p38 MAP Kinase Assay

For High Content Screening

For 5 x 96-well plates

Cat. No. HCS231

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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) p38 MAP kinase and its significance in cell biology and drug discovery

Protein kinases modulate a variety of cellular signal transduction pathways, and abnormal phosphorylation events can be a cause or contributor to disease progression in a variety of disorders. This has led to the emergence of protein kinases as an important class of drug targets [5, 6]. p38 mitogen-activated protein kinases (p38 MAPKs) are a group of serine/threonine protein kinases that together with ERK (extracellular signal-regulated kinases) and JNK (c-Jun N-terminal kinases) MAPKs act to convert extracellular signals into specific cellular responses through interacting with and phosphorylating downstream targets [7]. The p38 MAPK family is made up of 4 isoforms (α, β, γ, δ). Both p38α and p38β are ubiquitously expressed, while p38γ and p38δ have a more tissue-specific expression pattern, p38γ being expressed predominantly in skeletal muscle, while p38δ is highly expressed in lung, pancreas, kidney, small intestine and testis [8, 9].

p38 MAPK are regulated by a wide variety of stimuli, often associated with stress, such as pro-inflammatory cytokines like IL-1 and TNFα, UV radiation, reactive oxygen species, osmotic shock, chemical toxins such as anisomycin, and biological agents such as bacterial lipopolysaccharide (LPS) and viruses [10]. The p38 upstream activators include dual specificity MAPK kinases 3 & 6 (MKK3 and MKK6) and c-Jun N-terminal Kinase Kinase 1 (JNKK1) [7]. Upon stimulation, p38 MAPK are dually phosphorylated on their common TGY motif (Thr180/Tyr182 in human p38α), resulting in their activation and translocation from the cytoplasm to the nucleus [11]. Once activated, p38 is able to phosphorylate a wide array of targets, including numerous kinases such as MK2, MK3 (MAPK-activating protein kinases 2 & 3), PRAK (p38-related/activated protein kinase), MSK (mitogen- and stress-activated protein kinase), and MNK (MAPK-integrating kinase). p38 also phosphorylates many transcription factors, including STAT1, p53, NFAT, ATF-2, and MEF2 [7]. Additionally, p38 is known to activate the key antioxidant gene MnSOD as a response to the burden of oxidative stress [10].

p38 MAPK plays a key role in regulating the synthesis of pro-inflammatory mediators such as TNFα, IL-1 and COX2 [12], and has been implicated in many inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease, and psoriasis, where aberrant cytokine signaling is the driver of the disease [13]. As a point of convergence for multiple signaling processes that are activated during inflammation, p38 MAPK has become a key target for the pharmacological modulation of cytokine production [9]. p38α is believed to be the family member most responsible for regulation of inflammation [12]. The discovery and publication of p38α and a pyridinyl-imidazole-based p38α inhibitor initiated a huge effort to develop p38α inhibitors as potential treatments for inflammatory diseases [9]. Multiple compounds that selectively inhibit p38α and β relative to γ and δ have been demonstrated to have potent anti-inflammatory effects [12]. Thus, many companies have developed p38α inhibitors as potential treatments for inflammatory diseases [9]. Pharmacological inhibitors of p38 have entered Phase II clinical trials for inflammatory conditions such as rheumatoid arthritis, neuropathic pain, atherosclerosis, psoriasis, myelodysplastic syndromes and chronic obstructive pulmonary diseases (COPD) [13, 14]. p38 inhibition is also under consideration as a potential therapy for a number of other conditions, including Crohn's disease, surgery-induced tissue injury and osteoporosis [5, 12]. Additionally, emerging evidence suggests that the potential applications of p38-targeted therapies may broaden to include ischemic heart disease [14], neurodegenerative disease [5] and use as anti-tumor agents in cancer therapy [7, 8].

