# Nuclear Factor of Activated T cells (NFAT)
## Reporter Assay System

**3x 32 Assays in 96-well Format**
Product # IB18001-32

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**Technical Manual**
*(version 7.1)*

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INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

# NFAT Reporter Assay System
# 3x 32 Assays in 96-well Format

## *I. Description*

**▪ The Assay System ▪**

The five members of the Nuclear Factor of Activated T cells (NFAT1-5) family were initially identified as key regulators of genes involved in the activation, proliferation, differentiation, and apoptosis of cells, most notably the T cells and B cells of the immune system[1].

Inactive NFAT resides in the cytoplasm in a multi-phosphorylated form. Phospho-NFAT is converted to its active form through the action of calcineurin, a calcium-dependent phosphatase. Any physiological event that drives the influx of extra-cellular $Ca^{+2}$, or depletes internal $Ca^{+2}$ stores within the endoplasmic reticula, results in $Ca^{2+}$-activation of calcineurin and its subsequent dephosphorylation of phospho-NFAT. Activated NFAT translocates to the nucleus where it binds to specific genetic response element (GREs) sequences within the promoter/enhancer region of target genes. Functional transcription complexes form *via* co-operative association with other transcription factors, most notably AP-1[2].

Importantly, dysregulation of the calcineurin-NFAT pathway is strongly associated with autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis, thereby making it an important therapeutic target for small molecule drug development[2].

INDIGO's Reporter Cells contains an engineered luciferase reporter gene functionally linked to tandem NFAT/AP-1 GRE sequences positioned immediately upstream of a minimal promoter. Activated NFAT will bind to its corresponding GRE's to initiate the formation of a complete transcription complex that drives Luc gene expression. Thus, quantifying changes in luciferase activity in the treated reporter cells provides a sensitive surrogate measure of changes in NFAT 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 the calcineurin-NFAT signal transduction pathway.

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, cell titer adjustments, or the pre-incubation of reporter cells prior to assay setup.

INDIGO's Reporter Assays are all-inclusive cell-based assay systems. In addition to NFAT Reporter Cells, this kit provides two optimized media for use during cell culture and in diluting test samples, the calcineurin-NFAT pathway activator Ionomycin, Luciferase Detection Reagent, and a cell culture-ready assay plate.

[1] Crabtree GR, *et. al.* (2002) NFAT signaling: Choreographing the social lives of cells, *Cell.*: **109**, S67-79.
[2] Park JY, *et. al.* (2020) The Role of Calcium-Calcineurin-NFAT Signaling Pathway in Health and Autoimmune Disease, Frontiers in Immunology.:doi:10.3389/fimmu.2020.00195.

## ▪ The Assay Chemistry ▪

INDIGO's Reporter Assays 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 Reporter Assays 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 ▪

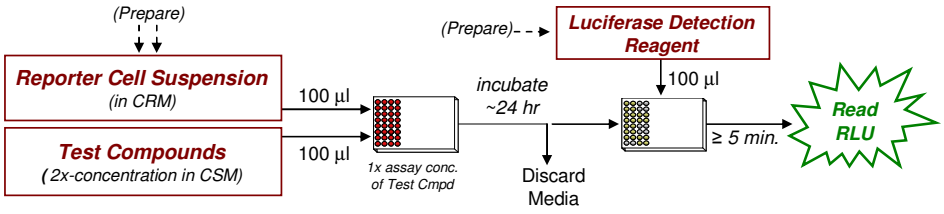
Small molecule test compounds are typically solvated in DMSO at high concentrations; ideally 1,000x-concentrated stocks relative to the highest desired treatment concentration in the assay. Using high-concentration stocks minimizes DMSO carry-over into the assay plates. NOTE: The final concentration of total DMSO carried over into assay reactions should never exceed 0.4%.

Immediately prior to setting up the assay plate(s) master stocks are serially diluted using **Compound Screening Medium (CSM**; as described in *Step 2 of the Assay Protocol*) to generate *2x-concentrated* treatment media.

> *NOTE:* CSM is formulated to help stabilize hydrophobic test compounds in the aqueous environment of the assay mixture. Nonetheless, high concentrations of hydrophobic test compounds diluted in CSM will lack long-term stability and/or solubility, especially if further stored at low temperatures. Hence, it is advised that test compound dilutions are prepared in CSM immediately prior to assay setup and are considered to be 'single-use' reagents.

