

FKHR Redistribution[®] Assay

For High-Content Analysis

008-01.03

Number	Description
R04-008-01	Recombinant U2OS cells stably expressing human FKHR/Foxo1 (GenBank Acc. NM_002015.3) fused to the N-terminus of enhanced green fluorescent protein (EGFP). U2OS cells are adherent epithelial cells derived from human osteosarcoma. Expression of FKHR-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 1.0×10^6 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 FKHR Redistribution[®] Assay

Forkhead proteins comprise a highly conserved family of transcription factors named after the original forkhead gene in *Drosophila*. Forkhead transcription factor family members (FKHR, FKHL1 and AFX) are known to control the expression of genes encoding proteins essential for insulin, apoptosis (e.g. Fas Ligand and Bim) and cell cycle (e.g. p27, p130 and GADD45) signaling. The activity of FKHR is regulated via its phosphorylation by the protein kinase Akt that is part of the phosphoinositide 3-kinase (PI3K) signaling pathway. Phosphorylated FKHR is sequestered in its inactive form within the cytosol by the so-called 14-3-3 protein. Un-phosphorylated and active FKHR reside in the nucleus. Furthermore, cellular localization of forkhead proteins is also dependent on the classical nuclear export sequence (NES)/Crm1 pathway [1]. Wortmannin inhibits PI3K signalling and hereby hinders FKHR phosphorylation and cytoplasmic sequestering, eventually resulting in nuclear accumulation of FKHR [2, 3]. In this assay wortmannin is used as reference compound. Test compounds are assayed for their ability to induce nuclear accumulation of FKHR. Test compounds causing accumulation of FKHR in the nucleus may interfere directly with FKHR import, act upstream of FKHR interfering with the PI3K/Akt1 signaling pathway or may be general nuclear import activators/nuclear export inhibitors. For further profiling of test compounds identified as positive in the FKHR assay, isoform selectivity can be determined by using the FKHL1 Redistribution[®] Assay [4]. Possible effect of positive test compounds upstream of Akt1, within the PI3K signaling pathway, can be determined using the Akt1 Redistribution[®] Assay [5]. General export inhibitor characteristics of compounds can be performed using the Rev1 Redistribution[®] Assay [6]. Finally, the FKHR Redistribution[®] Assay can be used also to detect compounds acting as general nuclear export inhibitors; in this case the nuclear export inhibitors Leptomycin B or Ratjadone A is used as reference compound.

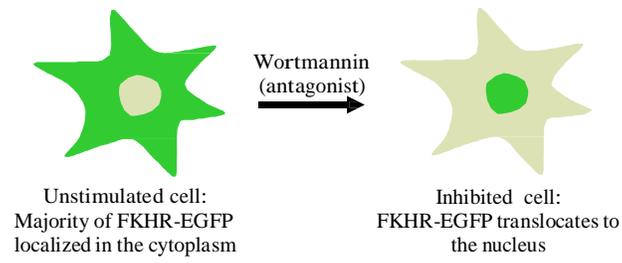


Figure 1: Illustration of the FKHR translocation.

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)
- 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)
- Wortmannin (EMD Chemicals, cat.# 681675)
- 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% FBS
- Cell Wash Buffer: DMEM supplemented with 2mM L-Glutamine, 1% Penicillin-Streptomycin, 5 mM HEPES Buffer and 1.4% FBS
- Assay Buffer: DMEM supplemented with 2mM L-Glutamine, 1% Penicillin-Streptomycin and 5 mM HEPES Buffer
- Control Compound Stock: 1 mM Wortmannin stock solution in DMSO. Prepare by dissolving 1 mg Wortmannin (MW=428.4) in 2334 μ l DMSO. Store at -20°C. Protect from light. Note that Wortmannin is unstable in aqueous solution. Dilute Wortmannin in aqueous solution just in time before addition to cells.
- 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 μ M 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 μ l 10 mM Hoechst 33258. Store at 4°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 37°C, 5% CO₂, 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 37°C.

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 37°C, 5% CO₂, 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 10⁶ cells per ml (5 cryogenic vials = 5 x 10⁶ 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 10⁶ 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 -80°C 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 21 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 30,000 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 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 37°C, 5% CO₂, 95% humidity incubator prior to starting the assay

Assay protocol

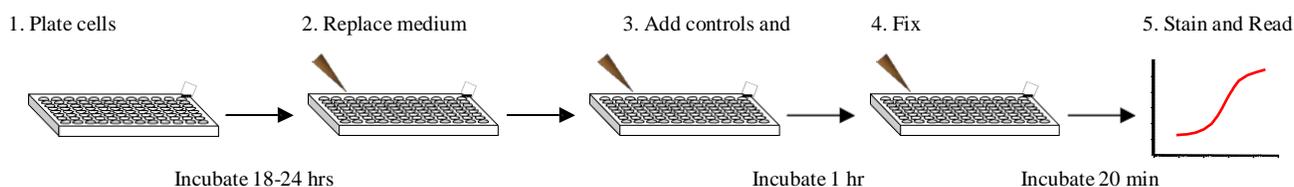


Figure 2: Quick assay workflow overview

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 20-37°C.

