

ER β Redistribution[®] Assay

For High-Content Analysis

063-01.02

Number	Description
R04-063-01	Recombinant U2OS cells stably expressing human Estrogen receptor β (ER β) (GenBank Acc. NM_001437) fused to the C-terminus of enhanced green fluorescent protein (EGFP). U2OS cells are adherent epithelial cells derived from human osteosarcoma. Expression of EGFP-ER β 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 ER β Redistribution[®] Assay

Estrogen is a regulator of normal endocrine functions. Signal transduction induced by estrogens such as estradiol, the main endogenous human estrogen, is mediated by the estrogen receptor (ER). ER is a nuclear receptor that upon ligand binding organizes into homo- and heterodimers of the ER α and ER β subtypes. Following ligand binding, ER acts as a transcription factor and regulates expression of several target genes such as cyclin D1 and IGF-1 [1]. Estrogens and estrogen receptors are implicated in development and progression of breast cancer. Moreover, environmental chemical contaminants with estrogenic activity are suggested to promote reproductive disorders.

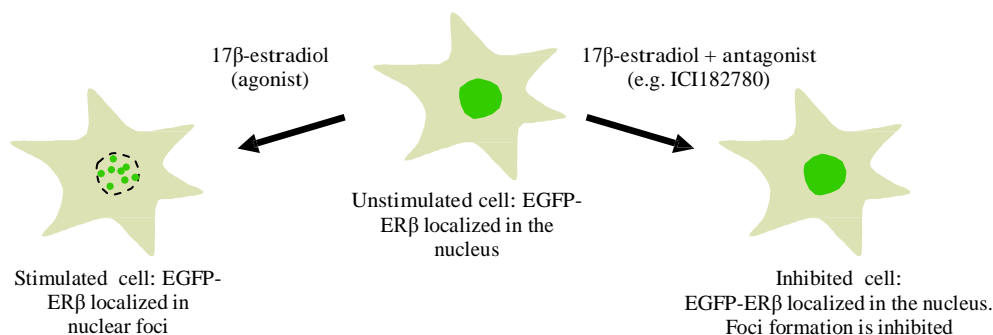


Figure 1. Illustration of the ER β translocation event.

The ERP Redistribution® assay monitors accumulation of an EGFP-ERP fusion protein in nuclear foci as a measure of ERP activation. The agonist version of the assay uses the potent estrogen, 17 β -estradiol, as a reference compound. The EC₅₀ of 17 β -estradiol in the assay is approximately 0.6 nM and corresponds well with EC₅₀ values reported by others [2]. The antagonist version of the assay uses the estrogen receptor antagonist ICI182,780 as a reference compound. The assay is developed in human osteosarcoma (U2OS) cells.

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)
- Charcoal/Dextran Treated Fetal Bovine Serum (CCS-FBS) (Thermo Scientific, Fisher Scientific cat.# SH3006802)
- 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)
- 17 β -estradiol (Sigma-Aldrich, cat.# E2758)
- ICI 182,780 (Tocris Biosciences, cat. # 1047)
Note: ICI 182,780 is only used in the antagonist mode of the assay.
- 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% CCS-FBS.
- Assay Buffer: DMEM supplemented with 2mM L-Glutamine and 1% Penicillin-Streptomycin.
- Control Compound Stock (agonist mode): 25 mM 17 β -estradiol stock solution in DMSO. Prepare by dissolving 5 mg 17 β -estradiol (MW = 272.4) in 734 μ l DMSO. Store at -20°C.
- Control Compound Working Solution (agonist mode): 250 μ M 17 β -estradiol stock solution in DMSO. Prepare by diluting 25 mM 17 β -estradiol 1:100 in DMSO.
- Control Compound Stock (antagonist mode): 25 mM ICI 182,780 stock solution in DMSO. Prepare by dissolving 10 mg ICI 182,780 (MW = 606.77) in 659 μ l DMSO. Store at -20°C.
Note: ICI 182,780 is only used in the antagonist mode of the assay.
- 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. Mr. Frosty, Nalgene Nunc, catalog no. 5100-0001) 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 up to passage 23, 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 100 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 - agonist mode

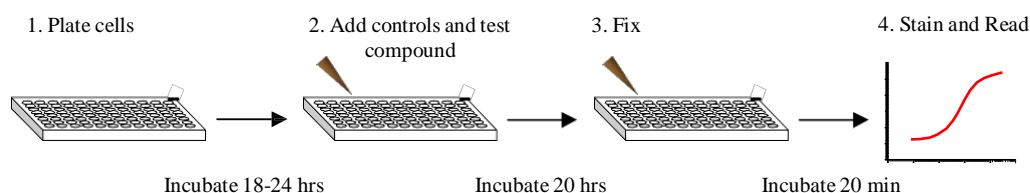


Figure 2: Quick assay workflow overview.

