

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

96-well Format Assays
Product # IB18001

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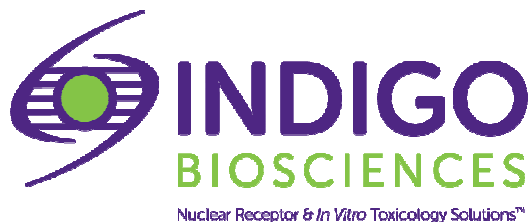
Technical Manual
(version 7.2i)

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NFAT Reporter Assay System 96-well Format Assays

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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 system¹.

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 Ca²⁺, or depletes internal Ca²⁺ stores within the endoplasmic reticula, results in Ca²⁺-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-1².

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 development².

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 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 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 A23187, 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 Mg²⁺-dependent reaction that consumes O₂ and ATP as co-substrates, and yields as products oxyluciferin, AMP, PP_i, CO₂, 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 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 samples: Chemical 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 7* and depicted in Appendix 1 for the reference activator, **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 carried over into assay wells should not exceed 0.4%. Significant DMSO-induced cytotoxicity can be expected above 0.4%.

Protein test samples: It is recommended that protein or antibody test samples are solvated in aqueous buffered solutions with carrier protein (*e.g.*, PBS + 0.1% BSA) at concentrations *no less* than 10x-concentrated relative to the highest desired treatment concentration. CSM is then used as the diluent to prepare treatment media.

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.

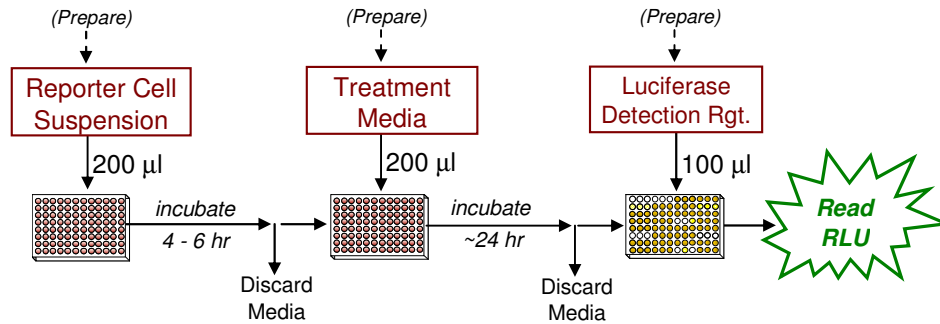
▪ **Considerations for Automated Dispensing** ▪

When processing a small number of assay plates, first carefully considered 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; 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 workflow. *In brief*, 200 μ l of Reporter Cells is 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 μ l/well of the prepared 1x-concentration treatment media are added. Following 22 - 24 hours incubation, treatment media are discarded, and Luciferase Detection Reagent is added. The intensity of light emission (in units of 'Relative Light Units'; RLU) from each assay well is quantified using a plate-reading luminometer.



