INSTRUCTIONS

NFκB Redistribution® Assay

For High-Content Analysis

091-01.03

Number	Description
R04-091-01	Recombinant U2OS cells stably expressing human NFκB (GenBank Acc. NM_021975) fused to the N-terminus of enhanced green fluorescent protein (EGFP). U2OS cells are adherent epithelial cells derived from human osteosarcoma. Expression of NFκB-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 NFκB p65 Redistribution® Assay

Nuclear factor-κB (NFκB) is a nuclear transcription factor which regulates the expression of a large number of genes critical for several processes, including apoptosis, viral replication, tumorigenesis, inflammation, and various autoimmune diseases. Activation of NFκB is part of a stress response, and it is activated by growth factors, cytokines, lymphokines, UV light, pharmacological agents, and stress. Five mammalian NF-kB family members are identified (p50, p52, p65, RelB and c-Rel). The transcription factor NF-κB works only when two members form a dimer. The most abundant form consists of a p50 or p52 subunit and a p65 subunit. In its inactive form, NFκB is located in the cytoplasm, bound by members of the IκB family of inhibitor proteins. Stimuli such as interleukin-1β or TNFα cause phosphorylation of IκB, which leads to its ubiquitination and subsequent degradation of the inhibitor protein. This results in nuclear translocation of NFκB and increased NFκB-mediated gene expression. NFκB nuclear translocation can be inhibited by the IκBα specific inhibitor RO 106-9920, which inhibits ubiquitination and degradation of IκBα and subsequent nuclear import of NFκB [1,2].

Pierce Biotechnology
3747 N. Meridian Road
PO Box 117
Rockford, IL 61105 USA
(815) 968-0747
(815) 968-7316 fax
www.thermoscientific.com/cellomics

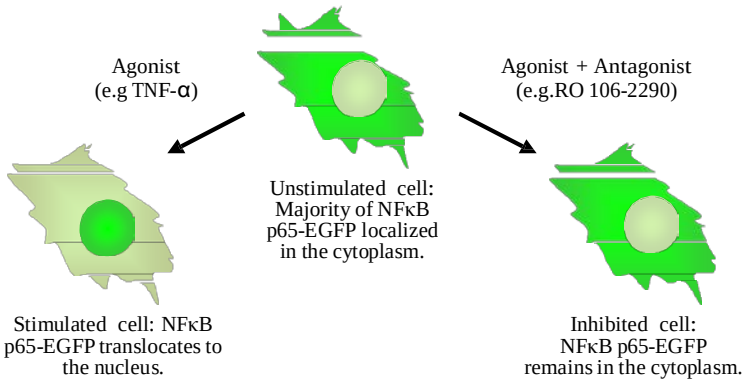


Figure 1: Illustration of the NFκB translocation event.

The NFκB Redistribution® assay is designed to measure NFκB activation by monitoring the translocation of the NFκB p65-EGFP fusion protein from the cytoplasm to the nucleus. The NFκB cell line is responsive to both interleukin 1 (IL-1) and to tumor necrosis factor alpha (TNFα). The assay has been optimized and validated in agonist format using TNFα as agonist.

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
- Recombinant Human Tumor Necrosis Factor-α (TNF-α) (Invitrogen, cat.# PHC3015)
- 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
- Cell Wash Bufer: DMEM supplemented with 2mM L-Glutamine, 1% Penicillin-Streptomycin and 1% FBS
- Assay Buffer: DMEM supplemented with 2mM L-Glutamine and 1% Penicillin-Streptomycin
- 10% BSA: 1 g BSA dissolved in purified water to a final volume of 10 ml
- Control Compound Stock: 10 µg/ml (571 nM) TNF-α stock solution in PBS containing 0.1% BSA. Prepare by dissolving 10 µg TNF-α (MW = 17.5 kDa) in 1 ml PBS containing 0.1% BSA. 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

