

**Human Interleukin-6 Receptor
Reporter Assay System
(IL-6R)**

96-well Format Assays
Product # IB53001

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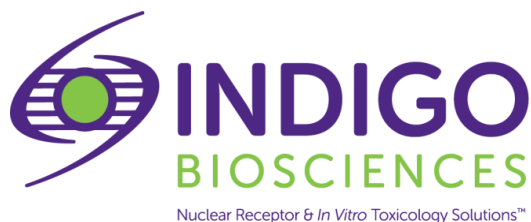
Technical Manual
(version 7.2i)

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Human IL-6R Reporter Assay System 96-well Format Assays

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I. Description

▪ The Assay System ▪

This assay utilizes proprietary human cells that provide constitutive expression of the **Human Interleukin-6 Receptor (IL-6R)**.

Interleukin-6 (IL-6) is considered one of the most prominent pro-inflammatory cytokines and causative agent of many autoimmune disorders ⁽¹⁾. Chronic overproduction of IL-6 has been associated with wide-ranging illnesses such as Rheumatoid Arthritis (RA), Systemic Lupus Erythematosus (SLE), Multiple Sclerosis (MS), Gastric and Prostate Cancers, Alzheimer's disease, Atherosclerosis, Cardiovascular disease and Stroke ⁽²⁾. In addition, the 'cytokine storm' caused by viral infections such as COVID have led to severe adverse outcomes ⁽³⁾. Considering its significant role and involvement in the progression of many types of disease, IL-6 and its receptor IL-6R continues to command much interest as a target for the development of novel, specific, and predominantly inhibitory drugs and antibodies. For example, blockade of IL-6R by the neutralizing monoclonal antibody tocilizumab has been approved in more than 100 countries for the treatment of rheumatoid arthritis ⁽²⁾.

IL-6 binds to a membrane-bound IL-6 receptor (IL-6R), and the complex of IL-6 and IL-6R associates with a second receptor protein, glycoprotein 130 kDa (gp130), which dimerizes and initiates signaling via the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) ⁽⁴⁾. Phosphorylation and activation of the transcription factor STAT3 is the signaling mechanism exploited by the reporter cells included in this kit.

INDIGO's Reporter Cells contain the luciferase reporter gene functionally linked to an upstream minimal promoter and tandem STAT3 genetic response element (GRE) sequences. Activated, dimeric STAT3^P binds the STAT3 GREs to initiate the formation of a complete transcription complex that drives luciferase gene expression. Quantifying relative changes in luciferase activity in the treated reporter cells relative to the untreated cells provides a sensitive, dose-dependent surrogate measure of drug- or antibody-induced changes in IL-6R activity. Accordingly, the primary application of this IL-6R Assay is to screen test materials for any functional activity, either agonistic or inhibitory, that they may exert against the IL-6R.

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.

INDIGO's IL-6R Assay kit provides the convenience of an all-inclusive cell-based assay system. In addition to Reporter Cells, provided are two optimized media for use in recovering the cryopreserved cells and for diluting test samples, the reference agonist human IL-6, Luciferase Detection Reagents, and a cell culture-ready assay plate.

¹ Kishimoto T. Interleukin-6: From basic science to medicine – 40 years in immunology. (2005) *Annu Rev Immunol*:**23**, 1-21.

² Tanaka T, *et al.* A new era for the treatment of inflammatory autoimmune diseases by interleukin-6 blockade strategy. (2014) *Semin Immunol*:**26(1)**, 88-96.

³ Moore JB, June CH. Cytokine release syndrome in severe COVID-19. (2020) *Science*: **368(6490)**:473-4, 184-188.

⁴ Schaper F, Rose-John S. Interleukin-6: Biology, signaling and strategies of blockade. (2015) *Cytokine Growth Factor Rev*: **26(5)**: 475-87.

▪ 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, 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 (RLUs).

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 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 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 that the final concentration of DMSO carried over into assay wells should not exceed 0.4%.

Stocks of test materials that are **Protein** or **Poly-peptide** ligands, or **Antibodies**, should be 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. The IL-6 stock included with this kit is prepared in PBS + 0.1% BSA at a 1,000x-concentration relative to the highest recommended treatment (refer to APPENDIX 1).

Immediately prior to setting up an assay, the above master stocks are serially diluted using one of two alternative strategies:

1.) For both **small-molecule** and **proteinaceous** test samples, **Compound Screening Medium (CSM)** may be used as the diluent to achieve the desired assay concentration series, as described in *Step 7* (pg. 10).

