# Zebrafish Glucocorticoid Receptor
## (nr3c1, zfGR)
## Reporter Assay System

**96-well Format Assays**
Product # Z00201

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**Technical Manual**
*(version 7.2)*


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INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

# Zebrafish GR Reporter Assay System
# 96-well Format Assays

**I. Description**

## *I. Description*

### ▪ The Assay System ▪

This assay utilizes proprietary mammalian cells engineered to provide constitutive high-level expression of full-length, unmodified **Zebrafish (*Danio rerio*) Glucocorticoid Receptor** (**nr3c1**), a ligand-dependent transcription factor, referred to herein as **zfGR**.

INDIGO's Reporter Cells include the luciferase reporter gene functionally linked to a GR-responsive promoter. Thus, quantifying changes in luciferase expression in the treated reporter cells provides a sensitive surrogate measure of the changes in zfGR activity. Luciferase gene expression occurs after ligand-bound zfGR undergoes DNA binding, recruitment and assembly of the co-activators and accessory factors required to form a functional transcription complex, culminating in expression of the reporter gene. Unlike some other cell-based assay strategies, the readout from INDIGO's reporter cells demands the same orchestration of all intracellular molecular interactions and events that can be expected to occur *in vivo.*

zfGR 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.

INDIGO Bioscience's receptor assays are all-inclusive cell-based assay systems. In addition to zfGR Reporter Cells, this kit provides two optimized media for use during cell culture and in diluting the user's test samples, the reference agonist Dexamethasone, Luciferase Detection Reagent, and a cell culture-ready assay plate.

### ▪ The Assay Chemistry ▪

INDIGO's nuclear receptor assays 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 assays 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 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 **Compound Screening Medium (CSM**; as described in *Step 7*) to achieve the desired assay concentrations.

> *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 regarded as 'single-use' reagents.

Alternatively, if test compound solubility is expected to be problematic, 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 never exceed 0.4%. Significant DMSO-induced cytotoxicity can be expected above 0.4%.

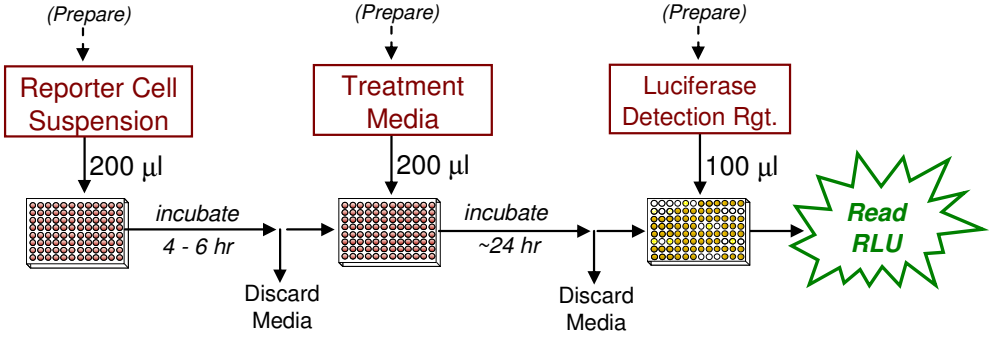
## ▪ Considerations for Automated Dispensing ▪

When processing a small number of assay plates, first carefully considered 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** 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 of reporter cell suspension is dispensed into wells of the assay plate and pre-incubated for 4-6 hours. Following the pre-incubation period, culture media are discarded and 200 µl/well of the prepared 1x-concentration treatment media are added. Following 22-24 hr incubation, treatment media are discarded, and Luciferase Detection Reagent is added. The intensity of light emission (in units of 'Relative Light Units'; RLU) from each assay well is quantified using a plate-reading luminometer.