Biochemical recombinant kinase assays and tumor necrosis factor alpha (TNFα) secretion assays are typically used to evaluate p38 inhibitors, but these assays do not provide insight into subcellular localization or proximal intracellular events [11]. Since phospho-specific antibody detection of phosphorylation at Thr180/Tyr182 provides a well-established measure of cellular p38 activation or inhibition [12], High Content Screening technology represents a particularly useful tool for cell-based analysis of p38 MAPK. An HCS-based approach to p38 screening has been used successfully by several groups for screening both activators and inhibitors of p38 in a variety of cellular models [11, 15, 16].

Application

Millipore's HCS231 p38 MAP Kinase Assay provides a complete solution for identifying and quantifying the phosphorylation state of endogenous cellular p38 MAPK at Thr180/Tyr182 via cellular imaging. The reagents in the kit have been specifically optimized for HCS applications.

The assay is designed to enable visualization and quantitative detection of phosphorylated p38, facilitating the identification and characterization of activators and inhibitors of p38. Key applications of this assay include p38 inhibitor screening, characterization of cellular stress and inflammatory responses, and *in vitro* toxicology applications. 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 for analysis of proximal cellular events, *e.g.*, for upstream or downstream signaling pathway profiling or for simultaneous screening for cytotoxicity.

The assay is immunofluorescence-based, and utilizes a high quality rabbit polyclonal antibody which identifies phospho-p38$^{(Thr180/Tyr182)}$ in human cells. Species cross-reactivity has also been observed with mouse and rat. 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 6), 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 phospho-p38$^{(Thr180/Tyr182)}$, a Cy3-conjugated secondary antibody, Hoechst HCS Nuclear Stain, HCS Fixation Solution, HCS Immunofluorescence Buffer, HCS Wash Buffer, and Plate Sealers. A positive control compound for p38-activation, the antibiotic Anisomycin, and a known p38 inhibitor, SB 202190, 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). Anisomycin, a protein synthesis inhibitor, has been shown to be a strong activator of stress-activated MAP kinases including p38 MAPK, as well as other signal transduction pathways [17, 18]. SB 202190, a pyridinyl imidazole, has been shown to be a potent, specific inhibitor of p38α and p38β MAP kinases [19].

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Kit Components

1. Rabbit Anti-Phospho-p38$^{(Thr180/Tyr182)}$ HCS Primary Antibody, 100X: - (Part No. CS201734) 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. Anisomycin, 2.5 mM in DMSO, 250X: - (Part No. 2007477) 1 vial containing 100 µL.
8. SB 202190, 2.5mM in DMSO, 250X: - (Part No. CS201733) 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.

Related Products Available from Millipore

19-134	p38/SAPK2 Inhibitor (SB 202190)	1 mg
19-135	p38/SAPK2 Inhibitor (SB 203580)	1 mg

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
Anisomycin	Anisomycin	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, Anisomycin and SB 202190 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 compound and dispense into appropriately sized aliquots. Store aliquots at -20ºC.)

Assay Instructions

Note: The HCS231 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, inhibitors, etc.) can be introduced at any point during this culture period, as appropriate for time-course of treatment of interest. Anisomycin is provided as a MAP kinase activation control compound, and SB 202190 as a known p38 inhibitor. 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. Both compounds are provided at 250X concentration (assuming a maximum treatment of 10 µM). Recommended treatment preparation involves half-log (1:√10) serial dilution of the 250X compound in DMSO, followed by dilution in Compound Dilution Buffer to 10X. 10-11 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 for a single compound, 0.8% DMSO for both compounds in a single well). Sample data is provided for 30 minutes of anisomycin treatment at 37°C prior to fixation, or 1 hour of SB 202190 pre-treatment followed by 30 minutes of anisomycin activation.

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 6).

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-Phospho-p38$^{(Thr180/Tyr182)}$ 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.

HCS231 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-Phospho-p38$^{(Thr180/Tyr182)}$ 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 – HCS231 p38 MAP Kinase Assay

Image acquisition and analysis

HCS231 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

HCS231 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.
Phospho-p38$^{(Thr180/Tyr182)}$ Expression	HCS Secondary Antibody, Cy3-conjugated	Nuclear region (600 nm emission channel). Measure Cy3 signal co-localizing with nuclear segmentation. Determine parameters such as average nuclear signal intensity, nuclear:cytoplasmic intensity ratio, etc.	Phospho-p38 expression may be modulated as a result of stress activation, cytokine response, kinase inhibition, etc.

