



High Content Screening Toolkit

For Use With Rabbit Primary Antibodies

For 5 x 96- or 384-well plates

Cat. No. HCS215

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

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Introduction

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 enables characterization of the subcellular distribution of fluorescent signals with labeled reagents. By combining the automated imaging of cells in microtiter plates with validated 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 [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 systemic 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 High Content Screening assays, the sample preparation protocol must be highly reproducible, and the reagents must exhibit minimal assay-to-assay variability.

Application

Millipore's HCS215 High Content Screening Toolkit provides a set of validated reagents to enable the end-user to create a reproducible HCS assay using any mouse primary antibody of their choice. The superior reagents provided with this kit allow the user to standardize their HCS assays, minimize assay-to-assay variability, and reproducibly generate images with a high signal-to-background ratio.

The Toolkit comprises a portfolio of validated cell staining reagents which have been specifically optimized for HCS applications. These reagents may also be used for fluorescence microscopy applications. The kit includes a FITC-conjugated anti-rabbit IgG antibody, a Cy3-conjugated anti-rabbit IgG antibody, Hoechst HCS Nuclear Stain, HCS Fixation Solution, HCS Immunofluorescence Buffer (a complete solution for blocking and permeabilization purposes), HCS Wash Buffer, and Plate Sealers.

The FITC- and Cy3-conjugated secondary antibodies provide the end-user with a choice of fluorophores for detection, and facilitate integration of a new parameter into a pre-existing assay. These secondary antibodies may be used with any mouse antibody specific for the target of interest. The nuclear dye (Hoechst 33342) may be used for measurements of cell number, DNA content and nuclear size. The suggested sample preparation and processing protocol takes less than 2.5 hrs after fixation. Reagents provided are sufficient for 5 x 96- or 384-well microplates.

As an added benefit, working solutions of the reagents have been demonstrated to be stable for at least 24 hours at room temperature (Figure 2), enabling large-scale screening applications. End-users must independently confirm the room temperature stability of their own primary antibody solutions of interest.

Note: Both secondary antibodies in this kit have been adsorbed against human, bovine, horse, goat, sheep, rat, chicken, guinea pig, hamster and mouse serum proteins, to minimize naturally occurring cross-reactivities.

Kit Components

1. HCS Secondary Antibody (donkey anti-rabbit IgG, FITC conjugate), 200X (Part No. CS201649) 1 vial containing 150 μ L.
2. HCS Secondary Antibody (donkey anti-rabbit IgG, Cy3 conjugate), 200X: (Part No. CS201659) 1 vial containing 150 μ L.
3. Hoechst HCS Nuclear Stain, 200X: (Part No. CS200438) 1 vial containing 150 μ L.
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. Plate Sealers: (Part No. CS200443) 10 each.

Materials Required But Not Provided

1. Rabbit primary antibody of choice.
2. Sterile, tissue culture-treated black/clear bottom microplates suitable for High-Content Imaging.
3. Cell type for assay.
4. Tissue culture instruments/supplies (including 37°C incubator, growth media, flasks/plates, detachment buffer, etc.) for cell type of interest.
5. 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, green or 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

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 and Plate Sealers should be stored at 2-8°C. HCS Secondary Antibodies and Hoechst HCS Nuclear Stain should be stored at -20°C, avoiding repeated freeze/thaw cycles. (Note: If kit is expected to be used for multiple experiments, rather than a single use, thaw antibodies and nuclear stain and dispense into appropriately sized aliquots. Store aliquots at -20°C.)

Discard any remaining reagents after the expiration date.

Suggested Protocol for Cell Fixation and 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 included in the kit are stable for at least 24 hours at RT, should long-duration automated handling be required (see Figure 2).

1. Prior to staining, pre-warm HCS Fixation Solution (2X) to room temperature (RT) or 37°C if desired (12 mL/96-well plate). In a chemical fume hood, add 100 μ L/well directly to culture media (assuming 100 μ L culture media volume) and allow to fix for 30 min at RT. Remove fixative/media. If proceeding immediately to staining, rinse each well twice with 200 μ L of HCS Immunofluorescence Buffer. Alternatively, if plates are to be stained at a later time, rinse twice with 200 μ L of Wash Buffer, then leave second rinse volume in wells and store plates tightly sealed at 4°C until staining.
2. If fixed samples have been stored at 4°C prior to staining, rinse twice with 200 μ L HCS Immunofluorescence Buffer before proceeding with staining.
3. Prepare working solution of primary antibody (in HCS Immunofluorescence buffer). Mix well. Remove previous Immunofluorescence Buffer rinse. Add 50 μ L of primary antibody solution to each well and incubate for 1 hour at RT.
4. Remove primary antibody solution. Rinse three times with 200 μ L HCS Immunofluorescence Buffer.
5. Prepare working solution of HCS Secondary Antibody/Hoechst HCS Nuclear Stain (6 mL/96-well plate) as follows: Add 30 μ L of thawed secondary antibody and 30 μ L 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 μ L of HCS Secondary Antibody/Hoechst HCS Nuclear Stain solution and incubate for 1 hour at RT, protected from light.
6. Remove HCS Secondary Antibody/Hoechst HCS Nuclear Stain solution. Rinse twice with 200 μ L HCS Immunofluorescence Buffer.
7. Remove previous HCS Immunofluorescence Buffer rinse. Rinse twice with 200 μ L of HCS Wash Buffer, leaving second rinse volume in wells.
8. Seal plate and image immediately, or store plate at 4°C protected from light until ready for imaging.

