# Instruction Manual
# for

# H2A.X (Ser139)
# Dual Detect CELISA Assay Kit
# (Fluorogenic Detection)

## Cat. No. 17-720

**FOR RESEARCH USE ONLY**
**Not for use in diagnostic procedures.**

upstate | CHEMICON

THE EXPERTISE OF UPSTATE® AND CHEMICON®
IS NOW A PART OF MILLIPORE

USA & Canada Phone: +1(800) 437-7500 Fax: +1 (951) 676-9209
Australia +61 3 9839 2000
www.millipore.com

## Application

The histone H2A.X protein is a variant member of the H2A family of histones that is distinguished from other H2A histones by a unique carboxy-terminal sequence. This unique sequence is highly conserved throughout eukaryotic evolution [reviewed in 1] and is rapidly phosphorylated by ATM or ATR at the fourth residue from the carboxy-terminus (Serine 139 in mammalian H2A.X) in response to DNA double-strand breaks (DSBs) [2]. Phosphorylation of H2A.X is important in the formation of a stable repair complex at the site of DNA damage.

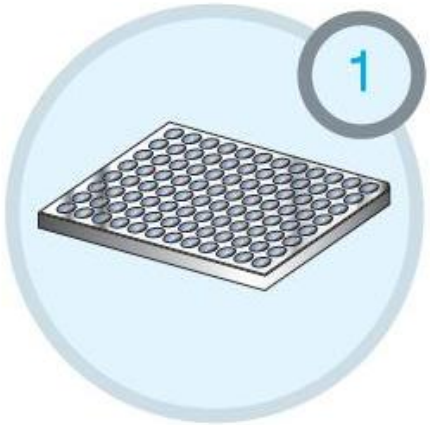
H2A.X phosphorylation is a very rapid response to DNA damage, occurring within as little as one minute after exposure to ionizing radiation [2]. Phosphorylation of H2A.X occurs irrespective of the cause of the DNA DSBs and phospho-H2A.X has been observed in response to environmental stresses that result in DSBs as well as programmed cellular events, including DNA rearrangement and apoptosis.

## Test Principle

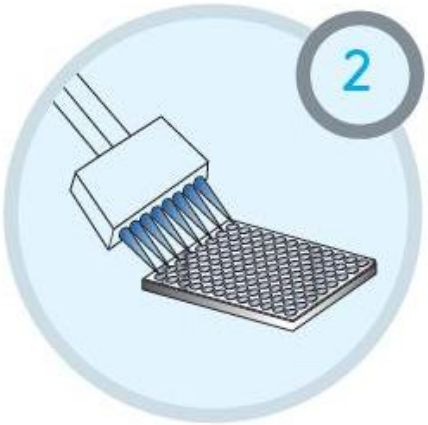
Dual Detect CELISA is a straightforward and efficient cell-based ELISA assay that eliminates the need to prepare cell lysates. It allows for the measurement of the relative expression levels of both the total and the phosphorylated (activated) forms of the target protein in whole cells in a convenient 96-well microplate format using dual fluorescent detection. This allows for the normalization of total protein between wells, thus allowing a more accurate measurement of phosphorylation levels among different cell and/or growth conditions.
Cells are cultured directly in the microplate and stimulated as desired. They are then fixed and simultaneously incubated with two primary antibodies. One is a phosphospecific antibody directed against the phosphorylated epitope of the target protein and the other is a total antibody that recognizes the target-protein, regardless of phosphorylation status. The levels of both the total target protein and the phosphorylated protein are measured simultaneously in a single well using a double immunoenzymatic labeling procedure, using both horseradish-peroxidase (HRP) and alkaline phosphatase (AP), and two spectrally distinct fluorogenic substrates for HRP and AP. The fluorescence of the phosphorylated protein is normalized to the total protein in each well thus reducing well-to-well variations. TheH2A.X (Ser139) kit contains the components required to run the Dual Detect CELISA using fluorogenic substrates to measure both total H2A.X and phosphorylated H2A.X (Ser139) in the context of whole cells.

