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ab118183
Fatty Acid Oxidation Human Flow Cytometry Kit

For the detection of key FAO enzymes (ACADVL, ACADM, HADHA) in live cells by flow cytometry.

View kit datasheet: www.abcam.com/ab118183
(use www.abcam.cn/ab118183 for China, or www.abcam.co.jp/ab118183 for Japan)

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

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1. Overview

Fatty Acid Oxidation Human Flow Cytometry Kit (ab118183) is a panel of antibodies against key enzymes of the mitochondrial fatty acid oxidation (FAO), specially designed to be used in flow cytometry. The kit measures the levels of three of the most studied enzymes in the FAO pathway: ACADVL (acyl-CoA dehydrogenase, very long chain specific), ACADM (acyl-CoA dehydrogenase, medium chain specific) and HADHA (long chain 3-hydroxyl-CoA dehydrogenase).

The assay combines the power of single cell analysis obtained from flow cytometry with the specificity of antibody-based immunostaining to quantify protein levels in cultured cells. Cells are harvested and fixed/permeabilized in suspension and targets of interest are detected with highly specific monoclonal antibodies, which are detected in turn with fluorescently-labeled secondary antibodies.

Fatty acid B-oxidation (FAO) pathway is a key metabolic pathway that plays an important role in energy homeostasis particularly in organs such as the liver, heart and skeletal muscle. Oxidation of fatty acids occurs inside the mitochondria where acyl-CoA esters (activated fatty acids) of various lengths are shortened into units of acetyl-CoA each time a cycle is fully completed. Each unit of acetyl-CoA is then oxidized by the mitochondria into CO_2 and H_2O via the citric acid cycle and the mitochondria respiratory chain (figure 1). The FAO pathway consists of a four-step process which involves: (1) dehydrogenation, (2) hydration, (3) second dehydrogenation and (4) thiolytic cleavage.

ACADVL (acyl-CoA dehydrogenase very long chain, VLCAD, EC 1.3.99.13) is a homodimer that carries out the first step toward esters of long-chain and very long chain fatty acids such as palmitoyl-CoA, myristoyl-CoA and stearoyl-CoA. Defects in ACADVL are the cause of ACADVL deficiency (ACADVLD) [MIM:201475].

ACADM (acy6l-CoA Dehydrogenase, MCAD, EC 1.3.8.7) is a homotetramer and it also carries out the first step in the pathway; however, as opposed to ACADVL, it is specific for acyl chain lengths of 4 to 16 carbons. Defects in ACADM are the cause of ACADM deficiency (ACADMD) [MIM:201450], the most common fatty acid oxidation disorder in humans. This is an autosomal recessive disease

which causes fasting hypoglycemia, hepatic dysfunction, and encephalopathy, often resulting in death in infancy.

The trifunctional enzyme (TFP, HADHA/HADHB) is an octamer of 4 alpha and 4 beta subunits that catalyzes three out of the four steps in beta oxidation pathway with a specific affinity for long chain substrates. The alpha subunit of the trifunctional protein (HDHA, EC 1.1.1.211) catalyzes the hydration of enoyl-CoA and the dehydrogenation of 3-hydroxyacyl CoA compounds. Defects in HADHA are a cause of TP deficiency [MIM:609015], long-chain 3-hydroxyl-CoA dehydrogenase deficiency (LCHAD deficiency) [MIM:609016] and maternal acute fatty liver of pregnancy (AFLP) [MIM:609016].

