Human NF-κB Reporter Assay System

3x 32 Assays in 96-well Format
Product # IB09001-32

Technical Manual
(version 7.3)

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I. Description

- The Assay System -

This assay kit utilizes HEK293t cells that express NF-κB (nuclear factor kappa-light-chain enhancer of activated B cells) and contain the luciferase reporter gene functionally linked to upstream NF-κB genetic response elements. Thus, quantifying changes in luciferase expression provides a sensitive surrogate measure of changes in the level of NF-κB activation.

NF-κB is a signal transduction dependent transcription factor. This NF-κB reporter cell line is validated to provide a robust dose-dependent activation response when treated with TNFα, or the Protein Kinase C activator Phorbol 12-myristate 13-acetate (PMA). As such, the principal application of this assay is in the screening of test samples to quantify any functional activities that they may exert to modulate, either induce or suppress, NF-κB activities.

INDIGO Bioscience’s assay kits are all-inclusive cell-based assay systems. In addition to NF-κB Reporter Cells, this kit provides two optimized media for use during cell culture and in diluting the user's test samples, a positive control activator of NF-κB, Luciferase Detection Reagent, and a cell culture-ready assay plate.

- The Assay Chemistry -

INDIGO’s cell-based assay format capitalizes on the extremely low background, high-sensitivity, and broad linear dynamic range of bio-luminescence reporter gene technology. Reporter Cells incorporate the cDNA encoding beetle luciferase, a 62 kD protein originating from the North American firefly (Photinus pyralis). Luciferase catalyzes the mono-oxidation of D-luciferin in a Mg\(^{2+}\)-dependent reaction that consumes O\(_2\) and ATP as co-substrates, and yields as products oxyluciferin, AMP, PP\(_i\), CO\(_2\), and photon emission. Luminescence intensity of the reaction is quantified using a luminometer, and is reported in terms of Relative Light Units (RLU’s).

INDIGO’s assay kits feature a luciferase detection reagent specially formulated to provide stable light emission between 5 and 90+ minutes after initiating the luciferase reaction. Incorporating a 5 minute reaction-rest period ensures that light emission profiles attain maximal stability, thereby allowing assay plates to be processed in batch. By doing so, the signal output from all sample wells, from one plate to the next, may be directly compared within an experimental set.

- Preparation of Test Compounds -

Test compounds are typically solvated at high-concentration in DMSO and stored frozen as master stocks. Immediately prior to setting up an assay, the master stocks are serially diluted using Compound Screening Medium (CSM; as described in Step 7) to achieve the desired assay concentrations. Do not use DMSO to further dilute test compound solutions. This method of dilution avoids the significant adverse effects of introducing high concentrations of DMSO into the assay. The final concentration of total DMSO carried over into assay reactions should never exceed 0.4%.

NOTE: CSM is formulated to help stabilize hydrophobic test compounds in the aqueous environment of the assay mixture. Nonetheless, high concentrations of extremely hydrophobic test compounds diluted in CSM may lack long-term stability and/or solubility, especially if further stored at low temperatures. Hence, it is recommended that test compound dilutions are prepared in CSM immediately prior to assay setup, and are considered to be ‘single-use’ reagents.
NOTE! The NF-κB assay protocol includes Day 1 steps and dispensed volumes that are different from the conventional INDIGO assay protocol that users may be accustomed to when setting up INDIGO's other Nuclear Receptor Assays.

*In brief,* 200 µl of Reporter Cells are dispensed into wells of the assay plate and pre-incubated for 4-6 hours. Following the pre-incubation period, culture media are discarded and 200 µl/well of the prepared treatment media are added. Following 22-24 hr incubation, treatment media are discarded and Luciferase Detection Reagent is added. Light emission (values of relative light units; RLU) from each assay well is quantified using a plate-reading luminometer.