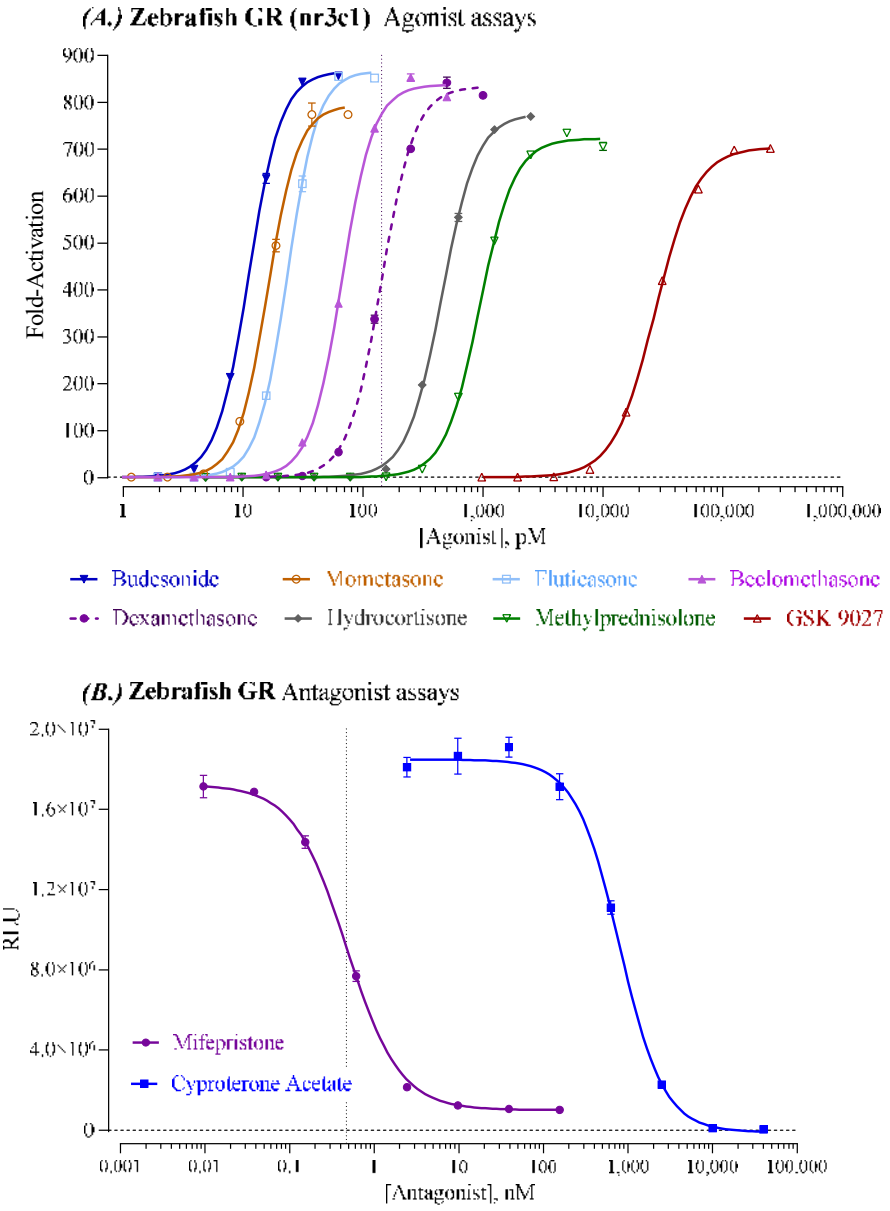



**Figure 2. Activity dose-response of zfGR using various reference agonists.**

All dose-response analyses of zfGR were performed according to the protocol provided in this Technical Manual. *(A.)* Agonist Dose-responses: Reporter cells were treated with the reference agonists Dexamethasone (provided), Beclomethasone dipropionate (Sigma), Mometasone furoate, Fluticasone propionate, Budesonide, Methylprednisolone, GSK 9027, and Hydrocortisone (all from Tocris). Luminescence was quantified at the 24 hr assay endpoint and values of average Fold-Activation and Z' were calculated as described by Zhang, *et al*. (1999)[1]. Data are plotted as Fold-Activation *vs.* $Log_{10}$[Agonist, pM]. The $EC_{50}$ for dexamethasone is ~ 150 pM, with the 500 pM treatment yielding ≥ 800-fold activation over the untreated reporter cells, and the corresponding Z'= 0.92.

*(B.)* Antagonist Dose-responses: Reporter cells were co-treated with a fixed $EC_{80}$ concentration of the agonist Dexamethasone and varying concentrations the GR antagonists Mifepristone(Tocris) and Cyproterone Acetate (Santa Cruz). Data are plotted as RLU *vs.* $Log_{10}$[Antagonist, nM]. The $IC_{50}$ for Mifepristone is ~ 0.5 nM; Z' = 0.84 at 150 nM.

Non-linear regression and $EC_{50}$ analyses were performed using GraphPad Prism software.

[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.

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

## *II. Product Components & Storage Conditions*

This Zebrafish GR assay kit contains materials to perform assays in a single 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 3* 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.

| *Kit Components* | *Amount* | *Storage Temp.* |
|---|---|---|
| ▪ zfGR 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 |
| ▪ Dexamethasone, 1.0 µM (in DMSO) (reference agonist for GR) | 1 x 30 µL | -20°C |
| ▪ Detection Substrate | 1 x 6.0 mL | **-80°C** |
| ▪ Detection Buffer | 1 x 6.0 mL | -20°C |
| ▪ 96-well, *collagen-coated* 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***

▪ dry ice bucket (*Step 2*)
▪ 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-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:* antagonist reference compound.
▪ *Optional:* clear 96-well assay plate, *collagen-coated*, sterile, for viewing cells on Day 2.

