

ab139412 – Cell Cycle In-Cell ELISA Kit (IR)

Instructions for Use

For measuring in high throughput, levels of cell cycle marker proteins Cdk2 phosphorylated at tyrosine 15 and Histone H3 phosphorylated at serine 10 in human and mouse cell lines.

This product is for research use only and is not intended for diagnostic use.

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1. Introduction

Principle: ab139412 is an In-Cell ELISA (ICE) assay kit that uses quantitative immunocytochemistry to measure levels of Cdk2 protein phosphorylated Tyr15 and Histone H3 protein phosphorylated Ser10 levels in cultured cells. Cells are fixed in a microplate and targets of interest are detected with highly specific, well-characterized antibodies. Relative target levels are quantified using IRDye®-labeled Secondary Antibodies and IR imaging such as a LI-COR® Odyssey® or Aeries® system. Antibody signal intensity can be normalized to the total cell amount using Janus Green stain.

Background: The Cdk2 (pTyr15) + Histone H3 (pSer10) In-Cell ELISA Kit (IR) (ab139412) is designed to study cell cycle effects in response to various stimuli. Monoclonal antibodies specific to Cdk2 (pTyr15) and Histone H3 (pSer10) are used in this high-throughput duplexing plate-based assay. Cdk2 (pTyr15) is elevated in G1/S phase of the cell cycle and Histone H3 (pSer10) is elevated in G2/M phase. Cyclin-dependent kinase 2 (Cdk2) is a nuclear protein kinase that functions in the G1/S phase of the cell cycle. Inhibitory phosphorylation occurs on residues Thr14 and Tyr15; activation of Cdk2 includes dephosphorylation of these residues by cdc25. Cdk2 can form a complex with Cyclin A, D or E. Phosphorylation of Cdk2 at Tyr15 indicates that a cell is at the G1/S transition.

Histone H3 is one of the four core histone proteins (H2A, H2B, H3 and H4) that pack DNA in nucleosomes. Post-translational modifications of histones include phosphorylation and acetylation

and are important for chromatin assembly and gene expression. Phosphorylation of Histone H3 at Ser10 is tightly correlated with chromosome condensation during mitosis. Hence, Histone H3 pSer10 signal indicates a mitotic cell with condensed DNA.

In-Cell ELISA (ICE) technology employed to perform quantitative immunocytochemistry of cultured cells with a near-infrared fluorescent dye-labeled detector antibody. The technique generates quantitative data with specificity similar to western blotting, but with much greater quantitative precision and higher throughput due to the greater dynamic range and linearity of direct fluorescence detection and the ability to run 96 samples in parallel. Because the Cdk2 (pTyr15) antibody is a rabbit antibody and the Histone H3 (pSer10) antibody is a mouse antibody, they can be measured simultaneously in the same well using the cocktail of provided primary antibodies and the provided cocktail of IRDye[®]-labeled species-specific secondary antibodies when using a LI-COR infrared imager. This method rapidly fixes the cells in situ, stabilizing the in vivo levels of proteins and their post-translational modifications, and thus eliminating changes during sample handling, such as in the preparation of protein extracts. Finally, the Cdk2 (pTyr15) and Histone H3 (pSer10) signals can be normalized to cell amount, measured by the provided Janus Green whole cell stain, to further increase the assay precision.

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2. Assay Summary

Seed cells in a microwell culture plate.



Fix cells with 4% paraformaldehyde for 10 minutes and wash.



Permeabilize cells for 10 minutes.



Block cells for 2 hours.



Incubate cells with primary antibodies for 2 hours at room temperature or overnight at 4°C.



