ELISA

HT γ-H2AX Pharmacodynamic Assay

Catalog Number 4418-096-K

High throughput ELISA to quantify γ-H2AX in human cultured cells, peripheral blood mononuclear cells, and tissue biopsies.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Histone H2AX is a 14 kDa ubiquitous member of the H2A histone family that contains an evolutionarily conserved SQ motif at the C-terminus in eukaryotes. In response to double-strand DNA damage and apoptosis, serine 139 within the SQ motif becomes rapidly phosphorylated by ATM and ATR kinases to yield γ-H2AX (1). γ-H2AX has been confirmed as an important pharmacodynamic (PD) marker (2) and genotoxicity endpoint (3). There are over 21 anti-cancer drugs that are known to result in γ-H2AX formation. As a result, γ-H2AX is an ideal PD surrogate marker to measure molecular responses to a large number of drugs (4-6). While many of these drugs have already garnered regulatory approval and are currently being used to manage various types of cancers, they are the subject of ongoing clinical studies to evaluate their efficacy when used alone or in combination with molecularly targeted drugs.

While methods such as western blots and immunohistochemistry are widely used, the ELISA method is the most quantifiable and easiest to validate. To address this need the quantitative Pharmacodynamic HT γ-H2AX ELISA Kit measures γ-H2AX levels in cellular extracts and phosphorylation of H2AX in response to therapeutic intervention. This assay quantifies differences of γ-H2AX levels in human peripheral blood mononuclear cells (PBMCs), cultured cells and tissue biopsies. It may be useful in future clinical trials, providing one of many needed tools to enable hypothesis-driven preclinical drug design strategies.

PRINCIPLE OF THE ASSAY

In this assay, immobilized γ-H2AX antibody in the wells of a 96-well plates is used to capture γ-H2AX from sample lysate. Incubation with a H2AX detection antibody, followed by addition of a Goat anti-Mouse IgM HRP Conjugate and a chemiluminescent HRP substrate yields relative light units (RLU) that directly correlate with the amount of γ-H2AX in the sample. This assay is ideal for quantification of γ-H2AX in human PBMCs, tissue biopsies, and cultured cells. Important features of the assay include: 1) chemiluminescent, non-radioactive format; 2) high throughput 96 test format with pre-coated capture antibody; 3) dynamic range from 10 pM to 800 pM; and, 4) sensitivity with 5 pM of γ-H2AX.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not mix or substitute reagents with those from other lots or sources.
- Variations in sample collection, processing, and storage may cause sample value differences.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.

PRECAUTION

The acute and chronic effects of overexposure to reagents in this kit are unknown. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

MATERIALS PROVIDED & STORAGE CONDITIONS

Do not use past expiration date.

PART	PART #	AMOUNT PROVIDED	STORAGE OF UNOPENED MATERIAL	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
γ-H2AX Microplate	4418-096-P	96-well polystyrene microplate (12 strips of 8 wells)	Store 2-8 °C.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.
γ-H2AX Assay Buffer	4418-096-02	50 mL	Store 2-8 °C.	Store 2-8 °C.
γ-H2AX Cell Lysis Reagent	4418-096-05	40 mL		
γ-H2AX 25X Wash Buffer	4418-096-07	40 mL		
PeroxyGlow™ A	4675-096-01	6 mL		
PeroxyGlow™ B	4675-096-02	6 mL		
γ-H2AX Standard, 1 µM	4418-096-01	20 µL	Store at ≤ -20 °C.	Store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.
H2AX IgM Detecting Antibody	4418-096-03	30 µL		
Goat anti-Mouse IgM HRP Conjugate	4418-096-04	15 µL		
NALM-6 Cell Lysate Control	4418-096-08	60 µL		
Plate Sealers	N/A	4 adhesive strips		

