INSTRUCTIONS

GR Redistribution® Assay

For High-Content Analysis

014-01.03

Number	Description
R04014-01	Recombinant U2OS cells stably expressing human glucocorticoid receptor receptor (GR) (GenBank Acc. NM_000176; GeneID: 2908) fused to the C-terminus of enhanced green fluorescent protein (EGFP). U2OS cells are adherent epithelial cells derived from human osteosarcoma. Expression of EGFP-GR is controlled by a standard CMV promoter and continuous expression is maintained by addition of G418 to the culture medium.

Quantity: 2 cryo-vials each containing 1.0 x 10^6 cells in a volume of 1.0 ml Cell Freezing Medium.

Storage: Immediately upon receipt store cells in liquid nitrogen (vapor phase).

Warning: Please completely read these instructions and the material safety data sheet for DMSO before using this product. This product is for research use only. Not intended for human or animal diagnostic or therapeutic uses. Handle as potentially biohazardous material under at least Biosafety Level 1 containment. Safety procedures and waste handling are in accordance with the local laboratory regulations.

CAUTION: This product contains Dimethyl Sulfoxide (DMSO), a hazardous material. Please review Material Safety Data Sheet before using this product.

Introduction

The Redistribution® Technology

The Redistribution® technology monitors the cellular translocation of GFP-tagged proteins in response to drug compounds or other stimuli and allows easy acquisition of multiple readouts from the same cell in a single assay run. In addition to the primary readout, high content assays provide supplementary information about cell morphology, compound fluorescence, and cellular toxicity.

The Glucocorticoid Receptor Redistribution® Assay

Glucocorticoids are the most potent and effective drugs for preventing and suppressing inflammation caused by mechanical, chemical, infectious, and immunological stimuli. They exert their activity through binding to the glucocorticoid receptor (GR) resulting in either activation or repression of a large set of glucocorticoid responsive genes. GR is a ligand-dependent transcription factor belonging to the superfamily of steroid hormone receptors, which includes the mineralocorticoid, thyroid hormone, sex hormone, retinoic acid, and vitamin D receptors [1]. The GR receptor contains a DNA binding site, a hormone-independent activation function (AF1) domain in the N-terminus, which is associated with transcriptional activity and binding to co-activators and transcription factors, and a hormone-dependent AF2 domain in the C-terminus responsible for hormone binding as well as binding of co-activators. In the inactive state, the GR is found in the cytoplasm in complex with heat shock proteins. Upon ligand binding, the GR translocates to the nucleus, dimerizes, and acts as a transcription factor to regulate the expression of its target genes.

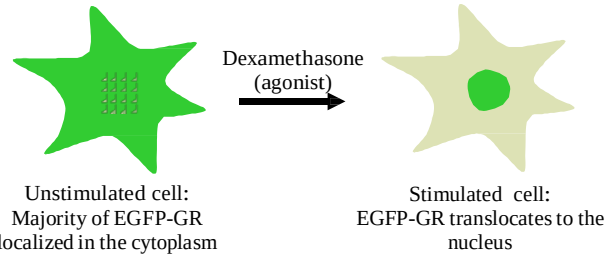


Figure 1: Illustration of the GR translocation.

GR agonists such as dexamethasone induce nuclear translocation and allow the receptor to bind co-activators with intrinsic histone acetylase and methylase activities, such as proteins from the SRC-1, p300/CBP, and PRMT families, whereas GR antagonists, which also induce nuclear translocation of GR, result in the recruitment of histone deacetylases through co-repressors of the N-CoR/SMRT family [2].

The GR Redistribution® Assay is designed to assay for compounds inducing GR translocation by monitoring the translocation of a GFP-GR fusion protein from the cytoplasm to the nucleus. GR nuclear translocation can be promoted by the agonist dexamethasone [3,4], which is used as reference compound in this assay. Compounds causing nuclear translocation of GR could be acting either as agonists or antagonists, or could be general nuclear import activators/nuclear export inhibitors. Compounds inducing nuclear accumulation of GR can be counter-screened for general export inhibitor characteristics using the Rev1 Redistribution® Assay (see related products).

Additional materials required

The following reagents and materials need to be supplied by the user.

