Human Nuclear Factor (erythroid-derived2)-like 2 (Nrf2; NFE2L2) Reporter Assay System

384-well Format Assays
Product # IB10002

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Technical Manual
(version 7.2)

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Human Nrf2 Reporter Assay System
384-well Format Assays

I. Description

▪ Background ▪

Nuclear Factor (erythroid-derived 2)-like 2 (Nrf2) is a ubiquitously expressed, basic leucine zipper transcription factor. It regulates the expression of a variety of genes encoding proteins that play critical roles in cyto-protection, as well as the detoxification and clearance of harmful endogenous and xenobiotic substances. In particular, Nrf2 regulates the expression of antioxidant proteins that confer cyto-protection against oxidative damage.

Under normal conditions Nrf2 resides in the cytoplasm in association with Keap1 and Cullin 3. Within the confines of this protein cluster Nrf2 is the target of ubiquitination and rapid turn-over *via* proteasomal degradation. However, under conditions of cellular oxidative stress the tight association of Nrf2 with Keap1 and Cullin 3 is broken, effectively disrupting the otherwise efficient process of Nrf2 degradation. Once non-ubiquitinated Nrf2 accumulates in the cytoplasm it translocates into the nucleus, whereupon it forms hetero-dimers with Maf. In this configuration Nrf2 binds to antioxidant response element (ARE) sequences resident in the promoter regions of some genes, initiating transcription complex formation, and culminating in the expression of antioxidant proteins.

▪ The Assay System ▪

INDIGO's Human Nrf2 Reporter Cells include the luciferase reporter gene functionally linked to a promoter containing tandem antioxidant response elements (AREs). Thus, quantifying changes in luciferase expression in the treated reporter cells provides a sensitive surrogate measure of the changes in Nrf2 activity. The principle application of this reporter assay system is in the screening of test samples to quantify any functional activity, either agonist or antagonist, that they may exert against human Nrf2.

Nrf2 Reporter Cells are prepared using INDIGO's proprietary **CryoMite™** process. This cryo-preservation method yields exceptional cell viability post-thaw, and provides the convenience of immediately dispensing healthy, division-competent reporter cells into assay plates. There is no need for cumbersome intermediate treatment steps such as spin-and-rinse of cells, viability determinations, or cell titer adjustments prior to assay setup.

INDIGO's Human Nrf2 assay kit is an all-inclusive system. In addition to Nrf2 Reporter Cells, this kit provides two optimized media for use during cell culture and in diluting the user's test samples, a reference activator of Nrf2, Luciferase Detection Reagent, and a cell culture-ready assay plate.

▪ The Assay Chemistry ▪

INDIGO's cell-based assay systems 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 (RLU's).

INDIGO's Nuclear Receptor Assay Systems feature a luciferase detection reagent specially formulated to provide stable light emission between 30 and 100+ minutes after initiating the luciferase reaction. Incorporating a 30 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 ▪

Most commonly, test compounds are solvated at high-concentration in DMSO, and these are stored as master stocks. Master stocks are then diluted to appropriate working concentrations immediately prior to setting up the assay. Users are advised to dilute test compounds to 2x-concentration stocks using **Compound Screening Medium (CSM)**, as described in *Step 2* of the **Assay Protocol**. This method avoids the adverse effects of introducing high concentrations of DMSO into the assay. The final concentration of total DMSO carried over into cell-based assays should never exceed 0.4%.

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 processing a small number of assay plates, first carefully consider the dead volume requirement of your dispensing 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.

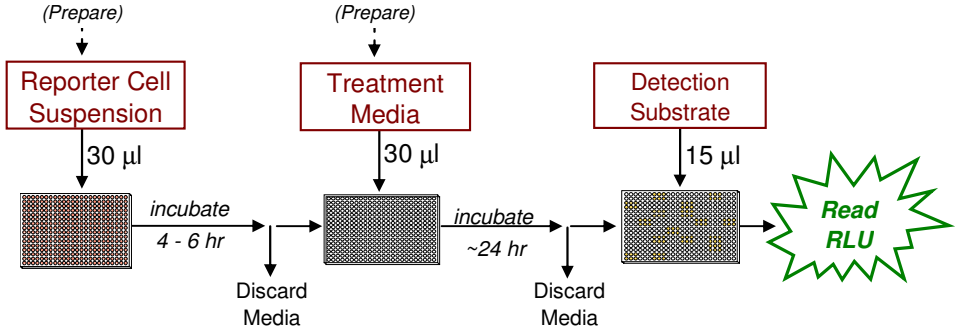
Stock Reagent & Volume provided	Volume to be Dispensed *(96-well plate)*	Excess rgt. volume available for instrument dead volume
Reporter Cell Suspension 12.5 ml *(prepared from kit components)*	30 µl / well 11.5 ml / plate	~1 ml
Detection Substrate 7.8 ml *(prepared from kit components)*	15 µl / well 5.8 ml / plate	~ 2 ml

▪ Assay Scheme ▪

Figure 1. Assay workflow.

