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

3x32 Assays in 96-well Plate Format
Product # IB18001-32

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Technical Manual
(version 7.2i)

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INDIGO BIOSCIENCES
Nuclear Receptor & *In Vitro* Toxicology Solutions™

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

I. Description

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▪ 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 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 receptor 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 to yield 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).

This assay kit features a luciferase detection reagent specially formulated to provide stable light emission between 10 and 90+ minutes after initiating the luciferase reaction. Incorporating a 10-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.

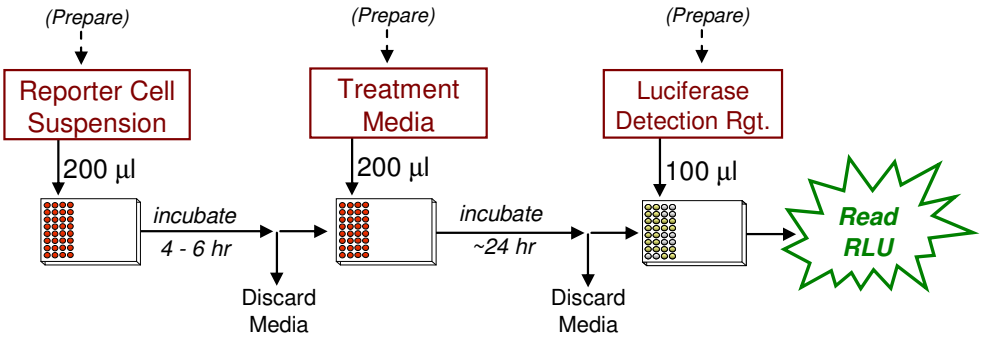
Regardless of the dilution method used, the final concentration of total DMSO carried over into assay wells should not exceed 0.4%. Significant DMSO-induced cytotoxicity can be expected above 0.4%.

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.

▪ Assay Scheme ▪

Figure 1. Assay workflow. *In brief*, 200 µl 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 µl/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 terms of 'Relative Light Units'; RLU) from each assay well is quantified using a plate-reading luminometer.



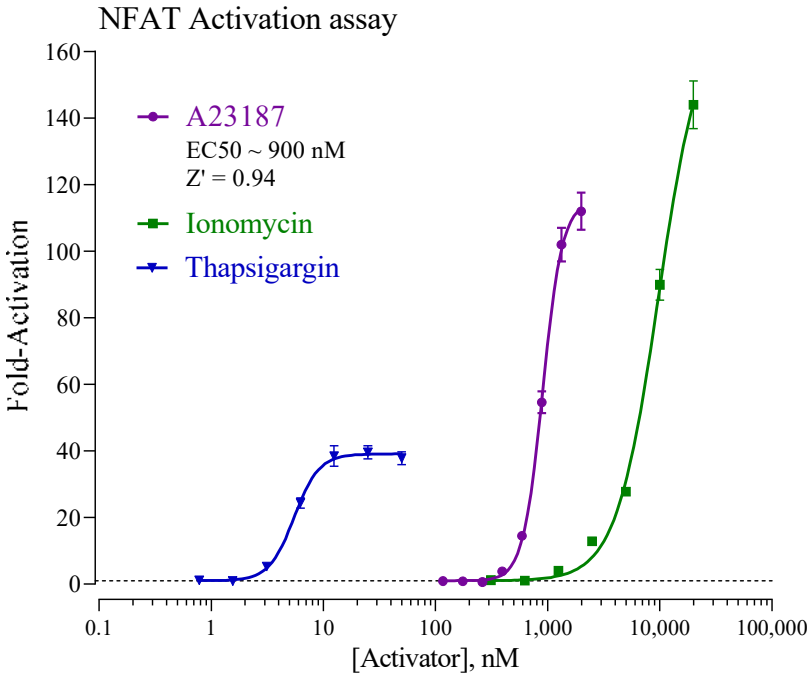


Figure 2. Dose-response activation of NFAT using various reference compounds. Analyses of NFAT activator dose-responses were performed according to the protocol provided in this Technical Manual. Reporter Cells were treated with the reference activator A23187 (provided), Ionomycin and Thapsigargin (Cayman 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 EC_{50} analyses were performed using GraphPad Prism software. EC_{50} 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.