Alternatively, if **small-molecule** test compound solubility is expected to be problematic, 2.) DMSO may be used to make serial dilutions to produce 1,000x-concentrated stocks for each independent test concentration. Treatment media are then prepared using CSM to make 1,000-fold dilutions of the prepared DMSO dilution series. NOTE: Do *not* use DMSO as the diluent for proteinaceous test compounds.

Regardless of the dilution method used, the concentration of total DMSO (or any organic solvent) carried over into the assay wells should not exceed 0.4%. Emerging cytotoxicity can be expected above 0.4% DMSO exposure over the 24-hour assay period.

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.

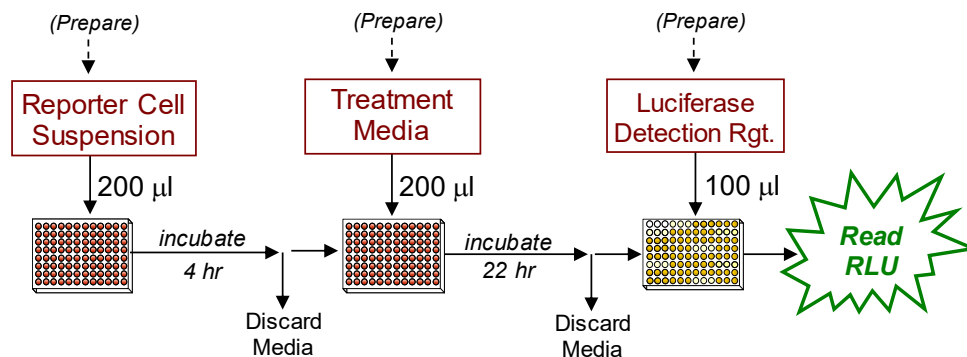
▪ Considerations for Automated Dispensing ▪

When 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 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 µl/well of Reporter Cells are dispensed into 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 hour incubation discard treatment media and add Luciferase Detection Reagent. The intensity of light emission (in units of 'Relative Light Units'; RLU) from each assay well is quantified using a plate-reading luminometer.