# Dual Detect CELISA

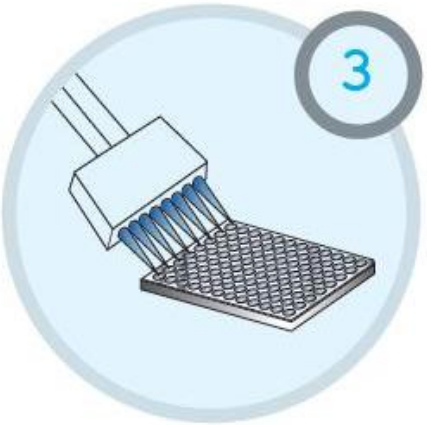
## Fluorescent Cell-Based Assay




Culture and treat 100µl of cells in each of the 96 wells.

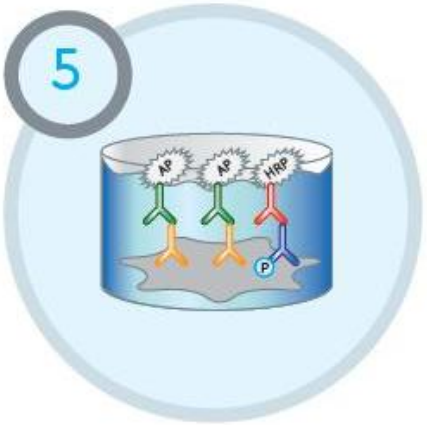



Wash and fix cells. (Plates can be stored up to 2 weeks @ 4°C).

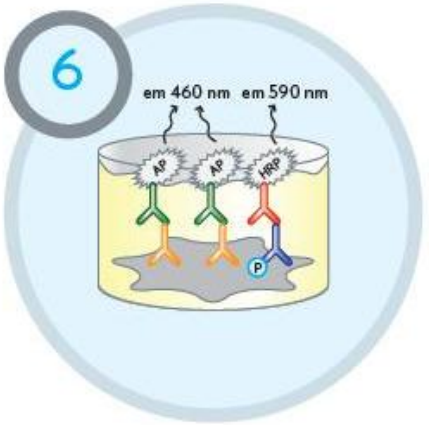



Quench and block wells in preparation for antibody incubation.


Add primary antibody solution containing antibodies to both the total target and the phospho-specific target.




Wash and incubate with detection antibodies solution.




Wash and add substrate mixture to each well. Perform dual fluorescent detection.

## Kit Components

1. Black Clear Bottom 96-well Cell Culture Plate: (Catalog # CS201104) One 96-well cell culture plate with clear (view) bottom.
2. 20% Tween® 20 (v/v): (Catalog # 20-246) One bottle containing 3 mL of 20% Tween® 20 in Milli-Q water.
3. 20X TBS: (Catalog # 20-190) One bottle containing 50 mL of 20X TBS.
4. 10% BSA in TBS (Blocking Buffer) (Catalog # 20-191) One bottle containing 10 mL 10% BSA in 1X TBS.
5. Substrate Diluent: (Catalog# CS200690) One bottle containing 20 mL of a proprietary buffer.
6. 500X anti-H2A.X (Total) Antibody: (Catalog # CS202605) One vial containing 15 μL of 500X total H2A.X antibody.
7. 500X anti-phospho-H2A.X (Ser139) Antibody: (Catalog # CS202604) One vial containing 15 μL of 500X phospho-H2A.X (Ser139) antibody.
8. 500X Goat anti-Mouse HRP Detection Reagent: (Catalog # CS200689) One vial containing 50 μL of 500X Goat anti-Mouse HRP Detection Reagent.
9. 500X Goat anti-Rabbit AP Detection Reagent: (Catalog # CS200674) One vial containing 50 μL of 500X Goat anti-Rabbit AP Detection Reagent.
10. 200X HRP Substrate: (Catalog # CS200673) One vial containing 60 μL of 200X HRP substrate.
11. 200X AP Substrate: (Catalog # CS200701) One vial containing 60 μL of 200X AP substrate.