OTHER SUPPLIES

Reagents

- Distilled water
- 200 mM Phenylmethyl Sulfonyl Fluoride (PMSF) in ethanol
- Protease Inhibitor Cocktail (optional)
- 1-200 µL and 100-1000 µL pipette tips
- 1X PBS
- Plasma Lyte A (optional)
- Trypsin for detaching adherent cells

Equipment

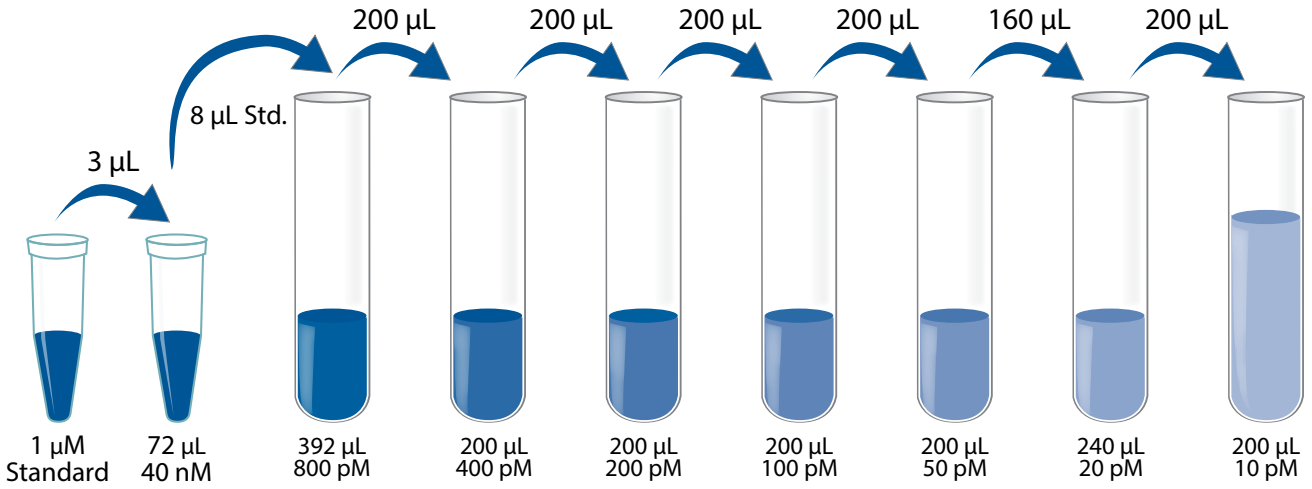
- Micropipettes and tips
- Multichannel pipettor 10-100 µL
- Wash bottle or microstrip wells plate washer (optional)
- 96-well chemiluminescent plate reader or luminometer
- Refrigerated centrifuge with swinging bucket rotor
- Microcentrifuge
- 15 mL and 50 mL screw cap centrifuge tubes
- 0.5 mL and 1.5 mL microtubes
- 25 mL solution reservoirs
- Incubator set at 25 °C

REAGENT PREPARATION

1X Wash Buffer - Prepare 1 liter of 1X Wash Buffer by diluting 25X Wash Buffer with distilled water in a wash bottle for washing strip wells.

γ-H2AX Standard - The kit contains 20 µL of γ-H2AX Standard at a concentration of 1 µM. Centrifuge before opening vial and aliquot to avoid repeated freeze/thaw cycles. Add 3 µL of γ-H2AX Standard to 72 µL Assay Buffer to prepare 40 nM of γ-H2AX Standard (diluted 1:24). Mix well.

Pipette 392 µL of Assay Buffer into the 800 pM tube. Pipette 200 µL or 240 µL into the remaining tubes indicated below. Use the γ-H2AX Standard to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 800 pM standard serves as the high standard. Assay Buffer serves as the zero standard (0 pM).
Note: *Diluted γ-H2AX standards should be used immediately and any remainder discarded.*



H2AX IgM Detecting Antibody - **Prepare and incubate on ice at 2-8 °C for 30 minutes before use.** Gently dilute the H2AX IgM Detecting Antibody 250-fold with Assay Buffer (**Note:** *Avoid vortexing or vigorous mixing*). A total of 50 µL/well of diluted H2AX IgM Detecting Antibody is required in the assay. For 96 wells, dilute 24 µL of H2AX IgM Detecting Antibody into 6 mL of Assay Buffer.