***DAY 2*** plate-reading luminometer.

## *IV. Assay Protocol*

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 actual bench work plus a 4 hr pre-incubation step. *Steps 12-17* 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 $EC_{50}$ – $EC_{80}$) of a known agonist AND the test compound(s) to be evaluated for antagonist activity. This zfGR assay kit includes a 1.0 µM stock solution of **Dexamethasone**, a potent agonist of zfGR that may be used to setup antagonist-mode assays. 250 pM Dexamethasone typically approximates $EC_{70\text{-}80}$ in this cell-based assay. Hence, it is a suitable assay concentration of agonist to be used when screening test compounds for inhibitory activity.

Add the challenge agonist (Dexamethasone) to a bulk volume of **CSM** at an $EC_{50}$ – $EC_{80}$ concentration. This medium is then used to prepare serial dilutions of test compounds to achieve the desired respective final assay concentrations. This is an efficient and precise method of setting up antagonist assays, and it is the method presented in *Step 7b* of this protocol.

**DAY 1 Assay Protocol:** All steps must 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 **Reporter Cells** from -80°C storage, place it directly into a dry ice bucket and transport the cells to the laminar flow hood. When ready, 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 - 10 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 disperse cell aggregates and gain a homogenous cell suspension. Transfer the cell suspension into a reservoir. Using an 8-chanel pipette, 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 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 clear 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% $CO_2$ ) for 4 - 6 hours.

**6.)** Near the end of the pre-incubation period 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 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. Agonist*-mode assays**: This assay kit includes a 1.0 µM stock solution of **Dexamethasone**, a potent agonist of zfGR. The following 7-point treatment series, with concentrations prepared in 2-fold decrements, provides a complete dose-response: 1.0, 0.50, 0.25, 0.125, 0.0625, 0.0313, and 0.0156 nM. Always include 'untreated' (or 'Vehicle only') control wells. **APPENDIX 1** provides guidance for generating this a dilution series.

*~ or ~*

***b. Antagonist*-mode assays**: When setting up antagonist assays, first supplement a bulk volume of CSM with the challenge agonist **Dexamethasone** to achieve an $EC_{50\text{-}80}$ concentration (*e.g.*, ~ 0.25 nM; refer to "*A word about antagonist-mode assay setup*", pg. 7). The agonist-supplemented CSM is then used to generate dilutions of test compound stocks to achieve the desired final assay concentrations.

**8.)** At the end of the cell pre-incubation period, 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 **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 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 **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.)** 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*, gently invert the tubes of Detection Substrate and Detection Buffer several times to ensure homogenous solutions, then transfer the entire volume of Detection Buffer into the vial of Detection Substrate, thereby generating a 12 ml volume of **Luciferase Detection Reagent (LDR)**. Mix gently to avoid foaming.

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

**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.

## *V. Related Products*

| Human GR Assay Products | |
|---|---|
| ***Product No.*** | ***Product Descriptions*** |
| IB00201-32 | Human GR Reporter Assay System<br>3x 32 assays in 8-well strips (96-well plate format) |
| IB00201 | Human GR Reporter Assay System<br>1x 96-well format assay |
| IB00202 | Human GR Reporter Assay System<br>1x 384-well format assays |
| **Mouse GR Assay Products** | |
| M00201-32 | Mouse GR Reporter Assay System<br>3x 32 assays in 8-well strips (96-well plate format) |
| M00201 | Mouse GR Reporter Assay System<br>1x 96-well format assay |
| **Rat GR Assay Products** | |
| R00201-32 | Rat GR Reporter Assay System<br>3x 32 assays in 8-well strips (96-well plate format) |
| R00201 | Rat GR Reporter Assay System<br>3x 32 assays in 8-well strips (96-well plate format) |
| **Zebrafish GR Assay Products** | |
| Z00201-32 | Zebrafish GR Reporter Assay System<br>3x 32 assays in 8-well strips (96-well plate format) |
| Z00201 | Zebrafish GR Reporter Assay System<br>3x 32 assays in 8-well strips (96-well plate format) |

| LIVE Cell Multiplex (LCM) Assay | |
|---|---|
| ***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 | Reagent in 5x-bulk volume to perform **480** Live Cell Assays performed in 5x 96-well plates |
| LCM-10 | Reagent in 10x-bulk volume to perform **960** Live Cell Assays performed in 10x 96-well plates |

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 ™ of INDIGO Biosciences, Inc. (State College, PA, USA)

Product prices, availability, specifications and claims are subject to change without prior notice.

# APPENDIX 1

Example scheme for the serial dilution of Dexamethasone reference agonist, and the setup of a Zebrafish GR dose-response assay.