ab131382
Human H2A.X (phospho S139) In-Cell ELISA Kit (IR)

Instructions for use:

For measuring in high throughput, levels of H2A.X protein phosphorylated at S139 in human cell lines.

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

Version 5 Last Updated 29 August 2019

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1. BACKGROUND

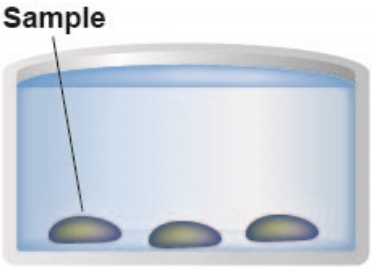
Principle: ab131382 is an In-Cell ELISA (ICE) assay kit that uses quantitative immunocytochemistry to measure levels of H2A.X protein phosphorylated Ser139 in cultured cells. Cells are fixed in a microplate and targets of interest are detected with a highly specific, well-characterized antibody. Relative target levels are quantified using an IRDye®-labeled Secondary Antibody and IR imaging using a LI-COR® Odyssey® or Aerius® system. Optionally, antibody signal intensity can be normalized to the total amount using Janus Green cell-stain.

Background: The Human H2A.X (phospho S139) In-Cell ELISA Kit (IR) (ab131382) is designed to study the induction of DNA damage in response to various stimuli. A rabbit monoclonal antibody specific to H2A.X (phospho S139) is used in this high-throughput plate-based assay. H2A.X (phospho S139) is a reliable readout for double-stranded DNA breaks.

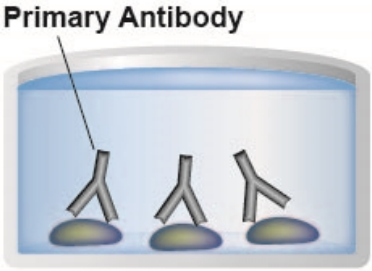
H2A.X is a variant in the H2A histone family. Upon DNA damage, H2A.X is phosphorylated at Serine 139 and localizes to sites of double-stranded DNA breaks. Also known as gamma-H2A.X, H2A.X (phospho S139) serves to mark DNA break sites and recruit repair enzymes. In mammals, ATM, ATR and DNA-PK kinases are all known to phosphorylate H2A.X.

In-Cell ELISA (ICE) technology is used 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 up to 96 samples in parallel. This method rapidly fixes the cells in situ, stabilizing the in vivo levels of proteins and their post-translational modifications, and thus essentially eliminates changes during sample handling, such as preparation of protein extracts. Finally, the H2A.X (phospho S139) signal can be normalized to cell amount, measured by the provided Janus Green whole-cell stain, to further increase the assay precision.

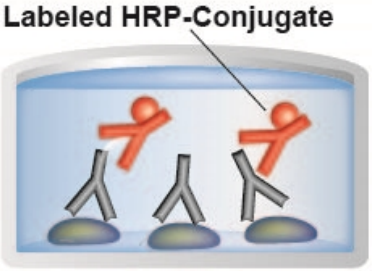
2. ASSAY SUMMARY



Seed cells and incubate overnight. Apply treatment activators or inhibitors. Fix cells with Fixing Solution. Incubate at room temperature. Add Quenching Buffer. Incubate at room temperature. Add Blocking Buffer. Incubate at 37ºC.



Add prepared primary antibody to each well used. Incubate at room temperature.



Empty and wash each well. Add prepared secondary antibody. Incubate at room temperature.

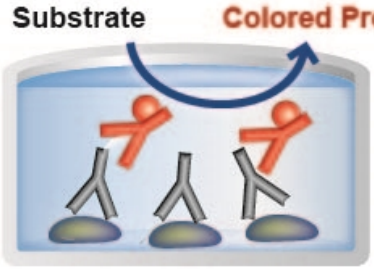


Image plate and analyze data. If desired, stain with Janus Green and measure relative cell seeding density in a microplate spectrophotometer or IR imager.
Calculate ratios and perform data analysis.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit at +4ºC in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature. **Reconstituted components are stable for 2 months.**

5. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
10X Phosphate Buffered Saline (PBS)	100 mL	4°C
100X Triton X-100 (10% solution)	500 µL	4°C
400X Tween-20 (20% solution)	2 mL	4°C
10X Blocking Solution	10 mL	4°C
100X Anti-H2A.X (phospho S139) Rabbit Primary Antibody	120 µL	4°C
1000X IRDye®-Labeled Secondary Antibody (anti-Rabbit IRDye680®)	24 µL	4°C
Janus Green Stain	17 mL	4°C

7. MATERIALS REQUIRED, NOT SUPPLIED

- A LI-COR® Odyssey® or Aerius® 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.