- Dulbecco's Modified Eagle Medium (DMEM), high glucose, without L-Glutamine, Sodium Pyruvate (Thermo Scientific, Fisher Scientific cat.# SH30081)
- L-Glutamine supplement, 200 mM (Thermo Scientific, Fisher Scientific cat.# SH30034)
- Fetal Bovine Serum (FBS) (Thermo Scientific, Fisher Scientific cat.# SH30071)
- Charcoal/Dextran Treated Fetal Bovine Serum (CCS-FBS) (Thermo Scientific, Fisher Scientific cat.# SH3006802)
- Penicillin/Streptomycin, 100X solution (Thermo Scientific, Fisher Scientific cat.# SV30010),
- Trypsin-EDTA, 0.05% (Thermo Scientific, Fisher Scientific cat.# SH30236)
- G418, 50mg/ml (Thermo Scientific, Fisher Scientific cat.# SC30069)
- Dimethylsulfoxide (DMSO) (Fisher Scientific, cat.# BP231)
- Dulbecco´s Phosphate-Buffered Saline (PBS), w/o calcium, magnesium, or Phenol Red (Thermo Scientific, Fisher Scientific cat.# SH30028)
- Hepes Buffer, 1 M, Free Acid (liquid) (Thermo Scientific, Fisher Scientific cat.# SH30237)
- Dexamethasone (EMD Chemicals, cat.# 265005)
- Hoechst 33258 (Fisher Scientific, cat.# AC22989)
- Triton X-100 (Fisher Scientific, cat.# AC21568)
- 10% formalin, neutral-buffered solution (approximately 4% formaldehyde) (Fisher Scientific, cat.# 23-305-510)
  Note: is not recommended to prepare this solution by diluting from a 37% formaldehyde solution.
- 96-well microplate with lid (cell plate) (e.g. Nunc 96-Well Optical Bottom Microplates, Thermo Scientific cat.# 165306)
- Black plate sealer
- Nunc EasYFlasks with Nunclon Delta Surface, T-25, T-75, T-175 (Thermo Scientific, cat.# 156367, 156499, 159910

Reagent preparation

The following reagents are required to be prepared by the user.

- Cell Culture Medium: DMEM supplemented with 2mM L-Glutamine, 1% Penicillin-Streptomycin, 0.5 mg/ml G418 and 10% FBS.
- Cell Freezing Medium: 90% Cell Culture Medium without G418 + 10% DMSO.
- Plate Seeding Medium: DMEM supplemented with 2mM L-Glutamine, 1% Penicillin-Streptomycin, 0.5 mg/ml G418 and 10% CCS-FBS.
- Assay Buffer: DMEM supplemented with 2mM L-Glutamine and 1% Penicillin-Streptomycin.
- Control Compound Stock: 4 mM Dexamethasone stock solution in DMSO. Prepare by dissolving 10 mg Dexamethasone (MW = 392.5) in 6369 µl DMSO. Store at -20^{o}C.
- Control Compound Working Solution: 40 µM Dexamethasone Working Solution in DMSO. Prepare by diluting the 4 mM Dexamethasone stock solution 1:100 in DMSO.
- Fixing Solution: 10% formalin, neutral-buffered solution (approximately 4% formaldehyde).
  Note: It is not recommended to prepare this solution by diluting from a 37% formaldehyde solution.
- Hoechst Stock: 10 mM stock solution is prepared in DMSO.
- Hoechst Staining Solution: 1 µM Hoechst in PBS containing 0.5% Triton X-100. Prepare by dissolving 2.5 ml Triton X-100 with 500 ml PBS. Mix thoroughly on a magnetic stirrer. When Triton X-100 is dissolved add 50 µl 10 mM Hoechst 33258. Store at 4^{o}C for up to 1 month.

The following procedures have been optimized for this cell line. As early as possible, create and store at least one aliquot of cells for back-up.

Cell thawing procedure

1. Rapidly thaw frozen cells by holding the cryovial in a 37°C water bath for 1-2 minutes. Do not thaw cells by hand, at room temperature, or for longer than 3 minutes, as this decreases viability.
2. Wipe the cryovial with 70% ethanol.
3. Transfer the vial content into a T75 tissue culture flask containing 25 ml Cell Culture Medium and place flask in a 37ºC, 5% CO_2, 95% humidity incubator.
4. Change the Cell Culture Medium the next day.

Cell harvest and culturing procedure

For normal cell line maintenance, split 1:8 every 3-4 days. Maintain cells between 5% and 95% confluence. Passage cells when they reach 80-95% confluence. All reagents should be pre-warmed to 37ºC.

