ab110217 MitoBiogenesis™ In-Cell ELISA Kit (Colorimetric)

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.

Version 11c Last Updated 06 February 2023

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1. BACKGROUND

In-Cell ELISA Assay Kits use 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.

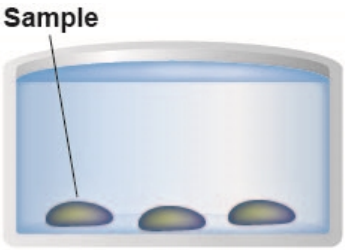
Each kit contains sufficient reagents to analyze two 96-well plates of fixed human, rat, mouse, or bovine cells. This kit utilizes colorimetric detection for use with standard plate readers. An alternate IR version of this kit is available which utilizes LI-COR® near-infrared IRDyes® for detection - MitoBiogenesis™ In-Cell ELISA Kit (IR) (ab110216).

ab110217 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.

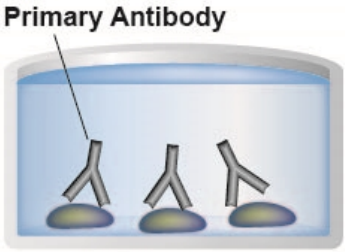
Cells (human, rat or mouse) are seeded in 96- or 384-well microplates, and after exposure to experimental compounds for several cell doublings, the levels of two mitochondrial proteins are measured simultaneously in each well. The two proteins are each subunits of a different oxidative phosphorylation 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, total protein levels can also be measured.

2. ASSAY SUMMARY

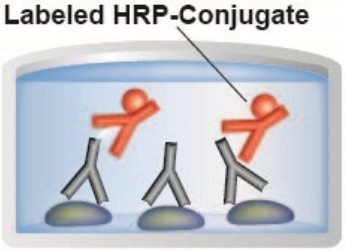
Unless specified the entire assay procedure is performed at room temperature.



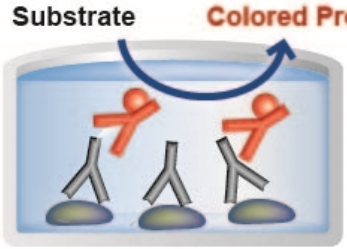
Seed cells into wells and incubate overnight at 37 C. Apply activators or inhibitors, and treat cells as desired at 37 C. Empty wells and fix cells with paraformaldehyde solution. Empty and wash wells. Empty wells and block endogenous alkaline phosphatase with acetic acid. Empty and wash wells. Empty wells and permeabilize cells with permeabilization buffer. Empty wells and block wells with blocking buffer.



Empty wells and incubate wells with prepared primary antibodies overnight at 4C.



Empty and wash wells. Incubate wells with prepared secondary AP/HRP conjugated antibodies.



Empty and wash wells. While recording absorbance at appropriate wavelength in kinetic mode, incubate wells sequentially with AP development solution and HRP development solution.

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 at least 6 months 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)	1.5 mL	+4°C
400X Tween – 20 (20% solution)	2 mL	+4°C
10X Blocking Buffer	15 mL	+4°C
200X Primary Antibodies	100 µL	+4°C
2500X AP-labelled Secondary Antibody	12 µL	+4°C
2500X HRP-labelled Secondary Antibody	12 µL	+4°C
1X AP Development Solution	24 mL	+4°C
1X HRP Development Solution	24 mL	+4°C
AP Development Reagent	139 mg	+4°C
1X Janus Green Stain	17 mL	+4°C
Plate Seals	2 EA	+4°C

7. MATERIALS REQUIRED, NOT SUPPLIED

- A microplate reader capable of reading 405 nm (for **AP detection of SDH-A**) and either 600 nm or 650 nm (for **HRP detection of COX-I)**.
- Two 96-flat bottom cell culture plates. Collagen or poly-lysine plates are highly recommended for improved cell seeding and data quality.
  NOTE: 384-well plates can also be used with minor protocol modifications – please see Appendix for details
- 20% paraformaldehyde
- Deionized water
- Acetic Acid
- Multichannel pipette (recommended)
- 0.5 M HCl (optional for Janus Green cell staining procedure)

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 PREPARATION

- Briefly centrifuge small vials at low speed prior to opening

9.1. **1X PBS**

Dilute 50 mL of 10X PBS in 450 mL deionized water. Mix well. Store at room temperature.