NOTE that this Nrf2 assay protocol includes steps and dispensed volumes that are different from the conventional INDIGO assay protocol that users may be accustomed to when setting up INDIGO's other Nuclear Receptor Assays.

In brief, 30 µl/well of Nrf2 Reporter Cells are dispensed into the assay plate and pre-incubated for 4-6 hr. Pre-incubation media is removed by aspiration or 'dumping' and 30 µl/well of prepared test compound treatment media are added. Following 22 -24 hr incubation, treatment media are discarded and 15 µl/well of prepared Luciferase Detection Reagent (LDR) is added. Light emission from each assay well is quantified using a plate-reading luminometer.



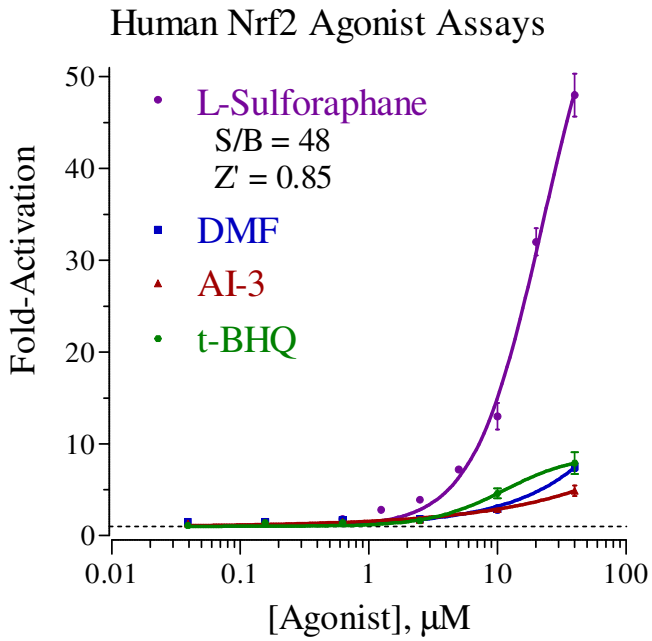


Figure 2. Agonist dose-response analyses of Human Nrf2.
Agonist analyses of Human Nrf2 Reporter Cells were performed according to the protocol described in this Technical manual, using the reference agonists L-Sulforaphane (provided), DMF (dimethyl fumarate; Tocris), t-BHQ (t-butylhydroquinone; Enzo), and AI-3 (1-Cl-6,7-dihydro-6,6-dimethyl-3-(methylsulfonyl)-benzo[c]thiophen-495H)-one; Tocris). Luminescence was quantified using a GloMax-Multi+ luminometer (Promega). Values of fold-activation of Nrf2 and Z' were calculated as described by Zhang, *et al*. (1999)[1]. Non-linear regression and EC_{50} analyses were performed using GraphPad Prism software.

The reference agonist L-Sulforaphane yielded a Z' value of 0.85, confirming the robust performance of this assay and it's suitability for HTS[1].

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

$$Z' = 1-[3*(SD^{Control} + SD^{Background}) / (RLU^{Control} - RLU^{Background})]$$

II. Product Components & Storage Conditions

This Human Nrf2 Reporter Assay System contains materials to perform assays in a single 96-well assay plate.

The aliquot of Nrf2 Reporter Cells is provided as a single-use reagent. Once thawed, reporter cells can NOT be refrozen or maintained in extended culture with any hope of retaining downstream assay performance. Therefore, extra volumes of these reagents should be discarded after assay setup.

Assay kits are shipped on dry ice. Upon receipt, individual kit components may be stored at the temperatures indicated on their respective labels. Alternatively, the entire kit may be further stored at -80°C.

To ensure maximal viability, Reporter Cells must be maintained at -80°C until immediately prior to use.

The date of product expiration is printed on the Product Qualification Insert (PQI) enclosed with each kit.

