Manganese Superoxide Dismutase (MnSOD) Assay

For High Content Screening

For 5 x 96-well plates

Cat. No. HCS232

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Not for use in diagnostic procedures.

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Introduction

(i) High Content Screening

High Content Screening (HCS) technology offers a major opportunity to improve the drug discovery and development process [1]. HCS enables the evaluation of multiple biochemical and morphological parameters in cellular systems and facilitates characterization of the subcellular distribution of fluorescent signals with labeled reagents. By combining automated imaging of cells with validated detection reagents and powerful image analysis algorithms, scientists can now acquire deeper knowledge of multiple pathways at the single-cell level, usually in a single assay, at an early stage in the development of new drugs [2]. HCS platforms such as the IN Cell Analyzer (GE Healthcare), ArrayScan (ThermoFisher Scientific), or Opera (Perkin Elmer), can be used to deliver detailed profiles of cellular responses [3].

Successful HCS assays rely on high quality reagents [4]. With the commercial availability of thousands of immunoreagents and fluorescent probes, large numbers of fixed-endpoint HCS assays are possible. However, incompatibility of reagents when integrated into a single assay can lead to a significant drop-off in assay performance. Immunoreagents for HCS assays carry special requirements. Strong antigen affinity is required, minimal non-specific binding must be observed, interactions between multiple primary or secondary immunoreagents must be minimized, and the signal to background ratio must be sufficient to ensure an adequate screening window [4]. Additionally, to enable scale up of HCS assays, the sample preparation protocol must be highly reproducible, and the reagents must exhibit minimal assay-to-assay variability.

(ii) Manganese superoxide dismutase (MnSOD) and its significance in cell biology and drug discovery

Reactive oxygen species (ROS), such as superoxide anion radicals and hydrogen peroxide, are potentially harmful by-products of normal cellular metabolism [5]. ROS normally exist in the cell in balance with antioxidant molecules. Oxidative stress occurs when this balance is disrupted due to depletion of antioxidants or excess accumulation of ROS, or both [6]. Many inducers of oxidative stress are known carcinogens, mutagens, and toxins. Accordingly, oxidative stress is a common denominator in many diseases and environmental insults and can lead to severe cellular damage leading to physiological dysfunction and cell death. When oxidative stress occurs, cells function to restore redox balance by resetting critical homeostatic parameters. One rapid and clear indicator of oxidative stress is the induction of antioxidant defenses [6].

To minimize the damaging effects of ROS, cells utilize an array of defenses. Enzymatic defenses, such as superoxide dismutases (SOD) and catalases (CAT), protect by scavenging superoxide radicals and hydrogen peroxide respectively, converting them to less reactive species. SOD guard against superoxide toxicity, protecting redox-sensitive cellular machinery from damage by catalyzing the disproportionation of superoxide anion to oxygen and hydrogen peroxide. Thus, the SOD and CAT serve, in tandem, as front-line antioxidant defenses [6].

The manganese-containing superoxide dismutase of the mitochondria (MnSOD) plays an essential role in oxidative stress protection [7], and has been shown by knockout mice studies to be essential for life [8]. MnSOD is an inducible antioxidant enzyme protecting mitochondria from oxidative damage [9]. Numerous studies have shown that MnSOD can be induced to protect against pro-oxidant insults resulting from cytokine treatment, ultraviolet light, irradiation, certain tumors, amyotrophic lateral sclerosis, and ischemia/ reperfusion [8]. Thus, it is important to know the status of both MnSOD protein levels and activity in order to assess its role as an important regulator of cell biology [8].

Mitochondrial reactive oxygen species (ROS) represent a target for drug discovery since their production is characteristic of early stages of apoptosis [10]. In fact, many antitumor agents, such as vinblastine, cisplatin, mitomycin C, doxorubicin, camptothecin, inostamycin, neocarzinostatin and many others exhibit antitumor activity via ROS-dependent activation of apoptotic cell death [5]. Since MnSOD expression is upregulated in response to ROS, quantitation of MnSOD expression represents a useful drug screening tool.

