

ab140359

Mitobiogenesis™ In-Cell ELISA Kit (Fluorescent)

Instructions for use:

For identifying inhibitors and activators of mitochondrial biogenesis in adherent cultured cells.

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

Table of Contents

INTRODUCTION	1
1. BACKGROUND	1
2. ASSAY SUMMARY	2
GENERAL INFORMATION	3
3. PRECAUTIONS	3
4. STORAGE AND STABILITY	3
5. LIMITATIONS	3
6. MATERIALS SUPPLIED	4
7. MATERIALS REQUIRED, NOT SUPPLIED	5
8. TECHNICAL HINTS	6
ASSAY PREPARATION	7
9. REAGENT PREPARTATION	7
ASSAY PROCEDURE	8
10. ASSAY PROCEDURE	8
DATA ANALYSIS	11
11. CALCULATIONS	11
12. TYPICAL DATA	12
RESOURCES	16
13. TROUBLESHOOTING	16
14. FAQ	18
15. NOTES	20

1. BACKGROUND

This In-Cell ELISA Kits employs quantitative immunocytochemistry to measure protein levels or post-translational modifications in cultured cells. Cells are fixed in a 96-well plate and targets of interest are detected with highly specific, well-characterized monoclonal antibodies and levels are quantified with enzyme-labeled secondary antibodies. Each kit contains sufficient reagents to analyze 96 samples of fixed human, rat, mouse, or bovine cells. This kit utilizes secondary antibodies conjugated to either horseradish peroxidase (HRP) or alkaline phosphatase (AP) which generate signal through the use of two spectrally distinct fluorogenic substrates. Fluorescence is measured using a standard fluorescent spectrophotometer and relative levels of target proteins are quantified.

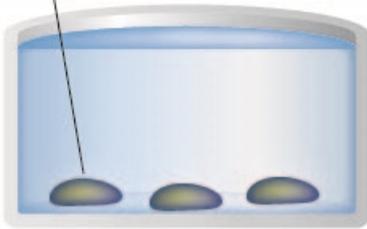
Alternative versions of this kit utilize: (1) colorimetric detection for use with standard plate readers for detection - MitoBiogenesis™ In-Cell ELISA Kit (Colorimetric) (ab110217). (2) LI-COR® near-infrared IRDyes® for detection - MitoBiogenesis™ In-Cell ELISA Kit (IR) (ab110216).

The MitoBiogenesis™ In-Cell ELISA Kit (ab140359) is designed to measure drug-induced effects on mitochondrial biogenesis early in the safety screening process. The MitoBiogenesis™ In-Cell ELISA Kit is a true duplexing 96/384-well assay that ratios both an mtDNA- and an nDNA-encoded protein in cultured or primary cells, and which requires very little sample prep and few overall steps.

Cells (human, rat or mouse) are seeded in 96- or 384-well microplates, and after exposure to experimental compounds for several cell doublings, the levels of two mitochondrial proteins are measured simultaneously in each well. The two proteins are each subunits of a different oxidative phosphorylation enzyme complex, one protein being subunit I of Complex IV (COX-I), which is mtDNA-encoded, and the other being the 70 kDa subunit of Complex II (SDH-A), which is nDNA-encoded. Complex IV includes several proteins which are encoded in the mitochondrion, while the proteins of Complex II are entirely encoded in the nucleus. Optionally, antibody signal intensity can be normalized to the total cell stain Janus Green.

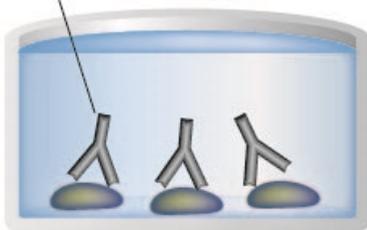
2. ASSAY SUMMARY

Sample



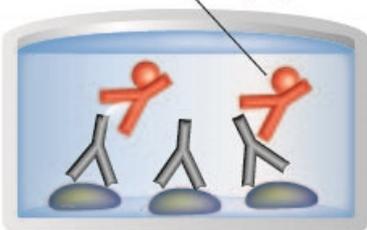
Seed cells and incubate overnight. Apply treatment activators or inhibitors. Fix cells with Fixing Solution. Incubate at room temperature. Add Quenching Buffer. Incubate at room temperature. Add Blocking Buffer. Incubate at 37°C.