- **Assay Scheme**

**Figure 1.** Assay workflow.
Figure 2a. TNFα and PMA dose-dependent activation of NF-κB
Activation of NF-κB is demonstrated by treating reporter cells with the activator TNFα (Tocris) and Phorbol 12-myristate 13-acetate (PMA; provided). Average relative light units (RLU) and corresponding standard deviation (SD) values were determined for each treatment concentration (n ≥ 6). Fold-activation and Z’ values were calculated as described by Zhang, et al. (1999)1. Non-linear regression and EC<sub>50</sub> analyses were performed using GraphPad Prism software. High Z’ scores confirm the robust performance of this assay, and its suitability for HTS<sup>1</sup>.


\[ Z' = 1 - \frac{3 \times (SD_{control} + SD_{background})}{RLU_{control} - RLU_{background}} \]

Figure 2b. QNZ dose-dependent inhibition of NF-κB
Human NF-κB Reporter Cells were treated with ~EC<sub>80</sub> of PMA and challenged with the antagonists QNZ (Abmole) or PS-1145 (Cayman). Both antagonists delivered > 4-fold reduction in PMA stimulated NF-κB activity.
II. Product Components & Storage Conditions

This Human NF-κB Assay kit contains materials to perform three distinct groups of assays in a 96-well plate format. Reagents are configured so that each group will comprise 32 assays. If desired, however, reagents may be combined to perform either 64 or 96 assays.

The aliquots of Reporter Cells are provided as a single-use reagents. Once thawed, reporter cells can NOT be refrozen or maintained in extended culture with any hope of retaining downstream assay performance. Therefore, extra volumes of these reagents should be discarded after assay setup.

Assay kits are shipped on dry ice. Upon receipt, individual kit components may be stored at the temperatures indicated on their respective labels. Alternatively, the entire kit may be further stored at -80°C.

To ensure maximal viability, “Reporter Cells” must be maintained at -80°C until immediately prior to use.

The date of product expiration is printed on the Product Qualification Insert (PQI) enclosed with each kit.

<table>
<thead>
<tr>
<th>Kit Components</th>
<th>Amount</th>
<th>Storage Temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-κB Reporter Cells</td>
<td>1 x 2.0 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>Cell Recovery Medium (CRM)</td>
<td>2 x 10.5 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>Compound Screening Medium (CSM)</td>
<td>1 x 45 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>PMA*, 30 µM (in DMSO)</td>
<td>1 x 30 µL</td>
<td>-20°C</td>
</tr>
<tr>
<td>Detection Substrate</td>
<td>3 x 2.0 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>Detection Buffer</td>
<td>3 x 2.0 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>Plate frame</td>
<td>1</td>
<td>ambient</td>
</tr>
<tr>
<td>Snap-in 8-well strips (white, sterile, cell culture treated wells)</td>
<td>12</td>
<td>-20°C</td>
</tr>
</tbody>
</table>

*PMA (Phorbol 12-myristate 13-acetate; CAS No. 16561-29-8) binds to, and is a potent activator of, Protein Kinase C (PKC), leading to the activation of NF-κB2.


III. Materials to be Supplied by the User

The following materials must be provided by the user, and should be made ready prior to initiating the assay procedure:

**DAY 1**
- cell culture-rated laminar flow hood.
- 37°C, humidified 5% CO₂ incubator for mammalian cell culture.
- 37°C water bath.
- 70% alcohol wipes
- 8- or 12-channel electronic, repeat-dispensing pipettes & sterile tips
- disposable media basins, sterile.
- sterile multi-channel media basins (such as the Heathrow Scientific "Dual-Function Solution Basin"), or deep-well plates, or appropriate similar vessel for generating dilution series of reference compound(s) and test compound(s).
- Optional: antagonist reference compound.
- Optional: clear 96-well assay plate, sterile, cell culture treated, for viewing cells on Day 2.

**DAY 2** plate-reading luminometer.
IV. Assay Protocol

Review the entire Assay Protocol before starting. Completing the assay requires an overnight incubation. Steps 1-12 are performed on Day 1, requiring less than 2 hours of bench work to complete, but including a 4 hr incubation step. Steps 13-18 are performed on Day 2, and require less than 1 hour to complete.