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 20-37°C.
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 2% DMSO final concentration.
 - Mix controls for 1x 96-well plate as indicated below:

	Assay Buffer	Control Working Solution	DMSO	2X concentration	Final assay concentration	Final DMSO concentration
Negative control	12 ml	----	60 µl	0.5% DMSO	----	0.25%
Positive control	12 ml	9.6 µl 250 µM 17β-estradiol	50.4 µl	200 nM 17β-estradiol	100 nM 17β-estradiol	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 20 hours in a 37°C, 5% CO₂, 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 4°C for up to 3 days in the dark.

Assay protocol - antagonist mode

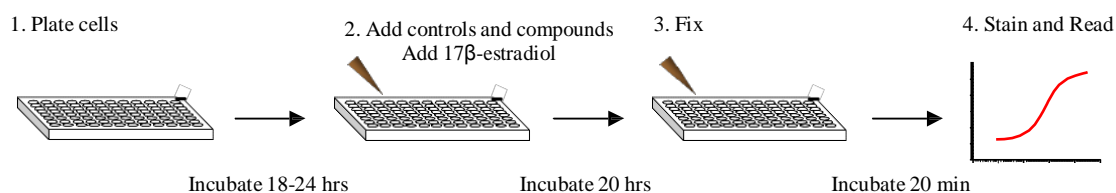


Figure 3: Quick assay workflow overview.

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 20-37°C.

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	12 ml	----	120 µl	1% DMSO	----	0.25%
Positive control	12 ml	1.92 µl ICI 182,780	118 µl	4 µM ICI 182,780	1 µM ICI 182,780	0.25%

3. Prepare 4X 17β-estradiol Agonist Solution (20 nM):

- Prepare fresh in 2 steps:
 - 25 µM 17β-estradiol is prepared by diluting the 25 mM 17β-estradiol Control Stock Solution 1:1000 in DMSO.
 - 20 nM 17β-estradiol Agonist Solution is prepared by diluting 4.8 µl 25 µM 17β-estradiol with 6 ml Assay Buffer. Use the 17β-estradiol Agonist Solution within 20 min after preparation

4. Add 50 µl 4X concentrated control or compound solution in Assay Buffer to appropriate wells of the cell plate.

5. Add 50 µl 4X 17β-estradiol Agonist Solution to all wells of the cell plate.

6. Incubate cell plate for 20 hours in a 37°C, 5% CO₂, 95% humidity incubator.

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

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

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

10. Decant PBS from last wash and add 100 µl 1 µM 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 EGFP-ER β 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 20x objective or higher magnification.

The primary output in the ER β Redistribution[®] assay is the formation of spots in the nucleus. The data analysis should therefore report an output that corresponds to number, area, or intensity of spots in the nucleus.

Imaging on Thermo Scientific Arrayscan HCS Reader

This assay has been developed on the Thermo Scientific Arrayscan HCS Reader using a 20x objective (0.63X coupler), 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 150 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:

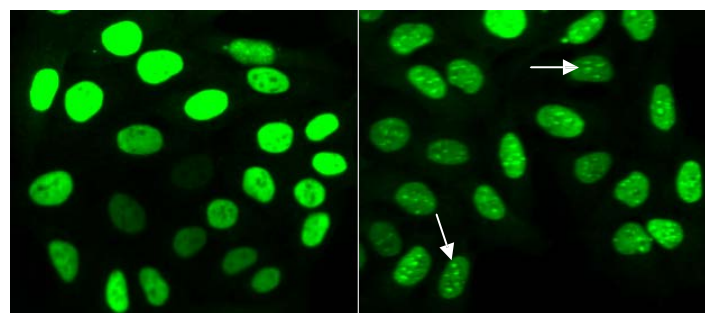
Nucleus size, shape, intensity:	Parameter used to identify DNA damage, effects on cell cycle and apoptosis.
Cell number, size, and shape:	Parameter for acute cytotoxicity and apoptosis.
Cell fluorescence intensity:	Parameter for compound cytotoxicity and fluorescence.

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 ER β Redistribution[®] assay monitors nuclear foci formation of EGFP-ER β . 17 β -estradiol is used as a reference ligand, and compounds are assayed for their ability to induce translocation of EGFP-ER β to nuclear foci. In antagonist format, compounds are tested for their ability to inhibit estradiol-induced nuclear foci formation. ICI 182,780 is used as a reference antagonist.

Representative images of ER β Redistribution[®] cells treated with 17 β -estradiol are shown in figure 4.



DMSO-treated cells

17 β -estradiol-treated cells

Figure 4. Nuclear foci formation of EGFP-ER β . Cells were treated with 100 nM 17 β -estradiol for 20 hrs. Arrows indicate the nuclear foci containing EGFP-ER β that are detected by the image analysis algorithm.