Goat anti-Mouse IgM HRP Conjugate - Gently dilute the Goat anti-Mouse IgM HRP Conjugate 1000-fold with Assay Buffer. A total of 50 µL/well of diluted Goat anti-Mouse IgM HRP Conjugate is required in the assay. For 96 wells, dilute 6 µL of Goat anti-Mouse IgM HRP Conjugate into 6 mL of Assay Buffer.

PeroxyGlow™ A and B Chemiluminescent Substrates - Allow PeroxyGlow™ A and B to come to room temperature before use. Immediately before addition, mix equal volumes of PeroxyGlow™ A and B together. A total of 100 µL is required per well. PeroxyGlow™ A and B are Horseradish Peroxidase (HRP) substrates, generating light that can be quantified in a suitable chemiluminescent plate reader. For 96 wells, mix 6 mL of PeroxyGlow™ A with 6 mL of PeroxyGlow™ B.

REAGENT PREPARATION *CONTINUED*

Cell Lysis Buffer - Immediately before use, prepare 1 mL of Cell Lysis Buffer by mixing the following and placing on ice:

Part	Volume
Cell Lysis Reagent	995 µL
200 mM PMSF (in ethanol)	5 µL
100X Protease Inhibitor Cocktail*	10 µL

*Optional; Cell Lysis Reagent contains phosphatase inhibitors.

NALM-6 Cell Lysate Control - NALM-6 Cell Lysate Control contains 100-400 pM γ-H2AX when diluted 1:10 in Assay Buffer. Store lysate at ≤ -20 °C in working aliquots and avoid repeated freeze/thaw cycles. Keep on ice after thawing.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Suspension Cells

1. Grow 1-5 x 10^6 suspension cells in complete medium in a suitable tissue culture plate or flask.
2. Transfer cells to pre-chilled 50 mL screw cap centrifuge tube. Centrifuge at 200 x g for 3 minutes at 2-8 °C and discard supernatant.
3. Suspend cells in 10 mL of ice-cold 1X PBS and perform cell count.
4. Centrifuge at 200 x g for 3 minutes at 2-8 °C and discard supernatant.
5. Suspend cell pellet in 1 mL of ice-cold 1X PBS and transfer to 1.5 mL microcentrifuge tube. Centrifuge at 10,000 x g or top speed for 10 seconds at 2-8 °C. Discard supernatant.
   Note: *If not used immediately, flash-freeze cell pellet in liquid nitrogen and store at ≤ -70 °C.*
6. Suspend cell pellet at 5 x 10^6 cells/mL in Cell Lysis Buffer. For example, add 1 mL cold Cell Lysis Reagent to a 5 x 10^6 cell pellet and pipette up and down to suspend cells.
7. Incubate on ice for 20 minutes and vortex periodically.
8. Centrifuge at 10,000 x g for 10 minutes at 2-8 °C. Carefully transfer supernatant to new 1.5 mL microcentrifuge tube.
9. Measure the protein concentration of extracts by BCA protein assay.
10. Protein extract can be assayed immediately or aliquoted and stored at ≤ -20 °C.

