HCS DNA Damage Kit

Catalog no. H10292

Table 1. Contents and storage information.

Material	Amount	Concentration	Storage*	Stability
Image-iT®Dead Green™ viability stain (Component A)	10 µL	1 mM solution in DMSO	• ≤–20˚C • Desiccate • Protect from light	When stored as directed this kit is stable for 1 year.
pH2AX mouse monoclonal antibody (Component B)	15 µL	1 mg/mL		
Alexa Fluor® 555 goat anti-mouse IgG (H+L) *highly cross-adsorbed* (Component C)	10 µL	2 mg/mL		
Hoechst 33342 (Component D)	25 µL	10 mg/mL		
*These storage conditions are for storing the kit upon receipt. For optimal storage of each component, see vial labels.				
Number of assays: Sufficient material is supplied for 2 × 96-well plates based on the protocol below.				
Approximate fluorescence excitation/emission maxima: Hoechst 33342: 350/461 nm bound to DNA; Image-iT® Dead Green™ viability stain: 488/515 nm; Alexa Fluor® 555 goat anti-mouse IgG: 555/565 nm.				

Introduction

In mammalian cells, double-strand break (DSB) in genomic DNA is a potentially lethal lesion.[1] One of the known responses to DSB formation is phosphorylation of H2A histones.[2] Specifically, DNA damaging agents induce phosphorylation of histone variant H2AX (Ser139) forming DNA foci at the site of DNA DSBs.[3] Phosphorylated H2AX aids in the recruitment of proteins responsible for DSB repair.[4] In mammalian cells, phosphatidylinositol 3-kinase-like protein kinases such as ATM (ataxia-telangiectasia mutated), ATR (ATM- and Rad3-related), and DNA-PKcs (DNA-dependent protein kinase catalytic subunit) phosphorylate histone variant, H2AX.[5]

The HCS DNA Damage Kit was developed to enable simultaneous quantitation of two cell health parameters, genotoxicity and cytotoxicity, by high content analysis in the same cell. DNA damage is measured as an indication of genotoxicity and accomplished by specific antibody-based detection of phosphorylated H2AX (Ser139) in the nucleus. Cytotoxicity is measured with the Image-iT® DEAD Green™ viability stain. The Image-iT® DEAD Green™ viability stain has a high affinity for DNA, is non-fluorescent, and impermeant but forms highly fluorescent and stable dye-nucleic acid complexes when bound to DNA. Staining of nuclear DNA cannot occur in live cells due to the impermeability of the plasma membrane for the stain. Drugs and test compounds leading to serious cell injuries, including plasma membrane permeability, allow entry of the Image-iT® DEAD Green™ viability stain. This property enables the discrimination of dead cells with Image-iT® DEAD Green™ viability stain. Hoechst 33342, which stains nuclear DNA in live and dead cells, is included in the kit as a segmentation tool for automated image analysis.

In examples described below, the HCS DNA Damage Kit was used to detect and quantitate phosphorylated H2AX and changes in cell permeability in A549 cells treated with valinomycin (Figures 1 and 2) and validated for robustness and signal change (Tables 2 and 3). Other treatments known to induce DNA damage including etoposide, hydrogen peroxide, and camptothecin have been validated using the HCS DNA Damage Kit in A549 and HeLa cells.

The HCS DNA Damage Kit contains sufficient material to perform the DNA damage assay on two 96-well plates when used as described in the protocol below. For larger quantities, inquire at www.invitrogen.com.

