Human Macrophage Colony-Stimulating Factor Receptor Reporter Assay System

(M-CSFR; CSF1R)

96-well Format Assays
Product # IB30201

▪

Technical Manual
(version 7.2)

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Nuclear Receptor & *In Vitro* Toxicology Solutions™

Human Macrophage Colony-Stimulating Factor Reporter Assay System 96-well Format Assays

I. Description

▪ Background ▪

Macrophage colony-stimulating factor (M-CSF) is a cytokine expressed in a wide range of cells and tissues. It stimulates progenitor cells from bone marrow and plays a role in the development, proliferation, and maintenance of phagocytes like monocytes, dendritic cells and microglia[1,2].

The activity of M-CSF is mediated through binding interactions with the M-CSF Receptor (M-CSFR; also known as CSF1R). M-CSF/M-CSFR activity is involved in various pathologies such as ovarian cancer, breast cancer, rheumatoid arthritis, and cutaneous lupus[1]. Recent phase II clinical trials have indicated that monoclonal antibody or small molecule antagonists targeting M-CSFR have reduced the inflammatory response often associated with rheumatoid arthritis[1].

In the brain, M-CSF is secreted by neurons, astrocytes and microglia and is involved in brain development[3,4]. M-CSF-deficient animals have severe brain deficits with abnormalities associated with the cerebral cortex. Other phenotypic traits associated with M-CSF deficiency include reduced body weight and skeletal defects[1]. Additionally, in many neurodegenerative diseases, microglia are becoming a key target for therapeutic purposes as they eliminate toxic elements from the brain and set the conditions for repair and remyelination[1]. Low level M-CSF/M-CSFR expression or receptor inhibition in microglia has been associated with pre-symptomatic Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis (MS) and frontotemporal dementia[1,3,4]. Consequently, M-CSF and its specific receptor, M-CSFR, command considerable interest in therapeutics development and drug safety screening.

▪ The Assay System ▪

This assay utilizes proprietary human cells that have been engineered to provide constitutive expression of the **Human Macrophage Colony-Stimulating Factor Receptor (M-CSFR)**.

Binding of M-CSF to its receptor M-CSFR activates the tyrosine kinase domains of the receptor and initiates intracellular signaling cascades that include RAS-MAPK pathways[2, 3]. For example, activation of the RAS-MAPK pathway leads to activation of ERK1/2 and subsequent phosphorylation and activation of the transcription factor Elk-1[3]. M-CSFR signal transduction *via* the RAS-MAPK-ERK1/2 cascade is exploited by the reporter cells provided in this kit.

INDIGO's M-CSFR Reporter Cells express a hybrid Elk-1 transcription factor in which the native Elk-1 DNA-binding domain (DBD) has been replaced with the yeast Gal4 DBD sequence. The luciferase reporter gene is functionally linked to an upstream Gal4 Upstream Activation Sequence (UAS). When activated, Elk-1 binds to the UAS elements to initiate the formation of a complete transcription complex that drives Luciferase gene expression. Quantifying changes in luciferase activity in the treated reporter cells relative to the untreated cells provides a sensitive, dose-dependent surrogate measure of drug-induced changes in M-CSFR activity. The principal application of this assay is in the screening of test materials to quantify any functional interactions, either activating or inhibitory, that they may exert against M-CSFR or the coupled RAS-MAPK pathway.

INDIGO's Reporter Cells are transiently transfected and prepared as frozen stocks using a proprietary **CryoMite™** process. This cryo-preservation method allows for the immediate dispensing of healthy, division-competent reporter cells into assay plates. There is no need for intermediate treatment steps such as spin-and-rinse of cells, viability determinations or cell titer adjustments prior to assay setup.

This assay kit provides the convenience of an all-inclusive cell-based reporter assay system for M-CSFR signaling. In addition to Reporter Cells, included are an optimized culture medium for use in reviving the cryopreserved cells, a culture medium for use in preparing test sample treatments, the physiological activator M-CSF as a positive control, Luciferase Detection Reagents, and a cell culture-ready assay plate.

▪ 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 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: Small molecules 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 directly as the diluent to prepare serial dilutions of test compounds to achieve the desired final assay concentration series.

Alternatively, if small-molecule 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%. DMSO-induced cytotoxicity can be expected above 0.4%.

> *NOTE:* CSM is formulated to help stabilize hydrophobic small molecule test compounds in the aqueous environment of the treatment media. Nonetheless, high concentrations of small organic molecules diluted in CSM may lack long-term stability and/or solubility, especially if further stored at low temperatures. Hence, it is recommended that compound dilutions are prepared in CSM immediately prior to assay setup and are then treated as 'single-use' reagents.

