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

& Ki-67 Assay

For High Content Screening

For 5 x 96-well plates

Cat. No. HCS209

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

USA & Canada
Phone: +1(800) 437-7500 • Fax: +1 (951) 676-9209
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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) Role of Phospho-Histone H3$^{(Ser10)}$ and Ki-67 in the cell cycle and proliferation and their significance for Drug Discovery

Phospho-Histone H3$^{(Ser10)}$

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 [6-8], illustrating the value of this approach in drug discovery applications.

Ki-67

The Ki-67 antigen (Ki-67) is a classic marker of cellular proliferation and has found widespread application in diagnostic, research and drug discovery applications [9-11]. The Ki-67 antigen was originally defined by the monoclonal antibody Ki-67, the name being derived from the city of origin (Kiel) and the number of the original clone in the 96-well plate [12]. Ki-67 antigen is preferentially expressed during late G1, S, G2 and M phase of the cell cycle, while resting, non-cycling cells (G0 phase) lack Ki-67 expression. Thus, Ki-67 is commonly used as a proliferation marker [9].

The proliferation index, defined as the percentage of proliferating cells in a population, is an important parameter in evaluation of the *in vitro* stimulation potency of growth factors and the antiproliferative activity of drugs [7], as well as being a sensitive marker of cytotoxicity [13, 14]. Signals that regulate cell proliferation represent potential targets for new cancer treatment strategies. Indeed, one of the most widely pursued strategies in drug discovery is the identification and characterization of therapeutic compounds able to inhibit cell proliferation [6, 15-17]. Additionally, application of Ki-67 as a pharmacodynamic marker of the effectiveness of cancer therapy has been reported to hold great promise for rapid evaluation of new drugs [10]. High-Content Screening is now well-established as an ideal tool with which to characterize cellular proliferation [6, 7, 13-17].

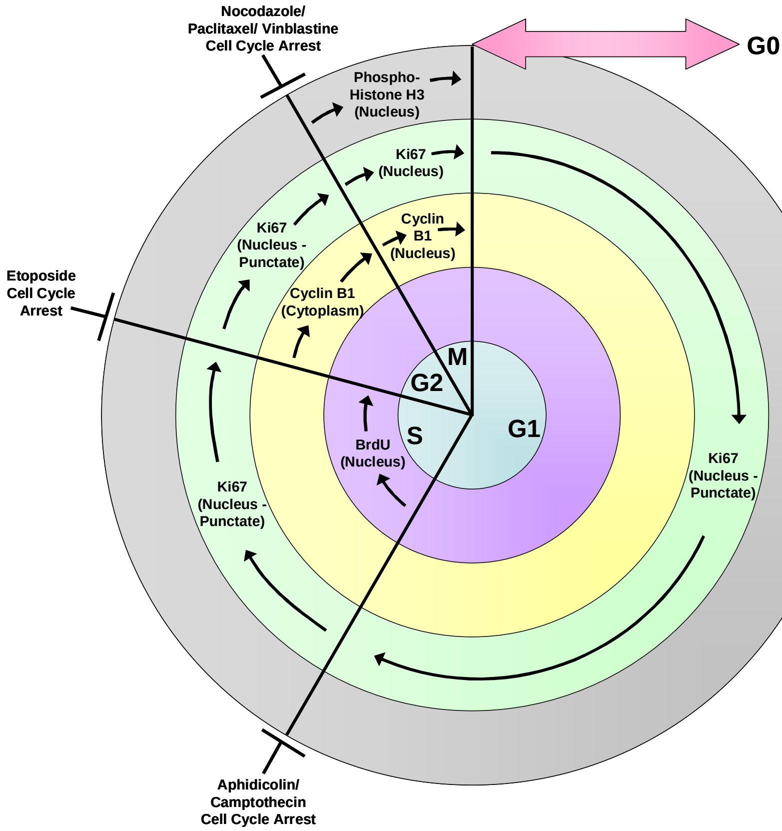

Figure 1. Eukaryotic cell cycle marker expression and effects of cell cycle phase blocking agents.