Several lines of evidence suggest the possibility of MnSOD as a target for new, effective and possibly tumor-selective anticancer treatments [9]. Elevated levels of MnSOD are found in several classes of human neoplasms, in a fashion which often correlates with the degree of their malignancy. Recent observations suggest that pharmacological inhibition of MnSOD may represent an effective strategy to selectively kill cancer cells and to circumvent their resistance to commonly used anticancer treatments [9]. Thus, MnSOD may serve a dual role in health and disease, in that oxidants should be eliminated by MnSOD in normal cells, but may prove useful once a tumor has developed, to promote the chemosensitivity of cancer cells [9].

Application

Millipore's HCS232 assay provides a complete solution for identifying and quantifying manganese superoxide dismutase (MnSOD) in cellular imaging studies. The reagents in the kit have been specifically optimized for HCS applications.

The assay is designed to enable visualization and quantitative detection of MnSOD, facilitating the characterization of the cellular oxidative stress response, and screening of compounds that may induce, inhibit or repair cellular injury. The nuclear dye (Hoechst 33342) may be used for measurements of cell number, DNA content and nuclear size. Additionally, the assay can be multiplexed with other probes, *e.g.*, for apoptotic pathway studies, oncology drug efficacy trials or *in vitro* toxicology applications.

The assay is immunofluorescence-based, and utilizes a high quality rabbit polyclonal antibody which identifies MnSOD in human cells. Species cross-reactivity has also been observed with mouse, rat and bovine tissue (not canine). Alternate species cross-reactivity must be confirmed by the end user.

The superior Millipore reagents provided with this kit enable the user to reproducibly generate images with a high signal-to-background ratio, greatly facilitating HCS. In addition, working solutions of the primary and secondary antibodies are stable for at least 24 hours at room temperature (Figure 3), a great benefit for large-scale screening applications. The straightforward sample preparation and processing protocol takes less than 2.5 hrs after fixation.

Reagents are provided for 5 x 96-well microplates – *i.e.*, sufficient to perform 480 separate experiments. The kit includes a primary antibody for manganese superoxide dismutase, a Cy3-conjugated secondary antibody, Hoechst HCS Nuclear Stain, HCS Fixation Solution, HCS Immunofluorescence Buffer, HCS Wash Buffer, and Plate Sealers. Two control compounds, the chemotherapeutic drugs camptothecin and etoposide, along with DMSO for Compound Serial Dilution and Compound Dilution Buffer, are also included in the kit, sufficient for duplicate 12-point dose response samples per plate (see Assay Instructions). Camptothecin, a DNA topoisomerase I inhibitor, and etoposide, a DNA topoisomerase II inhibitor [11, 12], have both been shown to induce ROS, to interfere with DNA replication/synthesis, and to induce double-strand DNA breaks.

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Kit Components

1. Rabbit Anti-Manganese Superoxide Dismutase (MnSOD) HCS Primary Antibody, 100X: - (Part No. CS201736) 1 vial containing 300 µL.

2. HCS Secondary Antibody (donkey anti-rabbit IgG, Cy3 conjugate), 200X: - (Part No. CS201659) 1 vial containing 150 µL.

3. Hoechst HCS Nuclear Stain, 200X: - (Part No. CS200438) 1 vial containing 150 µL.

4. HCS Fixation Solution with Phenol Red, 2X: - (Part No. CS200434) 1 bottle containing 100 mL.

5. HCS Immunofluorescence Buffer, 1X: - (Part No. CS200435) 1 bottle containing 1000 mL.

6. HCS Wash Buffer, 1X: - (Part No. 2007643) 1 bottle containing 500 mL.

7. Camptothecin, 2.5 mM in DMSO, 250X: - (Part No. CS201666) 1 vial containing 100 µL.

8. Etoposide, 25 mM in DMSO, 250X: - (Part No. CS200439) 1 vial containing 100 µL.

9. DMSO for Compound Serial Dilution: - (Part No. CS200441) 1 bottle containing 10 mL.

10. Compound Dilution Buffer: - (Part No. CS200442) 1 bottle containing 25 mL.

11. Plate Sealers: - (Part No. CS200443) 10 each.

Materials Not Supplied

1. Sterile, tissue culture-treated black/clear bottom microplates suitable for High-Content Imaging.
2. Cell-type for assay, *e.g.*, HeLa (human cervical adenocarcinoma, ATCC #CCL-2), A549 (human lung carcinoma, ATCC #CCL-185) or HepG2 (human hepatocellular carcinoma, ATCC #HB-8065).
3. Tissue culture instruments/supplies (including 37ºC incubator, growth media, flasks/plates, detachment buffer, etc.) for cell type of interest.
4. HCS imaging/analysis system, *e.g.*, GE Healthcare IN Cell Analyzer 1000 with Investigator software. System must be equipped with beam-splitters and filters capable of reading emission spectra in the blue and red ranges. Detailed image acquisition and analysis guidelines are provided in Table 2.