## ▪ Assay Scheme ▪

**Figure 1.** Assay workflow. *In brief*, Reporter Cells are dispensed into wells of the assay plate and then immediately dosed with the user's test compounds. Following 22 -24 hr incubation, treatment media are discarded, and prepared Luciferase Detection Reagent (LDR) is added. Light emission from each assay well is quantified using a plate-reading luminometer.



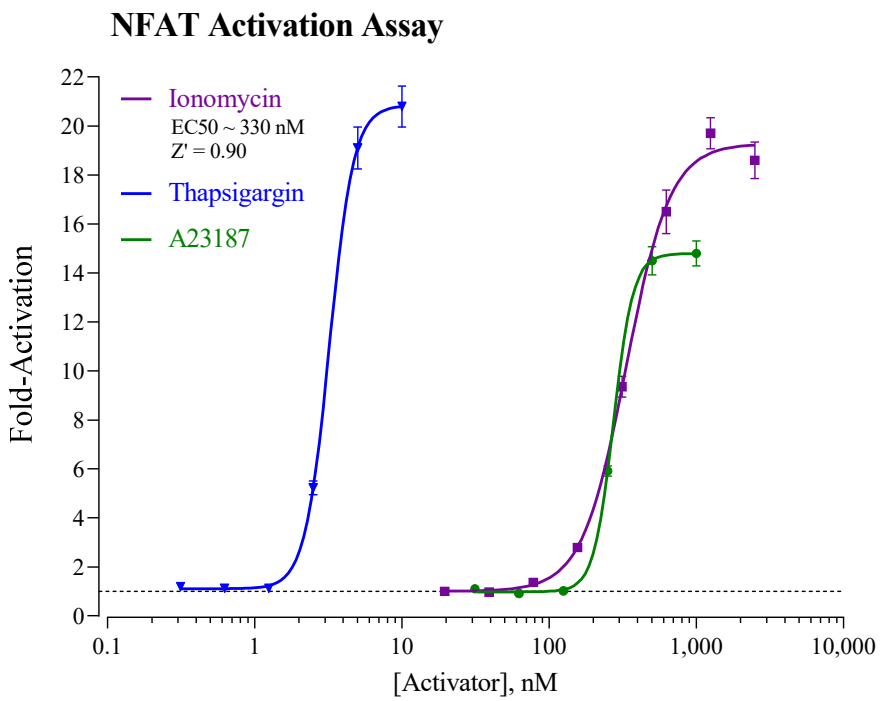



**Figure 2. Dose-response activation of NFAT using various reference compounds.**

Analyses of NFAT activator dose-responses were performed according to the protocol provided in this Technical Manual. Reporter Cells were treated with the reference activator Ionomycin (provided), A23187 and Thapsigargin (Cayman Chemicals). Average relative light units (RLU) and corresponding standard deviation (SD) values were determined for each treatment concentration (n = 3). Values of Fold-Activation and Z' were calculated as described by Zhang, *et al.* (1999)[1]. Non-linear regression and $EC_{50}$ analyses were performed using GraphPad Prism software.

RESULTS: $EC_{50}$ and Z' values for Ionomycin confirm the robust performance of this NFAT Assay and demonstrate its suitability for use in HTS applications.[3]

[3] 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.

