

DuoSet[®] IC

Human Phospho-Histone H2AX (S139)

Catalog Number **DYC2288-2**
DYC2288-5
DYC2288E

For the development of sandwich ELISAs to measure phosphorylated Histone H2AX (S139) in cell lysates.

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

TABLE OF CONTENTS

SECTION	PAGE
PRINCIPLE OF THE ASSAY.....	1
MATERIALS PROVIDED & STORAGE CONDITIONS	1
OTHER MATERIALS REQUIRED	2
SOLUTIONS REQUIRED.....	2
REAGENT PREPARATION.....	3
PREPARATION OF SAMPLES.....	3
PRECAUTION	4
TECHNICAL HINTS AND LIMITATIONS.....	4
GENERAL ELISA PROTOCOL	5
CALCULATION OF RESULTS	6
TYPICAL DATA.....	6
CALIBRATION	6
SPECIFICITY.....	7
QUANTIFICATION	8
PLATE LAYOUT	9

MANUFACTURED AND DISTRIBUTED BY:

USA & Canada | R&D Systems, Inc.

614 McKinley Place NE, Minneapolis, MN 55413, USA
TEL: (800) 343-7475 (612) 379-2956 FAX: (612) 656-4400
E-MAIL: info@RnDSystems.com

DISTRIBUTED BY:

UK & Europe | R&D Systems Europe, Ltd.

19 Barton Lane, Abingdon Science Park, Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449 FAX: +44 (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

China | R&D Systems China Co., Ltd.

24A1 Hua Min Empire Plaza, 726 West Yan An Road, Shanghai PRC 200050
TEL: +86 (21) 52380373 FAX: +86 (21) 52371001
E-MAIL: info@RnDSystemsChina.com.cn

PRINCIPLE OF THE ASSAY

This DuoSet[®] IC ELISA contains the basic components required for the development of sandwich ELISAs to measure phospho-Histone H2AX (S139) in cell lysates. An immobilized capture antibody specific for Histone H2AX binds both phosphorylated and unphosphorylated protein. After washing away unbound material, a biotinylated detection antibody specific for Histone H2AX phosphorylated at S139, is used to detect only phosphorylated protein, utilizing a standard Streptavidin-HRP format.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

DESCRIPTION	PART #	CATALOG # DYC2288-2	CATALOG # DYC2288-5	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Phospho-Histone H2AX (S139) Capture Antibody	843489	1 vial	2 vials	Store for up to 1 month at 2-8 °C or aliquot and store at ≤ -20 °C for up to 3 months in a manual defrost freezer.*
Phospho-Histone H2AX (S139) Detection Antibody	843490	1 vial	2 vials	
Phospho-Histone H2AX (S139) Standard	843491	3 vials	5 vials	Use within one hour of reconstitution. Use a fresh standard for each assay.
Streptavidin-HRP	890803	1 vial	1 vial	Store at 2-8 °C. DO NOT FREEZE.

* Provided this is within the expiration date of the kit.

DYC2288-2 contains sufficient materials to run ELISAs on at least two 96 well plates.[†]
DYC2288-5 contains sufficient materials to run ELISAs on at least five 96 well plates.[†]

This kit is also available in an Economy Pack (R&D Systems, Catalog # DYC2288E). Economy Packs contain sufficient materials to run ELISAs on 15 microplates.[†] Specific vial counts of each component may vary. Please refer to the literature accompanying your order for specific vial counts.

[†] Provided the following conditions are met:

- The reagents are prepared as described in this package insert.
- The assay is run as described in the General ELISA Protocol on page 5.
- The recommended microplates, buffers, diluents, substrates, and solutions are used.

