

**Human Bradykinin B2 Receptor
Reporter Assay System
(B2R)**

3x32 Assays in 96-well Plate Format
Product # IB51001-32

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Technical Manual
(version 7.2i)

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I. Description

▪ Background ▪

This assay utilizes proprietary human cells that provide constitutive expression of the receptors that comprise the **Bradykinin B2 Receptor (B2R)**.

Bradykinin receptors are class A G protein-coupled receptors (GPCRs) that respond to kinin peptides. These receptors play an important role in mediating pain, increasing vascular permeability, and vasodilation. The Bradykinin receptor family includes two main subtypes: the Bradykinin B1 Receptor (B1R) which is synthesized de novo following tissue injury, and the constitutively expressed Bradykinin B2 Receptor (B2R)¹.

B2R is ubiquitously expressed and found in most healthy human tissue. It can be activated by the nonapeptide Bradykinin and by the decapeptide Kallidin². Upon activation, B2R primarily signals through the Gq/11 pathway leading to calcium mobilization, triggering the release of vasodilators such as prostaglandins, which play a role in lowering blood pressure³. B2R can also form functional dimers with different GPCRs such as angiotensin II type 1 receptor (AT1R). The AT1-B2 heterodimers play a role in hypertensive cardiovascular disorders such as preeclampsia hypertension⁴. Unlike B1R, B2R undergoes rapid desensitization and internalization via β -arrestins following agonist stimulation⁵. Due to the many important roles played by B2R, dysregulation of the receptor can have pathological consequences such as cardiovascular diseases⁶, renal diseases⁷, and hereditary angioedema (HAE)⁸.

▪ The Assay System ▪

B2R activation of the PLC pathways leads to an increase in intracellular calcium and the concomitant activation of calcineurin, a calcium-dependent phosphatase. Ca^{+2} -calcineurin acts to dephosphorylate and activate the transcription factor NFAT⁹. B2R activation of the Ca^{+2} -calcineurin > NFAT cascade is the signal transduction pathway exploited by the reporter cells provided in this kit.

INDIGO's B2R Agonist Reporter Cells contain the luciferase reporter gene functionally linked to tandem NFAT consensus response element sequences upstream of a minimal promoter. Activated NFAT binds to these response elements to initiate the formation of a complete transcription complex that drives Luc 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-induced changes in B2R activity. Accordingly, the principal application of this reporter assay is in the screening of test materials to quantify any functional interactions, either activating or inhibitory, that they may exert against B2R, or the coupled Ca^{+2} -calcineurin/NFAT signal transduction pathway.

INDIGO's Reporter Cells are transiently transfected and prepared as cryopreserved 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 assay kits provide the convenience of an all-inclusive cell-based assay system. In addition to B2R Reporter Cells, provided are two optimized media for use in recovering the cryopreserved cells and for diluting test samples, the reference activator bradykinin, 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).

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 test compounds: Small-molecule 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 8* and depicted in Appendix 1 for the reference activator Bradykinin, 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.

▪ Assay Scheme ▪

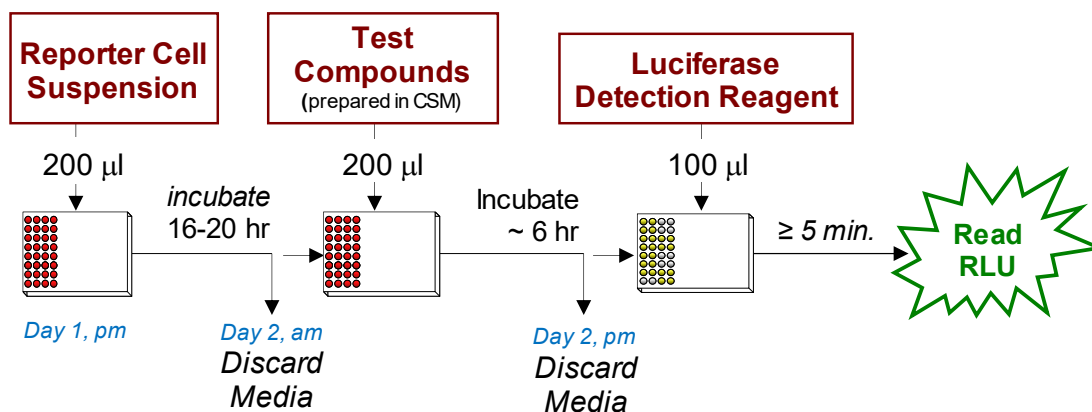


Figure 1. Assay workflows for B2R activation and inhibition assays. It is recommended to begin assay setups in the late afternoon (pm) of *Day 1*. In brief, 200 $\mu\text{l}/\text{well}$ of Reporter Cells are dispensed into the assay plate, which is then incubated overnight (16-20 hours). In the morning (am) of *Day 2*, the culture media are discarded and 200 $\mu\text{l}/\text{well}$ of the prepared treatment media are added. Following an incubation period of ~ 6 hours, 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.