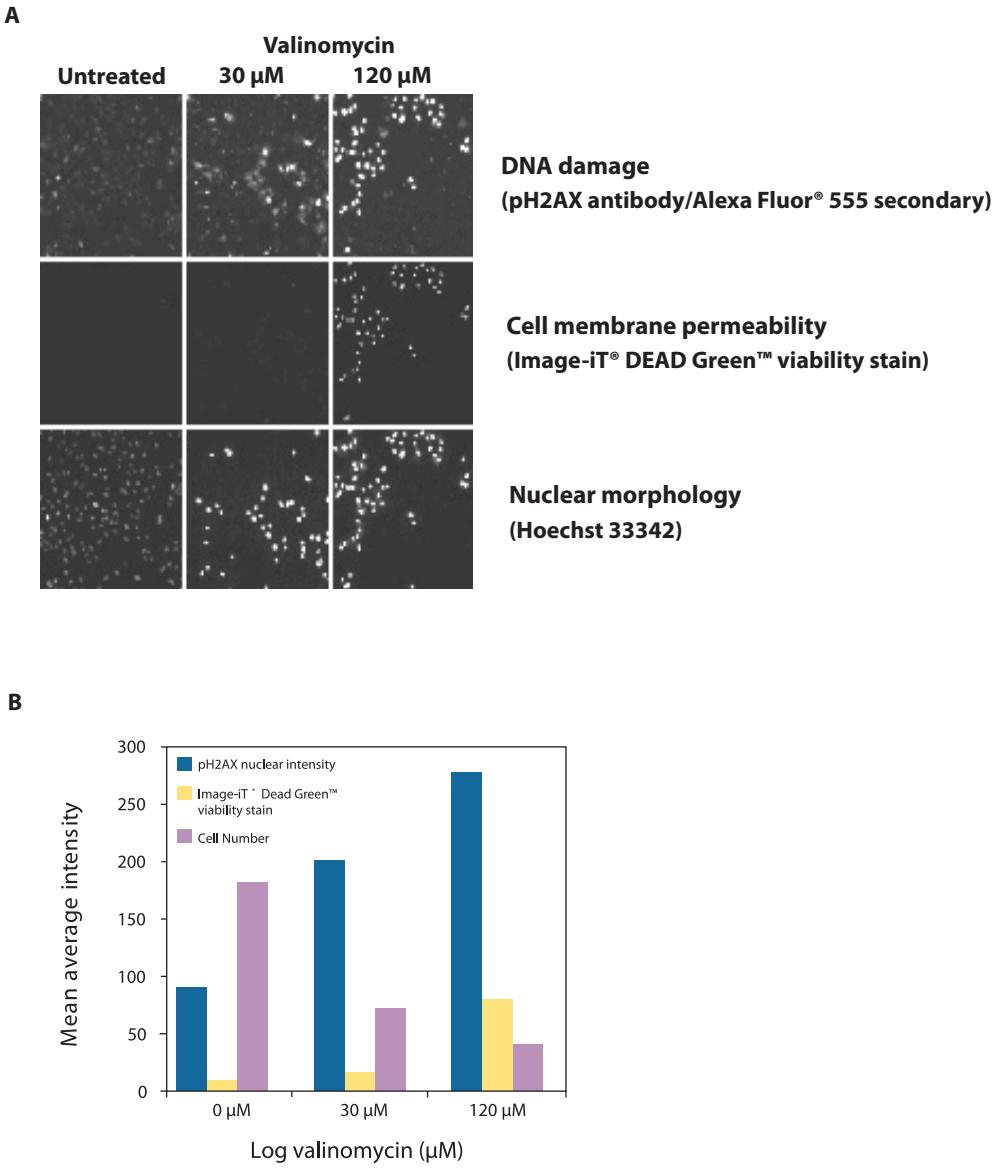

Figure 1. Imaging of genotoxicity and cytotoxicity of valinomycin in A549 cells using the HCS DNA Damage Kit. A549 cells were treated with 30 µM or 120 µM valinomycin for 24 hours at 37°C/5% CO_2 and toxicity was assayed using the HCS DNA Damage Kit. Imaging and analysis was performed using a 10X objective and the Compartmental Analysis Bioapplication with the Thermo Scientific Cellomics® ArrayScan® VTI platform. At 30 µM valinomycin, cells were positive for pH2AX, but not for Image-iT® DEAD Green™ viability stain indicating DNA damage, but not a compromise in plasma membrane integrity. At 120 µM valinomycin, cells showed genotoxic and cytotoxic effects as demonstrated by the positive pH2AX and Image-iT® DEAD Green™ viability stain fluorescence. Hoechst 33342 was used as a nuclear segmentation tool (A). The bar graph (B) demonstrates quantitative representation of valinomycin treated cells.

Table 2. Assay robustness.*

Analyzed parameter	CV of treated samples (%)
Cell membrane permeability	14.21 ± 3.5
pH2AX nuclear intensity	15.34 ± 3.0

*A549 cells were treated with 60 µM valinomycin for 24 hours at 37°C/5% CO_2 and the DNA damage was assayed using the HCS DNA Damage Kit. Quantitative analysis was performed using the Thermo Scientific Cellomics® ArrayScan® VTI and Compartmental Analysis Bioapplication. The data represent % CVs of the averages and standard deviations obtained from treated samples (Max) of three Min/Max plates. CV values were <20% for both parameters indicating consistency and robustness of the assay.