- A word about Antagonist-mode assay setup -

Receptor inhibition assays expose the Reporter Cells to a constant, sub-maximal concentration (typically between EC50 – EC85) of a known agonist AND the test compound(s) to be evaluated for antagonist activity. This NFκ-B assay kit includes a 30 µM stock solution of PMA, a potent activator of NF-κB that may be used to setup antagonist-mode assays. 1.5 nM PMA typically approximates EC80 in this cell-based assay. Hence, it presents a reasonable assay concentration of agonist to be used when screening test compounds for inhibitory activity.

Add the challenge activator, PMA, to a bulk volume of CSM at an EC50 – EC85 concentration. This medium is then used to prepare serial dilutions of test compounds to achieve the desired respective final assay concentrations. We find that this is an efficient and precise method of setting up NFκ-B antagonist assays, and it is the method presented in Step 7b of this protocol.

DAY 1 Assay Protocol: All steps must be performed using aseptic technique.

1.) Remove the 2 tubes of Cell Recovery Medium (CRM) from freezer storage, thaw and equilibrate to 37°C using a water bath.

2.) Rapid Thaw of the Reporter Cells: First, retrieve one or two tubes of CRM from the 37°C water bath and sanitize the outside surface(s) with a 70% ethanol swab. Second, retrieve Reporter Cells from -80°C storage: 1 tube for 32 assay wells, 2 tubes for 64 assay wells, or 3 tubes for 96 assay wells. Without delay, perform a rapid thaw of the frozen cells by transferring 6.4 ml of pre-warmed CRM into each tube of frozen cells. Recap the tube of Reporter Cells and immediately place it in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be 7.0 ml per tube.

3.) Retrieve the tube of Reporter Cell Suspension from the water bath and sanitize the outside surface with a 70% alcohol swab.

4.) If more than one tube of Reporter cells was thawed, combine them and gently invert several times to disperse cell aggregates and gain a homogenous cell suspension. Dispense 200 µl / well of cell suspension into the assay plate.

NOTE 4.1: Take special care to prevent cells from settling during the dispensing period. Allowing cells to settle during the transfer process, and/or lack of precision in dispensing uniform volumes across the assay plate will cause well-to-well variation (= increased Standard Deviation) in the assay.

NOTE 4.2: Users sometimes wish to examine the reporter cells using a microscope. If so, the extra volume of cell suspension provided with each kit may be dispensed into a clear 96-well cell culture treated assay plate. Continue to process the assay plate in identical manner to the white assay plate.

5.) Pre-incubate reporter cells: Place the assay plate into a 37°C, ≥ 85% humidity, 5% CO₂ incubator for 4 - 6 hours.
Near the end of the 4-6 hour pre-incubation period:

6.) Remove Compound Screening Medium (CSM) from freezer storage and thaw in a 37°C water bath.

7.) Prepare dilutions of test compound treatment media at the desired assay concentrations: Use CSM to prepare appropriate dilution series of test compound stocks. Prepare treatment concentrations at the desired final assay concentrations. In Step 9, the prepared treatment media are dispensed at 200 µl/well into the desired number of replicate assay wells. Manage dilution volumes carefully; this assay kit provides 45 ml of CSM.

   NOTE: Total DMSO carried over into assay reactions should never exceed 0.4%.

   a. Agonist-mode assays. This NF-κB Assay kit includes a 30 µM stock solution of Phorbol 12-myristate 13-acetate (PMA) a potent activator of Protein Kinase C, a critical intermediate in transduction pathways that converge on NF-κB activation. The following 7-point treatment series, prepared in serial 3-fold decrements, provides a suitable dose-response: 30.0, 10.0, 3.33, 1.11, 0.370, 0.123 and 0.0412 µM (final assay concentrations), and including a 'no treatment' control. APPENDIX 1 provides an example for generating such a dilution series.

