INSTRUCTIONS

Thermo SCIENTIFIC

Cellomics® NF-κB Activation HCS Reagent Kits

High-Content Screening Reagents

1797.1

Number	Description
K0100011	**NF-κB Activation Kit,** sufficient materials for 5 × 96 wells
R0105021	**NF-κB Activation Kit,** sufficient materials for 50 × 96 wells

Kit Contents:	K0100011	R0105021
NF-kB primary antibody (rabbit)	120 µl	1.22 ml
DyLight™ 488-Conjugated Goat Anti-Rabbit IgG	75 µl	1 ml
Hoechst Dye	30 µl	165 µl
Wash Buffer (10X)	100 ml	--
Wash Buffer II (10X)	100 ml	--
Permeabilization Buffer (10X)	100 ml	--
Thin Plate Seal Assembly	7/pack	--

Storage: Upon receipt store all kit components at 4°C. Keep vial containing DyLight 488 conjugated Goat Anti-Rabbit IgG protected from light. Allow buffers to warm to room temperature before use. See the **Solution Preparation** section for storage and stability of prepared solutions.

Warning: Please completely read these instructions and the accompanying material safety data sheets before using this product. The Cellomics Reagents are not for diagnostic use in humans or animals.

Introduction

The NFκB Activation HCS Reagent Kit has been optimized for quantification of NFκB activation by directly measuring the spatial translocation of NFκB from the cell cytoplasm to the nucleus. The kit provides a fixed end-point assay based on immunofluorescence detection of NFκB in cells grown on standard high-density microplates. The core reagents supplied include a rabbit primary antibody specific for NFκB and a DyLight 488-Conjugated Secondary Antibody. The cell nucleus is identified by DNA-specific Hoechst Dye, also included in the kit.

Nuclear factor kappa B (NFκB) is a transcription factor that is activated in response to ligands, such as IL-1α and tumor necrosis factor,[1] and is associated with the activation of many cellular defense genes[2]. Normally, NFκB is present in the cytoplasm as a complex with members of the IκB inhibitor family. Both the size of this complex and IκB masking of the nuclear localization sequence on NFκB, prevents NFκB entry into the nucleus. Upon phosphorylation and degradation of IκB, the nuclear localization sequence becomes accessible and NFκB translocates to the nucleus (Figure 1). NFκB must be in the nucleus to induce specific gene expression; therefore, translocation from the cytoplasm to the nucleus is a definitive measure of NFκB activation.

The NFκB Activation HCS reagents, in combination with the ArrayScan HCS Reader and the Cytoplasm to Nucleus Translocation BioApplication software afford automated plate handling, focusing, cell image acquisition, analysis, and quantification of NFκB activation. For a more detailed description of the image processing algorithm, see the Cytoplasm to Nucleus Translocation BioApplication Guide that accompanies the Cytoplasm to Nucleus Translocation BioApplication software.

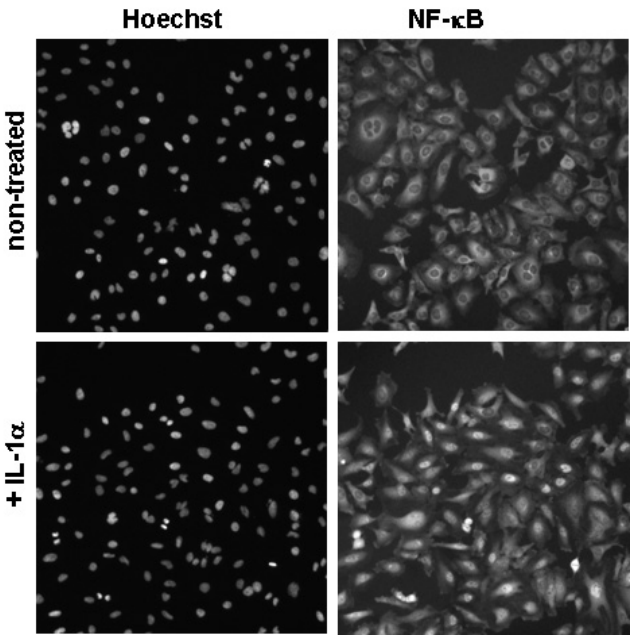

Figure 1. Stained HeLa cells before and after NFκB activation. HeLa cells were stimulated for 30 minutes with 1 ng/ml IL-1α. Non-treated cells are in top panel, while treated cells are in bottom panel.

Additional Materials Required

- IL-1α or other NFκB activator (R&D Systems, Product No. 200-LA)
- Paraformaldehyde (16%) (Thermo Scientific 16% Formaldehyde, Product No. 28906)
- Black, clear-bottom microplates (Packard ViewPlate®, Product No. 6005182)
- Note: For the screening size kit, Wash Buffer, Permeabilization Buffer, and Wash Buffer II are available separately (please contact customer service for more information).