Incubate cells for 2 hours with secondary antibodies diluted in 1X Incubation Buffer and wash



Scan the plate

3. Kit Contents

Item	Quantity
10X Phosphate Buffered Saline (PBS)	100 mL
100X Triton X-100 (10% solution)	500 μ L
400X Tween-20 (20% solution)	2 mL
10X Blocking Buffer	10 mL
100X Anti-Cdk2 (pTyr15) Primary Antibody (Rabbit Monoclonal)	120 μ L
100X Anti-Histone H3 (pSer10) Primary Antibody (Mouse Monoclonal)	120 μ L
1000X IRDye [®] -Labeled Secondary Antibody Cocktail	24 μ L
Janus Green Stain	17 mL

4. Storage and Handling

Upon receipt spin down the contents of the IRDye[®]-Labeled Secondary Antibody tube and protect from light. Store all components upright at 4°C. This kit is stable for at least 6 months from receipt.

5. Additional Materials Required

- A LI-COR® Odyssey® or Aeries® infrared imaging system.
- 96 or 384-well amine coated plate(s).
- 20% paraformaldehyde.
- Nanopure water or equivalent.
- Multi- and single-channel pipettes.
- 0.5 M HCl (optional for Janus Green cell staining procedure).
- Optional humid box for overnight incubation step.
- Optional plate shaker for all incubation steps.

6. Preparation of Reagents

- 6.1 Equilibrate all reagents to room temperature.
- 6.2 Prepare 1X PBS by diluting 45 mL of 10X PBS in 405 mL of nanopure water or equivalent. Mix well and store at room temperature.
- 6.3 Prepare 1X Wash Buffer by diluting 750 μ L of 400X Tween-20 in 300 mL of 1X PBS. Mix well and store at room temperature.
- 6.4 Immediately prior to use prepare 8% Paraformaldehyde Solution in PBS. To make 8% Paraformaldehyde combine 6 mL of nanopure water or equivalent, 1.2 mL of 10X PBS and 4.8 mL of 20% Paraformaldehyde.

Note – Paraformaldehyde is toxic and should be prepared and used in a fume hood. Dispose of paraformaldehyde according to local regulations.

- 6.5 Immediately prior to use prepare 1X Blocking Buffer by diluting 5 mL of 10X Blocking Buffer in 45 mL of 1X PBS.
- 6.6 Immediately prior to use prepare 1X Permeabilization Solution by diluting 150 μ L of 100X Triton X-100 in 15 mL of 1X Blocking Buffer. Mix well.

7. Sample Preparation

Note: The protocol below is described for a 96-well plate. If performing assay on a 384-well plate, adjust volumes accordingly. This assay has been optimized for use on adherent cells. For suspension cells, refer to section 11.4. Ensure the microplate does not dry out at any time before or during the assay procedure.

- 7.1 Seed adherent cells directly into an amine coated plate and allow them to attach for >6 hours or overnight. It is advised to seed in 100 μ L volume of the same media used to maintain the cells in bulk culture. The optimal cell-seeding density is cell-type dependent. The goal is to seed cells such that they are just reaching confluency (but not over-confluent) at the time of fixation. As an example, HeLa cells may be seeded at ~ 20,000 cells per well and cultured overnight for fixation the following day.

- 7.2 The attached cells can be treated if desired with a drug of interest. See section 10 of this protocol for suggested positive controls.
- 7.3 Fix cells by adding a final concentration of 4% Paraformaldehyde Solution. This can be achieved by one of two methods: (1) Add an equal volume of 8% Paraformaldehyde Solution to the culture volume (e.g. add 100 μ L 8% Paraformaldehyde to a well with 100 μ L media) or (2) gently remove/dump culture media from the wells and replace with 100 μ L 4% Paraformaldehyde Solution.

Note – If treatments cause cells to become loosely attached, the overlay fixation option above (1) is recommended. See also FAQ 11.3. Use of amine coated plates minimizes cell loss, even mitotic cells.

- 7.4 Incubate for 10 minutes at room temperature.
- 7.5 Gently aspirate or dump the Paraformaldehyde Solution from the plate and wash the plate 3 times briefly with 1X PBS. For each wash, rinse each well of the plate with 200 μ L of 1X PBS. Finally, add 100 μ L of 1X PBS to the wells of the plate. The plate can now be stored at 4°C for several days. Cover the plate with a lid or seal while stored. For prolonged storage supplement PBS with 0.02% sodium azide.