Table 2. Image Acquisition and Analysis Guidelines – HCS231 p38 MAP Kinase Assay

Sample Results

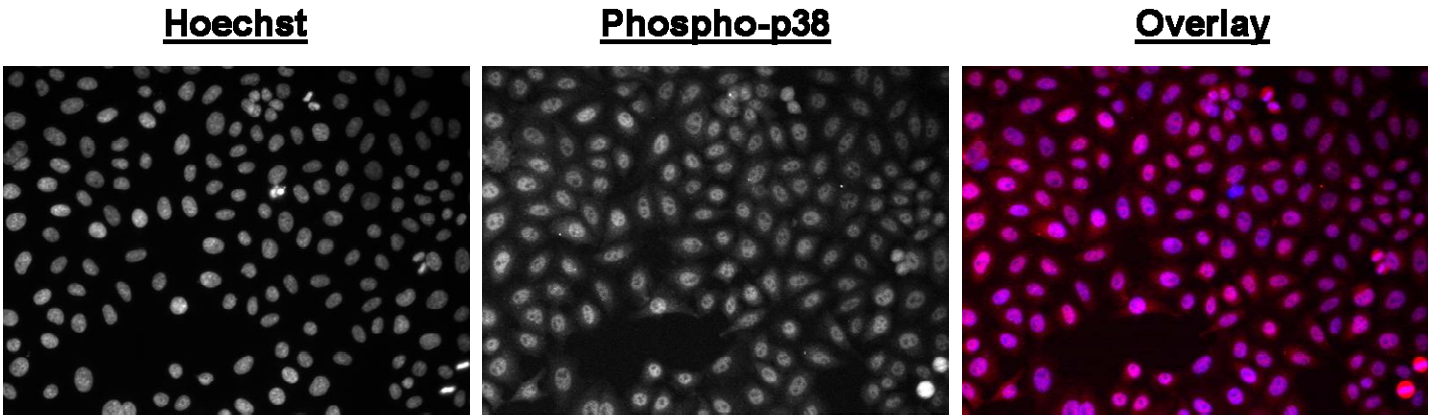

Figure 1. Immunofluorescence of anisomycin-treated HeLa cells.

HeLa cells were plated at 18,000 cells/cm^2 on 96-well plates in growth media and cultured for a total of 48 hours. Cells were treated with 10µM anisomycin for 30 minutes prior to fixation. Cell handling, fixation and immunostaining were performed according to HCS231 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 phospho-p38 fluorescence. *Right panel*: Fused image of Hoechst HCS Nuclear Stain (blue) and phospho-p38 fluorescence (red).