P**rotein samples** (*e.g.,* antibodies or activator polypeptides): For protein test samples it is recommended to solvate the test materials in aqueous buffered solutions supplemented with carrier protein (*e.g.,* PBS + 0.1% BSA) at concentrations *no less* than 10x relative to the highest desired treatment concentration. The M-CSF stock included with this kit is prepared in PBS + 0.1% BSA at a 100x-concentration relative to the highest recommended treatment (as depicted in APPENDIX 1).

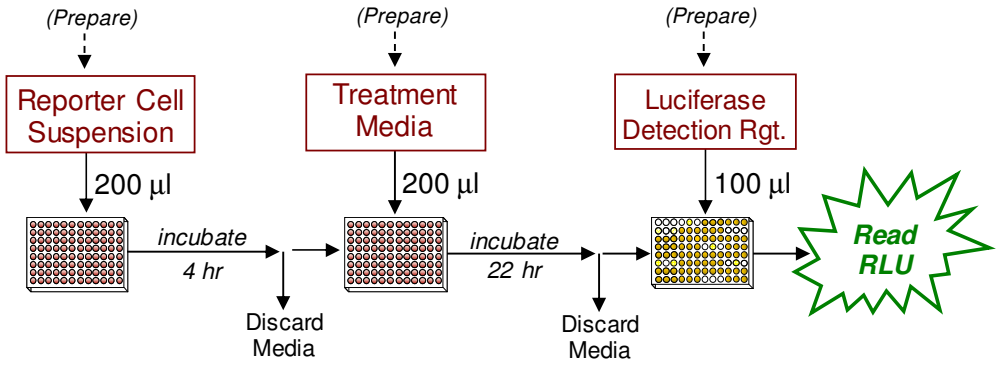
▪ Considerations for Automated Dispensing ▪

When using an automated dispensing instrument to process a small number of assay plates, first carefully consider the dead volume requirement of your 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 reagent 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 µl of Reporter Cells are dispensed into wells of the assay plate and incubated for 4-6 hours. Following the pre-incubation period, culture media are discarded and 200 µl/well of the prepared treatment media are added. Following 22-24 hours incubation discard the treatment media and add Luciferase Detection Reagent. The intensity of light emission from each assay well is quantified (in units of Relative Light Units; RLU) using a plate-reading luminometer.



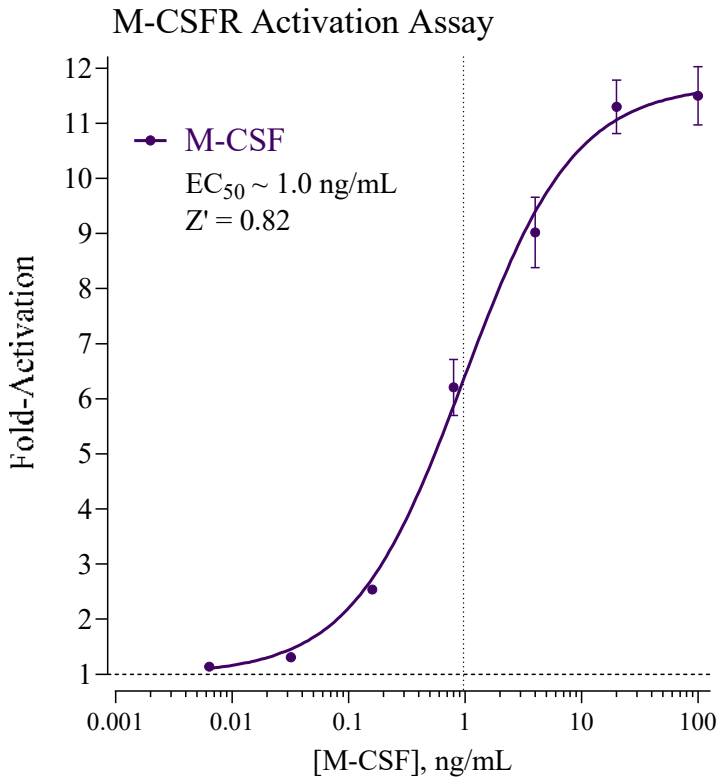


Figure 2. M-CSFR activation dose response analyses.
Activation dose-response assays were performed according to the protocol provided in this Technical Manual. 200 µl / well of M-CSFR Reporter Cell suspension was dispensed into the 96-well assay plate, which was then incubated for 4 hours. The concentrated stock of M-CSF (provided) was further diluted using CSM to produce treatment media at the desired assay concentrations. The pre-culture media were discarded from the assay wells and 200 µl per well of the prepared treatment media were dispensed (n = 3/conc.), including 'untreated control' wells. Following 22-hours incubation the treatment media were discarded, Luciferase Detection Reagent was added, and luminescence intensity per well was quantified. Values of average relative light units (RLU) and corresponding values of standard deviation (SD), percent coefficient of variation (%CV), Fold-Activation and Z'[5] were determined for each treatment concentration. GraphPad Prism software was used to perform the least-squares method of non-linear regression to plot Fold Activation *vs.* Log_{10}[ng/mL] and to determine EC_{50} values.