Precautions

1. This product contains hazardous materials. Refer to MSDS for further information.

Component	Hazardous Constituent	Warnings (See MSDS)
HCS Fixation Solution	Formaldehyde	Toxic, carcinogen, combustible, readily absorbed through skin
Hoechst HCS Nuclear Stain	Hoechst 33342	Harmful, potential mutagen
Camptothecin	Camptothecin	Toxic
Etoposide	Etoposide	Toxic
DMSO	Dimethyl sulfoxide	Combustible, readily absorbed through skin

2. For Research Use Only. Not for use in diagnostic procedures.

Storage

Store kit components under the conditions indicated on the labels. HCS Fixation Solution, HCS Immunofluorescence Buffer, HCS Wash Buffer, DMSO, and Compound Dilution Buffer should be stored at 2-8ºC. Plate Sealers may be stored at room temperature. HCS Primary Antibody, HCS Secondary Antibody, Hoechst HCS Nuclear Stain, Camptothecin and Etoposide should be stored at -20ºC, avoiding repeated freeze/thaw cycles. Discard any remaining reagents after 6 months.

(Note: If kit is expected to be used for multiple experiments, rather than a single use, thaw antibodies, nuclear stain and control compounds and dispense into appropriately sized aliquots. Store aliquots at -20ºC.)

Assay Instructions

Note: The HCS232 assay protocol has been optimized for HeLa human cervical carcinoma (ATCC #CCL-2), A549 human lung carcinoma (ATCC #CCL-185) and HepG2 human hepatocellular carcinoma (ATCC #HB-8065) cells. However, this kit is suitable for HCS analysis of a variety of human cell types. Alternate species reactivity must be confirmed by the end user.

Cell Preparation:

1. Prior to cell seeding for assay, culture HeLa, A549 or HepG2 cells in growth media until ~70-80% confluent.

2. Detach cells from culture flasks/plates via method appropriate for cell type of interest. If necessary, coat assay plate wells with extracellular matrix protein (*e.g.*, collagen I for HepG2) to enhance cell adhesion. Adjust cell density to 5-7 x 10^4 cells/mL (Hela/A549) or 1-2 x 10^5 cells/mL (HepG2) in growth media. Add 90 µL of this cell suspension to each well (for a 96-well plate, this is approximately equivalent to 15,000-21,000 HeLa or A549 cells/cm^2 of well surface, or 30,000-60,000 HepG2 cells/cm^2). After adding cells to plate, allow plate to sit on a level surface at room temperature for 15-30 min, which allows for even cell distribution. Following this period, incubate cells in growth media (37ºC/5% CO_2) for ~24-48 hours.

3. Cell treatments (control compounds, test compounds, etc.) can be introduced at any point during this culture period, as appropriate for time-course of treatment of interest. Camptothecin and Etoposide are provided as DNA Damage control compounds. Sufficient reagents are provided for duplicate 12-point dose response curves (including one DMSO-control set within the dose response) for all five 96-well plates. The compounds are provided at 250X concentration (assuming maximum treatments of 10 µM and 100 µM for camptothecin and etoposide, respectively). Recommended treatment preparation involves half-log (1:$\sqrt{10}$) serial dilution of the 250X compound in DMSO, followed by dilution in Compound Dilution Buffer to 10X. 10 µL of each treatment may then be added to the 90 µL of culture media already present in each well, for a final 1X concentration (0.4% DMSO). Sample data is provided for 4 or 24hrs of compound treatment at 37°C prior to fixation.

Cell Fixation and Immunofluorescent Staining:

Note: Staining time is ~2.5 hours post-fixation. Do not allow wells to dry out between staining steps. Aspiration and dispensation of reagents should be conducted at low flow rates to diminish any cell loss due to fluid shear. All recommended 'per well' volumes refer to a single well of a 96-well microplate. All recommended 'per 96-well plate' volumes include 25% excess for liquid handling volume loss. All staining steps are performed at room temperature (RT). All buffers and antibody solutions are stable for at least 24 hours at RT (see Figure 3).

