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Data Sheet

The Transfection Collection™ – ISRE Transient Pack

JAK/STAT Signaling Pathway

Catalog #: 79264

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 (IFNα) is a Type I interferon. Binding of IFNα 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 Transient Pack* is designed to provide the tools necessary for transiently transfecting and monitoring the activity of Type I interferon-induced JAK/STAT signaling pathway in cultured HEK293 cells. The pack contains transfection-ready vectors containing firefly luciferase as a JAK/STAT 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 ISRE Transient Pack is the 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 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 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.

Additionally, the pack includes cell culture medium (BPS Medium 1) that has been optimized for use with HEK293 cells*. BPS Medium 1 includes MEM medium, 10% fetal bovine serum, 1% non-essential amino acids, sodium pyruvate, and 1% Pen/Strep.

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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 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, but an alternate cell culture medium may be required for optimal cell growth.

Applications

- Monitor IFNα-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	Amount	Storage
Reporter (Component A) ISRE 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 ready-to-use for transient transfection. They are NOT SUITABLE for transformation and amplification in bacteria.

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Materials Required but Not Supplied

- HEK293 cells. Other mammalian cell lines may also 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.
- Opti-MEM I Reduced Serum Medium (Life Technologies, #31985-062)
- 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 provided on a per well basis.

1. One day before transfection, seed HEK293 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 transfection is: **1 µl of Reporter** (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 transfection is: **1 µl of Reporter** (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.

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Note: we recommend setting up at least triplicate assays for each condition, 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.

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 complexes to each well containing cells and medium. Mix gently by tapping the plate.

4. Incubate cells at 37°C in a CO_2 incubator. After ~24 hours of transfection, treat cells with test activators/inhibitors for additional 6 to 24 hours. 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 by 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 at least 15 minutes at room temperature. Measure firefly luminescence using a luminometer. The signal under these conditions will be stable for

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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 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 ISRE reporter, subtract the background luminescence, then calculate the ratio of firefly luminescence from ISRE 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 dose response of HEK293 cells transfected with ISRE reporter to IFNα

Additional materials required in this experiment setup

- Human Interferon Alpha A (IFNα) (R&D Systems # 11100-1)
- HEK293 cells
- 96-well tissue culture treated white clear-bottom assay plate (Corning, #3610)

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 µl of BPS Medium 1. Incubate cells at 37°C in a CO_2 incubator for overnight.

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

3. After ~24 hours of transfection, dilute IFNα in fresh BPS Medium 1and replace cell medium in the stimulated wells with 50 µl of diluted IFNα.

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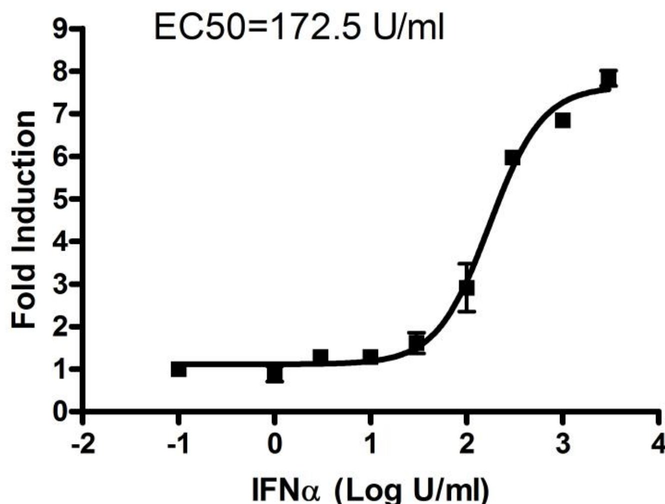
Change medium in the unstimulated control wells to 50 µl of BPS Medium 1; add 50 µl of BPS Medium 1 to cell-free control wells (for determining background luminescence). Set up each treatment in at least triplicate.

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

5. 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.

6. 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. To determine the fold induction, divide the value for the treated cells by the value for the untreated cells.

Figure 1. Dose response of ISRE reporter activity to IFNα 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 IFNα treatment.
The EC50 of IFNα is ~ 172.5 units/ml



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Sample protocol to determine the effects of JAK inhibitor on IFNα-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 (IFNα) (R&D Systems, #11100-1)

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 µl of BPS Medium 1. Incubate cells at 37°C in a CO_2 incubator for overnight.

2. The next day, transfect 1 µl 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 in BPS Medium 1. Replace the cell medium with 50 µl of diluted JAK inhibitor I. The final concentration of DMSO in the wells is 0.1%.

Change medium to 50 µl of 0.1% DMSO assay in BPS Medium 1 to wells without inhibitor and cell-free control wells (for determining background luminescence).

Incubate cells at 37°C in a CO_2 incubator for ~ 1 hours

4. Add 5 µl of diluted IFNα in BPS Medium 1 to stimulated wells (final IFNα concentration = 1000 U/ml).
Add 5 µl of BPS Medium 1 to the unstimulated control wells (cells without inhibitor and IFNα treatment) to determine the basal activity.
Add 5 µl of BPS Medium 1 to cell-free control wells.
Set up each treatment in at least triplicate.

5. Incubate 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 55 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 55 µl of Renilla Luciferase reagent per well, rock at room temperature for ~1 minute and measure Renilla luminescence.

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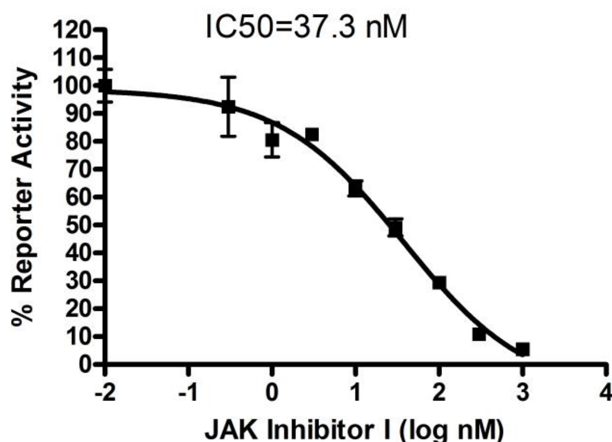
7. To obtain the normalized luciferase activity of ISRE reporter, subtract background luminescence then calculate the ratio of firefly luminescence from the ISRE reporter to *Renilla* luminescence from the control *Renilla* luciferase vector.

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 IFNα in the absence of JAK inhibitor I was set at 100%.

The IC50 of JAK inhibitor I is ~ 37. 3 μM.



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.

Refills

Product	Catalog #	Size
ISRE Reporter Kit	60613	500 rxns.
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
BPS Medium 1	79259	100 ml

Related Products

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Product	Catalog #	Size
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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