1. Remove medium and wash cells once with PBS (10 ml per T75 flask and 12 ml per T175 flask).
2. Add trypsin-EDTA (2 ml per T75 flask and 4 ml per T175 flask) and swirl to ensure all cells are covered.
3. Incubate at 37ºC for 3-5 minutes or until cells round up and begin to detach.
4. Tap the flask gently 1-2 times to dislodge the cells. Add Cell Culture Medium (6 ml per T75 flask and 8 ml per T175 flask) to inactivate trypsin and resuspend cells by gently pipetting to achieve a homogenous suspension.
5. Count cells using a cell counter or hemocytometer.
6. Transfer the desired number of cells into a new flask containing sufficient fresh Cell Culture Medium (total of 20 ml per T75 flask and 40 ml per T175 flask).
7. Incubate the culture flask in a 37ºC, 5% CO_2, 95% humidity incubator.

Cell freezing procedure

1. Harvest the cells as described in the “Cell harvest and culturing procedure”, step 1 – 5.
2. Prepare a cell suspension containing 1 x 10^6 cells per ml (5 cryogenic vials = 5 x 10^6 cells).
3. Centrifuge the cells at 250g (approximately 1100 rpm) for 5 minutes. Aspirate the medium from the cells.
4. Resuspend the cells in Cell Freezing Medium at 1 x 10^6 cells per ml until no cell aggregates remain in the suspension.
5. Dispense 1 ml of the cell suspension into cryogenic vials.
6. Place the vials in an insulated container or a cryo-freezing device (e.g. Nalgene "Mr. Frosty" Freezing Container, Thermo Scientific, Fisher Scientific cat.# 15-350-50) and store at -80ºC for 16-24 hours.
7. Transfer the vials for long term storage in liquid nitrogen.

Cell plating procedure

The cells should be seeded into 96-well plates 18-24 hours prior to running the assay. Do not allow the cells to reach over 95% confluence prior to seeding for an assay run. The assay has been validated with cells in up to passage 30 split as described in the “Cell harvest and culturing procedure”.

1. Harvest the cells as described in the “Cell harvest and culturing procedure”, step 1-5 using Plate Seeding Medium instead of Cell Culture Medium.
2. Dilute the cell suspension to 60,000 cells/ml in Plate Seeding Medium.
3. Transfer 100 µl of the cell suspension to each well in a 96-well tissue culture plate (cell plate). This gives a cell density of 6000 cells/well.
   Note: At this step, be careful to keep the cells in a uniform suspension.
4. Incubate the cell plate on a level vibration-free table for 1 hour at room temperature (20-25°C). This ensures that the cells attach evenly within each well.
5. Incubate the cell plate for 18-24 hours in a 37ºC, 5% CO_2, 95% humidity incubator prior to starting the assay.

Assay protocol

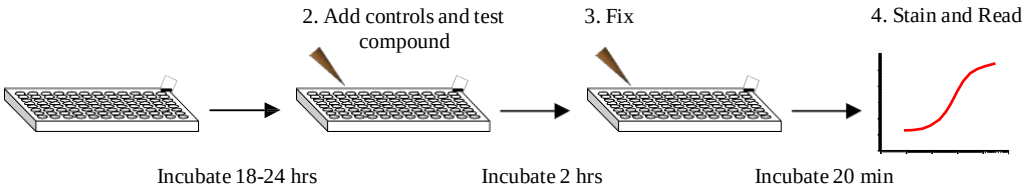


Figure 2: Quick assay workflow overview.

The following protocol is based on 1x 96-well plate.

1. Before initiating the assay:
    - Prepare Assay Buffer. Ensure Assay Buffer is pre-warmed to 20-37ºC.
2. Prepare controls and test compounds:
    - Dilute controls and test compounds in Assay Buffer to a 2X final concentration. (Volumes and concentrations are indicated below). A final DMSO concentration of 0.25% is recommended, but the assay can tolerate up to 2% DMSO final concentration.
    - Mix controls for 1x 96–well plate as indicated below:

	Assay Buffer	Control Working Solution	DMSO	2X concentration	Final assay concentration	Final DMSO concentration
Negative control	12 ml	----	60 µl	0.5% DMSO	----	0.25%
Positive control	12 ml	60 µl 40 µM Dexamethasone	----	200 nM Dexamethasone	100 nM Dexamethasone	0.25%

3. Add 100 µl 2X concentrated control or compound solution in Assay Buffer to appropriate wells of the cell plate.
4. Incubate cell plate for 2 hours in a 37ºC, 5% CO_2, 95% humidity incubator.
5. Fix cells by gently decanting the buffer and add 150 µl Fixing Solution per well.
6. Incubate cell plate at room temperature for 20 minutes.
7. Wash the cells 4 times with 200 µl PBS per well per wash.
8. Decant PBS from last wash and add 100 µl 1 µM Hoechst Staining Solution.
9. Seal plate with a black plate sealer. Incubate at room temperature for at least 30 minutes before imaging. The plate can be stored at 4ºC for up to 3 days in the dark.

