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## Data Sheet

### ***NF-κB Reporter Cellular Assay Pack (HCT-116)*** **Catalog #: 79326**

#### **Product Description**

The NF-κB Reporter Cellular Assay Pack provides all the key reagents required to monitor the activity of the nuclear factor Kappa B (NF-κB) signal transduction pathways. The pack contains the NF-κB Reporter (Luc)- HCT-116 Recombinant Cell Line, a luciferase reporter cell line that contains a firefly luciferase gene under the control of four copies of the NF-κB response element located upstream of the minimal TATA promoter. After activation by pro-inflammatory cytokines or stimulants of lymphokine receptors, endogenous NF-κB transcription factors bind to the DNA response elements, inducing transcription of the luciferase reporter gene. This cell line is validated for the response to TNFalpha and to treatment with NF-κB inhibitor, evodiamine.

Additionally, the pack includes cell culture medium (Thaw Medium 7) that has been optimized for use with HCT-116 cells. Thaw Medium 7 includes 10% fetal bovine serum and 1% Pen/Strep. Finally, the pack provides the ONE-Step™ Luciferase Detection System. This reagent provides highly sensitive, stable detection of firefly (*Photinus pyralis*) luciferase activity. The ONE-Step luciferase reagent can be used directly in cells in growth medium and can be detected with any luminometer; automated injectors are not required.

#### **Application**

The NF-κB reporter cell line is designed for screening inhibitors of NF-κB and for monitoring NF-κB signaling in response to stimulants such as the cytokines TNFα and IL-1β, pathogen-associated molecular pattern (PAMP) (i.e. flagellin) or endogenous damage-associated molecular pattern (DAMP) molecules (i.e. NOD1 ligand) (see application references). It is also suitable for establishing cell-based screens for inhibitors that target specific NF-κB stimulating molecules. This cell line can be further modified to allow investigation of downstream NF-κB activities as a result of targeted genetic mutation(s).

#### **Components**

<b>Cat. #</b>	<b>Component</b>	<b>Amount</b>	<b>Storage</b>
60623	NF-κB Reporter (Luc) – HCT-116 Cell Line	2 vials*	liquid nitrogen
60690-1	ONE-Step Luciferase Buffer ( <b>Component A</b> )	10 ml	-20°C
	ONE-Step Luciferase Reagent Substrate, 100x ( <b>Component B</b> )	100 µl	-20°C <i>Protect from light</i>
60185	Thaw Medium 7	100 ml	+4°C

\*Each vial contains ~3 X 10<sup>6</sup> cells in 1 ml of 10% DMSO in FBS.

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### General Culture conditions

**Thaw Medium 7 (BPS Bioscience #60185):** McCoy's 5A medium with 10% FBS, 1% Penicillin/Streptomycin.

**Complete Growth Medium:** Thaw Medium 7, 1 mg/ml Geneticin®, G418 Sulfate.

Cells should be grown at 37°C with 5% CO<sub>2</sub> using complete growth medium.

**To thaw the cells,** prepare a T-75 culture flask with 20 ml of pre-warmed Thaw Medium 7. Quickly thaw cells in a 37°C water bath with constant and slow agitation. After cleaning the outside of the vial with 70% ethanol, immediately transfer the entire content to Thaw Medium 7 (**no G418**). Avoid pipetting up and down, and gently rock the flask to distribute the cells. Incubate the cells in a humidified 37°C incubator with 5% CO<sub>2</sub>. 24-48 hours after incubation, change to fresh complete growth medium (**contains G418**), without disturbing the attached cells. Continue to change medium every 2-3 days until cells reach desired confluency. If slow cell growth occurs during resuscitation, increase FBS to 15% for the first week of culture. Cells should be split before they reach complete confluence.

**To passage the cells,** when cells reach 90% confluency, remove the medium and wash twice with PBS (without Magnesium or Calcium). Treat cells with 2-3 ml of 0.25% trypsin/EDTA and incubate for 2-3 minutes at 37°C. After confirming cell detachment by light microscopy, add 10 ml of pre-warmed complete growth medium and gently pipette up and down to dissociate cell clumps. Transfer cells to a 15 ml conical tube and centrifuge at 200 x g for 5 minutes. Remove the medium and resuspend cells in 10 ml pre-warmed growth medium. Dispense 2 ml of the cell suspension into a new T75 flask containing pre-warmed 18 ml complete medium (a subcultivation ratio of 1:2 to 1:10 is recommended). Incubate cells in a humidified 37°C incubator with 5% CO<sub>2</sub>. To freeze cells, re-suspend cell pellet in freezing medium (10% DMSO in FBS). Cells have been demonstrated to be stable for at least 15 passages; BPS Bioscience recommends preparing frozen stocks so cells are not used beyond passage 15.

