Human Activator Protein-1 (AP-1) Reporter Assay System

3x32 Assays in 96-well Plate Format
Product # IB24001-32

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Technical Manual
(version 7.4i)

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3x32 Assays in 96-well Plate Format

I. Description

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▪ The Assay System ▪

AP-1 is a dimeric transcription factor composed of homodimers or heterodimers of members of the JUN (c-Jun, JunB and JunD), FOS (c-Fos, FosB, Fra-1 and Fra-2), ATF (ATF2, ATF3,/LRF1, ATF4, ATF5, ATF6B, ATF7, B-ATF, B-ATF3, JDP1 and JDP2) and MAF (MafA, MAfB, cMAf, Nrl and MafF/G.K) families of proteins. These proteins share a characteristic basic leucine zipper (bZIP) DNA-binding domain[1]. The leucine zipper drives the dimer formation, while the basic region is required for binding to the DNA response element. The variety of AP-1 dimer configurations confer versatility to this transcription factor in regulating numerous physiological (cell proliferation, differentiation, apoptosis, migration) and pathological activities (cancer, inflammation, transplant rejection) in the cell[1].

The most studied form of AP-1 transcription factor is c-Jun/Fos heterodimer. Upon activation, this heterodimer binds a palindromic DNA binding sequence referred to as the TPA response element (TRE)[2,3].

AP-1 is activated by a variety of physiological and environmental stimuli such as growth factors, cytokines, stress, ultraviolet radiation, and bacterial and viral infections. The dimer composition of AP-1, the cell type and the stage of development are all important in the regulation of AP-1 activity. Upon stimulation, a cascade of kinases is activated leading ultimately to the activation of MAPK, ERK and/or JNK, which in turn regulate Fos and Jun on the transcriptional and post-translational level[3].

INDIGO's Reporter Cells contain an engineered luciferase reporter gene functionally linked to tandem TPA response element (TRE) sequences positioned immediately upstream of a minimal promoter. Activated AP-1 binds to its TRE to drive Luc gene expression. Thus, quantifying changes in luciferase activity in the treated reporter cells provides a sensitive surrogate measure of changes in AP-1 activity. Accordingly, the principal application of this reporter assay is in the screening of test compounds to quantify any functional activities, either activating or inhibitory, that they may exert against AP-1.

Reporter Cells are prepared using INDIGO's proprietary CryoMite™ process. This cryo-preservation method yields high cell viability post-thaw and provides the convenience of immediately dispensing healthy reporter cells into assay plates. There is no need for intermediate preparatory steps such as the spin-and-rinse of cells, viability determinations, or cell titer adjustments prior to assay setup.

INDIGO's Reporter Assays are all-inclusive cell-based assay systems. In addition to AP-1 Reporter Cells, this kit provides two optimized media for use during cell culture and in diluting test samples, the reference AP-1 activator Phorbol 12-myristate 13-acetate (PMA), Luciferase Detection Reagent, and a cell culture-ready assay plate.

[1] Bejjani F et. al., 2019, Biochim Biophys Acta Rev Cancer; 1872(1):11-23. doi:10.1016/j.bbcan.2019.04.003. Epub 2019 Apr 26. PMID: 31034924 Review
[2] Lee W, Mitchell P, Tjian R., 1987, Cell, 49(6): 741-752. doi: 10.1016/0092-8674(87)90612-x. PMID:3034433.
[3] Shaulian E and Karin M., 2002, Nature Cell Biology; 4(5):E131-6. doi:10.1038/ncb0502-e131. PMID: 11988758 Review

▪ 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 at least 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%.

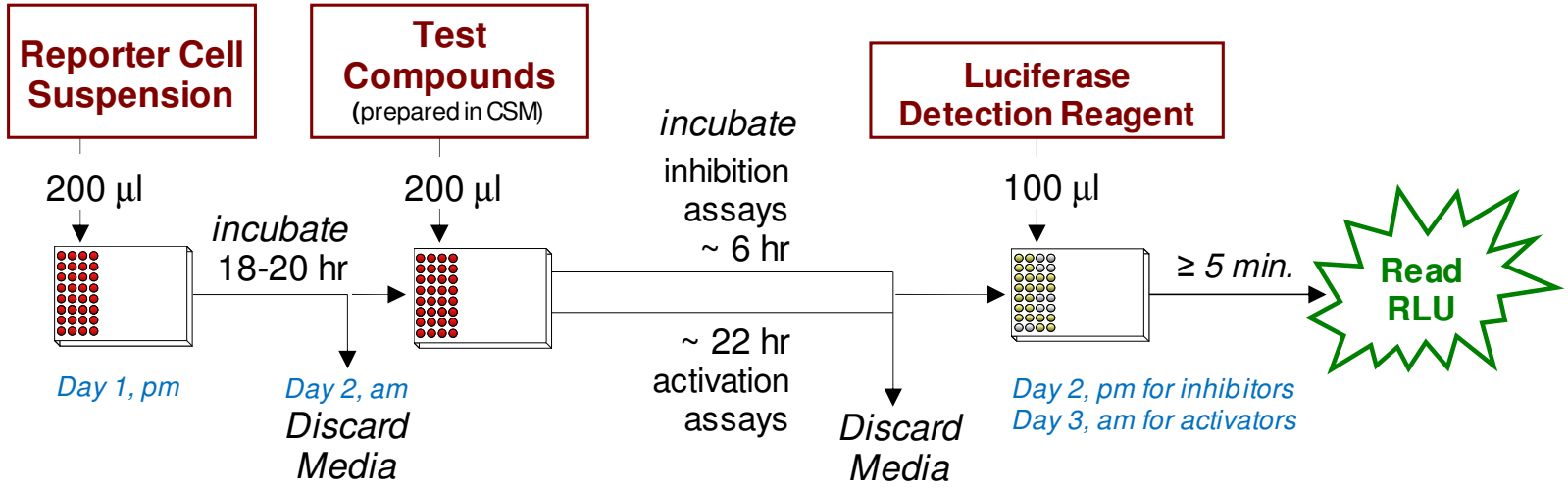
> *NOTE:* CSM is formulated to help stabilize hydrophobic test compounds in the aqueous environment of the assay mixture. Nonetheless, high concentrations of extremely hydrophobic test compounds diluted in CSM may lack long-term stability and/or solubility, especially if further stored at low temperatures. 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.

