Phospho-Histone H3$^{(Ser\ 10)}$ Assay

(Mouse primary antibody)

For High Content Screening

For 5 x 96-well plates

Cat. No. HCS203

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Not for use in diagnostic procedures.

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Introduction

(i) High Content Screening and Drug Discovery

The development of High Content Screening (HCS) technology represents a major step towards improving the drug discovery and development process [1]. HCS enables the evaluation of multiple biochemical and morphological parameters in cellular systems and enables characterization of the subcellular distribution of fluorescent signals with labeled reagents. By combining the automated imaging of cells in microtiter plates with high-quality detection reagents and powerful image analysis algorithms, scientists can now acquire deeper knowledge of multiple morphological or biochemical pathways at the single-cell level, usually in a single assay, at an early stage in the development of new drugs. 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 systemic responses [2].

(ii) Phospho-Histone H3$^{(Ser10)}$ in Cell Biology and Drug Discovery

Previously considered to be merely packing material for nuclear DNA, histones have recently been more rightly found to have key roles in regulating cellular responses to various stimuli [3]. The N-terminal tail of histone H3 protrudes from the globular nucleosome core and can undergo several different types of epigenetic modifications that influence cellular processes. These modifications include the covalent attachment of methyl or acetyl groups to lysine and arginine amino acids and the phosphorylation of serine or threonine.

Histone H3 phosphorylation plays an important regulatory role in mitosis, transcriptional activation and neoplastic cell transformation [3, 4]. Several kinases, including MAP and Aurora kinases, have been reported to phosphorylate histone H3 at serine 10 following mitogenic stimulation or stress [4, 5]. Since mitosis is accompanied by phosphorylation of histone H3 on serine 10, the presence of phosphorylated histone H3$^{(Ser10)}$ indicates that a cell is mitotic [4]. Upon exit from mitosis, a global dephosphorylation of histone H3 takes place. These phosphorylation and dephosphorylation events are well characterized and accordingly, phospho-histone H3 nuclear expression is widely used as a measure of mitotic index (the percentage of cells in M phase) in flow cytometry and HCS applications [5-9] (see Figure 1).

Due to its important regulatory role in cell proliferation and neoplastic cell transformation, histone H3 has been identified as a crucial target for cancer chemotherapy [3]. Accordingly, HCS of histone H3 phosphorylation is increasingly being used as a tool in drug discovery. HCS assays quantifying

phospho-histone H3 levels have recently been utilized to characterize Aurora kinase inhibitors [5]. Additionally, histone H3 phosphorylation has been used as a readout in cell cycle inhibitor profiling studies [7-9], illustrating the value of this approach in drug discovery applications.

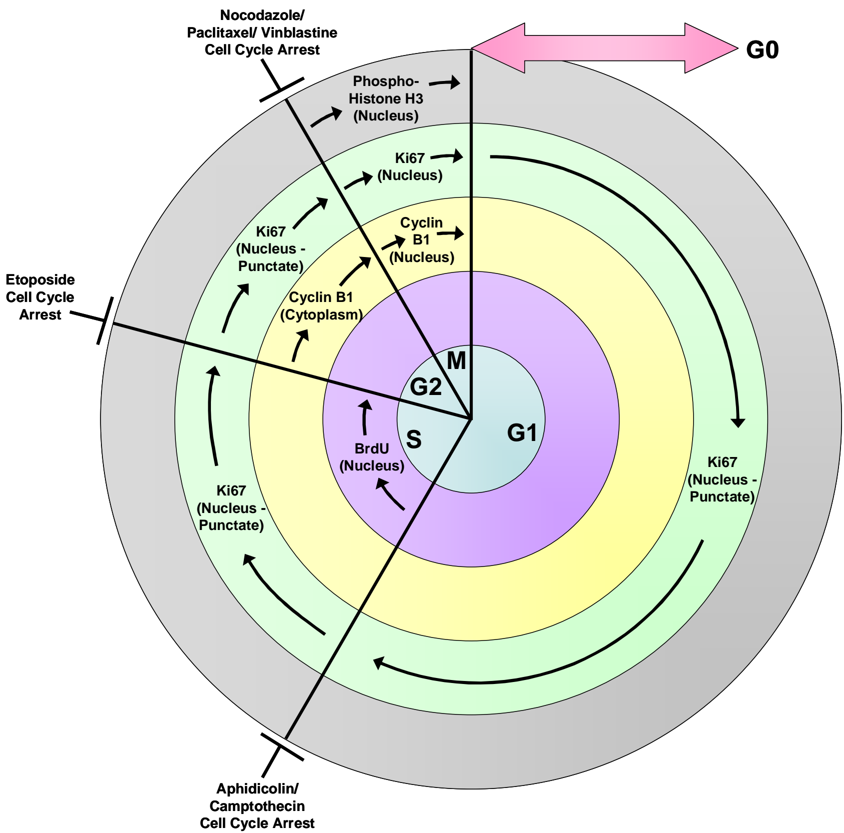

Figure 1. Eukaryotic cell cycle marker expression and effects of cell cycle phase blocking agents.

