Human NF-κB
Reporter Assay System

96-well Format Assays
Product # IB09001

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Technical Manual
(version 7.4i)

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Human NF-κB Reporter Assay System
96-well Format Assays

I. Description

▪ The Assay System ▪

This assay kit utilizes HEK293 cells that express **NF-κB** (nuclear factor kappa-light-chain enhancer of activated B cells). In addition, these cells have been engineered to 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 transcriptional activity.

NF-κB is a signal transduction dependent transcription factor. These reporter cells and assay kit components are validated to provide both robust activation and inhibition response when treated with the respective reference compounds (see Figures 2 and 3). The Protein Kinase C activator Phorbol 12-myristate 13-acetate (PMA) is a potent activator of NF-κB, and it is provided in this kit as a reference activator, and for use in setting up inhibition-mode assays.

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 activate or inhibit, NF-κB transcriptional activities.

INDIGO'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 collagen-coated cell culture-ready assay plate.

▪ The Assay Chemistry ▪

INDIGO's assay kits capitalize 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 ▪

Small molecule test compounds are typically solvated in DMSO at high concentrations; ideally 1,000x-concentrated stocks relative to the highest desired treatment concentration in the assay. Using high-concentration stocks minimizes DMSO carry-over into the assay plates. Immediately prior to setting up an assay, the master stocks are serially diluted using one of two alternative strategies:

1.) As described in *Step 8* and depicted in Appendix 1 for the reference activator PMA, **Compound Screening Medium (CSM)** may be used as the diluent to make serial dilutions of test compounds to achieve the desired final assay concentration series.

Alternatively, if test compound solubility is expected to be problematic,
2.) DMSO may be used to make serial dilutions, thereby generating 1,000x-concentrated stocks for each independent test concentration. Treatment media are then prepared using CSM to make final 1,000-fold dilutions of the prepared DMSO dilution series.

Regardless of the dilution method used, the final concentration of total DMSO (or any organic solvent) carried over into assay wells should not exceed 0.4%. Significant DMSO-induced cytotoxicity can be expected above 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 then considered to be 'single-use' reagents.

▪ Considerations for Automated Dispensing ▪

When processing a small number of assay plates, first carefully consider the dead volume requirement of your dispensing instrument before committing assay reagents to its setup. In essence, "dead volume" is the volume of reagent that is dedicated to the instrument plumbing; it will *not* be available for final dispensing into assay wells. The following Table provides information on reagent volume requirements, and available excesses.

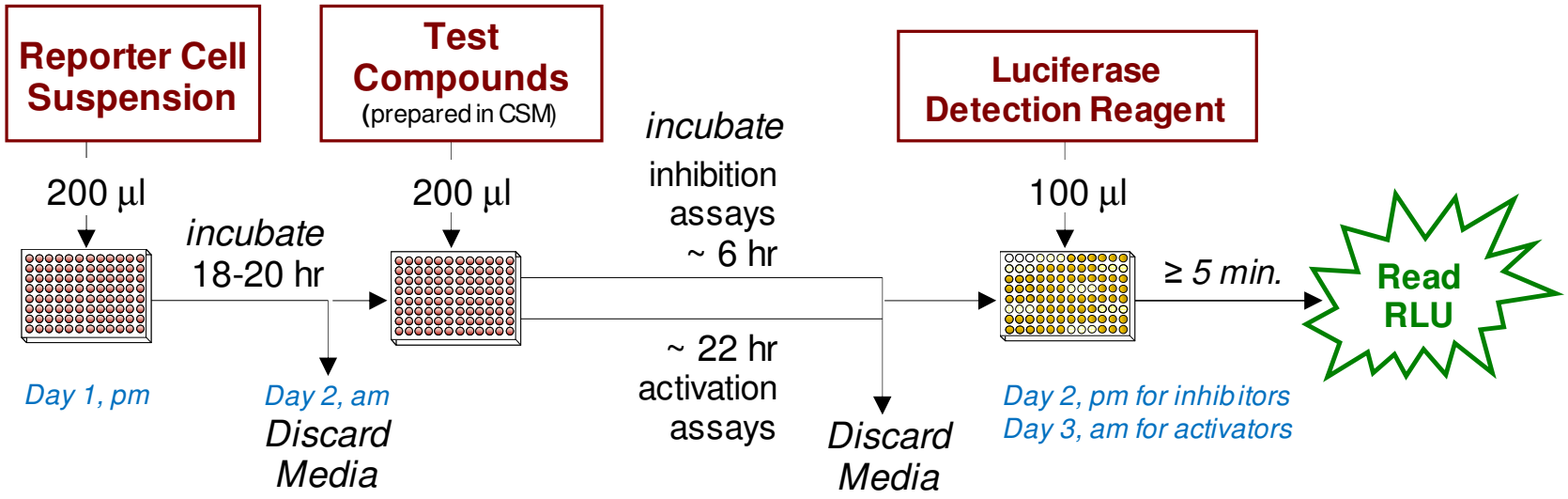
Stock Reagent & Volume provided	**Volume to be Dispensed** *(96-well plate)*	**Excess rgt. volume available for instrument dead volume**
Reporter Cell Suspension 21 ml *(prepared from kit components)*	200 µl / well 19.2 ml / plate	~ 1.8 ml
LDR 12 ml *(prepared from kit components)*	100 µl / well 9.6 ml / plate	~ 2.4 ml