2. Gently remove Plate Seeding Medium and wash cell plate once with 100 μ l Assay Buffer per well.

3. Add 100 μ l Assay Buffer per well.

4. 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 0.5% DMSO final concentration. Note that Wortmannin is unstable in aqueous solution. Dilute Wortmannin in aqueous solution just in time before addition to cells.
- 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 μ l	0.5% DMSO	----	0.25%
Positive control	12 ml	3.6 μ l Wortmannin	56.4 μ l	300 nM Wortmannin	150 nM Wortmannin	0.25%

5. Add 100 μ l 2X concentrated control or compound solution in Assay Buffer to appropriate wells of the cell plate.

6. Incubate cell plate for 1 hour in a 37°C, 5% CO₂, 95% humidity incubator.

7. Fix cells by gently decanting the buffer and add 150 μ l Fixing Solution per well.

8. Incubate cell plate at room temperature for 20 minutes.

9. Wash the cells 4 times with 200 μ l PBS per well per wash.

10. Decant PBS from last wash and add 100 μ l 1 μ M Hoechst Staining Solution.

11. Seal plate with a black plate sealer. Incubate at room temperature for at least 30 minutes before imaging. The plate can be stored at 4°C for up to 3 days in the dark.

Imaging

The translocation of FKHR-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 5-10x objective or higher magnification.

The primary output in the FKHR Redistribution[®] assay is the translocation from cytoplasm to nucleus of FKHR-EGFP. 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 100 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 FKHR Redistribution[®] Assay monitors inhibition of serum-induced FKHR translocation from the nucleus to the cytoplasm. Representative images of FKHR Redistribution cells treated in the absence or presence of 150 nM Wortmannin are shown in figure 3. A concentration response curve of the reference compound wortmannin is shown in figure 4 and concentration response curves of other PI3K pathway and nuclear export inhibitors are shown in figure 5. The EC₅₀ value of wortmannin in the assay is approximately 9 nM.

Figure 6 shows that the PI3K pathway in the FKHR Redistribution[®] assay cell line is regulated as expected after treatment with PI3K or Akt inhibitors.

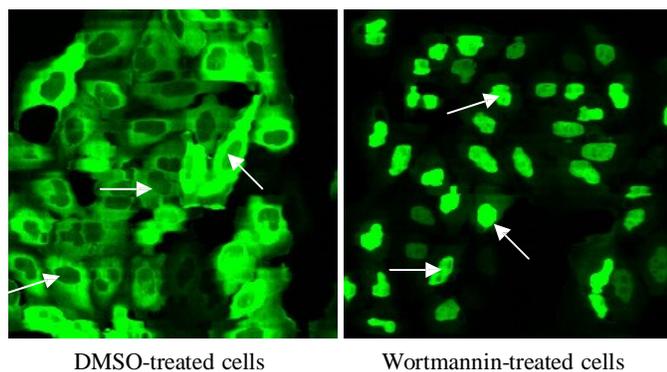


Figure 3. Translocation of FKHR-EGFP in response to wortmannin. Cells were treated with 0.25% DMSO or 150 nM wortmannin. Arrows indicate cytoplasm to nucleus translocation detected by the image analysis algorithm.

Wortmannin concentration response curve in FKHR assay

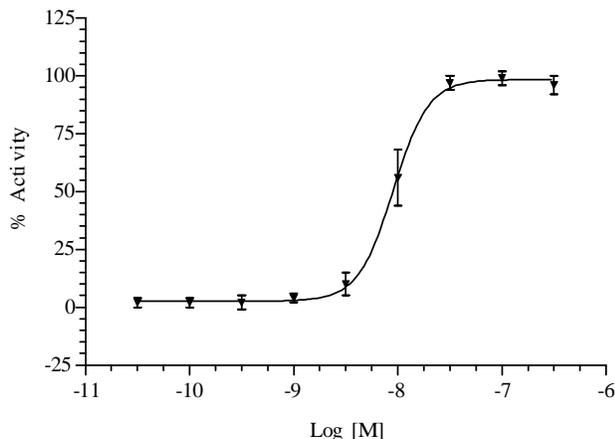


Figure 4. Wortmannin concentration response curve in the FKHR Redistribution[®] assay. Concentration response was measured in 9 point half log dilution series of wortmannin. Cells were incubated with wortmannin for 60 min. Cells were then fixed and the nucleus to cytoplasm translocation was measured using the Cellomics ArrayScan V[™] Reader and the RedistributionV3 BioApplication. % activity was calculated relative to the positive (150 nM wortmannin) and negative control (0.25% DMSO). The EC₅₀ of wortmannin is approximately 9 nM.