Note – The plate should not be allowed to dry at any point during or before the assay. Both paraformaldehyde and

sodium azide are toxic, handle with care and dispose of according to local regulations.

8. Assay Procedure

It is recommended to use a plate shaker (~200 rpm) during all incubation steps. Any step involving removal of buffer or solution should be followed by blotting the plate gently upside down on a paper towel before refilling wells. Unless otherwise noted, incubate at room temperature.

During development of this assay edge effect problems have not been observed. However if edge effects are of concern, the perimeter wells of the plate can be used as control wells (primary antibody omitted). Regardless, one well from which the primary antibodies are excluded to determine background signals of the assay is required.

- 8.1 Remove 1X PBS and add 100 μ L 1X Permeabilization Solution to each well of the plate. Incubate 10 minutes at room temperature.
- 8.2 Wash plate once with 200 μ L of 1X PBS solution.
- 8.3 Add 200 μ L of 1X Blocking Buffer and incubate for 2 hours at room temperature.
- 8.4 Prepare 1X Primary Antibody Cocktail Solution by diluting both of the primary antibodies by 100X into appropriate volume of 1X Blocking Buffer (i.e. 12 mL of 1X Blocking Buffer + 120 μ L of the 100X Mouse Anti- Histone H3

(pSer10) Primary Antibody + 120 μ L of the 100X Rabbit Cdk2 (pTyr15) Primary Antibody).

- 8.5 Remove 1X Blocking Buffer and add 100 μ L 1X Primary Antibody Cocktail Solution to each well of the plate. Incubate for 2 hours at room temperature or overnight at 4°C.

Note – To determine the background signal it is essential to omit primary antibody from at least one well containing cells for each experimental condition.

- 8.6 Remove Primary Antibody Cocktail Solution and wash the plate 3 times briefly with 1X Wash Buffer. For each wash, rinse each well of the plate with 200 μ L of 1X Wash Buffer.

Do not remove the last wash until step 8.8.

- 8.7 Prepare 1X Secondary Antibody Cocktail Solution by diluting 12 μ L of 1000X IRDye®-Labeled Secondary Antibody Cocktail into of 12 mL 1X Blocking Buffer. Protect labeled antibodies from light.

Note – The secondary antibody cocktail contains IRDye800®-labeled anti-mouse antibody and IRDye680®-labeled anti-rabbit antibody.

- 8.8 Remove 1X Wash Buffer and add 100 μ L 1X Secondary Antibody Cocktail Solution to each well of the plate. Incubate 2 hours at room temperature in the dark.

- 8.9 Remove 1X Secondary Antibody Cocktail Solution and wash 3 times briefly with 1X Wash Buffer. For each wash, rinse each well of the plate with 200 μ L of 1X Wash Buffer.

8.10 Wash 2 times with 1X PBS, using 200 μ L for each well.

Do not remove the last wash.

8.11 Wipe the bottom of the plate and the scanner surface with a damp lint-free cloth to clean before scanning the plate on the LI-COR[®] Odyssey[®] system. Collect data in both the 700 (IR680) and 800 (IR800) channels according to manufacturer's instructions. The optimal focus off-set for typical amine plates is 3.9. The Cdk2 (pTyr15) protein signal corresponds to the 700 channel (IRDye680[®]) and the Histone H3 (pSer10) protein signal corresponds to the 800 channel (IRDye800[®]).

Note – The absolute value of the IR signal is dependent on the 700 and 800 channel intensity settings. Value 7 is recommended for initial scanning. Adjust as needed so that neither signal is saturated in any well.

8.12 Remove the last wash and add 100 μ L of Janus Green Stain to each well of the plate. Incubate plate for 5 minutes at room temperature.

Note – The IR signal should be normalized to the Janus Green staining intensity to account for differences in cell seeding density.

8.13 Remove the dye and wash the plate 5 times in deionized water or until excess dye is removed.

8.14 Remove last water wash, blot to dry, add 200 μ L of 0.5 M HCl to each well of the plate and incubate for 10 minutes.

