INSTRUCTIONS

AR Redistribution® Assay

For High-Content Analysis

043-01.03

Number	Description
R04-043-01	Recombinant U2OS cells stably expressing human androgen receptor (AR) (GenBank Acc. NM_000044) fused to the C-terminus of enhanced green fluorescent protein (EGFP). U2OS cells are adherent epithelial cells derived from human osteosarcoma. Expression of EGFP-AR 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 AR Redistribution® Assay

The androgen receptor (AR) is a nuclear receptor transcription factor that mediates the cellular actions of androgens, the male sex steroids testosterone and dihydrotestosterone (DHT). Androgen signaling mediates male sexual differentiation, sexual maturation, and spermatogenesis. Androgens maintain normal prostate homeostasis but are also involved in prostate tumorigenesis [1]. Androgens exert their activity through binding to AR resulting in either activation or repression of androgen responsive genes. Ligand binding exposes a nuclear localization signal (NLS) allowing translocation of AR to the nucleus via a transport protein. Agonists as well as most antagonists induce translocation of AR to the nucleus. However, only agonists activate AR functions by interaction with androgen response elements (AREs). Moreover, AR agonists often induce nuclear localisation of AR with a punctuate distribution pattern, the so-called nuclear foci [1, 2, 3].

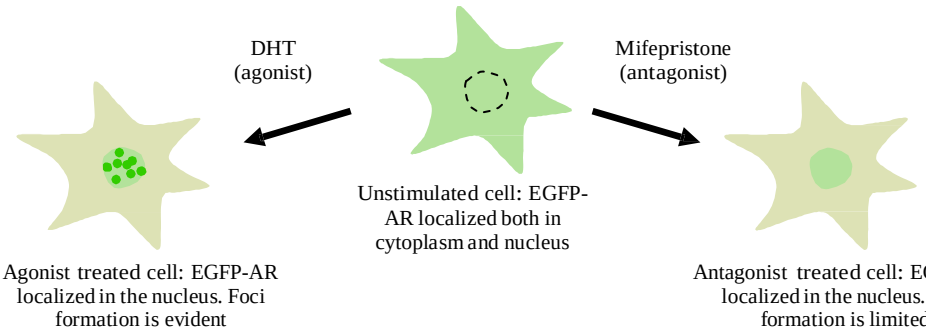


Figure 1. Illustration of the AR translocation event.

The AR Redistribution® Assay is designed to assay for compounds inducing EGFP-AR nuclear translocation and formation of nuclear foci. AR nuclear accumulation and foci formation is promoted by the agonist DHT, which is used as reference compound. Antagonists such as mifepristone also cause nuclear accumulation of the EGFP-AR fusion protein, but with no or less foci formation. This difference enables the discrimination of agonists from antagonist in the AR Redistribution® Assay. Further profiling of test compounds inducing nuclear accumulation of AR can be performed by counter-screening for general export inhibitor characteristics using the Rev Redistribution® Assay.

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)
- Dihydrotestosterone (DHT)/5α-Androstan-17β-ol-3-one) (Sigma-Aldrich, cat.# A8380)
- 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: 25 mM DHT stock solution in DMSO. Prepare by dissolving 3 mg DHT (MW = 290.44) in 413 µl DMSO. Store at -20 °C.
- Control Compound Working Solution: 25 µM DHT Working Solution in DMSO. Prepare by diluting the 25 mM DHT stock solution 1:1000 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°C for up to 1 month.

The following procedures have been optimized for this cell line. It is strongly recommended that an adequately sized cell bank is created containing cells at a low passage number.

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 up to passage 25, 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

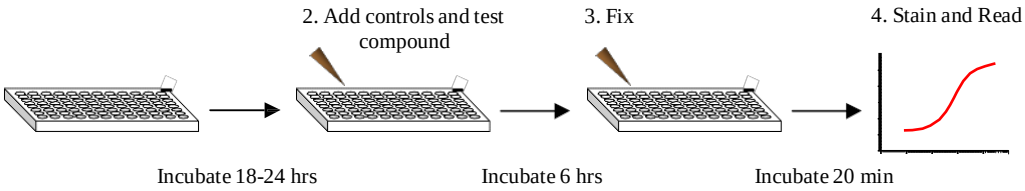


Figure 2: Quick assay workflow overview.

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.
    - 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	9.6 µl 25 µM DHT	50.4 µl	20 nM DHT	10 nM DHT	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 6 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.

The assay can also be performed with 24 hr compound incubation time if desired.