Phospho-histone H3$^{(Ser\ 10)}$ is expressed in the nuclei of cells during M-phase (mitosis). Compounds such as etoposide (a DNA topoisomerase II inhibitor) and vinblastine sulfate (a microtubule inhibitor) are capable of arresting the cell cycle at S/G2 and G2/M phases, respectively.

Application

Millipore's HCS203 assay provides a complete solution for identifying and quantifying phospho-histone H3$^{(Ser10)}$ expression in cellular imaging applications. The reagents in the kit have been specifically optimized for HCS applications.

The assay is designed to enable quantification and characterization of mitotic cells and identification of compounds that inhibit or induce mitosis via detection of phospho-histone H3$^{(Ser10)}$ staining. The nuclear dye (Hoechst) may be used for measurements of cell number, DNA content and nuclear size. The kit is suitable for many applications, including cancer drug screening, mitotic index determination, and Aurora kinase profiling.

The assay is immunofluorescence-based, and utilizes a high quality mouse monoclonal antibody which identifies histone H3 phosphorylated at Serine 10, with high sensitivity and specificity. Cross-reactivity in a wide variety of mammalian species is expected, including human and rat.

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 1), 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-histone H3$^{(Ser10)}$, 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 vinblastine sulfate 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 Procedure). Vinblastine sulfate, a microtubule inhibitor, and etoposide, a DNA topoisomerase II inhibitor, have both been demonstrated to induce mitotic block [10, 11], at G2/M phase and S/G2 phase, respectively. G2/M phase block causes an accumulation of phospho-histone H3$^{(Ser\ 10)}$ expressing cells, while S/G2 phase block diminishes the proportion of cells reaching M phase (see Figure 1).

Kit Components

1. Mouse Anti-Phospho-Histone H3$^{(Ser10)}$ HCS Primary Antibody, 100X: (Part No. CS200436) 1 vial containing 300 µL.
2. HCS Secondary Antibody (donkey anti-mouse IgG, Cy3 conjugate), 200X: (Part No. CS200437) 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. Etoposide (S/G2 Arrest Control Compound), 250X: (Part No. CS200439) 1 vial containing 100 µL.
8. Vinblastine Sulfate (G2/M Arrest Control Compound), 250X: (Part No. CS200440) 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 Required But Not Provided

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) or A549 (human lung carcinoma, ATCC #CCL-185).
3. Tissue culture instruments/supplies (including 37°C 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. Imaging system must be equipped with beam-splitters and filters capable of reading emission spectra in the blue and red ranges. Example filter ranges are shown in Table 2 below.

Warnings and 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
Etoposide	Etoposide	Toxic
Vinblastine Sulfate	Vinblastine sulfate	Harmful
DMSO	Dimethyl sulfoxide	Combustible, readily absorbed through skin

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

Stability and Storage

When stored under the conditions indicated on the labels, the kit components are stable up to the expiration date. HCS Fixation Solution, HCS Immunofluorescence Buffer, HCS Wash Buffer, DMSO, Compound Dilution Buffer, and Plate Sealers should be stored at 2-8ºC. HCS Primary Antibody, HCS Secondary Antibody, Hoechst HCS Nuclear Stain, Etoposide and Vinblastine Sulfate should be stored at -20ºC, avoiding repeated freeze/thaw cycles. (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.)

Discard any remaining reagents after the expiration date.

Assay Procedure

Note: *The HCS203 assay protocol has been optimized for HeLa human cervical carcinoma (ATCC #CCL-2) and A549 human lung carcinoma (ATCC #CCL-185) cells. However, this kit is suitable for proliferation/cell cycle 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 or A549 cells in growth media until ~70-80% confluent.

2. Detach cells from culture flasks/plates via method appropriate for cell type of interest. Adjust cell density to 5-7 x 10^4 cells/mL in growth media. Add 90 µL of this cell suspension (4500-6300 cells) to each well (for a 96-well plate, 4500-6300 cells/well is approximately equivalent to 15,000-21,000 cells/cm^2 of well surface). 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 48 hours.