4. At end of culture period, pre-warm HCS Fixation Solution (2X) to room temperature (RT) or 37ºC if desired (12 mL/96-well plate). In a chemical fume hood, add 100 µL/well directly to culture media and allow to fix for 30 min at RT. Remove fixative/toxin-containing media and dispose of in compliance with regulations for hazardous waste (see MSDS). If proceeding immediately to staining, rinse each well twice with 200 µL of HCS Immunofluorescence Buffer. Alternatively, if plates are to be stained at a later time, rinse twice with 200 µL of Wash Buffer, then leave second rinse volume in wells and store plates tightly sealed at 4ºC until staining.

5. If fixed samples have been stored at 4ºC prior to staining, rinse twice with 200 µL HCS Immunofluorescence Buffer before proceeding with staining protocol.

6. Prepare working solution of Rabbit Anti-MnSOD HCS Primary Antibody (6 mL/96-well plate) as follows: Add 60 µL of thawed Primary Antibody to 5.94 mL of HCS Immunofluorescence Buffer (see Table 1). Mix well. Remove previous Immunofluorescence Buffer rinse. Add 50 µL of Primary Antibody solution to each well and incubate for 1 hour at RT.

7. Remove Primary Antibody solution. Rinse three times with 200 µL HCS Immunofluorescence Buffer.

8. Prepare working solution of Cy3-donkey anti-rabbit IgG HCS Secondary Antibody/Hoechst HCS Nuclear Stain (6 mL/96-well plate) as follows: Add 30 µL of thawed Secondary Antibody and 30 µL of thawed Hoechst HCS Nuclear Stain to 5.94 mL of HCS Immunofluorescence Buffer (see Table 1). Mix well, protecting solution from light. Remove previous HCS Immunofluorescence Buffer rinse. Add 50 µL of HCS Secondary Antibody/Hoechst HCS Nuclear Stain solution and incubate for 1 hour at RT, protected from light.

9. Remove HCS Secondary Antibody/Hoechst HCS Nuclear Stain solution. Rinse twice with 200 µL HCS Immunofluorescence Buffer.

10. Remove previous HCS Immunofluorescence Buffer rinse. Rinse twice with 200 µL of HCS Wash Buffer, leaving second rinse volume in wells.

11. Seal plate and image immediately, or store plate at 4ºC protected from light until ready for imaging.

HCS232 Detection Reagent Specifications			
Primary Antibody working solution			
Reagent	**Required dilution of initial reagent**	**Vol. required for 1 well (50 µL)**	**Vol. required for 1 plate (6 mL) (includes ~25% excess)**
Rabbit Anti-MnSOD HCS Primary Antibody	1:100	0.5 µL	60 µL
HCS Immunofluorescence Buffer	None	49.5 µL	5.94 mL (5940 µL)
Secondary Antibody/Hoechst HCS Nuclear Stain working solution			
Reagent	**Required dilution of initial reagent**	**Vol. required for 1 well (50 µL)**	**Vol. required for 1 plate (6 mL) (includes ~25% excess)**
Cy3-Donkey Anti-Rabbit HCS Secondary Antibody	1:200	0.25 µL	30 µL
Hoechst HCS Nuclear Stain	1:200	0.25 µL	30 µL
HCS Immunofluorescence Buffer	None	49.5 µL	5.94 mL (5940 µL)

Table 1. Detection Reagent Specifications – HCS232 Rabbit Anti-MnSOD Assay

Image acquisition and analysis

HCS232 Image Acquisition Guidelines			
Detection Reagent	**Objective Lens**	**Excitation Filter Range [peak/bandwidth (nm)]**	**Emission Filter Range [peak/bandwidth (nm)]**
Hoechst HCS Nuclear Stain	10X	360/40	460/40 (or 535/50 if necessary)
HCS Secondary Antibody, Cy3-donkey anti-rabbit IgG	10X	535/50	600/50

HCS232 Image Analysis Guidelines			
Cell Parameter	**Detection**	**Segmentation/ Measurement**	**Rationale**
Cell Number	Hoechst HCS Nuclear Stain	Nuclear region (460 nm emission channel). Count number of nuclei. DNA content (nuclear intensity) or nuclear area analyses are also possible.	Use cell number, nuclear characteristics to determine cell loss, toxicity phenotypes, etc.
MnSOD Expression	HCS Secondary Antibody, Cy3-conjugated	Cytoplasmic region (600 nm emission channel). Can utilize Cy3 signal to determine cytoplasmic segmentation. Determine parameters such as average cytoplasmic intensity, total cytoplasmic intensity (cell area-dependent), etc.	MnSOD expression may be modulated as a result of DNA damage, toxic stresses, etc.