## Materials Not Supplied

- Cell culture media and supplies
- 37% formaldehyde
- 100% ethanol
- Acetic acid
- 30% Hydrogen Peroxide ($H_2O_2$)
- shaker or platform vortex
- Shaking incubator
- Variable volume (2-200 μL) pipet + tips
- Reagent troughs for multichannel pipettes
- Wash bottle or multichannel dispenser for washing
- Microtiter plate washer (optional)
- Microplate reader capable of excitation at 550 nm and emission at 590 nm as well as excitation at 360 nm and emission at 460 nm

## Precautions

The H2A.X Dual Detect CELISA kit is designed for research use only and is not recommended for internal use in humans or animals. All chemicals should be considered potentially hazardous and all principles of good laboratory practice should be followed.

## Storage

This kit is shipped at -20°C. Upon receipt, check individual components for storage conditions. The cell culture plate should be stored at room temperature. The 20X TBS (Catalog # 20-190), 20% Tween® 20 (Catalog # 20-246), 10% BSA (20-191) and Substrate Diluent (Catalog # CS200690) should be stored at 4°C. All other components are stored at -20°C. Aliquot the -20°C components, if necessary, to avoid freeze-thaw cycles.

## Stability

Kit components are stable for 3 months from date of shipment if stored and handled correctly.

## Handling Recommendations

For maximum recovery of product, prior to removing the cap, rapidly thaw the vial under cold water. Centrifuge briefly to collect the material and immediately place on ice or keep at room temperature, as recommended in the protocol.

## Assay Instructions

### I. Buffer/solution preparation

#### Common Reagents

**1X TBS**: Prepare 200 mL of 1X TBS by adding 10 mL of 20X TBS (Catalog # 20-190) to 190 mL of Milli-Q or distilled water. Store at room temperature.

**1X TBST (Wash Buffer)**: Prepare 800 mL of 1X TBST by adding 2.0 mL of 20% Tween® 20 (v/v) (Catalog # 20-246) and 40 mL of 20X TBS (Catalog # 20-190) to 758 mL of Milli-Q or distilled water. Store at room temperature.

**Blocking Buffer**: For each 96 well plate, prepare 30 mL of 3% BSA in TBST by adding 10 mL of 10% BSA in TBS (Catalog # 20-191) to 20 mL of 1X TBST. This solution is stable for several days at 4°C. Discard unused portion following assay completion.

#### Cell Fixation Solution (not provided)

**95% Ethanol, 5% Acetic acid (not provided)**

For each 96 well plate prepare 12 mL by mixing 11.4 mL of 100% Ethanol and 0.6 mL of Acetic Acid solution. Vortex to mix well.

**1% Formaldehyde in TBS (not provided)**

For each 96 well plate prepare 12 mL of a 1% formaldehyde solution by adding 324 µL 37% formaldehyde solution (formalin) to 11.676 mL 1X TBS. This solution must be prepared fresh. Discard unused portion following assay completion.

#### Quenching Buffer (not provided)

**1% $H_2O_2$ in TBST**: For each 96 well plate prepare 12 mL quenching buffer by adding 0.4 mL 30% $H_2O_2$ to 11.6 mL of 1X TBS-T. This solution must be prepared fresh. Discard unused portion following assay completion.

#### Primary and Detection Antibody Preparations

***Prepare immediately before use***

**Primary Antibody Solution**: Prepare 6 mL per plate of 1X Primary Antibody Solution by adding 12 µL each of 500X anti-H2A.X (Total) Antibody (Catalog # CS202605) and 500X anti-phospho-H2A.X (Ser139) Antibody (Catalog # CS202604) to 5.976 mL of Blocking Buffer.

**Gently mix the solution to ensure the antibodies are evenly dispersed in the solution.**

Store at 4°C. Discard unused portion following assay completion.

**Detection Antibody Solution**: Prepare 12 mL per plate of 1X Detection Antibody Solution by adding 24 µL each of 500X Goat anti-Mouse HRP Detection Reagent (Catalog # CS200689) and 500X Goat anti-Rabbit AP Detection Reagent (Catalog # CS200674) in 11.952 mL of Blocking Buffer.

**Gently mix the solution to ensure the antibodies are evenly dispersed in the solution.**

Store at 4°C. Discard unused portion following assay completion.