Kit Components	***Amount***	***Storage Temp.***
▪ Nrf2 Reporter Cells	1 x 2.0 mL	**-80°C**
▪ Cell Recovery Medium (CRM)	1 x 10.5 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x **45** mL	-20°C
▪ L-Sulforaphane, 40 mM (in DMSO) (positive control for Nrf2 activation)	1 x 30 µL	-20°C
▪ Detection Substrate	1 x 7.8 mL	**-80°C**
▪ 384-well assay plate (white, sterile, cell-culture ready)	1	ambient

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

▪ 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- *or* 12-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).

▪ antagonist reference compound (optional).

DAY 2 plate-reading luminometer.

IV. Assay Protocol

Review the entire Assay Protocol before starting. Completing the assay requires an overnight incubation. *Steps 1-8* are performed on **Day 1**, requiring less than 2 hours to complete. *Steps 9-15* are performed on **Day 2**, and require less than 1 hour to complete.

▪ A word about Antagonist-mode assay setup ▪

Receptor inhibition assays expose the Reporter Cells to a constant, sub-maximal concentration (typically between EC_{50} – EC_{85}) of a known agonist AND the test compound(s) to be evaluated for antagonist activity. This Nrf2 Reporter Assay System kit includes a 40 mM stock solution of **L-Sulforaphane**, an activator of Nrf2 that may be used to setup antagonist-mode assays. While an accurate EC50 value cannot be determined for L-Sulforaphane, a 20 µM treatment concentration yields the desired sub-maximal activation of Nrf2 with a suitably large assay window (see **Figure 2**). Hence, it presents a reasonable assay concentration to be used when screening test compounds for inhibitory activity to Nrf2.

We find that adding the challenge agonist to a bulk volume of CSM, at the desired final assay concentration, is the most efficient and precise method of setting up antagonist assays, and it is the method presented in *Step 7b* of the following protocol.

DAY 1 Assay Protocol:

All steps must be performed using proper aseptic technique.

Note: *Protocol steps designated with * represent modifications to the conventional INDIGO protocol that users may be accustomed to when performing INDIGO's other Nuclear Receptor Assays.*

1.) Remove the tube 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, *without delay*, perform a rapid thaw of the frozen cells by transferring the entire 10.5 ml volume of pre-warmed **CRM** into the 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 12.5 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 disperse cell aggregates and gain an homogenous cell suspension. Transfer the cell suspension into a reservoir. Using an 8-chanel pipette, dispense **30 µl / well** of cell suspension into the 96-well Assay Plate.

NOTE 4.1: Take special care to prevent cells from settling during the dispensing period. Allowing cells to settle during the transfer process, and/or lack of precision in dispensing uniform volumes across the assay plate *will* cause well-to-well variation (= increased Standard Deviation) in the assay.

NOTE 4.2: Users sometimes prefer to examine the reporter cells using a microscope. The extra volume of cell suspension provided with each kit may be dispensed into a clear assay plate, treated +/- test compounds as desired, and incubated overnight in identical manner to those reporter cells contained in the white assay plate.

5.)* Pre-incubate reporter cells: Place the assay plate into a 37°C, ≥ 85% humidity, 5% CO_2 incubator for 4 - 6 hours.

Near the end of the 4-6 hour pre-incubation period:

6.) Remove **Compound Screening Medium (CSM)** from freezer storage and thaw in a 37°C water bath.

7.)* **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 treatment media at the desired **final assay concentrations**. In *Step 9*, the prepared treatment media are dispensed at **30 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. Agonist*-mode assays**. This Nrf2 Reporter Assay System kit includes a 40 mM stock solution of **L-Sulforaphane**, an activator of Nrf2. The following 7-point treatment series, prepared in serial 2-fold decrements, provides a suitable dose-response: 40, 20, 10, 5.0, 2.5, 1.25, and 0.625 µM, and including a 'no treatment' control. **APPENDIX 1** provides an example for generating such a dilution series.

***b. Antagonist*-mode assays**. When setting antagonist assays, first supplement a bulk volume of CSM with the challenge agonist L-Sulforaphane to achieve the desired final assay-concentration (refer to "*A word about antagonist-mode assay setup*", pg. 8). The agonist-supplemented CSM is then used to generate dilutions of test compounds to achieve their final assay concentrations.

At the end of the cell pre-incubation period:

8.)* **Discard the culture 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 **30 µl** of each treatment media into appropriate wells of the assay plate.

10.) Transfer the assay plate into a 37°C, humidified 5% CO_2 incubator for 22 - 24 hours.

