# INSTRUCTIONS

# EGFR Redistribution® Assay

## For High-Content Analysis

082-01.03

| Number | Description |
|---|---|
| **R04-082-01** | Recombinant U2OS cells stably expressing human epidermal growth factor receptor (EGFR) (GenBank Acc. NM_005228) fused to the N-terminus of enhanced green fluorescent protein (EGFP). U2OS cells are adherent epithelial cells derived from human osteosarcoma. Expression of EGFR-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 Epidermal Growth Factor Receptor (EGFR) Antagonist Redistribution Assay

Epidermal growth factor receptor (EGFR, ErbB-1) is a member of the ErbB receptor family, a subfamily of four closely related tyrosine kinases [1]. The other members of the ErbB receptor family are HER2/c-neu (ErbB-2), HER3 (ErbB-3), and HER4 (ErbB-4). EGFR is a ubiquitous single-transmembrane receptor, which in its inactive state is present as monomers in the cell membrane.

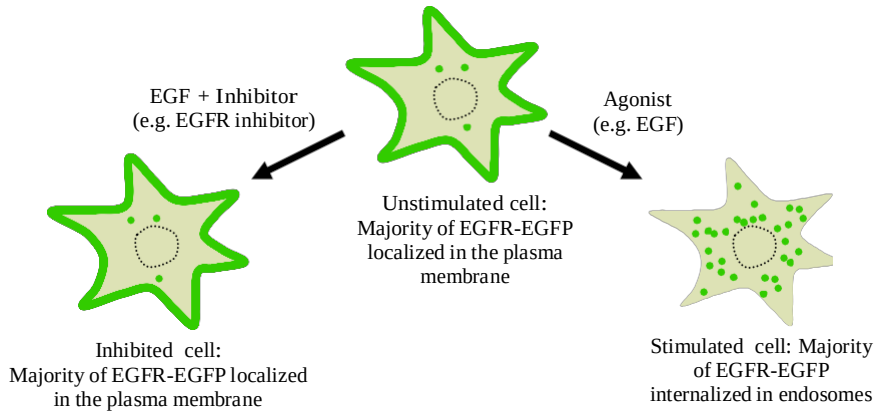



Figure 1. Illustration of the EGFR translocation event.

Upon activation by a ligand, the receptor dimerizes with another member of ErbB receptor family, e.g. another EGFR molecule or HER2. The homo- and heteromer ErbB receptor dimers have different ligands. EGFR dimers and EGFR/ErbB-2

heterodimers are activated by epidermal growth factor (EGF), transformation growth factor α (TGFα), and amphiregulin (AR). Binding of ligand to the receptor and subsequent dimerization leads to stimulation of the intrinsic tyrosine kinase activity of EGFR and activation of multiple downstream signaling pathways, including the MAP kinase pathway, the AKT pathway, and the STAT pathway. Following activation, the EGFR is internalized through clathrin coated pits and subsequently sorted to and degraded in lysosomes. The ubiquitin ligase c-Cbl targets the EGFR to endocytosis by tagging the receptor with multiple monoubiquitin molecules important for receptor down-regulation [2-4]. The EGFR and HER2 are involved in cell proliferation and differentiation, and they are often over-expressed or amplified in cancers, making them important targets for cancer therapy [5].

The EGFR_antagonist Redistribution® assay is designed to screen for antagonists of EGF-induced internalization of EGFR from the plasma membrane to endosomes of human U2OS osteosarcoma cells. EGFR inhibitor [6] is used as reference compound in the assay, and compounds are assayed for their ability to inhibit internalization of the EGFR.

## 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)
- Bovine Serum Albumin (BSA) Cohn Fraction V
- Acetic acid
- Epidermal Growth Factor, Human, Recombinant, E.Coli (rhEGF) (EMD Chemicals, cat.# 324831)
- EGFR Inhibitor (EMD Chemicals, cat.# 324674)
- 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.
- 10% BSA: 1g BSA dissolved in purified water to a final volume of 10 ml.
- Assay Buffer: DMEM supplemented with 2mM L-Glutamine and 1% Penicillin-Streptomycin.

- Control Compound Stock: 4 mM EGFR inhibitor stock solution in DMSO. Prepare by dissolving 1 mg EGFR inhibitor (MW = 413.4) in 605 µl DMSO. Store at -20°C, protect from light.
- rhEGF Agonist Stock: 100 µg/ml rhEGF stock solution prepared in acetic acid containing 0.1% BSA. Prepare by dissolving 200 µg rhEGF in 2 ml acetic acid containing 0.1% BSA.
- 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 20, 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 40-48 hours in a 37ºC, 5% $CO_2$, 95% humidity incubator prior to starting the assay.