8. TECHNICAL HINTS

- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Keep enzymes, heat labile components and samples on ice during the assay.
- Make sure all buffers and solutions are at room temperature before starting the experiment.
- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Make sure you have the right type of plate for your detection method of choice.
- Make sure the heat block/water bath and microplate reader are switched on before starting the experiment.

9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening
- Equilibrate all reagent to room temperature

9.1. 1X PBS

Prepare by diluting 45 mL of 10X PBS in 405 mL of nanopure water or equivalent. Mix well and store at room temperature.

9.2. 1X Wash Buffer

Prepare by diluting 750 µL of 400X Tween-20 in 300 mL of 1X PBS. Mix well and store at room temperature.

9.3. 8% Paraformaldehyde Solution

Immediately prior to use prepare 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.

9.4. 1X Permeabilization Solution

Immediately prior to use prepare by diluting 150 µL of 100X Triton X-100 in 15 mL of 1X PBS. Mix well.

9.5. 1X Blocking Buffer

Immediately prior to use prepare by diluting 5 mL of 10X Blocking Solution in 45 mL of 1X PBS.

10. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to correct temperature prior to use.**
- **It is recommended to assay all standards, controls and samples in duplicate.**
- **Prepare all reagents, working standards, and samples as directed in the previous sections.**

Seeding

10.1. 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 a 100 µL 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.

10.2. The attached cells can be treated if desired with a drug of interest. See section 12 of this protocol for suggested positive controls.

10.3. Fix cells by adding a final concentration of 4% Paraformaldehyde Solution. This can be achieved by one of two means:

(1) Add an equal volume of 8% Paraformaldehyde Solution to the culture volume (e.g. add 100 µL 8% Paraformaldehyde to a well with 100 µL media) *or*

(2) Gently remove/dump culture media from the wells and replace with 100 µL 4% Paraformaldehyde Solution.

10.4. Incubate for 10 minutes at room temperature.

10.5. 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 µL of 1X PBS. Finally, add 100 µL 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.*

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 we have not observed problems with edge effects. However if edge effects are of concern, the perimeter wells of the plate can be used as control wells (primary antibody omitted). Regardless, it is required to leave at minimum one well from which the primary antibodies are excluded to determine background signals of the assay.

10.6. Remove 1X PBS and add 100 µL 1X Permeabilization Solution to each well of the plate. Incubate 10 minutes at room temperature.

10.7. Wash plate 1 time with 200 µL of 1X PBS solution.

10.8. Add 200 µL of 1X Block Solution and incubate for 2 hours at room temperature.

10.9. Prepare 1X Primary Antibody Solution by diluting the 100X Anti-H2A.X (phospho S139) Rabbit Primary Antibody 100X into appropriate volume of 1X Blocking Buffer (i.e. 12 mL of 1X Blocking Solution + 120 µL of the 100X Anti-H2A.X (phospho S139) Rabbit Primary Antibody.)

10.10. Remove Blocking Solution and add 100 µL 1X Primary Antibody 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.*

10.11. Remove Primary Antibody Solution and wash the plate 3 times briefly with 1X Wash Buffer. For each wash, rinse each well of the plate with 200 µL of 1X Wash Buffer.

10.12. **Do not remove the last wash until step 10.14.**

10.13. Prepare 1X Secondary Antibody Solution by diluting 12 µL of 1000X IRDye®-Labeled Secondary Antibody Cocktail into of 12 mL 1X Blocking Buffer. Protect labeled antibodies from light. *Note – The secondary antibody is IRDye680®-Labeled anti-rabbit antibody.*

10.14. Remove 1X Wash Buffer and add 100 µL 1X Secondary Antibody Solution to each well of the plate. Incubate 2 hours at room temperature in the dark.

10.15. Remove 1X Secondary AntibodySolution and briefly wash 3 times with 1X Wash Buffer. For each wash, rinse each well of the plate with 200 µL of 1X Wash Buffer.

10.16. Wash 2 times with 1X PBS, using 200 µL for each well. **Do not remove the last wash.**

10.17. 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 the 700 (IR680) channel according to manufacturer's instructions. The optimal focus off-set for typical amine plates is 3.9. The H2A.X (phospho S139) protein signal corresponds to the 700 channel (IRDye680®).

***NOTE:** The absolute value of the IR signal is dependent on the intensity settings. Value 8 is recommended for initial scanning. Adjust as needed so that the signal is not saturated in any well.*

10.18. Remove the last PBS wash and add 100 µL 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.*

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

10.20. Remove last water wash, blot to dry, add 100 µL of 0.5 M HCl to each well of the plate and incubate for 10 minutes.

10.21. Measure OD595 nm using a standard microplate spectrophotometer or measure a signal in the 800 nm channel using a LI-COR® Odyssey® scanner.