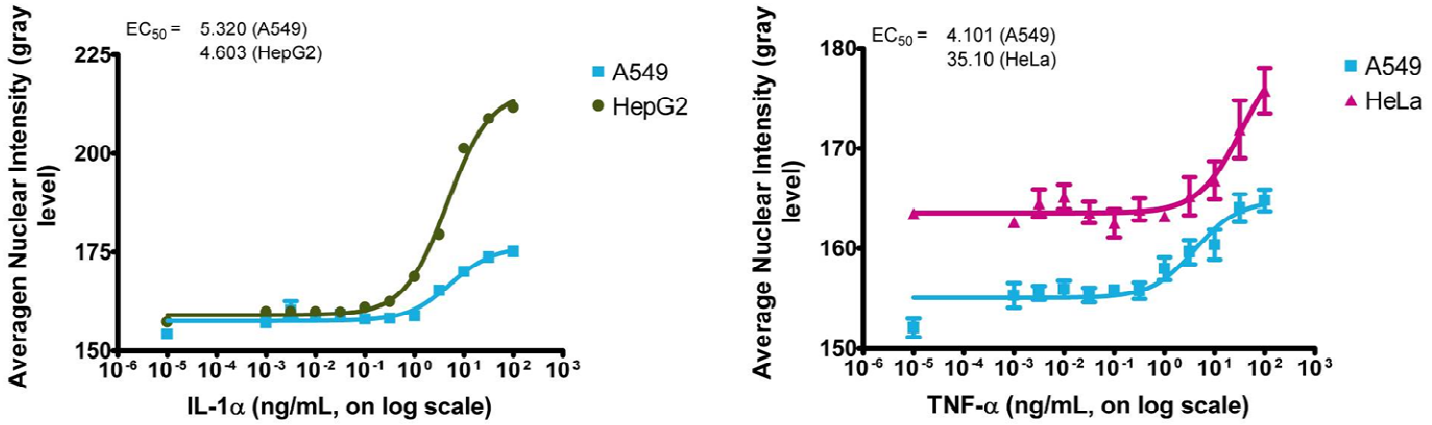

Figure 2. Dose response curves of inflammatory cytokine-induced p38 activation in A549, HeLa and HepG2 cells

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 a total of 48 hours. Cells to be treated with IL-1α or TNF-α were serum-starved for 24 hours prior to cytokine activation. Cells were incubated with serial dilutions of IL-1α (*left panel*, 15 minute treatment, max. concentration = 100 ng/mL) or TNF-α (*right panel*, 15 minute treatment, max. concentration = 100 ng/mL) prior to fixation. Cell handling, fixation and immunostaining were performed according to HCS231 assay protocols. 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. Data presented are mean ± SEM, *n* = 4.

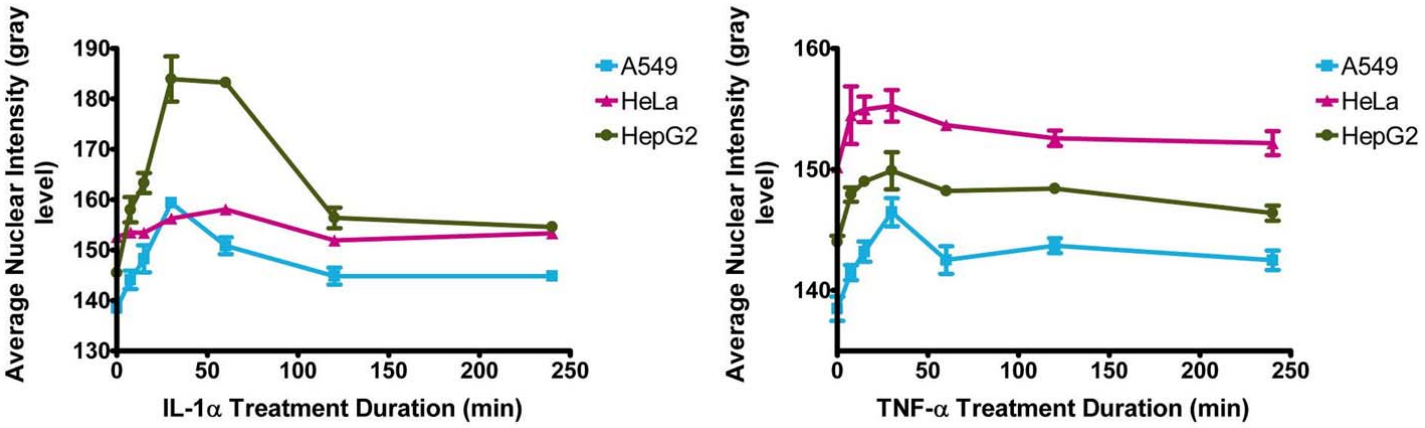

Figure 3. Timecourse of inflammatory cytokine-induced p38 activation in A549, HeLa and HepG2 cells

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. Cells were then serum-starved for an additional 24 hours prior to cytokine activation. Cells were treated with 100 ng/mL IL-1α (*left panel*) or 100 ng/mL TNF-α (*right panel*) for a series of treatment durations prior to fixation. Cell handling, fixation and immunostaining were performed according to HCS231 assay protocols. 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. Data presented are mean ± SEM, *n* = 3. Note peaks in p38 phosphorylation, generally following 30 minutes of cytokine treatment.