▪ Assay Performance ▪

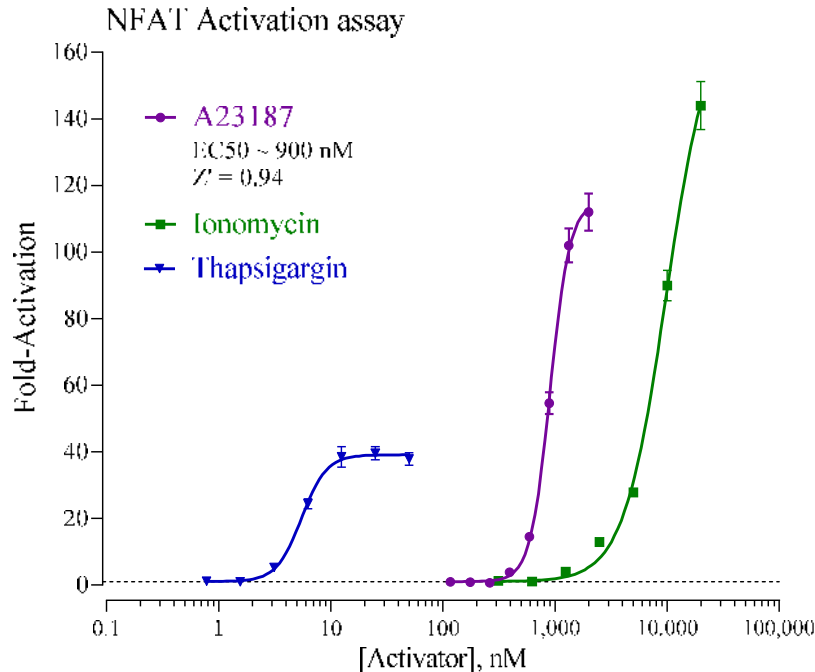


Figure 2. Dose-response activation of NFAT using various reference compounds.

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 A23187 (provided), Ionomycin 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 EC₅₀ analyses were performed using GraphPad Prism software. EC₅₀ and Z' values for A23187 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.

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

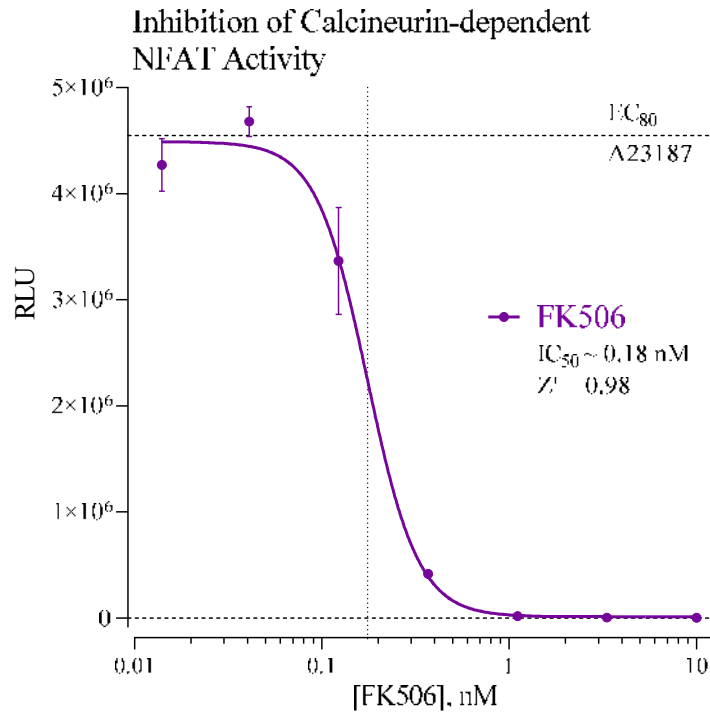


Figure 3. Calcineurin / NFAT Pathway Inhibition Assay.

NFAT reporter cells were co-treated with an EC_{80} concentration of A23187 (provided) and varying concentrations of the Calcineurin inhibitor FK506 (Cayman Chemical). INDIGO's Live Cell Multiplex (LCM) Assay confirmed that no treatment concentration was cytotoxic (data not shown). Non-linear regression analyses of RLU vs. $\text{Log}_{10}[\text{FK506}, \text{nM}]$ were plotted and an IC_{50} determination made using GraphPad Prism software.

II. Product Components & Storage Conditions

This NFAT Assay kit contains materials to perform assays in a single 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 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 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.

<u>Kit Components</u>	<u>Amount</u>	<u>Storage Temp.</u>
▪ NFAT 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
▪ A23187, 2.0 mM (in DMSO)	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, <i>collagen-coated</i> assay plate (white, sterile, cell-culture ready)	1	-20°C

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% CO₂ 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 (see Figure 3).
- *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**, 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 2.0 mM stock solution of **A23187**, (an Ionophore that increases cytoplasmic Ca^{2+} levels leading to calcineurin-dependent NFAT activation) that may be used to setup inhibition-mode assays. ~1,000 nM A23187 approximates EC_{80} in this assay. Hence, it is a suitable assay concentration of activator to be used when screening test compounds for inhibitory activity.

