INSTRUCTIONS

AT1R:NFATc1 Redistribution® Assay

For High-Content Analysis

078-01.03

Number	Description
R04-078-01	Recombinant U2OS cells stably expressing human angiotensin II Type-1 receptor (ATIR) (GenBank Acc. NM_000685) and human NFATc1 (GenBank Acc. NM_172389) fused to the C-terminus of enhanced green fluorescent protein (EGFP). U2OS cells are adherent epithelial cells derived from human osteosarcoma. Expression of NK1 receptor and EGFP-NFATc1 are controlled by a standard CMV promoter and continuous expression is maintained by addition of G418 and Zeocin 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.

Angiotensin II Type-1 (AT1) Receptor Activation Redistribution Assay

Angiotensin activates a wide spectrum of signaling responses via the Angiotensin II Type-1 receptor (AT1R) having immediate physiological effects on vasoconstriction and blood pressure regulation as well being implicated in inflammation, endothelial dysfunction, atherosclerosis, hypertension, and congestive heart failure. AT1R is a member of the superfamily of G protein–coupled receptors (GPCRs) and couples to Gαq [1,2]. In this assay, the AT1R has been transfected into the GPCR Reporter Assay for Gq-coupled Receptors, where receptor activation leads to release of cytoplasmic Ca2+, which in turn induces NFATc1 translocation. Binding of an agonist to the extracellular parts of AT1R causes a conformational change in the receptor. This leads to activation of heterotrimeric Gq proteins, subsequent release of Gαq from the beta-gamma subunit and activation of phospholipase C, which catalyzes the formation of DAG and IP3 from PIP2. Free IP3 diffuses into the cytoplasm, and activates IP3 receptors on the endoplasmic reticulum (ER) resulting in Ca2+ release from the ER into the cytoplasm. Elevated calcium levels leads to dephosphorylation of NFATc1 and rapid translocation from the cytoplasm to the nucleus [3,4]. Figure 1 illustrates the translocation of NFATc1 upon agonist stimulation of AT1R. The AT1R:NFATc1 assay is designed to screen for agonists causing AT1R activation and thereby nuclear translocation of GFP-NFATc1. Test compounds having activity in the assay are considered to be agonists of AT1R. Angiotensin I is used as reference compound in the assay.

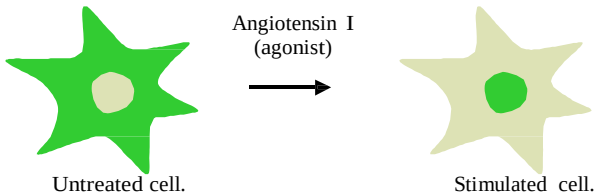

Figure 1. Illustration of the NFAT translocation in the AT1R:NFATc1 assay

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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)
- 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)
- Zeocin™ Selective Reagent 100 mg/ml (Invitrogen, cat.# R250-05)
- 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)
- Angiotensin I human acetate hydrate Sigma-Aldrich, cat.# A9650)
- 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, 1 mg/ml Zeocin and 10% FBS.
- Cell Freezing Medium: 90% Cell Culture Medium without G418 and Zeocin + 10% DMSO.
- Plate Seeding Medium: DMEM supplemented with 2mM L-Glutamine, 1% Penicillin-Streptomycin, 0.5 mg/ml G418, 1 mg/ml Zeocin, and 1% FBS.
- Assay Buffer: DMEM supplemented with 2mM L-Glutamine, and 1% Penicillin-Streptomycin.
- Control Compound Stock: 0.7 mM Angiotensin stock solution in H_2O. Prepare by dissolving 1 mg Angiotensin (MW = 1296.48) in 1102 µl H_2O. Store at -70°C.
- 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 24, 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 120,000 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 12,000 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

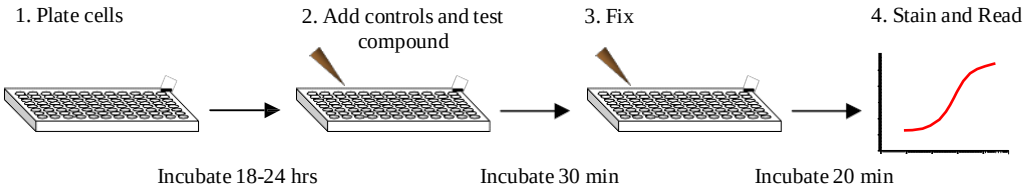


Figure 2: Quick assay workflow overview.

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 1% DMSO final concentration.
    - Mix controls for 1x 96–well plate as indicated below:

	Assay Buffer	Control Stock	DMSO	2X concentration	Final assay concentration	Final DMSO concentration
Negative control	12 ml	----	60 µl	0.5% DMSO	----	0.25%
Positive control	12 ml	10.3 µl Angiotensin	60 µl	600 nM Angiotensin	300 nM Angiotensin	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 30 minutes 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 EGFP-NFATc1 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 AT1R:NFATc1 Redistribution® assay is the translocation of EGFP-NFATc1 from the cytoplasm to the nucleus. 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 250 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:

