INSTRUCTIONS

FYVE Redistribution® Assay

For High-Content Analysis

074-01.03

Number	Description
R04-074-01	Recombinant U2OS cells stably expressing the FYVE finger from the human homologue of the hepatocyte growth factor-regulated tyrosine kinase substrate Hrs, duplicated in tandem (GenBank Acc. NM_004712) and fused to the C-terminus of enhanced green fluorescent protein (EGFP). U2OS cells are adherent epithelial cells derived from human osteosarcoma. Expression of EGFP-2xFYVE 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 FYVE Domain Redistribution® Assay

Vesicular trafficking and proper sorting of internalized proteins require the recruitment of cytosolic proteins to a specific endosomal membrane in a reversible and regulated manner. Many proteins become reversibly localized to membranes through interaction with specific lipid head groups. PtdIns(3)P is a product of the class III PI3K Vps34 that is constitutively produced and specifically localize to early endosomes. Several proteins bind specifically to the PtdIns(3)P head group through domains such as the FYVE-finger or the PX domain [1-5]. The FYVE-finger is a cysteine-rich double-zinc-binding domain, which is thought to localize FYVE-finger proteins to early endosomes through its interaction with PtdIns(3)P.

The FYVE domain Redistribution assay is designed to screen for inhibitors of FYVE endosomal localization by monitoring the dissociation of FYVE domains from early endosomal membranes. The EGFP-2xFYVE fusion protein used in this assay consists of the FYVE finger from the human homologue of the hepatocyte growth factor-regulated tyrosine kinase substrate Hrs, duplicated in tandem. Hrs is involved in sorting of ubiquitinated proteins into clathrin-coated microdomains of early endosomes.

The FYVE domain Redistribution® assay monitors translocation of EGFP-2xFYVE from its initial location bound to (PtdIns(3)P) in early endosomes to the cytoplasm, in response to test compounds.

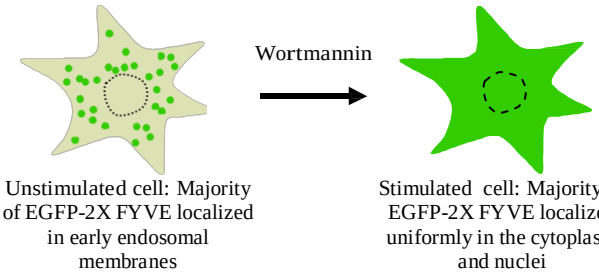


Figure 1: Illustration of the FYVE translocation event.

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

FYVE translocation can be promoted by the PI3K inhibitor wortmannin [6, 7], which inhibits Vps34 catalytic activity and thereby prevents (PtdIns(3)P) synthesis. In this assay, wortmannin is used as reference compound and compounds are assayed for their ability to dissociate FYVE domains from early endosomal membranes.

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)
- 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.
- Assay Buffer: DMEM supplemented with 2mM L-Glutamine, 1% Penicillin-Streptomycin, and 10% FBS.
- Control Compound 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. 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 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 80,000 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 8000 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

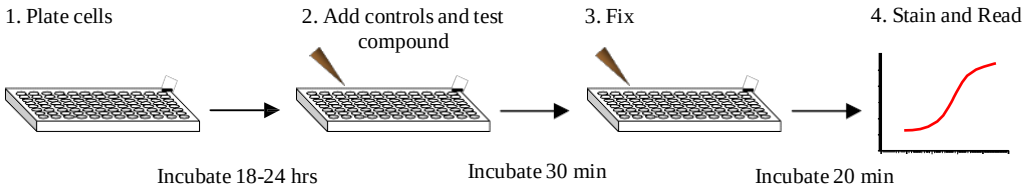


Figure 2: Quick assay workflow overview.

