

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

3x 32 Assays in 96-well Format
Product # IB53001-32

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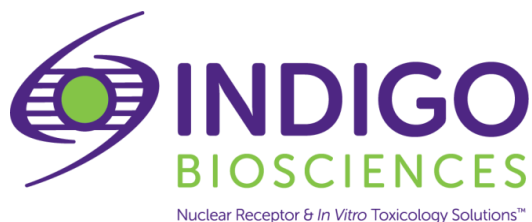
Technical Manual
(version 7.2i)

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Human IL-6R Reporter Assay System **3x 32 Assays in 96-well Format**

I. Description	
▪ The Assay System.....	3
▪ The Assay Chemistry.....	4
▪ Preparation of Test Compounds.....	4
▪ Assay Scheme.....	5
▪ Assay Performance.....	5
II. Product Components & Storage Conditions	7
III. Materials to be Supplied by the User.....	7
IV. Assay Protocol	
▪ A word about Inhibition-mode assay setup.....	8
▪ <i>DAY 1 Assay Protocol</i>	8
▪ <i>DAY 2 Assay Protocol</i>	10
V. Related Products.....	11
VI. Limited Use Disclosures.....	11
APPENDIX 1: Example Scheme for Serial Dilutions.....	12

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. 9).

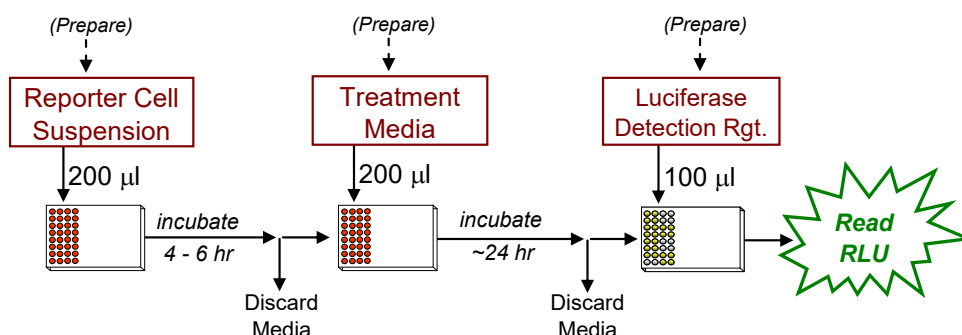
Alternatively, if **small-molecule** test compound solubility is expected to be problematic, 2.) DMSO may be used to make serial dilutions to produce 1,000x-concentrated stocks for each independent test concentration. Treatment media are then prepared using CSM to make 1,000-fold dilutions of the prepared 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 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.

▪ Assay Scheme ▪

Figure 1. Assay workflow. *In brief*, 200 μ l of Reporter Cells is dispensed into wells of the assay plate and for 4-6 hours. Following the pre-incubation period, culture media are discarded and 200 μ l/well of the prepared treatment media are added. Following 22-24 hr incubation, discard the 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.



▪ Assay Performance ▪

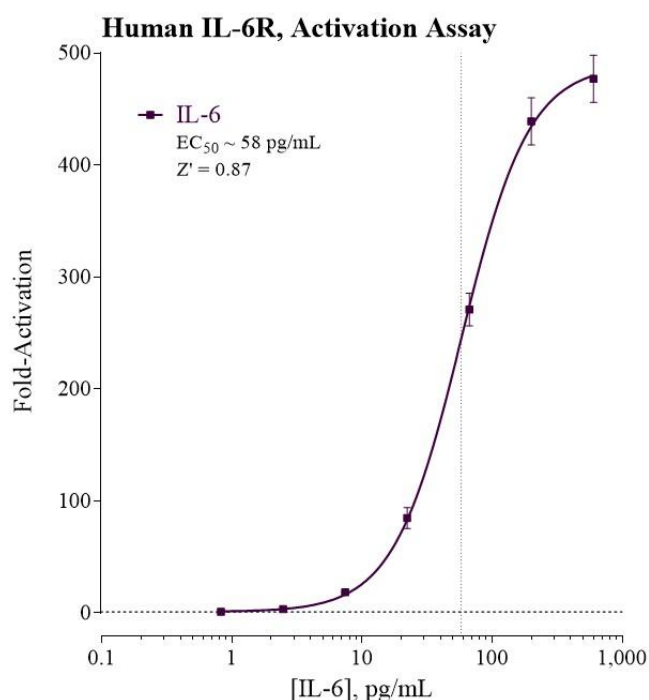


Figure 2. IL-6R Agonist dose-response assays. IL-6R activation assays were performed according to the protocol provided in this Technical Manual. 200 μ l / well of IL-6R Reporter Cell suspension was dispensed into the assay plate, which was then incubated for 4 hours. The reference peptide IL-6 (provided; ProspecBio) were 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 EC_{50} calculations were performed using GraphPad Prism software. Plots show Fold Activation vs. $\text{Log}_{10}[\text{pg/mL}]$ for the various treatment materials; error bars depict $\pm$ %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.