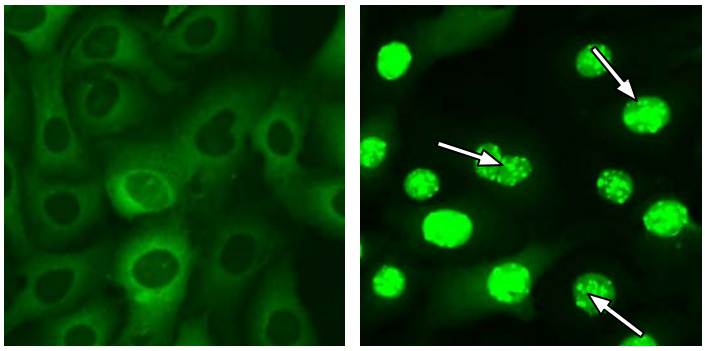
Nucleus size, shape, intensity:	Parameter used to identify DNA damage, effects on cell cycle and apoptosis.
Cell number, size, and shape:	Parameter for acute cytotoxicity and apoptosis.
Cell fluorescence intensity:	Parameter for compound cytotoxicity and fluorescence.

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 AT1R:NFATc1 Redistribution® assay monitors translocation of an EGFP-NFATc1 fusion protein from the cytoplasm to the nucleus in response to activation of the AT1 receptor. Angiotensin is used as the reference ligand, and compounds are assayed for their ability to induce nuclear localization of EGFP-NFATc1.

Representative images of AT1R:NFATc1 Redistribution® cells treated with angiotensin are shown in Figure 3.



Untreated (DMSO) 300 nM Angiotensin I

Figure 3. Nuclear translocation of EGFP-NFATc1. Cells expressing the AT1 receptor were treated with 300 nM angiotensin for 30 min. Activation of the receptor leads to nuclear translocation of EGFP-NFATc1, which can be detected by the image analysis algorithm.

A representative concentration response curve of the reference compound angiotensin in the AT1R:NFATc1 assay is shown in Figure 4. The EC_{50} of angiotensin is approximately 10 nM.

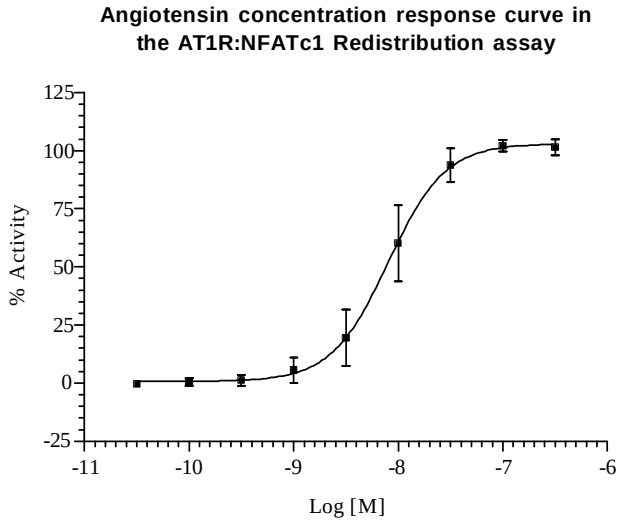


Figure 4. Angiotensin concentration response in the AT1R:NFATc1 assay. The EC_{50} of angiotensin is ~10 nM. Concentration response was measured in 9 point half log dilution series (n=16). Cells were treated with angiotensin for 30 min. Cells were then fixed and nuclear translocation was measured using the Cellomics ArrayScan V^{TI} Reader and the Redistribution V3 BioApplication. % activity was calculated relative to the positive (300 nM angiotensin) and negative control (0.25% DMSO).

Product qualification

Assay performance has been validated with an average Z'=0.83±0.05. 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
R04-017-02	Profiling & Screening	Gq-coupled GPCRs – NFATc1 Redistribution® Assay	U2OS
R04-045-02	Profiling & Screening	Gs/Gi-coupled GPCRs – PKA Redistribution® Assay	CHO-K1
R04-071-01	Profiling & Screening	AT1R Redistribution® Assay	U2OS
R04-046-01	Profiling & Screening	β2-AR:PKA Redistribution® Assay	CHO-K1
R04-047-01	Profiling & Screening	GlucagonR:PKA Redistribution® Assay	CHO-K1
R04-067-01	Profiling & Screening	S1P1:PKA Redistribution® Assay	CHO-K1
R04-088-01	Profiling & Screening	M1:NFATc1 Redistribution® Assay	U2OS
R04-072-01	Profiling & Screening	M2:PKA Redistribution® Assay	CHO-K1
R04-073-01	Profiling & Screening	M3:NFATc1 Redistribution® Assay	U2OS
R04-048-01	Profiling & Screening	NK1:NFATc1 Redistribution® Assay	U2OS
R04-079-01	Profiling & Screening	MCH1:NFATc1 Redistribution® Assay	U2OS
R04-081-01	Profiling & Screening	MOR1:PKA Redistribution® Assay	CHO-K1

References

1. de Gasparo M et al. Pharmacol Rev., 52, 415-472, 2000.
2. Mehta PK & Griendling KK, Am J Physiol Cell Physiol., 292:C82-97, 2007
3. Rao, A. et al. Annu. Rev. Immunol.; 15, 707-747, 1997.
4. Masuda, E.S. et al. Cell Signal.; 10, 599-611, 1998.

Licensing Statement

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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 AT1R:NFATc1 Redistribution cell line is genetically modified with vectors expressing AT1R and NFATc1 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.

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