- 8.15 Measure OD595 nm using a standard microplate spectrophotometer or measure a signal in the 700 nm channel using a LI-COR® Odyssey® scanner.

9. Data Analysis

- 9.1 Background subtraction. Determine the raw signal intensity (Integrated Intensity) values for the IR700 and IR800 channels for the wells that lacked primary antibodies. Subtract the mean IR700 and IR800 background values from all other IR700 and IR800 experimental values.
- 9.2 Janus Green normalization of both targets. Divide the background subtracted IR intensities (from 9.1) by the Janus Green value of the corresponding well. The result is the “normalized intensity”.

10. Assay Performance and Specificity

Assay performance and specificity were tested using HeLa cells treated with hydroxyurea and paclitaxel. Hydroxyurea inhibits DNA

synthesis and arrests cells at the G1/S-phase transition of the cell cycle. Paclitaxel is a microtubule stabilizer and arrests cells at the G2/M phase of the cell cycle.

Figures 1, 2 and 3 show typical results using ab139412. Note that Cdk2 pTyr15 and Histone H3 pSer10 are inversely related to each other as their signal is maximal in different stages of the cell cycle.

Since treatments that induce cell cycle arrest will decrease the relative cell number in treated wells compared to control wells, it is strongly advised to perform the Janus Green cell stain and analyze data according to section 9.2 above.

Antibody Specificity - Because confidence in antibody specificity is critical to ICE data interpretation, the primary antibodies in this kit were validated for specificity by Western blotting (Figure 4).

Reproducibility - ICE results provide accurate quantitative measurements of antibody binding and hence cellular antigen concentrations. The coefficient of the intra-assay of variation of this assay kit for HeLa cells is typically <10% for both analytes. For example, the mean coefficient of the intra-assay of variation of HeLa cells treated with Paclitaxel and Hydroxyurea in the experiment described in figures 1 and 2 was 3.9% for Histone H3 (pSer10) and 4.4% for Cdk2 (pTyr15).

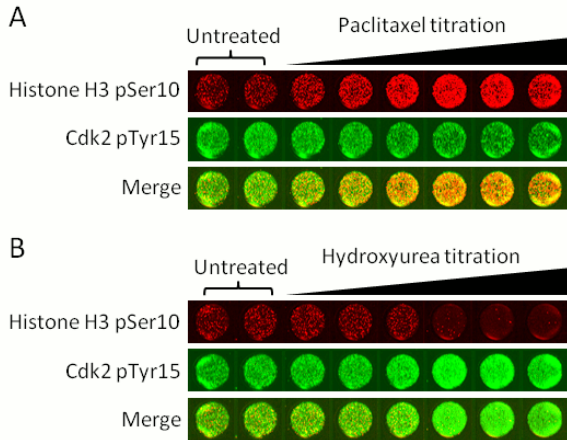


Figure 1. Sample IR data using ab139412 on HeLa cells treated with Paclitaxel and Hydroxyurea. (A) HeLa cells were treated for 24h with varying concentrations of paclitaxel (0.26 – 2000 μM). Histone H3 pSer10 (red) intensity increases with increasing paclitaxel whereas Cdk2 pTyr15 (green) intensity decreases. This is the expected result for paclitaxel treatment: mitotic arrest. (B) HeLa cells were treated for 24h with varying concentrations of hydroxyurea (0.002 – 5 mM). Histone H3 pSer10 (red) intensity decreases with increasing paclitaxel whereas Cdk2 pTyr15 (green) intensity increases. This is the expected result for hydroxyurea treatment: G1/S-phase arrest.