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 0.5% 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	2.4 µl Wortmannin	57.6 µl	200 nM Wortmannin	100 nM Wortmannin	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-2xFYVE 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 FYVE Redistribution® assay is the dissociation of spots in the cytoplasm. The data analysis should therefore report an output that corresponds to number, area, or intensity of spots in 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), High Resolution images, 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 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:

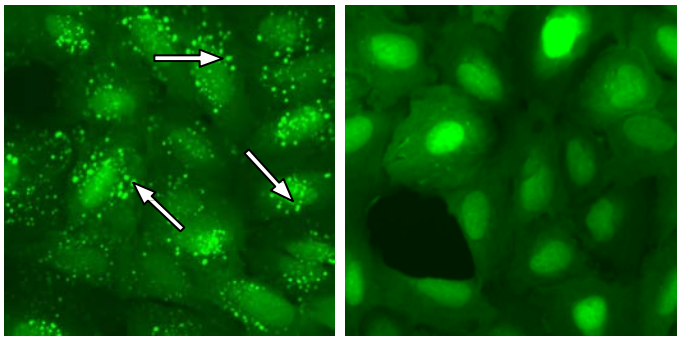
Nucleus size, shape, intensity:	Parameter used to identify DNA damage, effects on cell cycle and apoptosis.
Cell number, size, and shape:	Parameter for acute cytotoxicity and apoptosis.
Cell fluorescence intensity:	Parameter for compound cytotoxicity and fluorescence.

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 FYVE Redistribution® assay monitors the translocation of EGFP-2xFYVE from early endosomes to the cytoplasm. Wortmannin is used as a reference compound, and test compounds are assayed for their ability to dissociate FYVE domains from early endosomal membranes.

Representative images of FYVE Redistribution® cells treated with wortmannin are shown in Figure 3.



DMSO-treated cells Wortmannin-treated cells

Figure 3. Dissociation of EGFP-2xFYVE from early endosomes. Cells were treated with 100 nM wortmannin for 30 min. Arrows indicate the endosome-localized EGFP-2xFYVE detected by the image analysis algorithm.

Figure 4 shows a representative concentration response curve of the reference compound wortmannin in the FYVE assay. The EC_{50} of wortmannin is ~10 nM .

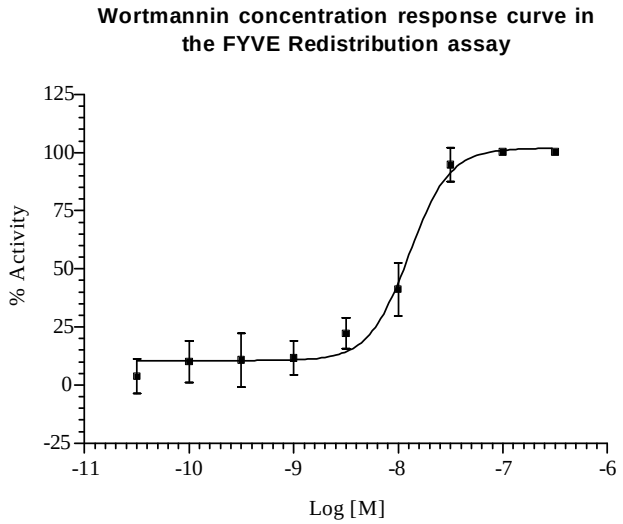


Figure 4. Wortmannin concentration response in the FYVE assay: The EC_{50} of wortmannin is ~10 nM. Concentration response was measured in 9 point half log dilution series (n=16). Cells were treated with wortmannin for 30 min. Cells were then fixed and nuclear translocation was measured using the Cellomics ArrayScan V^{TI} Reader and the SpotDetectorV3 BioApplication. % activity was calculated relative to the positive (100 nM wortmannin) and negative control (0.25% DMSO)

Product qualification

Assay performance has been validated with an average Z'=0.70±0.08. 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
R04-006-01	Profiling/Screening	Akt1-PH domain Redistribution Assay	CHO
R04-085-01	Profiling/Screening	Akt1 Redistribution Assay	CHO
R04-011-02	Profiling/Screening	Akt2 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. Stenmark H & Aasland R, J cell Sci., 112, 4175-4183, 1999.
2. Gillooly DJ et al., EMBO J., 19, 4577-4588, 2000.
3. Burd CG & Emr SD, Mol Cell, 2, 157-162, 1998.
4. Gaullier JM et al., Nature 394, 432-433, 1998.
5. Patki V et al., Nature., 394, 433-434, 1998.
6. Acaro A. & Wymann MP. Biochem J. 296, 297-301, 1993.
7. Burgering BM. & Coffer PJ. Nature 376, 599-602, 1995.

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For European customers:
The FYVE Redistribution cell line is genetically modified with a vector expressing 2x FYVE domain 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. LC06903402

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