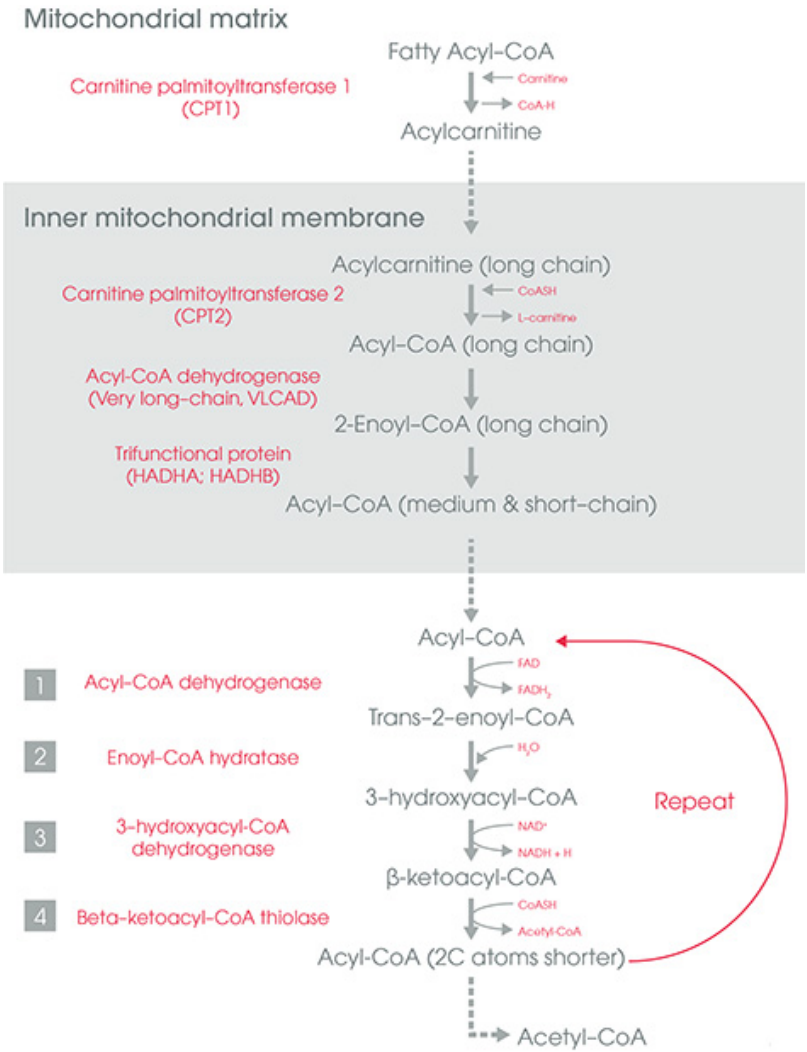
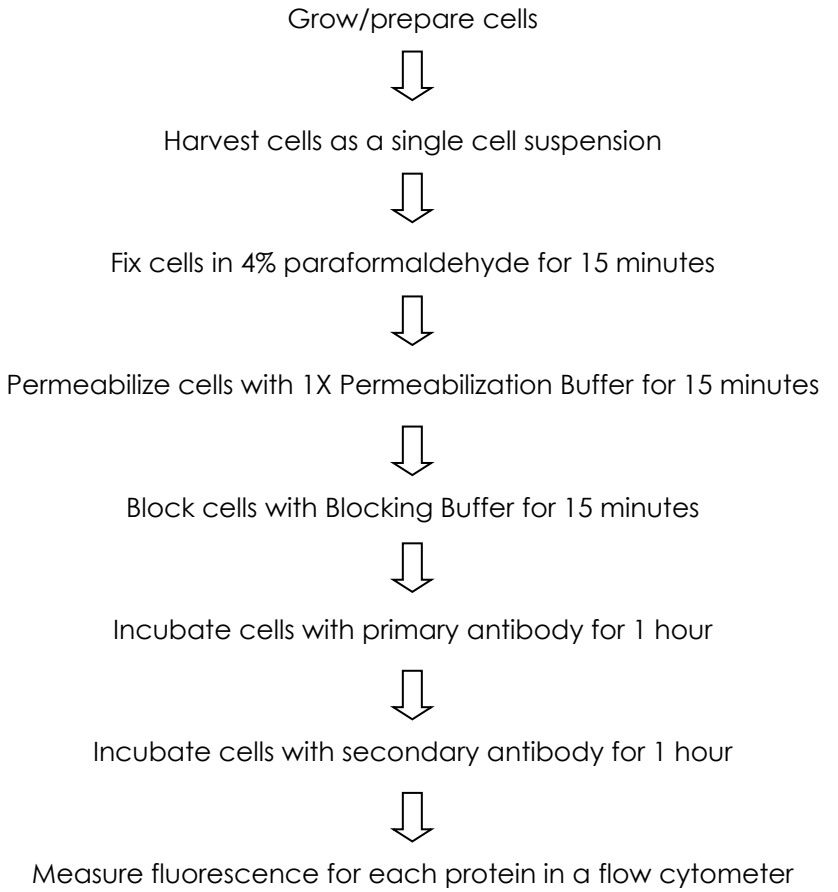