**Fluorogenic Substrate Solution**

***Prepare immediately before use***

**HRP and AP Substrate Solution:** Prepare 12 mL per plate of a 1:200 dilution substrate by adding 60 µL each of 200X HRP Substrate (Catalog # CS200673) and 200X AP Substrate (Catalog # CS200701) in 11.88 mL substrate diluent.

**This solution must be prepared immediately before use.**

Discard unused portion following assay completion.

## II. Assay Protocol

### A. General Notes

1. All kit reagents should be at room temperature (20°C to 25°C) prior to use.
2. Do not use reagents beyond the expiration date of the kit.
3. Do not mix or interchange reagents from various kit lots.
4. When performing washes manually, avoid introducing bubbles when dispensing liquids into the wells, and ensure each well is filled with buffer, but not overflowing to avoid cross-contamination between wells. Empty wells with a wrist-flick motion over an appropriate receptacle, and while still inverted, blot any remaining moisture onto clean absorbent paper. If an automated plate washer is used, follow manufacturer's recommendation for operation.
5. Agitation of wells during incubation of Blocking Buffer and Antibody steps is recommended to reduce non-specific background. Vigorous washing and complete removal of all liquid by aspiration at the end of each washing step is very important to obtain low background values. Gentle agitation during the wash steps or a 2-3 minute soak may reduce background values. If a microtiter plate agitator is not available, a platform vortex at a low setting can be used (e.g. level 1 of Fisher's Genie II platform vortex). If background problems occur, increase the number and/or duration of washes.
6. Do not allow the wells to dry out during the protocol.
7. Incubation temperatures for Primary Antibody Solution and Detection Antibody Solution can be varied and should be empirically determined.

### B. Culture, Stimulation, and Fixing Cells to 96-Well Cell Culture Plates

Fixing of the cells in the 96-well plates should be done as soon as the desired treatment has completed.

1. Day 1: Seed 100 μL of 10,000-20,000 adherent cells into each well of the black 96-well microplate with clear bottom included in the kit. Incubate plate overnight at 37°C in a cell culture incubator. The cell number used is dependent upon the cell line and the relative amount of protein phosphorylation. Optimal cell numbers should be determined by each laboratory for the assay.
2. Day 2: Replace the media with a low serum media (0.5% serum) and incubate overnight (16-18 hrs).
3. Day 3: Stimulate the cells as desired, then immediately proceed to step 4.
4. Fix cells by replacing medium with 100 µL/well of 95% Ethanol, 5% acetic acid Cell Fixation Solution. Add the fixing solution slowly to ensure cells do not detach from the plastic. Let stand for 7 minutes at room temperature.
5. Remove fixing solution from wells with a wrist-flick. While still inverted, tap the plate gently onto absorbent paper to remove any excess fixing solution still within the wells.
6. Add 100 µL/well of 1% formaldehyde in TBS. Add solution slowly to ensure cells do not dislodge from the wells. Let stand for 5 minutes at room temperature.
7. Remove formaldehyde solution from wells with a wrist flick. While still inverted, gently tap the plate onto absorbent paper to remove any excess liquid still in the wells. ***Warning:*** *Formaldehyde is highly toxic. Confine vapors to a chemical hood and wear appropriate gloves and eye protection when using this chemical. Refer to MSDS prior to use.*
8. Remove the formaldehyde solution and wash the cells 3 times with 200 μL per well of 1X Wash Buffer. Each wash step should be performed for 5 minutes with gentle agitation.
9. Remove the Wash Buffer and add 100 μL per well of Quenching Buffer. Add the plate cover and incubate for 20 minutes at room temperature.
10. Remove the Quenching Buffer and wash the cells 3 times with 200 μL per well of 1X Wash Buffer. Each wash step should be performed for 5 minutes with gentle agitation.
11. Remove the Wash Buffer, and add 100 μL per well of Blocking Buffer. Add the plate cover and incubate for 1 hour at room temperature with gentle agitation.