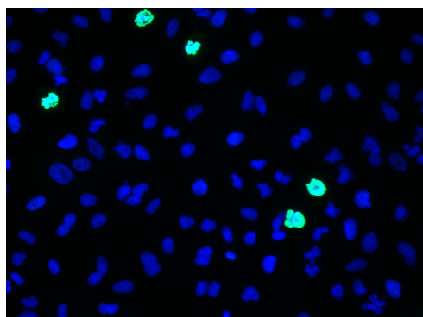
HCS215 Secondary Detection Reagent Specifications			
<i>Secondary Antibody/Hoechst HCS Nuclear Stain working solution</i>			
Reagent	Required dilution of initial reagent	Vol. required for 1 well (50 μL)	Vol. required for 1 plate (6 mL) (includes ~25% excess)
HCS Secondary Antibody	1:200	0.25 μ L	30 μ L
Hoechst HCS Nuclear Stain	1:200	0.25 μ L	30 μ L
HCS Immunofluorescence Buffer	None	49.5 μ L	5.94 mL (5940 μ L)

Table 1. Secondary Detection Reagent Specifications

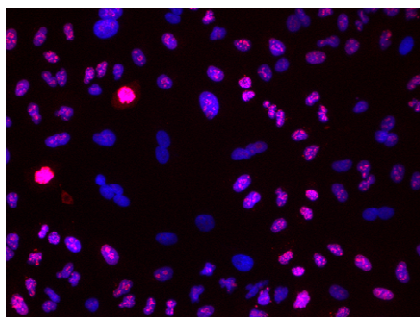
HCS215 Excitation/Emission Filter Guidelines		
Detection Reagent	Excitation Filter Range [peak/bandwidth (nm)]	Emission Filter Range [peak/bandwidth (nm)]
Hoechst HCS Nuclear Stain	360/40	460/40 (or 535/50 if necessary)
HCS Secondary Antibody, FITC-donkey anti-rabbit IgG	480/40	535/50
HCS Secondary Antibody, Cy3-donkey anti-rabbit IgG	535/50	600/50

Table 2. Excitation/Emission Filter Guidelines for Hoechst, FITC and Cy3

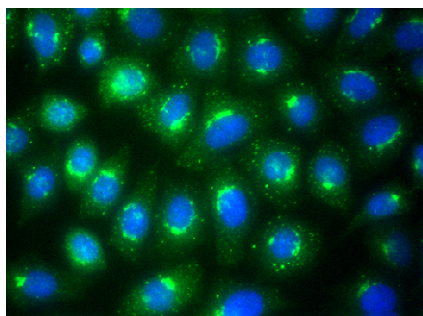
Sample Results



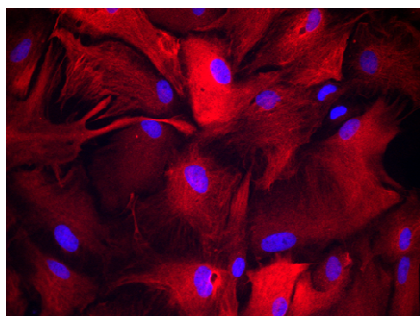
**Rabbit Anti-Phospho-Histone H3
(A549 Cells, 20X Objective
Magnification)**



**Rabbit Anti-Ki-67 (A549 Cells, 20X
Objective Magnification)**



**Rabbit Anti-Mannosidase II (Golgi
Apparatus) (HeLa, 40X)**



**Rabbit Anti-Glial Fibrillary Acidic
Protein (Rat Hippocampal
Astrocytes, 20X)**

Figure 1. Immunofluorescence of rabbit primary antibodies detected using HCS214 HCS toolkit components and protocols.

All samples were fixed and stained according to the suggested HCS215 kit protocol, using a variety of rabbit primary antibodies with FITC-conjugated (left panels) or Cy3-conjugated (right panels) secondary antibodies (with Hoechst nuclear stain, blue). Cells were imaged on the GE IN Cell Analyzer 1000 (3.3).