SAMPLE COLLECTION & STORAGE *CONTINUED*

Adherent Cells

1. Grow 1-5 x 10^6 adherent cells in complete medium in a suitable tissue culture plate until 70-80% confluent.
2. Remove media and gently wash cells with 5 mL of 1X PBS pre-warmed to 37 °C. Repeat PBS wash one more time. Detach the cells with trypsinization according to standard procedure.
3. Suspend cells in 10 mL of ice-cold 1X PBS and perform cell count.
4. Centrifuge at 200 x g for 3 minutes at 2-8 °C and discard supernatant.
5. Suspend cell pellet in 1 mL of ice-cold 1X PBS and transfer to 1.5 mL microcentrifuge tube. Centrifuge at 10,000 x g or top speed for 10 seconds at 2-8 °C. Discard supernatant.
   Note: *If not used immediately, flash-freeze cell pellet in liquid nitrogen and store at ≤ -70 °C.*
6. Suspend cell pellet at 5 x 10^6 cells/mL in Cell Lysis Buffer. For example, add 1 mL cold Cell Lysis Reagent to a 5 x 10^6 cell pellet and pipette up and down to suspend cells.
7. Incubate on ice for 20 minutes and vortex periodically.
8. Centrifuge at 10,000 x g for 10 minutes at 2-8 °C. Carefully transfer supernatant to new 1.5 mL microcentrifuge tube.
9. Measure the protein concentration of the extracts by BCA protein assay.
10. Protein extract can be assayed immediately or aliquoted and stored at ≤ -20 °C.

Peripheral Blood Mononuclear Cells (PBMC)

1. Withdraw 8 mL of blood into BD Vacutainer CPT tube containing sodium heparin as the anticoagulant. Mix by gently inverting 8-10 times.
2. Centrifuge at room temperature in a horizontal rotor (swinging bucket) with proper adaptor for 25 minutes at 1500 x g.
3. After centrifugation, carefully transfer PBMC layer into 50 mL centrifuge tube.
4. Add Plasma Lyte A to a 40 mL final volume and mix by inverting 5-8 times.
5. Centrifuge at 300 x g for 10 minutes at 25 °C and discard supernatant.
6. Suspend cell pellet in cold Plasma Lyte A to 10 mL final volume and perform cell count.
7. Centrifuge at 300 x g for 10 minutes at 25 °C and discard supernatant.
8. Suspend cell pellet in 1 mL Plasma Lyte A, transfer to 1.5 mL microcentrifuge tube and place on ice.
9. Centrifuge at 10,000 x g for 10 seconds at 2-8 °C and discard supernatant.
   Note: *If not used immediately, flash-freeze cell pellet in liquid nitrogen and store at ≤ -70 °C.*
10. Suspend cell pellet at 5x10^6 cells/mL in Cell Lysis Buffer. For example, add 1 mL Cell Lysis Reagent to 5 x 10^6 cell pellet and pipette up and down to suspend cells.
11. Incubate on ice for 20 minutes and vortex periodically.
12. Centrifuge at 10,000 x g for 10 minutes at 2-8 °C. Carefully transfer supernatant to new 1.5 mL microcentrifuge tube.
13. Measure protein concentration of extract by BCA protein assay.
14. Protein extract can be assayed immediately or aliquoted and stored at ≤ -20 °C.

SAMPLE COLLECTION & STORAGE *CONTINUED*

Tissue Biopsies

1. Biopsy of tissue samples are collected according to standard procedure and specimen sizes typically range from 5-20 mm in length, and/or 3-12 mg in mass. If not used immediately, snap-freeze tissue in liquid nitrogen and store at ≤ -70 °C until assayed.
2. Add 1 mL cold Cell Lysis Reagent to frozen tissue and mince completely with fine-point scissors. Vortex to mix and place on ice. Pipette up and down to suspend cells.
3. Incubate on ice for 20 minutes and vortex periodically.
4. If necessary, disrupt the extracts by sonication on ice three times for 10 seconds each cycle. Vortex and place on ice for 15 minutes.
5. Centrifuge at 10,000 x g for 10 minutes at 2-8 °C. Carefully transfer supernatant to new 1.5 mL microcentrifuge tube.
6. Measure protein concentration of extract by BCA protein assay. Protein extract can be assayed immediately or aliquoted and stored at ≤ -20 °C.

