

5-LOX Redistribution[®] Assay

For High-Content Analysis

083-01.03

Number	Description
R04-083-01	Recombinant CHO _h IR cells stably expressing human 5-Lipoxygenase (5-LOX) (GenBank Acc. NM_000698) fused to the N-terminus of enhanced green fluorescent protein (EGFP). CHO _h IR cells are adherent epithelial cells derived from Chinese hamster ovary expressing human insulin receptor. Expression of 5-LOX-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.

5-Lipoxygenase Redistribution[®] Assay

5-Lipoxygenase (5-LOX) is a lipid-peroxidizing enzyme that plays an essential role in the biosynthesis of leukotrienes, which mediate inflammatory and allergic reactions. The key regulatory steps in leukotriene biosynthesis following cell activation include Ca^{2+} mobilization and subsequent release of arachidonic acid (AA) from membrane phospholipids by phospholipase A2. In a Ca^{2+} - and ATP-dependent reaction, AA is then metabolized by 5-LOX to yield the epoxide intermediate leukotriene A4 (LTA4). This step is dependent upon the interaction of 5-LOX with the nuclear membrane protein 5-lipoxygenase activating protein (FLAP) and requires translocation of 5-LOX to the nuclear envelope. FLAP then presents AA to 5-LOX and thereby increases the catalytic potential of 5-LOX. In addition to being activated by an increase in intracellular Ca^{2+} concentration, 5-LOX can be activated by diacylglycerols as well as by phosphorylation by MAPKAP kinase-2 and ERK.

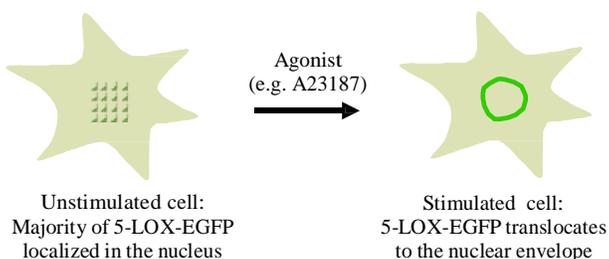


Figure 1: Illustration of the 5-LOX translocation event.

In addition to the classical application of leukotriene synthesis inhibitors in asthma and allergic disorders, leukotriene synthesis inhibitors might be of value for the treatment of cardiovascular diseases and osteoporosis. Moreover, dual 5-LOX/COX inhibitors are potential new drugs to treat inflammation, since they act by blocking the formation of both prostaglandins and leukotrienes. [1-4].

Figure 1 illustrates the translocation of 5-LOX upon agonist stimulation. The 5-LOX Redistribution® assay is designed to screen for agonists of 5-LOX by monitoring the translocation of 5-LOX to the nuclear envelope. The ionophore A23187 (also known as calicimycin) is used as reference compound in the assay, and compounds are assayed for their ability to induce translocation of 5-LOX to the nuclear envelope.

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)
- A23187, Free Acid, Streptomyces chartreusensis (EMD Chemicals, cat.#100105)
- 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.
- Assay Buffer: Ham's F12 with L-Glutamine, 1% Penicillin-Streptomycin and 1% FBS.
- Control Compound Stock: 100 mM A23187 stock solution in DMSO. Prepare by dissolving 10 mg A23187 (MW = 523.6) in 191 μ l DMSO. Store at -20°C.
- Control Compound Working Solution: 1 mM A23187 Working Solution in DMSO. Prepare by diluting the 100 mM A23187 Control Compound Solution 1:100 in DMSO. Store at -20°C.
- 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. 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% CO₂, 95% humidity incubator.
4. Change the Cell Culture Medium the next day.

Cell harvest and culturing procedure

For normal cell line maintenance, split 1:12 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% 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 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 100,000 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 10,000 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% CO₂, 95% humidity incubator prior to starting the assay.

Assay protocol

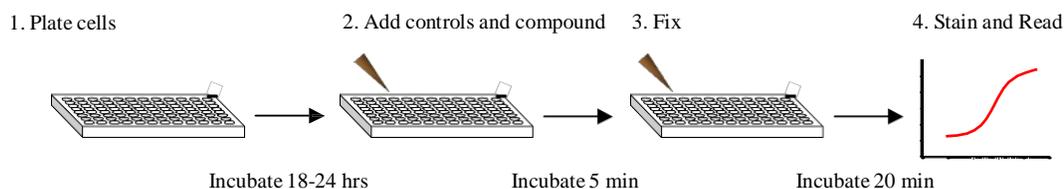


Figure 2: Quick assay workflow overview.

The following protocol is based on 1x 96-well plate.

1. Before initiating the assay:

- Prepare Assay Buffer. 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 1% 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	7.2 µl 1 mM A23187	52.8 µl	600 nM A23187	300 nM A23187	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 5 minutes 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.

Imaging

The translocation of 5-LOX-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 20x objective or higher magnification.

The primary output in the 5-LOX Redistribution[®] assay is the translocation from the nucleus to the nuclear envelope of 5-LOX-EGFP. The data analysis should therefore report an output relating to the GFP fluorescence intensities in the nucleus and the nuclear envelope.

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 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 400 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 5-LOX Redistribution[®] assay monitors the translocation of 5-LOX-EGFP to the nuclear envelope. A23187 is used as reference compound.

Representative images of 5-LOX Redistribution[®] cells treated with A23187 are shown in Figure 3.