Figure 1. Overview of long-chain fatty acid activation, import and oxidation.

2. Protocol Summary



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.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at 4°C in the dark immediately upon receipt. Kit has a storage time of 6 months from receipt.

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.

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	Quantity	Storage temperature (before prep)	Storage temperature (after prep)
10X Phosphate Buffer Saline (PBS)	100 mL	4°C	4°C
100X Triton X-100 (10% solution)	1.25 mL	4°C	4°C
400X Tween-20 (20% solution)	4 mL	4°C	4°C
Blocking Solution	25 mL	4°C	4°C
100X ACADVL primary antibody	120 µL	4°C	4°C
100X ACADM primary antibody	120 µL	4°C	4°C
100X HADHA primary antibody	120 µL	4°C	4°C
100X normal Mouse IgG	120 µL	4°C	4°C

7. Materials Required, Not Supplied

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

- Flow cytometer
- Double distilled water (ddH_2O)
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- 20% paraformaldehyde solution
    - Goat anti-mouse secondary detection antibody, labeled with fluorochrome(s) suitable for use in flow cytometer: we recommend Goat anti-Mouse IgG H&L (Alexa Fluor® 488) (ab150113) or Goat anti-Mouse IgG H&L ((Alexa Fluor® 647) (ab150115)

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.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.
- Samples which generate values that are greater than the most concentrated standard should be further diluted in the appropriate sample dilution buffer.
- Make sure all necessary equipment is switched on and set at the appropriate temperature.

9. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

9.1 10X Phosphate Buffered Saline (PBS):

Prepare 1X PBS by diluting 100X PBS in ddH_2O: make 1 L of 1X PBS by combining 100 mL of 10X PBS with 900 mL ddH_2O. Equilibrate to room temperature. Store at 4°C.

9.2 100X Triton X-100 (10% solution):

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C.

9.3 400X Tween-20 (20% solution):

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C.

9.4 Blocking Solution:

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C.

9.5 100X Triton X-100 (10% solution):

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C.

9.6 100X ACADVL primary antibody (mouse anti-ACADVL):

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C.

9.7 100X ACADM primary antibody (mouse anti-ACADM):

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C.

9.8 100X HADHA primary antibody (mouse anti-HADHA):

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C.

9.9 100X Normal Mouse IgG:

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C.

10. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all controls and samples in duplicate.
- Prepare all reagents and samples as directed in the previous sections.
- Cell culture and treatment conditions are dictated by the experiment at hand. As a general guideline, we recommend analyzing at least 10,000 events (cells) on the flow cytometer per sales/data point. Therefore, you need to collect at least 4 times that many cells per data point to ensure sufficient material at the end of the staining.
- For each cell type, prepare a negative antibody control sample using the Normal Mouse IgG provided in the kit. The signal from this antibody can be used to determine background fluorescence.

10.1 Harvest cells:

10.1.1 Collect cells:

10.1.1.1 Suspension cells: generate a single cells solution by pipetting up and down.

10.1.1.2 Adherent cells: fully dissociate cells with trypsin into a single cell suspension (trypsinize cells in trypsin/EDTA for 1-2 min and stop trypsinization by adding culture medium).