OTHER MATERIALS REQUIRED

- Aprotinin (Sigma # A6279)
- Leupeptin (Tocris # 1167)
- Pepstatin (Tocris # 1190)
- Phenylmethylsulfonylfluoride (PMSF) (Sigma # P7626)
- Sodium Azide (NaN_3) (Sigma # S2002)
- Sodium Fluoride (NaF) (Sigma # 201154)
- NP-40 Alternative (EMD/Calbiochem # 492016)
- Triton™ X-100 (Sigma # T9284)
- Sodium deoxycholate (Sigma # D6750)
- Pipettes and pipette tips
- Deionized or distilled water
- 96 well microplates [Costar EIA Plates (Catalog # 2592 or R&D Systems, Catalog # DY990) are suggested]
- Plate sealers (R&D Systems, Catalog # DY992)
- Squirt bottle, manifold dispenser, or automated microplate washer.
- Heating block capable of reaching 90 °C or equivalent

SOLUTIONS REQUIRED

PBS - 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , pH 7.2-7.4, 0.2 μm filtered.

Wash Buffer - 0.05% Tween® 20 in PBS, pH 7.2-7.4 (R&D Systems, Catalog # WA126).

Block Buffer - 1% BSA*, 0.05% NaN_3 in PBS, pH 7.2-7.4.

IC Diluent #1 - 1% BSA* in PBS, pH 7.2-7.4, 0.2 μm filtered.

IC Diluent #4** - 1 mM EDTA, 0.5% Triton X-100 in PBS, pH 7.2-7.4.

Note: *Approximately 50 mL of this diluent is required to run the assay on one plate.*

Lysis Buffer #15 - 50 mM Tris, 1% NP-40 Alternative, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 5 mM NaF, 10 $\mu\text{g}/\text{mL}$ Leupeptin, 10 $\mu\text{g}/\text{mL}$ Pepstatin, 100 μM PMSF, and 3 $\mu\text{g}/\text{mL}$ Aprotinin, pH 7.2-7.4.

Substrate Solution - 1:1 mixture of Color Reagent A (H_2O_2) and Color Reagent B (Tetramethylbenzidine) (R&D Systems, Catalog # DY999).

Stop Solution - 2 N H_2SO_4 (R&D Systems, Catalog # DY994).

*The use of R&D Systems Reagent Diluent Concentrate 2 (Catalog # DY995) or Millipore Bovine Serum Albumin, Fraction V, Protease free (Catalog # 82-045) is recommended. All buffers containing BSA must be stored at 2-8 °C.

**Sample Diluent Concentrate 1 (5X) (R&D Systems, Catalog # DYC001), prepared as described in the DYC001 package insert.

All trademarks and registered trademarks are the property of their respective owners.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Phospho-Histone H2AX (S139) Capture Antibody (Part 843489) - Each vial contains 1440 µg/mL of mouse anti-human Histone H2AX antibody when reconstituted with 200 µL of PBS.

Phospho-Histone H2AX (S139) Detection Antibody (Part 843490) - Each vial contains 18 µg/mL of biotinylated rabbit anti-human phospho-Histone H2AX (S139) antibody when reconstituted with 1.0 mL of IC Diluent #1.

Phospho-Histone H2AX (S139) Standard (Part 843491) - Each vial contains 110 ng/mL of recombinant human phospho-Histone H2AX (S139) when reconstituted with 500 µL of IC Diluent #4. **Use within one hour after reconstitution. A fresh standard should be used for each assay.** A seven point standard curve using 2-fold dilutions and a high standard of 20,000 pg/mL is recommended.

Streptavidin-HRP (Part 890803) - 1.0 mL of streptavidin conjugated to horseradish-peroxidase.

PREPARATION OF SAMPLES

Cell Lysates - Rinse cells two times with PBS, making sure to remove any remaining PBS after the second rinse. Solubilize cells at 1×10^7 cells/mL in Lysis Buffer #15 and allow samples to sit on ice for 15 minutes. Assay immediately or store at ≤ -70 °C. Before use, centrifuge samples at 2000 x g for 5 minutes and transfer the supernate to a clean test tube. Sample protein concentration may be quantified using a total protein assay. If needed, further dilutions should be made in IC Diluent #4. Prior to adding samples to the plate, heat for 1 minute at 90 °C followed by a centrifuge spin for 1 minute at 2000 x g.