**Alternative Protocol:** Seed the cells at **double** the density determined for the overnight incubation, early in the day, and proceed to the overnight (16-18 hrs) low serum (0.5%) starvation later the same day. Stimulate the cells as desired in step B 3 above. **Overnight growth for most cell lines is recommended. The one day growth procedure may not work for some cell lines.**

### C. Addition of Primary and Detection Antibodies

1. Remove blocking agent with a wrist flick, tap the plate gently over a stack of absorbent papers to remove excess liquid.
2. Wash the wells 3 times with 200 μL per well of Wash Buffer. Each wash step should be performed for 5 minutes with gentle agitation.
3. Remove Wash Buffer with a wrist flick. While the plate is still inverted, tap onto absorbent paper to remove any excess buffer within the wells.
4. Add 50 μL per well of **Primary Antibody Solution** or blocking buffer for the negative controls. Cover with a plate sealer and incubate overnight at 4°C with gentle agitation.
5. Remove Primary Antibody with a wrist flick, tap the plate gently over a stack of absorbent papers to remove excess liquid.

6. Wash the wells 3 times with 200 µL per well of Wash Buffer. Each wash step should be performed for 5 minutes with gentle agitation.
7. Remove Wash Buffer with a wrist flick, tap the plate gently over a stack of absorbent papers to remove excess liquid.
8. Add 100 µL per well of **Detection Antibody Solution**. Cover with a plate sealer and incubate for 1 hour at room temperature with gentle agitation.

   **Note:** The Detection Antibody Mixture is added into each well including the negative control wells.

### D. Fluorogenic Detection

1. Remove the Detection Antibody Mixture from each well and wash the cells 2 times with 200 µL per well of 1X Wash Buffer, followed by 2 washes with 200 µL per well of 1X TBS. Each wash step should be performed for 5 minutes with gentle agitation.
2. Remove the 1X TBS from the plate and add 100 µL per well of Substrate mixture to each well. Incubate for 20 to 60 minutes at room temperature. Protect the plate from direct light. A pink or rosy color may develop in the wells.
3. Read the plate using a fluorescence plate reader capable of excitation at 550 nm and emission at 590 nm to detect the HRP signal. Then read the plate using excitation at 360 nm and emission at 460 nm to detect the AP signal. The readings at 460 nm represent the amount of total H2A.X in the cells, while readings at 590 nm represent the amount of phosphorylated H2A.X (Ser139) in the cells.

   **Note**: The HRP can also use 530-550 nm as excitation and 580-600 nm as emission based on the available filters of each end user.

### E. Data Management

Control wells with no primary antibody (detection antibody only) should be included in each experiment. The fluorescence (counts per second) from these wells in the background fluorescence is subtracted from all sample wells. The phospho-antibody fluorescence at 590 nm in each well is normalized to that of the total antibody fluorescence at 460 nm of the same well. The normalized triplicate readings for each sample are then averaged.

## Representative Data

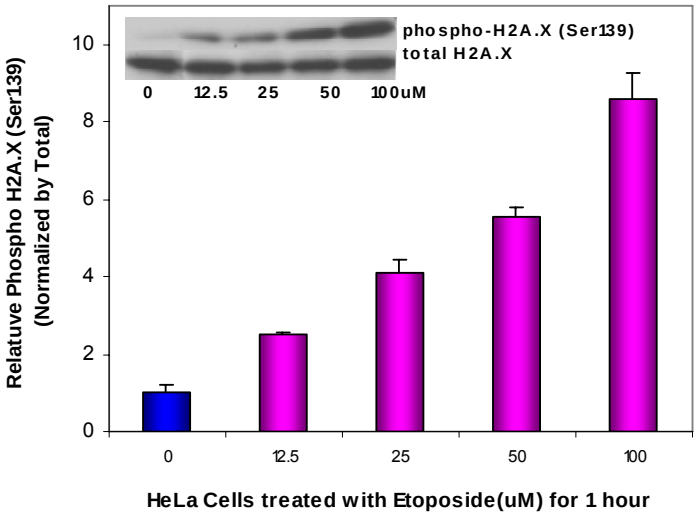
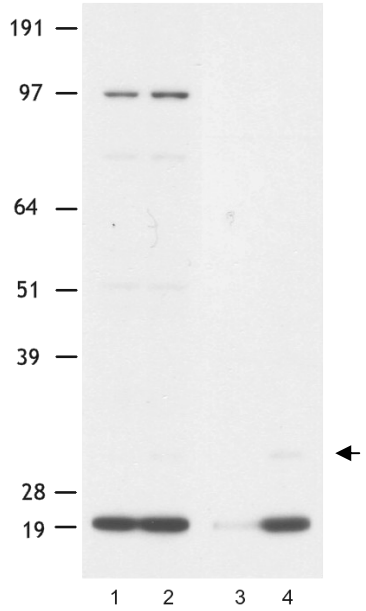