Table 2. Image Acquisition and Analysis Guidelines – HCS232 Rabbit Anti-MnSOD Assay

Sample Results

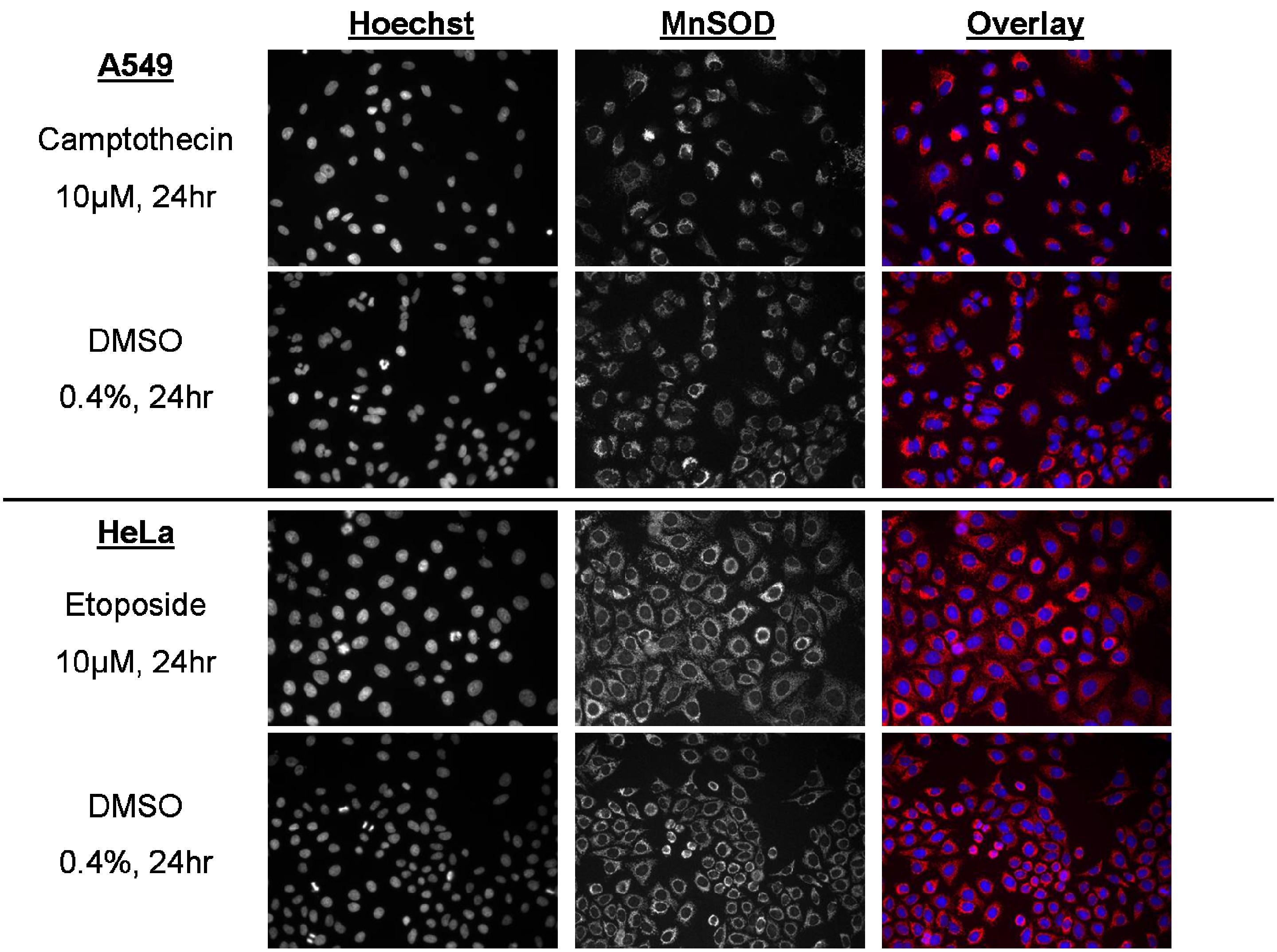

Figure 1. Immunofluorescence of untreated and toxin-stressed A549 and HeLa cells.