3. Cell treatments (cell-cycle arrest control compounds, test compounds, etc.) can be introduced at any point during this culture period, as appropriate for time-course of treatment of interest. Etoposide (S/G2 arrest) and vinblastine sulfate (G2/M arrest) are provided as cell-cycle arrest 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 100µM and 1µM for etoposide and vinblastine, respectively). 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. 10µL of each treatment may then be added to the 90µL of culture media already present in each well, for a final 1X concentration (0.4% DMSO). Sample data is provided for 4 hours 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 if long-duration automated handling is required (see Figure 1).*

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 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 Mouse Anti-Phospho-Histone $H3^{(Ser10)}$ 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 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.

HCS203 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)**
Mouse Anti-Phospho-Histone H3$^{(Ser10)}$ 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)**
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 - HCS203 Mouse Anti-Phospho-Histone H3$^{(Ser10)}$ Assay

Image Acquisition and Analysis

HCS203 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-mouse IgG	10X	535/50	600/50

HCS203 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 for cell cycle phase are also possible.	Use total cell number to determine percentage of phospho-histone $H3^{(Ser\ 10)}$-expressing cells.
Phospho-Histone $H3^{(Ser10)}$ Expression	HCS Secondary Antibody, Cy3-conjugated	Nuclear region (600 nm emission channel). Measure Cy3 signal co-localizing with nuclear segmentation. Threshold for positive (+) cells displaying Cy3 nuclear intensity above a user-defined limit. Determine parameters such as percentage/number of (+) or negative (-) cells, etc.	Phospho-histone $H3^{(Ser\ 10)}$ expression may be modulated as a result of proliferation rate changes, cell cycle arrest, etc.

Table 2. Image Acquisition and Analysis Guidelines – HCS203 Mouse Anti-Phospho-Histone $H3^{(Ser10)}$ Assay

Sample Results

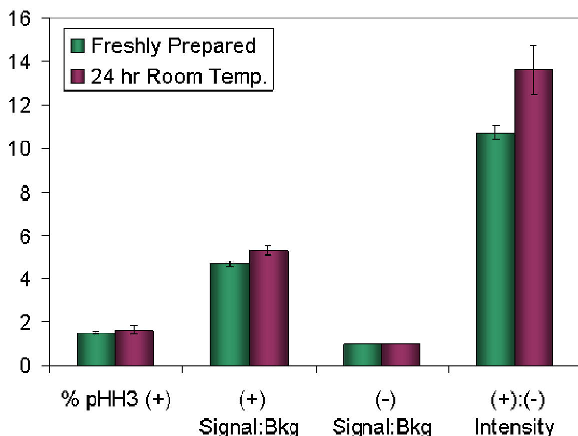

Figure 2. HCS203 Mouse Anti-Phospho-Histone H3$^{(Ser10)}$ Assay reagent stability.

A549 cells were seeded at 18,000 cells/cm^2 on 96-well plates in growth media and cultured for 48 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 (3.3) at 10X magnification (10 fields/well) and analyzed using the GE IN Cell Analyzer 1000 Workstation (3.4) Multi Target Analysis algorithm. The percentage of phospho-histone H3-positive (% pHH3 (+)) cells was determined by counting the number of Hoechst-segmented nuclei thresholded above an empirically-determined Cy3 nuclear intensity. Signal:background ratios for positive (+) and negative (-) cells were calculated by comparing Cy3 intensities in the nucleus and cytoplasm (as defined by a 3 µm wide perimeter surrounding the nucleus). (+):(-) Cy3 nuclear intensity ratios were calculated as a measure of contrast between phospho-histone H3-expressing and non-expressing cells. Data presented are mean ± SD; $n = 3$. No diminishment in signal quality was observed between freshly prepared and 24 hour samples.

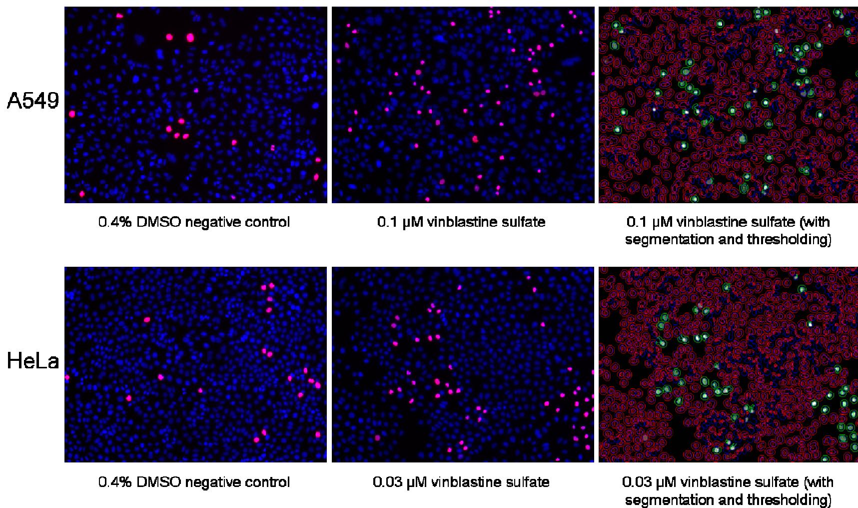

Figure 3. Immunofluorescence of untreated and cell cycle-arrested A549 and HeLa cells.

