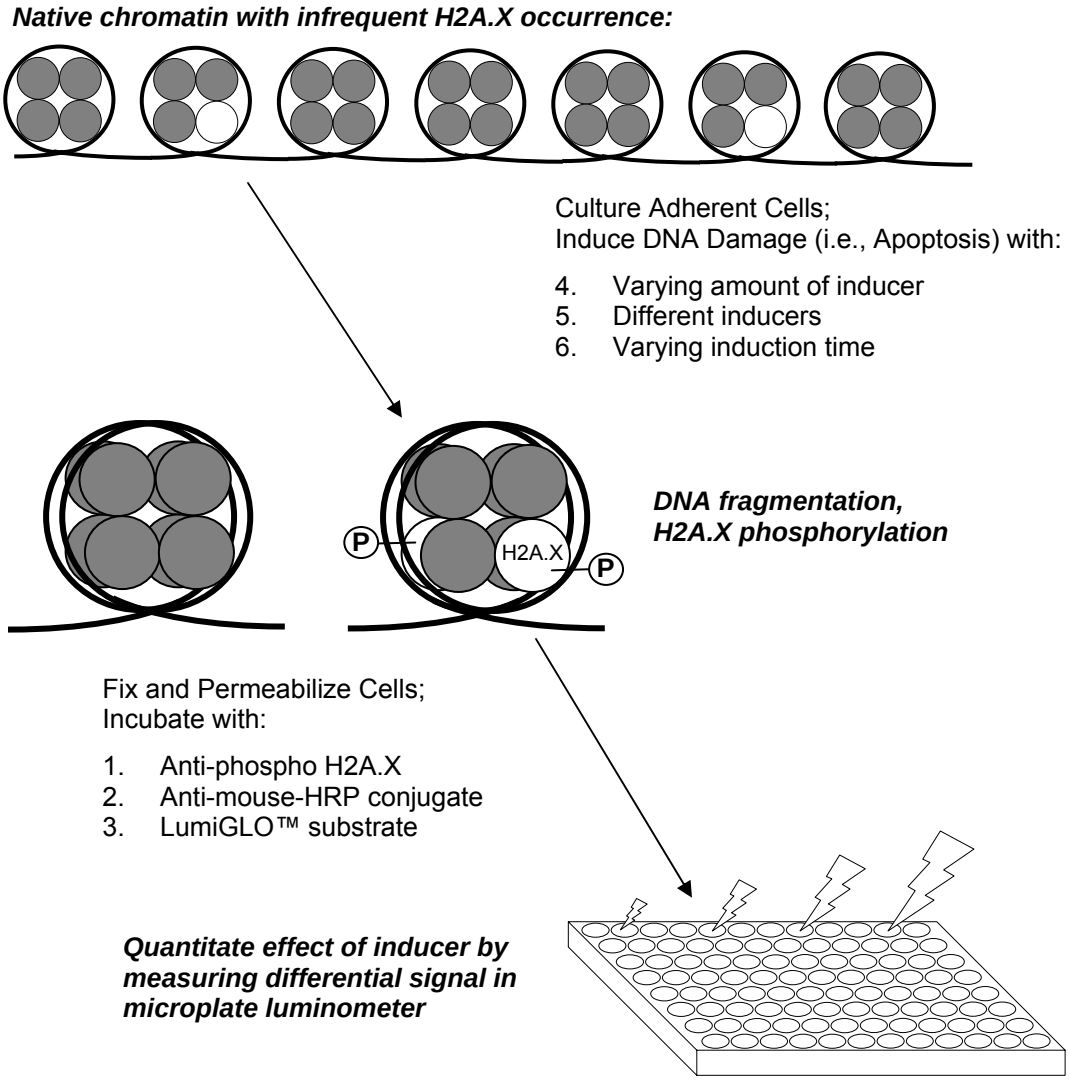
Instruction Manual

H2A.X Phosphorylation Assay Kit
Chemiluminescence Detection

Catalog #17-327



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Instruction Manual

H2A.X Phosphorylation Assay Kit
Chemiluminescence Detection

Catalog #17-327

Chemiluminescent ELISA System for Detection of
DNA Double-Strand Breaks and/or Apoptosis.

Sufficient reagents for two 96-well plate assays per kit.