High Throughput Screening

1. For high throughput screening purpose, cells can be seeded into a suitable 96-well plate at 10,000-50,000 cells/well. Culture cells per manufacturer's recommendation; adherent cells should be cultured to no more than 80% confluence.
2. Centrifuge the 96 well plate at 200 x g for 3 minutes at 2-8 °C using a 96-well plate adapter and discard supernatant. Suspend cells in ice-cold 1X PBS. Centrifuge at 200 x g for 3 minutes at 2-8 °C and discard supernatant. Repeat PBS wash. For adherent cells, directly remove media and gently wash cells with ice-cold 1X PBS. Gently remove PBS and repeat PBS wash one more time.
3. Centrifuge at 200 x g for 3 minutes at 2-8 °C and discard supernatant.
4. Immediately add 10-20 µL Cell Lysis Buffer to each well. Incubate on ice or at 2-8 °C for 20 minutes and carefully pipetting the lysate periodically to avoid air bubbles.
5. Transfer the lysate from the wells and assay immediately or store lysate at ≤ -20 °C for later use.

ASSAY PROTOCOL

Bring Assay Buffer, 1X Wash Buffer, and PeroxyGlow™ A and B to room temperature before use. It is recommended that all samples, controls, and standards be assayed in triplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Prepare dilutions of γ-H2AX Standard for standard curve and NALM-6 Cell Lysate Control for positive control.
4. Prepare dilutions of sample extracts to fall within standard curve using 25 °C Assay Buffer.

   NOTES:

   i. Recommend diluting cell sample extracts in the starting range of 20,000 cells/well to 50,000 cells/well. Cell samples are added in 50 µL volume. γ-H2AX levels in PBMC, suspension, and adherent sample extracts are reported as pM γ-H2AX per 10^7 cells/mL extract.

   ii. Recommend diluting tissue biopsy extracts in the starting range of 0.5 µg/well to 2 µg/well. Tissue biopsy samples are added in 50 µL volume. γ-H2AX levels in tissue cell extracts are reported as pM γ-H2AX per mg/mL of protein extract.

   iii. If samples generate values greater than the 800 pM standard, assay at a higher sample dilution. If samples generate values lower than 10 pM standard, assay at a lower sample dilution.
5. Add 50 µL/well of γ-H2AX Standards, diluted test samples, diluted NALM-6 Cell Lysate Control, and Assay Buffer (background control) to appropriate wells in triplicate. Apply film sealer and incubate at 25 °C for 2 hours. Gently dilute H2AX IgM Detecting Antibody 1:250 in Assay Buffer by pipetting (avoid vortexing or vigorous mixing) and **incubate on ice or 2-8 °C for 30 minutes before use.**
6. Gently remove plate sealer and rinse wells 4 times with 1X Wash Buffer (300 µL well). Ensure that all liquid is removed by tapping strip wells onto paper towels.
7. Add 50 µL per well of diluted H2AX IgM Detecting Antibody. Apply film sealer and incubate at 25 °C for 1 hour.
8. Gently remove the plate sealer and rinse wells 4 times with 1X Wash Buffer (300 µL/well). Ensure that all the liquid is removed by tapping strip wells onto paper towels.
9. Add 50 µL per well of diluted Goat anti-Mouse IgM HRP Conjugate. Apply film sealer and incubate at 25 °C for 1 hour. Place PeroxyGlow™ A and B reagents at 25 °C to pre-warm.
10. Gently remove the plate sealer and rinse wells 4 times with 1X Wash Buffer (300 µL/well). Ensure that all the liquid is removed by tapping strip wells onto paper towels.
11. Just before use, mix equal volumes of PeroxyGlow™ A and B together and add 100 µL per well. Immediately take chemiluminescent readings.

CALCULATION OF RESULTS

Average the triplicate readings for each standard, control, and sample then subtract the average background relative light units (RLU).

Plot the log of γ-H2AX Standard concentrations (nM) on the X-axis versus relative absorbance on the Y-axis.

