Technology
Biomyx
Innovation & Convenience for Life Sciences

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Stimulight™ Hippo Pathway *trans*-Reporting System

MATERIALS PROVIDED

Catalog #H-1001

Component	Part#	Plasmid Name	Conc.	Quantity
Reporter plasmid	P2136	pHTS-GAL4	0.5 mg/ml	40 μg
Activator plasmid	H1001a	pGAL4-TEAD4	25 ng/μl	2 μg
Negative control	P2138	pGAL4-DBD	25 ng/μl	2 μg
Positive control	H1001c	pM2H-YAP2A5	25 ng/μl	2 μg

KEY FEATURES OF THE PLASMIDS

Plasmid	Bacterial Selection	Eukaryotic Selection	Promoter	Gene of Interest
pHTS-GAL4	Ampicillin	Hygromycin	5xGBE[5] (CGGAGTACTGTCCTCCG AG).	Firefly Luciferase
pGAL4-TEAD4	Kanamycin	G418	CMV Early	Fusion protein of yeast GAL4 DBD (1-147) & TEAD4 (NM_003213, 74-434)
pGAL4-DBD	Kanamycin	G418	CMV Early	Yeast GAL4 DNA binding Domain (aa 1-147)
pM2H-YAP2A5	Kanamycin	G418	CMV Early	Constitutively Active Mutant of YAP2 (NM_006106)[1,2]

RECEIVING & STORAGE

Upon receiving, spin the vials briefly in a microcentrifuge to collect the contents. Store the products at 2-8^{0}C if used immediately or, store at –20^{0}C for extended storage.

PROPGATION OF THE PLASMIDS

If desired, the plasmids provided in the kit can be amplified by transforming 25 ng of the plasmid DNA into an *E. coli* strain (e.g. DH5a, XL10-GOLD, TOPO10 etc). Transformants containing pHTS-GAL4 should be plated on LB-Agar plates with 100μg/ml ampicillin or carbenicillin; *E. coli* transformed with pGAL4-TEAD4, pGAL4-DBD and pM2H-YAP2A5 plasmid should be plated on LB-Agar plates with 50μg/ml Kanamycin.

INTRODUCTION

The Stimulight™ Hippo Pathway *trans*-reporting system is designed for specific, rapid in vivo read-out of the activation of transcription factor TEAD4 and its upstream Hippo signaling pathway, which plays key roles in cell growth, proliferation, apoptosis and organ size control[1-4].

The system uses Gal4-TEAD4 fusion protein, which is expressed from the Activator Plasmid pGAL4-TEAD, as the sensor of the activation of Hippo kinase cascade. The transcription coactivator YAP works together with Gal4-TEAD4 to activate luciferase expression from pHTS-GAL4 vector when transfected into mammalian cells[2].

Activated Hippo kinase cascade in the cell results in the phosphorylation of YAP and its translocation to the cytoplasm. Less YAP in the nucleus, in turn, causes reduced luciferase expression. Inhibition of Hippo kinase cascade, however, results in elevated luciferase activity. This system is useful to asses whether a gene is involved or affecting the various components along the Hippo pathway. It can also be used to study the in vivo effects of growth factors, drug candidates, and extracellular stimuli.

THE ACTIVATOR PLASMID

The fusion *trans*-activator plasmid pGAL4-TEAD4 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.

The fusion *trans*-activator GAL4-TEAD4 consists of the DNA binding domain (DBD) of yeast GAL4 (residues 1-147) and the activation domain of human TEAD4 (NM_003213, residues M74-E434). The GAL4 DBD domain allows the specific binding of the fusion protein to the GAL4 binding elements (GBE) in the Reporter Plasmid (pHTS-GAL4); The TEAD4 activation domain, when activated by upstream YAP2, activates luciferase gene expression from pHTS-GAL4 plasmid.

The activator plasmids can be selected with Kanamycin that is conferred by the kanamycin-resistance gene under control of the prokaryotic β-lactamase promoter. The neomycin-resistance gene, driven by the SV40 early promoter, confers stable selection with G418 in mammalian cells.

REPORTER PLASMID

The reporter plasmid pHTS-GAL4 contains an expression cassette for the firefly luciferase under the control of GAL4-binding element (**CGGAGTACTGTCCTCCG** AG)$_5$. Luciferase expression is activated in mammalian cells when a *trans*-activator (e.g. GAL4-TEAD) binds to the GBE.