## Assay protocol

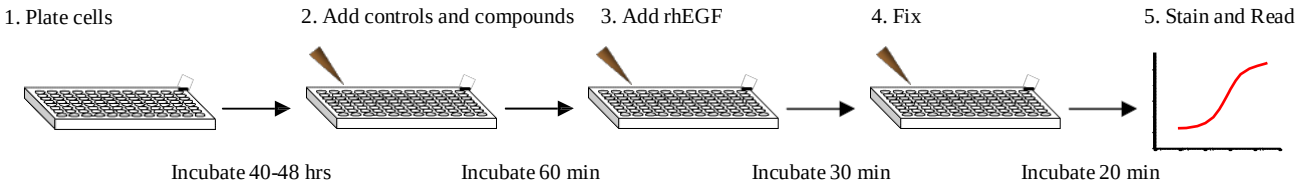



Figure 2: Quick assay workflow overview.

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 4X final concentration. (Volumes and concentrations are indicated below). A final DMSO concentration of 0.25% is recommended, but the assay can tolerate up to 1% 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 | ---- | 0.25% |
| Positive control | 6 ml | 18 µl EGFR inhibitor | 42 µl | 12 µM EGFR inhibitor | 3 µM EGFR inhibitor | 0.25% |

Important Note! This assay is sensitive to stress such as changes in temperature and $CO_2$. It is very important to treat the cells gently during the cell seeding and compound handling and to keep the cells in the incubator as long as possible.

3. Add 50 µl 4X concentrated control or compound solution in Assay Buffer to appropriate wells of the cell plate.
4. Incubate cell plate for 60 minutes in a 37ºC, 5% $CO_2$, 95% humidity incubator.
5. Prepare 4X rhEGF Agonist Solution (40 ng/ml):
   - Prepare fresh by mixing 2.4 µl 100 µg/ml rhEGF Agonist Stock with 6 ml Assay Buffer. Use the rhEGF Agonist Solution within 20 min after preparation.
6. Add 50 µl 4X rhEGF Agonist Solution to appropriate wells of the cell plate.
7. Incubate cell plate for 30 minutes in a 37ºC, 5% $CO_2$, 95% humidity incubator.
8. Fix cells by gently decanting the buffer and add 150 µl Fixing Solution per well.
9. Incubate cell plate at room temperature for 20 minutes.

10. Wash the cells 4 times with 200 µl PBS per well per wash.
11. Decant PBS from last wash and add 100 µl 1 µM Hoechst Staining Solution.
12. 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 EGFR-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 EGFR Redistribution® assay is the formation 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 450 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.

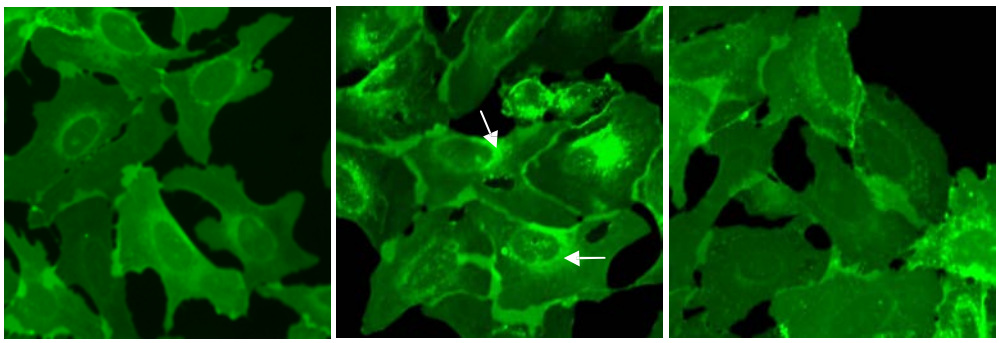
Examples of useful secondary high content outputs:

| | |
|---|---|
| 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 EGFR Redistribution® Assay monitors internalization of the epidermal growth factor receptor. An EGFR inhibitor [6] is used as the reference compound. Example images are shown in Figure 3.