Concentration response curves in FKHR assay

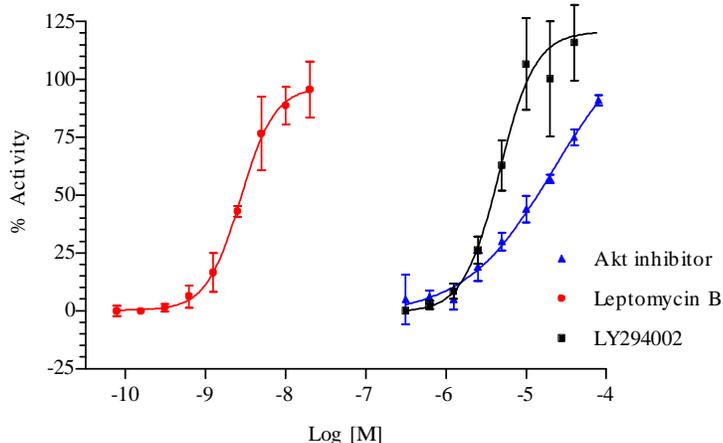


Figure 5. Concentration response curves in the FKHR Redistribution[®] assay. Concentration response was measured in 9 point 2x dilution series of Leptomycin B (nuclear export inhibitor), LY294009 (PI3K inhibitor) and an Akt inhibitor [4]. Cells were incubated with test compound for 60 min. Cells were then fixed and the nucleus to cytoplasm translocation was measured using image analysis. % activity was calculated relative to the positive (150 nM wortmannin) and negative control (0.25% DMSO). The EC₅₀ values are: Leptomycin EC₅₀= 2.7 nM, LY294002 EC₅₀=4.7 μM and Akt inhibitor EC₅₀=22

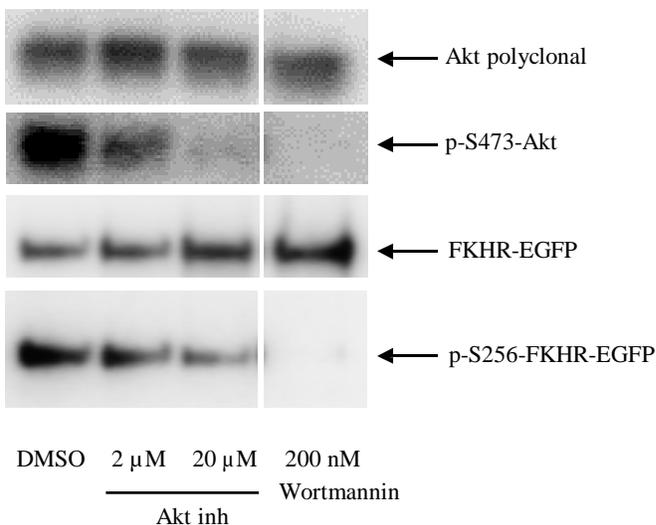


Figure 6. Western blot showing regulation of FKHR Redistribution[®] cells. FKHR Redistribution[®] cells were incubated with DMSO, wortmannin or an Akt inhibitor [4] for 60 min. Cells were then lysed and cell extracts were run on western blot. Akt polyclonal and FKHR-EGFP indicate the total amount of Akt1-3 and FKHR-EGFP in the assay cell line, respectively. P-S473-Akt indicates the amount of Akt that is activated by S473 phosphorylation. This activation is fully inhibited by wortmannin and the Akt inhibitor. P-S256-FKHR-EGFP indicates the amount of FKHR-EGFP that is phosphorylated on S256 (Akt site). FKHR phosphorylation is fully inhibited by

Product qualification

Assay performance has been validated with an average $Z' = 0.66 \pm 0.05$. 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
R04-008-01	Profiling and Screening	FKHR/Foxo1 Redistribution [®] Assay	U2OS
R04-009-02	Profiling and Screening	FKHRL1/Foxo3a Redistribution [®] Assay	U2OS
R04-090-01	Profiling and Screening	AFX/Foxo4 Redistribution [®] Assay	U2OS
R04-085-01	Profiling and Screening	Akt1 Redistribution [®] Assay	CHO
R04-011-02	Profiling and Screening	Akt2 Redistribution [®] Assay	CHO
R04-012-01	Profiling and Screening	Akt3 Redistribution [®] Assay	CHO

References

1. Burgering BMT. & Kops GJPL. Trends Biochem. Sci. 27, 352-360, 2002.
2. Acaro A. & Wymann MP. Biochem J. 296, 297-301, 1993.
3. Burgering BM. & Coffey PJ. Nature 376, 599-602, 1995.
4. Barnett, SF et al. Curr Top Med Chem 5, 109-125, 2005.

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