Cell Preparation Information

- Protocol optimized for HeLa cells (American Type Culture Collection, Product No. CCL-2).
- For routine culture of HeLa cells use EMEM complete media (HyClone, Product No. SH30024) supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 1X non-essential amino acids and 1 mM sodium pyruvate.
- Split cells when they reach 70-80% confluency (2-3 times per week) at a dilution of 1:2 to 1:6.
- For NFκB activation, harvest cells with trypsin-versene mixture (BioWhittaker, Product No. 17-161F), dilute into EMEM Complete Medium and determine cell density.
- Adjust cell density to 5×10^4 cells/ml in EMEM Complete Medium and add 100 µl of the cell suspension to each well of a 96-well microplate (= 5,000 cells/well).
- Incubate cells for 18-24 hours at 37°C in 5% CO_2.

NF-κB Activation Kit Protocol

A. Solution Preparation (per 96-well plate)

1X Wash Buffer	Add 20 ml 10X Wash Buffer to 180 ml ultrapure water for a final volume of 200 ml. Store buffer at 4°C for up to 7 days.
1X Permeabilization Buffer	Add 2 ml of 10X Permeabilization buffer to 18 ml of ultrapure water for a final volume of 20 ml. Store buffer at 4°C for up to 7 days.
1X Wash Buffer II	Add 20 ml 10X Wash Buffer II to 180 ml ultrapure water for a final volume of 200 ml. Store buffer at 4°C for up to 7 days.
Fixation Solution	Add 3.0 ml 16 % paraformaldehyde to 9.0 ml 1X Wash Buffer. Warm to 37°C prior to use. Prepare fresh for each assay.
Primary Antibody Solution	Add 24 µl of NF-κB antibody to 6.0 ml of 1X Wash Buffer.
Secondary Antibody Staining Solution	Add 3.0 µl of Hoechst Dye and 12.0 µl of the DyLight 488 Goat Anti-Rabbit to 6.0 ml of 1X Wash Buffer. Prepare solution just before each assay.

B. Procedure

Note: Use 100 µl per well volume unless indicated otherwise. This protocol requires ~3 hours to perform once compound incubation has been completed.

1. Dilute IL-1α to 5 ng/ml in culture medium. Add 25 µl/well and mix thoroughly. Incubate 30 minutes at 37°C. [For an agonist screen, compound would replace stimulator. For an antagonist screen, compound addition should precede addition of stimulator].
2. Aspirate culture medium and add 100 µl of prewarmed Fixation Solution to each well. Incubate in fume hood at room temperature for 10 minutes. Pre-warming fixative is critical to maintaining cell integrity.
3. Aspirate Fixation Solution and wash plate once with 100 µl of 1X Wash Buffer.
4. Aspirate Wash Buffer, add 100 µl of 1X Permeabilization Buffer, and incubate for 10 minutes.
5. Aspirate Permeabilization Buffer and wash plate twice with 100 µl of 1X Wash Buffer.
6. Aspirate Wash Buffer and add 50 µl/well of Primary Antibody Solution. Incubate plate for 1 hour.
7. Aspirate Primary Antibody Solution and add 100 µl of 1X Wash Buffer II. Incubate for 15 minutes.
8. Aspirate Wash Buffer II, then wash twice with 100 µl of 1X Wash Buffer.
9. Aspirate Wash Buffer and add 50 µl/well of Staining Solution. Incubate for 1 hour protected from light.
10. Aspirate Staining Solution and then add 100 µl of 1X Wash Buffer II. Incubate for 10 minutes.
11. Aspirate Detergent Buffer and replace with 100 µl of 1X Wash Buffer.
12. Aspirate Wash Buffer and replace with 200 µl of 1X Wash Buffer.
13. Seal plate and evaluate on ArrayScan HCS Reader.
14. Store sealed plates in the dark at 4°C.

Additional Information

A. Dose-response curve and time-course experiment. Cells were analyzed using the Cytoplasm to Nucleus Translocation BioApplication and an ArrayScan Reader.

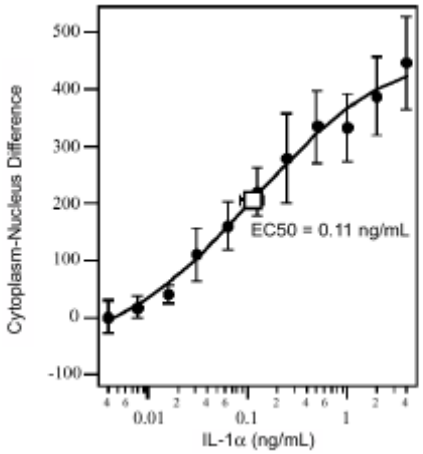

Figure 2. Dose-response curve of HeLa cells treated with IL-1α. Cells were stimulated with IL-1α for 40 minutes at a range of 4 pg/ml to 4 ng/ml. An EC_{50} of 0.11 ng/ml was determined.

