INSTRUCTIONS

Thermo SCIENTIFIC

Cellomics® Heme Oxygenase 1 and Phospho-p38 Activation Kit

High-Content Screening Reagents

2049.0

Number	Description
8405901	**Heme Oxygenase 1 and Phospho-p38 Activation Kit,** sufficient materials for 1 × 96 wells
8405902	**Heme Oxygenase 1 and Phospho-p38 Activation Kit,** sufficient materials for 5 × 96 wells

Kit Contents:	8405901	8405902
Phospho-p38 Primary Antibody (rabbit)	24 µl	120 µl
Heme Oxygenase 1 Primary Antibody (mouse)	15 µl	75 µl
DyLight™ 549 Conjugated Goat Anti-Rabbit IgG	14 µl	72 µl
DyLight 488 Conjugated Goat Anti-Mouse IgG	14 µl	72 µl
Hoechst Dye	30 µl	30 µl
Wash Buffer (10X Dulbecco's PBS)	100 ml	100 ml
Wash Buffer II (10X Dulbecco's PBS with Tween®-20)	100 ml	100 ml
Permeabilization Buffer (10X Dulbecco's PBS with 1% Triton® X-100)	100 ml	100 ml
Blocking Buffer (10X)	85 ml	85 ml
Thin Plate Seal Assembly	7/pack	7/pack

Storage: Upon receipt immediately store the Heme Oxygenase 1 and Phospho-p38 Primary Antibodies at -20°C. Store all other components at 4°C. Keep vials containing the fluorescent antibody and Hoechst Dye solutions 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 Cellomics Heme Oxygenase 1 and Phospho-p38 Activation Kit contains reagents for measuring activation in cells for high-content screening (HCS) assays. The kit contains a rabbit polyclonal antibody that detects only phosphorylated p38, a mouse monoclonal antibody for heme oxygenase 1, DyLight-conjugated Secondary Antibodies and various other reagents and buffers required for immunofluorescence detection.

Heme oxygenase 1 is a microsomal enzyme (32 kDa) that catalyzes the oxidation of heme to antioxidants, biliveridin and carbon monoxide and protects cells from wide variety of stress conditions through activation of p38 mitogen activated protein kinase (MAPK). Carbon monoxide produced during oxidation of heme by heme oxygenase 1 activates p38 kinase, which confers tissue protection through inhibition of cytokine production.[1, 2] Phosphorylation at Thr180/Tyr182 activates p38 kinase. Phosphorylated p38 translocates to nucleus where it phosphorylates and activates several substrates involved in cell stress, inflammation, growth and differentiation. Heme oxygenase 1 and p38 is activated by stressors including oxidative stress, hypoxia, heat shock, heavy metals and cytokines.

In normal cells, heme oxygenase 1 protein levels are low and p38 kinase is inactivated (i.e., non-phosphorylated); however, when cells are treated with copper sulfate, heme oxygenase 1 is induced and p38 is phosphorylated. Heme oxygenase 1 induction increases protein staining in the nucleus and cytoplasm (Figure 1). Phospho-p38 translocates to nucleus and is measured by the average nuclear intensity or difference between nuclear and cytoplasmic intensities. The assay was developed in A549 cells and optimized using the Thermo Scientific ArrayScan® HCS Reader and Compartmental Analysis Bioapplication Software Module.[3] Cells stained using this kit also can be imaged using fluorescence or confocal microscopy.