9.2. **1X Wash Buffer**

Dilute 500 µL of 400X Tween-20 in 199.5 mL of 1X PBS. Mix well. Store at room temperature.

9.3. **4% paraformaldehyde**

Dilute 2.5 mL 20% paraformaldehyde in 12.5 mL 1X PBS. Note – Paraformaldehyde is toxic and should be prepared and used in a fume hood. Dispose of paraformaldehyde according to local regulations.

9.4. **0.5% acetic acid**

Dilute 60 µL 100% acetic acid in 12 mL water.

9.5. **1X Permeabilization Buffer**

Dilute 150 µL 100X Triton X-100 in 14.85 mL 1X PBS.

9.6. **2X Blocking Buffer**

Dilute 5 mL 10X Blocking Buffer in 20 mL 1X PBS.

9.7. **1X Incubation Buffer**

by diluting 2.5 mL 10X Blocking Buffer in 22.5 mL 1X PBS.

9.8. **AP Development Reagent**

Dissolve the AP Development Reagent in 500 µL H_2O for a 50X solution. Immediately prior to use prepare a 1X AP Development solution by adding 240 µL of this 50X Solution into 11.76 mL 1X AP Development Buffer. Store the remaining 50X AP Development Solution at -20°C.

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.**

NOTE: It is recommended to use a plate shaker (~300 rpm) during incubation step.

10.1. 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 - 50,000 adhered cells per well.

10.3. Verify that cells have adhered to the bottom of the plate. Gently aspirate off medium into a container for proper disposal. Immediately add 4% paraformaldehyde to the wells of the plate. Gently trickle solution down the sides of each well to avoid dislodging cells. Incubate for 20 minutes.

10.4. Gently aspirate the paraformaldehyde solution from the plate and wash 3 times briefly with 300 µL 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 provided seal. Note: The plate should not be allowed to dry at any point during or before the assay. Dispose of paraformaldehyde according to local regulations. Remove PBS and blot plate upside down on a paper towel.

10.5. Add 100 µL of freshly prepared 0.5% acetic acid to each well of the plate for 5 minutes to block endogenous alkaline phosphatase activity.

10.6. Remove 0.5% acetic acid and wash plate once with 200 µL 1X PBS.

10.7. Remove PBS and add 100 µL of freshly prepared 1X Permeabilization Buffer to each well of the plate. Incubate 30 minutes.

10.8. Remove 1X Permeabilization Buffer and add 200 µL of 2X Blocking Buffer to each well of the plate. Incubate 2 hours.

10.9. Prepare 1X Primary Antibody Solution by diluting the 200X Primary Antibodies into 1X Incubation Buffer. Use 1 volume of the 200X stock per 199 volumes of 1X Incubation Buffer.

10.10. Remove 2X Blocking Buffer and add 100 µL diluted Primary Antibody Solution to each well of the plate. Incubate overnight at 4°C.

10.11. Remove Primary Antibody Solution and wash 3 times briefly in 1X Wash Buffer. For each wash, rinse each well of the plate with 250 µL of 1X Wash Buffer. Do not remove the last wash until step 10.13

10.12. Prepare 1X AP/HRP-labelled Secondary Antibody Solution by adding both 4.8 µL of 2500X AP-labeled and 4.8 µL of 2500X HRP-labeled Secondary Antibody into 12 mL 1X Incubation Buffer.

10.13. Remove the 1X Wash Buffer and add 100 µL 1X AP/HRP-labelled Secondary Antibody Solution to each well of the plate. Incubate for 1 hr at room temperature.

10.14. Remove Secondary Antibody Solution and wash 4 times briefly in 1X Wash Buffer. For each wash, rinse each well of the plate with 250 µL of 1X Wash Buffer

10.15. **AP Development for detection of SDH-A:**

Remove final Wash and blot plate face down to remove excess liquid. Add 100 µL AP Development Solution supplemented with AP Reagent (described in Step 9.8) to each empty well. Pop any bubbles and immediately record the yellow color development in the microplate reader prepared as follows:

Mode	Kinetic
Wavelength	405 nm
Time	15 minutes
Interval	20 seconds – 1 minute
Shaking	Shake between readings

Alternative – In place of a kinetic reading, at a user-defined time record the endpoint OD data at 405 nm.

