ab168540 – MitoBiogenesis™ Flow Cytometry Kit

Instructions for Use

For the identification of inhibitors and activators of mitochondrial biogenesis in cultured and circulating cells.

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

Version 1a Last Updated 06 July 2021

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1. BACKGROUND

Abcam's MitoBiogenesis™ Flow Cytometry kit is designed to evaluate drug-induced effects on mitochondrial biogenesis early in the safety screening process. The assay combines the power of single cell analysis obtained with flow cytometry to evaluate the ratio between two important mitochondrial proteins, one encoded by the mitochondrial DNA (mtDNA) and one encoded by the nuclear DNA (nDNA).

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.

Human, rat and mouse cells are treated for several doublings then are harvested, fixed and permeabilized in suspension. The in-vivo levels of the target proteins are detected simultaneously in each cell with highly specific, well-characterized, monoclonal antibodies that are labeled with Alexa® fluorophores. Thus, minimizing potential changes during sample preparation and handling, such as preparation of protein extracts.

2. ASSAY SUMMARY

Harvest cells as single cell suspension

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Fix cells with paraformaldehyde at room temperature

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Pellet cells and aspirate supernatant

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Permeabilize cells with cold methanol. Incubate at -20ºC then pellet and wash

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Block cells with 1X blocking buffer at room temperature and pellet

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Add antibodies diluted in 1X BSA blocking buffer, incubate at room temperature, protected from light.

↓

Pellet, aspirate supernatant and wash cells twice with 1X PBS

↓

Pellet, aspirate supernatant and resuspend cells in 1X PBS

↓

Read with flow cytometer

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 immediately upon receipt.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in section 9. Reagent Preparation.

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
10X PBS	100 mL	4ºC
10X Blocking Buffer	6 mL	4ºC
100X MTCO1 AlexaFluor® 488 + SDHA AlexaFluor® 647 Cocktail	100 µL	4ºC

Note: Enough reagents are provided for 100 tests in a 100 µL volume.

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Cells of interest and media
- Microcentrifuge tubes
- Microcentrifuge
- Methanol
- Nanopure water or equivalent
- Paraformaldehyde solution
- Flow cytometer with appropriate light source and filters to view Alexa® 488 and Alexa® 647 signals:

	Absorption Max (nm)	Emission Max (nm)	Emission Color	Flow Channel
MTCO1 Alexa® 488	346	442	Green	FL1
SDHA Alexa® 647	650	668	Far-Red	FL4

7. 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.

8. TECHNICAL HINTS

- Prepare blocking buffer fresh every time.
- Sample integrity is very important for proper analysis by flow cytometry. It is important to handle the samples gently and pipette slowly during wash steps and centrifugations.
- Always include negative control (no primary) to set proper gates for flow cytometer analysis.

9. REAGENT PREPARATION

Equilibrate all reagents to room temperature (18-25°C), or other specified temperature prior to use.

9.1 Chill methanol to -20°C

9.2 **1X PBS**

Prepare 1X PBS by diluting 100 mL of 10X PBS in 900 mL Nanopure water or equivalent. Mix well. Cool a small portion for sample preparation on ice. Store excess 1X PBS at room temperature.

9.3 **1X Blocking Buffer**

Immediately prior to use prepare 1X Blocking Buffer by adding 1mL 10X Blocking Solution to 9 mL 1X PBS. Any excess should be stored at 4ºC for no more than 24 hours.

10. SAMPLE PREPARATION

Cell culture and treatment conditions are dictated by the experiment at hand. As a general guideline, it is advisable to analyze at least 10,000 events (cells) on the flow cytometer per sample/data point. Therefore at least four to ten times that many cells should be collected per data point to ensure sufficient material at the end of the staining.

10.1 For suspension cells:
Generate a single cell solution by pipetting cell suspension solution up and down.

10.2 For adherent cells:
Fully dissociate cells (e.g. trypsin) into single cell suspension. Passaging the cell line the day before the experiment onto a fresh culture plate may help improve single cell dissociation on the day of the experiment.

10.3 Maintain cells resuspended in the culture treatment media, at approximately $1x10^6$ cells/mL.
Overlay paraformaldehyde on the cell suspension so that the final concentration of paraformaldehyde is 4%, gently mix by inverting the tube and incubate at room temperature for 15 minutes.
Note: paraformaldehyde is toxic: handle with care and dispose of according to local requirements

10.4 Pellet cells at 350 - 500 x g for 5 minutes (depending on cell size) and decant supernatant.

10.5 Dislodge the pellet by gently tapping the bottom of the tube and resuspend the cells in a small volume of cold 1X PBS (100 µL PBS per $1x10^6$ cells).

10.6 Add 9X volumes of methanol (final concentration is 90% methanol) and store at -20°C for a minimum of 30 minutes. Cells may be kept frozen for up to 1 month. It is recommended to store cells aliquoted at $1x10^5$ per vial/assay tube.

11. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
- **It is recommended to assay all standards, controls and samples in duplicate.**

11.1 Prepare all reagents, working standards, and samples as directed in the previous sections.

11.2 Pellet prepared cells (see Section 10) at 350 – 500 x g for 5 minutes (depending on cell size) and aspirate supernatant.

11.3 Dislodge the pellet by gently tapping the bottom of the tube and resuspend with 1mL of 1X PBS per assay tube.

11.4 Repeat steps 11.2 and 11.3

11.5 Pellet the sample as described in step 11.2, aspirate supernatant, dislodge the pellet by gently tapping the bottom of the tube and add 50 µL of 1X Blocking Buffer per tube (optimal concentration is $2x10^4$ cells/µL). Mix by gently inverting the tube and incubate at room temperature for 15 minutes.

11.6 Prepare 50 µL per assay tube of 2X Primary Antibody Cocktail Solution in 1X Blocking Buffer (1:50 dilution) so that it can overlay the 50 µL cell suspension for a final 1X Antibody Cocktail Solution. Incubate at room temperature for at least 1 hour, **protected from light**.

11.7 Pellet cells at 350 - 500 x g for 5 minutes (depending on cell size) and aspirate supernatant.

11.8 Dislodge the pellet by gently tapping the bottom of the tube and resuspend with 1mL of 1X PBS per assay tube.

11.9 Repeat steps 11.7 and 11.8.

11.10 Pellet the sample as described in step 11.7, dislodge the pellet by gently tapping the bottom of the tube and add 100 µL of 1X PBS to each assay tube.

11.11 Use appropriate settings on flow cytometer to capture data.

12. CALCULATIONS

Specific methods depend on the available flow cytometer. It is important to appropriately establish forward and side scatter gates to exclude debris and cellular aggregates from analysis. Certain treatments may generate subpopulations of cells that are apparent from the forward/side scatter plots. Under these circumstances it is recommended to adequately gate the subpopulation of interest before capturing events. If the histogram does not generate a perfect normal distribution, use the median measurement to prevent artifacts from skewing the data.

- A total of 10,000 events should be collected in the correct forward and side scatter gates.

	Absorption Max (nm)	Emission Max (nm)	Emission Color	Flow Channel
MTCO1 Alexa®488	346	442	Green	FL1
SDHA Alexa®647	650	668	Far-Red	FL4

13. TYPICAL DATA

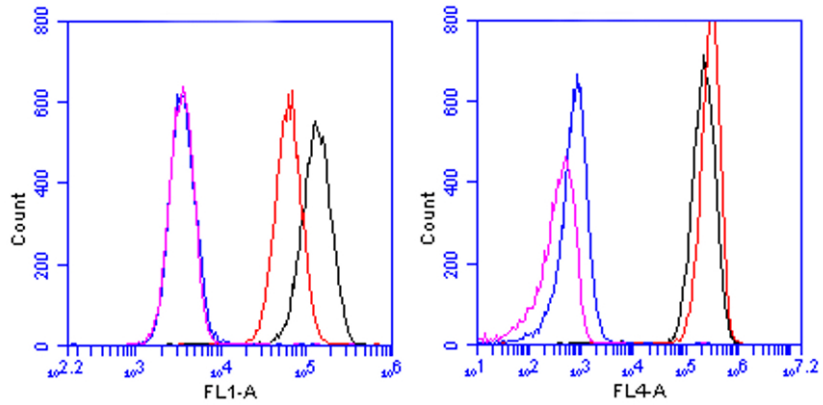

Figure 1. Histograms generated from flow cytometry data**.** A total of 10,000 gated events were captured for analysis of HeLa cells with or without chloramphenicol (16 µM, 6-days). The results show MTCO1 Alexa® 488, FL1(A) and SDHA Alexa® 647, FL4(B) co-staining for untreated, no anitobdies (blue); untreated with antibodies (black); chloramphenicol, no antibodies (purple); and chloramphenicol with anitbodies (red).

Example Data

Chloramphenicol	Total Events in Gate	Mean FL1 (MTCO1 Alexa®488)	Mean FL4 (SDHA Alexa®647)	Relative FL1	Relative FL4
0.25 µM	10,000	139,298	257,487	1.00	1.00
1 µM	10,000	133,262	261,987	0.96	1.02
4 µM	10,000	118,173	286,884	0.85	1.11
8 µM	10,000	105,637	326,623	0.76	1.27
32 µM	10,000	65,618	317873	0.46	1.23
64 µM	10,000	53,616	292,026	0.37	1.13

Example data collected of HeLa cells ± chloramphenicol (6 days)

