HUMAN CONSTITUTIVE ANDROSTANE RECEPTOR, SPLICE VARIANT 1 (CAR1, NR1I3) ACTIVATION ASSAY SYSTEM

Technical Manual for 96-Well Format Assay
Catalog# hCAR1-96-001

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HUMAN CAR1 NUCLEAR RECEPTOR ACTIVATION ASSAY, WITH ASSESSMENT OF CELL VIABILITY IN A 96-WELL FORMAT

This technical manual is available in its most current version at: www.puracyp.com. Please contact Puracyp if you have questions concerning the use of the assay system. 760-929-9508 ext. 113; Email: sales@puracyp.com

I. DESCRIPTION

Puracyp's nuclear receptor activation kits are all-inclusive cell-based assay systems. The hCAR1 kits incorporate mycoplasma-free, single-use, transiently-transfected cells derived from a hepatoma cell line. The kits also include a cell culture-ready assay plate, optimized cell culture medium, cell dosing medium, a positive control specific for the nuclear receptor, CellTiter-Fluor™ for determining cell viability and luciferase detection reagents. The primary application of these assay kits is to identify agents with the ability to activate hCAR1, thereby inducing drug-metabolizing enzymes and ABC transporters.

Puracyp's *Human CAR1 Activation Assay System* utilizes hepatoma cells that have been transiently transfected with the full length human CAR, splice variant 1, (NR1I3) and a luciferase reporter gene linked to two promoters identified in the human CYP3A4 gene, namely XREM and PXRE. The hCAR1 cells included in the assay kit remain highly viable upon thawing, and can be immediately dispensed into 96-well plates. There is no need for intermediate spin-and-wash steps. However, determining viability and cell titer is recommended.

Puracyp's *Human CAR1 Activation Assay System* also includes CellTiter-Fluor™, a cell viability detection reagent, and ONE-Glo™, a luciferase detection reagent, from Promega Corporation. Puracyp's hCAR1 assay gives extremely low background, high sensitivity, and a broad linear dynamic range typical of bio-luminescence reporter gene technology. Further details on the use of CellTiter-Fluor™ and ONE-Glo™ can be found at www.promega.com.

In a typical assay, hCAR1 cells are first dispensed into the wells of the assay plate, and then placed in a CO_2 incubator at 37^{o}C to equilibrate overnight. The following day, the cells are treated with user's test compounds and the appropriate positive control. Since the hCAR1 cells included with the kit are transiently transfected, test treatment should take place within 24 h of plate seeding. Once treated, the cells are returned to the CO_2 incubator at 37^{o}C for 24 h. The dosing medium is then discarded, cell viability is assessed fluorimetrically with CellTiter-Fluor™, and luciferase activity then measured using ONE-Glo™. The luminescence light intensity is directly proportional to the extent of hCAR1 activation and accompanying gene transcription in the hCAR1 cells.

II. ASSAY KIT COMPONENTS & STORAGE CONDITIONS

Puracyp's *Human CAR1 Activation Assay System* (Product: hCAR1-96-001) contains materials to perform reporter assays in a 96 well plate format. The kits are constructed so that 32 reporter assays can be performed in triplicate. Individual aliquots of hCAR1 cells, media and detection reagents are intended for a single-use. Once hCAR1 cells have been thawed, they can neither be refrozen nor cultured for future use. Therefore, if an entire 96-well plate is not seeded, the remaining cells should be discarded.

Assay kits are shipped on dry ice and hCAR1 cells are shipped in a liquid nitrogen shipper or in a separate dry-ice container. **If the hCAR1 cells will not be used immediately, they require storage in liquid nitrogen.** The other kit components can be stored at -20^{o} C for up to 90 days. Individual expiration dates are provided with each kit.

