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## **Data Sheet**

### **ISRE Reporter Kit JAK/STAT Signaling Pathway Catalog #: 60613**

#### **Background**

The JAK (Janus kinase) /STAT (Signal Transducer and Activator of Transcription) pathway is activated by various cytokines and growth factors and plays a critical role in cell growth, hematopoiesis, and immune response. In mammals, there are four JAKs (JAK1, JAK2, JAK3 and TYK2) and seven STAT proteins. Interferon alpha (IFN $\alpha$ ) is a Type I interferon. Binding of IFN $\alpha$  to its receptor leads to the activation of JAK1 and TYK2, which in turn phosphorylate and activate STAT1 and STAT2. The phosphorylated STAT1 and 2 form a heterodimer and bind to IRF9/p48, forming a protein complex ISGF3. This complex translocates to the nucleus and binds to the ISRE (Interferon Stimulated Response Element) in the promoter region thereby promoting transcription of interferon-inducible genes.

#### **Description**

The ISRE Reporter kit is designed for monitoring the activity of Type I interferon-induced JAK/STAT signaling pathway in the cultured cells. The kit contains transfection-ready ISRE luciferase reporter vector, which is a JAK/STAT pathway-responsive reporter. This reporter contains a firefly luciferase gene under the control of multimerized ISRE responsive element located upstream of a minimal promoter. The ISRE reporter is premixed with constitutively-expressing *Renilla* luciferase vector, which serves as an internal control for transfection efficiency.

The kit 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 a firefly luciferase gene under the control of a minimal promoter, without any additional response elements. The negative control is critical to determining pathway specific effects and background luciferase activity.

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## Applications

- Monitor IFN $\alpha$ -induced JAK/STAT pathway activity.
- Screen activators or inhibitors of the JAK/STAT signaling pathway.
- Study effects of RNAi or gene overexpression on the activity of the JAK/STAT pathway.

## Components

Component	Specification	Amount	Storage
<b>Reporter (Component A)</b>	ISRE luciferase reporter vector + constitutively expressing <i>Renilla</i> luciferase vector	500 $\mu$ l (60 ng DNA/ $\mu$ l)	-20°C
<b>Negative Control Reporter (Component B)</b>	Non-inducible luciferase vector + constitutively expressing <i>Renilla</i> luciferase vector	500 $\mu$ l (60 ng DNA/ $\mu$ l)	-20°C

These vectors are ready-to-use for transient transfection. They are NOT SUITABLE for transformation and amplification in bacteria.

## Materials Required but Not Supplied

- Mammalian cell line and appropriate cell culture medium
- 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 (Invitrogen # 11668027). However, other transfection reagents work equally well.
- Opti-MEM I Reduced Serum Medium (Invitrogen #31985-062)
- TWO-step luciferase assay system:  
TWO-Step Luciferase (Firefly & Renilla) Assay System (BPS Bioscience #60683): This system assays cells directly in growth medium. It can be used with any luminometer. Automated injectors are not required.

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- Luminometer

## 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 condition should be optimized according to the cell type and study requirements.

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

1. One day before transfection, seed HEK293 cells at a density of 30,000 cells per well in 100  $\mu$ l of growth medium 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  $\mu$ l of Opti-MEM I medium (antibiotic-free). Mix gently. Depending upon the experimental design, the DNA mixtures may be any of following combinations:
    - **1  $\mu$ l of Reporter** (component A); in this experiment, the control transfection is **1  $\mu$ l of Negative Control Reporter** (component B).
    - **1  $\mu$ l of Reporter** (component A) + experimental vector expressing gene of interest; in this experiment, the control transfection is: **1  $\mu$ l of Reporter** (component A) + negative control expression vector, **1  $\mu$ l of Negative Control Reporter** (component B) + experimental vector expressing gene of interest, and **1  $\mu$ l of Negative Control Reporter** (component B) + negative control expression vector.
    - **1  $\mu$ l of Reporter** (component A) + specific siRNA; in this experiment, the control transfection is: **1  $\mu$ l of Reporter** (component A) + negative control siRNA, **1  $\mu$ l of Negative Control Reporter** (component B) + specific siRNA, and **1  $\mu$ l of Negative Control Reporter** (component B) + negative control siRNA.

Note: we recommend setting up at least triplicate assays for each condition, and preparing transfection cocktail for multiple wells.

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b. Mix Lipofectamine 2000 gently before use, then dilute 0.35  $\mu$ l of Lipofectamine 2000 in 15  $\mu$ l 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.

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. Add the 30  $\mu$ l of complexes to each well containing cells and medium. Mix gently by tapping the plate.

