INSTRUCTIONS

Akt2 Redistribution® Assay

For High-Content Analysis

011-02.03

Number	Description
R04-011-02	Recombinant CHOhIR cells stably expressing human Akt2 (GenBank Acc. NM_001626) fused to the N-terminus of enhanced green fluorescent protein (EGFP). CHOhIR cells are adherent epithelial cells derived from Chinese hamster ovary cells with stable expression of human insulin receptor. Expression of Akt2-EGFP 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 Akt2 Redistribution® Assay

The Akt/protein kinase B (PKB) family of serine/threonine-specific protein kinases comprises three highly homologous members in mammalian cells (Akt1/PKBα, Akt2/PKBβ and Akt3/PKBγ). The Akt family members are activated by diverse stimuli such as hormones and growth factors (e.g. insulin and IGF-I). The Akt protein kinases function within the phosphoinositide 3-kinase (PI3K) signaling pathway. PI3K generates phosphatidylinositol-3,4,5-trisphosphate (PIP3), a lipid second messenger essential for the translocation of Akt to the plasma membrane. Following translocation to the membrane Akt is phosphorylated and activated by the phosphoinositide-dependent kinase-1 (PDK-1). Active PKB phosphorylates and regulates the function of many cellular proteins essential for metabolism, apoptosis, and proliferation. The Akt2 Redistribution® assay monitors translocation of an EGFP-human Akt2 fusion protein from the cytoplasm to the plasma membrane. Insulin-like growth factor-I (IGF-1) is used as reference agonist.

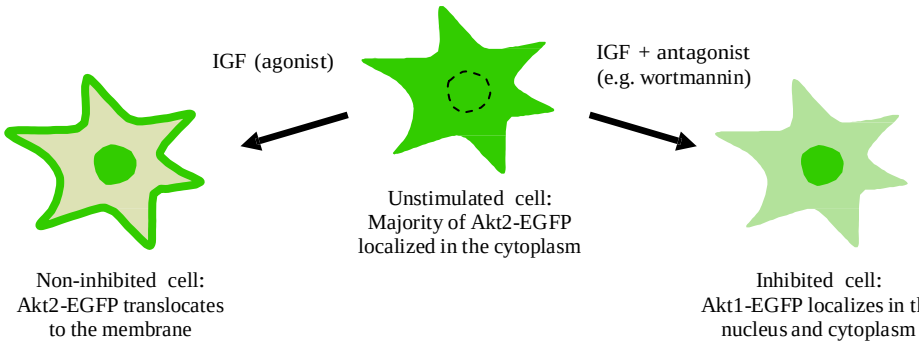


Figure 1:Illustration of the Akt2 translocation.

Test compounds are assayed for their ability to inhibit IGF-1-stimulated membrane translocation of Akt2. The PI3K inhibitor wortmannin [1, 2] is used as reference antagonist. Compounds inhibiting IGF-I-induced translocation of Akt2 may interfere directly with Akt2 translocation (e.g. via PH-domain binding) or act upstream of Akt2. For further profiling of test compounds that inhibit membrane translocation of Akt2, analysis of isoform selectivity can be performed by using the Akt1 and Akt3 Redistribution® assays (see related products).

Additional materials required

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

- Ham's F12 with L-Glutamine (Thermo Scientific, Fisher Scientific cat.# SH30026)
- 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)
- 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)
- Bovine Serum Albumin (BSA) Cohn Fraction V (MP Biomedicals, cat.# ICN841032)
- Long□R^{3}IGF-1 (IGF-1) (Sigma-Aldrich, cat.# 85580)
- Wortmannin (EMD Chemicals cat.# 681675)
- Acetic acid
- 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: Ham's F12 with 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: Ham's F12 with L-Glutamine, 1% Penicillin-Streptomycin, 0.5 mg/ml G418 and 10% FBS, 0.25% DMSO.
- Assay Buffer: Ham's F12 with L-Glutamine, 0.1% BSA, 5 mM HEPES and 1% Penicillin-Streptomycin.
- Cell Wash Buffer: Ham's F12 with L-Glutamine, 0.1% BSA, 5 mM HEPES and 1% Penicillin-Streptomycin, 0.1% FBS, 0.25% DMSO.
- 10% BSA: 1 g BSA dissolved in purified water to a final volume of 10 ml.
- Control Stock: 1 mM Wortmannin stock solution in DMSO. Prepare by dissolving 1 mg Wortmannin (MW=428.4) in 2334 µl DMSO. Store at -20ºC. Protect from light.
- Agonist Stock: 1 mg/ml IGF-1 (110µM) in 100 mM Acetic acid. Prepare by dissolving 5 mg IGF-1 in 5 ml 100 mM 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^{o}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 1:12 to 1:24 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 27 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 200 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