KIT COMPONENT	***AMOUNT***	***STORAGE TEMPERATURE***
Tube **A** – hCAR1 Cells	5 million x 1 vial	Liquid Nitrogen
Tube **B** - Positive Control (20 mM)	1 x 10 µl	-20^{o} C
Tube **C** - Culture Media	1 x 15 mL	-20^{o} C
Tube **D** - Dosing Media	1 x 35 mL	-20^{o} C
Tube **E** –CTF Buffer	1 x 10 mL	-20^{o} C
Tube **F** - ONE-Glo™ Assay Buffer	1 x 10 mL	-20^{o} C
Tube **G** - ONE-Glo™ Assay Substrate, Lyophylized	1 vial	-20^{o} C
Tube **J** - CellTiter-Fluor™	1 x 5 µl	-20^{o} C
96-Well Microtiter Plate	1	Ambient

III. MATERIALS TO BE SUPPLIED BY USER

The following materials are required by the end-user, and should be available prior to starting the assay procedure.

FOR RECEIPT OF CELLS

- Cell culture-rated hood
- Humidified 5% CO_2 /37°C incubator suitable for mammalian cell culture
- 37^{o} C water bath
- Sterile multi-channel media basins or reservoirs
- 70% ethanol mixture
- Multi-channel (8- or 12-channel) pipette & sterile tips

FOR DAY 1

- Cell culture-rated hood
- Humidified 5% CO_2 /37°C incubator
- 37^{o}C water bath
- Sterile 1.7 ml tubes for DMSO dilutions
- Sterile 96 deep well plate (2 ml/well capacity, for example, Eppendorf cat.# 9400002001) to make test compound dilutions in media
- DMSO or other solvent for use as a test compound diluent and as the negative control
- Multi-channel (8 or 12) pipette & sterile tips

FOR DAY 2 (Receptor Activation)

- Multi-mode microplate reader with capacity to read luminescence and fluorescence. The luminometer portion of the instrument needs to be able to accumulate luminescence RLUs over a 5 sec/well read. Instruments that average the counts per second will produce low counts resulting in low induction and increased well-to-well variability.
- 2 x Sterile Media reservoirs
- 15 ml sterile conical centrifuge tube
- Multi-channel pipette & sterile tips

IV. ASSAY PROCEDURE

NOTE: It is advisable to review the following section before proceeding with the assay

Puracyp's *Human CAR1 Activation Assay* incorporates one overnight and one 24 h incubation. *Steps 1-11* are performed upon receipt of cells, and require about **30 min** to complete. *Steps 12-21* are performed on Day 1, and need **1.5 - 4.5 h** to complete, depending on the number of dilutions and compounds to be tested. *Steps 22-32* are performed on Day 2, and require **1.5-3 h** to perform.

RECEIPT OF CELLS: ALL STEPS MUST BE PERFORMED USING PROPER ASEPTIC TECHNIQUE

1. Wipe down tissue culture hood with 70% alcohol solution.
2. Remove 96-well plate from kit and place inside tissue culture hood.
3. Place sterile media reservoir or basin in hood.
4. Prepare multi-channel pipette with sterile tips.
5. Place Tube C from kit directly into a 37^{o} C water bath until medium has thawed and warmed to 37^{o}C, and transfer **12 ml** into a sterile reservoir.
6. Place Tube A containing the hCAR1 cells into a 37^{o}C water bath for 1-2 min, or until cell suspension has thawed. **Do Not Overheat**
7. Sterilize outside of Tube A containing the cells with 70% alcohol solution. Using a 2 ml pipette, aspirate the cell mixture up and down twice to mix, then transfer cells to the sterile trough containing 12 ml of medium from Tube C.
8. Remove 2 ml of the cell-medium mixture, and use it to rinse out Tube A. Transfer contents back to the media trough. It is recommended that the cells be counted to ensure there are 5 million viable cells/vial.
9. Uniformly mix cells with medium by pipetting up and down at least 10X with a 10 ml serological pipette.
10. Using the multi-channel pipette, transfer 100 μl of cell medium suspension from the reservoir to the individual wells of 96-well plate. If a re-pipettor is not used during the seeding process, ensure that the cells remain suspended in media by mixing the suspension between the seeding of each row.
11. Carefully place seeded plate into humidified 5% CO_2 /37°C incubator overnight.

DAY 1

12. Place Tube B from kit at room temperature.
13. Place Tube D from kit at room temperature, and allow to thaw.
14. Wipe down tissue culture hood with 70% alcohol solution.

