

# Btk-PH Redistribution<sup>®</sup> Assay

For High-Content Analysis

007-01.03

Number	Description
R04-007-01	Recombinant CHO <sub>h</sub> IR cells stably expressing human Btk PH-domain, Btk(1-180) (GenBank Acc. NM_000061) fused to the N-terminus of enhanced green fluorescent protein (EGFP). CHO <sub>h</sub> IR cells are adherent epithelial cells derived from Chinese hamster ovary cells with stable expression of human insulin receptor. Expression of Btk-PH-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  $1.0 \times 10^6$  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<sup>®</sup> Technology

The Redistribution<sup>®</sup> 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 Btk-PH Redistribution<sup>®</sup> Assay

Bruton's tyrosine kinase (Btk) is a non-receptor cytoplasmic tyrosine kinase belonging to the Tec family of kinases. Btk mediates B lymphocyte proliferation, differentiation and signaling. Btk translocates to the plasma membrane via binding of its pleckstrin homology (PH) domain to phosphatidylinositol-3,4,5-triphosphate (PIP3) following growth factor stimulation and cross linking of the B cell receptor. Mutations in the Btk gene cause X-linked agammaglobulinemia (XLA). This disorder is characterized by lack of mature B cells resulting in primary immunodeficiency [1]. The Btk-PH Redistribution<sup>®</sup> assay monitors the translocation of the Btk-PH-EGFP fusion protein from the cytoplasm to the plasma membrane. Insulin-like growth factor-I (IGF-I) is used as the reference agonist.

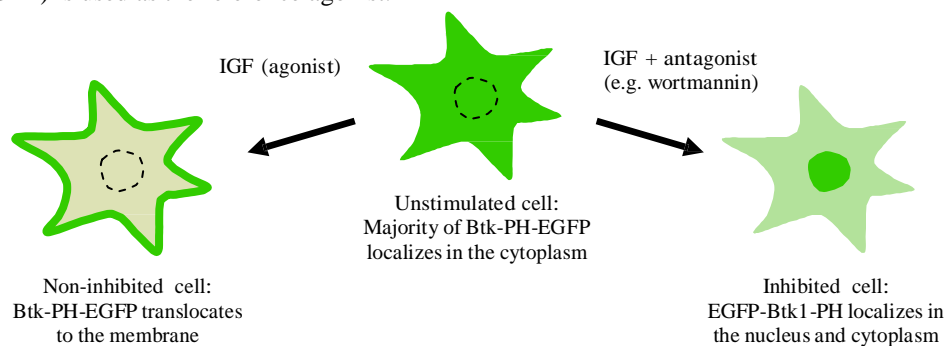


Figure 1: Illustration of the Btk-PH translocation.

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Test compounds are assayed for their ability to inhibit IGF-1-stimulated membrane translocation of the Btk-PH-EGFP fusion protein. Wortmannin inhibits the phosphatidylinositol 3-kinase (PI3K) [2,3] and hereby decreases the production of PIP3. Thus, wortmannin is used in the assay as reference antagonist. Test compounds inhibiting IGF-1-induced cytoplasm to membrane translocation of Btk may interfere directly with Btk-PH translocation or may act upstream of Btk. The Btk Redistribution® assay can be used as a selectivity assay for the Akt Redistribution® assays (see related products) to deselect or identify compounds working upstream of Akt at the level of PI3K.

## 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 $\alpha$ R<sup>3</sup>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  $\mu$ l DMSO. Store at -20°C. Protect from light.
- Agonist Stock: 1 mg/ml IGF-1 (110 $\mu$ M) 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  $\mu$ 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  $\mu$ l 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 37°C, 5% CO<sub>2</sub>, 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 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% CO<sub>2</sub>, 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  $1 \times 10^6$  cells per ml (5 cryogenic vials =  $5 \times 10^6$  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  $1 \times 10^6$  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 -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 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 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 37°C, 5% CO<sub>2</sub>, 95% humidity incubator prior to starting the assay

## Assay protocol

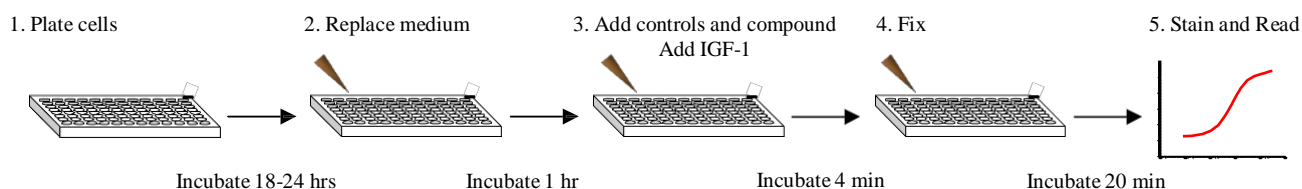


Figure 3: Quick assay workflow overview

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 3 times with 100 µl Assay Buffer per well.
3. Add 100 µl Assay Buffer per well.
4. Incubate cell plate for 60 minutes in a 37°C, 5% CO<sub>2</sub>, 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), but the assay can tolerate up to 0.625% DMSO final concentration.
  - 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	12 µl Wortmannin	48 µl	2 µM Wortmannin	500 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 4°C for up to 3 days in the dark.