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

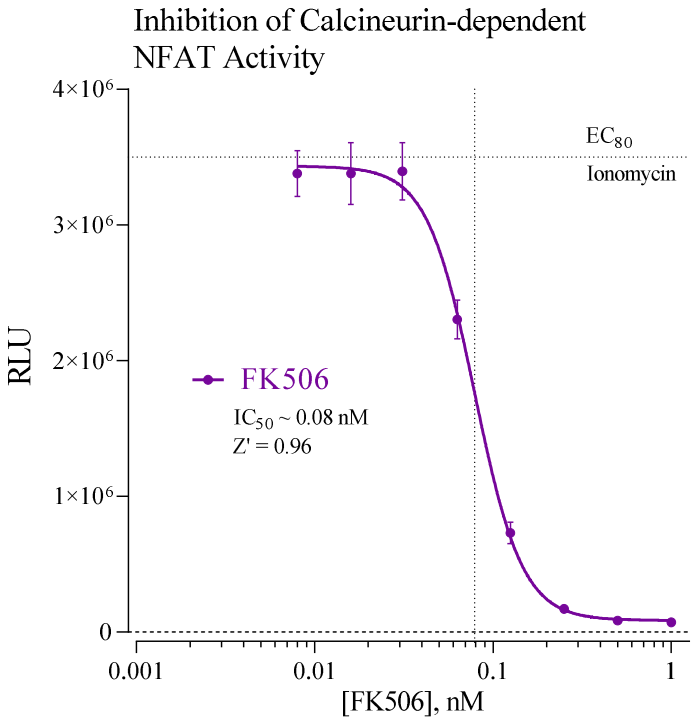



**Figure 3. Calcineurin / NFAT Pathway Inhibition Assay**

Inhibition of NFAT activation was performed using the potent calcineurin inhibitor FK506 (Cayman Chemical). The bulk suspension of reporter cells was supplemented with a 2x-$EC_{80}$ concentration of Ionomycin and dispensed into the assay plate at 100 µl / well. Cells were then treated by adding 100 µl/well of the serially diluted inhibitor. The assay plate was incubated for 24 hr, then processed to quantify luciferase activity.

## *II. Product Components & Storage Conditions*

This NFAT 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 tubes of cells must be maintained at -80°C until immediately prior to the rapid-thaw procedure described in *Step 3* 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 into 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.*** |
|---|---|---|
| ▪ NFAT Reporter Cells | 3 x 0.60 mL | **-80°C** |
| ▪ Cell Recovery Medium (CRM) | 1 x 10.5 mL | -20°C |
| ▪ Compound Screening Medium (CSM) | 1 x 35 mL | -20°C |
| ▪ Ionomycin, 3.0 mM (in DMSO) | 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, cell-culture ready) | 12 | ambient |

## *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 3*)

▪ 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 reservoirs, sterile.

▪ 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).

▪ *Optional:* Inhibitor 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 1-8* are performed on ***Day 1***, typically requiring less than 2 hours to complete. *Steps 9-14* are performed on ***Day 2*** and require less than 1 hour to complete.

**▪ A word about Inhibition-mode assay setup ▪**

Inhibition assays expose the Reporter Cells to a constant, sub-maximal concentration (typically between $EC_{50}$ – $EC_{80}$) of a known activator AND the test compound(s) to be evaluated for inhibition activity. This NFAT Assay kit includes a 3.0 mM stock solution of **Ionomycin,** (an Ionophore that increases cytoplasmic $Ca^{2+}$ levels leading to calcineurin-dependent NFAT activation) that may be used to setup inhibition-mode assays. 500 nM Ionomycin approximates $EC_{70\text{-}80}$ in this assay. Hence, it is a suitable assay concentration of activator to be used when screening test compounds for inhibitory activity.

Adding a 2x-$EC_{80}$ concentration of challenge activator (Ionomycin) to the bulk suspension of Reporter Cells is the most efficient and precise method of setting up antagonist assays, and it is the method presented in *Step 5b* of the following protocol. Note that, in *Step 6*, 100 µl of treatment media is combined with 100 µl of pre-dispensed [Reporter Cells + Ionomycin]. Consequently, one must prepare the bulk suspension of Reporter Cells to contain a 2x-$EC_{80}$ concentration of Ionomycin (~ 1.0 µM). **APPENDIX 1** provides a dilution scheme that may be used as a guide when preparing cell suspension supplemented with a 2x-concentration of Ionomycin.

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

**1.)** Remove **Cell Recovery Medium (CRM)** and **Compound Screening Medium (CSM)** from freezer storage and thaw in a 37°C water bath.

**2.) Prepare dilutions of treatment compounds** (first consider *Note 5.4*): Prepare Test Compound treatment media for *Agonist-* or *Inhibitor-mode* screens.

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

Note that, in *Step 6*, 100 µl of the prepared treatment media is added into assay wells that have been pre-dispensed with 100 µl of Reporter Cells. Hence, to achieve the desired *final* assay concentrations one must prepare treatment media with a 2x-concentration of the test and reference material(s). Use **CSM** to prepare the appropriate dilution series. Manage dilution volumes carefully. This assay kit provides 35 ml of CSM.

**Preparing the positive control:** This assay kit includes a 3.0 mM stock solution of **Ionomycin**, a potent ionophore that drives $Ca^{2+}$ influx and (*via* calcineurin activation) the activation of NFAT. The following 7-point treatment series, with concentrations presented in 2-fold decrements, provides a complete dose-response: 3.0, 1.5, 0.75, 0.375, 0.188, 0.094, and 0.047 µM. Always include a 'no treatment', or 'vehicle', control. **APPENDIX 1** provides an example for generating such a dilution series.