Imaging

The translocation of GR can be imaged on most HCS platforms and fluorescence microscopes. The filters should be set for Hoechst (350/461 nm) and GFP/FITC (488/509 nm) (wavelength for excitation and emission maxima). Consult the instrument manual for the correct filter settings.

The translocation can typically be analyzed on images taken with a 10x objective or higher magnification.

The primary output in the GR Redistribution® assay is the translocation from cytoplasm to nucleus of EGFP-GR. The data analysis should therefore report an output relating to the GFP fluorescence intensities in the nucleus and the cytoplasm.

Imaging on Thermo Scientific Arrayscan HCS Reader
This assay has been developed on the Thermo Scientific Arrayscan HCS Reader using a 10x objective (0.63X coupler), XF100 filter sets for Hoechst and FITC and the Redistribution V3 BioApplication. The output used was MEAN_CircRingAvgIntenRatioLog (Log of the ratio of average fluorescence intensities of nucleus and cytoplasm (well average)). The minimally acceptable number of cells used for image analysis in each well was set to 200 cells.

Other BioApplications that can be used for this assay include Molecular TranslocationV2, CompartmentalAnalysisV2, NucTransV2 and ColocalizationV3.

High Content Outputs
In addition to the primary readout, it is possible to extract secondary high content readouts from the Redistribution® assays. Such secondary readouts may be used to identify unwanted toxic effects of test compounds or false positives. In order to acquire this type of information, the cells should be stained with a whole cell dye which allows for a second analysis of the images for determination of secondary cell characteristics.

Examples of useful secondary high content outputs:

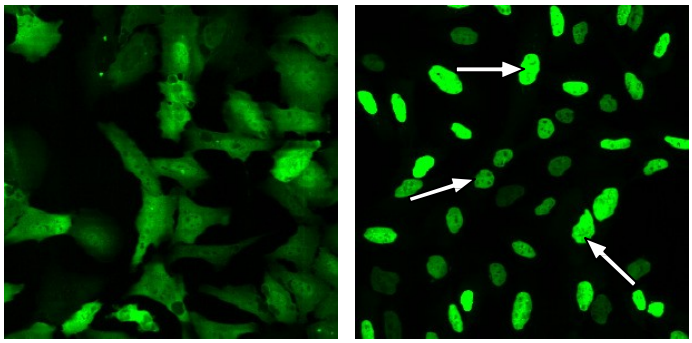
Nucleus size, shape, intensity:	Parameter used to identify DNA damage, effects on cell cycle and apoptosis.
Cell number, size, and shape:	Parameter for acute cytotoxicity and apoptosis.
Cell fluorescence intensity:	Parameter for compound cytotoxicity and fluorescence.

The thresholds for determining compound cytotoxicity or fluorescence must be determined empirically. Note that the primary translocation readout in some cases may affect the secondary outputs mentioned above.

Representative Data Examples

The GR Redistribution® assay monitors nuclear translocation of EGFP-GR. Dexamethasone is used as a reference ligand, and compounds are assayed for their ability to induce nuclear translocation of EGFP-GR. Compounds causing nuclear translocation of GR could be acting either as agonists or antagonists or could be general nuclear import activators/nuclear export inhibitors. Compounds inducing nuclear accumulation of GR can be counter-screened for general export inhibitor characteristics using the Rev1 Redistribution® Assay (see related products).

Representative images of GR Redistribution® assay cells treated with dexamethasone are shown in figure 3.



DMSO-treated cells | Dexamethasone-treated cells

Figure 3. Nuclear translocation of EGFP-GR. Cells were treated with or without 100 nM dexamethasone for 2 hours. Arrows indicate the nuclear translocation of EGFP-GR detected by the image analysis algorithm.

Figure 4 shows representative concentration response curves of the reference compound dexamethasone and mifepristone in the GR assay. The EC_{50} of dexamethasone is ~1 nM and the EC_{50} of mifepristone is ~0.5 nM .