Add Ionomycin to a bulk volume of **CSM**, as described above. This activator-supplemented medium is then used to prepare serial dilutions of test material stocks to achieve the desired respective final assay concentrations. This is an efficient and precise method of setting up NFAT inhibition assays, and it is the method presented in *Step7b* 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 $37^{\circ}C$ using a water bath.

2.) Rapid Thaw of the Reporter Cells: *First*, retrieve the two tubes of **CRM** from the $37^{\circ}C$ water bath and sanitize their outside surfaces with a 70% ethanol swab.

Second, retrieve the tube of **Reporter Cells** from $-80^{\circ}C$ storage, place it directly into dry ice for transport to the laminar flow hood. When ready to begin, 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 $37^{\circ}C$ CRM into the tube of frozen cells. Place the tube of Reporter Cells in a $37^{\circ}C$ water bath for 5 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 reservoir. Using an 8-channel pipette, dispense **200 μ l / 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 '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 4.2: Increased well-to-well variation (= increased standard deviation!) will occur if care is not taken to prevent cells from settling in the reservoir 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, preferably collagen-coated, 96-well assay plate. Continue to process this 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, $\geq 70\%$ humidity, 5% CO₂) for 4 - 6 hours.

6.) Near the end of the pre-incubation period remove Compound Screening Medium (CSM) from freezer storage and thaw in a 37°C water bath.

7.) **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 9*, the prepared treatment media will be dispensed at 200µl / well into the assay plate. Manage dilution volumes carefully; this assay kit provides **45 ml** of CSM.

NOTE: Total DMSO carried over into assay reactions should not exceed 0.4%.

a. Activation-mode assays. Preparing the positive control: This assay kit includes a 2.0 mM stock solution of **A23187**, a potent ionophore that drives Ca²⁺ influx, calcineurin activation, and the activation of NFAT. The following 8-point treatment series, with concentrations presented in 2-fold decrements, provides a complete dose-response: 2.0, 1.33, 0.889, 0.593, 0.395, 0.263, 0.176, and 0.117 µM. Always include a 'no treatment', or 'vehicle', control. **APPENDIX 1** provides an example for generating such a dilution series.

~ or ~

b. Inhibition-mode assays. When setting inhibition-mode assays, first supplement a bulk volume of CSM with the challenge activator Ionomycin to achieve the desired final assay-concentration (refer to "*A word about inhibition-mode assay setup*", pg. 8). The A23187 supplemented CSM is then used to generate dilutions of test compound stocks to achieve their final assay concentrations.

8.) At the end of the 4-6 hours pre-culture period, discard the media. The preferred method is to use a 'wrist flick' to eject media 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.

9.) 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. Simply remove 1 tip from the 8-channel dispenser and dispense 100 µl of sterile water into each of the seven inter-well spaces per column of wells.

10.) Transfer the assay plate into a cell culture incubator for 22 - 24 hours.

NOTE: Ensure a high-humidity ($\geq 70\%$) 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 **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.

12.) 30 minutes before intending to quantify receptor 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.

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.

13.) 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*.

14.) *Immediately before proceeding to Step 12*, 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 the volume of into a media reservoir.

15.) Following 22 - 24 hours of incubation discard all 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.

16.) Dispense 100 μ l of **LDR** to each well of the assay plate. Allow the assay plate to rest at room temperature for 5- 10 minutes. Do not shake the assay plate during this period.

17.) Quantify luminescence.

V. Related Products

Product No.	Product Descriptions
NFAT Assays	
IB18001-32	NFAT Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
IB18001	NFAT Reporter Assay System 1x 96-well format assay
IB18002	NFAT Reporter Assay System 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 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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VI. Limited Use Disclosures

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