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

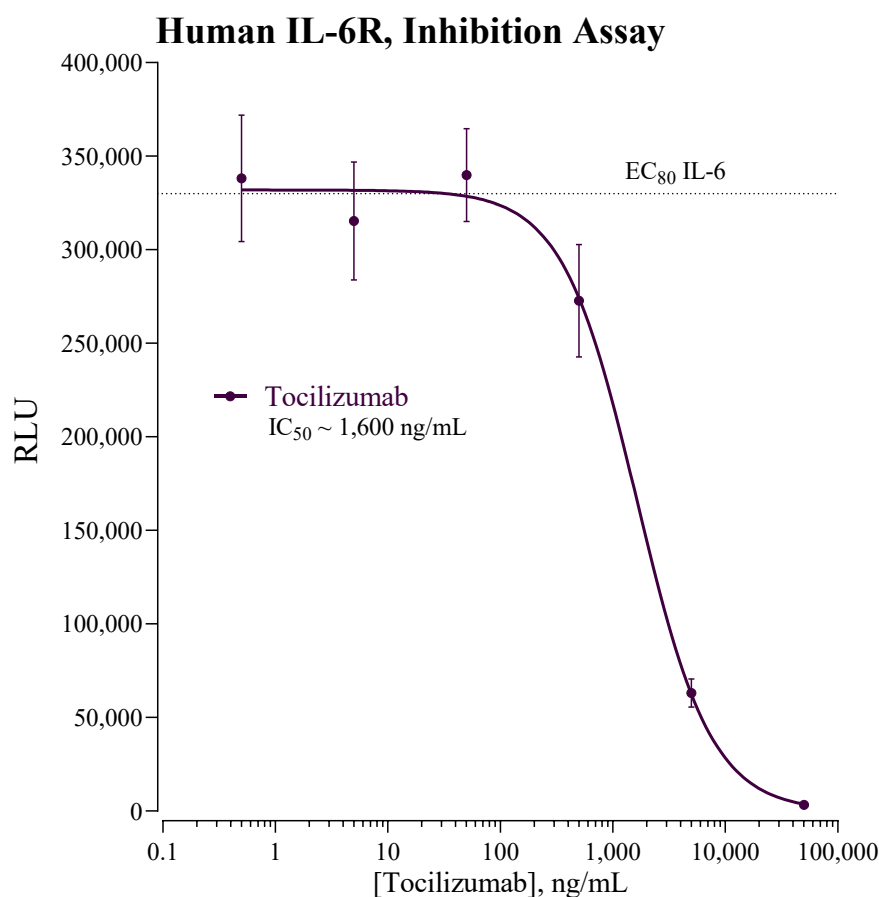


Figure 3. IL-6R Inhibition assays. 200 μ l / well of IL-6R Reporter Cell suspension was dispensed into 96-well assay plates, which were then incubated for 4 hours. For inhibition-mode assays, prior to the end of the pre-culture period, CSM was supplemented with ~ 125 pg IL-6/ml (an approximate EC_{80} 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 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.

Cryopreserved mammalian cells are temperature sensitive! To ensure maximal viability the tubes 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 aliquots of Reporter Cells are 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><i>Kit Components</i></u>	<u><i>Amount</i></u>	<u><i>Storage Temp.</i></u>
▪ IL-6R 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
▪ IL-6, 600 ng/ml (in PBS+0.1%BSA) (physiological activator of IL-6R)	1 x 40 µL	-20°C
▪ Detection Substrate (Note: contains DTT)	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 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 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 (e.g., Figure 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 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 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 EC₅₀ – EC₈₅) 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 EC₈₀ 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 **Reporter Cells** from -80°C storage and place them directly into dry ice to transport them 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, during the 5 - 10 minutes incubation period, work in the cell culture hood to *carefully* mount four sterile 8-well strips into the blank assay plate frame. Strip-wells are fragile. Note that they have keyed ends (square and round), hence, they will fit into the plate frame in only one orientation.

3.) Retrieve the tube(s) 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 disperse cell aggregates and 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 'blank' wells (meaning cell-free but containing 'CSM') must be included in the assay plate to allow quantification of fluorescence background (refer to the LCMA Technical Manual).

NOTE 4.2: Increased well-to-well variation 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, collagen-coated 96-well assay plate. Continue to process the assay 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% CO₂) 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*, 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 never exceed 0.4%.

a. Activation-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. Inhibition-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. 8). 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 cell pre-culture 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 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 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 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 or in a **fume hood**.

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. 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.

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 and throughout the 'plate rest' period (*Step 16*).

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 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.

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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