A549 or HeLa cells were plated at 18,000 cells/cm^2 on 96-well plates in growth media and cultured for 24 hours. Cells were subsequently treated for 24 hours with camptothecin, etoposide or 0.4% DMSO (negative control). Cell handling, fixation and immunostaining were performed according to HCS232 assay protocols. Cells were imaged on the GE IN Cell Analyzer 1000 (3.4) at 20X objective magnification. *Left and center panels*: Monochromatic images of Hoechst HCS Nuclear Stain and MnSOD fluorescence. *Right panel*: Fused images of Hoechst HCS Nuclear Stain (blue) and MnSOD fluorescence (red).

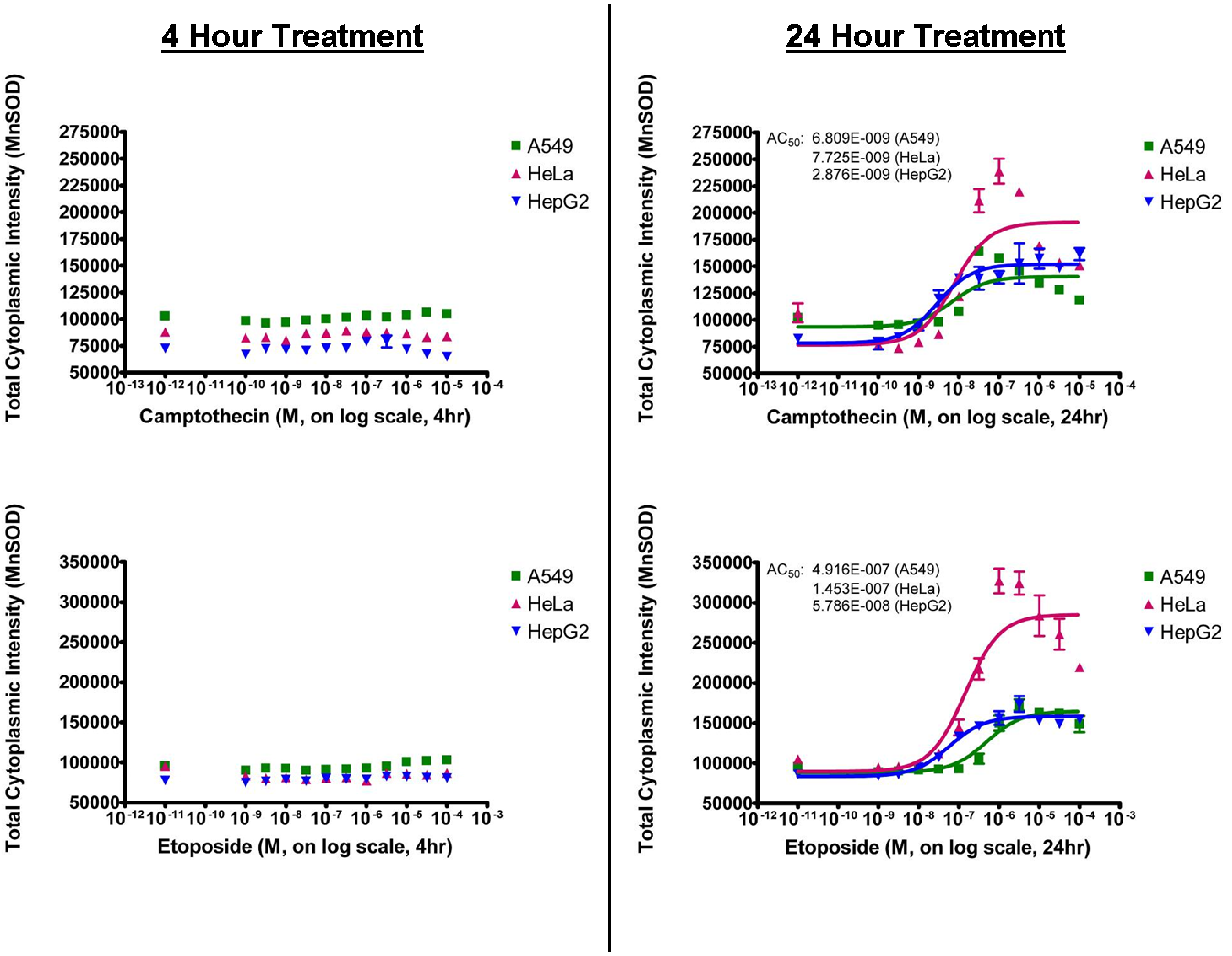

Figure 2. MnSOD dose responses of A549, HeLa and HepG2 cells to toxic stresses.