4. Incubate cells at 37°C in a CO<sub>2</sub> incubator. After ~24 hours of transfection, treat cells with test activators/inhibitors for additional 6 to 24 hours. Perform the TWO-Step Luciferase Assay System following the protocol on the BPS data sheet (BPS Bioscience #60683).

### **Sample protocol to determine the dose response of HEK293 cells transfected with ISRE reporter to IFN $\alpha$**

Additional materials required in this experiment setup

- Human Interferon Alpha A (IFN $\alpha$ ) (R&D Systems # 11100-1)
- HEK293 growth medium: MEM/EBSS (with L-glutamine) (Hyclone #SH30024.01) + 10% FBS + 1% non-essential amino acids + 1 mM Na pyruvate + 1% Pen/Strep
- Assay medium: same as HEK293 growth medium
- TWO-Step Luciferase (Firefly & Renilla) Assay System (BPS Bioscience #60683)

1. One day before transfection, seed HEK293 cells at a density of 30,000 cells per well into a white clear-bottom 96-well plate in 100  $\mu$ l of growth medium. Incubate cells at 37°C in a CO<sub>2</sub> incubator for overnight.

2. Next day, transfect 1  $\mu$ l of ISRE luciferase reporter (component A) into cells following the procedure in **Generalized Transfection and Assay Protocols**.

3. After ~24 hours of transfection, dilute IFN $\alpha$  in assay medium and replace cell medium in the stimulated wells with 50  $\mu$ l of diluted IFN $\alpha$ .

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Change medium in the unstimulated control wells to 50µl of assay medium; add 50 µl of assay medium to cell-free control wells (for determining background luminescence).

Set up each treatment in at least triplicate.

4. Incubate cells at 37° in a CO<sub>2</sub> incubator for ~ 6 hours.

5. Perform TWO-step luciferase assay using TWO-Step Luciferase (Firefly & Renilla) Assay System (BPS Bioscience #60683): Dilute 100x Firefly Luciferase Reagent Substrate (Component B) into Firefly Luciferase Reagent Buffer (Component A). 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 (Component D) into Renilla Luciferase Reagent Buffer (Component C). Add 50 µl of Renilla Luciferase reagent per well, rock at room temperature for ~1 minute and measure Renilla luminescence.

7. To obtain the normalized luciferase activity for ISRE reporter, subtract background luminescence, then calculate the ratio of firefly luminescence from the ISRE reporter to *Renilla* luminescence from the control *Renilla* luciferase vector.

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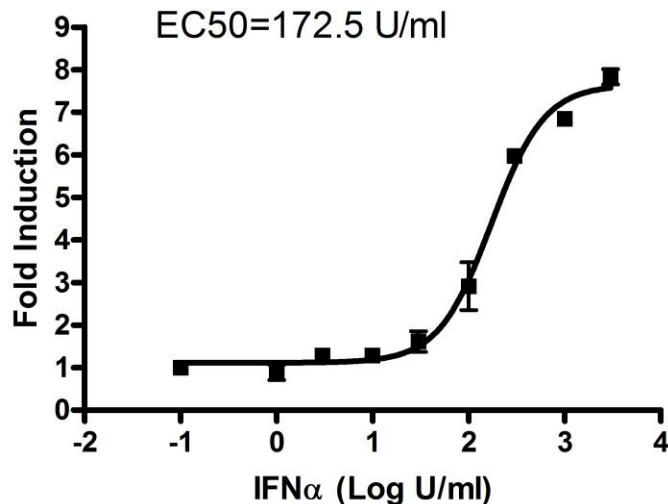
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**Figure 1. Dose response of ISRE reporter activity to IFN $\alpha$  in HEK293**

The results are shown as fold induction of normalized ISRE luciferase reporter activity. Fold induction was determined by comparing values against the mean value for control cells without IFN $\alpha$  treatment.

The EC<sub>50</sub> of IFN $\alpha$  is ~ 172.5 units/ml



**Sample protocol to determine the effects of JAK inhibitor on IFN $\alpha$ -induced ISRE reporter activity in HEK293 cells**

Additional materials required in this experiment setup

- JAK Inhibitor I (Pyridone 6) (Calbiochem # 420099): inhibitor of JAKs. Prepare stock solution in DMSO.
- Human Interferon Alpha A (IFN $\alpha$ ) (R&D Systems # 11100-1)
- HEK293 growth medium: MEM/EBSS (with L-glutamine) (Hyclone #SH30024.01) + 10% FBS + 1% non-essential amino acids + 1 mM Na pyruvate + 1% Pen/Strep
- Assay medium: same as HEK293 growth medium

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- TWO-Step Luciferase (Firefly & Renilla) Assay System (BPS Bioscience #60683)

1. One day before transfection, seed HEK293 cells at a density of 30,000 cells per well into a white clear-bottom 96-well plate in 100  $\mu$ l of growth medium. Incubate cells at 37°C in a CO<sub>2</sub> incubator for overnight.