CONTROL PLASMIDS

The negative control plasmid pGAL4-DBD expresses the GAL4 DBD, which can bind to GBE in pHTS-GAL4 plasmid but is incapable of activation luciferase expression. The positive control plasmid pM2H-YAP2A5 is a constitutively active human YAP2 mutant that activates TEAD4 independent of activation of upstream components in the Hippo signaling pathway[1, 2].

TRNASFECTION AND SELECTION OF CELL

The provided plasmids are highly purified and ready for transfection into mammalian cells with any standard transfection methods such as LipofectAmine®, electroporation or Calcium Phosphate Precipitation. Refer to the manufacturers' instruction manuals for details of these transfection methods.

Different types of mammalian cells vary widely in endogenous signaling proteins and other properties. Hence, the sensitivity of the assay may vary widely as well, depending on the properties of the cell and the culture conditions. Experimental parameters should be determined experimentally for each cell type.

EXPERIMENTAL DESIGN

Experiments should be designed with the proper controls according to the purpose of the study. The examples given below can serve as a starting point. The exact conditions, especially the amount of positive control plasmid and the experimental plasmid and extracellular stimuli should be determined experimentally.

The examples given are based on 24-well tissue culture plate. If plates of different sizes are being used, the amount of all reagents should be adjusted according to the area of the wells or dishes. Ideally, each sample should be run in triplicate.

Example Experiment

Mix different plasmids as listed below and then proceed to transfection. Amount of plasmid DNA is shown as ng.

Sample#	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
pHTS-GAL4	400	400	400	400	400	400	400	400
pGAL4-TEAD4	25	25	25	25	25	25	25	-
Experimental Plasmid(e.g. pM2H-Mst2)	25	50	100	-	-	-	-	50
Experimental Plasmid w/o GOI (e.g. pM2H)	-	-	-	25	50	100	-	-
Positive control	-	-	-	-	-	-	25	-
pGAL4-DBD	-	-	-	-	-	-	-	25

Notes: *1) The plasmid used in #4-6 is ideally the parental plasmid used for expression of the gene of interest. 2) Sample #7 is the positive control that assesses the efficacy of the assay. 3)*

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pGAL4-DBD in sample #8 does not express GAL4-TEAD4 and is a negative control. 4) Ideally, a carrier DNA (e.g. pUC18) should be used to make all the samples have the same amount of DNA. 5) The exact amount (or the range) of the experimental plasmid should be determined experimentally.

LUCIFERASE ACTIVITY ASSAY

There're various commercial sources for reagents prepared for luciferase extraction and activity assay (e.g. Promega, Stratagene). The following protocol is provided for quick reference only.

1. Remove media from cell and rinse twice with PBS and remove residual PBS.
2. Add 1x Lysis Buffer (e.g. 400μl per well of a six-well plate, see below for buffer components). Incubate the plate for 15 minutes at room temperature (RT) with gentle rocketing.
3. The lysates could be used in luciferase assay or be transferred to microcentrifuge tubes and stored at -80^{0}C.
4. Mix 5-20μl of cell lysate with 100μl of 1X Luciferase Assay Buffer in an appropriate tube (e.g. Falcon® 2054 polystyrene tube). All reagents should be brought to RT for assay.
5. Measure the light emission with a luminometer with an integration time of 10-30 seconds.

Buffers for Luciferase Activity Assay: *(Final concentrations are shown)*

Lysis Buffer (5X)		**Assay Buffer (1X)**	
40mM	Tricine (pH7.8)	40mM	Tricine (pH7.8)
50mM	NaCl	0.5mM	ATP
2mM	EDTA	10mM	$MgSO_4$
1mM	$MgSO_4$	0.5mM	EDTA
5mM	DTT	10mM	DTT
1%	Triton® X-100	0.5mM	Coenzyme A
		0.5mM	luciferin

SELECTED REFERENCES

1. Zhao B. et al., 2007. Genes Dev., 21: 2747-61
2. Zhao B. et al., 2008. Genes Dev., 22: 1962-1971
3. Dong J. et al., 2007, Cell, 130: 1120-1133
4. Zhang L. et al., Dev Cell, Epub March 2008, doi:10.1016/j.devce.2008.01.006
5. Sadowski, I. and Ptashne, M. (1989) *Nucleic Acids Res* 17(18):7539.
6. Wu S. et al., Dev Cell, Epub March 2008, doi:10.1016/j.devce.2008.01.006

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