# INSTRUCTIONS
# NFκB p65 Redistribution® Assay

For High-Content Analysis

002-01.04

| Number | Description |
|---|---|
| R04-002-01 | Recombinant CHOhIR cells stably expressing human NKκB receptor (GenBank Acc. NM_021975) fused to the N-terminus of green fluorescent protein variant 3FP. Human TNFαR1 (GenBank Acc. NM_001065) is co-expressed in the cell line. CHOhIR cells are adherent epithelial cells derived from Chinese hamster ovary expressing human insulin receptor. Expression of NKκB-3FP 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 p65 and increased NFκB-mediated gene expression. NFκB p65 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 p65 [1,2].

The NFκB p65 Redistribution® assay is designed to identify antagonists of NFκB activation by monitoring the translocation of an NFκB p65-GFP fusion protein from the cytoplasm to the nucleus. In addition, the NFκB p65-GFP cell line is co-transfected with the human tumor necrosis factor receptor 1 (TNFR1). Interleukin-1β is used as the reference agonist and test compounds are assayed for their ability to inhibit interleukin-1β-induced cytoplasm-to-nucleus translocation of NFκB p65. RO 106-9920 is used as reference antagonist. Compounds which inhibit interleukin-1β-induced translocation possibly interfere directly with NFκB p65 translocation, act upstream of NFκB p65, or may be general nuclear import inhibitors/activators of nuclear export.

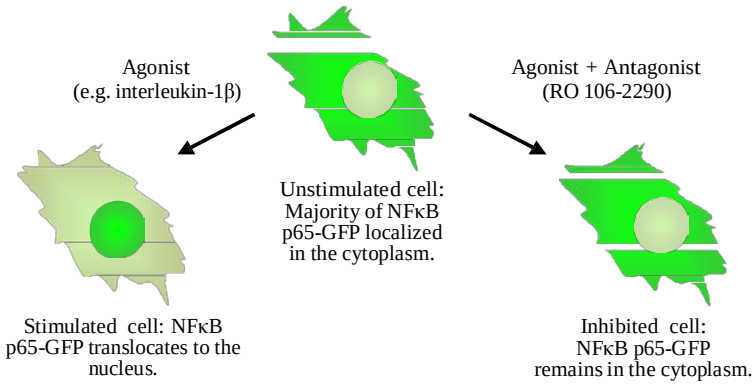



Figure 1: Illustration of the NFκB p65 translocation event.

## 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)
- Blasticidin (Fisher Scientific, cat.# BP2647)
- 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)
- Hepes Buffer, 1 M, Free Acid (liquid) (Thermo Scientific, Fisher Scientific cat.# SH30237)
- Fibronectin (BD Biosciences, Fisher Scientific cat.# CB-40008)
- Bovine Serum Albumin (BSA) Cohn Fraction V (MP Biomedicals, cat.# ICN841032)
- Interleukin-1β (IL-1β), Human Recombinant, E.Coli (EMD Chemicals, cat.# 407615)
- Ro106-9920 (EMD Chemicals cat.# 557550)
- 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 supplemented with 1% Penicillin-Streptomycin, 0.5 mg/ml G418, 5 µg/ml Blasticidin and 10% FBS.
- Cell Freezing Medium: 90% Cell Culture Medium without G418 and Blasticidin + 10% DMSO.
- Plate Seeding Medium: Ham's F12 supplemented with 1% Penicillin-Streptomycin, 0.5 mg/ml G418, 5 µg/ml Blasticidin and 10% FBS.
- Fibronectin Working Solution: 10 µg/ml Fibronectin in PBS. Prepare by dissolving the 1 mg/ml stock solution 100X in PBS.
- 10% BSA: 1 g BSA dissolved in purified water to a final volume of 10 ml.
- Assay Buffer: Ham's F12 supplemented with 1% Penicillin-Streptomycin, 10 mM Hepes and 0.1% FBS.
- Control Compound Stock: 100 mM Ro106-9920 stock solution in DMSO. Prepare by dissolving 5 mg Ro106-9920 (MW = 245.3) in 204 µl DMSO. Store at -20°C.
- IL-1β Agonist Stock: 10 µg/ml IL-1β stock solution in PBS containing 0.1% BSA. Prepare by dissolving 5 µg IL-1β in 495 µl PBS + 5 µl 10% BSA. Store at -70°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. 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 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 1:12 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 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 fibronectin coated 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. Add 100 µl Fibronectin Working Solution to each well in a 96-well tissue culture plate (cell plate) and incubate for 1 hour at room temperature. Remove remaining material from the wells by washing 2X with PBS (100 µl/well). Decant PBS and allow the plates to dry without lids. The plates can be used immediately or can be stored at 4 °C.
2. Harvest the cells as described in the “Cell harvest and culturing procedure”, step 1-5 using Plate Seeding Medium instead of Cell Culture Medium.
3. Dilute the cell suspension to 40,000 cells/ml in Plate Seeding Medium.
4. Transfer 200 µl of the cell suspension to each well in the 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.
5. 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.
6. Incubate the cell plate for 18-24 hours in a 37ºC, 5% $CO_2$, 95% humidity incubator prior to starting the assay.