A549 or HeLa cells were plated at 18,000 cells/cm^2 on 96-well plates in growth media and cultured for 48 hours. Cells were treated for the last 4 hours of culture with either vinblastine sulfate (a G2/M phase cell cycle arresting agent) or 0.4% DMSO (negative control). Cell handling, fixation and immunostaning were performed as according to HCS203 assay protocols. Cells were imaged on the GE IN Cell Analyzer 1000 (3.3) at 10X objective magnification. *Left and center panels*: Merged images of staining with Hoechst HCS Nuclear Stain (blue) and Mouse Anti-Phospho-Histone H3$^{(Ser10)}$ HCS Primary/Secondary Antibodies (red). Note the increase in the percentage of phospho-histone H3-expressing cells following vinblastine sulfate-induced arrest of cells in M phase. *Right panel*: Monochromatic images of phospho-histone H3 fluorescence overlaid with segmentation and thresholding generated by the GE IN Cell Analyzer 1000 (3.4) Workstation Multi Target Analysis algorithm (inner ring = nuclear segmentation, outer ring = 3 µm wide nuclear perimeter for cytoplasmic analysis; phospho-histone H3 (-) cells outlined in red, (+) cells outlined in green).

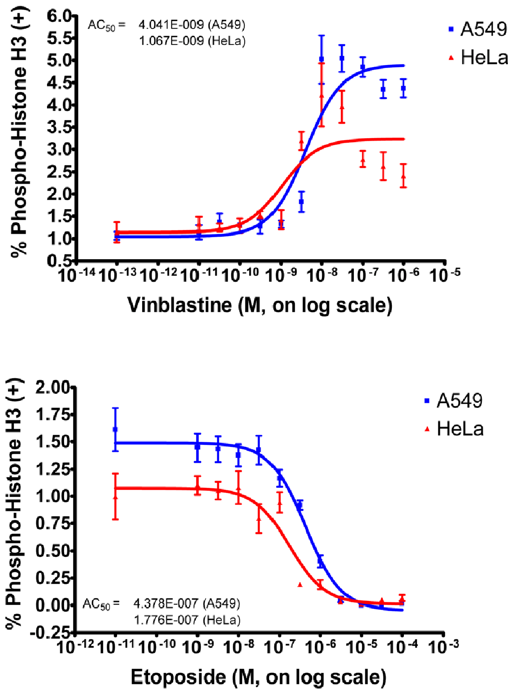

Figure 4. Dose response of A549 and HeLa cells to cell-cycle arresting agents.

A549 or HeLa cells were plated at 18,000 cells/cm^2 on 96-well plates in growth media and cultured for 48 hours. Cells were treated for the last 4 hours of culture with serial dilutions of either vinblastine sulfate (*top panel*, G2/M phase arrest, max. concentration = 1 μM) or etoposide (*bottom panel*, S/G2 phase arrest, max. concentration = 100 μM). Cell handling, fixation and immunostaning were performed as according to HCS203 assay protocols. Cells were imaged on the GE IN Cell Analyzer 1000 (3.3) at 10X (10 fields/well) and analyzed (nuclear segmentation and (+)/(-) intensity thresholding) using the GE IN Cell Analyzer 1000 Workstation (3.4) Multi Target Analysis algorithm. Data presented are mean ± SEM, $n = 4$.

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 0.2µm 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.

Poor nuclear segmentation/ intensity thresholding 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). Cy3 nuclear intensity thresholding may vary between experiments, depending on cell type, exposure time, duration between staining and imaging, etc. – adjust threshold intensity between (+) and (-) cells for each experiment accordingly.
No/low percentage of phospho-histone H3-expressing cells	Percentages of cells expressing phospho-histone H3 will vary with cell type, doubling time, seeding density, time in culture, passage number, etc. Optimize culture conditions to maximize expression.
No dose response observed/partial response curve	Efficacy of cell cycle arrest 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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Cat No. HCS203

12/2009
Revision C, 4003713