**3.) Rapid Thaw of the Reporter Cells:** *First*, retrieve the tube of **CRM** from the 37°C water bath and sanitize the outside with a 70% ethanol swab.

*Second*, retrieve **Reporter Cells** from -80°C storage and immerse them in dry ice for transport to the laminar-flow hood: retrieve 1 tube for 32 assay wells, 2 tubes for 64 assay wells, and 3 tubes for 96 assay wells. When ready, transfer the tube(s) of frozen cells into a rack and, *without delay*, perform a rapid thaw of the frozen cells by transferring a 3.0 ml volume of 37°C CRM into each tube of frozen cells. Recap the tube of cells and immediately place it in a 37°C water bath for 5 - 10 minutes. If only one tube of reporter cells is thawed (32 assays), the resulting volume of cell suspension will be 3.6 ml.

*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.

**4.)** Retrieve the tube of Reporter Cell Suspension from the water bath. Sanitize the outside surface of the tube(s) with a 70% alcohol swab, then transfer it into the cell culture hood. If more than one tube of cells was thawed, pool the individual tubes into a common reservoir.

**5.)** ***a. Activation*-mode assays**. *Gently* invert the tube of cells several times to gain a homogenous cell suspension. Dispense 100 µl / well of cell suspension into the assay plate.

*~ or ~*

***b. Inhibition*-mode assays**. *Gently* invert the tube of cells several times to gain a homogenous cell suspension. Supplement the bulk suspension of Reporter Cells with the desired 2x-concentration of Ionomycin (refer to "*A word about inhibition-mode assay setup*", pg. 8). Dispense 100 µl of cell suspension into each well of the assay plate.

*NOTE 5.1:* If INDIGO's Live Cell Multiplex Assay is to be incorporated, a minimum of 3 'cell 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).

*NOTE 5.2:* 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 5.3:* 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 (100 µl/well) into a clear 96-well cell culture treated assay plate, followed by 100 µl/well of CSM. Incubated overnight in identical manner to those reporter cells contained in the white assay plate.

*NOTE 5.4:* For logistical reasons, some users find it more convenient to first dispense the prepared suspension of reporter cells (starting at *Step 3*) *before* preparing their test compound dilutions. That strategy works equally well. Once plated, cells may be placed in an incubator for up to 3 hours before proceeding to *Step 6*.

**6.)** Dispense 100 µl of 2x-concentration treatment media into appropriate assay wells.

**7.)** Transfer the assay plate into a cell culture incubator (37°C, humidified 5% $CO_2$) for 22 - 24 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.

**8.)** For greater convenience on *Day 2*, retrieve the appropriate number of vials of **Detection Substrate** *and* **Detection Buffer** from freezer storage and place them in a dark refrigerator (4°C) to thaw overnight.

**DAY 2 Assay Protocol:** Subsequent manipulations do *not* require special regard for aseptic technique and may be performed on a bench top.

**9.)** 30 minutes before intending to quantify NFAT activity, remove the tubes of **Detection Substrate** and **Detection Buffer** from the refrigerator and place them in a low-light area so that they may equilibrate to room temperature.

*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.

**10.)** 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*.

**11.)** *Immediately before proceeding to Step 12*: 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.

**12.)** After 22-24 hours of incubation, remove media contents from each well.

*NOTE:* Because the assay plate is composed of a frame with snap-in strip-wells, the practice of physically ejecting media is NOT advised. 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. Removal of the treatment 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).

**13.)** 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. Do not shake the assay plate during this period.

**14.)** Quantify luminescence.

## *V. Related Products*

| NFAT Assay Products | |
|---|---|
| **Product No.** | **Product Descriptions** |
| IB18001-32 | NFAT Reporter Assay System<br>3x 32 assays in 96-well format |
| IB18001 | NFAT Reporter Assay System<br>1x 96-well format assay |
| IB18002 | NFAT Reporter Assay System<br>1x 384-well format assays |
| Bulk volumes of 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 contained in 5 x 96-well assay plates |
| LCM-10 | Reagent in **10x bulk volume** to perform **960** Live Cell Assays contained in 10 x 96-well assay plates |

Please refer to INDIGO Biosciences website for updated product offerings.

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## *VI. Limited Use Disclosures*

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Product prices, availability, specifications, claims and technical protocols are subject to change without prior notice. The printed Technical Manual provided in the kit box will always be the most current version available.

**APPENDIX 1**

Example scheme for the serial dilution of Ionomycin reference activator, and the setup of an NFAT dose-response assay.