THE FOLLOWING STEPS ARE PERFORMED IN A STERILE HOOD

15. Prepare positive control (Tube B) dilutions as described in **Appendix 1.**
16. Prepare Test Compound dilutions in the same manner as the positive control (triplicate determinations are suggested). Prepare the vehicle control (0.1% DMSO) by adding 1 μl of DMSO to 1 ml of medium from Tube D. Remove 96-well plate containing the cells from the incubator and place into the hood.
17. Prepare the plate for test agent addition by marking those wells that will receive test articles, positive controls, and vehicle control on the lid.
18. Carefully remove the liquid from each well using a multi-channel pipette. *Avoid touching the cells with the pipette tip but ensure that all media is removed; tilting the plate will aid this***.**
19. Gently add 100 μl of medium containing test agent, positive control or vehicle control to the plate wells. *Medium addition is performed by gently pipetting the liquid against the well walls rather than directly onto the cells attached to the well bottom.*
20. Return the plate to the incubator, and continue to incubate at 37°C for 24 h.

DAY 2 hCAR1 Activation (hCAR1-96-001 Kit)

21. Allow Tubes E, F, G & J to reach room temperature.
22. Add the contents of Tube F (ONE-Glo™ Assay Buffer) to Tube G (ONE-Glo™ Assay Substrate), cap, and invert the bottle 3X to mix.
23. Transfer 10 ml from Tube E to a sterile 15 ml conical tube, and add 5 μl from Tube J (CellTiter-Fluor™). Mix by inverting tube.
24. Remove 96-well plate containing the cells from the incubator.
25. *Carefully* remove the liquid from each well using a multi-channel pipette. Again, avoid touching the cells with the pipette tips but ensure that all media is removed; tilting the plate will aid this.
26. Pour contents of 15 ml conical tube (containing CellTiter-Fluor™ reagent) into a sterile media trough. Using a multi-channel pipette, *gently* add 100 μl of CellTiter-Fluor™ reagent into each well.
27. Place 96-well plate in the incubator for 60 min.

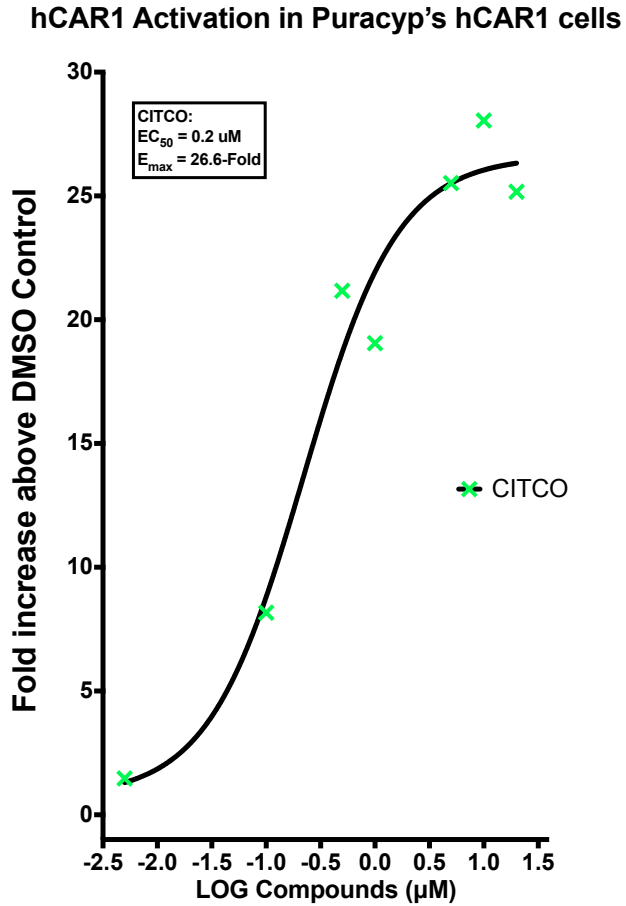
The Following Steps Do Not Require Sterile Conditions

28. Remove 96-well plate from the incubator and measure fluorescence of individual wells with a microplate reader in fluorescence mode using an excitation wavelength of 380-400 nm and an emission wavelength of 505 nm. Read plate at 10 msec/well using low sensitivity.
29. Pour ONE-Glo™ Assay reagent (prepared in Step #22) into a media reservoir, and add 100 μl of this reagent into each well of the plate.
30. Carefully agitate the plate to mix the reagents contained in the wells and incubate at room temperature for 5 min.