Primary Antibody



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

Labeled HRP-Conjugate



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

Substrate

Colored Product



Empty and wash each well. Add the fluorogenic substrates to each well. Incubate at room temperature. Add Stop Solution to each well. Immediately begin recording the color development.

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 +4°C 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.

GENERAL INFORMATION

6. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
10X Phosphate Buffered Saline (PBS)	100 mL	+4°C
100X Triton X-100 (10% solution)	1.5 mL	+4°C
400X Tween – 20 (20% solution)	2 mL	+4°C
10X Blocking Buffer	15 mL	+4°C
100X Primary Antibody Cocktail Stock	120 µL	+4°C
1000X AP-Labeled Secondary Antibody (anti-Mouse IgG1)	20 µL	+4°C
1000X HRP-Labeled Secondary Antibody (anti-mouse IgG2a)	20 µL	+4°C
400X Fluorescent Substrate Cocktail	50 µL	+4°C
Fluorescent Substrate Buffer	12 mL	+4°C
8000X H ₂ O ₂	50 µL	+4°C
10X Quenching Solution	1.5 mL	+4°C
1X Janus Green Stain	17 mL	+4°C

7. MATERIALS REQUIRED, NOT SUPPLIED

- Fluorescent spectrophotometer.
- 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).
- Sodium Azide (preservative)
- 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.
- 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 PREPARTION

- Equilibrate all reagents to room temperature

9.1. 1X PBS

Dilute 50 mL of 10X PBS in 450 mL of Nanopure water or equivalent and mix well. Store at room temperature.

9.2. 1X Wash Buffer

Dilute 1.25 mL of 400X Tween-20 in 500 mL of 1X PBS and mix well. Store at room temperature.

9.3. 4% paraformaldehyde solution

Combine 12.5 mL of 1X PBS and 2.5 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 Quenching solution

Dilute 1.2 mL of 10X quenching solution in 10.8 mL of nanopure water and mix well. Store at room temperature.

9.5. 1X Permeabilization Solution

Add 150 μ L of 100X Triton X-100 to 14.85 mL of 1X PBS and mix well.

9.6. 2x Blocking Buffer

Add 5 mL of 10X Blocking Buffer to 20 mL of 1X PBS and mix well.

9.7. 1X Incubation Solution

Add 2.5 mL of 10X Blocking Buffer to 22.5 mL of 1X PBS and mix well.

9.8. Development Solution

Add 30 μ L of 400X Fluorescent Substrate Cocktail and 1.5 μ L of 8000X H₂O₂ to 12 mL of Fluorescent Substrate Buffer and mix well. Discard any excess after completing the experiment.

ASSAY PROCEDURE

10. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to correct temperature prior to use.**
 - **It is recommended to assay all controls and samples in duplicate.**
 - **Prepare all reagents and samples as directed in the previous sections.**
- 10.1. Seed adherent cells directly into an amine coated plate and allow them to attach for >6 hours or overnight. Cell seeding density, culture surface treatment needed for optimal attachment, culture medium and growth conditions are cell-type specific and will be defined by your experimental demands. To determine the background signal it is essential to omit primary antibody from at least one well containing cells for each experimental condition. For suggestions and general guidelines, see Appendix.
 - 10.2. In general, ICE analysis is optimal when the final fixed cell density is approximately 20,000 to 50,000 adhered cells per well in a 100 μ L volume of the same media used to maintain the cells in bulk culture.
 - 10.3. The attached cells can be treated, if desired, with a drug of interest.
 - 10.4. Fix cells by adding a final concentration of 4% Paraformaldehyde Solution. This can be achieved by one of two means: (1) Add a volume of 8% Paraformaldehyde Solution equal to that of 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.
 - 10.5. Incubate for 10 minutes at room temperature.
 - 10.6. 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.
 - 10.7. Add 100 μ L of 1X PBS with 0.02% sodium azide and store the plate overnight at 4°C. Sodium azide will preserve the plate for long storage and it will decrease the peroxidase background normally

