



6042 Cornerstone Court W, Ste B  
San Diego, CA 92121  
**Tel:** 1.858.829.3082  
**Fax:** 1.858.481.8694  
**Email:** info@bpsbioscience.com

## Data Sheet

### The Transfection Collection™ – NF-κB Transient Pack

#### *NF-κB Signaling Pathway*

Catalog #: 79268

#### **Background**

NF-κB (Nuclear Factor-KappaB) is a heterodimeric protein composed of different combinations of members of the Rel family of transcription factors. The NF-κB / Rel family of transcription factors (p50, p65, c-Rel, etc.) are involved in stress, immune, and inflammatory responses. In unstimulated cells, the NF-κB dimers are sequestered in the cytoplasm by inhibitory IκB proteins. Proinflammatory cytokines, LPS, growth factors, and antigen receptors activate IκB kinase (IKK), which phosphorylates the IκB proteins. Phosphorylation of IκB leads to its degradation, freeing NF-κB complexes to translocate to the nucleus, bind to NF-κB DNA response elements, and induce the transcription of the target genes.

#### **Description**

The *NF-κB Transient Pack* is designed to provide the tools necessary for transiently transfecting and monitoring the activity of the NF-κB signaling pathway in cultured HEK293 cells. The kit contains transfection-ready vectors containing firefly luciferase as a NF-κB 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 NF-κB Transient Pack is the NF-κB luciferase reporter vector. This reporter contains a firefly luciferase gene under the control of multimerized NF-κB responsive element located upstream of a minimal promoter. The NF-κB 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. Finally, the pack provides the Dual Luciferase (Firefly-Renilla) Assay System. These reagents provide highly sensitive, stable detection of firefly luciferase activity and *Renilla* luciferase activity.

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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 NF-κB pathway activity.
- Screen activators or inhibitors of the NF-κB signaling pathway.
- Study effects of RNAi or gene overexpression on the activity of the NF-κB pathway.

### Components

Component	Amount	Storage
<b>Reporter (Component A)</b> NF-κB luciferase reporter vector* + constitutively expressing Renilla luciferase vector*	500 µl (60 ng DNA/ µl)	-20°C
<b>Negative Control Reporter (Component B)</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 <i>Protect from light</i>
Renilla Luciferase Reagent Buffer	10 ml	Room Temp.
Renilla Luciferase Reagent substrate (100x)	100 µl	-20°C <i>Protect from light</i>
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.*

### 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)

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- 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)
- Luminometer

### Generalized Transfection and Assay Protocols

The following procedure is designed to transfet the reporter into HEK293 cells using Lipofectamine 2000 in a 96-well format. To transfet 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.

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.

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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 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 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 ~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 and mix well.  
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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: To obtain the normalized luciferase activity for the NF-κB reporter, subtract the background luminescence (wells with medium and luciferase reagent only), then calculate the ratio of firefly luminescence from NF-κB 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 NF-κB reporter to TNFα**

Additional materials required in this experiment setup

- TNFα (BPS Bioscience, #90244-A)
- HEK293 cells

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<sub>2</sub> incubator for overnight.

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

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

Change medium in the unstimulated control wells to 50 µl of fresh 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<sub>2</sub> 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

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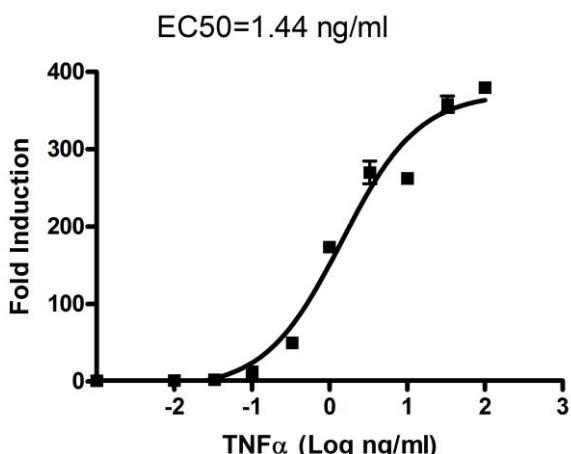
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Luciferase Reagent Buffer that has been equilibrated to room temperature . Add 50  $\mu$ l of Firefly Luciferase reagent per well and rock at room temperature for ~15 minutes, then measure firefly luminescence using a luminometer. Dilute 100x Renilla Luciferase Reagent Substrate into Renilla Luciferase Reagent Buffer . Add 50  $\mu$ l 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 NF- $\kappa$ B reporter, subtract background luminescence, then calculate the ratio of firefly luminescence from the NF- $\kappa$ B 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 NF- $\kappa$ B reporter activity to TNF $\alpha$  in HEK293**

The results are shown as fold induction of normalized NF- $\kappa$ B luciferase reporter activity. Fold induction was determined by comparing values against the mean value for control cells without TNF $\alpha$  treatment. The EC50 of TNF $\alpha$  is ~ 1.44 ng/ml.



**References**

1. Pessara, U., Koch, N. (1990). TNF alpha regulates expression of the major histocompatibility complex class II-associated invariant chain by binding of an NF-kappa B-like factor to a promoter element. *Mol Cell Biol.* **10(8)**:4146-4154.
2. Baeuerle, P.A. (1998). Pro-inflammatory signaling: last pieces in the NF-kappaB puzzle? *Curr Biol.* **8(1)**:R19-R22.

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### Refills

<u>Product</u>	<u>Cat. #</u>	<u>Size</u>
BPS Medium 1	79259	100 ml
NF-κB Reporter Kit (NF-κB Signaling Pathway)	60614	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

### Related products

<u>Product</u>	<u>Cat. #</u>	<u>Size</u>
NF-κB Reporter (Luc) - HEK293 Cell Line	60650	2 vials
NF-κB Reporter (Luc) - Jurkat Cell Line	60651	2 vials
NF-κB Reporter (Luc) - HCT116 Cell Line	60623	2 vials
NF-κB Reporter (Luc) - CHO-K1 Cell Line	60621	2 vials
TNF $\alpha$ (human)	90244-A	10 $\mu$ g
TNF $\alpha$ (human)	90244-B	50 $\mu$ g
TNF $\alpha$ (mouse)	90246-A	5 $\mu$ g

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