▪ Assay Scheme ▪

Figure 1. Assay workflows for NF-κB activation and inhibition assays. It is recommended to begin assay setups in the late afternoon (pm) of *Day 1*. In brief, 200 µl/well of Reporter Cells are dispensed into the assay plate, which is then incubated overnight (**18-20 hours**). In the morning (am) of *Day 2*, the culture media are discarded and 200 µl/well of the prepared treatment media are added. Following an incubation period*, treatment media are discarded, and Luciferase Detection Reagent is added. The intensity of light emission (in terms of 'Relative Light Units'; RLU) from each assay well is quantified using a plate-reading luminometer.

*For optimal **NF-κB activation** responses it is recommended to incubate the reporter cells with treatment media for 22-24 hours. Hence, for NF-κB *activation* assays the assay plate is processed in the morning of *Day 3* to quantify luciferase activities.

For **NF-kB inhibition** assays it is recommended to incubate the reporter cells with treatment media for 6 hours. We find that longer treatment periods lead to significant reporter cell toxicity that degrades, or obliterates, inhibition-mode assay performance. Hence, for NF-κB *inhibition* assays the assay plate is processed in the afternoon of *Day 2* to quantify luciferase activities.



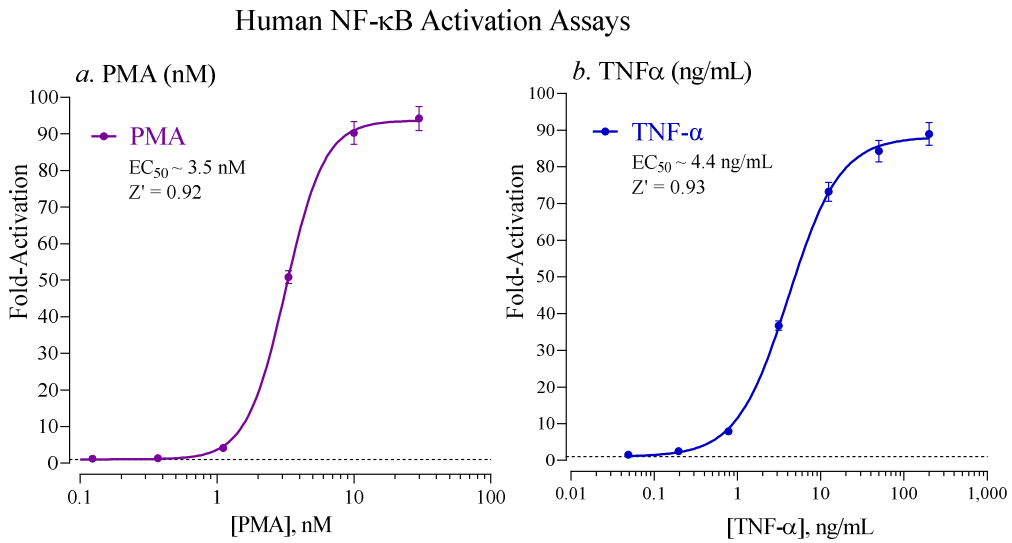


Figure 2. PMA and TNFα activation of Human NF-κB.
Activation of NF-κB is demonstrated by treating reporter cells with *(a.)* Phorbol 12-myristate 13-acetate (PMA; provided), and *(b.)* TNFα (Tocris) for 22 hours, following the protocol for activation assays depicted in Figure 1. Average relative light units (RLU) and corresponding standard deviation (SD) values were determined for each treatment concentration ($n \geq 4$). Fold-activation and Z' values were calculated as described by Zhang, *et al*. (1999)[1]. Non-linear regression and EC_{50} analyses were performed using GraphPad Prism software. High Z' scores confirm the robust performance of this assay, and its suitability for HTS[1].