## Imaging

The translocation of Btk 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 10-20x objective or higher magnification.

The primary output in the Btk Redistribution<sup>®</sup> assay is the translocation of Btk 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<sup>®</sup> 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 Btk-PH Redistribution<sup>®</sup> assay monitors translocation of a Btk-PH-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 Btk-PH.

Representative images of Btk-PH Redistribution cells treated with IGF-I in the absence or presence of 500 nM Wortmannin are shown in figure 3.

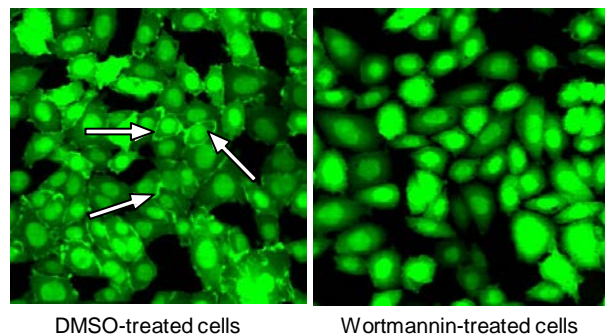


Figure 3. Membrane translocation of Btk-PH-EGFP. Cells were treated with 10 nM IGF-1 with and without addition of 500 nM wortmannin. Arrows indicate IGF-1 induced membrane translocation of EGFP-PDK1 detected by the image analysis algorithm.

A representative concentration response curve of the reference compound wortmannin in the Btk-PH assay is shown in figure 4. The  $EC_{50}$  of wortmannin in the assay is approximately 45 nM. Note that the  $EC_{50}$  may change depending on the choice of image analysis algorithm.

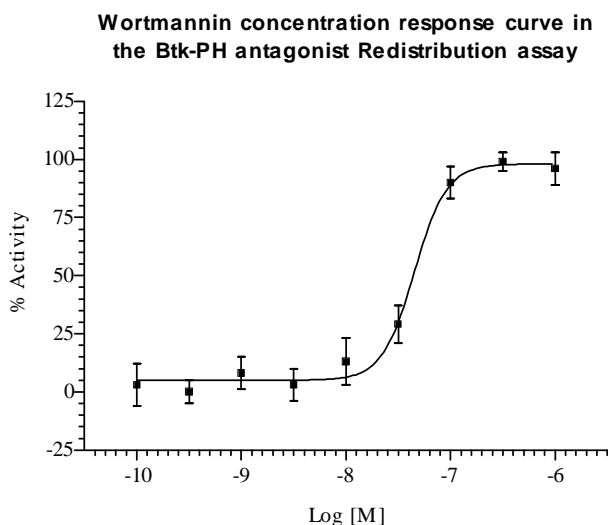


Figure 4. Concentration response curve in the Btk-PK antagonist assay. Wortmannin concentration response curve in the Btk-PH antagonist Redistribution assay stimulated by 100 nM IGF-1 (n=8). The  $EC_{50}$  of wortmannin is 45 nM. Concentration response was measured in 9 point half log dilution series. Cells were incubated with 100 nM IGF-1 and wortmannin for 4 min. Cells were then fixed and membrane translocation was measured using the Cellomics ArrayScan V<sup>TI</sup> Reader and the CytoCellMemTrans.V2 BioApplication. % activity was calculated relative to the positive (500 nM wortmannin) and negative control (0.25% DMSO).

## Product qualification

Assay performance has been validated with an average  $Z' = 0.49 \pm 0.05$ . The cells have been tested for viability. The cells have been tested negative for mycoplasma.

## Related Products

Product #	Type	Product description	Cell line
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
R04-013-01	Profiling/Screening	PDK1 Redistribution assay	CHO
R04-008-01	Profiling/Screening	FKHR Redistribution assay	U2OS
R04-009-02	Profiling/Screening	FKHRL1 Redistribution assay	U2OS
R04-090-01	Profiling/Screening	AFX/Foxo4 Redistribution assay	U2OS

## References

1. Rosen H & Goetzl EJ, Nature Rev Immunol., 5, 560-570, 2005.
2. Kostenis E, J Cell Biochem., 92, 923-936, 2004.
3. Okamoto H et al., J Biol Chem., 273, 27104-27110, 1998.
4. Jo E et al., Chem Biol., 12, 703-715, 2005.



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