INSTRUCTIONS
PKC ε Redistribution® Assay

For High-Content Analysis

099-01.03

Number	Description
R04-099-01	Recombinant U2OS cells stably expressing human PKC ε (GenBank Acc. NM_005400) fused to the N-terminus of enhanced green fluorescent protein (EGFP). The human neurokinin-1 (NK1) receptor (GenBank Acc. NM_001058) is coexpressed as a model receptor for activation of PKC ε. U2OS cells are adherent epithelial cells derived from human osteosarcoma. Expression of PKCε-EGFP is controlled by a standard CMV promoter and continuous expression of PKCε-EGFP and NK1 receptor 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.

The PKC ε Redistribution Assay

Protein Kinase C is an expanding family of at least 10 enzymes that can be divided into three classes. The conventional kinases (α, βI, βII,γ), the novel (δ,ε,η,θ,μ), and the atypical (ζ,ι,λ). The kinases are localized to the cytoplasm, but upon activation they translocate to an organelle or the plasma membrane. The conventional PKC isoforms are activated by Ca^{2+} and 1,2-diacylglycerol (DAG), the novel isofoms require DAG for activation, and activation of the atypical isorforms is less described. A common mechanism of Ca^{2+} and DAG release is through activation of G_q-coupled GPCRs. A large number of PKC substrates are then phosphorylated and thereby regulated by PKC.

PKC ε has been reported to be involved many different cellular functions, for example apoptosis, heat shock response, macrophage activation, neurite development, and insulin signaling [1-3].

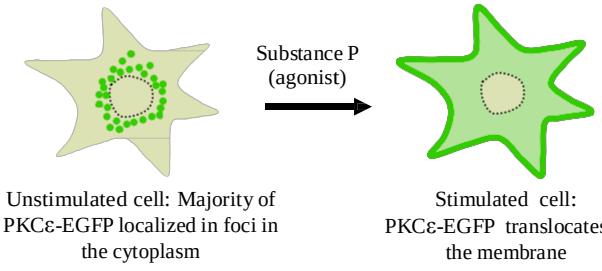

Figure 1. Illustration of the PKC ε translocation event.

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The PKC ε Redistribution assay is developed using Substance P as agonist. Substance P activates coexpressed NK1 receptors, which activate G_q, PLC, and subsequently PKC ε [4]. Test compounds can be assayed for agonistic effects on the PKC ε pathway. The assay can be reformatted to an antagonist assay format for testing PKC ε pathway inhibitors. We do not recommend using PMA as agonist in an antagonist assay format, since PMA is a direct PKC activator.

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)
- Acetic acid
- Substance P (EMD Chemicals, cat.# 05-23-0600)
- 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 10% FBS.
- Assay Buffer: DMEM supplemented with 2mM L-Glutamine and 1% Penicillin-Streptomycin.
- Control Compound Stock: 100 µM Substance P stock solution. Prepare by dissolving 1 mg Substance P (MW = 1347.6) in 7421 µl 5% acetic acid. Aliquot and store at -20°C.
- Control Compound Working Solution: 10 µM Substance P Working Solution. Prepare by diluting 100 µM Substance P 1:10 in 5% acetic acid.
- 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 37°C 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 20,000 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 2000 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 37°C, 5% CO_2, 95% humidity incubator prior to starting the assay.

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Assay protocol

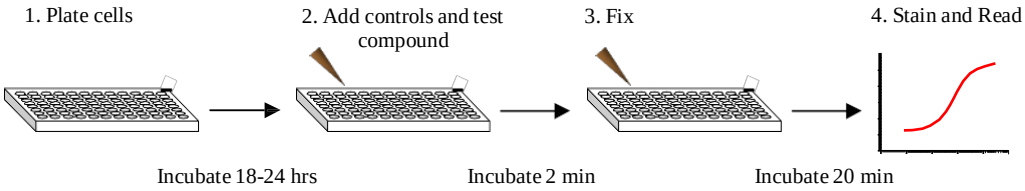


Figure 2: Quick assay workflow overview.

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	7.2 µl 10 µM Substance P	60 µl	6 nM Substance P	3 nM Substance P	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 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 PKCε -EGFP 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 20x objective or higher magnification.

The primary output in the PKC ε Redistribution® assay is the dispersion of spots in the cytoplasm. The data analysis should therefore report an output that corresponds to number, area, or intensity of spots.

Imaging on Thermo Scientific Arrayscan HCS Reader
This assay has been developed on the Thermo Scientific Arrayscan HCS Reader using a 20x 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 150 cells.

