

Cellomics[®] Heme Oxygenase 1 Activation Kit

High-Content Screening Reagents

2043.0

Number	Description
8405801	Heme Oxygenase 1 Activation Kit, sufficient materials for 1 × 96 wells
8405802	Heme Oxygenase 1 Activation Kit, sufficient materials for 5 × 96 wells

Kit Contents:	8405801	8405802
Heme Oxygenase 1 Primary Antibody	15 µl	75 µl
DyLight™ 549 Conjugated Goat Anti-Mouse IgG	30 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 Primary Antibody 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. Kit is shipped with an ice pack.

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 Activation Kit measures activation of heme oxygenase 1 in cells. The kit contains a mouse monoclonal heme oxygenase 1 primary antibody, a DyLight 549-Conjugated Secondary Antibody, and various other reagents and buffers required for immunofluorescent detection for high-content screening (HCS) assays.

Heme oxygenase 1 is a microsomal enzyme that catalyses the oxidation of heme to antioxidants, biliveridin and carbon monoxide and protects cells from wide variety of stress conditions through activation of p38 MAPK. Carbon monoxide produced during oxidation of heme by heme oxygenase 1 activates p38 MAPK, which confers tissue protection through inhibition of cytokine production.^{1,2} Heme oxygenase 1 can be induced by oxidative stress, hypoxia, heat shock, heavy metals and cytokines.

To measure heme oxygenase 1 levels, A549 cells were treated with copper sulfate. In normal cells, heme oxygenase 1 protein levels are low. Upon activation, heme oxygenase 1 increases in the cytoplasm and nucleus, as evidenced by increased staining (Figure 1). The assay was developed in A549 cells and optimized using the Thermo Scientific ArrayScan[®] HCS Reader and Compartmental Analysis Bioapplication Software Module.³ Stained cells also can be imaged using fluorescence or confocal microscopy.

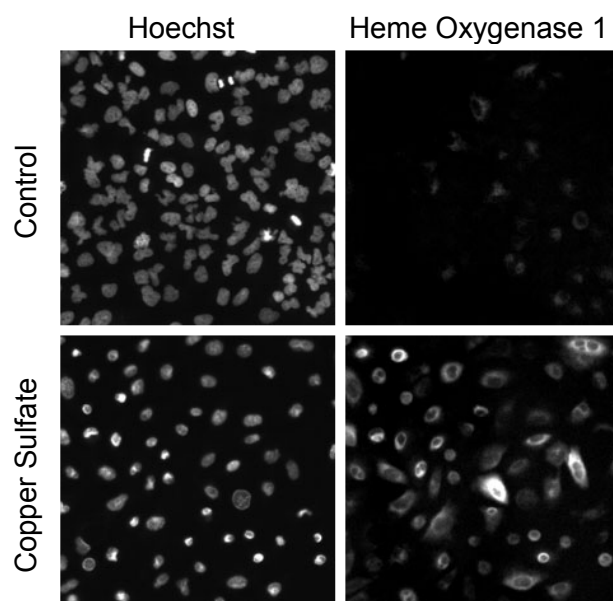


Figure 1. Staining of Heme oxygenase 1 in A549 cells treated with or without 0.5 mM copper sulfate for 24 hours. Cells were stained according to the kit protocol and imaged using the ArrayScan HCS Reader.

Additional Materials

- Formaldehyde (16%) (Thermo Scientific 16% Formaldehyde, Product No. 28906)
- Packard View 96-well microplates (e.g., Perkin-Elmer, Product No. 6005182)
- Positive control compound such as 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 will require protocol optimization.
- For routine culture of cells, EMEM medium containing the following supplements: 10% fetal bovine serum, 1 mM sodium pyruvate, non-essential amino acids and 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 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% CO₂ before drug treatment.

Heme Oxygenase 1 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 15 µl of 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 and 12 µl of the DyLight 549 Goat Anti-Mouse Antibody to 6 ml of 1X Blocking Buffer without FBS. Prepare solution just before each assay.

Procedure

1. Prepare 2X solution of copper sulfate (1 mM) and add 100 µl to the cells and incubate for 24 hours at 37°C.
2. Add 65 µl/well of 16% formaldehyde and incubate plate in a fume hood at room temperature for 15 minutes.
3. Aspirate formaldehyde and wash plate twice with 100 µl/well of 1X Wash Buffer.
4. Aspirate Wash Buffer, 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% fetal bovine serum (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 Curve

The heme oxygenase 1 in A549 cells was measured as described in the procedure using different doses of copper sulfate treatment for 24 hours (Figure 2).

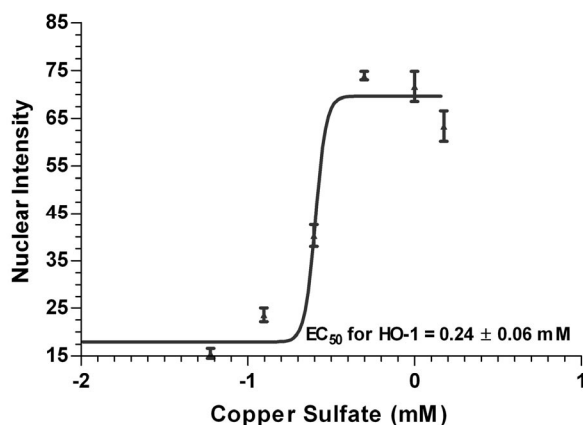


Figure 2. Dose response curve of heme oxygenase 1 in A549 cells treated with copper sulfate for 24 hours. Data represents mean \pm SD from three plates (eight wells per 96-well plate per dose of copper sulfate).

B. Performance Robustness

The robustness of the kit was ascertained by determining the Z' for the average nuclear intensity in nontreated and copper sulfate (0.5 mM for 24 hours) treated wells.⁴ The Z' factor was calculated using three plates of HeLa cells treated identically and was 0.54 ± 0.05 .

DMSO tolerance: The assay performance was robust when compounds were added with up to 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 549 Conjugates = 550/568 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 the 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 CO₂ 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.

<u>Kit Component</u>	<u>96-Well Plates</u> (<u>µl/well</u>)	<u>384-Well Plates</u> (<u>µl/well</u>)	<u>24-Well Plates</u> (<u>µl/well</u>)
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

S50-5019-1 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. *Methods 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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