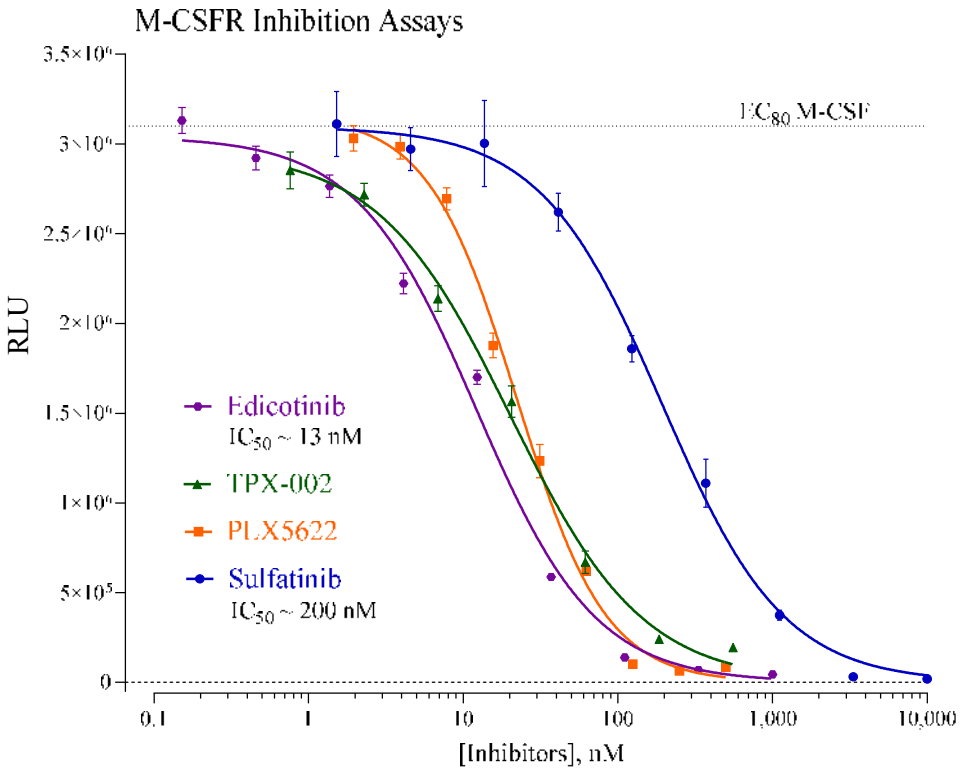


Figure 3. M-CSFR Inhibition dose-response analyses. M-CSFR reporter cells were co-treated with an EC_{80} concentration of the reference activator M-CSF (provided) and varying concentrations of the small molecule M-CSFR inhibitors Edicotinib, TPX-002, PLX5622 and Sulfatinib. The range of determined IC_{50} values is shown; (n = 3 / conc.). INDIGO's Live Cell Multiplex (LCM) Assay was performed to confirm that no treatment concentrations were cytotoxic (data not shown). Non-linear regression analyses of RLU *vs.* Log_{10}[Inhibitor, nM] were plotted and IC_{50} determinations were made using GraphPad Prism software. All compounds were obtained from Cayman Chemical, Ann Arbor MI, USA.

II. Product Components & Storage Conditions

This Macrophage Colony Stimulating Factor Receptor (M-CSFR) Assay kit contains materials to perform assays in a single collagen-coated 96-well assay plate.

Cryopreserved mammalian cells are temperature sensitive! **To ensure maximal viability the tube of Reporter 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, please 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.*
▪ M-CSFR 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
▪ M-CSF, 10 µg/ml (in PBS/0.1%BSA) (activator of M-CSFR)	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 one 96-well assay plate in which the assay wells have been collagen-coated and dried; the assay plate should be stored frozen (-20°C or colder) 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

▪ container of dry ice (see *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* deep-well plates, *or* appropriate similar vessel for generating dilution series of reference compound(s) and test compound(s).