Untreated cells | EGF-treated cells | EGFR inhibitor-treated cells

Figure 3. Internalization of EGFR-EGFP in response to EGF. Cells were treated with 10 ng/ml EGF for 30 min in the presence or absence of EGFR inhibitor. Arrows indicate the EGFR-EGFP internalization that is detected by the image analysis algorithm.

Figures 4 and 5 show the response of the assay cell line to EGF, and a concentration response curve of the EGFR inhibitor reference compound [6] using EGF as agonist. The $EC_{50}$ of EGF is ~0.2 ng/ml, and the $EC_{50}$ of the EGFR inhibitor is approximately 1 µM.

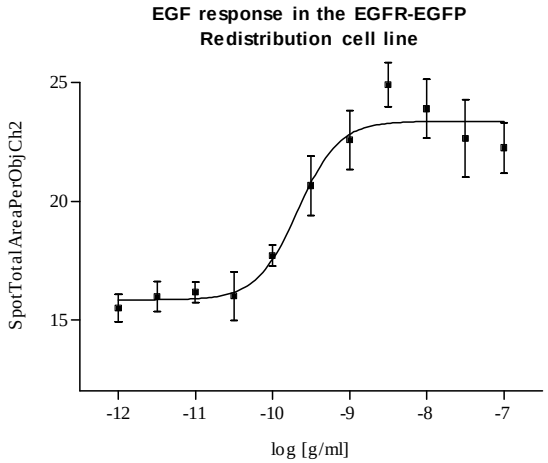



Figure 4. Concentration response of EGF in the EGFR Redistribution Assay (n=8). Cells were treated with EGF for 30 min. Cells were then fixed and receptor internalization was measured using the Cellomics ArrayScan V$^{TI}$ Reader and the SpotDetectorV3 BioApplication. The $EC_{50}$ of EGF is ~0.2 ng/ml.

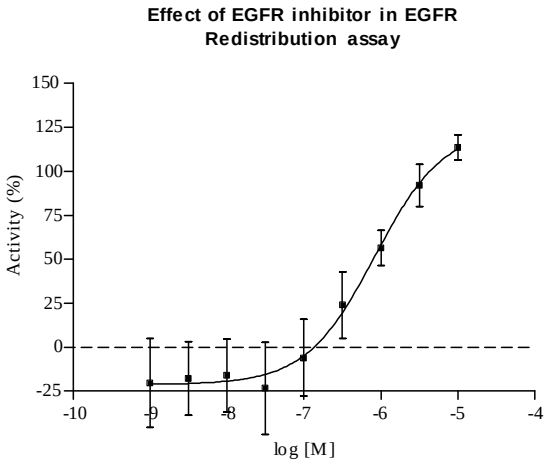



Figure 5. Concentration response of the EGFR inhibitor compound in the EGFR Redistribution assay (n=16). Cells were incubated with compound for 1 hr, followed by EGF treatment for 30 min. Cells were then fixed and receptor internalization was measured using the Cellomics ArrayScan V$^{TI}$ Reader and the SpotDetectorV3 BioApplication. The $EC_{50}$ of the EGFR inhibitor is ~1 µM.

## Product qualification

Assay performance has been validated with an average Z'=0.37±0.2. 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 |
|---|---|---|---|
| R04-016-01 | Profiling & Screening | STAT3 EGF-stimulated Redistribution® Assay | MDA-MB-468 |
| R04-049-01 | Profiling | Ras activation Redistribution® Assay | CHO |
| R04-019-01 | Profiling & Screening | ERF, MAPK pathway reporter Redistribution® Assay | T24 |
| R04-022-01 | Profiling & Screening | ERF, MAPK pathway reporter Redistribution® Assay | U2OS |
| R04-087-01 | Profiling & Screening | ERK2 Redistribution® Assay | U2OS |

## References


1. Normanno N et al., Gene. 17, 2-16, 2006.
2. Mosesson Y et al., J Biol Chem., 278, 21323–21326, 2003.
3. Soubeyran P et al. Nature, 416, 183–187, 2002.
4. Jiang X and Sorkin A. Traffic, 4, 529–543, 2003.
5. Johnston JB et al., Curr Med Chem. 13, 3483-3492, 2006.
6. Calbiochem, Cat no. 324674, Cyclopropanecarboxylic acid-(3-(6-(3-trifluoromethyl-phenylamino)-pyrimidin-4-ylamino)-phenyl)-amide

## 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 EGFR Redistribution cell line is genetically modified with a vector expressing EGFR 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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