ASSAY PROCEDURE

found on fixed cells. If longer storage is desired: Add 1x PBS with 0.02% sodium azide to final volume of 200 μ L per well and wrap plates with parafilm to reduce evaporation. Store plates at 4°C.

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*

- 10.8. Remove 1X PBS with 0.02% sodium azide and add 100 μ L of 1X Quenching Solution. Incubate for 10 minutes at room temperature. The 1X Quenching Solution will decrease the phosphatase background normally found on fixed cells.
- 10.9. Wash the plate 3 times with 1X PBS and proceed immediately to step 10.10.
- 10.10. Remove 1X PBS and add 100 μ L of 1X Permeabilization Solution to each well of the plate. Incubate 30 minutes at room temperature.
- 10.11. Remove 1X Permeabilization Solution and add 200 μ L of 2x Blocking Buffer to each well of the plate. Incubate 2 hours at room temperature.
- 10.12. Prepare 1X Primary Antibody Cocktail Solution by diluting the 100X Primary Antibody Cocktail Stock 1:100 into appropriate volume of 1X Incubation Buffer.
- 10.13. Remove 2X 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.*

- 10.14. Remove 1X 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 10.16.**

ASSAY PROCEDURE

- 10.15. Prepare 1X Secondary Antibody Cocktail Solution by diluting both 12 μL of 1000X HRP-Labeled Secondary Antibody (COX-1) and 12 μL of 1000X AP-Labeled Secondary Antibody (SDH-A) into 12 mL 1X Incubation Buffer.
- 10.16. Remove 1X Wash Buffer and add 100 μL 1X Secondary Antibody Cocktail Solution to each well of the plate. Incubate 2 hours at room temperature.
- 10.17. 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 μL of 1X Wash Buffer.
- 10.18. Wash 2 times with 200 μL per well of 1X PBS.
- 10.19. Remove PBS and add 100 μL per well of Development Solution and immediately begin recording with the following settings:

Mode:	Kinetic	End Point
Excitation spectra	AP substrate = 360 ± 5 nm HRP substrate = 530 - 570 nm	AP substrate = 360 ± 5 nm HRP substrate = 530 - 570 nm
Emission spectra:	AP substrate = 449 ± 10 nm HRP substrate = 585 - 600nm	AP substrate = 449 ± 10 nm HRP substrate = 585 - 600nm
Time:	up to 45 minutes	AP signal = between 20 – 45 min HRP signal = between 15 – 25 min
Interval:	1 - 5 minutes	n/a
Shaking:	Shake between readings	n/a

- 10.20. Remove the Development solution and add 100 μL of Janus Green Stain to each well of the plate. Incubate plate for 5 minutes at room temperature.

NOTE– The RFU signal should be normalized to the Janus Green staining intensity to account for differences in cell density.

- 10.21. Remove the dye and wash the plate 5 times in deionized water or until excess dye is removed.
- 10.22. 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 in a plate shaker.
- 10.23. Measure OD 595 nm using a standard microplate spectrophotometer.

11. CALCULATIONS

- 11.1. Background subtraction. Determine the raw RFU signal values for each substrate. Subtract the mean background values from all other RFU experimental values respectively.
- 11.2. Janus Green normalization of both targets. Divide the background subtracted RFU intensities (from 9.1) by the Janus Green value of the corresponding well. The result is the “normalized intensity”.
- 11.3. HRP and AP labeled antibody targets. The HRP signal corresponds to levels of COX-1 mtDNA-encoded protein present and the AP signal corresponds to levels of SDH-A nDNA-encoded protein present in the cells.
- 11.4. Intra-assay variation (%CV). The reported intra-assay variation of the assay was approximately 4.62% for HRP (COX-1) signal and 6.02% for AP (SDH-A) signal.