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.
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 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 30,000 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 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 37ºC, 5% CO_2, 95% humidity incubator prior to starting the assay

Assay protocol

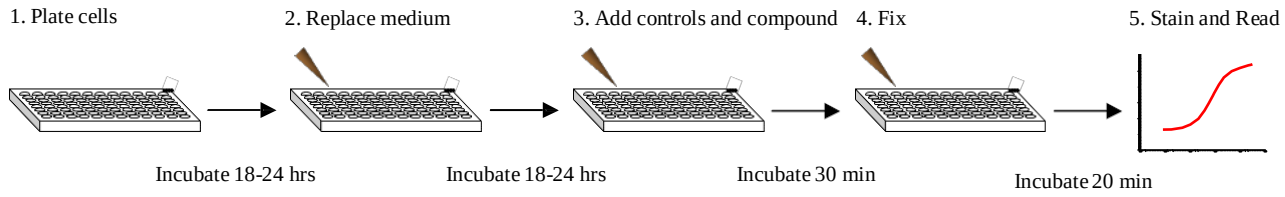


Figure 2: Quick assay workflow overview

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.

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

1. Before initiating the assay:
    - Prepare Cell Wash Buffer. Ensure Cell Wash Buffer is pre-warmed to 20-37ºC.
2. Gently remove Plate Seeding Medium and wash cell plate twice with 100 µl Cell Wash Buffer per well.
3. Add 180 µl Cell Wash Buffer per well.
4. Incubate cell plate for 18-24 hours in a 37ºC, 5% CO_2, 95% humidity incubator.
5. Prepare controls and test compounds
    - Dilute controls and test compounds in Assay Buffer to a 10X final concentration. (Volumes and concentrations are indicated below). A final DMSO concentration of 0.25% is recommended, but the assay can tolerate up to 0.5% DMSO final concentration.
    - Mix controls for 1x 96–well plate as indicated below:

	Assay Buffer	Control Stock	DMSO	10X concentration	Final assay concentration	Final DMSO concentration
Negative control	3 ml	----	75 µl	2.5% DMSO	----	0.25%
Positive control	3 ml	30 µl TNF-α	75 µl	100 ng/ml TNF-α	10 ng/ml TNF-α	0.25%

6. Add 20 µl 10X concentrated control or compound solution in Assay Buffer 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 4ºC for up to 3 days in the dark.

Imaging

The translocation of NFκB p65-GFP 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 NFκB Redistribution® assay is the translocation of NFκB p65-GFP from the cytoplasm to the nuleus. The data analysis should therefore report an output relating to the GFP fluorescence intensities in the nucleus and 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), 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 700 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.

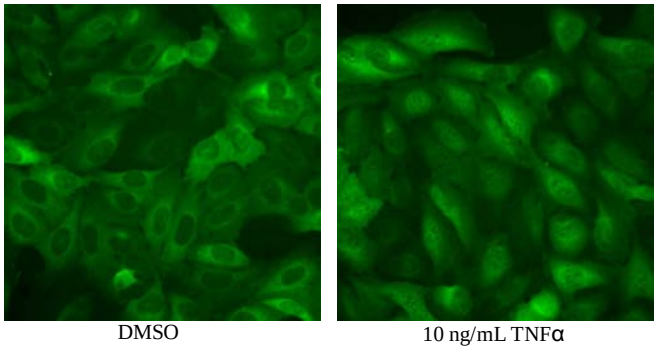
Examples of useful secondary high content outputs:

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 NFκB Redistribution® Assay monitors NFκB translocation from the cytoplasm to the nucleus induced by TNFα or IL-1. Representative images of NFκB Redistribution cells treated in the absence or presence of 10 ng/ml TNFα are shown in figure 3. Concentration response curves of TNFα and IL-1 are shown in figure 4 and a concentration response curve of the inhibitor RO106-2290 is shown in figure 5. The EC_{50} value of TNFα in the assay is approximately 0.7 ng/ml.