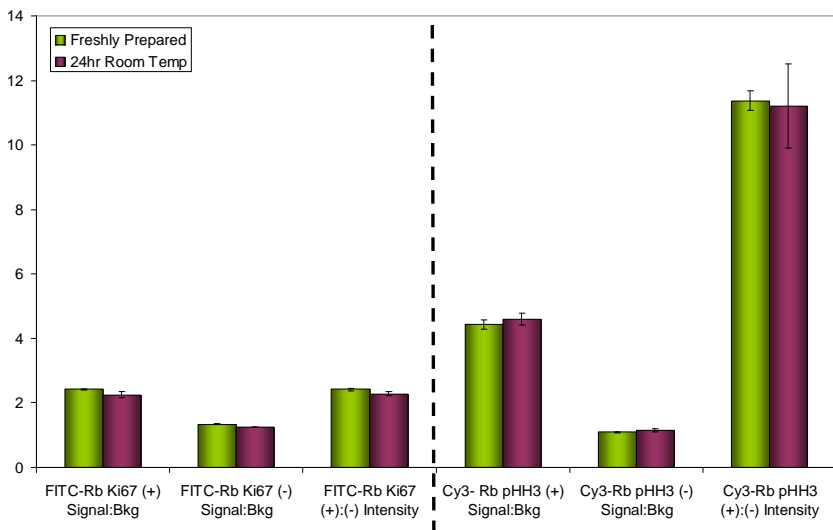


Figure 2. HCS215 High Content Screening Toolkit reagent stability with mouse primary antibodies.

A549 cells were seeded at 18,000 cells/cm² on 96-well plates in growth media and cultured for 48 hours. Samples were fixed and stained according to the suggested HCS215 kit protocol, using rabbit primary antibodies against the proliferation markers Ki-67 (left side of graph, with FITC-conjugated secondary antibody) or phospho-histone H3^(Ser10) (right side of graph, with Cy3-conjugated secondary antibody). Staining utilized 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. Ki-67 and phospho-histone H3-positive cells were determined by thresholding Hoechst-segmented nuclei 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 μ m wide perimeter surrounding the nucleus). (+):(-) nuclear intensity ratios were calculated as a measure of contrast between cells that did or did not express Ki-67/phospho-histone H3. Data presented are mean \pm SD; $n = 3$. Significant differences in signal quality were not observed between freshly prepared and 24 hour samples.

Troubleshooting

<u>Problem</u>	<u>Potential Explanations/Solutions</u>
Weak FITC/Cy3/ Hoechst signal	<p>Improper storage or preparation of Primary/Secondary antibody or Nuclear Stain – retry stain with fresh antibody/dye solution.</p> <p>Inadequate primary/secondary antibody or Nuclear Stain concentrations for cell type – titrate dilutions to optimize signal.</p> <p>Signal may diminish in extremely dense cultures – decrease cell seeding concentration or increase primary/secondary antibody or Hoechst concentration.</p> <p>Optimize exposure times and/or fluorescence filters appropriate to fluorophore.</p>
Excessive background	<p>Improper reagent storage or preparation – retry with fresh reagent (antibodies/dyes and/or buffers). Contaminated buffers/solutions may require 0.2µm filter sterilization.</p> <p>Samples may have dried during staining – retry stain on fresh samples.</p> <p>Excessive primary or secondary antibody concentrations for cell type – titrate dilutions to optimize signal.</p> <p>Check for autofluorescence of microplate.</p>
Excessive FITC/Cy3/ Hoechst signal	<p>Improper preparation of antibody/dye – retry stain with fresh antibody/dye solutions.</p> <p>Inappropriate antibody/dye concentrations for cell type – titrate dilutions to optimize signal.</p> <p>Optimize exposure times and/or fluorescence filters appropriate to fluorophore.</p>

Cell loss	<p>Optimize liquid aspiration/dispensation rate to reduce shear.</p> <p>Consider protein-coating to improve cell adhesion.</p> <p>Optimize cell seeding concentrations for better cell adhesion.</p>
Poor nuclear segmentation/ intensity thresholding during analysis	<p>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).</p> <p>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.</p>

References

1. Giuliano KA, Haskins JR, Taylor DL. Advances in high content screening for drug discovery. *Assay Drug Dev Technol.* 2003;1:565–577.
2. Giuliano KA, Johnston PA, Gough A, Taylor DL. Systems cell biology based on high-content screening. *Methods Enzymol.* 2006;414:601-619.
3. Gough AH, Johnston PA. Requirements, features, and performance of high content screening platforms. *Methods Mol Biol.* 2007;356:41-61.
4. Giuliano KA. Optimizing the integration of immunoreagents and fluorescent probes for multiplexed high content screening assays. *Methods Mol Biol.* 2007;356:189-193.

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