Component**	**Concentration**	**Quantity**
	HLR-CHOP *trans*-Reporting Cell Line	—	1×10^6 viable cells
	pFC-CMV negative control plasmid	25 ng/µl	5 µg
	pFC-MEK3 plasmid (positive control)	25 ng/µl	5 µg
Catalog #800065	**Component**	**Concentration**	**Quantity**
	HLR-CREB *trans*-Reporting Cell Line	—	1×10^6 viable cells
	pFC-CMV negative control plasmid	25 ng/µl	5 µg
	pFC-PKA plasmid (positive control)	25 ng/µl	5 µg

STORAGE CONDITIONS

Control Plasmids: –20°C

Cell lines: Liquid Nitrogen

Revision A

ADDITIONAL MATERIALS REQUIRED

Growth medium (See *Preparation of Media and Reagents*)
Luciferase assay kit
5-ml BD Falcon polystyrene round-bottom tubes (BD Biosciences Catalog #352054)
Calcium- and magnesium-free PBS
Tissue culture plasticware
Transfection reagents
Luminometer
Hygromycin
G418 Sulfate

SAFETY CONSIDERATION

The American Type Culture Collection (ATCC) has designated CCL-2 HeLa cell lines as Biosafety Level 2. Appropriate caution should be used while handling any human-derived cell line. For more information, see *Biosafety in Microbiological and Biomedical Laboratories (4th edition)*. It is available on the Web sites of both the National Institutes of Health at *http://bmbl.od.nih.gov* and the Office of Health and Safety of the Centers for Disease Control and Prevention at *www.cdc.gov/od/ohs* (Click the link to *Biosafety Information*).

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INTRODUCTION

The Stratagene PathDetect reporter cell lines are designed for specific, rapid, and convenient analysis of the in vivo activation of transcriptional activators and upstream signal transduction pathways.[1,2] Two types of stable reporter cell lines have been developed for use with the PathDetect system using ATCC, CCL-2 HeLa cells. The first type is the HeLa Luciferase Reporter (HLR) cell line, which contains a stably integrated luciferase reporter gene. The second type includes three HLR *trans*-reporting cell lines, which contain both the luciferase reporter gene and the gene for a *trans*-acting GAL4 fusion protein (Elk1, CHOP, or CREB). The cell lines are useful for examining whether and where a newly cloned gene is involved in a signal transduction pathway or to study the effects of growth factors, drug candidates, and extracellular stimuli on signal transduction pathways converging at these transcription factors.

The PathDetect HeLa Luciferase Reporter (HLR) cell line contains an integrated synthetic promoter consisting of five direct repeats of the yeast GAL4 binding site that controls expression of the luciferase gene from *Photinus pyralis* [American firefly (*see Appendix I: Plasmid Information*)]. The HLR cell line also contains an expression cassette for hygromycin-resistance. To assay for involvement of a gene in a particular pathway, a plasmid encoding a fusion *trans*-activator protein consisting of the GAL4 DNA binding domain (DBD) fused to a target activation domain for the gene of interest is cotransfected into cells with an expression vector containing the gene of interest. If the gene of interest directly or indirectly phosphorylates the activation domain of the fusion *trans*-activator protein, this will activate transcription of luciferase. Expression (or activity) levels of luciferase reflect the activation status of the signaling events. The HLR cell line can also serve as the reporter cell line for many other applications using the GAL4 DNA binding domain, such as testing protein-protein interactions with mammalian two-hybrid vectors.

Each PathDetect HLR *trans*-reporting cell line contains the luciferase reporter cassette found in the HLR cell line and also expresses a unique, stably integrated, *trans*-acting fusion protein. Each fusion protein consists of the activation domain of one of three transcriptional activators: Elk1,[3–6] CREB,[6,7] or CHOP,[2,8] that are fused to the yeast GAL4 DBD (residues 1–147).[9,10] The transcriptional activator domains of Elk1, CHOP, and CREB are activated upon phosphorylation by mitogen-activated protein kinase (MAPK), p38 MAP kinase, or cyclic AMP-dependent kinase (PKA), respectively. Transcriptional activation of the reporter in these cell lines thus reflects in vivo activation of these kinases and their corresponding signal transduction pathways. Constitutive expression of the *trans*-acting fusion genes in the PathDetect cell lines is driven by the human cytomegalovirus (CMV) immediate early promoter. The *trans*-reporting cell lines are resistant to both G418 and hygromycin.