▪ **Assay Scheme** ▪

Figure 1. Assay workflows for AP-1 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 **AP-1 activation** responses it is recommended to incubate the reporter cells with treatment media for 22 - 24 hours. Hence, for *activation* assays the assay plate is processed in the morning of *Day 3* to quantify luciferase activities.

For **AP-1 inhibition** assays it is recommended to incubate the reporter cells with treatment media for 6 hours. Longer treatment periods can lead to significant reporter cell toxicity that degrades, or obliterates, inhibition-mode assay performance. Hence, for AP-1 *inhibition* assays the assay plate is processed in the afternoon of *Day 2* to quantify luciferase activities.



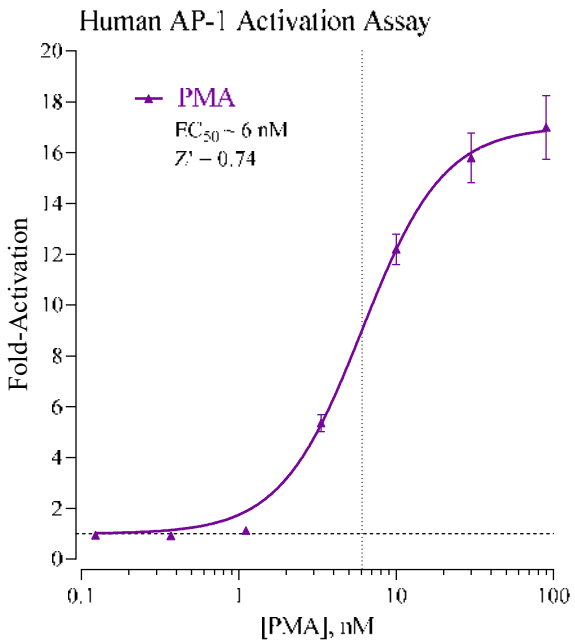


Figure 2. Dose-response activation of AP-1 using PMA.
Activation of AP-1 is demonstrated by treating reporter cells with Phorbol 12-myristate 13-acetate (PMA; provided) for 22 hours, following the protocol for activation assays depicted in Figure 1. Average relative light units (RLU) and corresponding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

[4] 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.