Other BioApplications that can be used for this assay include CompartmentalAnalysisV2 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:

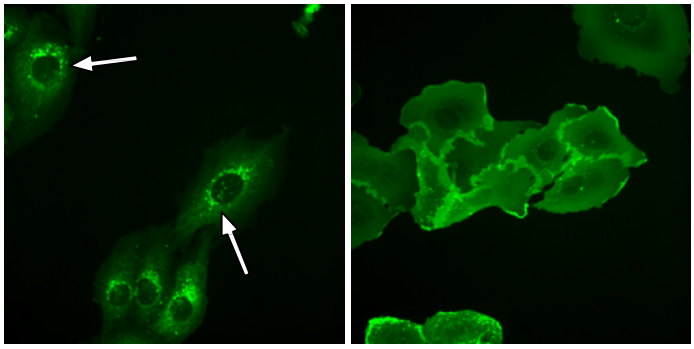
Nucleus size, shape, intensity:	Parameter used to identify DNA damage, effects on cell cycle and apoptosis.
Cell number, size, and shape:	Parameter for acute cytotoxicity and apoptosis.
Cell fluorescence intensity:	Parameter for compound cytotoxicity and fluorescence.

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 PKC ε Redistribution® assay monitors translocation of PKCε-EGFP from cytoplasmic foci to the plasma membrane in response to activation of the NK1 receptor. Substance P is used as reference ligand.

Representative images of PKCε-EGFP Redistribution® cells treated with Substance P are shown in Figure 3.



No treatment (DMSO) 3 nM Substance P

Figure 3. Translocation to the plasma membrane of PKCε-EGFP. U2OS cells expressing the NK1 receptor and PKCε-EGFP were treated with 3 nM Substance P for 2 min. Activation of the NK1 receptor leads to release of Ca^{2+} and DAG which in turn induces translocation of PKCε-EGFP from cytoplasmic foci to the plasma membrane. Arrows indicate cytoplasmic foci detected by the image analysis algorithm.

A representative concentration response curve of the reference compound Substance P in the PKC ε assay is shown in Figure 4. The EC_{50} of Substance P in the assay is approximately 230 pM. The assay may be reformatted to antagonist format for testing of PKC ε pathway antagonists.

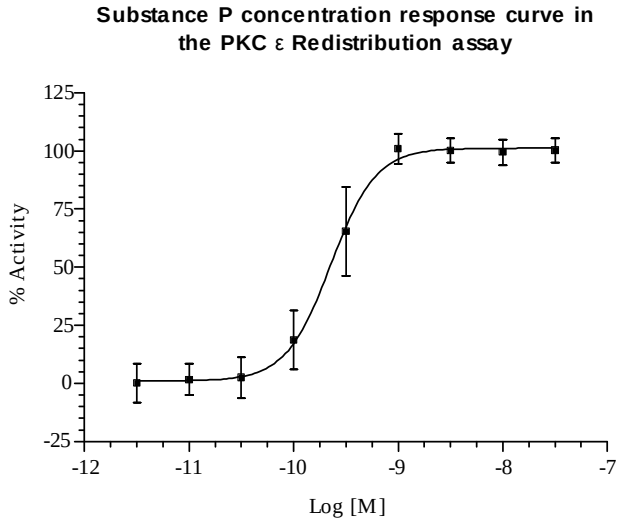


Figure 4. Substance P concentration response in the PKC ε assay. Concentration response was measured in 9 point half log dilution series (n=16). The EC_{50} of Substance P is approximately 230 pM. Cells were treated with Substance P for 2 min. Cells were then fixed and disappearance of cytoplasmic foci (membrane translocation) was measured using the Cellomics ArrayScan V^{TI} Reader and the SpotDetectorV3 BioApplication. % activity was calculated relative to the positive (3 nM Substance P) and negative control (0.25% DMSO).

Product qualification

Assay performance has been validated with an average Z'=0.64±0.10. 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
R04-098-01	Profiling & Screening	PKC β Redistribution® Assay	U2OS
R04-096-01	Profiling & Screening	MARCKS Redistribution® Assay	U2OS
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
R04-048-01	Profiling & Screening	NK1:NFATc1 Redistribution® Assay	U2OS

References

1. Steinberg et al, J Biol Chem. 2007 Nov 2;282(44):32288-97
2. Castrillo et al, J. Exp. Med. 194: 1231-1242, 2001.
3. Wu et al, J Biol Chem. 2003 Dec 19;278(51):51143-9
4. Akita, J. Biochem. 2002 Dec;132(6):847-52.

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 PKC ε Redistribution cell line is genetically modified with a vector expressing PKC ε 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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