Table 3. Quantitation of genotoxicity and cytotoxicity.*

Analyzed parameter	Signal change by treatment (-fold)
Cell membrane permeability	12.28 ± 0.8
pH2AX nuclear intensity	11.06 ± 1.37

*A549 cells were treated with 60 µM valinomycin for 24 hours at 37°C/5% CO_2. DNA damage was assayed with HCS DNA Damage Kit and quantitative analysis was performed using the Thermo Scientific Cellomics® ArrayScan® VTI and Compartmental Analysis BioApplication. The average intensities and standard deviations were calculated for each output parameter. The data shown represents the average fold change in signal intensities of treated samples (Max) from three Min/Max plates. The fold change in signal between treated samples and control was >3-fold for both parameters indicating cytotoxicity and genotoxicity.

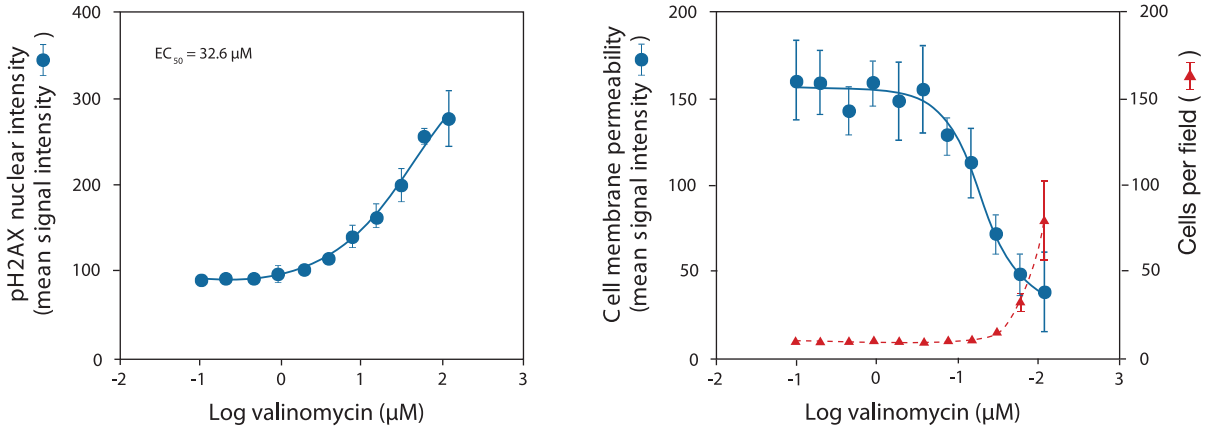

Figure 2. Dose response for valinomycin in A549 cells using the HCS DNA Damage Kit. A549 cells were treated with valinomycin at final concentrations between 0.12–120 µM and incubated for 24 hours. Imaging and analysis was performed using a 10X objective and the Compartmental Analysis Bioapplication with the Thermo Scientific Cellomics® ArrayScan® VTI platform. The dose response curves, generated by non-linear regression with GraphPad PRISM®, were used to determine an EC_{50} value for valinomycin-induced DNA damage (left) and to illustrate loss of cells and plasma membrane integrity with increasing concentrations of valinomycin (right). **Note:** Since valinomycin is less soluble in DMSO at concentrations above 120 µM, data points beyond this concentration are not shown in the figure.

Before You Begin

Materials Required but Not Provided

- Phosphate buffered saline (PBS, Invitrogen Cat. no. 14190-144)
- Cell culture medium
- Paraformaldehyde 16% aqueous solution (Polysciences Cat. no. 18814)
- Flat-bottom 96-well microplates
- Triton® X-100
- Bovine serum albumin (BSA)

Caution

- Hoechst 33342 (Component D) is a known mutagen. Use the dye with appropriate precautions.
- DMSO (in Component A), is known to facilitate the entry of organic molecules into tissues. Handle reagents containing DMSO using equipment and practices appropriate for the hazards posed by such materials. Dispose of the reagents in compliance with all pertaining local regulations. Always wear protective laboratory clothing and gloves when handling this reagent.

Preparing Cells

Plate cells in appropriate medium the day before adding the test compound. For adherent cells, optimize the cell number and plate coating requirements for the chosen cell model and time span of test compound treatment before performing assay.

Preparing Stock Solutions

Prepare the following solutions **fresh** on the day of the assay. The following protocol prepares sufficient material to stain one 96-well plate.