NOTE: Ensure a high-humidity (≥ 85%) 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** from freezer storage and place 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 Nrf2 activity, remove **Detection Substrate** from the refrigerator and place in a low-light area so that it may equilibrate to room temperature. Once at room temperature, gently invert the tube several times to ensure an homogenous solution.

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.) Following 22 - 24 hours of incubation, retrieve the assay plate from the incubator. Discard all media contents 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.

15.) Add 15 µl of **Detection Substrate** to each well of the assay plate.

16.) Allow the plate(s) to rest at room temperature for 30 minutes. Do not shake the assay plate(s) during this period.

NOTE: the luminescent signal is unstable during the first 30 minutes of the luciferase reaction, however, after the initial 30 minute reaction period the luminescence signal achieves a stable emission output.

17.) Quantify luminescence.

V. Related Products

Human Nrf2 Assay Kit Products	
Product No.	*Product Descriptions*
IB10001-32	3x 32 Nrf2 assays; strip-wells in 96-well plate frame
IB10001	1x 96-well format Nrf2 assays
IB10002	1x 384-well format Nrf2 assays
Bulk assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

LIVE Cell Multiplex (LCM) Assay Products	
Product No.	*Product Descriptions*
LCM-01	Reagents to perform **96** Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well assay plate formats
LCM-05	Reagents in 5x-bulk volume to perform **480** Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats
LCM-10	Reagent in 10x-bulk volume to perform **960** Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats

Please refer to INDIGO Biosciences website for updated product offerings.

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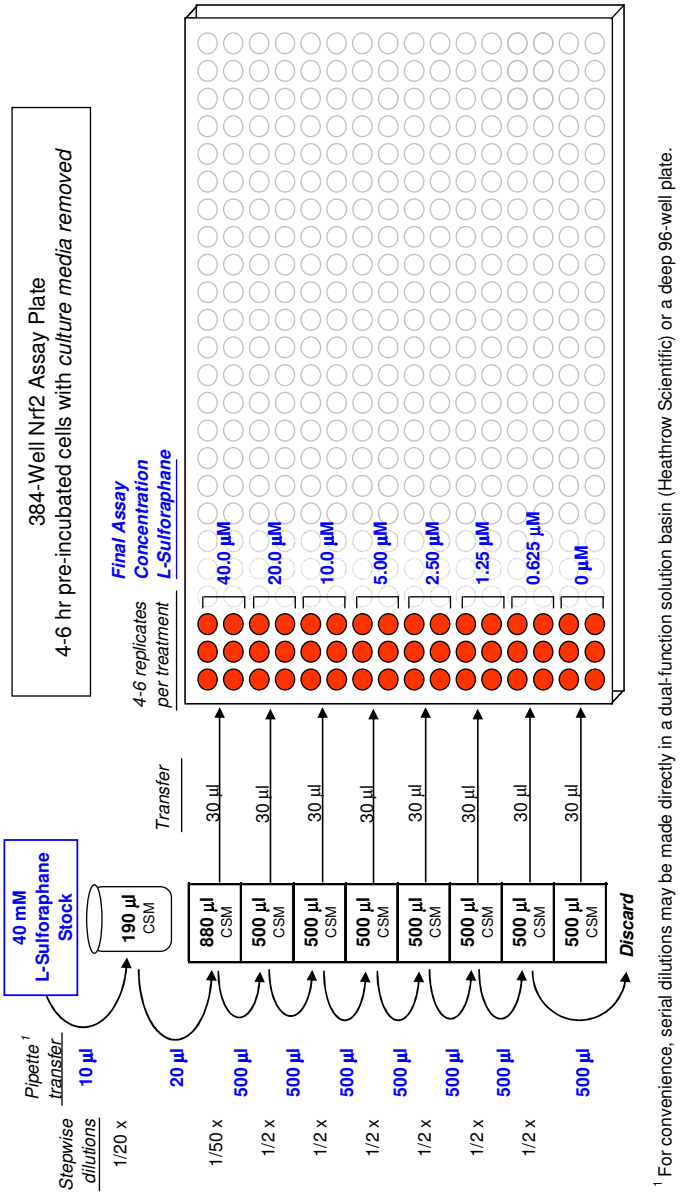
APPENDIX 1

Example scheme for the serial dilution of L-Sulforaphane reference agonist, and the setup of an Nrf2 dose-response assay.



[1] For convenience, serial dilutions may be made directly in a dual-function solution basin (Heathrow Scientific) or a deep 96-well plate.