The standard curve is a 2nd order polynomial function represented by the equation: $y = a + bx + cx^2$, where y is the relative absorbance, x is the log of γ-H2AX concentration in nM and a, b and c are coefficients. Calculate the γ-H2AX sample concentrations using the polynomial equation or interpolation from the standard curve.

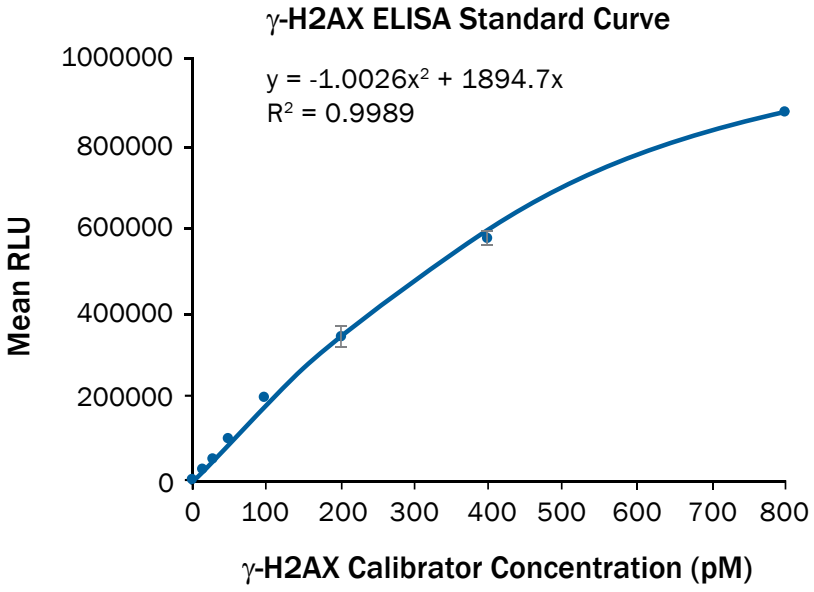
If desired, % B/B_0 can be calculated by dividing the corrected RLU for each standard or sample by the corrected B_0 RLU and multiplying by 100.

Calculate the concentration of γ-H2AX corresponding to the mean absorbance from the standard curve.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

STANDARD CURVE

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



PERFORMANCE CHARACTERISTICS

Intra-Assay Precision: The intra-assay precision (within-run precision): three samples of known concentration were tested nine times in a single run. The overall intra-assay coefficient of variation was calculated to be <10%.

Inter-Assay Precision: The inter-assay precision (between-run precision): three samples of known concentration were tested nine times in three separate runs. The overall inter-assay coefficient of variation was calculated to be <10%.

Sensitivity: The LLD (low limit of detection) of γ-H2AX was calculated to be 5 pM.

Spike and Recovery: Test samples were spiked with three different levels of γ-H2AX and analyzed for recovery before and after spiking. The calculated overall mean of assay accuracy is between 100% ± 10%.

Dilution Linearity: Test samples were serially diluted in the Assay Buffer and subsequently measured by the assay. Dilution recovery is assessed by comparing observed vs. expected values based on undiluted samples. The calculated overall mean of dilution recovery is between 100% ± 10%.

Specificity: The assay cross-reactivity testing is performed by comparing phosphorylated peptide and non-phosphorylated H2AX peptide. The assay only specifically detects phosphorylated peptide and has no cross reactivity with non-phosporylated H2AX peptide.

REFERENCES

1. Rogakou, E.P. *et al.* (1998) The Journal of Biol. Chem. **273**:10.
2. Wu, J. *et al.* (2013) Clinical Cancer Res. **19**:721.
3. Watters G.P. *et al.* (2009) Mutat. Res. **679**:50.
4. Redon, C. E. *et al.* (2010) Clin. Cancer Res. **16**:4532.
5. Kinders R.J. *et al.* (2010) Clin. Cancer Res. **16**:5447.
6. Redon C.E. *et al.* (2011) Aging **3**:168.

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