A549 or HeLa cells were plated at 18,000 cells/cm^2 (37,000 cells/cm^2 for HepG2) on 96-well plates in growth media and cultured for 24 hours. Maintaining 48 hours of total culture time, cells were subsequently treated for 4 hours (*left panel*) or 24 hours (*right panel*) with serial dilutions of either camptothecin (max. concentration = 10 μM) or etoposide (max. concentration = 100 μM). Cell handling, fixation and immunostaining were performed according to HCS232 assay protocols. Cells were imaged on the GE IN Cell Analyzer 1000 (3.4) at 10X (10 fields/well) and analyzed (nuclear/cytoplasmic segmentation) using the GE IN Cell Analyzer 1000 Workstation (3.5) Multi Target Analysis algorithm. Data presented are mean ± SEM, *n* = 4. Note the minimal MnSOD response after just 4 hours of toxin treatment, compared to the 24 hour time point.

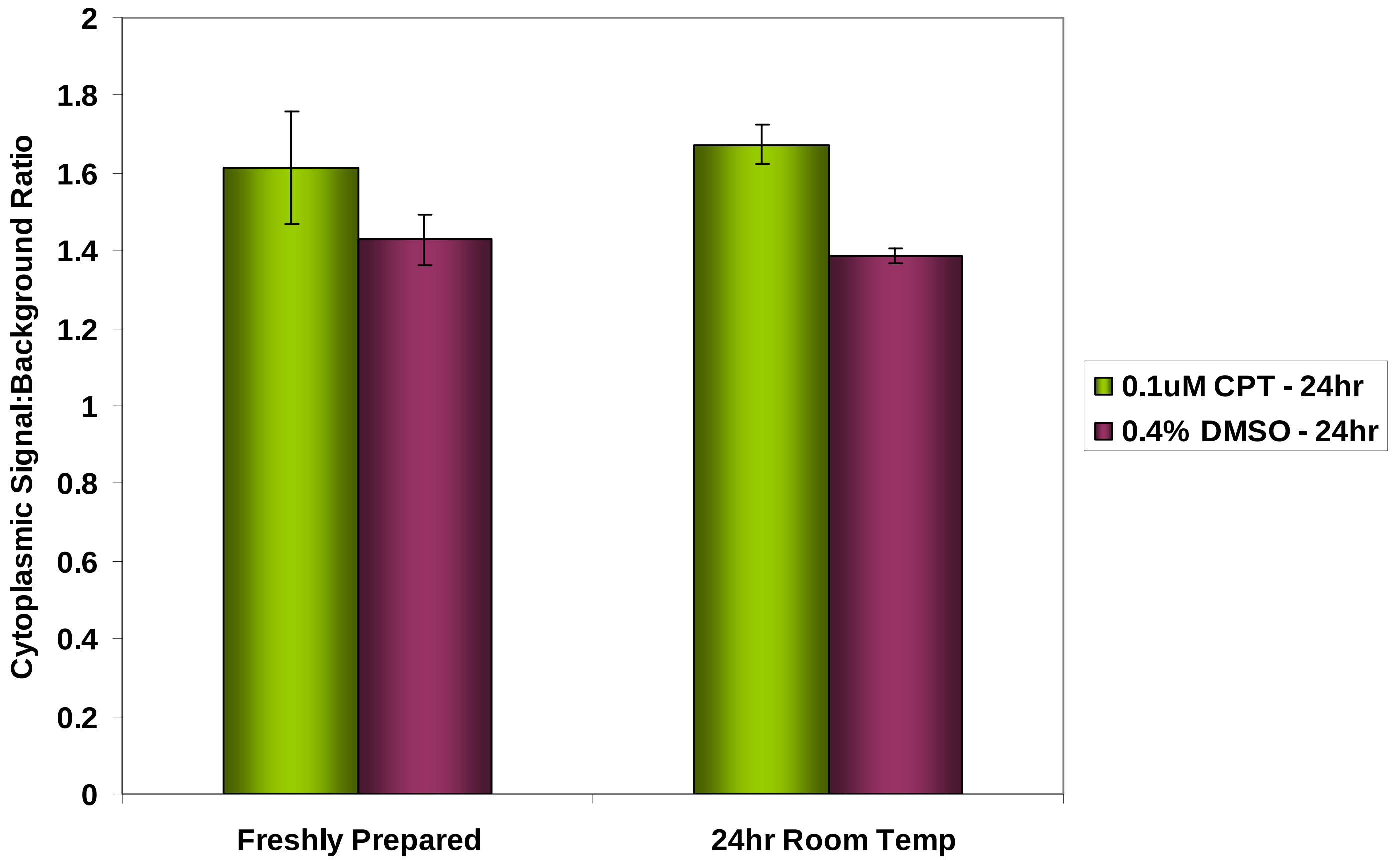

Figure 3. HCS232 Rabbit Anti-MnSOD Assay reagent stability.

HeLa cells were seeded at 18,000 cells/cm^2 on 96-well plates in growth media and cultured for 24 hours, followed by treatment with 0.1 µM camptothecin (CPT) or 0.4% DMSO (negative control) for an additional 24 hours. Samples were fixed and stained under kit conditions, using either fresh buffers and antibody/Hoechst solutions, or buffers and antibody/Hoechst solutions that had been allowed to sit at room temperature (protected from light) for 24 hours prior to staining. Cells were imaged on the GE IN Cell Analyzer 1000 (3.4) at 10X magnification (10 fields/well) and analyzed (nuclear/cytoplasmic segmentation) using the GE IN Cell Analyzer 1000 Workstation (3.5) Multi Target Analysis algorithm. Cytoplasmic signal:background ratios (*i.e.*, Cy3 cytoplasmic:background intensity ratios) were measured to observe MnSOD expression differences between camptothecin and DMSO-treated cells. Data presented are mean ± SD; *n* = 3. No significant differences in signal quality were observed between freshly prepared and 24 hour samples.

Troubleshooting

Problem	Potential Explanations/Solutions