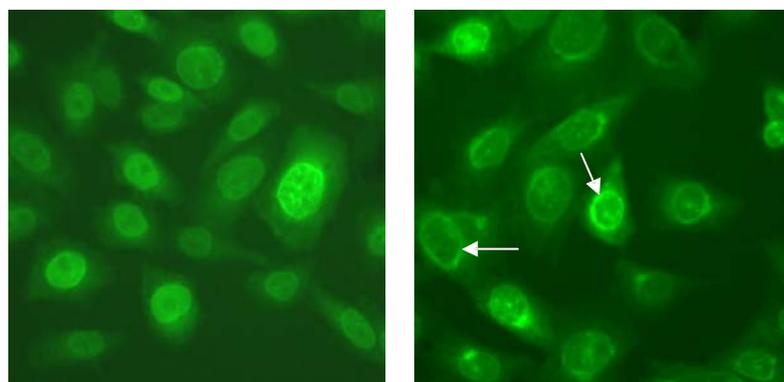


Figure 3. Translocation of 5-LOX-EGFP to the nuclear envelope. Cells were treated with 300 nM A23187 for 5 min. Arrows indicate the nuclear envelope localization detected by the image analysis algorithm.

DMSO-treated cells

A23187-treated cells

Figure 4 shows representative concentration response curves of A23187 and ionomycin in the 5-LOX Redistribution[®] assay. The EC₅₀ of A23187 is ~35 nM, and the EC₅₀ of ionomycin is ~30 nM.

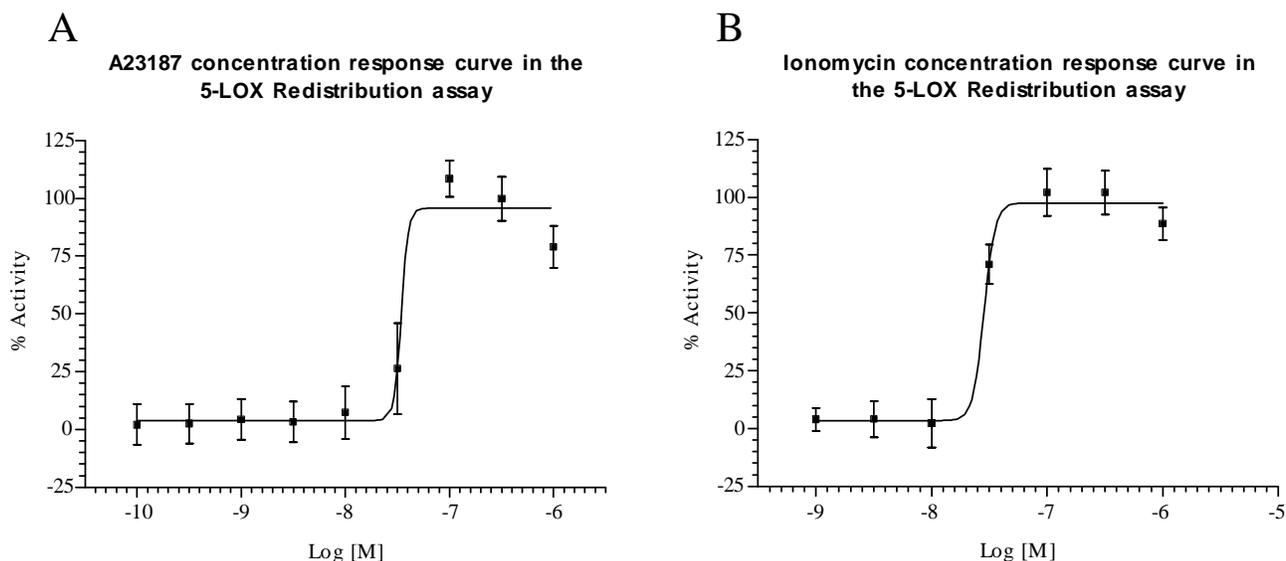


Figure 5. Concentration response curves in the 5-LOX assay: A) A23187 concentration response. The EC₅₀ is approximately 35 nM. Concentration response was measured in 9 point half log dilution series (n=16). Cells were treated with A23187 for 5 min. Cells were then fixed and translocation to the nuclear envelope was measured using the Cellomics ArrayScan V^{TI} Reader and the Redistribution V3 BioApplication. % activity was calculated relative to the positive (300 nM A23187) and negative control (0.25% DMSO). B) Ionomycin concentration response. The EC₅₀ is approximately 30 nM. Concentration response was measured in 7 point half log dilution series (n=16). Cell treatment and image analysis was performed as in A).

Product qualification

Assay performance has been validated with an average $Z' = 0.54 \pm 0.07$. The cells have been tested for viability. The cells have been tested negative for mycoplasma.

Related Products

Product #	Type	Product description	Cell line
R04-099-01	Profiling & Screening	PKC ϵ Redistribution [®] Assay	U2OS
R04-098-01	Profiling & Screening	PKC β Redistribution [®] Assay	U2OS
R04-096-01	Profiling & Screening	MARCKS Redistribution [®] Assay	U2OS
R04-017-02	Profiling & Screening	Gq-coupled GPCRs – NFATc1 Redistribution [®] Assay	U2OS
R04-045-02	Profiling & Screening	Gs/Gi-coupled GPCRs – PKA Redistribution [®] Assay	CHO-K1

References

1. Peters-Golden M. Am J Respir Crit Care Med., 157, S227-S231, 1998
2. Peters-Golden M & Brock T, Am J Respir Crit Care Med., 161, S36-S40, 2000
3. Flamand N et al. J Biol Chem., 281, 129-136, 2006.
4. Radmark O & Samuelsson B, Biochem Biophys Res Commun., 338, 102-110, 2005

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For European customers:

The 5-LOX Redistribution cell line is genetically modified with a vector expressing 5-LOX 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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