10.1.2 Pellet cells at 350-500 x*g* for 5 minutes at 4°C in a cold microcentrifuge and aspirate supernatant.

Δ Note: leaving no more than 100 µL of complete media over the cell pellet will help the fixation process.

10.1.3 Resuspend cell pellet to a final concentration of 2-5x10^6 cells/mL into a suitable volume of PBS.

10.2 Prepare working solutions:

10.2.1 Immediately prior use, prepare the following solutions:

- 1X Wash Buffer: 0.05% Tween-20, 1% Blocking Buffer in PBS
- 1X Permeabilization Buffer: 0.1% Triton X-100 in PBS
- 1X Incubation Buffer: 10% Blocking Buffer, 0.1% Triton X-100 in PBS

Δ Note: any remaining working solution can be stored at 4°C. Do not store for longer than 24 hours.

10.3 Cell fixation:

10.3.1 Add 20% paraformaldehyde solution (not provided) to the cells so that the final concentration in the sample is 4%. Gently mix by inverting the tube and incubate at room temperature for 15 minutes.

10.3.2 Pellet cells at 350-500 x*g* for 5 minutes at 4°C in a cold microcentrifuge and decant supernatant.

Δ Note: paraformaldehyde is toxic; handle with care and dispose of it according to local requirements.

10.3.3 Dislodge the pellet by gently tapping the bottom of the tube and add 1X Permeabilization Buffer (Step 10.2.1) to a final concentration of 1-2 x 10^6 cells/mL. Mix by gently inverting the tube and incubate at room temperature for 15 minutes.

10.3.4 Pellet cells at 350-500 x*g* for 5 minutes at 4°C in a cold microcentrifuge and aspirate supernatant.

10.3.5 Dislodge the pellet by gently tapping the bottom of the tube and add 50 µL 1X Incubation Buffer (Step 10.2.1) to a final concentration of 2 x 10^4 cells/µL. Mix by gently inverting the tube and incubate at room temperature for 15 minutes.

10.4 Antibody staining:

10.4.1 For each primary antibody (ACADVL, ACADM, HADHA, Normal Mouse IgG), prepare a 2X Primary Antibody Solution by diluting 100X Primary Antibody Solution in 1X Incubation Buffer. Prepare 50 µL solution per assay tube.

Δ Note: all antibodies are mouse in origin and should not be mixed.

10.4.2 Add 50 µL2X Primary Antibody Solution to the 50 µL cell suspension and mix by gently inverting the tube. Incubate at room temperature for at least 1 hour.

10.4.3 Pellet cells at 350-500 x*g* for 5 minutes at 4°C in a cold microcentrifuge and decant supernatant.

10.4.4 Dislodge the pellet by gently tapping the bottom of the tube.

10.4.5 Wash pellet twice by centrifugation with 1 mL 1X Wash Buffer.

10.4.6 Prepare 100 µL Goat Anti-Mouse Secondary Antibody (not provided) solution in 1X Incubation Buffer per assay tube.

10.4.7 After the final wash, dislodge the pellet by gently tapping the bottom of the tube and add 100 µL Secondary Antibody solution per assay tube.

10.4.8 Incubate samples for at least 1 hour at room temperature in the dark.

10.4.9 Pellet cells at 350-500 x*g* for 5 minutes at 4°C in a cold microcentrifuge and aspirate supernatant.

10.5 Detection by flow cytometry:

10.5.1 Dislodge the pellet by gently tapping the bottom of the tube.

10.5.2 Wash pellet twice by centrifugation with 1 mL 1X Wash Buffer.

10.5.3 After the final wash, dislodge the pellet by gently tapping the bottom of the tube.

10.5.4 Resuspend cell pellet in 100 µL PBS.

10.5.5 Analyze cells by flow cytometry, using the channel appropriate for the fluorochrome.

Δ Note: analyze cells within 3 hours of staining.