Note: *Heating samples allows for linear dilution and improved detection of H2AX (S139).*

PRECAUTION

The Stop Solution suggested for use with this kit is an acidic solution. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

TECHNICAL HINTS AND LIMITATIONS

- This DuoSet IC ELISA should not be used beyond the expiration date on the kit label.
- Individual results may vary due to differences in technique, plasticware, and water sources.
- It is important that the diluents selected for reconstitution and for dilution of the standard reflect the environment of the samples being measured. The diluent suggested in this protocol should be suitable for most cell lysates.
- The type of enzyme and substrate and the concentrations of capture/detection antibodies used can be varied to create an immunoassay with a different sensitivity and dynamic range. A basic understanding of immunoassay development is required for the successful use of these reagents in immunoassays.
- A thorough and consistent wash technique is essential for proper assay performance. Wash Buffer should be dispensed forcefully and removed completely from the wells by aspiration or decanting. Remove any remaining Wash Buffer by inverting the plate and blotting it against clean paper towels.
- Use a fresh reagent reservoir and pipette tips for each step.
- It is recommended that all standards and samples be assayed in duplicate.
- Avoid microbial contamination of reagents and buffers. This may interfere with the sensitivity of the assay. Buffers containing protein should be made under aseptic conditions and stored at 2-8 °C or be prepared fresh daily.

GENERAL ELISA PROTOCOL

Plate Preparation

1. Dilute the Capture Antibody to a working concentration of 8.0 µg/mL in PBS, without carrier protein. Immediately coat a 96 well microplate with 100 µL per well of the diluted Capture Antibody. Seal the plate and incubate overnight at room temperature.
2. Aspirate each well and wash with Wash Buffer, repeating the process two times for a total of 3 washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by inverting the plate and blotting it against clean paper towels.
3. Block plates by adding 300 µL of Block Buffer to each well. Incubate at room temperature for 1-2 hours.
4. Repeat the aspiration/wash as in step 2. The plates are now ready for sample addition.

Assay Procedure

1. Add 100 µL of sample or standard in IC Diluent #4 per well. Use IC Diluent #4 as the zero standard. Cover with a plate sealer and incubate 2 hours at room temperature.
Note: *A seven point standard curve using 2-fold serial dilutions and a high standard of 20,000 pg/mL is recommended.*
2. Repeat the aspiration/wash as in step 2 of Plate Preparation.
3. Immediately before use, dilute the Detection Antibody to a working concentration of 500 ng/mL in IC Diluent #1. Prepare only as much Detection Antibody as required to run each assay. Add 100 µL of the diluted Detection Antibody to each well. Cover with a new plate sealer and incubate 2 hours at room temperature.
4. Repeat the aspiration/wash as in step 2 of Plate Preparation.
5. Immediately before use, dilute the Streptavidin-HRP to the working concentration specified on the vial label using IC Diluent #1. Add 100 µL of the diluted Streptavidin-HRP to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.
6. Repeat the aspiration/wash as in step 2 of the Plate Preparation.
7. Add 100 µL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.
8. Add 50 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
9. Determine the optical density of each well immediately, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