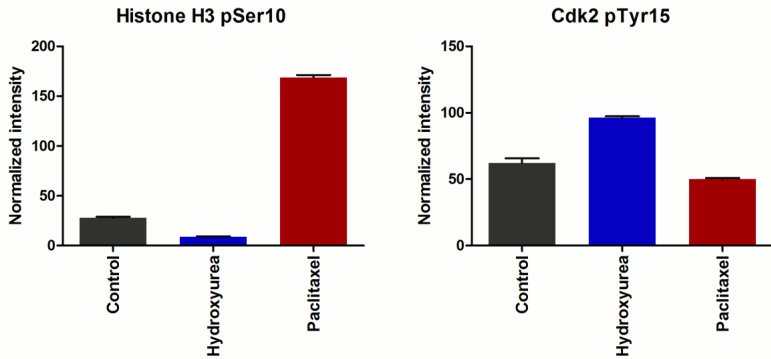


Figure 2. Sample experiment using ab139412 on HeLa cells treated with paclitaxel and hydroxyurea. Quantification of the data shown in Figure 1. Data shown is for 24 hour treatment with 1 mM hydroxyurea, 333 nM paclitaxel and untreated (Control). (Normalized intensity is described in section 9.)

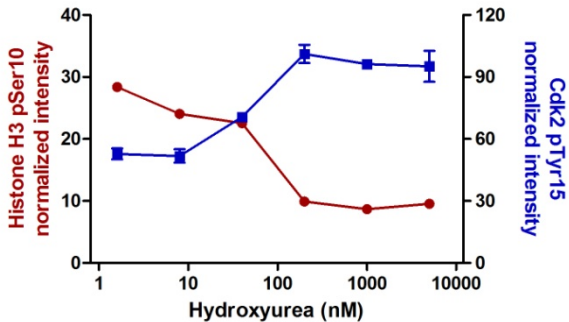


Figure 3. Sample data using ab139412 on HeLa cells treated with a titration of hydroxyurea. Quantification of the data shown in Figure 1B. Cdk2 pTyr15 intensity increases with Hydroxyurea treatment dose whereas Histone H3 pSer10 intensity decreases.

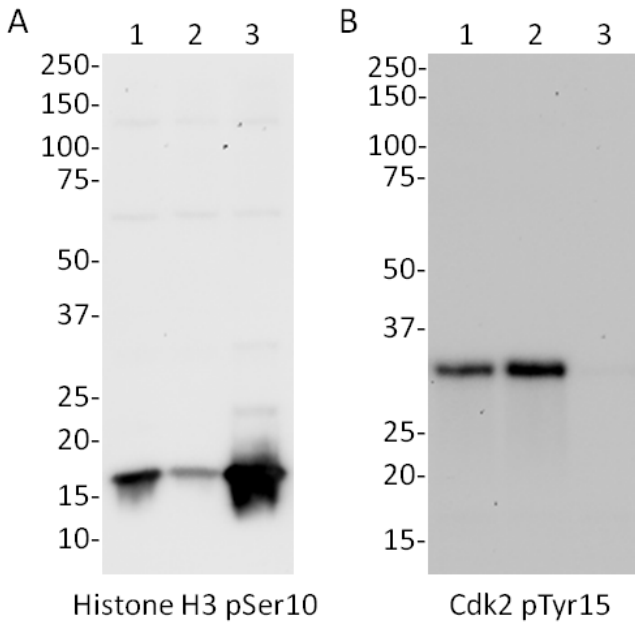


Figure 4. Antibody specificity demonstrated by Western Blot Analysis. Whole cell lysates from HeLa cells were analyzed by western blot with the primary antibodies used in this assay kit. (A) Histone H3 pSer10 antibody: untreated (lane 1), hydroxyurea = G1/S arrest (lane 2), paclitaxel = G2/M arrest (lane 3). (B) Cdk2 pTyr15 antibody: untreated (lane 1), thymidine = G1/S arrest (lane 2), nocodazole = G2/M arrest (lane 3).