31. After 5 min, read the luminescence of individual wells using a luminometer set for a 5 sec pre-shake with a **5 sec/well** read time. A high gain (sensitivity) setting should be used. Ensure that the luminometer is accumulating RLUs over the 5 sec/well read and not averaging the counts/sec.

QUANTITATION OF HCAR1 RECEPTOR ACTIVATION

1. Determine the average Relative Luminescence Units (RLU) of the three replicates for each test compound at each dosage. In addition, determine the average Relative Fluorescence Units (RFU) of the three replicates for each test compound at each dosage. RFU values should range between 5000-9000. If the values exceed 9000 RFU, adjust sensitivity or read time/well of the fluorimeter.
2. Determine the mean RLU and RFU for the 0.1% DMSO vehicle control replicates. RLU values for DMSO or media only should range between 200-6000. If values are less than 200, increase sensitivity of the luminescence readout and ensure that the luminometer is accumulating counts over the 5 sec/well read and providing a readout for the 5 sec, not as an average expressed as 1 sec/well.
3. Normalize the luciferase activity for cell viability by dividing the average RLU by the average RFU for each test compound at each dosage as well as for the vehicle control.
4. hCAR1 receptor activation at the individual test compound doses is calculated by dividing the normalized luciferase activity (RLU/RFU) for the test compound doses by that of the normalized DMSO vehicle control. The final data is expressed as fold activation relative to the vehicle control. The use of 5 or more doses of test compound and positive control allows for the derivation of EC_{50} and E_{MAX} values from nonlinear regression analysis of the log dose-response curves. Graphpad Prism (Graphpad Software, La Jolla, CA) and SigmaPlot (Systat Software, San Jose, CA) are among the software programs with built-in equations for deriving these CAR activation kinetic parameters.
5. Test compound values should be compared to those obtained for the positive (CITCO) control. An example of a typical hCAR1 activation dose-response curve generated with 7 concentrations of 6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO) is shown below.


hCAR1 Activation in Puracyp's hCAR1 cells
CITCO:
EC50 = 0.2 uM
Emax = 26.6-Fold
Fold increase above DMSO Control
30
25
20
15
10
5
0
-2.5 -2.0 -1.5 -1.0 -0.5 0.0 0.5 1.0 1.5
LOG Compounds (μM)
CITCO

TROUBLESHOOTING GUIDE

SYMPTOM	POSSIBLE CAUSE	RESOLUTION
High Well-to-Well Variation	Cells Not Evenly Dispersed Among Wells	**Mix cells thoroughly with medium before seeding.** To facilitate even plating, ensure the cells in Tube A are pipetted up/down at least twice prior to transfer to culture medium. Upon transfer, mix cells with culture medium by pipetting up/down 10 times.
Weak Activation with Potent Positive Control	Inadequate cell number	**Ensure that there are 5 million viable cells per vial.** Each well of a 96 well plate should receive at least 40,000 cells/well. To ensure that the cells arrived in good condition following shipping, it is recommended that the cells be counted and viability checked prior to seeding in the 96 well plate.
	Luminometer Settings	**The luminometer used should be one that can be set to accumulate counts over a 5 second/well read.** If the luminometer averages the counts per second, RLU values will be extremely low causing high well-to-well variability and low induction. Ensure that your instrument is set to 5 sec/well read time and is set on the highest sensitivity (gain).
Weak Activation with Test Compounds	Poor Test Compound Solubility	**Test compounds dissolved in DMSO and other solvents precipitate out of solution when added to culture medium**. This often occurs at higher concentrations. Weak activation stemming from poor solubility would be indicated by: a) a peak activation response occurring midway in the dose-response curve, followed by decreasing responses at higher concentrations and; b) no change in cell viability throughout the entire concentration curve.