2. The next day, transfect 1  $\mu$ l of ISRE luciferase reporter (component A) into cells following the procedure in **Generalized Transfection and Assay Protocols**.

3. After ~24 hours of transfection, prepare threefold serial dilution of JAK Inhibitor I assay medium. Replace the cell medium with 50  $\mu$ l of diluted JAK inhibitor I. The final concentration of DMSO in the wells is 0.1%. Change medium to 50  $\mu$ l of 0.1% DMSO assay medium to wells without inhibitor and cell-free control wells (for determining background luminescence).

Incubate cells at 37°C in a CO<sub>2</sub> incubator for ~ 1 hours

4. Add 5  $\mu$ l of diluted IFN $\alpha$  in assay medium to stimulated wells (final IFN $\alpha$  concentration = 1000 U/ml). Add 5  $\mu$ l of assay medium to the unstimulated control wells (cells without inhibitor and IFN $\alpha$  treatment) to determine the basal activity. Add 5  $\mu$ l of assay medium to cell-free control wells. Set up each treatment in at least triplicate.

5. Incubate at 37°C in a CO<sub>2</sub> incubator for ~6 hours.

6. Perform TWO-step luciferase assay using TWO-Step Luciferase (Firefly & Renilla) Assay System (BPS Bioscience #60683): Dilute 100x Firefly Luciferase Reagent Substrate (Component B) into Firefly Luciferase Reagent Buffer (Component A). Add 55  $\mu$ l 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 (Component D) into Renilla Luciferase Reagent Buffer (Component C). Add 55  $\mu$ l of Renilla Luciferase reagent per well, rock at room temperature for ~1 minute and measure Renilla luminescence.

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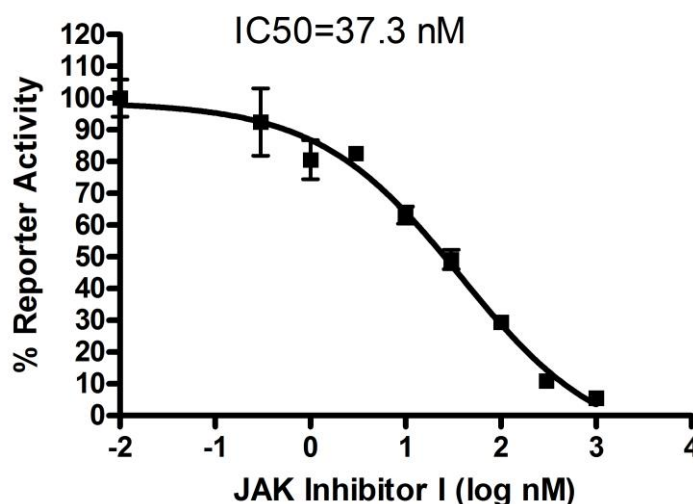


7. To obtain the normalized luciferase activity of ISRE reporter, subtract background luminescence then calculate the ratio of firefly luminescence from the ISRE reporter to *Renilla* luminescence from the control *Renilla* luciferase vector.

### Figure 2. JAK Inhibitor I inhibition dose response curve

The results are shown as the percentage of ISRE reporter activity. The normalized luciferase activity for cells stimulated with IFN $\alpha$  in the absence of JAK inhibitor I was set at 100%.

The IC<sub>50</sub> of JAK inhibitor I is ~ 37.3  $\mu$ M.



### References

1. Hebenstreit, D., *et al.* (2005). JAK/STAT-dependent gene regulation by cytokines. *Drug News Perspect* **18** (4): 243–249.
2. Pedranzini, L., *et al.* (2006). Pyridone 6, a pan-Janus-activated kinase inhibitor, induces growth inhibition of multiple myeloma cells. *Cancer Res.* **66** (19):9714-9721.

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## **Related Products**

<b><u>Product</u></b>	<b><u>Catalog #</u></b>	<b><u>Size</u></b>
TWO-Step Luciferase (Firefly & Renilla) Assay System	60683-1	10 mL
TWO-Step Luciferase (Firefly & Renilla) Assay System	60683-2	100 mL
TWO-Step Luciferase (Firefly & Renilla) Assay System	60683-3	1 L
ISRE Reporter - HEK293 Cell Line	60510	2 vials
Jak2 (JH2 domain) Enzyme	40449	10 µg
Jak2 (JH2 domain) Enzyme	40450	10 µg
Jak2 (JH1, JH2 domain) Enzyme	40451	10 µg
Jak3 Enzyme	40452	10 µg

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