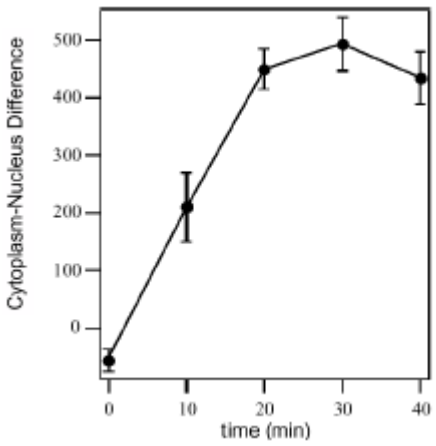

Figure 3. Time-course HeLa cells treated with Il-1α. Cells were treated with 1 ng/ml IL-1α for 40 minutes. HeLa cells responded to 1 ng/ml IL-1α with a t½ of 8-12 min. Maximal translocation occurred by ~30 minutes. Ding *et al.*[1] reported a t½ of 7-8 min for IL-1α or TNF stimulated HeLa using an ArrayScan HCS Reader; and chondrocytes responded with a t½ of 12-13 minutes.

B. Microscope Information

Cells prepared and labeled according to these instructions can be used and analyzed by fluorescence microscopes using the appropriate filter set(s) or confocal microscopy. Optimization may be required when using slides, coverslips or multi-well chamber slides. Use image-processing software to quantify the targets. The approximate absorption/emission maxima of the fluorescent dyes are as follows:

DyLight 488 Conjugates = 494/532 nm

Hoechst Dye = 350/461 nm

C. Recommendations for Automation

- Plating Cells: To improve the uniformity and throughput of plating cells, use a liquid handling system such as Thermo Scientific Multidrop® Combi or WellMate® Dispensers.
- Dead Volumes: Every piece of automation instrumentation has a non-recoverable dead volume associated with it. Be aware of these dead volumes, priming volumes and rinsing volumes when calculating your reagent requirements.
- Nonspecific Binding: Because of the potential of reagent interaction with large surface areas inherent to tubing, syringes and peristaltic pumps, pre-priming with reagents or pre-coating with protein blockers may be warranted.
- Mixing: Gentle mixing may be required when adding a DMSO-based solution to keep overly concentrated solutions from lying on top of the cell layer. Be careful not to dislodge cells or beads during mixing procedures.
- Cell Washing: Use an automated plate washer designed to gently wash attached cells. Be careful not to dislodge cells or beads during cell washing.
- Incubation: Minimize the time when plates with live cells are out of a controlled CO_2 environment. For best results, use an automated incubator to deliver plates to a pipetting deck.
- Exposure: Minimize operator exposure to fixative by some form of containment. Some reagents and compounds are light-sensitive; be aware of these constraints when scaling up for an automated run.

- Adapting to other plate formats: When using different plate types, adjust reagent volumes as needed. Some suggested starting volumes are listed in Table 1.

Table 1. Suggested volumes to use for different cell culture plates.

Kit Component	96-Well Plates (µl/well)	384-Well Plates (µl/well)	24-Well Plates (µl/well)
Fixation Solution	100	25	400
1X Wash Buffer	100	25	400
Wash Buffer II	100	25	400
1X Permeabilization Buffer	100	25	400
Antibody Solution	50	12.5	200
Staining Solution	50	12.5	200
1X Wash Buffer (final wash)	150	37.5	200

Compatible BioApplication Software Modules

S50-5001-1 or S50-2001-1 **Cytoplasm to Nucleus Translocation BioApplication**

S50-5019-1 or S50-2019-1 **Molecular Translocation BioApplication**

S50-5011-1 or S50-2011-1 **Target Detection BioApplication**

S50-5017-1 or S50-2017-1 **Compartmental Analysis BioApplication**

References

1. Ding G. J., *et al.* (1998). *J Biol Chem* **273:**28897-905.
2. Baldwin A. S. (1996). *Annu Rev Immunol* **14:**649-681.
3. Taylor, D.L., *et al.* (2007). High content screening: A powerful approach to systems cell biology and drug discovery. *Method Mol Biol* **356.** Humana Press, Totowa, N.J.
4. Zhang, J.H., *et al.* (1999). A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen* **4:**67-73.

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Thermo Scientific Cellomics Reagent Kits are developed and manufactured at the same Thermo Fisher Scientific Inc. facility as Pierce Protein Research Products and are supported by Pierce Technical Support (see contact information in page footer). These kits are part of the Cellomics Total Solution Platform for HCS, which also includes Cellomics ArrayScan and other HCS Instrumentation, BioApplication Image Analysis Software and High-Content Informatics. For more information, visit www.thermo.com/cellomics or call 800-432-4091 (toll free) or 412-770-2500.

Current versions of product instructions are available at www.thermo.com/pierce. For a faxed copy, call 800-874-3723 or contact your local distributor.