	Effects on Cell Viability	**Test compound cytotoxicity** can result in weak hCAR1 activation. Cell viability should be assessed using CellTiter-Fluor™ reagent, and the fold hCAR1 activation normalized to the viable cell number for a given test compound. A decline in receptor activation **and** cell viability over the concentration range examined would indicate that the test compound is toxic to the hCAR1 cells.
False Positives	Borderline Activation by Non-hCAR Ligands	The low levels of hCAR1 activation observed with certain compounds may not be indicative of "false positives". Cut-off values, commonly expressed as % of CITCO, should be established to differentiate negative, weak, moderate and potent CAR activators. For example, compounds giving activation < 15% of that observed with 10 μM CITCO would be considered negative. Agents giving 15 - 40% of the response observed with 10 μM CITCO would be considered weak activators while those eliciting 41 - 69% of the CITCO response would be moderate activators. Only those compounds giving **>** 70% of the response noted with 10 μM CITCO would be considered strong or potent CAR activators.

For any additional questions and/or problems with Puracyp's *Human CAR1 Activation Assay System*, please contact us by phone at 760-929-9508 or by email at sales@puracyp.com.

V. LIMITED USE DISCLOSURES

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ONE-Glo™ and CellTiter-Fluor™ are subject to patents pending by Promega Corporation.

The method of recombinant expression of Coleoptera luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.

Additional information on ONE-Glo™ and CellTiter-Fluor™ can be found at www.promega.com.

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VI. REFERENCES

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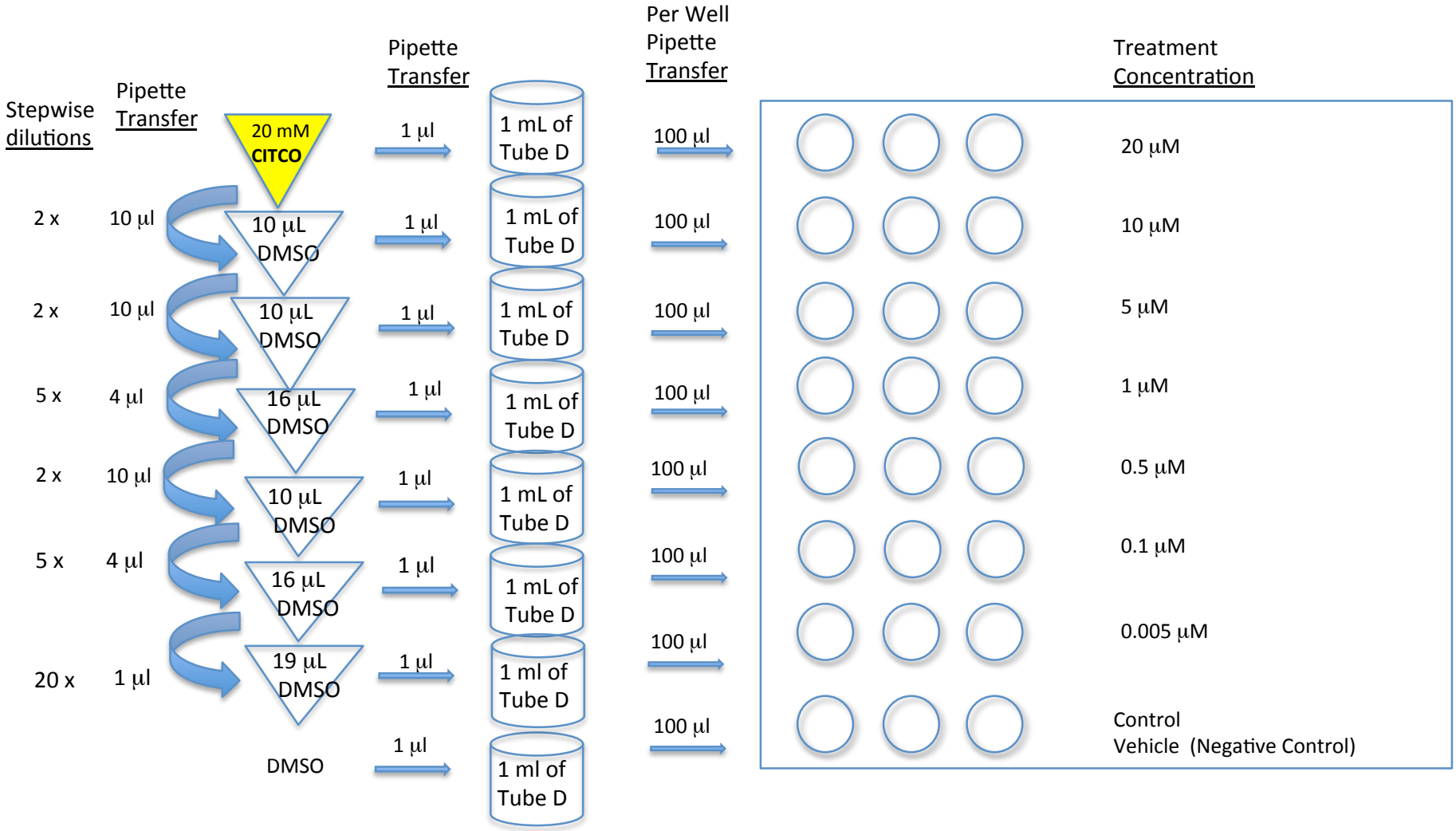
APPENDIX 1 FOR HCAR1-96-001