FOR RESEARCH USE ONLY.
NOT RECOMMENDED OR INTENDED FOR DIAGNOSIS OF DISEASE IN HUMANS.
DO NOT USE IN HUMANS.

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I. ASSAY OVERVIEW

The histone H2A.X protein is a variant member of the H2A family of histones and 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.

Table 1. Phospho-H2A.X detection in response to DNA DSBs

Treatment	**Cell Type**
Ionizing Radiation	CHO [3], IMR90 [3], SF268 [2], HeLa [2], HL60 [2]
UV Irradiation	HBL100 [4], MCF-7 [4]
Hydroxyurea	HBL100 [4], MCF-7 [4]
TNFα	NIH3T3 [6]
anti-Fas	Jurkat [5], NIH3T3 [6]
Staurosporine	HL60 [5], Jurkat [5]
Etoposide	Jurkat [5], 3T3A31[7]
Cytolethal Distending Toxin	HeLa [8]

Anti-phospho-Histone H2A.X (Ser139), clone JBW301 (Catalog #05-636) is a specific mouse monoclonal antibody that recognizes phospho-H2A.X (Ser139) and is useful in monitoring and measuring the extent of DNA DSBs. The H2A.X Phosphorylation Assay Kit is a cell-based ELISA that is intended for rapid and simple quantitation of relative levels of phosphorylated H2A.X in microplate cell cultures using chemiluminescent detection. Briefly, adherent cells are cultured in black-wall/clear-bottom microplates (provided with the kit), treated with agents that induce DNA damage/H2A.X phosphorylation, and are then fixed and permeabilized. Histone H2A.X phosphorylated at serine 139 is detected by the sequential addition of the anti-phospho-H2A.X (Ser139), clone JBW301 and an anti-mouse-HRP (horseradish peroxidase) conjugate. The chemiluminescent HRP substrate LumiGLO™ is then added, and signal is measured in a microplate luminometer. Our research indicates that on two instruments (a PerkinElmer/Wallac Victor^2™ and a PerkinElmer/Packard TopCount® NXT™), optimal results are obtained with the plates provided, however other instruments may provide better results with other plate types, either black- or white-wall, solid- or clear-bottom.

II. STORAGE AND STABILITY

Storage: This kit is shipped at 4°C. Upon receipt, see individual components for storage conditions. Catalog #17-327A contains two cell culture plates (Catalog #30-010), which should be stored at room temperature. Catalog #05-636 and #2003482 may be stored at 4°C for up to one month; storage at -20°C is recommended for prolonged periods. All other components are stored at 4°C.

Stability: Catalog #05-636 and #2003482 are stable for 1 year at -20°C and at least 1 month at 4°C from date of shipment. Other components are stable for 6 months from date of shipment if stored and handled correctly.

III. SYSTEM COMPONENTS

A. PROVIDED KIT COMPONENTS

Black View 96-well Cell Culture Plates
Catalog #30-010
Two black 96 well plates with clear (view) bottom and cover.

20% Tween-20™ (v/v)
Catalog #20-246
One vial containing **3 mL** of 20% Tween-20™ in sterile Milli-Q water.

Anti-mouse IgG Secondary Antibody, HRP Conjugate
Catalog #2003482
Two vials containing **30 µL** of goat IgG conjugated to horseradish peroxidase.

20X TBS
Catalog #20-190
One vial containing **50 mL** of 20X TBS.

Anti-phospho Histone H2A.X (Ser 139), clone JBW301
Catalog #05-636
One vial containing **200µg** of mouse IgG.

10% BSA in TBS (Blocking Buffer)
Catalog #20-191B
One vial containing **25 mL** 10% BSA in 1X TBS.

LumiGLO™ Chemiluminescent Substrate Reagent A
Catalog #20-212C
One vial containing **10 mL** of substrate.

LumiGLO™ Chemiluminescent Substrate Reagent B
Catalog #20-212D
One vial containing **10 mL** of substrate.

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B. REQUIRED MATERIALS NOT PROVIDED

- Cell culture flasks for growing and splitting cells.
- Cell culture media.
- Reagent troughs for multichannel pipettes.
- 95% EtOH/5% Acetic Acid for first fixing step.
- 1% formaldehyde in TBS for second fixing step.
- 30% hydrogen peroxide (H_2O_2) for quenching step.
- Microtiter plate washer (optional), shaker or a platform vortex.
- Shaking incubator.
- Wash bottle or multichannel dispenser for washing.
- Timer
- Microplate luminometer
- Variable volume (2-200µl) pipet + tips

C. STOCK SOLUTIONS

Prepare the following solutions in advance of performing the DNA Damage Detection Kit Assay:

1X TBS: Dilute 50 mL 20X TBS (Catalog #20-190) with 950 mL sterile water to create a working solution of 1X TBS. Store at room temperature.