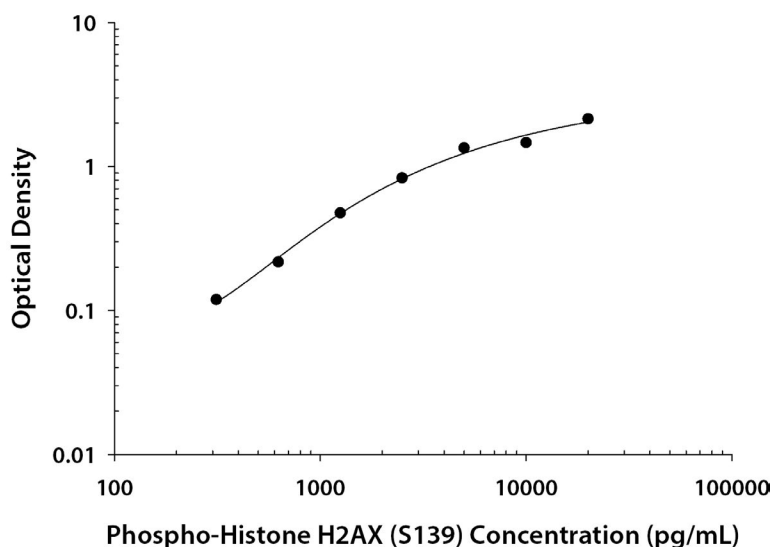
CALCULATION OF RESULTS

Average the duplicate readings for each standard and sample, then subtract the average zero standard optical density (O.D.). Results may be normalized to total protein or cell number.

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the phospho-Histone H2AX (S139) concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

TYPICAL DATA

A standard curve should be generated for each set of samples assayed. The graph below represents typical data generated when using this Human Phospho-Histone H2AX (S139) DuoSet IC ELISA. The standard curve was calculated using a computer generated 4-PL curve-fit. This standard curve is for demonstration purposes only.



CALIBRATION

The Human Phospho-Histone H2AX (S139) DuoSet IC ELISA is calibrated against a highly purified *E. coli*-expressed recombinant human phospho-Histone H2AX (S139) produced at R&D Systems. Samples containing natural phospho-Histone H2AX (S139) showed linear dilution parallel to the standard curve obtained using the Phospho-Histone H2AX (S139) Standard. These results indicate that O.D. values from this DuoSet IC ELISA can be used to determine the relative concentration of Phospho-Histone H2AX (S139) in natural samples.

SPECIFICITY

The Human Phospho-Histone H2AX (S139) DuoSet IC ELISA specifically recognizes Histone H2AX phosphorylated at S139. Specificity was demonstrated using both peptide competition and cross-reactivity analysis.