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

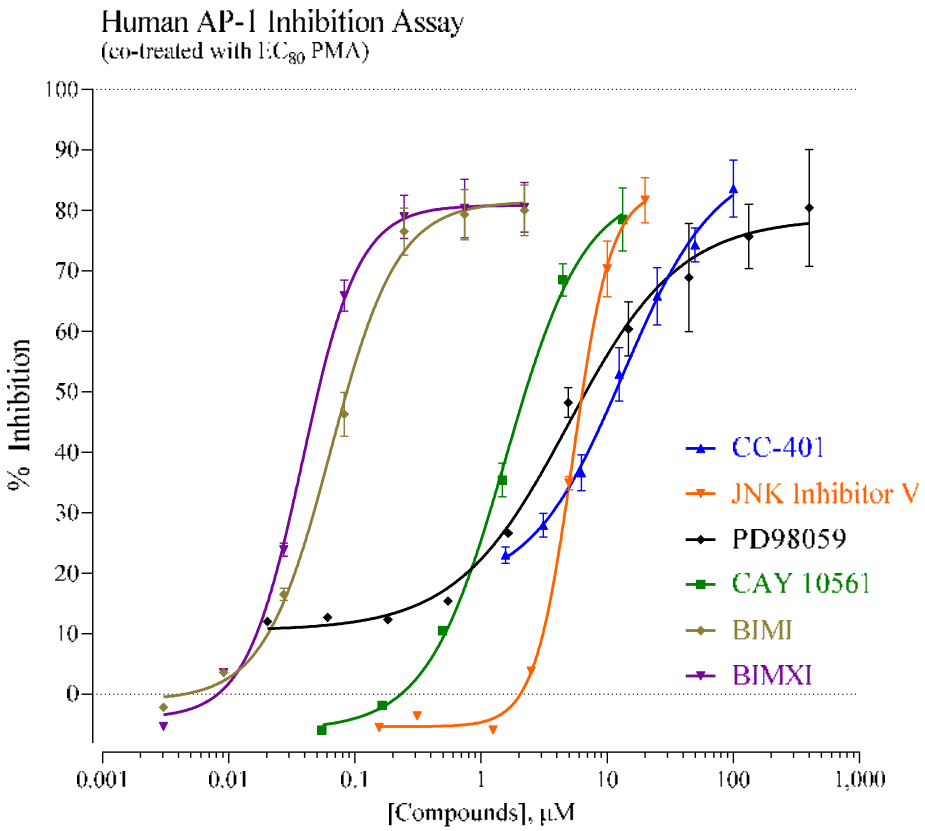


Figure 3. Dose-dependent inhibition of AP-1.
Human AP-1 Reporter Cells were treated with an ~EC_{80} concentration of PMA (10 nM) and then challenged with the inhibitors JNK Inhibitor V, CC-401, PD98059, CAY 10561, and PKC inhibitors Bisindolylmaleimide I and XI (all from Cayman Chemical, USA). Reporter cells were treated for 6 hours, following the protocol for inhibition assays depicted in Figure 1.

II. Product Components & Storage Conditions

This Human AP-1 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.

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.*
▪ AP-1 Reporter Cells	3 x 0.6 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*, 90 µM (in DMSO) (reference for AP-1 activation *via* PKC pathways)	1 x 30 µL	-20°C
▪ Detection Substrate	3 x 2.0 mL	**-80°C**
▪ Detection Buffer	3 x 2.0 mL	-20°C
▪ Plate frame	1	ambient
▪ Snap-in, 8-well strips (white, sterile, collagen-coated wells)	12	**-20°C**

NOTE: This assay kit contains a 12x 8-well strips that have 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 AP-1[5].

[5] Serkkola, E. and Hurme, M. (1993) Synergism between protein-kinase C and cAMP-dependent pathways in the expression of the interleukin-1β gene is mediated *via* the activator protein-1 (AP-1) enhancer activity. Eur. J. Biochem.:**213**, 243-249

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:* AP-1 inhibitor reference compound (refer to Figure 3)
- *Optional:* clear 96-well assay plate, sterile, cell culture treated, and collagen-coated, 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 one 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**, AP-1 *Inhibition*-assays are performed using a 6-hour treatment period, with the quantification of luciferase activity in the afternoon of *Day 2*.

AP-1 *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 AP-1 activator AND the test compound(s) to be evaluated for inhibitory activities. This assay kit includes a 90 µM stock solution of **PMA**, a potent activator of AP-1 that may be used to setup inhibition-mode assays. 10 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.

Add the challenge activator, PMA, 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. This is an efficient and precise method of setting up AP-1 inhibition assays, and it is the method presented in *Step 8b* 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 tubes of **Reporter Cells** from -80°C storage and place them directly into dry ice for transport to the laminar flow hood: 1 tube for 32 assay wells, 2 tubes for 64 assay wells, or 3 tubes for 96 assay wells. When ready to begin, transfer the tube(s) of reporter cells into a rack and, *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.

Third, during the 5 - 10 minutes incubation period, work in the cell culture hood to *carefully* mount four sterile 8-well strips into the blank assay plate frame. Strip-wells are fragile. Note that they have keyed ends (square and round), hence, they will fit into the plate frame in only one orientation.

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 gain a homogenous cell suspension. Dispense **200 µl / well** of cell suspension into the 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 'Compound Screening Media') must be included in the assay plate to allow quantification of plate-specific fluorescence background; refer to the LCMA Technical Manual.

(continued …)

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.

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.

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 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 AP-1 Assay kit includes a 90 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 AP-1 activation. The following 7-point treatment series, prepared in serial 3-fold decrements, provides a complete dose-response: 90, 30, 10, 3.33, 1.11, 0.370, and 0.123 nM. Always include a 'no treatment' control. **APPENDIX 1** provides an example for generating such a dilution series.

~ 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. 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 either a single-tip, or 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 cells and greatly increased well-to-well variability.

10.) Dispense **200 µl / well** of each prepared treatment media into 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*: To read 32 assay wells, transfer the entire volume of 1 vial of Detection Buffer into 1 vial of Detection Substrate, thereby generating a 4 ml volume of **Luciferase Detection Reagent (LDR)**. Mix gently to avoid foaming.

15.) Following the treatment period*, remove media contents from each well of the assay plate (as before in *Step 9*).

(*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 AP-1 Assay Kit Products	
IB24001-32	3x 32 AP-1 assays; strip-wells in 96-well plate frame
IB24001	1x 96-well format AP-1 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 AP-1 activation dose-response assay.