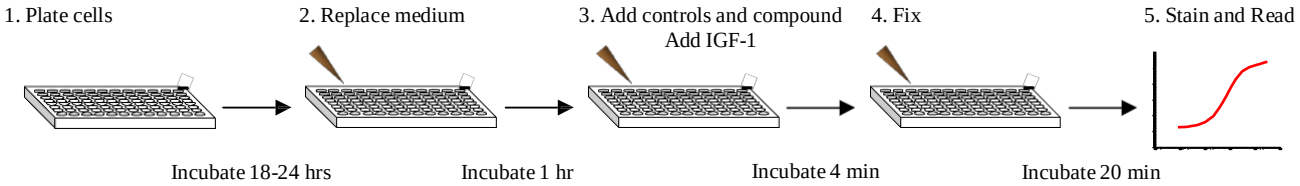


Figure 2. Quick assay workflow overview.

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

1. Before initiating the assay:
    - Prepare Assay Buffer and Cell Wash Buffer. Ensure Assay Buffer and Cell Wash Buffer are pre-warmed to 20-37ºC.
2. Gently remove Plate Seeding Medium and wash the plate 3 times with 100µl Cell Wash Buffer per well.
3. Add 100µl Cell Wash Buffer per well.
4. Incubate cell plate for 60 minutes in a 37ºC, 5% CO_2, 95% humidity incubator.
5. In the meantime, prepare controls and test compounds
    - Dilute controls and test compounds in Assay Buffer to a 4X final concentration. (Volumes and concentrations are indicated below). A final DMSO concentration of 0.375% is recommended (including the DMSO present in the Cell Wash buffer).
    - Mix controls for 1x 96–well plate as indicated below:

	Assay Buffer	Control Stock	DMSO	4X concentration	Final assay concentration	Final DMSO concentration
Negative control	6 ml	----	60 µl	1% DMSO	----	0.375%
Positive control	6 ml	7.2 µl Wortmannin	52.8 µl	1.2 µM Wortmannin	300 nM Wortmannin	0.375%

6. Prepare 4X IGF-1 Agonist Solution (400 nM):
    - Prepare fresh by mixing 27.3 µl 1 mg/ml (110 µM) IGF-1 Agonist Stock with 7.5 ml Assay Buffer. Final assay concentration is 100 nM IGF-1. Use the IGF-1 Agonist Solution within 20 minutes after preparation.
7. Add 50 µl 4X concentrated control or compound solution in Assay Buffer to appropriate wells of the cell plate.
8. Add 50 µl 4X Agonist Solution to each well in the cell plate and gently mix the solution once at a low pipetting speed.
9. Incubate cell plate with lid for 4 minutes at room temperature (starting when Agonist Solution is added).
10. Fix cells by gently removing the buffer and add 150 µl Fixing Solution per well.
11. Incubate cell plate at room temperature for 20 minutes.
12. Wash the cells 4 times with 200 µl PBS per well per wash
13. Decant PBS from last wash and add 100 µl 1 µM Hoechst Staining Solution per well.
14. Seal plate with 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 Akt2-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 10x objective or higher magnification.

The primary output in the Akt2 Redistribution® assay is the translocation of Akt2-EGFP from cytoplasm to membrane spots. The data analysis should therefore report an output that corresponds to number, area, or intensity of these spots in the membrane.

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 CytoCellMemTrans.V2 BioApplication. The output parameter used was MEAN_%MemColoc. The minimally acceptable number of cells used for image analysis in each well was set to 100 cells.

Other BioApplications that can be used for this assay 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:

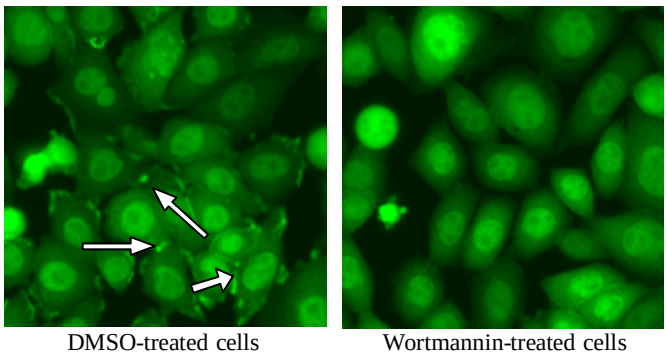
Nucleus size, shape, intensity:	Parameter used to identify DNA damage, effects on cell cycle and apoptosis.
Cell number, size, and shape:	Parameter for acute cytotoxicity and apoptosis.
Cell fluorescence intensity:	Parameter for compound cytotoxicity and fluorescence.