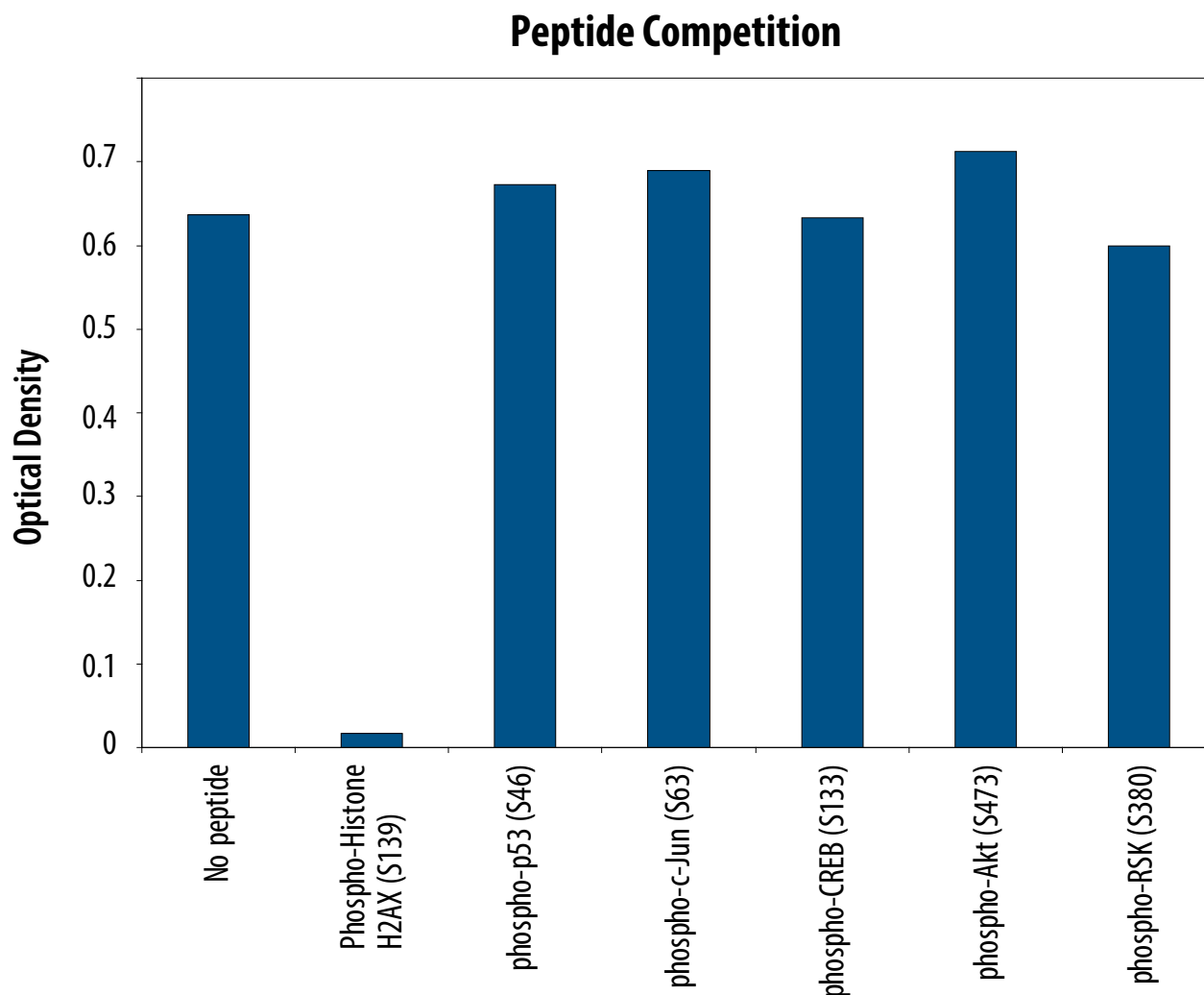


Figure 1: Lysate prepared from Jurkat human acute T cell leukemia cells treated with 1 μ M of camptothecin (CPT) (Tocris, Catalog # 1100) for three hours was analyzed with this DuoSet IC ELISA. The Phospho-Histone H2AX (S139) Detection Antibody was untreated (no peptide) or pre-incubated with peptides containing Phospho-Histone H2AX (S139), Phospho-p53(S46), Phospho-c-Jun (S63), Phospho-CREB (S133), Phospho-Akt (S473), or Phospho-RSK (S380). Peptides were used at 40 ng/mL. Only the peptide containing the Histone H2AX (S139) phosphorylation site blocked the signal, indicating that this ELISA is specific for Histone H2AX phosphorylated at S139.

Cross-reactivity experiments were performed with this DuoSet IC ELISA to further determine specificity. Unphosphorylated recombinant human (rh) Histone H2AX, rhHistone Macro H2A1, and rhHistone Macro H2A2 were assayed at 200 ng/mL and did not cross-react or interfere in the assay.

QUANTIFICATION

Amounts of phosphorylated Histone H2AX, as quantified by the Human Phospho-Histone H2AX (S139) DuoSet IC ELISA, are consistent with the relative amounts of phosphorylated Histone H2AX determined by qualitative Western blot analysis.