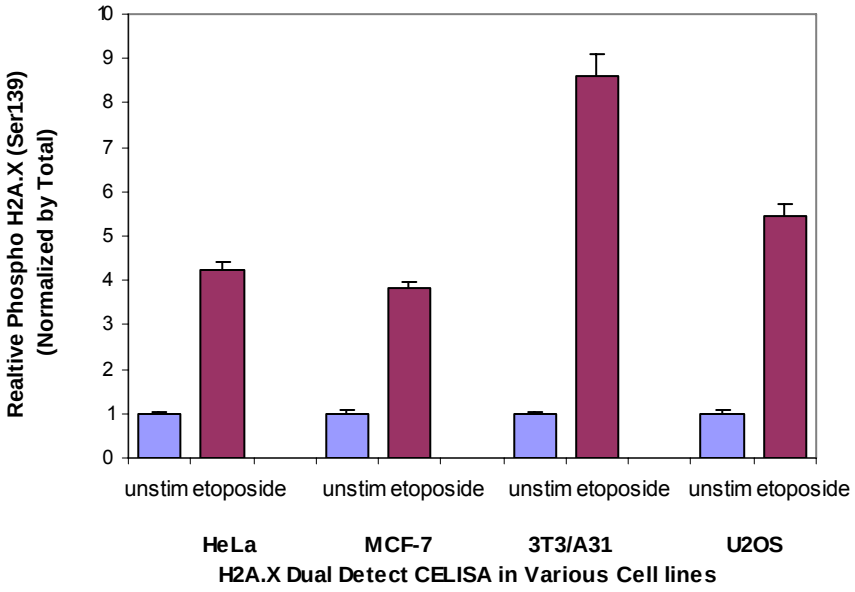
**Figure 1.** HeLa cells were seeded at 1x10$^4$ cells per well, cultured for 24 hr, serum starved 16 hr, and stimulated for 1 hr with Etoposide (0, 12.5, 25, 50, 100 μM). Total and phospho H2A.X levels were determined using H2A.X (Ser139) Dual Detect CELISA Kit. Phosphorylated H2A.X levels are normalized to the total H2A.X.
Insert shows the corresponding Western Blot Data. Lysates were prepared from the stimulated HeLa cells, resolved by electrophoresis and western blotted. H2A.X was detected using antibodies to either total H2A.X or phospho-H2A.X (Ser139).

**Figure 2. Antibody Quality and Specificity:** HeLa cells were grown and either untreated (lanes 1 and 3) or treated with Etoposide (100 μM for 2hr) (lanes 2 and 4). Lanes 1 and 2 were probed with the anti-H2A.X antibody used in the kit to detect total H2A.X levels. Lanes 3 and 4 were probed with anti-phospho-H2A.X (Ser139) that is used in the assay. This figure shows the high level of specificity of the antibodies and any non-specific bands which is imperative to the assay and its data. Arrow shows the monoubiquitinated H2A.X phosphorylated at Ser139.




**Note:** Total H2A.X antibody also recognizes a weak high band around 100KD which does not interfere the relative phospho-H2A.X after normalization. Phospho-H2A.X antibody also picks up the monoubiquitinated H2A.X phosphorylated at Ser139 in treated cells only.

**Figure 3. H2A.X Dual Detect CELISA assay in various cell lines.**
HeLa, MCF-7, 3T3/A31 and U2OS cells were seeded at 1x10$^4$ cells per well, cultured for 24 hr, serum starved 16 hr, and stimulated for 1 hr with 100 μM Etoposide .Total and phospho H2A.X levels were determined using H2A.X (Ser139) Dual Detect CELISA Kit. Phosphorylated H2A.X levels are normalized to the total H2A.X.