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

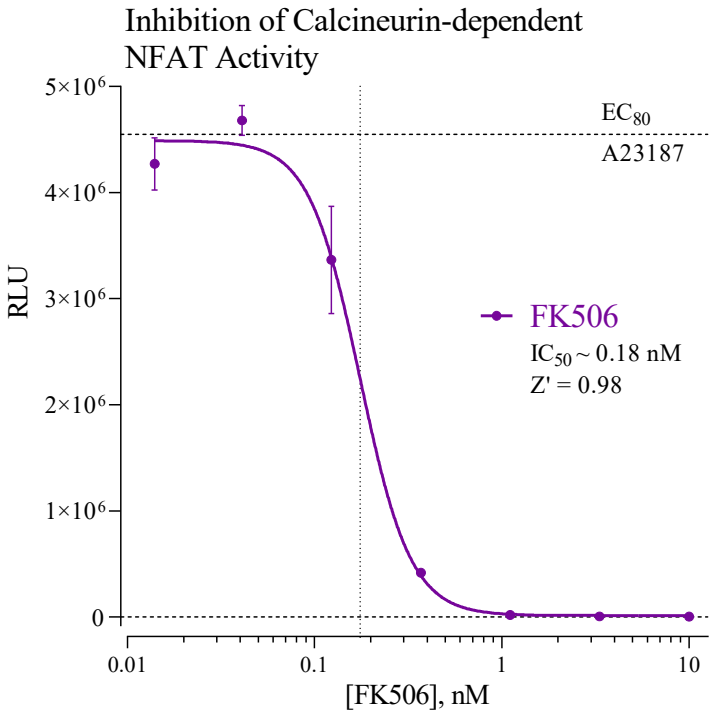


Figure 3. Calcineurin / NFAT Pathway Inhibition Assay.
NFAT reporter cells were co-treated with an EC_{80} concentration of A23187 (provided) and varying concentrations of the Calcineurin inhibitor FK506 (Cayman Chemical). INDIGO's Live Cell Multiplex (LCM) Assay confirmed that no treatment concentration was cytotoxic (data not shown). Non-linear regression analyses of RLU *vs.* Log_{10}[FK506, nM] were plotted and an IC_{50} determination made using GraphPad Prism software.

II. Product Components & Storage Conditions

This NFAT Reporter 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 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.

Kit Components	*Amount*	*Storage Temp.*
▪ NFAT 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
▪ A23187, 2.0 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, collagen-coated wells)	12	**-20°C**

NOTE: This Assay kit contains 8-well strips that have been collagen-coated and dried; these strip wells should be stored frozen (-20°C or -80°C) until use.

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.
▪ 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:* reference antagonist (refer to Fig. 3)
▪ *Optional:* clear 96-well assay plate, sterile, *collagen-coated*, for viewing cells on *Day 2*.

DAY 2 plate-reading luminometer.

IV. Assay Protocol

Please review the entire protocol before starting. Completing the assay requires an overnight incubation. *Steps 1-11* are performed on ***Day 1***, requiring less than 2 hours of bench work and a 4-hour incubation step to complete. *Steps 12-17* are performed on ***Day 2*** and require less than 1 hour to complete.

▪ A word about Inhibition-mode assay setups ▪

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 A23187 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°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, 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 '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, ≥ 70% humidity, 5% CO_2) 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 treatment media at the desired **final assay concentrations**. In *Step 9*, **200 µl / well** of the prepared treatment media are dispensed into the strip-wells of 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. **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 Ca^{2+} 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 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 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.

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% CO_2 incubator 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.

11.) 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.

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 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 22 - 24 hours incubation in treatment media, remove media contents from each well of the assay plate (as before in *Step 8*).

16.) Add 100 µl of **LDR** to each well of the assay plate. Allow the assay plate to rest at room temperature for 5 - 10 minutes following the addition of LDR. 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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Product prices, availability, specifications, and claims are subject to change without prior notice.

APPENDIX 1

Example scheme for the serial dilution of A23187 reference activator, and the setup of an NFAT dose-response assay.