INSTRUCTIONS

M2 Redistribution® Assay

For High-Content Analysis

075-01.03

Number	Description
R04-075-01	Recombinant U2OS cells stably expressing human Muscarinic acetylcholine receptor 2 (M2) (GenBank Acc. NM_001006630) fused to the N-terminus of enhanced green fluorescent protein (EGFP). U2OS cells are adherent epithelial cells derived from human osteosarcoma. Expression of M2-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.

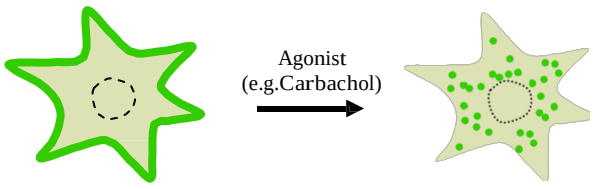
Introduction

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.

Muscarinic Acetylcholine Receptor 2 Redistribution Assay

Muscarinic acetylcholine receptors mediate their effect by exerting metabotropic actions of acetylcholine in the nervous system. The receptors belong to the G-protein-coupled receptor (GPCR) superfamily that transduces signals through multiple intracellular signaling cascades to regulate a wide variety of physiological responses. At present, five receptor subtypes have been identified, where the odd-numbered receptors (M1, M3, M5) preferentially couple to $G\alpha_q$ and activate phospholipase C, thereby initiating the phosphatidylinositol trisphosphate (PIP3) cascade leading to intracellular Ca^{2+} mobilization and activation of protein kinase C. The even-numbered receptors M2 and M4 couple to $G\alpha_{i/o}$ and inhibit adenylate cyclase activity.



Unstimulated cell: Majority of M2-EGFP localized in the plasma membrane

Stimulated cell: Majority of M2-EGFP internalized in endosomes

Figure 1: Illustration of the M2 translocation event.

Drugs targeting muscarinic acetylcholine receptors are currently used in the treatment of chronic obstructive pulmonary disorder and overactive bladder, but these receptors also represent potential therapeutic targets for cognitive disorders such as Alzheimer's disease and schizophrenia. Selective M1 agonists or mixed M1 agonists/M2 antagonists may provide an approach to the treatment of cognitive disorders, while M3 antagonism or mixed M2/M3 antagonists are approved for the treatment of contractility disorders including overactive bladder and chronic obstructive pulmonary disease [1-3].

Figure 1 illustrates the translocation of M2 upon agonist stimulation of the receptor. The M2 assay is designed to screen for agonists causing internalization of M2. Carbachol is used as reference compound in the assay and ligands/compounds are assayed for their ability to induce M2 internalization by a spot detecting image analysis algorithm.

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)
- Carbamoylcholine chloride (Carbachol) (Sigma-Aldrich, cat.# C4382)
- 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 1% FBS.
- Control Compound Stock: 400 mM Carbachol stock solution in purified water. Prepare by dissolving 100 mg Carbachol (MW = 182.65) in 1369 µl purified water. 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 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 26, 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

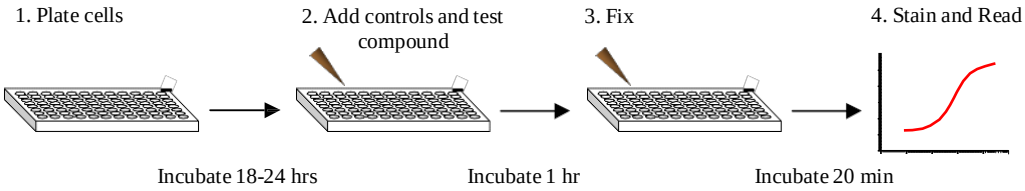


Figure 2: Quick assay workflow overview.

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 1% 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	60 µl Carbachol	60 µl	2 mM Carbachol	1 mM Carbachol	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 1 hour 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 M2-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 M2 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 700 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.