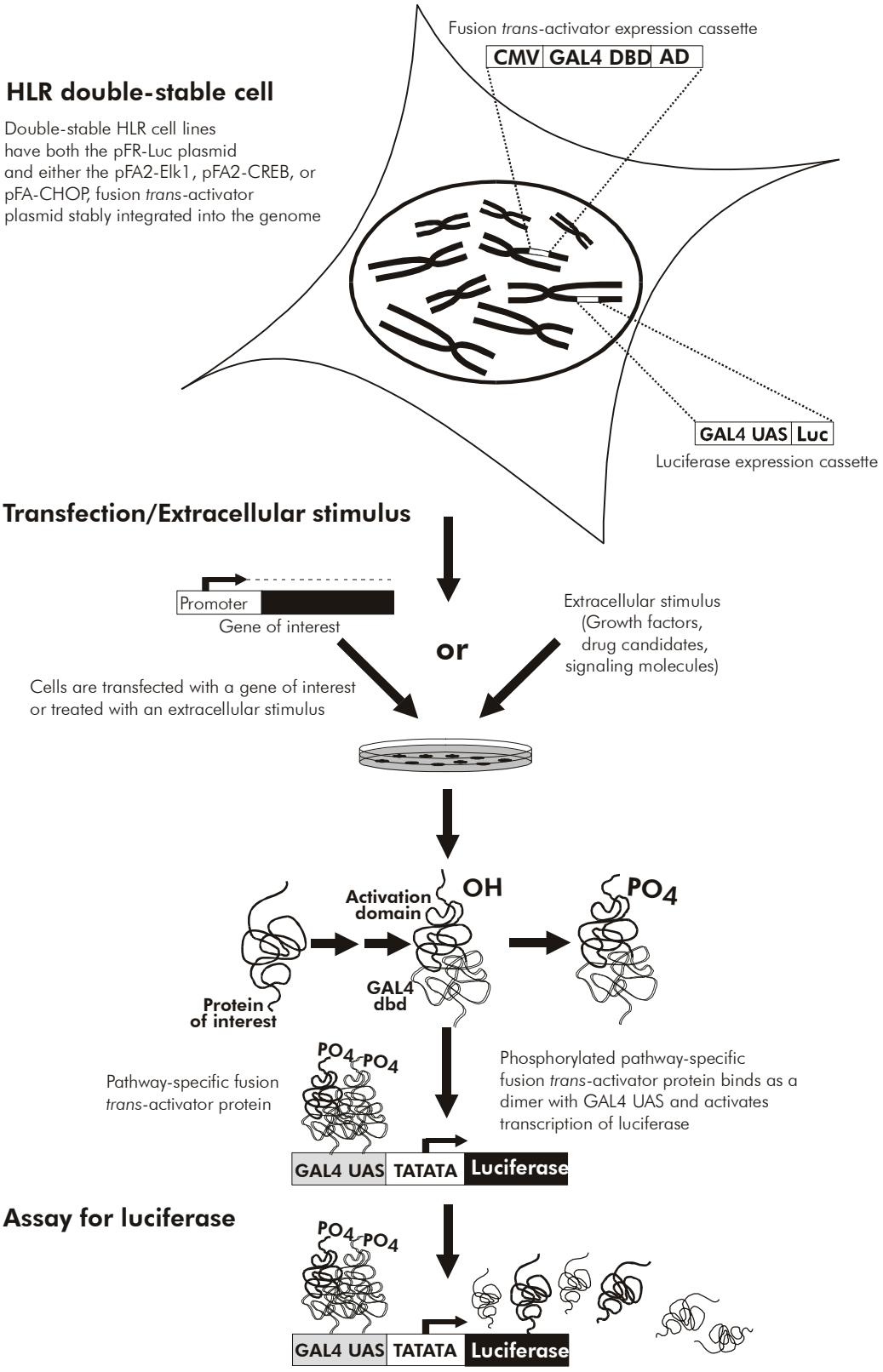


Figure 1 Schematic diagram for use of the PathDetect double-stable *trans*-reporting cell lines.

PREPROTOCOL CONSIDERATIONS

Choosing a Transfection Method

As with all transfection assays, the sensitivity of an assay using a PathDetect signal transduction pathway stable reporter cell line 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 with the provided positive control plasmid before performing the assays. Sufficient amounts of control plasmids are 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.

Tissue Cultureware

The protocols given are based on 24-well tissue culture dishes with a well diameter of ~15 mm and a surface area of ~1.88 cm^2.