▪ Assay Performance ▪

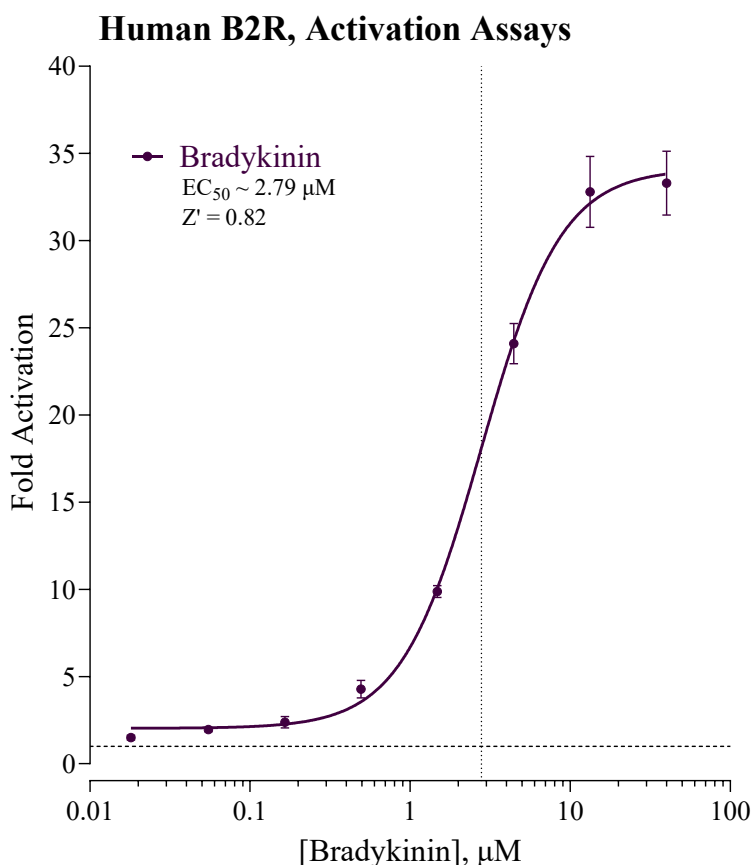


Figure 2. Activation of B2R. Assay was performed using the reference compound Bradykinin (provided; Cayman Chemical, Ann Arbor, MI). Luminescence was quantified and values of average ($n=3$) relative light units (RLU), corresponding standard deviation (SD), Fold-Activation, and Z' ¹⁰ values were calculated. GraphPad Prism software was used to plot data using the least-squares method of non-linear regression for Fold-Activation vs. Log_{10} [Bradykinin, μM], and to determine the EC_{50} value.

II. Product Components & Storage Conditions

This Human B2R 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 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>
▪ B2R 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
▪ Bradykinin (20 mM in DMSO)	1 x 30 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:

- dry ice bucket (*Step 2*)
- cell culture-rated laminar flow hood.
- 37°C, humidified 5% CO₂ incubator for mammalian cell culture.
- 37°C water bath.
- 70% alcohol wipes
- 8-channel electronic, repeat-dispensing pipettes & sterile tips
- disposable media 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).
- plate-reading luminometer.
- *Optional:* clear 96-well assay plate, sterile, collagen-coated, for viewing cells on *Day 2*.
- Plate-reading luminometer

IV. Assay Protocol

Review the entire Assay Protocol before starting. As noted in **Figure 1** (page 5), it is recommended that *Steps 1-6* are performed in the late afternoon on **Day 1**; these will require less than one hour of bench work to complete. An overnight incubation (16-20 hours) is required. *Steps 7-17* are performed in the morning of **Day 2**; approximately 2 hours of preliminary benchwork is required.

It is recommended that B2R *Activation* and *Inhibition*-assays are performed using a 6-hour treatment period, with the quantification of luciferase activity in the afternoon of **Day 2**.

▪ A word about Inhibition-mode assay setup ▪

Inhibition assays expose the Reporter Cells to a constant, sub-maximal concentration (typically between EC₅₀ – EC₈₅) of a B2R activator AND varying concentrations of the test compound(s) to be evaluated for inhibitory activities. This assay kit includes a 20 mM stock solution of **Bradykinin**, a potent activator of B2R that may be used to setup inhibition-mode assays. ~6.0 µM Bradykinin typically approximates EC₈₀ in this cell-based assay. Hence, it presents a suitable co-treatment concentration to be used to screen test compounds for inhibitory activity.