11. CALCULATIONS

- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).

11.1. Background subtraction. Determine the raw signal intensity (Integrated Intensity) values for the IR680 channel for the wells that lacked primary antibody. Subtract the mean IR800 background values from all other IR800 experimental values.

11.2. 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".

12. TYPICAL DATA

Assay performance and specificity were tested using HeLa cells treated with camptothecin, etoposide and staurosporin. Camptothecin and Etoposide are DNA topoisomerase inhibitors and Staurosporin is a non-specific protein kinase inhibitor.

Figure 1 shows typical results using ab131382. Note that Camptothecin, Etoposide and Staurosporin induces H2A.X (phospho S139) to different extents and at different effective concentrations.

Antibody Specificity - Because confidence in antibody specificity is critical to ICE data interpretation, the primary antibody in this kit was validated for specificity by fluorescence immunocytochemistry and Western blotting (Figures 2, and 3 and 4, respectively).

In Figure 2 note the nuclear localization of the H2A.X (phospho S139) in Camptothecin treated cells and the absense of staining in untreated cells.

In Figure 3 note that the H2A.X (phospho S139) band is cell treatment specific. Figure 4 demonstrates that the H2A.X (phospho S139) is phospo-specific because the signal disappears when the lysate is incubated with lambda phosphatase.

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 example, the mean coefficient of the intra-assay of variation of HeLa cells treated with staurosporin in the experiment described in Figures 1 is 4% for H2A.X (phospho S139).

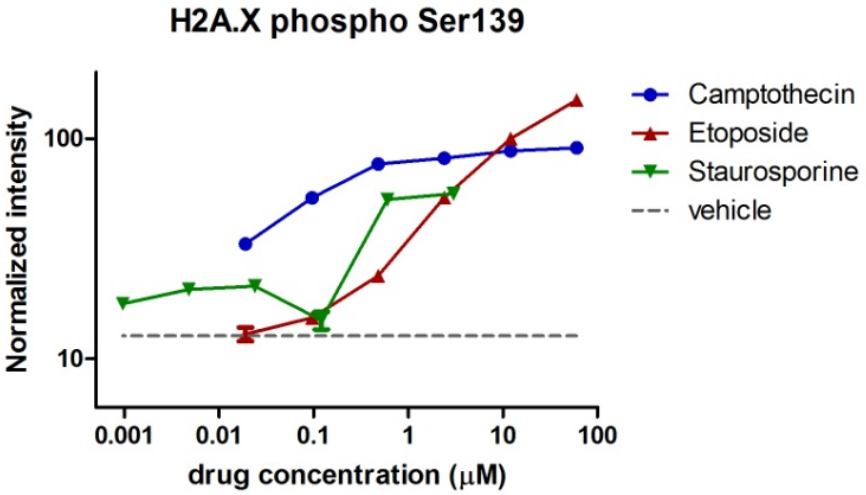


Figure 1. Sample experiment using ab131382 on HeLa cells following drug treatment: H2A.X (phospho S139) readout. HeLa cells were treated for 4 hours with dose titrations of camptothecin, Etoposide and Staurosporin. The data (presented as mean ± SD) were analyzed as described in Data Analysis section. The dashed grey line indicates the vehicle control signal.

