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Data Sheet

The Transfection Collection™ – AP1 Transient Pack (JNK Signaling Pathway)

Catalog #: 79266

Background

The stress-activated protein kinase/c-jun N-terminal kinase (SAPK/JNK) family of proteins includes mitogen-activated protein kinases (MAPKs) that are activated by stress, inflammatory cytokines, mitogens, oncogenes, and inducers of cell differentiation and morphogenesis. Upon activation of the SAPK/JNK pathway, MAP Kinase Kinases phosphorylate and activate JNKs. The activated JNKs translocate to the nucleus where they phosphorylate and activate transcription factors such as c-Jun. The activated c-Jun forms homodimers or heterodimers with fos family proteins which bind to the activator protein-1(AP1) response element and induce target gene transcription.

Description

The *AP1 Transient Pack* is designed to provide the tools necessary for transiently transfecting and monitoring the activity of the JNK signaling pathway and the transcriptional activity of AP1 in cultured HEK293 cells. The kit contains transfection-ready vectors containing firefly luciferase as a JNK pathway-responsive reporter and constitutively expressing Renilla luciferase as a transfection control. It also includes the Dual Luciferase detection reagents to detect both luciferase activities and specialized medium for growing and assaying HEK293 cells.

The key to the AP1 Transient Pack is the AP1 luciferase reporter vector. This reporter contains the firefly luciferase gene under the control of multimerized AP1 responsive elements located upstream of a minimal promoter. The AP1 reporter is premixed with a constitutively-expressing *Renilla* luciferase vector that serves as an internal control for transfection efficiency.

The pack also includes a non-inducible firefly luciferase vector premixed with constitutively-expressing *Renilla* luciferase vector as a negative control. The non-inducible luciferase vector contains the firefly luciferase gene under the control of a minimal promoter, without any additional response elements. The negative control is critical for determining pathway-specific effects and the background luciferase activity.

Additionally, the pack includes cell culture medium (BPS Medium 1) that has been optimized for use with HEK293 and HeLa cells*. BPS Medium 1 includes MEM medium, 10% fetal bovine serum, 1% non-essential amino acids, sodium pyruvate, and 1% Pen/Strep. Finally, the pack provides the Dual Luciferase (Firefly-Renilla) Assay System. These luciferase reagents provide highly sensitive, stable detection of firefly luciferase activity and Renilla luciferase activity. The

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dual luciferase reagents can be used directly in cells in growth medium, and can be detected with any luminometer; automated injectors are not required.
*Note: the kit may be used with other cell lines than HEK293 or HeLa, but an alternate cell culture medium may be required for optimal cell growth,

Applications

- Monitor JNK signaling pathway activity and AP1-mediated activity.
- Screen for activators or inhibitors of the JNK signaling pathway.
- Study effects of RNAi or gene overexpression on the activity of the JNK pathway.

Components

Component	Amount	Storage
Reporter (Component A) AP1 luciferase reporter vector* + constitutively expressing Renilla luciferase vector*	500 µl (60 ng DNA/ µl)	-20°C
Negative Control Reporter (Component B) Non-inducible luciferase vector*+ constitutively expressing Renilla luciferase vector*	500 µl (60 ng DNA/ µl)	-20°C
Firefly Luciferase Reagent Buffer	10 ml	-20°C
Firefly Luciferase Reagent Substrate (100x)	100 µl	-20°C *Protect from light*
Renilla Luciferase Reagent Buffer	10 ml	Room Temp.
Renilla Luciferase Reagent substrate (100x)	100 µl	-20°C *Protect from light*
BPS Medium 1	100 ml	+4°C

Note: These vectors are designed for transient transfection. They are NOT SUITABLE for transformation and amplification in bacteria.

Materials Required but Not Supplied

- HEK293 or HeLa cells. Other mammalian cell lines can be used, but an alternate cell culture medium may be required for optimal cell growth
- 96-well tissue culture plate or 96-well tissue culture-treated white clear-bottom assay plate (Corning, #3610)
- Transfection reagent for mammalian cell line [We use Lipofectamine™ 2000 (Life Technologies #11668027). However, other transfection reagents work equally well.]