Phospho-histone H3$^{(Ser\ 10)}$ is expressed in the nuclei of cells during M-phase (mitosis). Ki-67 is expressed throughout all phases of the cell cycle except G0. The protein exhibits punctate nuclear localization during the G1, S and G2 phases, then becomes homogeneously expressed throughout the nucleus during M phase. Etoposide (a DNA topoisomerase II inhibitor) and camptothecin (a DNA topoisomerase I inhibitor) are capable of arresting the cell cycle at S/G2 and G1/S phase, respectively. Paclitaxel (a microtubule stabilizer) and vinblastine sulfate (a microtubule inhibitor) are capable of arresting the cell cycle at G2/M phase.

Application

Millipore's HCS209 assay provides a complete immunofluorescence-based solution for identifying and quantifying phospho-histone $H3^{(Ser10)}$ and Ki-67 expression in cellular imaging applications. The assay may be used to study agents which modulate the cell cycle, mitotic index, for cancer drug screening, and for *in vitro* toxicology studies. The reagents in the kit have been specifically optimized for HCS applications.

Through phospho-histone $H3^{(Ser10)}$ immunostaining, the assay enables quantification and characterization of mitotic cells and identification of compounds that inhibit or induce mitosis. Phospho-histone $H3^{(Ser10)}$ may also be used as a readout in Aurora Kinase profiling. Though Ki-67 immunostaining, the assay enables quantification of the proliferation index, characterization of proliferating cells and classification of compounds that inhibit or induce cellular proliferation. .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 correlating mitotic index, cell cycle, and/or proliferation dependency with drug efficacy or toxicity.

Two high quality primary antibodies against phospho-histone $H3^{(Ser10)}$ and Ki-67 are provided with this kit. Phospho-histone $H3^{(Ser10)}$ is identified with high sensitivity and specificity in cells from all vertebrate species and drosophila using a rabbit polyclonal antibody which identifies histone H3 phosphorylated at Serine 10. Ki-67 is identified with high sensitivity and specificity in human cells using a mouse monoclonal antibody; other species reactivity must be determined 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 2), 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 multi-labeling experiments. The kit includes primary antibodies for phospho-histone $H3^{(Ser10)}$ (rabbit), Ki-67 (mouse), a FITC-conjugated anti-rabbit IgG secondary antibody, a Cy3-conjugated anti-mouse IgG secondary antibody, Hoechst HCS Nuclear Stain, HCS Fixation Solution, HCS Immunofluorescence Buffer, HCS Wash Buffer, and Plate Sealers. Four control compounds, the chemotherapeutic drugs etoposide, paclitaxel, camptothecin and vinblastine sulfate, 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).

Etoposide (a DNA topoisomerase II inhibitor) and camptothecin (a DNA topoisomerase I inhibitor) are capable of arresting the cell cycle at S/G2 and G1/S phase, respectively. Paclitaxel (a microtubule stabilizer) and vinblastine sulfate (a microtubule inhibitor) are capable of arresting the cell cycle at G2/M phase [18-24]. G1/S and S/G2 phase block diminish the proportion of cells reaching G2/M phase (decreased phospho-histone $H3^{(Ser10)}$ expression and decreased homogeneous nuclear Ki-67). G2/M phase block causes an accumulation of cells demonstrating both phospho-histone $H3^{(Ser10)}$ and characteristic M phase-Ki-67 expression.. (See Figure 1).