## F. Instrument Settings

**Filter Selection:** The HRP fluorescent substrate has an Ex max. of ~560 nm, and an Em max. of ~580 nm. The AP fluorescent substrate has an Ex max. of ~360 nm, and an Em max. of 460 nm. For optimal results, the excitation filter wavelength and emission filter wavelength chosen should be as close as possible to the maxima for the dye. Additionally, a narrower bandpass filter is preferred to a larger bandpass filter. Using filters with a larger bandpass could result in lower signal to background ratios.

*It should be noted that bandpass definitions might vary depending on the filter manufacturer.*

As an example, settings used to read this assay on a Molecular Devices Analyst AD™ plate reader are provided in Table 1 and settings used to read this assay on a Wallac Victor$^2$ 1420 (PerkinElmer) plate reader are provided in Table 2. Other plate readers will need to have settings optimized by the end user.

**Table 1: Molecular Devices Analyst AD™ Settings**

| Parameter | Setting |
|---|---|
| Mode | Fluorescence intensity |
| HRP Excitation filters | 530-25 nm |
| HRP Emission filters | 590-20 nm |
| AP Excitation filters | 360-35 nm |
| AP Emission filters | 460-35 nm |
| Dichroic mirror | 50/50 beamsplitter |
| Z-height | 3 mm |
| Attenuator | None |
| Integration time | 1000 μsec |
| Lamp | Continuous |
| Readings per well | One |
| PMT setup | SmartRead sensitivity 0 |
| Units | Counts/sec |

**Table 2: Wallac Victor2 1420 (PerkinElmer) Settings**

| Parameter | Setting |
|---|---|
| Mode | Fluorescence intensity |
| HRP Excitation filters | 550-9 nm |
| HRP Emission filters | 590-10 nm |
| AP Excitation filters | 355-25 nm |
| AP Emission filters | 460-25 nm |
| CW Lamp Energy | 10000 |
| Readings per well | One |
| Integration time | 1 sec |
| Units | Fluorescent Units |

## References


1. Redon, C., Pilch, D., Rogakou, E., Sedelnikova, O., Newrock, K., Bonner, W. Histone H2A variants H2AX and H2AZ, *Curr. Opin. Genet. Dev*. **12**:162-169, 2002.
2. Rogakou, E.P., Pilch, D.R., Orr, A.H., Ivanova, V.S., Bonner, W.M. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139, *J. Biol. Chem*. **273**:5858-5868, 1998.

## Warranty

**Millipore Corporation** (“Millipore”) warrants its products will meet their applicable published specifications when used in accordance with their applicable instructions for a period of three months from shipment of the products. **MILLIPORE MAKES NO OTHER WARRANTY, EXPRESSED OR IMPLIED. THERE IS NO WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE.** The warranty provided herein and the data, specifications and descriptions of Millipore products appearing in Millipore’s published catalogues and product literature may not be altered except by express written agreement signed by an officer of Millipore. Representations, oral or written, which are inconsistent with this warranty or such publications are not authorized and if given, should not be relied upon.

In the event of a breach of the foregoing warranty, Millipore’s sole obligation shall be to repair or replace, at its option, the applicable product or part thereof, provided the customer notifies Millipore promptly of any such breach. If after exercising reasonable efforts, Millipore is unable to repair or replace the product or part, then Millipore shall refund to the Company all monies paid for such applicable Product. **MILLIPORE SHALL NOT BE LIABLE FOR CONSEQUENTIAL, INCIDENTAL, SPECIAL OR ANY OTHER DAMAGES RESULTING FROM ECONOMIC LOSS OR PROPERTY DAMAGE SUSTAINED BY ANY COMPANY CUSTOMER FROM THE USE OF ITS PRODUCTS.**



Unless otherwise stated in our catalog or other company documentation accompanying the product(s), our products are intended for research use only and are not to be used for any other purpose, which includes but is not limited to, unauthorized commercial uses, in vitro diagnostic uses, ex vivo or in vivo therapeutic uses or any type of consumption or application to humans or animals.