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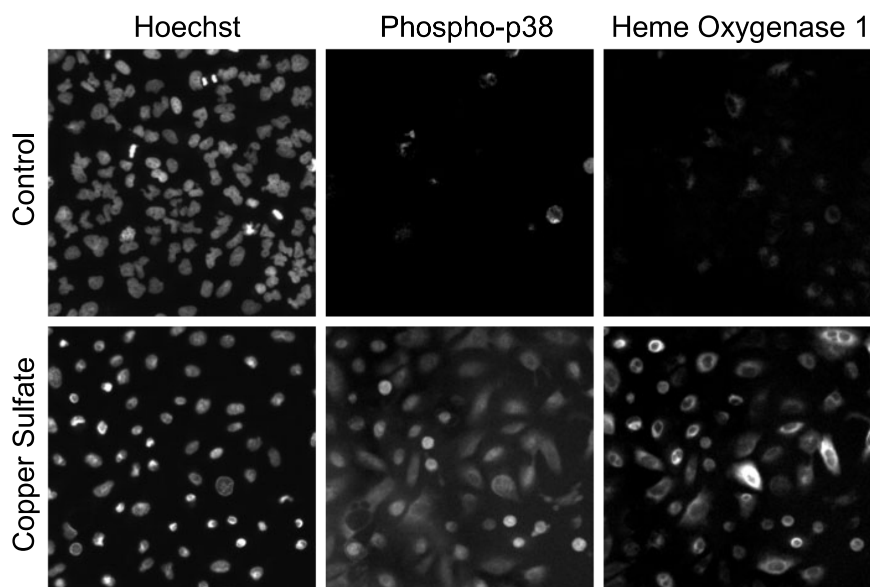

Figure 1. Staining of phospho-p38 and heme oxygenase 1 in A549 cells treated with or without copper sulfate (0.5 mM) for 24 hours. Cells were stained according to the kit protocol and imaged using the ArrayScan HCS Reader. Copper sulfate treatment leads to activation of p38 and heme oxygenase 1 and increased nuclear staining.

Additional Materials

- Formaldehyde (16%) (Thermo Scientific 16% Formaldehyde, Product No. 28906)
- 96-well microplates (e.g., Perkin-Elmer, Product No. 6005182)
- Positive control compound (e.g., copper sulfate)
- Fetal bovine serum (FBS)

Cell Preparation Information

- This protocol is optimized for A549 cells (American Type Culture Collection # CCL-85). HepG2 and HeLa cells also have been used successfully in this assay. Using cells other than A549 cells will require optimization.
- For routine culture of cells, use EMEM Medium containing the following supplements: 10% fetal bovine serum, 1 mM sodium pyruvate, non-essential amino acids, 100 units/ml penicillin and 100 µg/ml streptomycin (EMEM Complete Medium).
- Split cells when they reach 90% confluence at a dilution of 1:3. Use cells at a passage number ≤ 20.
- For phospho-p38 and heme oxygenase 1 detection, harvest cells by trypsinization, dilute into EMEM complete medium, and determine cell density. Dilute cells to 10^5 cells/ml in EMEM complete medium and add 100 µl of the cell suspension per well of a 96-well microplate to achieve the recommended plating density of 10,000 cells/well.
- Incubate cells overnight at 37°C in 5% CO_2 before drug treatment.

Heme Oxygenase 1 and Phospho-p38 Activation Kit Protocol

Solution Preparation (per 96-well plate)

1X Wash Buffer	Add 20 ml 10X Wash Buffer to 180 ml ultrapure water. Store buffer at 4°C for up to 7 days.
1X Wash Buffer II	Add 6 ml of 10X Wash Buffer II to 54 ml ultrapure water. Store buffer at 4°C for up to 7 days.
1X Permeabilization Buffer	Add 1.5 ml of 10X Permeabilization Buffer to 13.5 ml of the 1X Wash Buffer. Store this buffer at 4°C for up to 7 days.
1X Blocking Buffer	Add 5 ml of 10X Blocking Buffer to 44 ml of 1X Wash Buffer and 1 ml of fetal bovine serum for a final volume of 50 ml. Store this buffer at 4°C for up to 7 days.
Primary Antibody Solution	Add 24 µl of Phospho-p38 Primary Antibody and 15 µl of Heme Oxygenase 1 Primary Antibody to 6 ml of 1X Blocking Buffer without FBS. Prepare solution just before each assay.
Secondary Antibody/ Staining Solution	Add 0.6 µl of Hoechst Dye, 12 µl of DyLight 549 Goat Anti-Rabbit and 12 µl of the DyLight 488 Goat Anti-Mouse to 6.0 ml of 1X Blocking Buffer. Prepare solution just before each assay.