10.16. **HRP Development for detection of COX-I:**

Having saved the AP data from step 10.15, completely remove AP Development Solution blot plate face down to remove excess liquid. Add 100 µL HRP Development Solution. Pop any bubbles and immediately record the blue color development in the microplate reader prepared as follows:

Mode	Kinetic
Wavelength	600 nm
Time	15 minutes
Interval	20 Seconds – 1 minute
Shaking	Shake between readings

Alternative – In place of a kinetic reading, at a user-defined time record the endpoint OD data at 600 nm, or 650 nm.

10.17. Save the HRP data from step 10.16. Analyze both data sets using Microplate reader software. Tips on data analysis are given below in Appendix

10.18. **Whole Cell Staining with Janus Green (optional)**

10.19. Completely remove HRP Development Solution from the microplate wells and blot to dry. Add 50 µL of 1X Janus Green Stain per well. Incubate plate for 5 minutes at room temperature.

10.20. Remove dye, wash plate 5 times in ultrapure water or until excess dye is removed.

10.21. Remove last water wash, blot to dry, add 100 µL of 0.5 M HCl and incubate for 10 minutes.

10.22. Shake the plate for 10 minutes, and measure the OD 595 nm using a standard microplate spectrophotometer.

10.23. The AP and HRP data can be normalized to the Janus Green staining intensity to account for differences in cell seeding density.

11. TYPICAL DATA

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

11.1. Utility

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

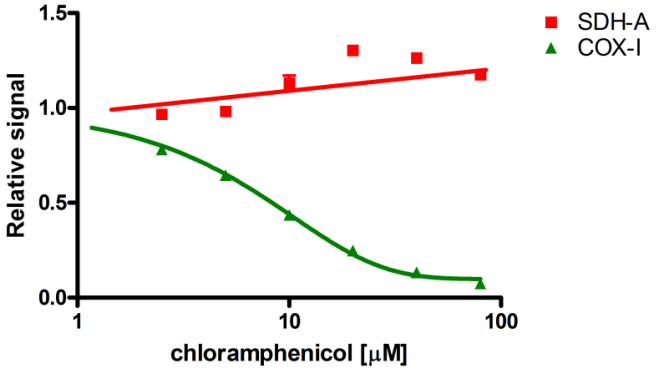


Figure 1. Inhibition of mitochondrial biogenesis by chloramphenicol:

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 3 cell doublings in a drug dilution series and then the relative amounts of COX-I, and SDH-A were measured in each well. Chloramphenicol inhibits mtDNA-encoded COX-I protein synthesis relative to nuclear DNA-encoded SDH-A protein synthesis by 50% at 8.1 µM.

11.2. Reliability

In-Cell 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 Abcam's 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 ab110217 MitoBiogenesis™ In-Cell ELISA Kit are shown in Figures 2a and 2b.

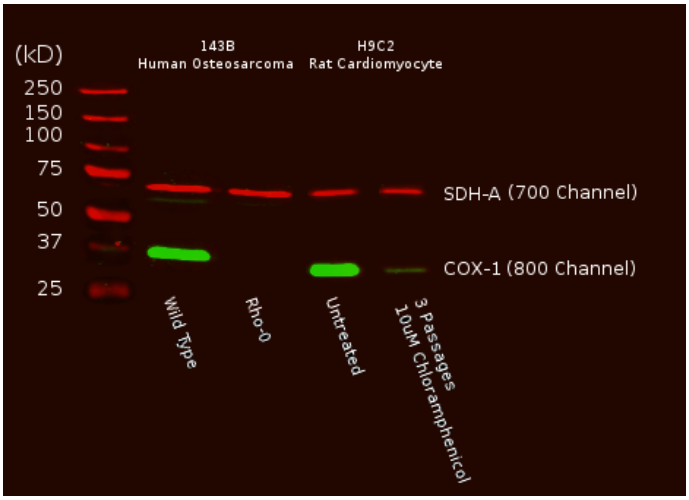


Figure 2a. Antibody specificity demonstrated by western Blot: A western blot of total cell protein (10 µg) from human or rat cultured cells was probed with the primary and secondary antibodies and scanned with a LI-COR® Odyssey® imager. The two mitochondrial proteins targeted by the two primary mAbs 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 both result in specific reduction of COX-I protein while nuclear DNA-encoded SDH-A is unaffected.