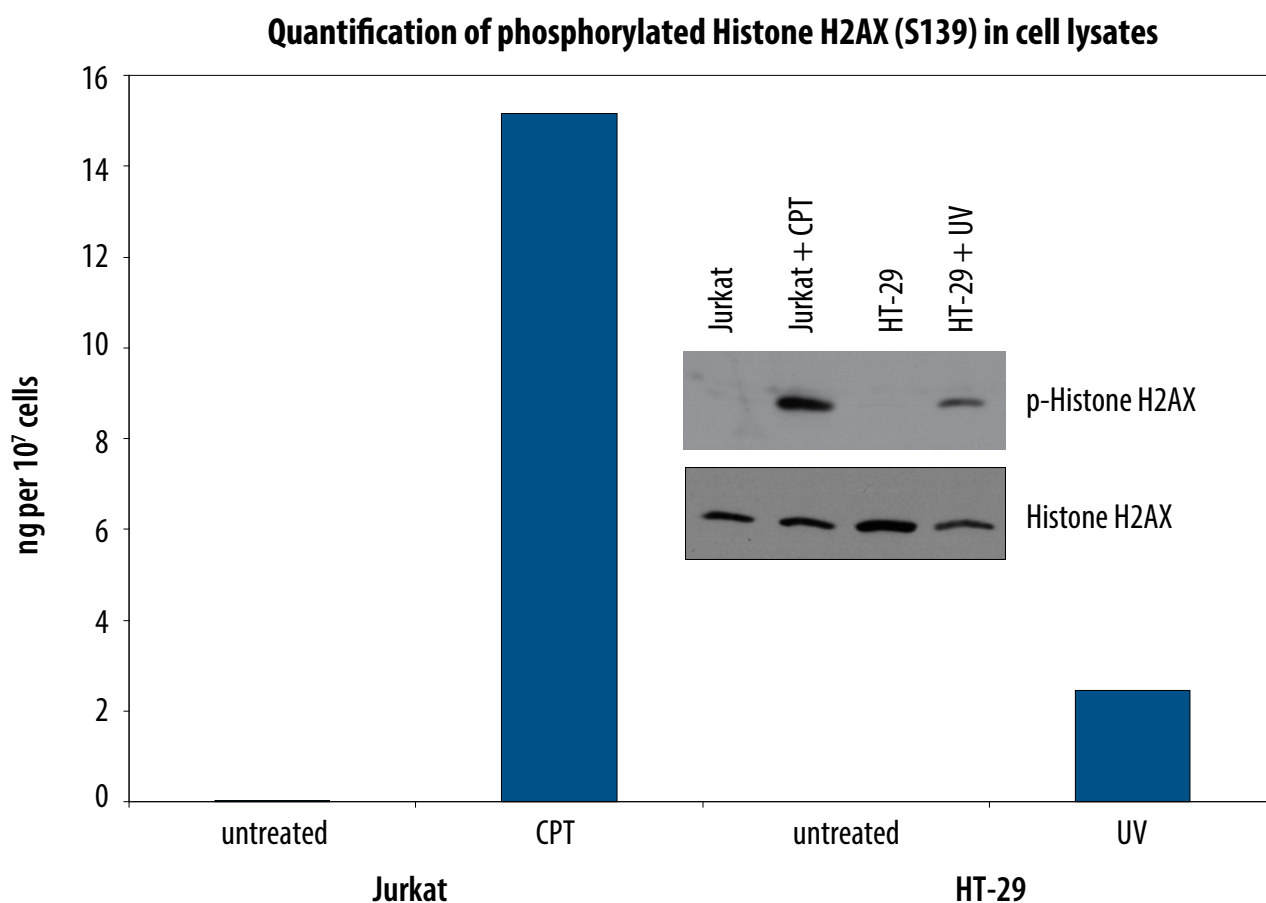


Figure 2: Lysates prepared from Jurkat human acute T cell leukemia cells treated with 1 μM of CPT for three hours and HT-29 human colon adenocarcinoma cells treated with 20 mJ/cm^2 UV were quantified with this DuoSet IC ELISA. The same lysates were also immunoblotted (inset) with either anti-phospho-Histone H2AX (R&D Systems, Catalog # AF2288) or anti-Histone H2AX (R&D Systems, Catalog # MAB3406) antibodies. The DuoSet IC ELISA results correlate well with the relative amounts of phosphorylated Histone H2AX detected by Western blot. The immunoblot with anti-Histone H2AX antibody indicates that total levels of Histone H2AX remained constant during the induction.

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.

12								
11								
10								
9								
8								
7								
6								
5								
4								
3								
2								
1								
	A	B	C	D	E	F	G	H

NOTES