**To freeze down the cells,** rinse cells with phosphate buffered saline (PBS), and detach cells from culture vessel with Trypsin/EDTA. Add complete growth medium and transfer to a tube, spin down cells, and resuspend in freezing medium (10% DMSO + 90% FBS). Place at -80°C overnight and place in liquid nitrogen the next day. Alternatively, vials may be placed directly in liquid nitrogen.

### Mycoplasma testing

The cell line has been screened using the MycoAlert™ Mycoplasma Detection Kit (Lonza, #LT07-118) to confirm the absence of Mycoplasma contamination. MycoAlert Assay Control Set (Lonza, #LT07-518) was used as a positive control.

### Assay performance

The following assays are designed for 96-well format. To perform the assay in different tissue culture formats, cell number and reagent volume should be scaled appropriately.

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### Materials Required but Not Supplied

- IL-1 $\beta$  (BPS Bioscience, #90168)
- TNF $\alpha$ , human (BPS Bioscience, #90244)
- IFN-gamma (BPS Bioscience, #90162)
- IFN-alpha (BPS Bioscience, #90158)
- 96-well tissue culture treated white clear-bottom assay plate (Corning, #3610)
- Luminometer

### A. TNF $\alpha$ dose response

1. Harvest NF- $\kappa$ B reporter (Luc)-HCT-116 cells from culture in complete growth medium and seed cells at a density of 5,000 cells per well into white opaque 96-well microplate in 45  $\mu$ l of Thaw Medium 7 (no G418).
2. Incubate cells at 37°C with 5% CO<sub>2</sub> overnight.
3. The next day, prepare threefold dilutions of TNF $\alpha$  in Thaw Medium 7 and add 5  $\mu$ l to TNF $\alpha$  stimulated wells.

Add 5  $\mu$ l of Thaw Medium 7 to the unstimulated control wells (for measuring uninduced level of NF- $\kappa$ B reporter activity).

Add 50  $\mu$ l of Thaw Medium 7 to cell-free control wells (for determining background luminescence).

4. Incubate at 37°C with 5% CO<sub>2</sub> for 7-8 hours.

Perform the luciferase detection assay using the ONE-Step™ Luciferase Assay System according to the protocol below:

### Luciferase Detection Procedure

5. Thaw Luciferase Reagent Buffer (**Component A**) by placing the reagent in a room temperature water bath. Equilibrate the buffer to room temperature and mix well before use. Note: Luciferase Reagent Buffer must be at ~ room temperature (20- 25°C) before use.
6. Calculate the amount of Luciferase Reagent needed for the experiment (**Component A + Component B**). Immediately prior to performing the experiment, prepare the luciferase assay working solution by diluting Luciferase Reagent Substrate (**Component B**) into Luciferase Reagent Buffer (**Component A**) at a 1:100 ratio and mix well. Avoid exposing to excessive light. *Only use enough of each component for the experiment, remaining **Component A** and **Component B** should be stored separately at -20°C.*
7. Remove multi-well plate containing mammalian cells from incubator. *Note: plates must be compatible with luminescence measurement with luminometer being used.*
8. Add 50  $\mu$ l of luciferase assay working solution (**Component A + Component B**) to the culture medium in each well.

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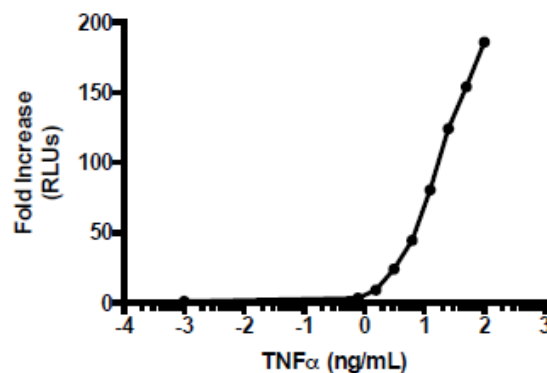
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9. Gently rock the plates for  $\geq 15$  minutes at room temperature. Measure firefly luminescence using a luminometer.