1.1 Prepare the Image-iT® Dead Green™ viability stain (Component A) working solution by adding 1.8 µL of Component A to 6 mL of complete medium.

1.2 Prepare the fixative solution by adding 1.5 mL 16% aqueous paraformaldehyde solution to 4.5 mL PBS to obtain a fixative solution of 4% paraformaldehyde solution.

1.3 Prepare the permeabilization solution by adding 15 µL of Triton® X-100 to 6 mL PBS.

1.4 Prepare the blocking buffer by dissolving 0.25 g BSA in 25 mL PBS. Mix well.

1.5 Prepare the primary antibody solution by adding 6 µL pH2AX antibody (Component B) to 6 mL blocking buffer.

1.6 Prepare the secondary antibody/counterstain solution by adding 3 µL of Alexa Fluor® 555 goat anti-mouse IgG (H+L) (Component C) and 1 µL of Hoechst 33342 (Component D) to 6 mL blocking buffer.

Experimental Protocol

Labeling Cells in 96-well Plates for Imaging

This labeling protocol was developed using A549 and HeLa cells. For other cell types, you

may need to modify the protocol appropriately. See Figure 3 for HCS DNA Damage Kit workflow.

2.1 Add test compound or drug to cells to a total volume of 100 µL and incubate for the desired period of time under normal cell culture conditions.

Note: DMSO concentrations should not be higher than 0.5% in the incubation with live cells. When adding drugs or test compounds at this step, consider the additional 0.06% DMSO introduced with the Image-iT® Dead Green™ viability stain. If other organic solvents are used for preparing drug stock solutions, determine their compatibility with cells.

2.2 Do not remove the incubation medium from wells of the 96-well plate after the test compound or drug treatment.

2.3 Add 50 µL of Image-iT® Dead Green™ viability stain working solution (prepared in step 1.1) for a total volume of 150 µL and incubate the plate for 30 minutes under normal cell culture conditions.

2.4 Remove medium.

2.5 Add 100 µL fixative solution (prepared in step 1.2) to each well and incubate for 15 minutes at room temperature.

2.6 Remove fixative solution and rinse wells once with PBS.

2.7 Incubate cells with permeabilization solution (prepared in step 1.3) for 15 minutes at room temperature.

2.8 Remove permeabilization solution and rinse wells once with PBS.

2.9 Add 100 µL blocking buffer (prepared in step 1.4) to each well and incubate for 60 minutes at room temperature. Remove blocking solution.

2.10 Add 50 µL of the primary antibody solution (prepared in step 1.5) and incubate for 60 minutes at room temperature.

2.11 Remove primary antibody solution and rinse wells three times with PBS.

2.12 Add 50 µL of the secondary antibody/counterstain solution (prepared in step 1.6) and incubate for 60 minutes at room temperature, **protected from light**.

2.13 Remove secondary antibody/counterstain solution and rinse wells three times with PBS.

2.14 Add 100 µL of PBS to each well and proceed to **Imaging and Analysis**.

Imaging and Analysis

Scan the plate using an automated imaging platform equipped with filters appropriate for DAPI/Hoechst, FITC, and TRITC. The nucleus is characterized by Hoechst 33342 in the Hoechst channel. Cell membrane permeability is assessed by determining signal intensity increase in the nucleus in the FITC channel. DNA damage is measured by the signal increase in the TRITC channel in the region defined as the nucleus.

When using the Thermo Scientific Cellomics® ArrayScan® VTI platform, use the Compartmental Analysis BioApplication. In channel 1, define the nucleus with Hoechst 33342 (the segmentation tool) as object with Hoechst/XF93 filters. In channel 2, assess the nuclear fluorescence intensity of Image-iT® DEAD Green™ viability stain with FITC/XF93 filters. In channel 3, determine the fluorescence intensity of pH2AX signal using TRITC/XF93 filters in the nucleus outlined in channel 1. For examples of results, see Figure 1.