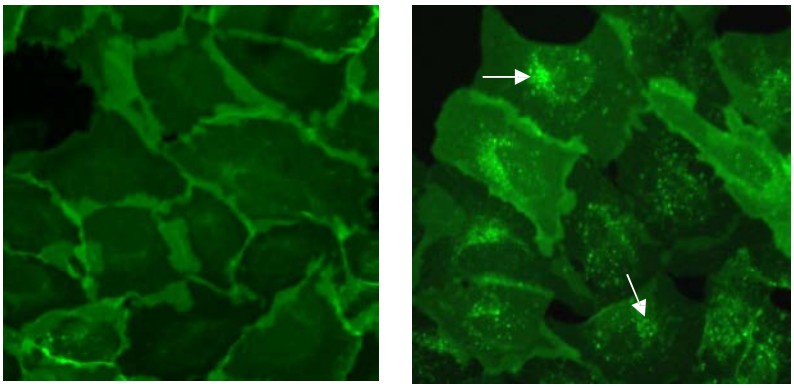
Examples of useful secondary high content outputs:

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 M2 Redistribution® Assay monitors internalization of M2-EGFP. Carbachol is used as the reference compound. Example images are shown in Figure 3.



DMSO-treated cells Carbachol -treated cells

Figure 3. Internalization of M2-EGFP stimulated with carbachol. Cells were treated with 1 mM carbachol for 1 hr. Arrows indicate the M2-EGFP internalization that is detected by the image analysis algorithm.

Figure 4 shows a concentration response curve of the reference ligand carbachol in the M2 Redistribution® assay. The EC_{50} of carbachol is approximately 40 µM.

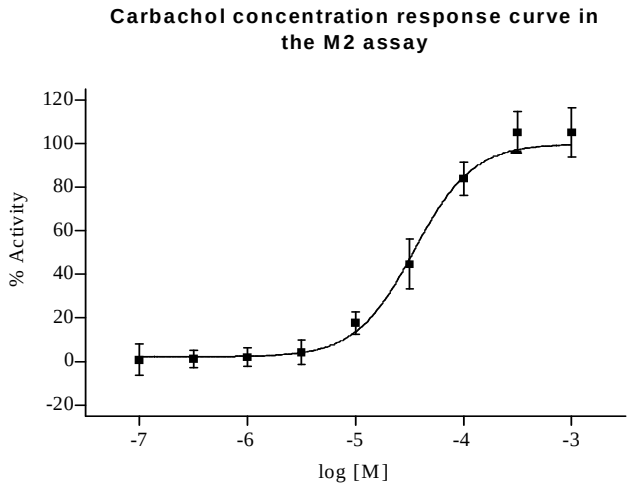


Figure 4. Carbachol concentration response in the M2 assay. Concentration response was measured in 9 point half log dilution series (n=16). The EC_{50} of carbachol is ~40 µM. Cells were incubated with carbachol for 1 hr. Cells were then fixed and internalization was measured using the Cellomics ArrayScan V^{TI} Reader and the SpotDetectorV3 BioApplication. % activity was calculated relative to the positive (1 mM carbachol) and negative control (0.25% DMSO).

Product qualification

Assay performance has been validated with an average Z'=0.55±0.13. 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
R04-067-01	Profiling	CXCR4 Redistribution® Assay	U2OS
R04-094-01	Profiling & Screening	GRPR Redistribution® Assay	U2OS
R04-054-01	Profiling	MCH1 Redistribution® Assay	U2OS
R04-039-01	Profiling & Screening	$S1P_1$ Redistribution® Assay	U2OS
R04-095-01	Profiling	$S1P_3$ Redistribution® Assay	U2OS
R04-086-01	Profiling	M1 Redistribution® Assay	U2OS
R04-076-01	Profiling & Screening	M3 Redistribution® Assay	U2OS
R04-057-01	Profiling	MC4 Redistribution® Assay	U2OS
R04-053-01	Profiling	FSHR Redistribution® Assay	U2OS
R04-093-01	Profiling & Screening	CRTH2 Redistribution® Assay	U2OS
R04-051-01	Profiling & Screening	CB1 Redistribution® Assay	U2OS
R04-061-01	Profiling & Screening	CB2 Redistribution® Assay	U2OS
R04-097-02	Profiling & Screening	GLP1R 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
R04-088-01	Profiling & Screening	M1:NFATc1 Redistribution® Assay	U2OS
R04-072-01	Profiling & Screening	M2:PKA Redistribution® Assay	CHO-K1
R04-073-01	Profiling & Screening	M3:NFATc1 Redistribution® Assay	U2OS

References

1. Ishii et al. Curr. Pharm. Des.; 12, 3573-81, 2006.
2. Krejci A et al. Physiol Res.; 53, S131-40, 2004.
3. Eglen RM Auton Autacoid Pharmacol.; 26, 219-33, 2006.

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For European customers:
The M2 Redistribution cell line is genetically modified with a vector expressing M2 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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