[1] Zhang JH, Chung TD, Oldenburg KR. (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen.:**4**(2), 67-73.

$$Z' = 1 - [3*(SD^{Ref\ EC100} + SD^{Untreated}) / (RLU^{Ref\ EC100} - RLU^{Untreated})]$$

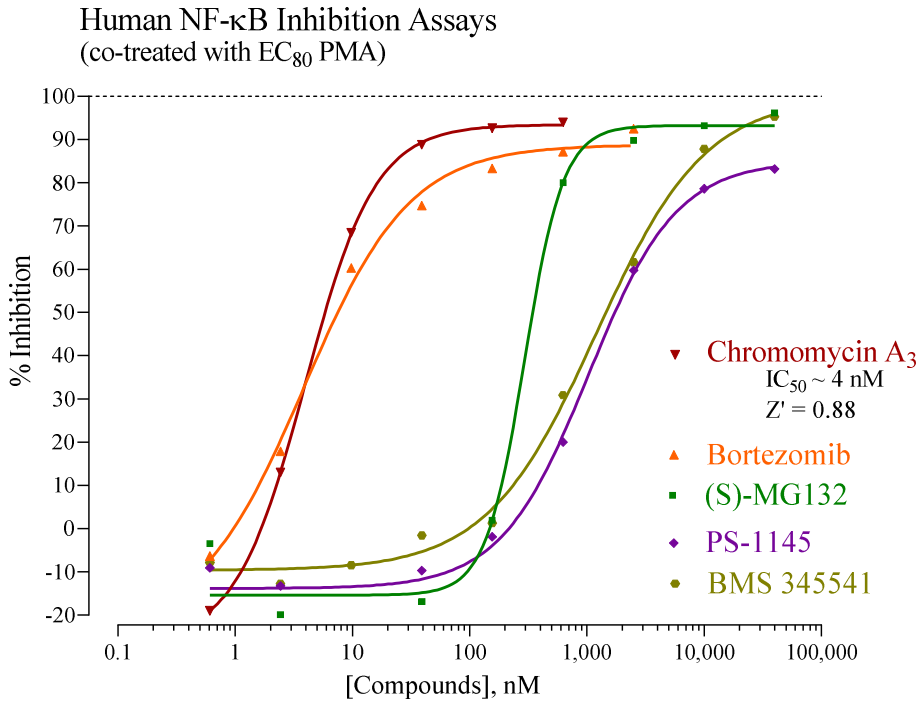


Figure 3. Dose-dependent inhibition of NF-κB.
Human NF-κB Reporter Cells were treated with ~EC_{80} of PMA and challenged with the inhibitors Chromomycin A_3, Bortezomib, (S)-MG132, PS-1145 and BMS 345541 (all from Cayman Chemical). Reporter cells were treated for 6 hours, following the protocol for inhibition assays depicted in Figure 1.

II. Product Components & Storage Conditions

This Human NF-κB Assay kit contains materials to perform assays in a single collagen-coated 96-well assay plate.

Reporter cells are temperature sensitive! To ensure maximal viability the tube of cells must be maintained at -80°C until immediately prior to the rapid-thaw procedure described in *Step 2* of this protocol.

Assay kits are shipped on dry ice. Upon receipt of the kit transfer it to -80°C storage. If you wish to first inspect and inventory the individual kit components be sure to first transfer and submerge the tube of reporter cells in dry ice.

The aliquot of Reporter Cells is provided as a single-use reagent. Once thawed, reporter cells can NOT be refrozen, nor can they be maintained in extended culture with any hope of retaining downstream assay performance. Therefore, extra volumes of these reagents should be discarded after assay setup.

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

Kit Components	***Amount***	***Storage Temp.***
▪ NF-κB Reporter Cells	1 x 2.0 mL	**-80°C**
▪ Cell Recovery Medium (CRM)	2 x 10.5 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 45 mL	-20°C
▪ PMA*, 30 µM (in DMSO) (reference for NF-κB activation *via* PKC pathways)	1 x 30 µL	-20°C
▪ Detection Substrate	1 x 6.0 mL	**-80°C**
▪ Detection Buffer	1 x 6.0 mL	-20°C
▪ 96-well, *collagen-coated* assay plate (white, sterile, cell-culture ready)	1	**-20°C**

NOTE: This assay kit contains a 96-well assay plate that has been collagen-coated and dried; store frozen (-20°C or colder) until use.

*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-κB[2].

[2] Moscat J, Diaz-Meco MT, and Rennert P. (2003) NF-kB activation by protein kinase C isoforms and B-cell function. EMBO Reports:**4**(1), 31-36.