11. Data Analysis

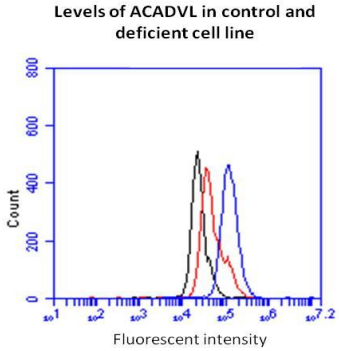
The following are general guidelines. Specific methods of analysis will vary with different flow cytometer analysis programs.

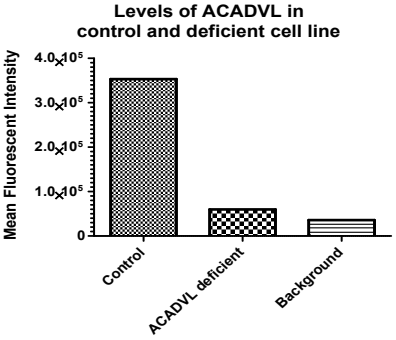
- Establish appropriate FSC vs SSC gates to exclude debris and cell aggregates.
- Use signal from Normal Mouse IgG staining to determine background fluorescence.
- Collect signal fluorescence in the appropriate channel.

12. Typical data

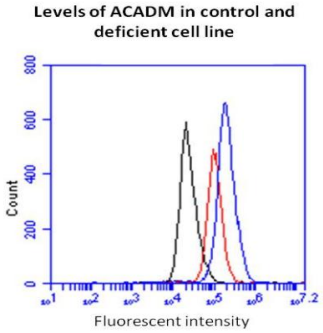
Data provided for **demonstration purposes** only.

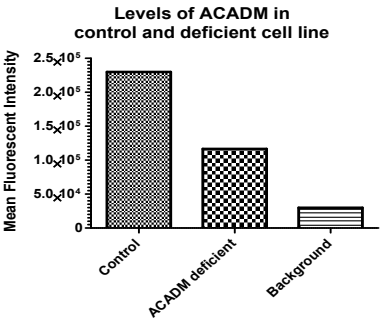
a) ACADVL





b) ACADM





c) HADHA

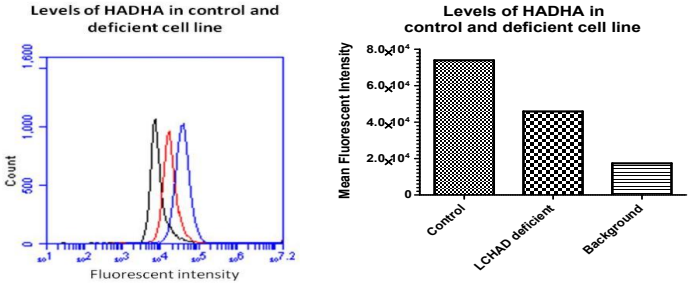


Figure 2. Expression of FAO key enzymes in primary fibroblasts. Left panel: Expression levels of ACADVL (a), ACADM (b), and HADHA (c) was determined in primary fibroblasts by flow cytometry. Blue: control cell line. Red: deficient cell lines extracted from patients with well characterized mutations in each of the enzymes (a: ACADVL:p[N122D]; b: ACADM:p[K604E]; c: HADHB:p[R61H];[R247H]). Background fluorescence was determined with the supplied negative control (black).

Δ Note: althoug the mutation in cell line used in (c) was in the HADHB gene, levels of HADHA were found depleted by flow cytometry, immunocytochemistry and western blot perhaps due to post-transcriptional instability of the assembled trifunctional protein TFP (HADHA + HADHB).

Right panel: After background subtraction, the ACADVL deficient cell line shows a 93% reduction in the level of the ACADVL protein; the ACADM deficient cell line shows a 57% reduction in the level of the ACADM protein, and the LCHAD deficient cell line should a 50% reduction in the level of the HADHA protein.