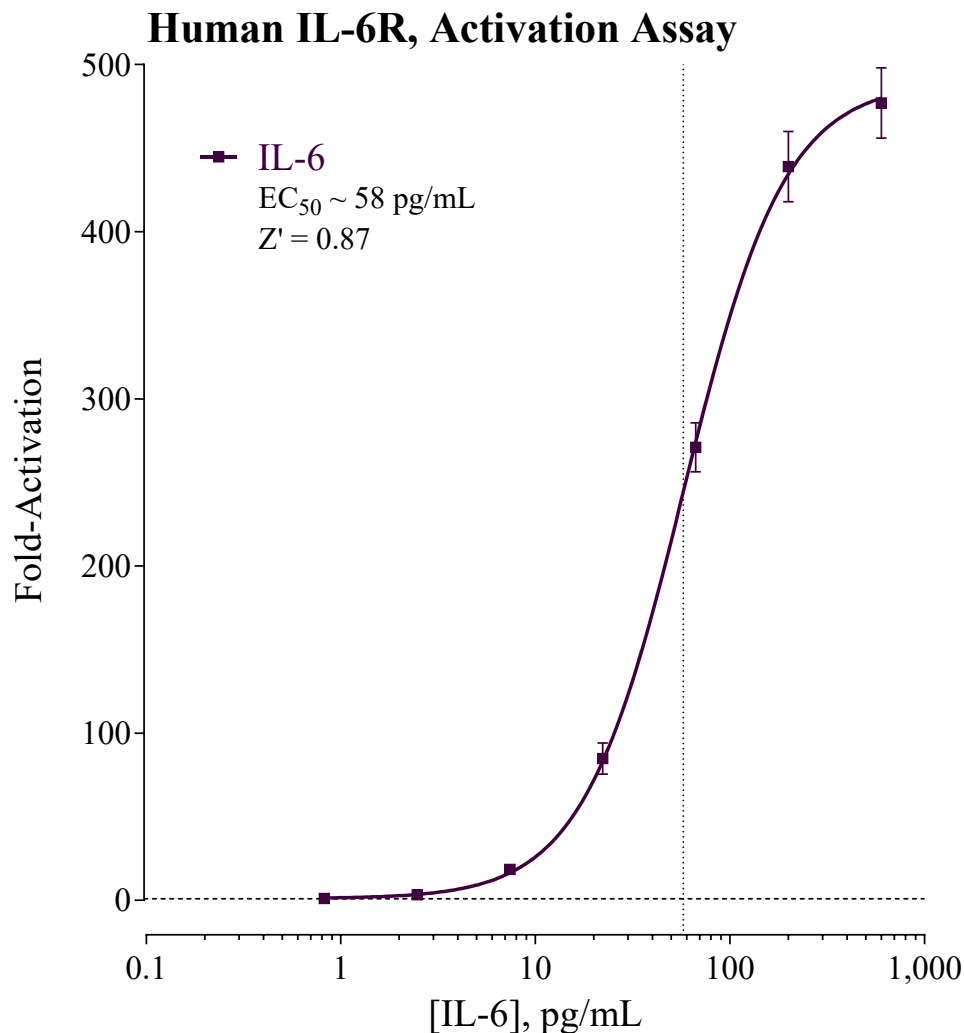


Figure 2. IL-6R Agonist dose-response assays. IL-6R activation assays were performed according to the protocol provided in this Technical Manual. 200 μ l / well of IL-6R Reporter Cell suspension was dispensed into the 96-well assay plate, which was then incubated for 4 hours. The reference peptide IL-6 (provided; ProspeBio) was diluted using CSM to produce treatment media at the desired assay concentrations. The pre-culture media were discarded from the assay wells and 200 μ l per well of respective treatment media were dispensed (n = 4/conc.). Following a 22-hour incubation period 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 % CV and Z'⁵ were determined. Non-linear regression analyses and EC₅₀ calculations were performed using GraphPad Prism software. Plots show Fold Activation vs. Log₁₀[pg/mL] for the various treatment materials; error bars depict +/- %CV.

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.