   Alternatively, Tumor Necrosis Factor alpha (TNFα) is also a potent activator of NF-κB, and is commonly used as a reference for NF-κB activation studies (see Figure 2).

   ~ or ~

   b. Antagonist-mode assays. When setting antagonist assays, first supplement a bulk volume of CSM with the challenge activator, PMA, to achieve the desired final assay-concentration (refer to "A word about antagonist-mode assay setup", pg. 7). The PMA-supplemented CSM is then used to generate dilutions of test compound samples to achieve their final assay concentrations.

8.) At the end of the cell pre-incubation period: Discard the culture media.

   Because the assay plate is composed of a frame with snap-in strip-wells, the practice of physically ejecting media is NOT advised. Complete removal of the media is efficiently performed by tilting the plate on edge and aspirating media using an 8-pin manifold (e.g., Wheaton Science Microtest Syringe Manifold, # 851381) affixed to a vacuum-trap apparatus. Do not touch the well bottom, or run the tip of the aspiration device around the bottom circumference of the assay well. Such practices will result in destruction of the reporter cells and greatly increased well-to-well variability.

9.) Dispense 200 µl of each treatment media into appropriate wells of the assay plate.

10.) Transfer the assay plate into a 37°C, humidified 5% CO2 incubator for 22 - 24 hours.

   NOTE: Ensure a high-humidity (≥ 85%) environment within the cell culture incubator. This is critical to prevent the onset of deleterious "edge-effects" in the assay plate.

11.) For greater convenience on Day 2, retrieve from freezer storage the appropriate number of vials of Detection Substrate and Detection Buffer and place them in a dark refrigerator (4°C) to thaw overnight.
13.) 30 minutes before intending to quantify NF-κB activity, remove Detection Substrate and Detection Buffer from the refrigerator and place them in a low-light area so that they may equilibrate to room temperature. Once at room temperature, gently invert each tube several times to ensure homogenous solutions.

   \textit{NOTE:} Do NOT actively warm Detection Substrate above room temperature. If these solutions were not allowed to thaw overnight at 4°C, a room temperature water bath may be used to expedite thawing.

14.) Set the plate-reader to "luminescence" mode. Set the instrument to perform a single 5 second "plate shake" prior to reading the first assay well. Read time may be set to 0.5 second (500 mSec) per well, or less.

15.) \textbf{Immediately before proceeding to Step 16}, transfer the entire volume of Detection Buffer into the vial of Detection Substrate, thereby generating a 12 ml volume of Luciferase Detection Reagent (LDR). Mix gently to avoid foaming.

16.) Following 22 - 24 hours incubation in treatment media, retrieve the assay plate from the incubator. Discard all media contents by ejecting it into an appropriate waste container. Gently tap the inverted plate onto a clean absorbent paper towel to remove residual droplets. Cells will remain tightly adhered to well bottoms.

17.) Add 100 µl of LDR to each well of the assay plate. Allow the assay plate to rest at room temperature for at least 5 minutes following the addition of LDR. Do not shake the assay plate during this period.

18.) Quantify luminescence.
V. Related Products

<table>
<thead>
<tr>
<th>Human NF-κB Assay Kit Products</th>
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<tbody>
<tr>
<td><strong>Product No.</strong></td>
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<td>IB09001-32</td>
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<td>IB09001</td>
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<td>IB09002</td>
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Bulk assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.

<table>
<thead>
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<th>LIVE Cell Multiplex (LCM) Assay Products</th>
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<tr>
<td><strong>Product No.</strong></td>
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<td>LCM-10</td>
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Please refer to INDIGO Biosciences website for updated product offerings.

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VI. Limited Use Disclosures

Products commercialized by INDIGO Biosciences, Inc. are for RESEARCH PURPOSES ONLY – not for therapeutic or diagnostic use in humans.

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APPENDIX 1
Example scheme for the serial dilution of Phorbol 12-myristate 13-acetate (PMA), and the setup of an NF-κB activation dose-response assay.