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.

**Data Analysis:** Background luminescence is a characteristic of luminometer performance, therefore, background luminescence must be subtracted from all readings for accuracy. Obtain the background-subtracted luminescence by NF- $\kappa$ B subtracting the average background luminescence (cell-free control wells) from the luminescence reading of all wells.

The fold induction of luciferase reporter expression = background-subtracted luminescence of TNF $\alpha$ -stimulated well / average background-subtracted luminescence of unstimulated control wells



**Figure 1. Analysis of NF- $\kappa$ B / HCT-116 reporter activity in response to TNF $\alpha$ .** NF- $\kappa$ B-Luciferase HCT-116 cells were seeded on a white opaque 96-well plate overnight at 5000 cells/well in complete growth medium. Cells were treated with human TNF $\alpha$  in growth medium and incubated for 7 hours at 37°C before the addition of ONE-Step™ Luciferase assay system. Luminescence was read using a luminometer and readings were normalized to wells that only contain medium to obtain the Relative Luminescence Units (RLUs). Error bar = standard deviation (SD), n=3.

#### **B. Analysis of NF- $\kappa$ B/HCT-116 reporter activity in response to various stimuli.**

1. Harvest NF- $\kappa$ B reporter (Luc)-HCT-116 cells from culture in complete growth medium and seed cells at a density of 5,000 cells per well into white opaque 96-well microplate in 45  $\mu$ l of Thaw Medium 7 (no G418). Note: Thaw Medium 7 contains 10% fetal bovine serum. Note: For some cytokines, use of serum free medium may result in greater induction of the HCT-116 cells.
2. Incubate cells at 37°C with 5% CO<sub>2</sub> overnight.

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3. Add 5  $\mu$ l of Thaw Medium 7 with cytokine to wells. Incubate cells overnight at 37°C with 5% CO<sub>2</sub>. We used IL-1 $\beta$ , 10, 50, or 100 ng/ml; IFN $\gamma$ , 2  $\mu$ g/ml; IFN $\alpha$ , 10<sup>4</sup> U/ml; TNF $\alpha$ , 0.8 ng/ml.  
Add 5  $\mu$ l of Thaw Medium 7 to the unstimulated control wells.  
Add 50  $\mu$ l of Thaw Medium 7 to cell-free control wells.

Incubate at 37°C with 5% CO<sub>2</sub> for 7-8 hours.

4. Perform the luciferase detection assay using the ONE-Step™ Luciferase Assay System according to the protocol below:

#### Luciferase Detection Procedure

5. Thaw Luciferase Reagent Buffer (**Component A**) by placing the reagent in a room temperature water bath. Equilibrate the buffer to room temperature and mix well before use. Note: Luciferase Reagent Buffer must be at ~ room temperature (20- 25°C) before use.
6. Calculate the amount of Luciferase Reagent needed for the experiment (**Component A + Component B**). Immediately prior to performing the experiment, prepare the luciferase assay working solution by diluting Luciferase Reagent Substrate (**Component B**) into Luciferase Reagent Buffer (**Component A**) at a 1:100 ratio and mix well. Avoid exposing to excessive light. *Only use enough of each component for the experiment, remaining **Component A** and **Component B** should be stored separately at -20°C.*
7. Remove multi-well plate containing mammalian cells from incubator. *Note: plates must be compatible with luminescence measurement with luminometer being used.*
8. Add 50  $\mu$ l of luciferase assay working solution (**Component A + Component B**) to the culture medium in each well.
9. Gently rock the plates for  $\geq$ 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.

**Data Analysis:** Background luminescence is a characteristic of luminometer performance, therefore, background luminescence must be subtracted from all readings for accuracy. Obtain the background-subtracted luminescence by subtracting the average background luminescence (cell-free control wells) from the luminescence reading of all wells.