Dilution Scheme for ***Dosing Positive Control***



All test compound dilutions are made with neat DMSO, after which the individual solutions are added to dosing media at a ratio of 1:1000.

APPENDIX 2: QUICK REFERENCE CARD

HUMAN CAR1 NUCLEAR RECEPTOR ACTIVATION ASSAY WITH ASSESSMENT OF CELL VIABILITY IN A 96-WELL FORMAT (*hCAR1*-96-001)

USE STERILE TECHNIQUE FOR STEPS 1-17

KIT RECEIPT DAY

1. Thaw Tube C by warming in a 37^{o} C water bath, and transfer 12 ml into a media reservoir.
2. Place Tube A containing hCAR1 cells in a 37^{o} C water bath until cell suspension has thawed.
3. Pipette cells into the media trough containing 12 ml of Reagent C, and mix by pipetting up-and-down 10 times. Count cells and determine viability.
4. Transfer 100 µl of cell mixture into plate wells, keeping cells suspended while pipetting.
5. Place seeded plate into a humidified CO_2 incubator at 37^{o} C overnight.

DAY 1

6. Thaw Tube D in a 37^{o}C water bath.
7. Allow Tube B to thaw at room temperature.
8. Prepare series of Test Compound and Positive Control dilutions in DMSO (see Appendix 1) followed by 1:1000 dilutions in dosing medium (Tube D).
9. Remove plate from the incubator, and carefully aspirate and discard the media from each well. Do not disturb the cells during the aspiration process.
10. Transfer 100 µl of each test compound concentration into pre-marked wells. Repeat with positive controls and vehicle controls.
11. Return plate to the incubator for a 24 h exposure to the treatments.

DAY 2

12. Thaw Tube J containing CellTiter-Fluor™.
13. Thaw Tube E and transfer 10 ml to a sterile 15 ml centrifuge tube. Add 5 µl of CellTiter-Fluor™. Mix by inversion and pour into media trough.
14. Thaw Tube F containing ONE-Glo™ Luciferase Assay Buffer, and add to Tube G containing ONE-Glo™ Assay Substrate; mix vial several times by inversion. Pour into media trough.
15. Remove plate from the incubator, and carefully aspirate and discard all dosing medium containing test compounds, positive controls, and negative (vehicle) controls.
16. Add 100 µl of Tube E containing the CellTiter-Fluor™ reagent (prepared in Step 13) to each well.
17. Return plate to the incubator for 60 min.
18. After incubation, measure fluorescence of individual wells with a multi-mode microplate reader[A] set for fluorescence using an excitation wavelength of 380-400 nm and an emission wavelength of 505 nm.
19. Remove plate from instrument, and add 100 µl of ONE-Glo™ Assay reagent to each well. Agitate plate to mix, and incubate for 5 min at room temperature.
20. Measure luminescence of individual wells with the multi-mode reader set for a 5 sec pre-shake and a **5 sec/well** read. A high instrument gain (sensitivity) setting should be used. Ensure that the luminometer is accumulating RLUs over the entire 5 sec/well read time.
21. Receptor activation is calculated according to the method given in the *Complete Protocol*.

[A]A stand-alone fluorimeter and luminometer can also be used