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- Assay medium: Opti-MEM I (Life Technologies #31985-062) + 0.5% FBS + 1% Non-essential amino acids + 1 mM sodium pyruvate + 1% Pen/Strep
- Luminometer
- Activator of AP1 such as Phorbol 12-myristate 13-acetate (PMA), (Sigma, P1585), 1 mM in DMSO

Generalized Transfection and Assay Protocols

The following procedure is designed to transfect the reporter into HEK293 cells using Lipofectamine 2000 in a 96-well format. To transfect cells in different tissue culture formats, adjust the amounts of reagents and cell number in proportion to the relative surface area. If using a transfection reagent other than Lipofectamine 2000, follow the manufacturer's transfection protocol. Transfection conditions should be optimized according to the cell type and study requirements.

All amounts and volumes in the following setup are provided on a per-well basis.

1. One day before transfection, seed cells at a density of ~ 30,000 cells per well in 100 µl of BPS Medium 1 so that cells will be 90% confluent at the time of transfection.

2. The next day, for each well, prepare complexes as follows:
   a. Dilute DNA mixtures in 15 µl of Opti-MEM I medium (antibiotic-free). Mix gently. Depending upon the experimental design, the DNA mixtures may be any of following combinations:
      - **1 µl of Reporter** (component A); in this experiment, the control transfection is **1 µl of Negative Control Reporter** (component B).
      - **1 µl of Reporter** (component A) + experimental vector expressing gene of interest; in this experiment, the control transfections are: **1 µl of Reporte**r (component A) + negative control expression vector, **1 µl of Negative Control Reporter** (component B) + experimental vector expressing gene of interest, and **1 µl of Negative Control Reporter** (component B) + negative control expression vector.
      - **1 µl of Reporter** (component A) + specific siRNA; in this experiment, the control transfections are: **1 µl of Reporte**r (component A) + negative control siRNA, **1 µl of Negative Control Reporter** (component B) + specific siRNA, and **1 µl of Negative Control Reporter** (component B) + negative control siRNA.

   Note: we recommend setting up each condition in at least triplicate, and preparing transfection cocktail for multiple wells to minimize pipetting errors.

   b. Mix Lipofectamine 2000 gently before use, then dilute 0.35 µl of Lipofectamine 2000 in 15 µl of Opti-MEM I medium (antibiotic-free). Incubate for 5 minutes at room temperature.
   Note: Prepare this dilution cocktail in volumes sufficient for the whole experiment.

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c. After the 5 minute incubation, combine the diluted DNA with diluted Lipofectamine 2000. Mix gently and incubate for 25 minutes at room temperature.

3. Carefully remove and discard 30 µl of media from each of the wells of cell culture, taking care not to disturb the cells or touch the bottom of the well with the pipet tip. Add the 30 µl of the complexes to each well containing 70 µl cells and medium. Mix gently by tapping the plate.

4. Incubate cells at 37°C in a CO_2 incubator for overnight.

5. The next day, change medium to assay medium (Opti-MEM I, 0.5% FBS, 1% non-essential amino acids, 1 mM Na-pyruvate,1% Pen/Strep) containing an activator of AP1 such as PMA. Incubate cells at 37°C in a CO_2 incubator for ~ 6 to 24 hours. After treatment, perform the Dual Luciferase Assay System (below).

Dual Luciferase Assay Procedure

1. Thaw **Firefly Luciferase Reagent Buffer** by placing the reagent in a room temperature water bath. Equilibrate the buffer to room temperature and mix well before use. *Note: It is important that the **Firefly Luciferase Reagent Buffer** be at room temperature before use.*
2. Calculate the amount of Firefly Luciferase Assay Working solution needed for the experiment (**Firefly Luciferase Reagent Buffer** + **Firefly Luciferase Reagent Substrate**). Immediately prior to performing the experiment, prepare the Firefly Luciferase Assay Working Solution by diluting **Firefly Luciferase Reagent Substrate** into **Firefly Luciferase Reagent Buffer** at a 1:100 ratio and mix well. Avoid exposing to excessive light*. Only use enough of each component for the experiment, remaining* **Firefly Luciferase Reagent Buffer** *and* **Firefly Luciferase Reagent Substrate** *should be stored separately at* -20°C*.*
3. Remove multi-well plate containing mammalian cells from incubator. *Note: plates must be compatible with luminescence measurement with luminometer being used.*
4. Add equal volume of Firefly Luciferase Assay Working Solution (step 2) to the culture medium in each well. Example: 96-well plate with 100 µl of culture medium requires 100 µl of Firefly Luciferase Assay Working Solution per well.