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 Akt2 Redistribution® assay monitors translocation of an Akt2-EGFP fusion protein from the cytoplasm to the plasma membrane. Insulin-like growth factor-I (IGF-I) is used as reference agonist, and compounds are assayed for their ability to inhibit IGF-I-stimulated membrane translocation of Akt2.

Representative images of Akt2 Redistribution cells treated with IGF-I in the absence or presence of 300 nM Wortmannin are shown in figure 3.



DMSO-treated cells | Wortmannin-treated cells

Figure 3. Membrane translocation of Akt2-EGFP. Cells were treated with 100 nM IGF-1 with and without addition of 300 nM wortmannin. Arrows indicate IGF-1 induced membrane translocation of Akt2-EGFP detected by the image analysis algorithm.

A representative concentration response curve of the reference compound wortmannin in the Akt2 assay is shown in figure 4. The EC_{50} of wortmannin in the assay is approximately 34 nM. Note that the EC_{50} value may change depending on the choice of image analysis algorithm.

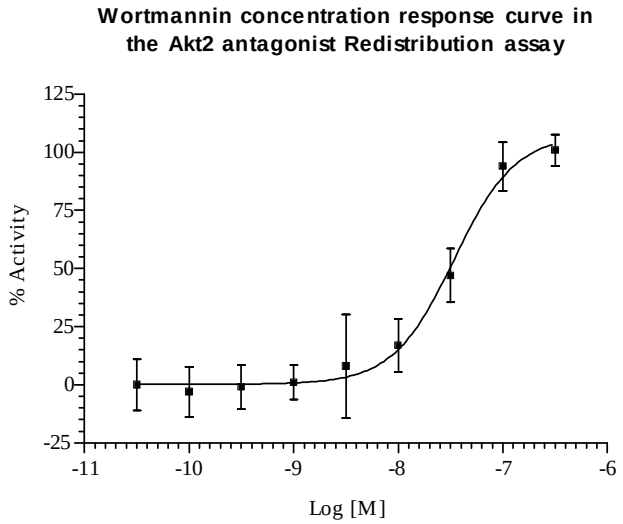


Figure 4. Concentration response curve in the Akt2 antagonist assay. Wortmannin concentration response curve in the Akt2 antagonist Redistribution assay stimulated by 100 nM IGF-1 (n=16). The EC_{50} of wortmannin is 34 nM. Concentration response was measured in 9 point half log dilution series. Cells were pre-incubated with 100 nM IGF-1 for 60 min. and treated with wortmannin for 4 min. Cells were then fixed and membrane translocation was measured using the Cellomics ArrayScan V^{TI} Reader and the CytoCellMemTrans.V2 BioApplication. % activity was calculated relative to the positive (300 nM wortmannin) and negative control (0.25% DMSO).

Product qualification

Assay performance has been validated with an average Z'=0.48±0.08. The cells have been tested for viability. The cells have been tested negative for mycoplasma.

Related Products

Product #	Type	Product description	Cell line
R04-006-01	Profiling/Screening	Akt1-PH domain Redistribution Assay	CHO
R04-085-01	Profiling/Screening	Akt1 Redistribution Assay	CHO
R04-012-01	Profiling/Screening	Akt3 Redistribution Assay	CHO
R04-008-01	Profiling/Screening	FKHR (FOXO1) Redistribution Assay	U2OS
R04-009-02	Profiling/Screening	FKHRL1 (FOXO3) Redistribution Assay	U2OS
R04-090-01	Profiling/Screening	AFX (FOXO4) Redistribution Assay	U2OS
R04-013-01	Profiling/Screening	PDK1 Redistribution Assay	CHO

References

1. Acaro A. & Wymann MP. Biochem J. 296, 297-301, 1993.
2. Burgering BM. & Coffer PJ. Nature 376, 599-602, 1995.

Licensing Statement

Use of this product is limited in accordance with the Redistribution terms and condition of sale.
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 Akt2 Redistribution cell line is genetically modified with a vector expressing Akt2 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.

The Thermo Scientific Redistribution assays are part of the Thermo Scientific High Content Platform which also includes Thermo Scientific HCS Reagent Kits, Thermo Scientific Arrayscan HCS Reader, Thermo Scientific CellInsight Personal Cell Imager, Thermo Scientific ToxInsight IVT platform, BioApplication image analysis software and high-content informatics. For more information on Thermo Scientific products for high content and Cellomics, visit www.thermoscientific.com/cellomics, or call 800-432-4091 (toll free) or 412-770-2500. LC06403402

Pierce Biotechnology
3747 N. Meridian Road
PO Box 117
Rockford IL 61105
USA
(815) 968-0747
(815) 968-7316 fax
www.thermoscientific.com/cellomics