1X TBS/T (Wash Buffer): Dilute 1.5 mL 20% Tween®-20 (v/v) (Catalog #20-246) in 600 mL 1X TBS to create a working solution of 1XTBS with 0.05% Tween®-20 (v/v). Store at room temperature.

Blocking Buffer: For each 96 well plate dilute 12 mL 10% BSA in TBS (Catalog # 20-191B) with 28 mL of 1X TBS to make a working solution of 3% BSA in TBS. This solution is stable for several days at 4°C. Discard unused portion following assay completion.

1% Formaldehyde in TBS: For each 96 well plate dilute 405.5 µL 37% formaldehyde solution (formalin) in 15 mL 1X TBS. This solution must be prepared fresh. Discard unused portion following assay completion.

1% H_2O_2 in TBST (Quenching Buffer): For each 96 well plate dilute 0.4 mL 30% H_2O_2 into 11.6 mL of 1X TBST. This solution must be prepared fresh. Discard unused portion following assay completion

95% EtOH/5% Acetic Acid: For each 96 well plate dilute 750 µL acetic acid in 14.25 mL of ethanol. This solution must be prepared fresh. Discard unused portion following assay completion.

Primary Antibody Solution: Prepare a 1:8000 dilution of Primary Antibody (Catalog #05-636) in Blocking Buffer. Vortex the solution to ensure antibody is evenly diluted in the solution. Discard unused portion following assay completion. Store at 4°C.

Detection Antibody Solution: Prepare a 1:500 dilution of Detection Antibody by diluting 20 µL Goat Anti-Mouse HRP (Catalog #2003482) in 10 mL Blocking Buffer**.** Vortex the solution to ensure antibody is evenly diluted in the solution. Discard unused portion following assay completion. Store at 4°C.

IV. DNA DAMAGE DETECTION ASSAY PROTOCOL

Safety Warnings and Precautions: The DNA Damage Detection assay kit is designed for research use only and not recommended for internal use in humans or animals. All chemicals should be considered potentially hazardous and principles of good laboratory practice should be followed.

A. General Notes

1. 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.
2. Agitation of wells during incubation of Blocking Buffer and Antibody steps is recommended to reduce non-specific background. If 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, simply increase the number and/or duration of washes.
3. A brief 1X TBS rinse is recommended prior to the addition of the LumiGLO™ substrate to remove any traces of the Tween-20™ which can interfere with HRP activity.

4. It is recommended that the plates are read between 10 and 20 minutes after the addition of the LumiGLO™ substrate. Waiting longer than 20 minutes will produce weaker signals due to the luminescence signal fading.
5. Do not allow the wells to dry out during the protocol.
6. Incubation temperatures for Primary Antibody and Detection Antibody can be varied and should be empirically determined.
7. Control wells with no primary antibody (secondary antibody alone) should be included in each experiment. The average background luminescence (counts per second) from these wells should be subtracted from all sample wells.

B. 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 is completed. A sample stimulation protocol using etoposide is described in the appendix.

1. Aspirate media from the wells, leaving approximately 10 µL of media. Avoid touching the bottom of the well and removing cells.
2. Immediately add 100 µL/well of 95% EtOH 5% acetic acid fixing agent. Add the fixing solution slowly to ensure cells do not detach from the plastic. Let stand for 7 minutes at room temperature.
3. Remove fixing agent from wells with a wrist-flick. While still inverted, tap the plate gently onto absorbent paper to remove any excess fixing agent still within the wells.
4. Add 100 µL/well of 1% formaldehyde in TBS. Add solution slowly to ensure cells are not dislodged from the wells. Let stand for 5 minutes at room temperature.
5. 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.
6. Wash cells 3 times with 200µL/well Wash Buffer for ~5 minutes each with gentle agitation. Remove Wash Buffer after each wash with a wrist flick. While still inverted, gently tap the plate onto clean absorbent paper to remove any excess liquid.
7. Add 100 µL/well of Quenching Buffer. Add the plate cover and incubate for 20 minutes at room temperature.
8. Wash cells 3 times with 200µL/well Wash Buffer for ~5 minutes each with gentle agitation. Remove Wash Buffer in-between each wash with a wrist flick.
9. Add 100 µL/well Blocking Buffer (3% BSA in TBS) and incubate for 1 hour at 37°C or overnight at 4°C.