Kit Components

1. Rabbit Anti-Phospho-Histone H3$^{(Ser10)}$ HCS Primary Antibody, 100X: (Part No. CS201661) 1 vial containing 300 µL.
2. HCS Secondary Antibody (donkey anti-rabbit IgG, FITC conjugate), 200X (Part No. CS201649) 1 vial containing 150 µL.
3. Mouse Anti-Ki-67 HCS Primary Antibody, 100X: (Part No. CS201642) 1 vial containing 300 µL.
4. HCS Secondary Antibody (donkey anti-mouse IgG, Cy3 conjugate), 200X: (Part No. CS200437) 1 vial containing 150 µL.
5. Hoechst HCS Nuclear Stain, 200X: (Part No. CS200438) 1 vial containing 150 µL.
6. HCS Fixation Solution with Phenol Red, 2X: (Part No. CS200434) 1 bottle containing 100 mL.
7. HCS Immunofluorescence Buffer, 1X: (Part No. CS200435) 1 bottle containing 1000 mL.
8. HCS Wash Buffer, 1X: (Part No. 2007643) 1 bottle containing 500 mL.
9. Etoposide (S/G2 Arrest Control Compound), 250X: (Part No. CS200439) 1 vial containing 100 µL.
10. Paclitaxel (G2/M Arrest Control Compound), 250X: (Part No. CS201655) 1 vial containing 100 µL.
11. Camptothecin (G1/S Arrest Control Compound), 250X: (Part No. CS201666) 1 vial containing 100 µL.
12. Vinblastine Sulfate (G2/M Arrest Control Compound), 250X: (Part No. CS200440) 1 vial containing 100 µL.
13. DMSO for Compound Serial Dilution: (Part No. CS200441) 1 bottle containing 10 mL.
14. Compound Dilution Buffer: (Part No. CS200442) 1 bottle containing 25 mL.
15. 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 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, green and red ranges. Detailed image acquisition and analysis guidelines are provided in Table 2.

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
Camptothecin	Camptothecin	Toxic
Vinblastine Sulfate	Vinblastine sulfate	Harmful
Etoposide	Etoposide	Toxic
Paclitaxel	Paclitaxel	Harmful, potential mutagen
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 Antibodies, HCS Secondary Antibodies, Hoechst HCS Nuclear Stain, Camptothecin, Vinblastine Sulfate, Etoposide and Paclitaxel 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 HCS209 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), camptothecin (G1/S arrest), vinblastine sulfate and paclitaxel (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 100 µM (etoposide), 10 µM (camptothecin), and 1 µM (vinblastine and paclitaxel)). 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 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 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 (see Figure 2).

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-Histone $H3^{(Ser10)}$/Mouse Anti-Ki-67 HCS Primary Antibodies (6 mL/96-well plate) as follows: Add 60 µL of <u>each</u> thawed primary antibody to 5.88 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 FITC/Cy3 HCS Secondary Antibodies/Hoechst HCS Nuclear Stain (6 mL/96-well plate) as follows: Add 30 µL of <u>each</u> thawed secondary antibody and 30 µL of thawed Hoechst HCS Nuclear Stain to 5.91 mL of HCS Immunofluorescence Buffer (see Table 1). Mix well, protecting solution from light. Remove previous HCS Immunofluorescence Buffer rinse. Add 50 µL of secondary antibody/nuclear stain solution to each well 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.

HCS209 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-Histone H3$^{(Ser10)}$ HCS Primary Antibody	1:100	0.5 µL	60 µL
Mouse Anti-Ki-67 HCS Primary Antibody	1:100	0.5 µL	60 µL
HCS Immunofluorescence Buffer	None	49 µL	5.88 mL (5880 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)**
FITC-Donkey Anti-Rabbit HCS Secondary Antibody	1:200	0.25 µL	30 µL
Cy3-Donkey Anti-Mouse 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	49.25 µL	5.91 mL (5910 µL)

Table 1. Detection Reagent Specifications - HCS209 Rabbit Anti-Phospho-Histone H3$^{(Ser10)}$/Mouse Anti-Ki-67 Assay

Image Acquisition and Analysis

HCS209 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, FITC-donkey anti-rabbit IgG	10X	480/40	535/50
HCS Secondary Antibody, Cy3-donkey anti-mouse IgG	10X	535/50	600/50

HCS209 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 $H3^{(Ser\ 10)}$- or Ki-67 (punctate and/or homogeneous)-expressing cells.
Phospho-Histone $H3^{(Ser10)}$ Expression	HCS Secondary Antibody, FITC-conjugated	Nuclear region (535 nm emission channel). Measure FITC signal co-localizing with nuclear segmentation. Threshold for positive (+) cells displaying FITC nuclear intensity above a user-defined limit. Determine	Phospho-histone $H3^{(Ser\ 10)}$ expression may be modulated as a result of proliferation rate changes, cell cycle arrest, etc.