When measuring the whole cell EGFP-AR intensity readout, a whole cell stain should be performed, e.g. 0.5 µM DRAQ5 (BioStatus) in PBS containing 1 mM $MgSO_4$:

1. Decant solution from the cell plate.
2. Add 100 µl 0.5 µM DRAQ5 in PBS containing 1 mM $MgSO_4$
3. Seal plate with a black plate sealer. Incubate at room temperature for at least 30 minutes before imaging.

Imaging

Three primary outputs can be derived from the AR Redistribution® assay: formation of spots in the nucleus, translocation of EGFP-AR into the nucleus, and whole cell intensity of EGFP-AR. The data analysis should therefore report outputs that correspond to number, area, or intensity of spots in the nucleus, an output relating to the GFP fluorescence intensities in the nucleus and the cytoplasm, and the total fluorescence intensity in the whole cell.

The translocation of EGFP-AR 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.

For measuring total GFP intensity the cells should be stained with a whole cell stain e.g. DRAQ5. The filters should be set for DRAQ5/Cy5 (647/670 nm) and GFP/FITC (488/509 nm) (wavelength for excitation and emission maxima). Consult the instrument manual for the correct filter settings.

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

Imaging on Thermo Scientific Arrayscan HCS Reader

Formation of spots: 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 SpotDetectorV3 BioApplication. The output parameter used was SpotTotalAreaPerObject. 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 output feature include CompartmentalAnalysisV2 and ColocalizationV3.

Translocation from cytoplasm to nucleus: 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 RedistributionV3 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 output feature include Molecular Translocation V2, Compartmental AnalysisV2, NucTransV2, and ColocalizationV3.

Whole cell intensity: This assay has been developed on the Thermo Scientific Arrayscan HCS Reader using a 10x objective (0.63X coupler), XF110 filter for Cy5, XF100 filter for FITC, and the RedistributionV3 BioApplication. The minimally acceptable number of cells used for image analysis in each well was set to 1000 cells.

Other BioApplications that can be used for this output feature include 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:

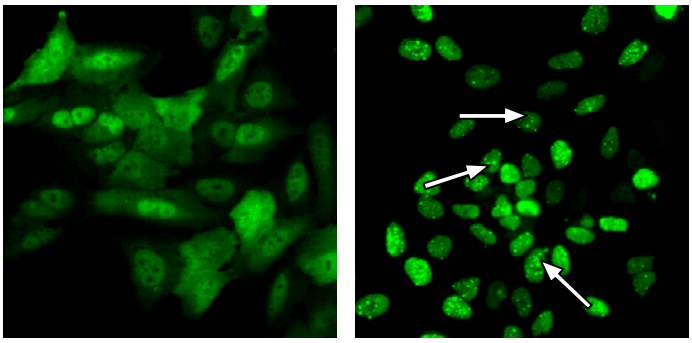
Nucleus size, shape, intensity:	Parameter used to identify DNA damage, effects on cell cycle and apoptosis.
Cell number, size, and shape:	Parameter for acute cytotoxicity and apoptosis.

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

Representative Data Examples

The AR Redistribution® assay monitors nuclear translocation and nuclear foci formation of EGFP-AR. Dihydrotestosterone (DHT) is used as the reference compound. Test compounds can be assayed for three different outputs: translocation of EGFP-AR to the nucleus, formation of fluorescent spots in the nucleus, and changes in the whole cell intensity of EGFP-AR.

Representative images of AR Redistribution® assay cells treated with DHT are shown in Figure 3.



DMSO-treated cells DHT-treated cells

Figure 3. Nuclear translocation of EGFP-AR. Cells were treated with or without 10 nM DHT for 6 hr. Arrows indicate the nuclear translocation and foci formation of EGFP-AR detected by the image analysis algorithm.

Figure 4 shows representative concentration response curves of DHT and mifepristone in the AR assay. Three different readouts are shown for each compound: translocation, spot formation, and intensity. The EC_{50} of the agonist DHT is ~0.2 nM for all three readouts. The antagonist mifepristone has an EC_{50} of ~3 nM for the translocation readout, while in the spot formation readout it is less potent (EC_{50} of ~9 nM) and has a considerably reduced maximal activity. Thus the multiple readouts enable discrimination between AR agonists and antagonists in a single assay run.