Weak Cy3/Hoechst signal	Improper storage or preparation of Primary/Secondary antibody or Nuclear Stain – retry stain with fresh antibody/dye solution. Inadequate primary/secondary antibody or Nuclear Stain concentrations for cell type – titrate dilutions to optimize signal. Signal may diminish in extremely dense cultures – decrease cell seeding concentration or increase primary/secondary antibody or Hoechst concentration. Optimize exposure times and/or fluorescence filters appropriate to fluorophore.
Excessive background	Improper reagent storage or preparation – retry with fresh reagent (antibodies/dyes and/or buffers). Contaminated buffers/solutions may require 0.2 µm filter sterilization. Samples may have dried during staining – retry stain on fresh samples. Excessive primary or secondary antibody concentrations for cell type – titrate dilutions to optimize signal. Check for autofluorescence of microplate.
Excessive Cy3/ Hoechst signal	Improper preparation of antibody/dye – retry stain with fresh antibody/dye solutions. Inappropriate antibody/dye concentrations for cell type – titrate dilutions to optimize signal. Optimize exposure times and/or fluorescence filters appropriate to fluorophore.
Cell loss	Optimize liquid aspiration/dispensation rate to reduce shear. Consider protein-coating to improve cell adhesion to microplate. Optimize cell seeding concentrations for better cell adhesion. Cell loss due to toxic treatments may hinder statistically relevant analysis; alter toxin dosages/treatment times to reduce cell loss levels.
Poor nuclear/cytoplasmic segmentation during analysis	Effective segmentation parameters can be HCS system/software-dependent. Consider decreasing cell seeding concentrations for difficulty in analysis of dense cultures (separation of multiple nuclei).

Problem	Potential Explanations/Solutions
No dose response observed/partial response curve	Efficacy of control compounds may vary with cell type, cell species, or quality of reagent storage. Use fresh compound, choose alternate maximum/minimum treatment concentrations, or select more appropriate control compounds for cell type of interest. Perform time-course experiments to determine kinetics of compound effects for cell type of interest. Shorter/longer treatment durations may be required.

References

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Cat No. HCS232

April 2009
Revision A