DMSO 10 ng/mL TNFα

Figure 3. Translocation of NFκB -EGFP in response to TNFα. Cells were treated with 0.25% DMSO or 10 ng/ml TNFα. The cytoplasm to nucleus translocation is detected by the image analysis algorithm.

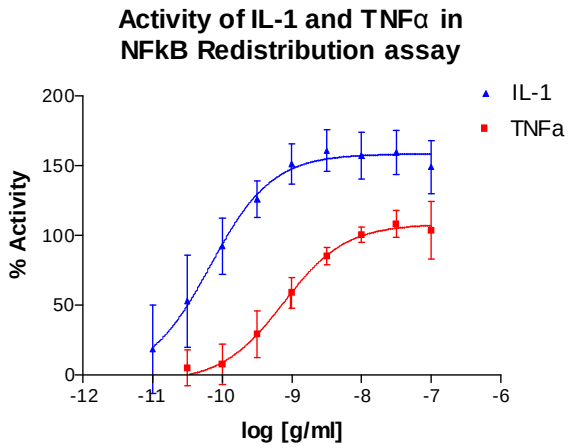


Figure 4. IL-1 and TNFα concentration response curves in the NFκB Redistribution® assay. Concentration response was measured in 9 point half log dilution series of IL-1 or TNFα. Cells were incubated with test samples for 30 min. Cells were then fixed and the nucleus to cytoplasm translocation was measured using the Cellomics ArrayScan V^{TI} Reader and the Redistribution V3 BioApplication. % activity was calculated relative to the positive (10 ng/ml TNFα) and negative control (0.25% DMSO). The EC_{50} of IL-1 is ~0.07 ng/ml and the EC_{50} of TNFα is ~0.7 ng/ml.

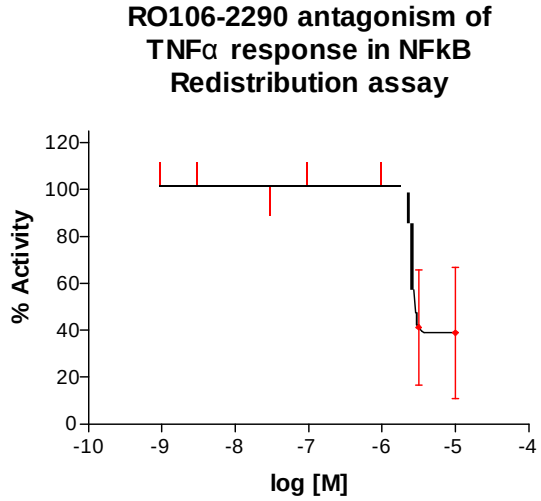


Figure 5. RO106-2290 concentration response curve in the NFκB Redistribution® assay (antagonist format). Concentration response was measured in 9 point half log dilution series of RO106-2290. Cells were incubated with RO106-2290 for 30 min in the presence of 5 ng/ml TNFα. Cells were then fixed and the nucleus to cytoplasm translocation was measured using the Cellomics ArrayScan V^{TI} Reader and the RedistributionV3 BioApplication. % activity was calculated relative to the positive (10 ng/ml TNFα) and negative control (0.25% DMSO). The EC_{50} of RO106-2290 is ~2.5 µM.

Product qualification

Assay performance has been validated with an average Z'=0.50±0.12. 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
R04-037-01	Profiling and Screening	MK2 Redistribution® Assay	U2OS
R04-038-01	Profiling and Screening	MK2EE Redistribution® Assay	U2OS

References

1. Swinney DC et al., J Biol Chem 277, 23573-23581, 2002.
2. Schmid JA et al., J Biol Chem 275, 17035-17042, 2000.

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For European customers:
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Pierce Biotechnology 3747 N. Meridian Road | PO Box 117 Rockford, IL 61105 USA | (815) 968-0747 (815) 968-7316 fax | www.thermoscientific.com/cellomics