C. Addition of Primary and Secondary Antibodies

1. Remove blocking agent with a wrist flick.
2. Wash cells 3 times with 200 µL/well Wash Buffer for ~5 minutes each wash, using gentle agitation. Remove Wash Buffer after each wash with a wrist flick. While the plate is still inverted, tap onto absorbent paper to remove any excess buffer within the wells.
3. Add 100 µL/well of **Primary Antibody Solution** (prepared as described in Section IIIC) and incubate overnight at 4°C.
4. Remove Primary Antibody with a wrist flick.
5. Wash cells 3 times with 200µl/well Wash Buffer for ~5 minutes each with gentle agitation. Remove Wash Buffer after each wash with a wrist flick.

6. Add 100 µL/well of **Detection Antibody** (prepared as described in Section IIIC) and incubate for 1 hour at room temperature.

D. Luminescence Detection

1. Remove Detection Antibody with a wrist flick.
2. Wash wells 3 times with 200 µL/well Wash Buffer for ~5 minutes with gentle agitation. Remove Wash Buffer after each wash with a wrist flick.
3. During last wash, prepare LumiGLO™ substrate. For each plate, prepare 8 mL of 1:1 (v/v) mixture of **LumiGLO™ Chemiluminescent Substrate Reagent A** (Catalog #20-212C) and **LumiGLO™ Chemiluminescent Substrate Reagent B** (Catalog #20-212D).
4. After last wash with Wash Buffer, rinse wells once with 300 µL/well **1X TBS.** Remove with a wrist flick and tap onto absorbent paper. Ensure that that no liquid remains in the well since it may alter the luminescence signal.
5. Add 75 µL/well of prepared LumiGLO™ substrate. Read plate on 96-well microplate luminometer between 10 and 20 minutes after addition of LumiGLO™ substrate.

V. REFERENCES

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6. Talasz, H., Helliger, W., Sarg, B., Debbage, P.L., Puschendorf, B., Lindner, H. Hyperphosphorylation of histone H2A.X and dephosphorylation of histone H1 subtypes in the course of apoptosis, Cell Death Differ. **9**:27-39, 2002.
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VI. APPENDIX

Etoposide stimulation of HeLa cells will induce the phosphorylation of H2A.X. Other inducing agents may also be suitable (Table 1). The fold induction of phospho-H2A.X in the stimulated wells vs. non-stimulated wells after subtracting secondary antibody only wells should be greater than 2.0.

Materials Required:

1. MEM growth medium (Fisher/Hyclone, SH30310) with 10% FBS, 1x Pen/Strep, 1x Sodium Pyruvate, 1x Non-Essential Amino Acids.

2. Fetal Bovine Serum (FBS): Fisher, SH3005503.

3. Pen/Strep: (Specialty Media, TMS-AB2-C) 100X.

4. Sodium Pyruvate: (Specialty Media, TMS-005-C), 100mM, 100X

5. Non-Essential Amino Acid: (Sigma, M7145), 100X

6. 100X Etoposide: #90533, a 10 mM stock in DMSO. Final concentration will be 100 μM.

Cell Culture and Stimulation

Fixing of the cells in the 96-well plates should be done as soon as the desired treatment is completed, per section B above.

Day1:

1. Seed 100 μL of 10,000 HeLa cells into each well of the black 96-well microplate with clear bottom and incubate overnight at 37° C in a cell culture incubator.

   Note: 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 each assay.

Day2:

2. At the end of the day, discard the culture medium and add 100 μL PBS/well (room temperature) to rinse the cells. Replace the PBS with 100 μL/well 0.5% serum medium to starve the cells for 16-18 hrs.

Day3:

3. Prepare 200 μM etoposide by diluting the 10 mM stock 1:50 in medium with 0.5% serum. Add 100 μL/well to have a total 200 μl medium at 100 μM etoposide. Stimulate the cells for 2 hours in the 37° C incubator. Include some control wells without etoposide.