Designing the Experiment

Studying the Effects of a Gene Product using HLR *trans*-reporting Cell Lines

The Elk1, CHOP, and CREB reporter cell lines include known upstream activators that can be used as positive controls for transactivation. The experimental plasmid minus the gene of interest can be used as a negative control to ensure that the introduction of viral promoters (e.g., CMV, RSV, or SV40) or other proteins expressed from the plasmid does not activate the fusion *trans*-activator protein. 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 experimental parameters for studying the effects of a gene product using the PathDetect *trans*-reporting cell lines are outlined in Table I. As all assays are to be run in triplicate, eight samples will utilize one 24-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
#	Negative control plasmid	Positive control	Experimental plasmid with gene of interest	Experimental plasmid without insert	Carrier DNA
1[a]	—	—	—	10 ng	240 ng
2[b]	—	—	—	20 ng	230 ng
3[c]	—	—	—	100 ng	150 ng
4[d]	—	—	10 ng	—	240 ng
5[e]	—	—	20 ng	—	230 ng
6[f]	—	—	100 ng	—	150 ng
7[g]	—	25 ng (1 µl)	—	—	225 ng
8[h]	25 ng (1 µl)	—	—	—	225 ng

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

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

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

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

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

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

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

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

Studying the Effects of Extracellular Stimuli using HLR *trans*-reporting Cell Lines

The PathDetect *trans*-reporting cell lines may be used to study the effects of extracellular stimuli, such as growth factors or drug candidates, on corresponding signal transduction pathways (see Table II). To perform such assays, cells are first treated with the compound of interest. Luciferase expression from the reporter indicates activation of the fusion *trans*-activator protein and, therefore, the presence of the endogenous protein kinase (e.g., MAPK, p38 MAPK, PKA, or an uncharacterized upstream activator).

TABLE II

Sample Experiment to Study the Effects of Extracellular Stimuli

#	Extracellular stimulus
1	Serum (10%)
2	PMA (10 ng/ml)
3	PMA (60 ng/ml)
4	EGF (100 ng/ml)
5	AC Toxin (10 µg/ml)
6	Medium (negative control)

CELL CULTURE PROTOCOLS

Starting the Cells in Culture

Note *All procedures from this point should be performed using sterile technique.*

1. Prepare the growth medium[§] with either G418 (250 µg/ml) and/or hygromycin (100 µg/ml), depending on cell type, and transfer 10 ml to a 15-ml conical tube.

2. Thaw the frozen cryovial of cells within 40–60 seconds by rapid agitation in a 37°C water bath. Remove the cryovial from the water and immediately immerse the cryovial in 70% (v/v) ethanol at room temperature.

3. Transfer the cell suspension to the conical tube containing the growth medium.

4. Centrifuge the cells at 200 × *g* for 5 minutes at room temperature and remove the growth medium from the conical tube by aspiration.

5. Resuspend the cells in the conical tube in 5 ml of growth medium containing the appropriate selective agent.

6. Add 10 ml of growth medium containing the appropriate selective agent to a 75-cm^2 tissue culture flask. Transfer the cell suspension to the tissue culture flask. Place the cells in a 37°C incubator at 5% CO_2.

Passaging the Cells

Note *Split the cells once the cell monolayer is ~80–90% confluent or approximately every 3–5 days using the following protocol.*

1. Remove the medium by aspiration, rinse the cells with 1× PBS,[§] and remove the PBS by aspiration.

2. Add 1.5 ml of trypsin–EDTA[§] to each 75-cm^2 tissue culture flask. Incubate the cells at room temperature for ~2–5 minutes. Tap the flask to release any adherent cells.

Note *Incubate the cells in the trypsin–EDTA for the minimum time necessary for the adherent cells to release from the flask. Overtrypsinization can kill the cells.*

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

3. **If using HLR cells,** perform step 3a. **If using double stable cells**, perform step 3b.

   a. Dilute the cells with growth medium containing hygromycin (100 µg/ml) to a final volume of 5–10 ml.

   b. Add growth medium containing G418 (250 µg/ml) and hygromycin (100 µg/ml) to a final volume of 5–10 ml.

4. The serum in the medium will inactivate the trypsin. Transfer the cells to the desired number of 75-cm^2 or 175-cm^2 tissue culture flasks. Depending on the experiment, use an inoculum of 1×10^4–1×10^6 cells. Add growth medium to the flasks to a final volume of 15 ml for 75-cm^2 tissue culture flasks or to a final volume of 30 ml for 175-cm^2 tissue culture flasks. Place the cells in a 37°C incubator at 5% CO_2.