		parameters such as percentage/number of (+) or negative (-) cells, etc.	
Ki-67 Expression	HCS Secondary Antibody, Cy3-conjugated	Nuclear region (600 nm emission channel). Measure Cy3 signal co-localizing with nuclear segmentation. Threshold for (+)/(-) cells displaying Cy3 nuclear intensity above/below a user-defined limit. Limits will likely differ for thresholding of cells displaying punctate (G1/S/G2) vs. homogeneous (M) expression. Determine parameters such as percentage/number of (+) or (-) cells, etc.	G0, G1/S/G2 and M phase Ki-67 expression may be modulated as a result of proliferation rate changes, cell cycle arrest, etc.

Table 2. Image Acquisition and Analysis Guidelines – HCS209 Rabbit Anti-Phospho-Histone H3$^{(Ser10)}$/Mouse Anti-Ki-67 Assay

Sample Results

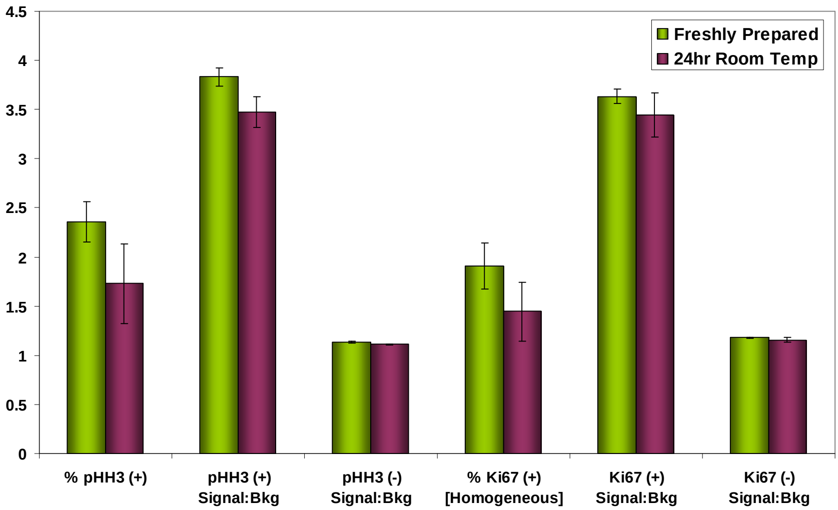

Figure 2. HCS209 Rabbit Anti-Phospho-Histone H3$^{(Ser10)}$/Mouse Ki-67 Assay reagent stability.

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 cells expressing phospho-histone H3 or homogeneous nuclear Ki-67 (M phase) was determined by counting the number of Hoechst-segmented nuclei thresholded above an empirically-determined FITC or Cy3 nuclear intensity, respectively. Signal:background ratios for positive (+) and negative (-) cells were calculated by comparing fluorescence intensities in the nucleus and cytoplasm (as defined by a 3 µm wide perimeter surrounding the nucleus). Data presented are mean ± SD; *n* = 3. Significant differences in signal quality were not observed between freshly prepared and 24 hour samples.