12. TYPICAL DATA

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

Assay utility can be demonstrated using chloramphenicol, a drug known to disturb mitochondrial biogenesis and specifically reduce levels of mtDNA-encoded proteins. Chloramphenicol also has well-known mitochondrial toxicity, the mechanism of which is the aforementioned reduction in mtDNA-encoded protein levels, and thus reduced OXPHOS function, through inhibition of mtDNA-encoded protein synthesis on mitochondrial ribosomes, which are structurally similar to bacterial ribosomes. In contrast, protein synthesis of nuclear-DNA-encoded transcripts on cytosolic ribosomes is unaffected by chloramphenicol.

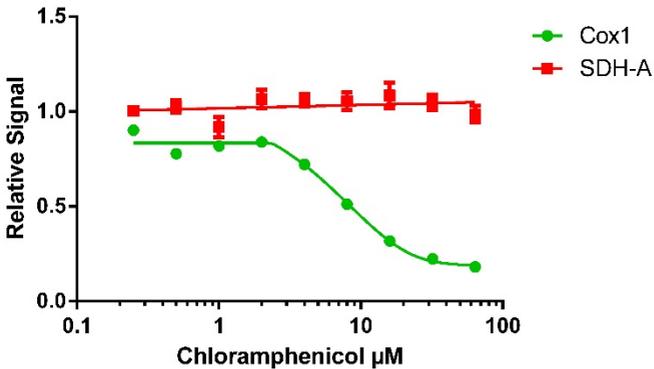


Figure 1. Inhibition of mitochondrial biogenesis by chloramphenicol

The IC_{50} of a drug's effect on mitochondrial protein translation can be determined quickly using the MitoBiogenesis™ ICE Kit. In this example, cells were seeded at 6,000 cells/well, allowed to grow for 6 days in a drug dilution series and then relative amounts of COX-I and SDH-A were measured in each well. Chloramphenicol inhibits mtDNA-encoded (HRP signal) COX-I protein synthesis relative to nuclear DNA-encoded (AP signal) SDH-A protein synthesis by 50% at 8.1 μM , %CV = 4.33% for HRP (COX-1) signal and 3.13% for AP (SDH-A) signal.

DATA ANALYSIS

Reliability – In-Cell ELISA results provide accurate quantitative measurements of cellular antigen concentrations. However, In-Cell ELISA does not provide internal confirmation of antibody binding specificity with each experiment, unlike traditional Western blots or immunocytochemistry, which allow confirmation by molecular weight or subcellular localization respectively. Therefore, confidence in antibody specificity is critical to In-Cell ELISA data interpretation and reliability. All of MitoSciences' In-Cell ELISA-qualified antibodies have been screened rigorously for specificity by Western blotting and by fluorescence immunocytochemistry under the conditions used for the In-Cell ELISA assay. Examples demonstrating the Western blot and immunocytochemical specificities of the two monoclonal antibodies used in the MitoBiogenesis™ In-Cell ELISA Kit are shown in Figures 2a and 2b.

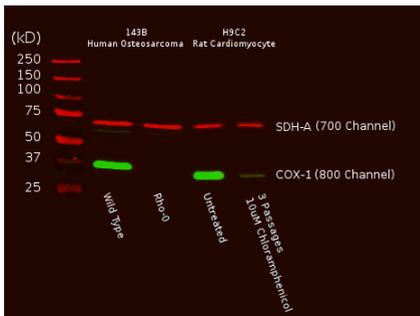
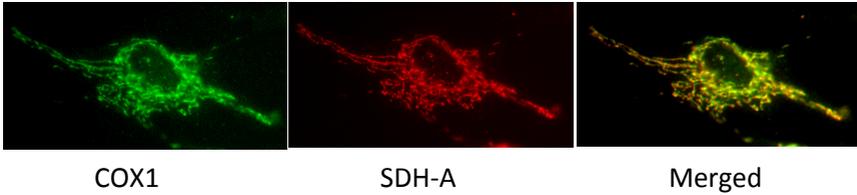


Figure 2a. Antibody specificity demonstrated by Western Blot.