▪ *Optional:* clear 96-well assay plate, cell culture treated, 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 benchwork 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 ▪

Receptor inhibition assays expose the Reporter Cells to a fixed, sub-maximal concentration (typically between EC_{50} – EC_{85}) of a reference activator AND varying concentrations of the test compound(s) to be evaluated for inhibitory activity. This M-CSFR Assay kit includes a 10 µg/mL stock solution of M-CSF, a potent physiological activator of M-CSFR that may be used to set up inhibition-mode assays. 3.8 ng/mL M-CSF approximates EC_{80} in this assay and is, therefore, a suitable concentration to use when screening test materials for inhibitory activities.

Add M-CSF 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 M-CSFR inhibition assays, and it is the method presented in *Step 7b* of this protocol.

DAY 1 Assay Protocol: All steps should 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 M-CSFR **Reporter Cells** from -80°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°C CRM into the tube of frozen cells. Place the tube of Reporter Cells in a 37°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 electronic, repeat-dispensing 8-channel pipette, dispense **200 µl / well** of cell suspension into wells of 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 CSM) 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 an 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-culture period: Remove **Compound Screening Medium (CSM)** from freezer storage and thaw in a 37°C water bath.

7.) Prepare the Test Compound(s) and Reference Compound treatment media: 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*, 200 µl / well of respective prepared treatment media will be dispensed at 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**. This M-CSFR Assay kit includes a concentrated stock of M-CSF, 10µg/ml prepared in PBS/0.1% BSA. The following 7-point treatment series, with concentrations generated using serial 5-fold dilutions, provides a complete dose-response: 100, 20.0, 4.00, 0.800, 0.160, 0.032 and 0.0064 ng/ml. Always include 'no treatment' control wells. **APPENDIX 1** provides guidance 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 Insulin to achieve an EC_{50} – EC_{80} concentration (refer to "*A word about inhibition-mode assay setup*", pg. 9). The M-CSF-supplemented CSM is then used to generate dilutions of test compound stocks to achieve the desired series of treatment 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-chanel 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 (≥ 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.) Approximately 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. Program the instrument to perform a single 5 second "plate shake" prior to reading the first assay well. Read time is set to 0.5 second (500 mSec) per well, *or less*.

14.) Immediately before proceeding to *Step 15*, prepare **Luciferase Detection Reagent** (**LDR**). Combine 'Detection Buffer' and 'Detection Substrate' by pouring-over their entire volumes into a media basin; rock the basin gently to mix the reagent. The resulting volume of LDR is 12 ml.

15.) Following 22 - 24 hours incubation in treatment media, 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.

16.) Add 100 µl of the prepared **LDR** into all wells of the assay plate. Allow the assay plate to rest at room temperature for at least 5 minutes following the addition of LDR. Do not shake the assay plate during this period.

17.) Quantify luminescence.

18.) Data analyses.

V. Related Products

Product No.	*Product Descriptions*
Macrophage Colony-Stimulating-Factor Receptor (M-CSFR; CSF1R) Assay Products	
IB30201	M-CSFR Reporter Assay System 1x 96-well format assay
IB30202	M-CSFR Reporter Assay System 1x 384-well format assays
Bulk volumes of reporter cells and assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

Product No.	*Product Descriptions*
Granulocyte-Macrophage Colony-Stimulating-Factor Receptor (GM-CSFR; CSF2R) Assay Products	
IB30001	GM-CSFR Reporter Assay System 1x 96-well format assay
IB30002	GM-CSFR Reporter Assay System 1x 384-well format assays
Bulk volumes of reporter cells and 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
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.

www.indigobiosciences.com

VI. Citations

[1] Pons, *et al*. (2018). New Therapeutic Avenues of mCSF for Brain Diseases and Injuries. Frontiers in Cellular Neuroscience. Volume 12. Article 499.

[2] Ségaliny *et al*. (2015). IL-34 and M-CSF form a novel heteromeric cytokine and regulate the M-CSF receptor activation and localization. Cytokine. **76**, 170-181.

[3] Stanley *et al*. (2014). CSF-1 Receptor Signaling in Myeloid Cells. Cold Spring Harbor Perspectives in Biology. **6**(6): a021857.

[4] Hu *et al*. (2021). Insights Into the Role of CSF1R in the Central Nervous System and Neurological Disorders. Frontiers in Aging Neuroscience. Volume 13; Article 789834.

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

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

VII. Limited Use Disclosures

Products offered by INDIGO Biosciences, Inc. are for RESEARCH PURPOSES ONLY – not for therapeutic, diagnostic, or contact use in humans or animals.

"CryoMite" is a Trademark ™ of INDIGO Biosciences, Inc. (State College, PA, USA).

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 recently updated version available.

APPENDIX 1

Example scheme for the serial dilution of M-CSF and the setup of an M-CSFR dose-response assay.