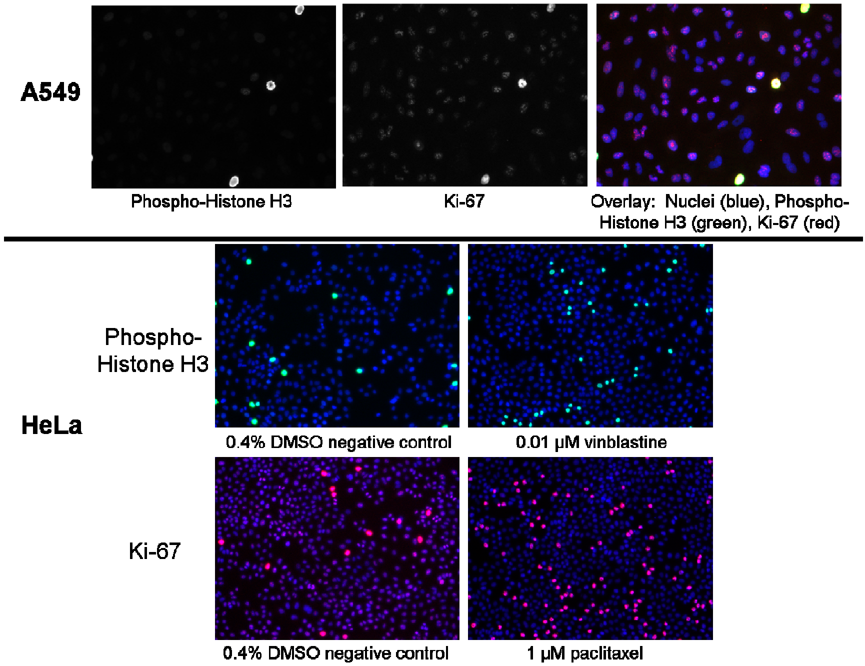

Figure 3. Immunofluorescence of untreated and cell cycle-arrested A549 and HeLa cells.

A549 or HeLa cells were plated at 18,000 cells/cm^2 on 96-well plates in growth media and cultured for 48 hours. HeLa cells were treated for the last 4 hours of culture with either vinblastine sulfate or paclitaxel (G2/M phase cell cycle arresting agents) or 0.4% DMSO (negative control). Cell handling, fixation and immunostaining were performed according to HCS209 assay protocols. Cells were imaged on the GE IN Cell Analyzer 1000 (3.3). *Top panel*: Monochromatic images of untreated A549 cells displaying phospho-histone H3 or Ki-67 fluorescence. Merging with Hoechst nuclear fluorescence results in a three-color overlay (20X objective magnification). *Bottom panel:* Merged images of staining with Hoechst HCS Nuclear Stain (blue) and Rabbit Anti-Phospho-Histone H3$^{(Ser10)}$ HCS Primary/Secondary Antibodies (green) or Mouse Anti-Ki-67 HCS Primary/Secondary Antibodies (red). Note the increase in the percentage of cells expressing phospho-histone H3 or homogeneous nuclear Ki-67 following vinblastine sulfate- or paclitaxel-induced arrest of cells in M phase, respectively (10X objective magnification).

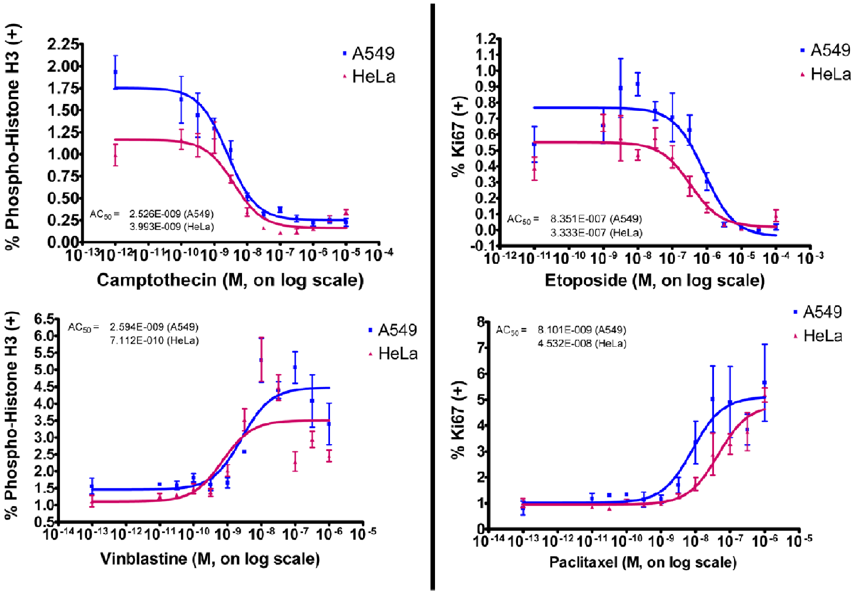

Figure 4. Phospho-histone H3 and homogeneous nuclear Ki-67 dose responses of A549 and HeLa cells to cell cycle-arresting agents.