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
- ▪ dry ice bucket (*Step 2*)
- ▪ cell culture-rated laminar flow hood.
- ▪ 37°C, humidified 5% CO_2 incubator for mammalian cell culture.
- ▪ 37°C water bath.
- ▪ 70% alcohol wipes
- ▪ 8-channel electronic, repeat-dispensing pipettes & sterile tips
- ▪ disposable media basins, sterile.

DAY 2
- ▪ sterile multi-channel media basins (such as the Heathrow Scientific "Dual-Function Solution Basin"), *or* sterilized 96 deep-well blocks (*e.g.,* Axygen Scientific, #P-2ML-SQ-C-S), *or* appropriate similar vessel for generating dilution series of reference and test compound(s).
- ▪ plate-reading luminometer.
- ▪ *Optional:* NF-κB inhibitor reference compound (refer to Figure 3)
- ▪ *Optional:* clear 96-well assay plate, sterile, cell culture treated, for viewing cells on *Day 2*.

IV. Assay Protocol

Review the entire Assay Protocol before starting. It is recommended that *Steps 1-6* are performed in the late afternoon on ***Day 1***; these will require less than a hour of bench work to complete. An overnight incubation (18-20 hours) is required. *Steps 7-17* are performed in the morning of ***Day 2***; approximately 2 hours of preliminary benchwork is required.

As depicted in **Figure 1**, NF-κB *Inhibition*-assays are performed using a 6-hour treatment period, with the quantification of luciferase activity in the afternoon of *Day 2*.

NF-κB *Activation*-assays are performed using a 22 - 24 hour treatment period, with the quantification of luciferase activity the following morning on *Day 3*.

▪ A word about Inhibition-mode assay setup ▪

Inhibition assays expose the Reporter Cells to a constant, sub-maximal concentration (typically between EC_{50} – EC_{85}) of a known NF-κB activator AND the test compound(s) to be evaluated for inhibitory activities. This assay kit includes a 30 µM stock solution of **PMA**, a potent activator of NF-κB that may be used to setup inhibition-mode assays. 1.5 nM PMA typically approximates EC_{80} in this cell-based assay. Hence, it presents a suitable co-treatment concentration to be used to screen test compounds for inhibitory activity.

Alternatively, some users may prefer to use Tumor Necrosis Factor alpha (TNFα) as the challenge activator; that strategy works equally well.

Add the challenge activator, PMA (or user-provided TNFα), to a bulk volume of **CSM** at an EC_{50} – EC_{85} 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 inhibition assays, and it is the method presented in *Step 7b* of this protocol.

DAY 1 Assay Protocol: It is recommended to begin mid- to late afternoon. 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 the two tubes of **CRM** from the 37°C water bath and sanitize their outside surfaces with a 70% ethanol swab.

Second, retrieve the tube of **Reporter Cells** from -80°C storage, place it directly into a dry ice bucket and transport the cells to the laminar flow hood. When ready, transfer the tube of reporter cells into a rack and, *without delay*, perform a rapid thaw of the cells by transferring 9.5 ml from ***each* of the 2 tubes** of 37°C CRM into the tube of frozen cells. Place the tube of Reporter Cells in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be **21 ml**.

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

4.) Gently invert the tube of Reporter Cells several times to gain a homogenous cell suspension. Transfer the cell suspension into a media basin and, using an 8-chanel pipette, dispense 200 µl/well of cell suspension into the collagen-coated assay plate.

NOTE 4.1: If INDIGO's Live Cell Multiplex Assay is to be incorporated, a minimum of 3 'blank' wells (meaning cell-free, but containing 'CSM') must be included in the assay plate to allow quantification of fluorescence background (refer to the LCMA Technical Manual).

NOTE 4.2: Increased well-to-well variation (= increased standard deviation!) will occur if care is not taken to prevent cells from settling in the basin during the dispensing period. Likewise, take care to ensure precision in dispensing exact volumes across the assay plate.

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NOTE 4.3: 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, *collagen-coated* 96-well assay plate. Continue to process the clear plate in identical manner to the white assay plate.

NOTE 4.4: If well-to-well variation due to 'edge-effects' is a concern this problem *may* be mitigated by dispensing sterile liquid into the *inter-well* spaces of the assay plate. Simply remove 1 tip from the 8-chanel dispenser and dispense 100 µl of sterile water into each of the seven inter-well spaces per column of wells.

5.) Pre-incubate reporter cells. Place the assay plate into a cell culture incubator (37°C, ≥ 70% humidity, 5% CO_2) for 18 - 20 hours.

NOTE: Ensure a high-humidity (≥ 70%) environment within the cell culture incubator. This is critical to prevent the onset of deleterious "edge-effects" in the assay plate.