   Gently rock the plates for ≥15 minutes at room temperature. Measure firefly luminescence using a luminometer. The signal under these conditions will be stable for more than 2 hours at room temperature. For maximal light intensity, measure samples within 1 hour of reagent addition.
5. Calculate the amount of Renilla Luciferase Assay Working Solution needed for the experiment (**Renilla Luciferase Reagent Buffer** + **Renilla Luciferase Reagent Substrate**). Prepare the Renilla Luciferase Assay Working Solution by diluting **Renilla Luciferase Reagent Substrate** into **Renilla Luciferase Reagent Buffer** at a 1:100 ratio

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and mix well. Avoid exposing to excessive heat or light. *Only use enough of each component for the experiment,*

6. Add equal volume of Renilla Luciferase Assay Working Solution (step 5) to each well. Example: 96-well plate with 100 µl of culture medium + 100 µl Firefly Luciferase Reagent requires 100 µl of Renilla Luciferase Assay Working Solution per well.
7. Gently rock the plates for ~1 minute at room temperature. Measure Renilla luminescence using a luminometer.
8. Data analysis: subtract background (wells with medium and luciferase reagent only) from all the readings. To obtain the normalized luciferase activity for the AP1 reporter, subtract the background luminescence, then calculate the ratio of firefly luminescence from AP1 reporter to Renilla luminescence from the control Renilla luciferase vector. To determine the fold induction, divide the value for the treated cells by the value for the untreated cells.

Sample protocol to determine the effect of PMA on AP1 reporter activity in HEK293 or HeLa cells

1. One day before transfection, seed HEK293 at a density of 30,000 cells per well into white clear-bottom 96-well plate in 100 µl of BPS Medium 1. Incubate cells overnight at 37°C in a CO_2 incubator.

2. The next day, transfect 1 µl of AP1 reporter (component A) into cells following the procedure in **Generalized Transfection and Assay Protocols.**

3. Incubate cells at 37°C in a CO_2 incubator overnight.

4. The next day after transfection, prepare 1 mM stock solution of PMA in DMSO. Dilute the PMA stock in assay medium. Change cell medium to 50 µl of diluted PMA in assay medium to induce the AP1 reporter. For unstimulated control wells, treat cells with 50 µl of assay medium (without PMA). Add 50 µl of assay medium (without PMA) to cell-free control wells to determine the background luminescence.
   Set up each treatment in at least triplicate.

5. Incubate cells at 37°C in a CO_2 incubator for ~ 6 hours.

6. Perform dual luciferase assay as described above in Dual Luciferase Assay Procedure. Briefly, dilute 100x Firefly Luciferase Reagent Substrate into Firefly Luciferase Reagent Buffer that has been equilibrated to room temperature . Add 50 µl of Firefly Luciferase reagent per well and rock at room temperature for ~15 minutes, then measure firefly luminescence using a luminometer. Dilute 100x Renilla Luciferase Reagent Substrate into Renilla Luciferase Reagent Buffer. Add 50 µl of Renilla Luciferase reagent per well, rock at room temperature for ~1 minute and measure Renilla luminescence.

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7. To obtain the normalized luciferase activity for the AP1 reporter, subtract the background luminescence, then calculate the ratio of firefly luminescence from AP1 reporter to *Renilla* luminescence from the control *Renilla* luciferase vector. To determine the fold induction, divide the value for the treated cells by the value for the untreated cells.