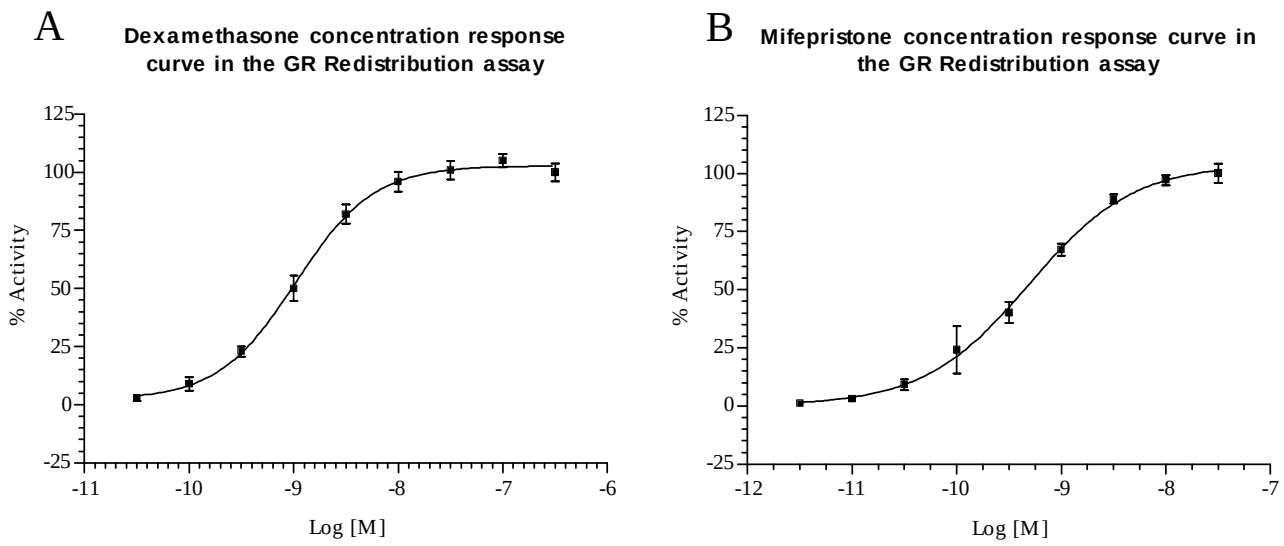


Figure 4. Concentration response curves in the GR assay: A) Dexamethasone concentration response in the GR assay (n=6). The EC_{50} is approximately 1 nM.. B) Mifepristone concentration response in the GR assay (n=3). The EC_{50} is approximately 0.5 nM.
Concentration response was measured in 9 point half log dilution series. Cells were treated with compound for 2 hrs. Cells were then fixed and imaging was performed using an image algorithm detecting nuclear translocation. % activity was calculated relative to the positive (100 nM dexamethasone) and negative control (0.25% DMSO)

Product qualification

Assay performance has been validated with an average Z'=0.84±0.03. The cells have been tested for viability. The cells have been tested negative for mycoplasma and authenticated to be U2OS cells by DNA fingerprint STR analysis.

Related Products

Product #	Type	Product description	Cell line
R04-043-01	Profiling & Screening	AR Redistribution® Assay	U2OS
R04-060-01	Profiling & Screening	VDR Redistribution® Assay	U2OS
R04-056-01	Profiling & Screening	ERα Redistribution® Assay	U2OS
R04-063-01	Profiling & Screening	ERβ Redistribution® Assay	U2OS
R04-064-01	Profiling & Screening	PR Redistribution® Assay	U2OS
R04-077-01	Profiling & Screening	PXR Redistribution® Assay	U2OS
R04-062-01	Profiling & Screening	REV Redistribution® Assay	U2OS

References

1. Bamberger CM et al. Endocrin Rev. 17, 245-261, 1996.
2. Rosenfeld MG & Glass CK, J. Biol. Chem. 276, 36865-36868, 2001.
3. Hardt FD, et al. Lancet. 2, 255-257, 1959.
4. Levinson BB et al. Science 175, 189-190, 1972.

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For European customers:
The GR Redistribution cell line is genetically modified with a vector expressing GR fused to EGFP. As a condition of sale, use of this product must be in accordance with all applicable local legislation and guidelines including EC Directive 90/219/EEC on the contained use of genetically modified organisms.