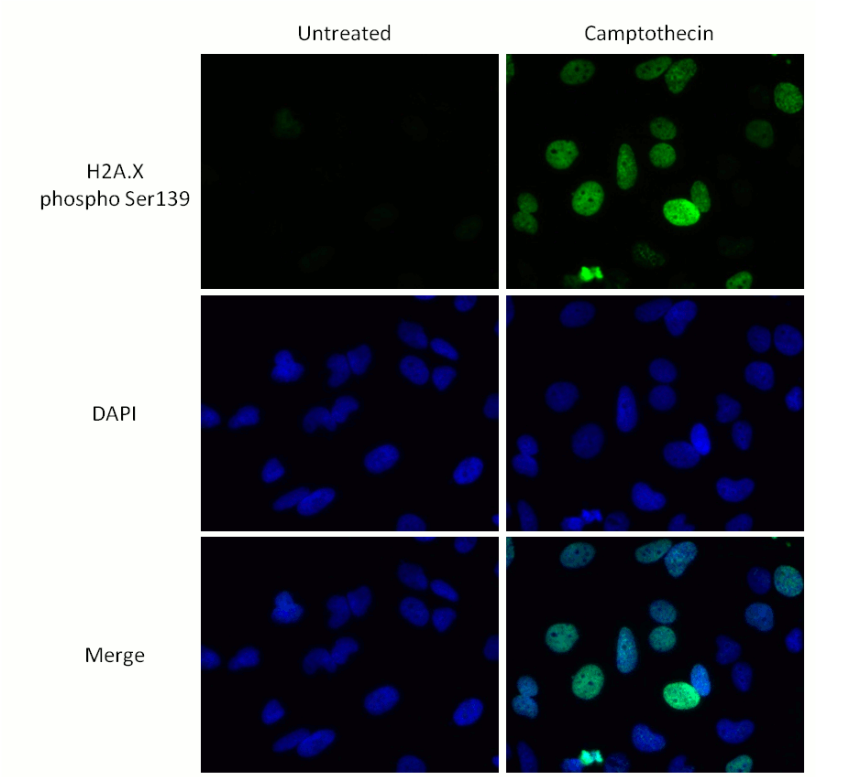


Figure 2. Specificity of H2A.X (phospho S139) antibody demonstrated by immunocytochemistry. The primary antibody used in this assay kit was validated by staining HeLa cells treated with 10 µM camptothecin or vehicle for 4 hours and imaged by fluorescent microscopy. Note the absence of H2A.X (phospho S139) signal in the untreated cells.

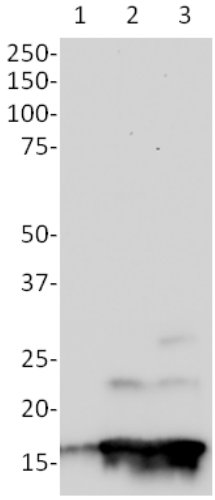


Figure 3. Antibody specificity demonstrated by Western Blot Analysis. Whole cell lysates from Jurkat cells were analyzed by western blot with the H2A.X (phospho S139) antibody used in this assay kit. Untreated cells (lane 1), camptothecin treated cells (lane 2) and UV exposed cells (lane 3) were analyzed.

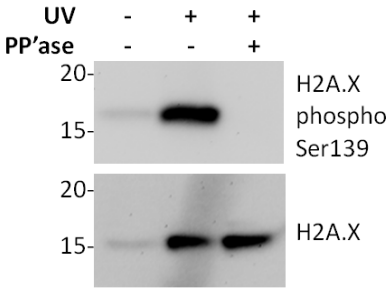


Figure 4. Antibody specificity demonstrated by Western Blot Analysis: H2A.X (phospho S139) is phospho-specific. *Jurkat cells were stimulated with UV light exposure to induce* H2A.X (phospho S139) *and then the UV treated lysate was treated with lambda protein phosphatase. Top panel: H2A.X (phospho S139) is induced by UV treatment and the western blot band is sensitive to phosphatase treatment. Lower panel: In contrast, total H2A.X levels are not sensitive to phosphatase treatment (ab124781).*

13. TROUBLESHOOTING

Problem	Cause	Solution
Assay not working	Use of ice-cold buffer	Buffers must be at room temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different 96-well plate	Clear plates
Sample with erratic readings	Samples not deproteinized (if indicated on protocol)	Use PCA precipitation protocol for deproteinization
	Cells/tissue samples not homogenized completely	Use Dounce homogenizer, increase number of strokes
	Samples used after multiple free/ thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Use of old or inappropriately stored samples	Use fresh samples or store at - 80°C (after snap freeze in liquid nitrogen) till use
	Presence of interfering substance in the sample	Check protocol for interfering substances; deproteinize samples
Unanticipated results	Measured at incorrect wavelength	Check equipment and filter setting
	Samples contain interfering substances	Troubleshoot if it interferes with the kit
	Sample readings above/ below the linear range	Concentrate/ Dilute sample so it is within the linear range

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

14. FAQ

14.1. 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 to not 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.

14.2. 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.

14.3. 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.

14.4. 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 cross-linking 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 µL 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 µL of PBS (preferred) or media. After the cells are transferred to the amine plate follow immediately the fixation procedure as described in section 14.3.

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.

14.5. **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.

14.6. **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.

14.7. **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).

15. NOTES

RESOURCES

UK, EU and ROW
Email: technical@abcam.com | Tel: +44-(0)1223-696000

Austria
Email: wissenschaftlicherdienst@abcam.com | Tel: 019-288-259

France
Email: supportscientifique@abcam.com | Tel: 01-46-94-62-96

Germany
Email: wissenschaftlicherdienst@abcam.com | Tel: 030-896-779-154

Spain
Email: soportecientifico@abcam.com | Tel: 911-146-554

Switzerland
Email: technical@abcam.com
Tel (Deutsch): 0435-016-424 | Tel (Français): 0615-000-530

US and Latin America
Email: us.technical@abcam.com | Tel: 888-77-ABCAM (22226)

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Email: ca.technical@abcam.com | Tel: 877-749-8807

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