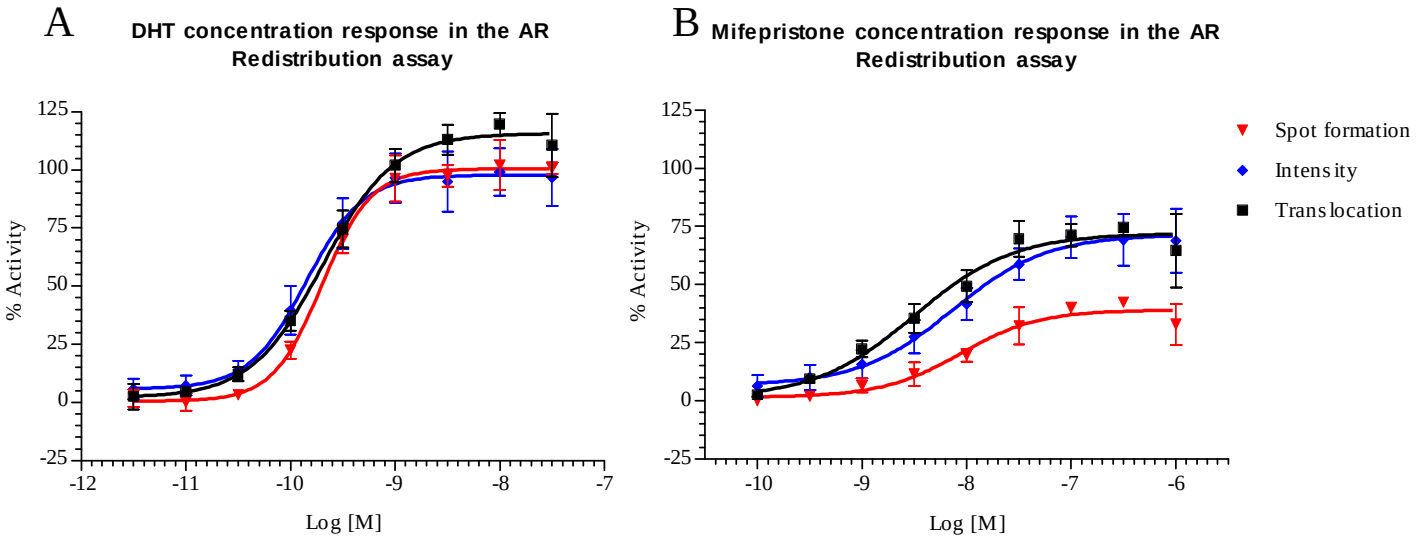


Figure 4. Concentration response curves in the AR assay: A) DHT concentration response (n=4). The EC_{50} is approximately 3 nM for all three readouts. B) Mifepristone concentration response (n=4). Mifepristone is less potent in the spot formation readout than in the translocation readout (EC_{50} of 9 nM and 3 nM respectively); there is also a clear reduction in the maximal activity in the sport formation readout.
Concentration response was measured in 9 point half log dilution series. Cells were treated with compound for 6 hrs. Cells were then fixed and imaging was performed using the Cellomics ArrayScan V^{TI} Reader. The RedistributionV3 and SpotDetectorV3 BioApplications were used to generate three different outputs. % activity for all three outputs was calculated relative to the positive (10 nM DHT) and negative control (0.25% DMSO).

Product qualification

Assay performance has been validated with an average Z'=0.63±0.02 for formation of spots (6 hours incubation), Z'=0.54±0.06 for nucleus translocation (6 hours incubation) and Z'=0.59±0.08 for total GFP intensity (6 hours incubation). 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
R04-060-01	Profiling & Screening	VDR Redistribution® Assay	U2OS
R04-056-01	Profiling & Screening	ERα Redistribution® Assay	U2OS
R04-063-01	Profiling & Screening	ERβ Redistribution® Assay	U2OS
R04-014-01	Profiling & Screening	GR 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. McEwan IJ, Endocrin-Related Cancer 11, 281-293, 2004.
2. Rosenfeld MG & Glass CK, J. Biol. Chem. 276, 36865-36868, 2001.
3. Mainwaring WI, Monogr. Endocrinol. 10, 1-178, 1977.

Licensing Statement

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The CMV promoter and its use are covered under U.S. Pat. Nos. 5,168,062 and 5,385,839 owned by the University of Iowa Research Foundation, Iowa City, Iowa, and licensed for research purposes use only. Any other use of the CMV promoter requires a license from the University of Iowa Research Foundation, 214 Technology Innovation Center, Iowa City, IA 52242, USA.

This product and/or its use is subject of patent nos. US 6,518,021; EP 1,199,564; EP 0,986,753; US 6,172,188; EP 0,851,874 including continuations, divisions, reissues, extensions, and substitutions with respect thereto, and all United States and foreign patents issuing therefrom to Fisher BioImage ApS, and the patents assigned to Aurora/ The Regents of the University of California (US5,625,048, US6,066,476, US5,777,079, US6,054,321, EP0804457B1) and the patents assigned to Stanford (US5,968,738, US5,804,387) including continuations, divisions, reissues, extensions, and substitutions with respect thereto, and all United States and foreign patents issuing therefrom.

For European customers:
The AR Redistribution cell line is genetically modified with a vector expressing AR 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.