Freezing the Cells for Long-term Storage

1. Remove the growth medium by aspiration, rinse the cells with 1× PBS, and remove the PBS by aspiration.

2. Add 1.5 ml of trypsin–EDTA to each 75-cm^2 tissue culture flask. Incubate the cells at room temperature for ~2–5 minutes. Tap the flask to release the adherent cells.

   Note *Incubate the cells in the trypsin–EDTA for the minimum time necessary for the adherent cells to release from the flask. Overtrypsinization can kill the cells.*

3. Dilute the cells in 8.5 ml of growth medium. The serum in the medium inactivates the trypsin.

4. Transfer the cell suspension to a 15-ml conical tube. Centrifuge the cells at 200 × *g* for 5 minutes at room temperature. Remove the growth medium from the tube by aspiration.

5. Resuspend the cells in 1–2 ml of freezing medium.[§]

6. Transfer the cell suspension to cryovials and place the cryovials in a chilled controlled-freezing container. Incubate the cryovials at –80°C overnight.

7. The following day, transfer the cryovials to liquid nitrogen for long-term storage. If properly stored, the cells should remain stable for >1 year.

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

PREPARING THE CELLS FOR TRANSFECTION

1. Seed 5×10^4 cells in 1 ml of growth medium without selective agents in each well of a 24-well tissue culture dish.

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

PREPARING PLASMIDS FOR TRANSFECTION

Note *The DNA used for transfections must be of high quality (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.*

Studying the Effects of a Gene Product

Prepare plasmid DNA mixtures in sterile BD Falcon polystyrene tubes as indicated by samples 1–8 in Table I. As each assay is run in triplicate, the amount of plasmid DNA in each tube should be sufficient for four transfections. See Table I for the appropriate amounts. For example, to prepare sample #1 **in triplicate** as indicated in Table I, combine the following components in a microcentrifuge tube and then proceed to *Transfecting the Cells*:

30 ng of experimental plasmid without an insert
720 µg of unrelated plasmid DNA

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.

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

If studying the effects of extracellular stimuli, 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 Preparing Cell Lysates for Luciferase Activity Assay.

PREPARING CELL LYSATES FOR LUCIFERASE ACTIVITY ASSAY

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

2. Remove as much 1× PBS as possible from the wells with a Pasteur pipet. Add 100 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 12,000 × *g* for 5 minutes at 4°C. Remove the supernatant and 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 1× luciferase assay reagent[§] 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.

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

TROUBLESHOOTING

Observation	Suggestions
Cells do not survive resuspension in growth medium following storage in liquid nitrogen	Cells are not stored properly. Store the cryovials of cells in liquid nitrogen immediately on receipt
	Cells are not thawed properly. Thaw the cells quickly and immediately dilute the cells in the growth medium
Cells do not survive passaging	Cells are over-diluted during passaging. When passaging, inoculate a fresh tissue culture flask with 1×10^4–1×10^6 cells
	Cells are over-tyrpsinized during passaging. Incubate the cells with trypsin–EDTA for the minimum time necessary for the cells to release from the flask
	Split the cells when the monolayer of cells is at most 90% confluent. Cells grown to 100% confluence are starved for growth medium
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
	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
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

Luciferase Reporter Cassette

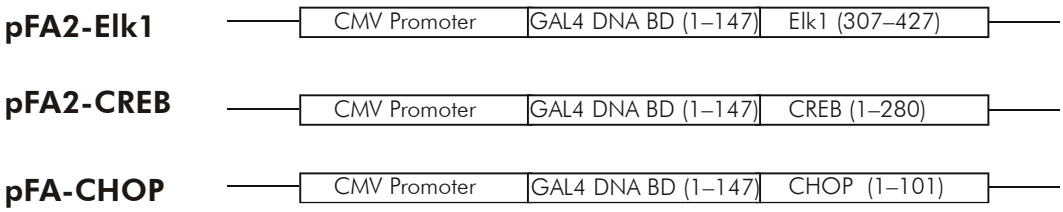
Sequence of GAL4 Binding Element in the pFR-Luc Plasmid