## Assay protocol

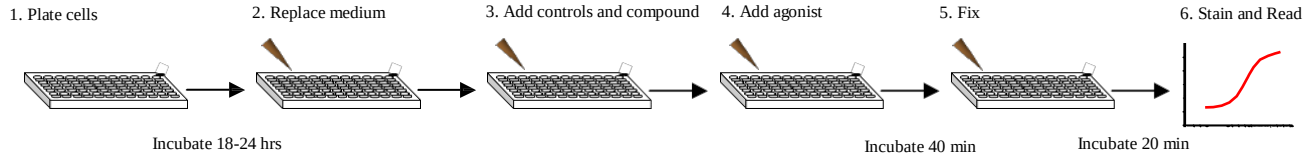



Figure 2: Quick assay workflow overview

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 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 | 24 µl Ro106-9920 | 96 µl | 200 µM Ro106-9920 | 50 µM Ro106-9920 | 0.25% |

3. Prepare 4X IL-1β Agonist Solution (20 ng/ml):
    - Prepare fresh by mixing 12 µl 10 µg/ml IL-1β Agonist Stock with 6 ml Assay Buffer. Use the IL-1β Agonist Solution within 20 min after preparation.
4. Gently remove Plate Seeding Medium and wash cell plate 3 times with 100 µl Assay Buffer per well.
5. Add 100 µl Assay Buffer per well.
6. Add 50 µl 4X concentrated control or compound solution in Assay Buffer to appropriate wells of the cell plate.
7. Add 50 µl 4X IL-1β Agonist Solution to appropriate wells of the cell plate.
8. Incubate cell plate for 40 minutes in a 37ºC, 5% $CO_2$, 95% humidity incubator.
9. Fix cells by gently decanting the buffer and add 150 µl Fixing Solution per well.
10. Incubate cell plate at room temperature for 20 minutes.
11. Wash the cells 4 times with 200 µl PBS per well per wash.
12. Decant PBS from last wash and add 100 µl 1 µM Hoechst Staining Solution.
13. 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 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 p65 Redistribution® assay is the translocation of NFκB p65 from nucleus to cytoplasm. 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 200 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.

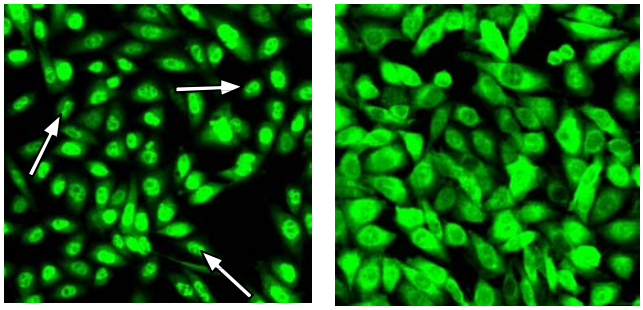
Examples of useful secondary high content outputs:

| | |
|---|---|
| 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_CHO Redistribution® Assay monitors inhibition of IL-1-induced NFκB translocation. Representative images of the NFκB Redistribution® Assay are shown in Figure 3. Figure 4 shows a concentration response curve of the reference compound RO106-9920 (an IκBα specific inhibitor) in the NFκB assay. The $EC_{50}$ value of RO106-9920 is approximately 5 µM in the assay.



5 ng/ml IL-1
DMSO-treated cells

5 ng/ml IL-1
RO106-9920-treated cells

Figure 3. Translocation of NFκB-3FP stimulated with IL-1 in response to RO106-9920. Cells were treated with 5 ng/mL IL-1 in the absence or presence of 50 µM RO106-9920. Arrows indicate IL-1-mediated nuclear translocation detected by the image analysis algorithm.

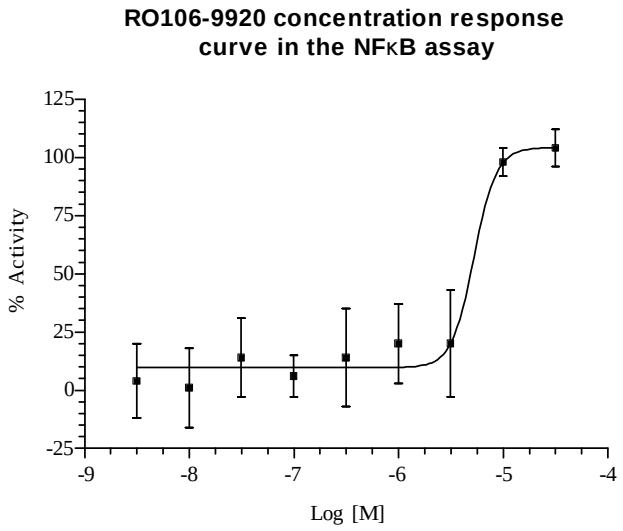



Figure 4: RO106-9920 concentration response curve in the NFκB Redistribution® assay. Concentration response was measured in 9 point half log dilution series of RO106-9920. Cells were then fixed and the nucleus to cytoplasm translocation was measured using the Cellomics ArrayScan V$^{TI}$ Reader and RedistributionV3 BioApplication. % activity was calculated relative to the positive (50 µM RO106-9920) and negative control (0.25% DMSO). The $EC_{50}$ value of RO106-9920 is approximately 5 µM in the assay.

## Product qualification

Assay performance has been validated with an average Z'=0.27±0.21. The cells have been tested for viability. The cells have been tested negative for mycoplasma.

## Related Products

| Product # | Type | Product description | Cell line |
|---|---|---|---|
| R04-037-01 | Profiling and Screening | MK2 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.

## 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 NFκB p65 Redistribution cell line is genetically modified with a vector expressing NFκB p65 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.