A Western blot of total cell protein (10 μ g) from human or rat cultured cells was probed with the primary and secondary antibodies and scanned with a LI-COR® Odyssey® imager. The two mitochondrial proteins targeted by the two primary mAbs were labeled and visualized specifically despite the presence of thousands of other proteins. Furthermore, reduction of mtDNA levels in human Rho0 (mtDNA-depleted) cells, or inhibition of mitochondrial protein translation by chloramphenicol in rat cells result in specific reduction of COX-I protein while nuclear DNA-encoded SDH-A is unaffected.

DATA ANALYSIS



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gure 2b. Antibody specificity demonstrated by immunocytochemistry.

Two-color immunocytochemical labeling of cultured cells with the two ab140359 primary monoclonal antibodies specific for COX-I and SDH-A. The two antibodies exhibit striking and specific co-localization in the mitochondria, consistent with the known mitochondrial expression of both proteins.

Flexibility – HeLa cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum. The cells were seeded into a 96-well cell culture treated plate at the described seeding densities (Fig 3). The plate was processed according to this protocol and data recorded in SpectraMax microplate reader.

DATA ANALYSIS

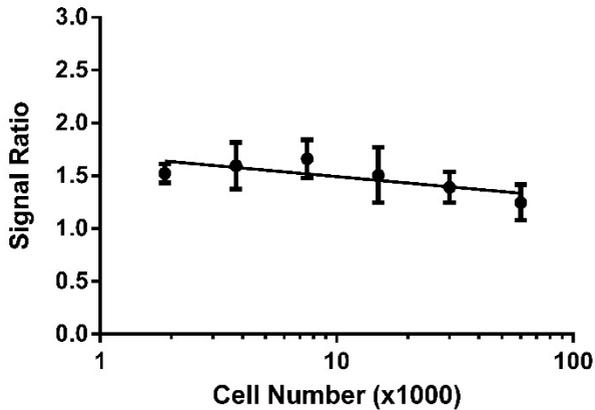


Figure 3. Quantitative measurement of the COX-I/SDH-A protein expression ratio. At all cell concentrations, a consistent ratio of mtDNA-encoded protein expression COX-I (HRP signal) to nuclear DNA-encoded mitochondrial protein expression SDH-A (AP signal) is observed in untreated cells. Therefore, normalizing COX-I levels to SDH-A levels simplifies data analysis and eliminates the need to perform all tests at the same cell concentration.

RESOURCES

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
Lower/ Higher readings in samples and Standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Allowing reagents to sit for extended times on ice	Always thaw and prepare fresh reaction mix before use
	Incorrect incubation times or temperatures	Verify correct incubation times and temperatures in protocol

RESOURCES

Problem	Cause	Solution
Standard readings do not follow a linear pattern	Pipetting errors in standard or reaction mix	Avoid pipetting small volumes (< 5 μ L) and prepare a master mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the tubes
	Standard stock is at incorrect concentration	Always refer to dilutions on protocol
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

14. FAQ

- **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.

- **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.

- **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.

RESOURCES

- **Can I use suspension cells for ICE?**

The In-Cell ELISA can be easily adapted for use with suspension cells. 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 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 follow immediately 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 cross-linking to the plate may be compromised.

- **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.

- **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.

- **Is Janus Green normalization necessary?**

Janus Green is a whole-cell stain that is useful to determine if a decrease in RFU 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 it is useful in the 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)

Canada

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

China and Asia Pacific

Email: hk.technical@abcam.com | Tel: 108008523689 (□□□□)

Japan

Email: technical@abcam.co.jp | Tel: +81-(0)3-6231-0940