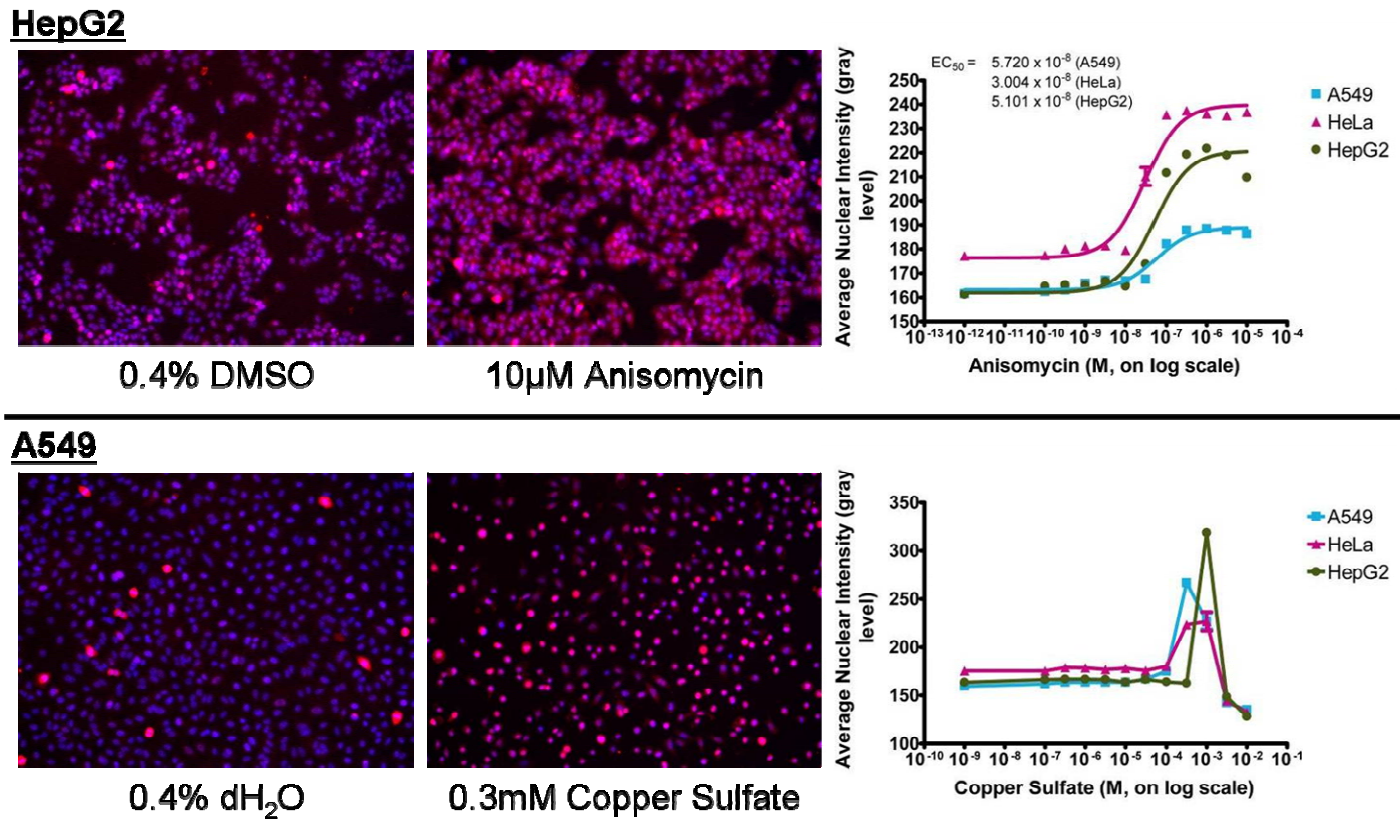

Figure 4. p38 activation screening – dose-response curves of toxic stress-induced p38 activation in A549, HeLa and HepG2 cells