The fold induction of NF- $\kappa$ B luciferase reporter expression = background-subtracted luminescence of cytokine-stimulated well / average background-subtracted luminescence of unstimulated control wells

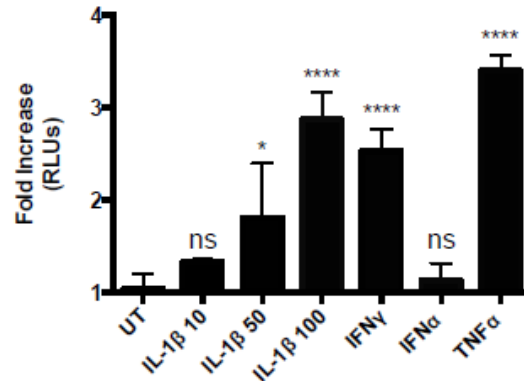
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**Figure 2. Analysis of NF-κB / HCT-116 reporter activity in response to cytokine stimulation.** NF-κB-Luciferase HCT-116 cells were seeded on a white opaque 96-well plate overnight at 5000 cells/ well in complete growth medium. Cells were treated with human cytokines in growth medium (IL-1β, 10, 50, or 100 ng/ml; IFNγ, 2 μg/ml; IFNα, 10<sup>4</sup> U/ml; TNFα, 0.8 ng/ml) and incubated for 7 hours at 37°C, followed by the addition ONE-Step™ Luciferase assay system. Luminescence was read using a luminometer and readings were normalized to wells containing only medium to determine the Relative Luminescence Unit (RLU). Fold increase is calculated with respect to untreated control cells (UT). Error bar = standard deviation (SD), n=3. \* P < 0.05, \*\*\*\* P < 0.0001, ns = not significant. One way ANOVA.

#### Application References Application References

1. Samuel T *et.al.* (2014) Variable NF-κB pathway responses in colon cancer cells treated with chemotherapeutic drugs. *BMC Cancer* **14**: 599.
2. Arabi A *et.al.* (2012) Proteomic screen reveals Fbw7 as a modulator of the NF-κB pathway. *Nature Communication* **3**: 976.
3. Clemo NK *et.al.* (2008) BAG-1 is up-regulated in colorectal tumour progression and promotes colorectal tumour cell survival through increased NF-κB activity. *Carcinogenesis* **29**: 849.
4. Tikhvatulin AI *et.al.* (2011) An *In Vitro* and *In Vivo* Study of the ability of NOD1 Ligands to Activate the Transcriptional Factor NF-κB. *Acta Naturae* **3**: 77.

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### Refills

<u>Product</u>	<u>Cat. #</u>	<u>Size</u>
NF- $\kappa$ B Reporter (Luc) - HCT116 Recombinant Cell Line	60623	2 vials
ONE-Step Luciferase Assay Detection System	60690-1	10 ml
ONE-Step Luciferase Assay Detection System	60690-2	100 ml
ONE-Step Luciferase Assay Detection System	60690-3	1 L
Thaw Medium 7	60185	100 ml

### Related Products

<u>Product</u>	<u>Cat. #</u>	<u>Size</u>
NF- $\kappa$ B Reporter (Luc) - Jurkat Recombinant Cell Line	60651	2 vials
NF- $\kappa$ B Reporter (Luc)-HEK293 Recombinant Cell Line	60650	2 vials
NF- $\kappa$ B Reporter (Luc)-CHO-K1 Recombinant Cell Line	60622	2 vials
NF- $\kappa$ B Reporter (Luc) – A549 Recombinant Cell Line	60625	2 vials
Transfection Collection™ : NF- $\kappa$ B Transient Pack	79268	500 rxns
NF- $\kappa$ B Reporter Kit	60614	500 rxns
TLR9/ NF- $\kappa$ B Reporter – HEK293 Recombinant Cell Line	60485	2 vials
OX40/ NF- $\kappa$ B Reporter – HEK293 Recombinant Cell Line	60482	2 vials
GITR/ NF- $\kappa$ B Reporter – HEK293 Recombinant Cell Line	60546	2 vials
CD40/ NF- $\kappa$ B Reporter – HEK293 Recombinant Cell Line	60626	2 vials
Interleukin-1 beta (IL-1 $\beta$ ), human	90168-B	10 $\mu$ g
TNF $\alpha$ , human	90244-A	10 $\mu$ g
TNF $\alpha$ , mouse	90246-B	20 $\mu$ g
IFN-alpha	90158-A	20 $\mu$ g
IFN-gamma	90162-A	20 $\mu$ g

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