Figure 1. PMA induced the expression of AP1 reporter. The results are shown as fold induction of normalized AP1 reporter activity. Fold induction is determined by comparing values against the mean value for unstimulated control cells.

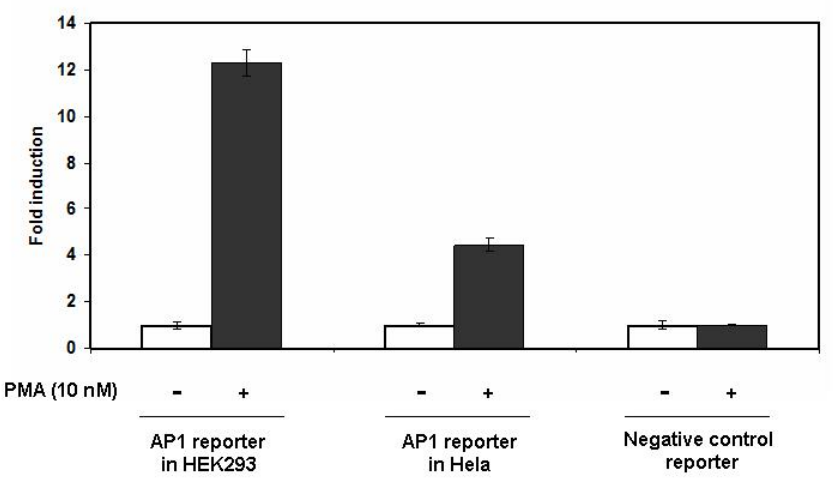
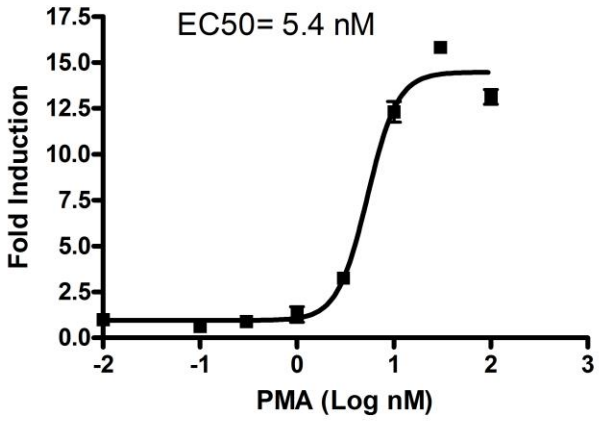


Figure 2. Dose response of AP1 reporter activity to PMA in HEK293 cells. The results are shown as fold induction of normalized AP1 reporter activity. Fold induction is determined by comparing values against the mean value for unstimulated control cells. The EC50 of PMA is ~5.4 nM



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Sample protocol to determine the effect of JNK pathway inhibitor on AP1 reporter activity

1. One day before transfection, seed cells at a density of 30,000 cells per well into white clear-bottom 96-well plate in 100 µl of BPS Medium 1. Incubate cells overnight at 37°C in a CO_2 incubator.

2. The next day, transfect 1 µl of AP1 reporter (component A) into cells following the procedure in **Generalized Transfection and Assay Protocols.**

3. Incubate cells at 37°C in a CO_2 incubator for overnight.

4. The next day after transfection, prepare 10 mM stock solution of JNK inhibitor V in DMSO. Dilute the inhibitor in assay medium to a final concentration of 10 µM. Carefully remove the medium from wells and add 45 µl of diluted inhibitor in assay medium to the wells.
   Add 45 µl of assay medium (without inhibitor) to inhibitor control wells or unstimulated control wells. Add 45 µl of assay medium (without inhibitor) to cell-free control wells (for determining background luminescence). Set up each treatment in at least triplicate.

5. Incubate cells at 37°C in a CO_2 incubator for 1 hour.

6. Add 5 µl of diluted PMA in assay medium to wells. The final PMA concentration for the cells is 10 nM.
   Add 5 µl of assay medium without PMA to the unstimulated control wells (cells without inhibitor and without PMA treatment for determining the basal activity).
   Add 5 µl of assay medium without PMA to cell-free control wells.