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 a total of 48 hours. Cells were treated with serial dilutions of either anisomycin (*top panel*, 30 minute treatment, max. concentration = 10 µM) or copper (II) sulfate (*bottom panel*, 24 hour treatment, max. concentration = 10 mM) prior to fixation. DMSO and dH_2O were used as vehicle negative controls for anisomycin and copper sulfate, respectively. Cell handling, fixation and immunostaining were performed according to HCS231 assay protocols. 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. Data presented are mean ± SEM, *n* = 4. Images are at 10X, displaying Hoechst HCS Nuclear Stain (blue) and phospho-p38 fluorescence (red).

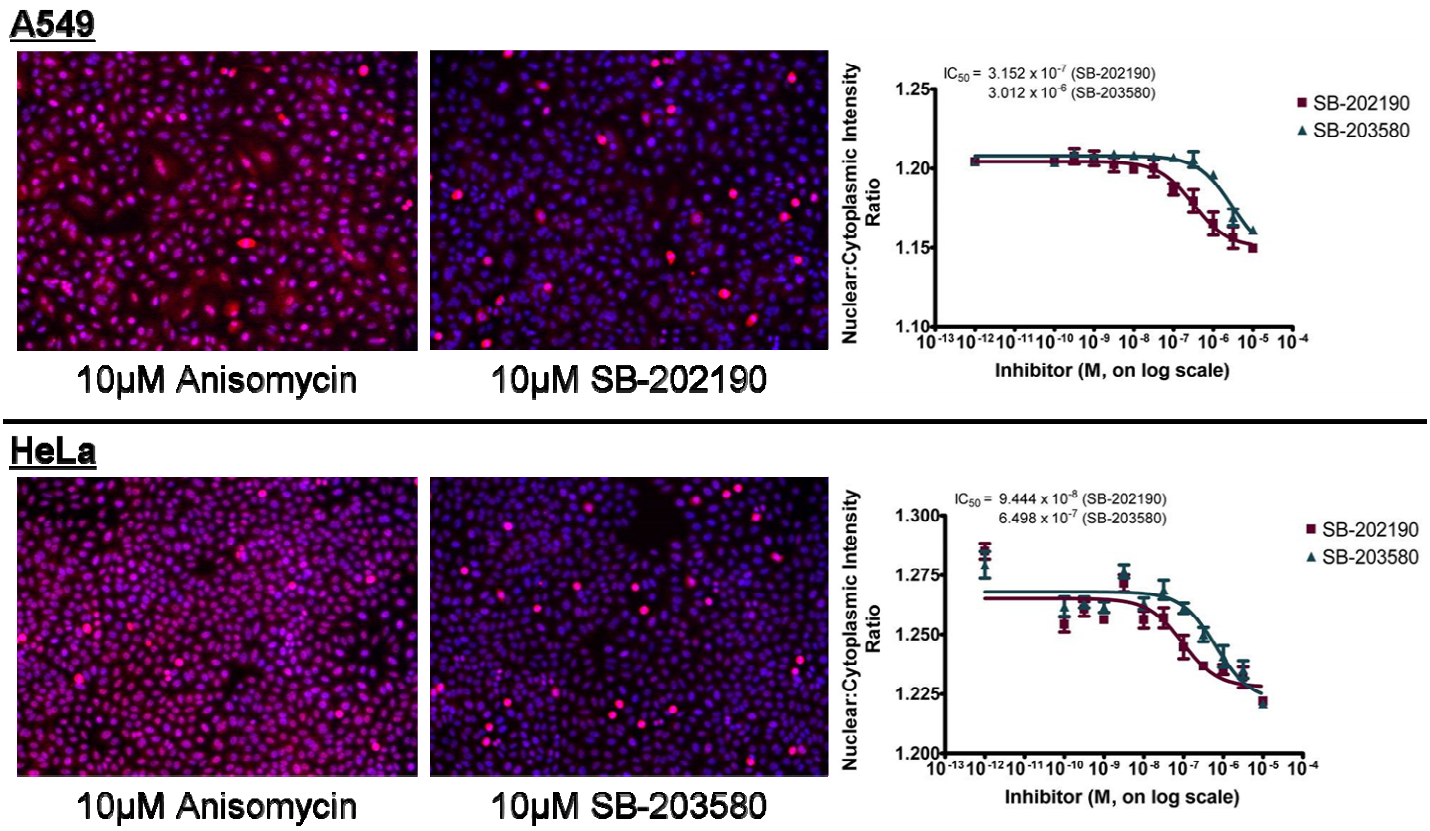

Figure 5. p38 inhibitor screening – quantitation of inhibition of anisomycin-induced p38 activation in A549 and HeLa cells.

A549 (*top panel*) or HeLa (*bottom panel*) cells were plated at 18,000 cells/cm^2 on 96-well plates in growth media and cultured for a total of 48 hours. Cells were pre-incubated with serial dilutions of the p38 inhibitors SB 202190 or SB 203580 (max. concentration = 10 µM) for 1 hour prior to 30 minute treatment with the p38 activator anisomycin (10 µM). Cell handling, fixation and immunostaining were performed according to HCS231 assay protocols. 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. Data presented are mean ± SEM, *n* = 4. Images are at 10X, displaying Hoechst HCS Nuclear Stain (blue) and phospho-p38 fluorescence (red). Note the enhanced inhibitory activity of SB 202190 compared to SB 203580 in both cell types.

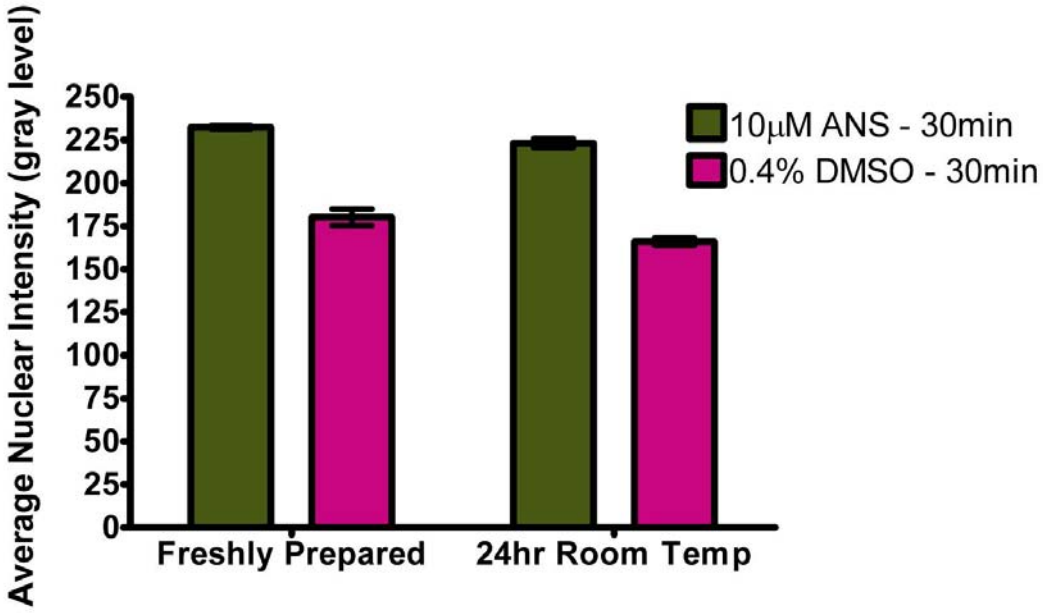

Figure 6. HCS231 p38 MAP Kinase Assay reagent stability.

HeLa cells were plated at 18,000 cells/cm^2 on 96-well plates in growth media and cultured for a total of 48 hours. Cells were treated with 10 µM anisomycin (ANS) or 0.4% DMSO (negative control) for 30 minutes prior to fixation. 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. Average nuclear intensities were measured to observe phospho-p38 expression differences between anisomycin and DMSO-treated cells. Data presented are mean ± SEM; *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.

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