13. Assay Performance and Specificity

All the antibodies used in this assay are IP positive and has been previously validated by mass spectrometry using the immunoprecipitated protein (data not shown, but available upon request). Assay specificity was demonstrated by using primary fibroblasts cell lines from patients with a well characterized enzymatic deficiency in one of the FAO enzymes.

Confidence in antibody specificity is critical to Flow data interpretation. Therefore, all antibodies present in this kit have also been tested for specificity in fluorescence immunocytochemistry under the same conditions used for the Flow assay. Furthermore, levels of the proteins have been independently validated by western blot in control and deficient cell lines. The antibody against ACADVL is only suitable for ICC and IP and therefore the levels of ACADVL in the deficient cell line were validated with anti-ACADVL antibody (ab54698). Examples demonstrating the immunocytochemical and Western blot specificities of the primary antibodies used in this panel are shown in Figures 3 and 4.

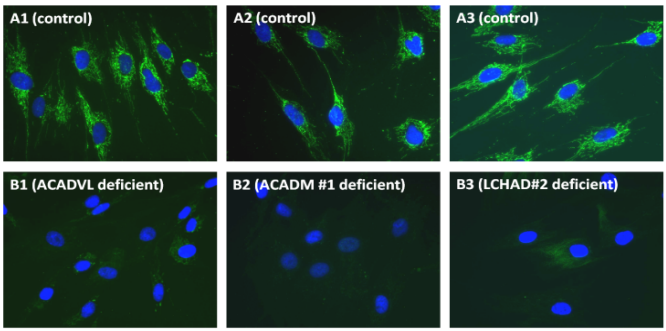


Figure 3. Antibody specificity demonstrated by immunocytochemistry. Cells were processes with the Flow cytometry protocol as explained above. Panel A shows control fibroblasts and panel B shows deficient fibroblasts. Left panel shows staining with anti-ACADVL antibody, center panel with anti-ACADM antibody and right panel with anti-HADHA antibody.

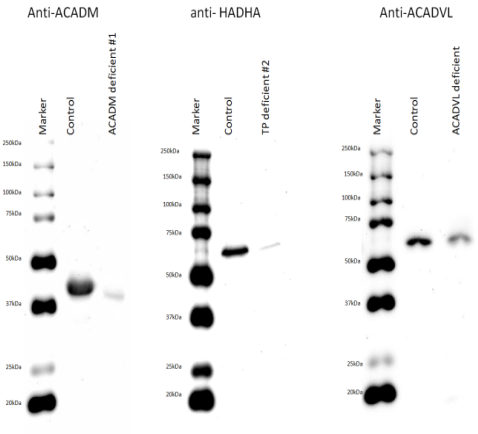


Figure 4. Validation of antibodies and cell lines by WB. Western blot was run on a 10-20% gradient acrylamide gel. Samples were loaded at 40 µg/lane for anti-ACADM and anti-HADHA antibodies and at 20 µg/lane for the anti-ACADVL antibody. Deficient samples used were the same as those used for the flow cytometry assay.

14. Troubleshooting

Problem	Reason	Solution
Low signal	Signal not correctly compensated	Check positive 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
	Insufficient secondary antibody	Increase concentration of antibody
	Secondary antibody is not compatible with primary antibody	Use a secondary antibody raised against mouse for this kit
High side scatter background	Cells lysed	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 at least 10^6 cells/mL
	Cells clumped	Ensure single cell suspension. Sieve clumps using a 30 µm nylon mesh
High event rate	High number of cells	Dilute cells to 10^5-10^6 cells/mL

15. FAQs

Q. Does this kit cross-react with rat or mouse?

A. The antibodies used in the kit have been validated in human samples.

Both anti-ACADM and anti-ACADVL antibodies will cross-react with rat, but the HADHA-antibody is specific for human. If you want to use this product in rat, you will need to purchase anti-HADHA/HADHB antibody (ab110302), which has been shown to work in rat.

16. Notes

Technical Support

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