Add the challenge activator, Bradykinin, to a bulk volume of CSM at an EC₅₀ – EC₈₅ concentration. This medium is then used to prepare serial dilutions of test compounds to achieve the desired respective final assay concentrations. We find that this is an efficient and precise method of setting up B2R inhibition assays, and it is the method presented in *Step 8b* of this protocol.

DAY 1 Assay Protocol: It is recommended to begin mid- to late afternoon. 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 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 '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 (= increased standard deviation!) will occur if care is not taken to prevent cells from settling in the basin during the dispensing period. Likewise, take care to ensure precision in dispensing exact volumes across the assay plate.

(continued ...)

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 clear 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, \geq 70% humidity, 5% CO₂) for 16 - 20 hours.

NOTE: Ensure a high-humidity (\geq 70%) environment within the cell culture incubator. This is critical to prevent the onset of deleterious "edge-effects" in the assay plate.

6.) For greater convenience on *Day 2*, transfer **Compound Screening Medium (CSM)** from freezer storage into a refrigerator (+4°C) to thaw overnight.

DAY 2 Assay Protocol: It is recommended to begin first thing in the morning.

7.) Near the end of the preliminary overnight incubation period remove **Compound Screening Medium (CSM)** from the refrigerator and allow it to warm to room temperature.

8.) 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 10*, the prepared treatment media will be dispensed at 200 μ l / well into the assay plate. Manage dilution volumes carefully; this assay kit provides **45 ml** of CSM.

NOTE: Total DMSO (or any organic solvent) carried over into assay reactions should not exceed 0.4%.

a. Activation-mode assays. This B2R Assay kit includes a 20 mM stock solution of (Bradykinin) a potent activator of B2R. The following 8-point treatment series, prepared in serial 3-fold decrements, provides a complete dose-response: 40.0, 13.3, 4.44, 1.48, 0.494, 0.165, 0.055, and 0.018 μ M. Always include a 'no treatment' (or 'Vehicle only') control. **APPENDIX 1** provides an example 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, Bradykinin, to achieve the desired final assay-concentration (refer to "*A word about inhibition-mode assay setup*", pg. 7). The Bradykinin-supplemented CSM is then used to generate dilutions of test compound stocks to achieve their final assay concentrations.

9.) 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.

10.) Dispense **200 μ l / well** of each prepared treatment media into the assay plate.

(continued...)

11.) Transfer the assay plate into a cell culture incubator (37°C, ≥70% humidity, 5% CO₂). Incubate the assay plate 6 hours for **activation** or **inhibition** assays.

NOTE: At this time, retrieve **Luciferase Detection Buffer** and **Luciferase Detection Reagent** from freezer storage and place them in a low-light area so that they may thaw and equilibrate to room temperature for ready use in *Step 13*. Do NOT actively warm Detection Substrate above room temperature; if needed, a room temperature water bath may be used to expedite thawing.

12.) Near the end of the 6-hour treatment period, turn on the plate-reader and set the instrument to "luminescence" mode. Program the instrument to perform a single 5 second "plate shake" prior to reading the first assay well. Set the read-time per assay well to 0.5 second (500 mSec), *or less*.

13.) Immediately before proceeding to *Step 14*: 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 15*).

14.) Following 6 hours incubation in treatment media, remove media contents from each well of the assay plate (as before in *Step 9*)

15.) Use an 8-channel pipette to dispense 100 µl of **LDR** to each well of the assay plate. Allow the plate to rest at room temperature for 5-10 minutes following the addition of LDR. Do not shake the plate during this period.

16.) Quantify luminescence.

17.) Data analyses.

V. Related Products

<i>Product No.</i>	<i>Product Descriptions</i>
Human B2R Assays	
IB51001-32	Human B2R Reporter Assay System 3x 32-well format assay
IB51001	Human B2R Reporter Assay System 1x 96-well format assay
Human B1R Assays	
IB50001-32	Human B1R Reporter Assay System 3x 32-well format assay
IB50001	Human B1R Reporter Assay System 1x 96-well format assay
Bulk volumes of B2R 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
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 available in 10 mL, 25 mL, 50 mL, 500 mL, or larger custom volumes.

Please refer to INDIGO Biosciences website for updated product offerings.

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VI. 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.

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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 current version available.

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VII. Citations

¹ Marceau F, *et al.* (2020) Bradykinin receptors: Agonists, antagonists, expression, signaling, and adaptation to sustained stimulation. *Int. Immunopharmacol.* 2020 May;82:106305. doi: 10.1016/j.intimp.2020.106305.

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