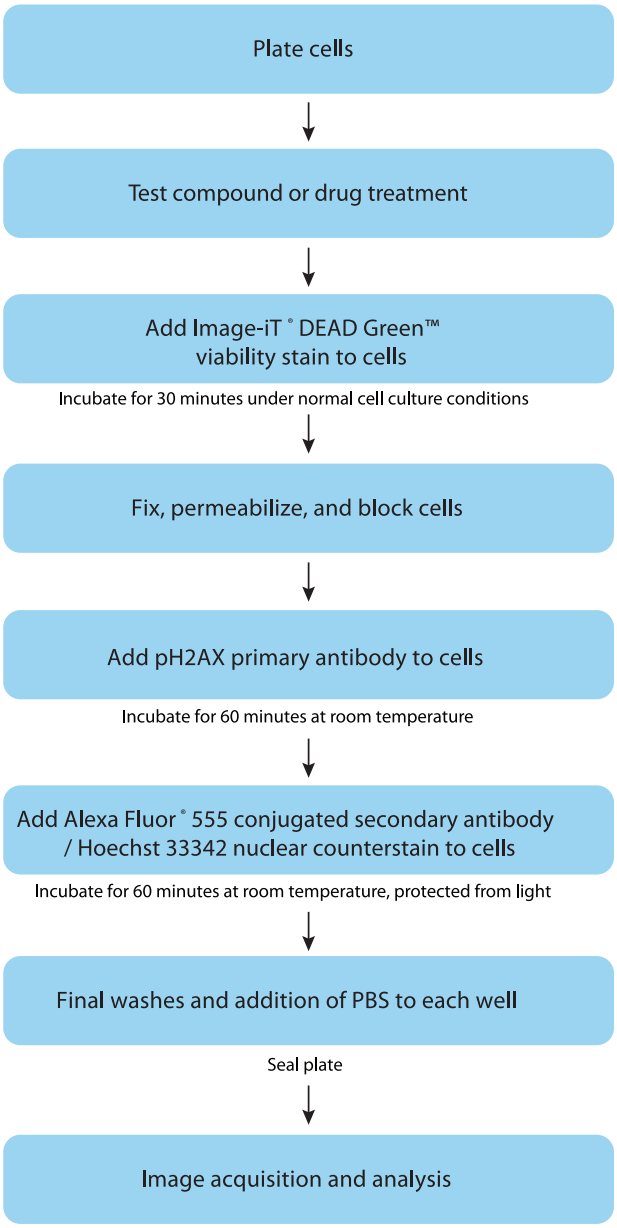

Figure 3. Work Flow for the HCS DNA Damage Kit.

References

1. Science 290, 138 (2001); **2.** Crit Rev Toxicol 27, 155 (1997); **3.** Nature 406, 697 (2000); **4.** Curr Opin Genet Dev 10, 144 (2000); **5.** Nature Genet 27, 247 (2001).

Product List **Current prices may be obtained from our website or from our Customer Service Department.**

Cat. no.	Product Name	Unit Size
H10292	HCS DNA Damage Kit *2-plate size*	1 kit
Related Products		
C10289	Click-iT® AHA Alexa Fluor® 488 Protein Synthesis HCS Assay *2-plate size*	1 kit
C10045	CellMask™ Orange plasma membrane stain *5 mg/mL solution in DMSO	100 µL
C10046	CellMask™ Deep Red plasma membrane stain *5 mg/mL solution in DMSO	100 µL
H10295	HCS Mitochondrial Health Kit *2-plate size*	1 kit
H32711	HCS CellMask™ Red cytoplasmic/nuclear stain *5 mM solution in DMSO* *for high content screening* *for cellular imaging*	125 µL
H34558	HCS CellMask™ Blue cytoplasmic/nuclear stain *for high content screening* *for cellular imaging*	1 set
H34560	HCS CellMask™ Deep Red cytoplasmic/nuclear stain *for high content screening* *for cellular imaging*	1 set
H34157	HCS LipidTOX™ Phospholipidosis and Steatosis Detection Kit *for high content screening* *for cellular imaging* *2-plate size*	1 kit
H34158	HCS LipidTOX™ Phospholipidosis and Steatosis Detection Kit *for high content screening* *for cellular imaging* *2-plate size*	1 kit
H34350	HCS LipidTOX™ Green phospholipidosis detection reagent *1000X aqueous solution* *for cellular imaging* 10-plate size*	each
H34351	HCS LipidTOX™ Red phospholipidosis detection reagent *1000X aqueous solution* *for cellular imaging* 10-plate size*	each
H34475	HCS LipidTOX™ Green neutral lipid stain *solution in DMSO* *for cellular imaging*	each
H34476	HCS LipidTOX™ Red neutral lipid stain *solution in DMSO* *for cellular imaging*	each
H34477	HCS LipidTOX™ Deep Red neutral lipid stain *solution in DMSO* *for cellular imaging*	each
I10291	Image-iT® DEAD Green™ viability stain *1 mM solution in DMSO*	each

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