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

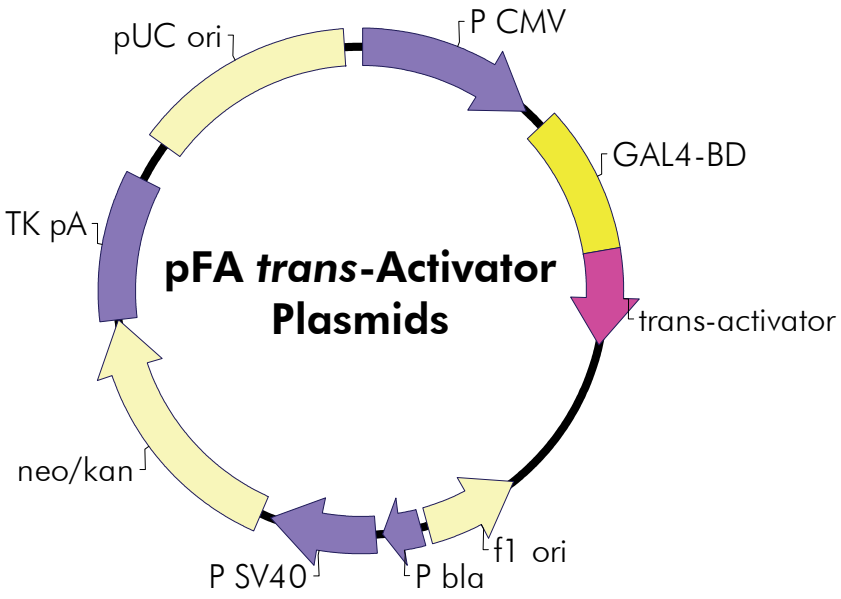
5× GAL4 Binding Element

Luciferase

Fusion *trans*-Activator Cassettes



The pFA *trans*-Activator Plasmids



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

Phosphate-buffered Saline (1× PBS)

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

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

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

Freezing Medium

Dulbecco's Modified Eagle Medium (DMEM) (high glucose, without L-glutamine, without sodium pyruvate)
20% (v/v) fetal bovine serum
10% (v/v) dimethylsulfoxide (DMSO)

Trypsin–EDTA

0.53 mM tetrasodium ethylenediaminetetraacetic acid
0.05% trypsin

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ENDNOTES

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MSDS INFORMATION

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

STRATAGENE

An **Agilent Technologies** Division

PathDetect Cell Lines

Catalog #800050, #800055, #800060, and #800065

QUICK-REFERENCE PROTOCOL

Starting the Cells in Culture

- Transfer 10 ml of growth medium to a 15-ml conical tube
- Thaw the frozen cells quickly in a 37°C water bath
- Transfer the cell suspension to the conical tube containing the growth medium
- Centrifuge the cells at 200 × g for 5 minutes at room temperature and remove the growth medium by aspiration

    a. For the HLR cell line, add 10 ml of growth medium containing hygromycin (100 µg/ml) to a 75-cm^2 tissue culture flask

    b. For HLR trans-reporting cell lines, add 10 ml of growth medium containing G418 (250 µg/ml) and hygromycin (100 µg/ml) to a 75-cm^2 tissue culture flask

- Resuspend the cells in the conical tube in 5 ml of growth medium containing the appropriate selective agent(s), and transfer the cell suspension to the tissue culture flask
- Place the cells in a 37°C incubator with 5% CO_2

Passaging the Cells

- Remove the growth medium, rinse the cells with 1× PBS, and remove the PBS by aspiration
- Add 1.5 ml of trypsin–EDTA to each 75-cm^2 tissue culture flask, incubate the cells at room temperature for ~2–5 minutes, and tap the flask to release the adherent cells
- Dilute the cells with the growth medium containing hygromycin (100mg /ml) and, for double stables, G418 (250 µg/ml) and transfer the cells to the desired number of tissue culture flasks
- Place the cells in a 37°C incubator with 5% CO_2

Freezing the Cells for Long-term Storage

- Remove the growth medium, rinse the cells with 1× PBS, and remove the PBS by aspiration
- Add 1.5 ml of trypsin–EDTA to each 75-cm^2 tissue culture flask containing cells, incubate the cells at room temperature for ~2–5 minutes, and tap the flask to release the adherent cells
- Dilute the cells with 8.5 ml of growth medium
- Transfer the cell suspension to a 15-ml conical tube, centrifuge at 200 × g for 5 minutes at room temperature, and remove the growth medium by aspiration
- Resuspend the cells in 1–2 ml of freezing medium
- Transfer the cells to cryovials, place the cryovials in a chilled controlled-freezing container, and incubate the cryovials at –80°C overnight
- The following day, transfer the cryovials to liquid nitrogen for long-term storage