Figure 5 shows a representative concentration response curve of the reference compound 17 β -estradiol in the ER β agonist assay and the reference compound ICI 182,780 in the ER β antagonist assay. The EC₅₀ of 17 β -estradiol is ~0.6 nM and the EC₅₀ of ICI 182,780 is ~15 nM.

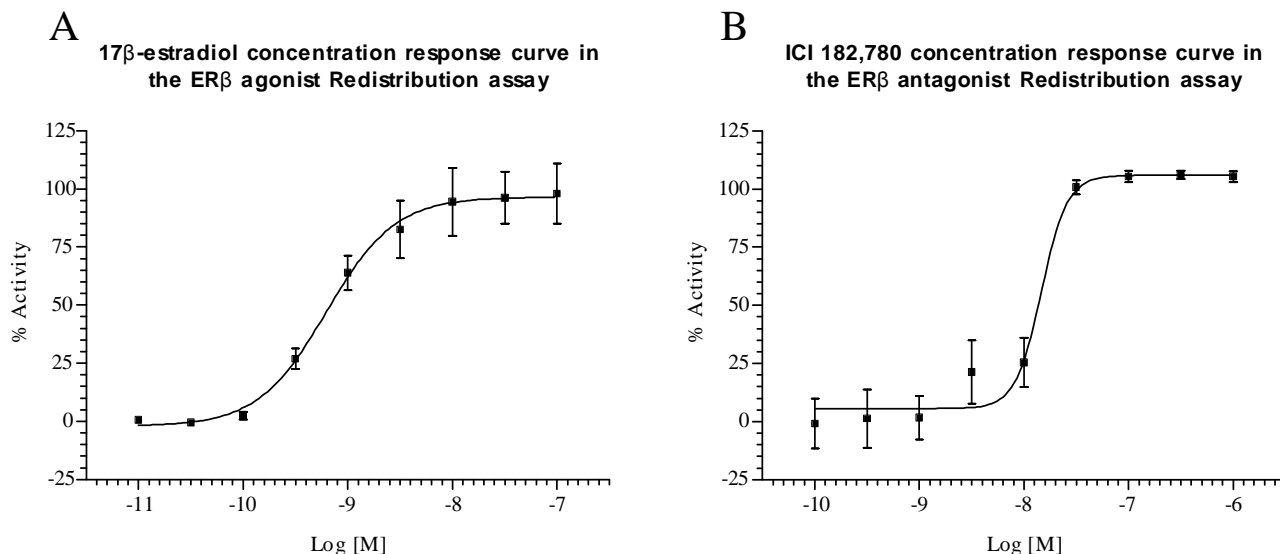


Figure 5. Concentration response curves in the ER β assay: A) 17 β -estradiol concentration response in the ER β agonist assay (n=16). The EC₅₀ is approximately 0.6 nM. Concentration response was measured in 9 point half log dilution series. Cells were treated with 17 β -estradiol for 20 hr. Cells were then fixed and nuclear foci formation was measured using the Cellomics ArrayScan V^{II} Reader and the SpotDetectorV3 BioApplication. % activity was calculated relative to the positive (100 nM 17 β -estradiol) and negative control (0.25% DMSO). B) ICI 182,780 concentration response in the ER β antagonist assay (n=16). The EC₅₀ is approximately 15 nM. Cells were treated with 17 β -estradiol in the presence of a half log dilution series of ICI 182,780 for 20 hr. Cells were then fixed and nuclear foci formation was measured using the IN Cell Analyzer 3000 (GE Healthcare). % activity was calculated relative to the positive (1 μ M ICI 182,780) and negative control (0.25% DMSO)

Product qualification

Assay performance has been validated with an average Z' \pm 0.55 \pm 0.12 (agonist format), Z' \pm 0.38 \pm 0.06 (antagonist format). 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
R04-043-01	Profiling & Screening	AR Redistribution [®] Assay	U2OS
R04-060-01	Profiling & Screening	VDR Redistribution [®] Assay	U2OS
R04-063-01	Profiling & Screening	ER β Redistribution [®] Assay	U2OS
R04-014-01	Profiling & Screening	GR Redistribution [®] Assay	U2OS
R04-064-01	Profiling & Screening	PR Redistribution [®] Assay	U2OS
R04-077-01	Profiling & Screening	PXR Redistribution [®] Assay	U2OS
R04-062-01	Profiling & Screening	REV Redistribution [®] Assay	U2OS

References

1. Ellis R Levin. Molecular Endocrinology. 19(8):1951-1959, 2005.
2. Wilson S V. et al. Toxicological Sciences. (81):69-77, 2004.

Licensing Statement

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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 ER β Redistribution cell line is genetically modified with a vector expressing ER β 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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