7. Incubate cells at 37°C in a CO_2 incubator for 6 hours.

8. Perform dual luciferase assay as described above in **Dual Luciferase Assay Procedure**. Briefly, dilute 100x Firefly Luciferase Reagent Substrate into Firefly Luciferase Reagent Buffer that has been equilibrated to room temperature. Add 50 µl of Firefly Luciferase reagent per well and rock at room temperature for ~15 minutes, then measure firefly luminescence using a luminometer. Dilute 100x Renilla Luciferase Reagent Substrate into Renilla Luciferase Reagent Buffer . Add 50 µl of Renilla Luciferase reagent per well, rock at room temperature for ~1 minute and measure Renilla luminescence.

9. To obtain the normalized luciferase activity for the AP1 reporter, subtract the background luminescence, then calculate the ratio of firefly luminescence from the AP1 reporter to *Renilla* luminescence from the control *Renilla* luciferase vector. To determine the fold induction, divide the value for the treated cells by the value for the untreated cells.

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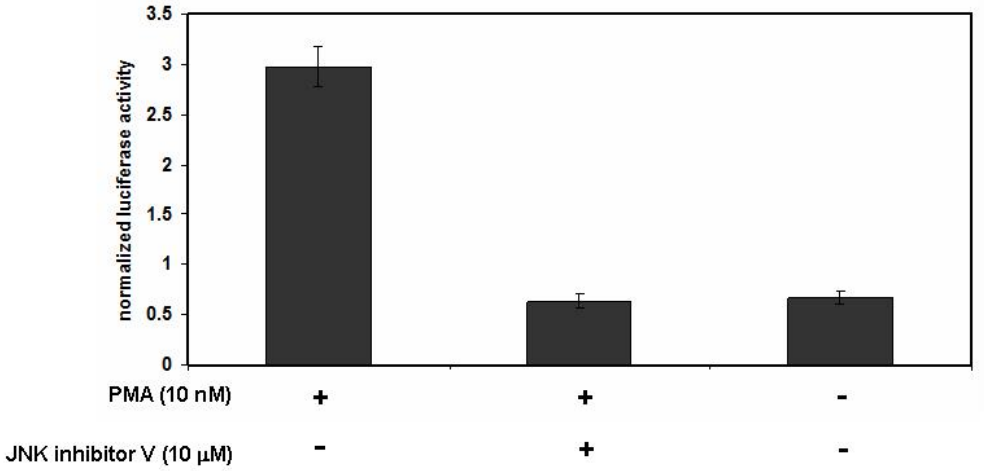
Figure 3. Inhibition of PMA-induced AP1 reporter activity by JNK inhibitor V. The results are shown as normalized AP1 reporter luciferase activity.



References

1. Zhou H. *et. al*. (2005) Frequency and distribution of AP-1 sites in the human genome. *DNA Research.* **11:** 139-150.
2. Gaillard P. *et.al.* (2005) Design and synthesis of the first generation of novel potent, selective, and in vivo active (benzothiazol-2-yl)acetonitrile inhibitors of the c-Jun N-terminal kinase. *J Med Chem*. **48(14):**4596-4607.

Refills

Product Name	Catalog #	Size
AP1 Reporter Kit (JNK Pathway)	60612	500 rxns.
BPS Medium 1	79259	100 ml
Dual Luciferase (Firefly-Renilla) Assay System	60683-1	10 mL
Dual Luciferase (Firefly-Renilla) Assay System	60683-2	100 mL
Dual Luciferase (Firefly-Renilla) Assay System	60683-3	1 L

Related Products

Product Name	Catalog #	Size
AP1 Reporter – HEK 293 cell line	60405	2 vials
SRE Reporter Kit (MAPK/ERK Signaling Pathway)	60511	500 rxns.
MAPK10 (JNK3), human	40092	10 µg
JNK1-β1(K55M), human	40871	100 µg
JNK1, mouse	40071	10 µg
JNK2, human	40113	10 µg
JNK3, human	40114	10 µg
MAPKAPK2 (MK2), human	40088	100 µg