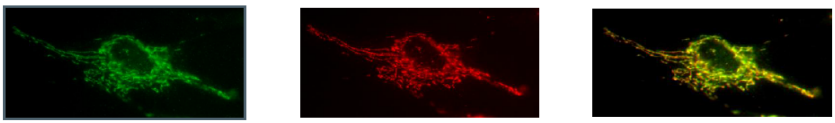


Figure 2b. Antibody specificity demonstrated by immunocytochemistry: Two-color immunocytochemical labeling of cultured cells with the two ab110217primary monoclonal antibodies specific for COX-I and SDH-A. The two antibodies exhibit striking and specific co-localization in the mitochondria, consistent with the known mitochondrial expression of both proteins.

11.3. **Flexibility**

HepG2 lymphocytes were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum, with or without 10 µM chloramphenicol to reduce levels of mtDNA-encoded proteins. The cells were allowed to divide three times over five days, harvested by trypsinization and then loaded into a 96-well cell culture treated plate at the described seeding densities. The plate was processed according to this protocol and data recorded in SpectraMax microplate reader.

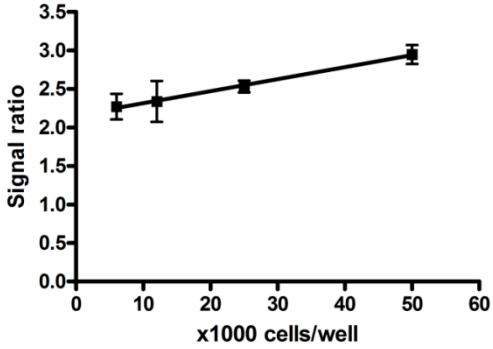


Figure 3. Quantitative measurement of the COX-I/SDH-A protein expression ratio. At all cell concentrations, a consistent ratio of mtDNA-encoded protein expression (COX-I) to nuclear DNA-encoded mitochondrial protein expression (SDH-A) is observed in untreated cells. Therefore, normalizing COX-I levels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

12. SPECIFICITY

Species Cross-Reactivity: Human, rat, mouse and bovine

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

Problem	Cause	Solution
Standard readings do not follow a linear pattern	Pipetting errors in standard or reaction mix	Avoid pipetting small volumes (< 5 µL) 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. APPENDIX

Clear-bottomed collagen or lysine coated plates are highly recommended to improve data quality, cell adhesion and seeding homogeneity.

In-Cell ELISA experiments give robust signal when the cell density is in the range 20,000-50,000 cells / well. Working on the high end of this range will generate stronger signals and allow greater reductions to be measured accurately. It is essential to omit primary antibodies from some wells containing cells to provide background control signals. As an example, when using HeLa cells, an endogenous alkaline phosphatase activity has been observed immediately after fixing the cells increasing the background signal. However in all other cells tested to date this was not the case but it is still a recommended to establish the background signal in this way.

A typical work flow is to seed the cells at low density directly into 96-well microplates and then allow them to divide to the desired cell density. Particular care must be taken when using in-well treatments that cause significant cell toxicity, loss of adhesiveness, apoptotic cell detachment or a decline in the rate of cell division. For this reason this kit takes a ratiometric approach to determine mitochondrial biogenesis. In addition this kit includes a method to determine the relative cell density per well by cell staining using the provided Janus Green Stain.

An alternate experimental work flow is to grow cells, both treated and untreated, in large cell culture flasks or dishes, e.g. 15 cm diameter dishes. The cells can then be harvested by trypsinization and should then be counted before seeding into the 96-well plate where they must be allowed to adhere, e.g., 4 hours for fibroblasts.

For a 384-well plate format, seed ¼ of the number of cells specified in this protocol. Prepare all solutions and wash buffers as described, but dispense ¼ of the specified volumes into the wells at the relevant steps.

Data analysis using either kinetic or endpoint data:
Calculate the average of all replicate background measurements from each experimental condition. Subtract this background reading from all experimental values of the same condition. COX-I signal and SDH-A signal can be plotted independently or as a ratio (COX-I/SDH-A). These data can also be normalized to Janus green staining intensity if desired.

15. NOTES

Technical Support

For all technical or commercial enquiries please go to:

www.abcam.com/contactus

www.abcam.cn/contactus (China)

www.abcam.co.jp/contactus (Japan)