Frequently Asked Questions

11.1 How many cells do I seed per well?

The cell seeding density varies by cell type and depends both on the cell size and the abundance of the target protein. The cell seeding will likely need to be determined experimentally by microscopic cell density observation of serially diluted cells. For adherent cells, prepare serial dilution of the cells in a plate and allow them to attach prior to observation. The goal is to have cells that are just confluent at the time of fixation. Overly confluent cells may have compromised viability and tend not to adhere as well to the plate. Under-seeded cells may yield too low a signal, depending on the analyte. Keep in mind that drug treatments or culture conditions may affect cell density/growth.

11.2 Do I have to use an amine-coated microplate?

We have tested black-wall-amine and cell-culture treated microplates and found that amine coated plates improve reproducibility and specificity in comparison to standard plates. In addition, multiple cell types appear to have the most favorable growth and even seeding on amine plates. The assay performance is only guaranteed with amine plates.

- 11.3 A treatment causes cells detachment. Is there a way to prevent the lost of detaching cells?

Loss of floating cells can be easily prevented by inserting two centrifugation steps into the protocol: (1) Immediately prior the addition of Paraformaldehyde Solution (step 7.3) centrifuge the microtiter plate at 500 x g for 5-10 minutes, (2) Immediately after the addition of Paraformaldehyde Solution centrifuge the microtiter plate again at 500 x g for 5-10 minutes. Continue in the fixation for a total of 15-20 minutes. For examples using detaching cells in ICE, refer to ab110215 Product Booklet.

- 11.4 Can I use suspension cells for ICE?

The In-Cell ELISA can be easily adapted for use with suspension cell. In this case an amine plate must be used. To ensure efficient crosslinking of the suspension cells to the amine plate, cells must be grown and treated in a different plate or dish of choice. The treated suspension cells are then transferred to the amine plate in 100 μ L of media per well. The cell seeding density of the amine plate is cell type-dependent. If necessary, cells can be concentrated by centrifugation and re-suspended in PBS (preferred) or in media to desired concentration. As an example, HL-60 and Jurkat cells should be seeded, respectively, at 300,000 and 200,000 cells per well in 100 μ L of PBS (preferred) or media. After the cells are transferred to the amine plate immediately follow the fixation procedure as described in section 11.3.

For examples using suspension cells in ICE, refer to ab110215 Product Booklet.

Note – With suspended cells, the media should contain no more than 10% fetal serum otherwise efficiency of the suspension cell crosslinking to the plate may be compromised.

- 11.5 I grow my cells in 15% FBS, will this interfere with the cell fixation?

Culture media containing up to 15% fetal serum does not interfere with the cell fixation and cross-linking to the plate.

- 11.6 How do I measure the assay background?

It is essential to omit primary antibody in at least one well (3 wells recommended) to provide a background signal for the experiment which can be subtracted from all measured data. This should be done for each experimental condition.

- 11.7 Can I measure each target in an individual well instead measure both targets simultaneously in a single well?

If the user prefers to use the Cdk2 (pTyr15) antibody and the Histone H3 (pSer10) antibody separately that may be done as well. Sufficient amounts of the secondary antibody cocktail and all buffers are provided to use each of the primary antibodies separately.

11.8 Is Janus Green normalization necessary?

Janus Green is a whole-cell stain that is useful to determine if a decrease in IR intensity in a well is due to a relevant down-regulation or degradation of the target analyte OR if it is a function of decreased cell number (e.g. due to cytotoxic effect of a treatment). As such it is not a required readout, but is useful in analysis to determine a normalized intensity value (section 9.2).

11. Troubleshooting

Problem	Cause	Solution
Low Signal	Too brief incubation times	Ensure sufficient incubation times
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
	Insufficient cells	Increase seeding density of cells; goal is newly confluent cells at time of fixation.
	Cell detachment	Refer to section 11.3
High CV	Plate is insufficiently washed	Review the manual for proper washing. If using a plate washer, check that all ports are free from obstruction
	Contaminated wash buffer	Make fresh wash buffer
	Artifacts creating increased signal on IR	Troughs used for multichannel pipetting could be dirty.
	Edge effects	Do not use the edges of the plate. Incubate in a humid box
	Variable cell seeding	Plate cells with care and normalize with Janus Green

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