6.) For greater convenience on *Day 2*, transfer **Compound Screening Medium (CSM)** from freezer storage into a refrigerator (+4°C) to thaw overnight.

DAY 2 Assay Protocol: It is recommended to begin first thing in the morning.

7.) Near the end of the preliminary overnight incubation period remove **Compound Screening Medium (CSM)** from the refrigerator and allow it to warm to room temperature.

8.) Prepare the Test Compound and Reference Compound treatment media at the desired final assay concentrations. Use **CSM** to prepare an appropriate dilution series of the reference and test compound stocks. Prepare all treatment media at the desired final assay concentrations. In *Step 10*, the prepared treatment media will be dispensed at 200 µl / well into the assay plate. Manage dilution volumes carefully; this assay kit provides **45 ml** of CSM.

NOTE: Total DMSO (or any organic solvent) carried over into assay reactions should not exceed 0.4%.

***a. Activation*-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 complete dose-response: 30.0, 10.0, 3.33, 1.11, 0.370, 0.123 and 0.0412 µM. Always include a 'no treatment' control. **APPENDIX 1** provides an example for generating such a dilution series.

Alternatively, TNFα is also a potent activator of NF-κB and is commonly used in NF-κB activation (see Figure 2b) and inhibition studies.

~ or ~

***b. Inhibition*-mode assays**. When setting up inhibition 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 inhibition-mode assay setup*", pg. 9). The PMA-supplemented CSM is then used to generate dilutions of test compound stocks to achieve their final assay concentrations.

9.) At the end of the 18 - 20 hour cell incubation period discard the culture media. The preferred method is to use a 'wrist flick' to manually eject media into an appropriate waste collection container. *Gently* tamp the inverted plate onto a clean absorbent paper towel to remove residual droplets. Cells will remain tightly adhered to well bottoms.

10.) Dispense **200 µl / well** of each prepared treatment media into the assay plate.

NOTE: If well-to-well variation due to 'edge-effects' is a concern this problem *may* be mitigated by dispensing sterile liquid into the *inter-well* spaces of the assay plate.

11.) Transfer the assay plate into a cell culture incubator (37°C, ≥ 70% humidity, 5% CO_2). Incubate the assay plate 6 hours for **inhibition** assays, or 22 - 24 hours for **activation** assays (refer to Figure 1).

12.) Near the end of the treatment period*, retrieve **Luciferase Detection Buffer** and **Luciferase Detection Reagent** from freezer storage and place them in a low-light area so that they may thaw and equilibrate to room temperature. Do NOT actively warm Detection Substrate above room temperature; if needed, a room temperature water bath may be used to expedite thawing.

(*6 hours treatment for *inhibition* assays; 22-24 hours treatment for *activation* assays.)

13.) Turn on the plate-reader and set the instrument to "luminescence" mode. Set the instrument to perform a single 5 second "plate shake" prior to reading the first assay well. Set the read-time per assay well to 0.5 second (500 mSec), *or less*.

14.) Immediately before proceeding to *Step 15*, 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, then transfer LDR to a media basin.

15.) Following the treatment period*, discard the media contents by manually 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.

(*6 hours incubation for *inhibition* assays; 22-24 hours incubation for *activation* assays.)

16.) Use an 8-channel pipette to dispense 100 µl of **LDR** to each well of the assay plate. Allow the plate to rest at room temperature for 5-10 minutes following the addition of LDR. Do not shake the plate during this period.

17.) Quantify luminescence.

V. Related Products

Product No.	*Product Descriptions*
Human NF-κB Assay Kit Products	
IB09001-32	3x 32 NF-κB assays; strip-wells in 96-well plate frame
IB09001	1x 96-well format NF-κB assays
IB09002	1x 384-well format NF-κB assays
Bulk assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

LIVE Cell Multiplex (LCM) Assay	
LCM-01	Reagent volumes sufficient to perform **96** Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well assay plate formats
LCM-05	Reagent in **5x bulk volume** to perform **480** Live Cell Assays performed in 5 x 96-well assay plates
LCM-10	Reagent in **10x bulk volume** to perform **960** Live Cell Assays performed in 10 x 96-well assay plates
INDIGlo Luciferase Detection Reagent	
LDR-10, -25, -50, -500	INDIGlo Luciferase Detection Reagents in 10 mL, 25 mL, 50 mL, and 500 mL volumes

Please refer to INDIGO Biosciences website for updated product offerings.

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Product prices, availability, specifications, and claims are subject to change without prior notice.

APPENDIX 1

Example scheme for the serial dilution of PMA, and the setup of an NF-κB activation dose-response assay.