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

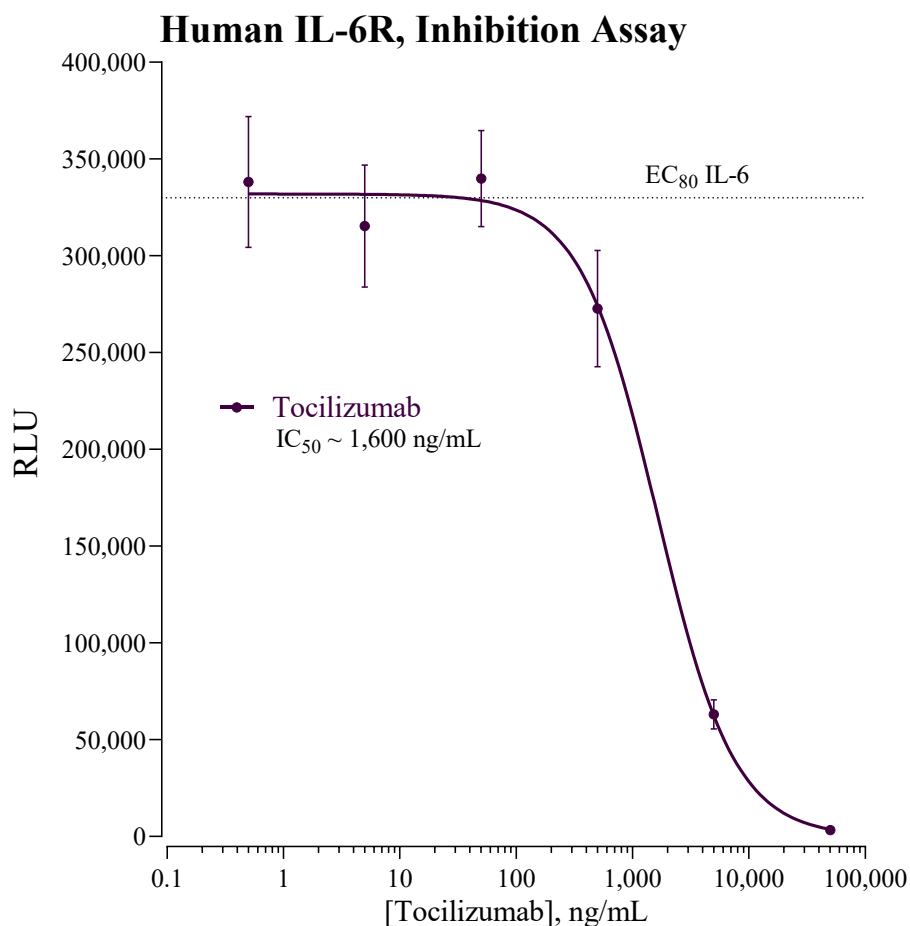


Figure 3. IL-6R Inhibition assays. 200 μ l / well of IL-6R Reporter Cell suspension was dispensed into 96-well assay plates, which were then incubated for 4 hours. For inhibition-mode assays, prior to the end of the pre-culture period, CSM was supplemented with $\sim$ 125 pg IL-6/ml (an approximate EC_{80} concentration). The IL-6-supplemented CSM was then used to prepare the various treatment media. The anti-IL-6R antibody Tocilizumab (Sino Biological) was diluted using IL-6-supplemented CSM to produce final assay concentrations beginning at 50 μ g/mL and proceeding with serial 10-fold decrements. Pre-culture media were discarded and 200 μ l/well of the prepared treatment media were dispensed ($n = 5/\text{conc.}$), including ‘vehicle only’ control wells. After 22 hr incubation treatment media were discarded, Luciferase Detection Reagent was added, and RLU/well were quantified. Plots are RLU vs. $\text{Log}_{10}[\text{ng/mL}]$ for the various test materials. Error bars depict $\pm$ %CV.

II. Product Components & Storage Conditions

This Human IL-6R Assay kit contains materials to perform assays in a single collagen-coated 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 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.

<u>Kit Components</u>	<u>Amount</u>	<u>Storage Temp.</u>
▪ IL-6R 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
▪ IL-6, 600 ng/mL (in PBS/0.1%BSA) (reference activator of IL-6R)	1 x 40 µL	-20°C
▪ Detection Substrate (Note: contains DTT)	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

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% CO₂ 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:* antagonist reference compound / antibody (*e.g.*, Fig. 3.)
- *Optional:* clear 96-well assay plate, cell culture treated, collagen-coated, for viewing cells on Day 2.

DAY 2 plate-reading luminometer.

IV. Assay Protocol

The Day 1 Assay protocol begins on the next page. Please review the entire Assay 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 hr 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 known agonist AND varying concentrations of the test compound(s) to be evaluated for chemical inhibition or neutralizing antibody activities. This IL-6R Assay kit includes a 600 ng/mL stock solution of **IL-6**, a potent physiological agonist of IL-6R, that may be used to set up inhibition-mode assays. ~ 125 pg IL-6/ml typically approximates an EC_{80} concentration in this assay. Hence, it presents a suitable concentration of agonist to use when screening test materials for inhibitory activities.

Add IL-6 to a bulk volume of **CSM**, as described above. This agonist-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 IL-6R 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 **IL-6R 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 '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-culture reporter cells: Place the assay plate into a 37°C, ≥ 70% humidity, 5% CO₂ incubator for 4 - 6 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.) 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*, 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 carried over into assay reactions should not exceed 0.4%.

a. Agonist-mode assays. This IL-6R Assay kit includes a concentrated (1000x) stock of IL-6, 600 ng/mL prepared in PBS/0.1%BSA. The following 7-point treatment series, with concentrations generated using serial 3-fold dilutions, provides a complete dose-response: 600, 200, 66.7, 22.2, 7.41, 2.47 and 0.823 pg/ml. **APPENDIX 1** provides guidance for generating such a dilution series. Always include 'no treatment' (or 'vehicle') controls.

~ or ~

b. Antagonist-mode assays. When setting up antagonist assays, first supplement a bulk volume of CSM with the challenge agonist **IL-6** to achieve an EC₅₀ – EC₈₀ concentration (refer to "*A word about inhibition-mode assay setups*", pg. 9). The agonist-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 pre-culture period, discard the media by 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.

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 37°C, humidified 5% CO₂ 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.) 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*, 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.

NOTE: 'Detection Substrate' contains a high concentration of DTT, which produces a strong odor that some users may find objectionable. It is advised to work in a **fume hood** when preparing LDR, and subsequently when dispensing it into the assay plate followed by a 'plate rest' period (*Step 16*).

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

<i>Product No.</i>	<i>Product Descriptions</i>
Human IL-6R Assay Products	
IB53001-32	Human IL-6R Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
IB53001	Human IL-6R Reporter Assay System 1x 96-well format assay
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
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. Limited Use Disclosures

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

“CryoMite” is a Trademark TM 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 currently updated version.

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APPENDIX 1

Example scheme for the serial dilution of the reference agonist IL-6, and the setup of an IL-6R dose-response assay.