Procedure

1. Prepare 2X solution of copper sulfate (1 mM) and add 100 µl to the cells. Incubate for 24 hours at 37°C.
2. Add 65 µl/well of 16% formaldehyde. Incubate plate in a fume hood at room temperature for 15 minutes.
3. Aspirate the formaldehyde, and wash plate twice with 100 µl/well of 1X Wash Buffer.
4. Aspirate Wash Buffer and add 100 µl/well of 1X Permeabilization Buffer and incubate for 15 minutes at room temperature.
5. Aspirate Permeabilization Buffer and wash plate twice with 100 µl/well of 1X Wash Buffer.
6. Aspirate Wash Buffer, add 100 µl/well of 1X Blocking Buffer supplemented with 2% FBS and incubate at room temperature for 15 minutes.
7. Aspirate Blocking Buffer and add 50 µl/well of Primary Antibody Solution. Incubate for 1 hour at room temperature.
8. Aspirate Primary Antibody Solution and wash plate twice with 100 µl/well of 1X Wash Buffer II.
9. Aspirate Wash Buffer II and wash plate once with 100 µl/well of 1X Wash Buffer.
10. Aspirate Wash Buffer and add 50 µl/well of Secondary Antibody/Staining Solution. Incubate for 45 minutes protected from light at room temperature.
11. Aspirate Staining Solution and wash plate twice with 100 µl/well of 1X Wash Buffer II.
12. Aspirate Wash Buffer II and wash plate twice with 100 µl/well of 1X Wash Buffer.
13. Aspirate Wash Buffer and replace with 200 µl/well of 1X Wash Buffer.
14. Seal plate and evaluate on the ArrayScan HCS Reader. Store plates at 4°C.

Additional Information

A. Dose Response Curves

Phospho-p38 and heme oxygenase 1 in A549 cells were measured as described in the procedure in response to different doses of copper sulfate treatment for 24 hours (Figure 2).

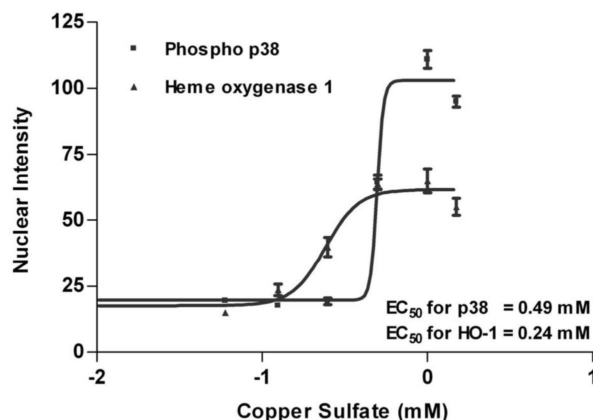

Figure 2. Dose response curves of phospho-p38 and heme oxygenase 1 in A549 cells. The average nuclear intensities for phospho-p38 and heme oxygenase 1 are plotted. Data represents mean ± SD from three plates (eight wells per 96-well plate per dose of copper sulfate).

B. Performance Robustness

Assay robustness was ascertained by determining the Z′ factors[4] for the average nuclear intensity (heme oxygenase 1) or difference between nuclear and cytoplasmic intensities (phospho-p38) in nontreated- (vehicle) and copper sulfate- (0.5 mM for 24 hours) treated wells. The Z′ factors were calculated using three plates of A549 cells treated identically and were as follows:

heme oxygenase 1: 0.56 ± 0.03

phospho-p38: 0.68 ± 0.04 for

DMSO tolerance: The assay performance was robust when compounds were added in DMSO up to a maximum concentration of 1% DMSO.

C. Microscope Information

Cells prepared and labeled according to this kit protocol can be used and analyzed by fluorescence microscopes using the appropriate filter set(s) or confocal microscopy. Optimization might be required when using slides, cover slips 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 649 Conjugates = 646/674 nm
DyLight 488 Conjugates = 494/532 nm
Hoechst Dye = 350/461 nm

A. Recommendations for Automation

- Plating Cells: To improve the uniformity and throughput of plating cells, use a liquid handling system such as a 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 might be needed.
- 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
1X Blocking 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**
S50-5017-1 or S50-2017-1	**Compartmental Analysis BioApplication**

References

1. Maines, M.D. (1997). The Heme oxygenase system: A regulator of second messenger gases. *Ann Rev Pharmacol Toxicol* **37**:517-54.
2. Lavin, M.F., *et al.* (2006). Heme Oxygenase-1/Carbon Monoxide: from basic science to therapeutic applications. *Physiol Rev* **86:**583-650.
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.

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