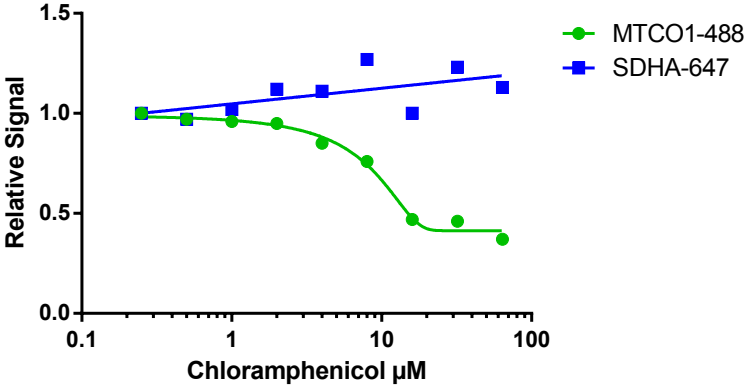

Figure 2. Relative expression levels of MTCO1 & SDHA in HeLa cells treated chloramphenicol using the MitoBiogenesis™ flow cytometry kit. HeLa cells were treated with 2-fold serial dilution of chloramphenicol for 6 days. The relative levels of the mean FL-1 fluorescence (MTCO1 Alexa® 488) and FL-4 fluorescence (SDHA Alexa® 647); a total of 10,000 events were collected.

14. SPECIES REACTIVITY

This antibodies provided in this kit have will detect the expression of MTCO1 and SDHA proteins in mouse, rat, cow, human, and Caenorhabditis elegans samples only.

15. ASSAY SPECIFICITY

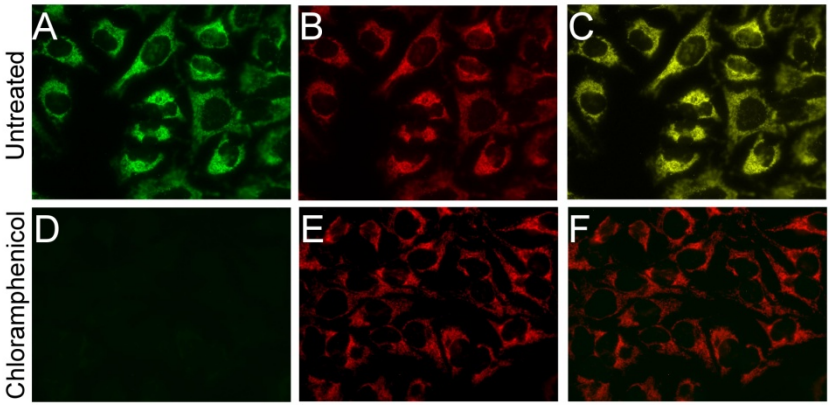

Figure 3. HeLa cell ICC staining using MitoBiogenesis™ flow cytometry kit antibodies. Cells were treated with 30 µM chloramphenicol for a period fo 6 days. Results of immunostaining of HeLa cells showing MTCO1 Alexa® 488 antibody (ab154477, green) on untreated (A) and chloramphenicol treated (D). SDHA Alexa® 647 antibody (ab168536, red) on untreated (B) and chloramphenicol treated (E). Merge of color channels for untreated (C) and chloramphenicol treated (F) cells.

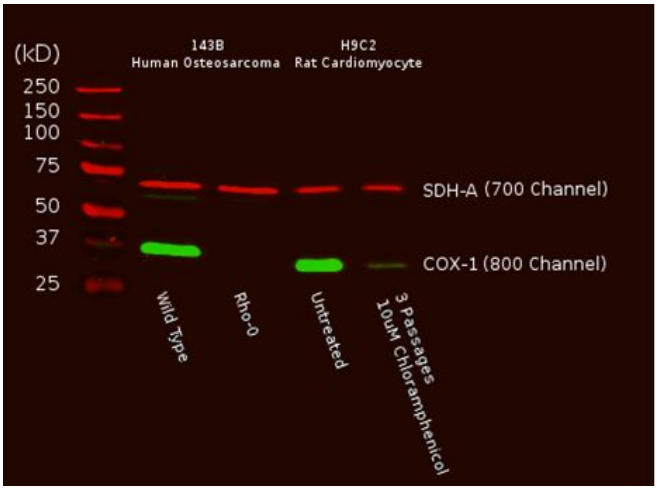

Figure 4. A Western blot of total cell protein. A total of 10 µg from human or rat cultured cells were probed with the primary and secondary antibodies and scanned with a LI-COR® Odyssey® imager. Reduction of mtDNA levels in human Rho0 (mtDNA-depleted) cells, or inhibition of mitochondrial protein translation by chloramphenicol in rat cells result in specific reduction of COX-I protein while nuclear DNA-encoded SDH-A is unaffected.

16. TROUBLESHOOTING

Problem	Cause	Solution
Low Signal	Signal not correctly compensated	Check positive single color control is set up correctly on flow cytometer and gated/compensated correctly to capture all events
	Lasers not aligned	Run flow check beads and adjust alignment if necessary
High side scatter back-ground	Cells lysed	Ideally samples should be freshly prepared. Do not vortex or shake the sample at any stage. Do not exceed 500 x g for centrifugation
	Bacterial contamination	Ensure sample is not contaminated
Low event rate	Low number of cells	Run $1x10^6$ cells/mL
	Cells clumped	Ensure a single cell suspension. Sieve clumps (30 µL nylon mesh)
High event rate	High number of cells/mL	Dilute between $1x10^5$ cells/mL and $1x10^6$ cells/mL

17. NOTES

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