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 camptothecin or etoposide (G1/S and S/G2 phase arrest, max. concentration = 10 µM and 100 µM, respectively) or vinblastine sulfate or paclitaxel (G2/M phase arrest, max. concentration = 1 µM). Cell handling, fixation and immunostaining were performed according to HCS209 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 for phospho-histone H3 (*left panel*) or homogeneous nuclear Ki-67 (*right panel*)) using the GE IN Cell Analyzer 1000 Workstation (3.4) Multi Target Analysis algorithm. Data presented are mean ± SEM, n = 4.

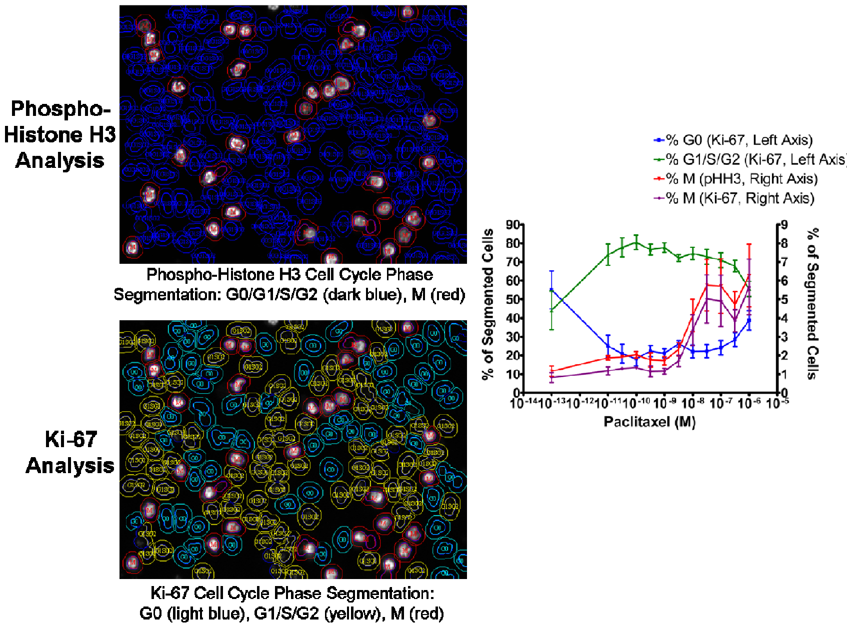

Figure 5. Cell cycle phase analysis of paclitaxel-treated A549 cells.

A549 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 paclitaxel (G2/M phase arrest, max. concentration = 1 µM). Cell handling, fixation and immunostaining were performed according to HCS209 assay protocols. *Left/top panel*: 20X monochromatic image 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). Segmented cells were classified for nuclear phospho-histone H3 expression (M-phase) or no expression (G0, G1, S or G2) using an empirically-determined intensity threshold. *Left/bottom panel*: 20X monochromatic image of Ki-67 fluorescence overlaid with segmentation and thresholding. Segmented cells were classified for homogeneous nuclear Ki-67 expression (M), punctate nuclear expression (G1, S or G2) and no expression (G0) using empirically-determined intensity thresholds. *Right panel*: The percentages of cells in each of the phase segments are plotted as a function of paclitaxel concentration. Data presented are mean ± SEM, *n* = 4, generated using 10X images from the GE IN Cell Analyzer 1000 (3.3) (10 fields/well).

Troubleshooting

Problem	**Potential Explanations/Solutions**
Weak FITC/Cy3/ Hoechst signal	Improper storage or preparation of primary/secondary antibodies 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 FITC/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). FITC/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/Ki-67-expressing cells	Percentages of cells expressing phospho-histone H3 or Ki-67 (homogeneously or punctate within the nucleus) 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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