abcam®
discover more

ab118182 –

Fatty Acid Oxidation Human In-Cell ELISA Kit

(ACADVL, ACADM, HADHA)

Instructions for Use

For measuring in high throughput very long chain specific acyl-CoA dehydrogenase, medium-chain specific acyl-CoA dehydrogenase and long-chain 3-hydroxyl-CoA dehydrogenase

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

Table of Contents

1. Introduction

Principle: ab118182 uses quantitative immunocytochemistry to measure protein levels or post-translational modifications in cultured cells. Cells are fixed in a microplate and targets of interest are detected with highly specific, well-characterized monoclonal antibodies, and levels are quantified with IRDye®-labeled Secondary Antibodies. IR imaging and quantitation is performed using a LI-COR® Odyssey® or Aerius® system.

Background: The 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. The FAO pathway consists of a four step process which involves: (1) dehydrogenation, (2) hydration, (3) second dehydrogenation and (4) thiolytic cleavage. This kit measures the levels of three of the most studied enzymes in this pathway: ACADVL, ACADM, HADHA. These enzymes are not only important in the field of inborn errors of metabolism, but also in metabolic syndrome, type 2-diabetes and compound toxicity.

ACADVL is a homodimer that carries out the first step toward esters of long-chain and very long chain fatty acids such as palmitoyl-CoA, mysritoyl-CoA and stearoyl-CoA. It can accommodate substrate acyl chain lengths as long as 24 carbons, but shows little activity for substrates of less than 12 carbons. Defects in ACADVL are the cause of acyl-CoA dehydrogenase very long chain deficiency (ACADVLD) [MIM:201475]. It is clinically heterogeneous, with three major phenotypes: (1)cardiomyopathy (2)a hypoketotic hypoglycemia and (3) rhabdomyolysis triggered by exercise or fasting.

ACADM is a homotetramer and it also carries out the first step in the pathway but, as opposed to ACADVL, it is specific for acyl chain lengths of 4 to 16 carbons. Defects in ACADM are the cause of acyl-CoA dehydrogenase medium-chain 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 (TP) is an octomer (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. This kit measures the alpha subunit of the trifunctional protein which catalyzes the hydration of enoyl-CoA and the dehydrogenation of 3-hydroxyacyl CoA compounds (steps 2 and 3). 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].

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 96 samples in parallel. This method rapidly fixes the cells in situ, stabilizing the in vivo levels of proteins, and thus essentially eliminates changes during sample handling, such as preparation of protein extracts. Finally, the ACADVL, ACADM and HADHA signal can all 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 in the provided microwell cultured plate.

Fix cells with 4% paraformaldehyde for 15 minutes and wash.

Permeabilize/Block the cells with 200 µL/well of Blocking/permeabilization buffer. Incubate for 2 hours at RT.

Add primary antibodies diluted in 1X incubation buffer overnight and wash.

Add secondary antibodies diluted in 1X incubation buffer for 2 hours and wash.

Scan the plate.

3. Kit Contents

Item	Quantity
10X Phosphate Buffered Saline (PBS)	100 mL
33X Triton X-100 (10% solution)	5 mL
400X Tween – 20 (20% solution)	4 mL
10X Blocking Buffer	20 mL
100X ACADVL Primary antibody	0.12 mL
100X ACADM Primary Antibody	0.12 mL
100X HADHA Primary Antibody	0.12 mL
1000X IRDye800®-labeled Secondary Antibody	0.036 mL
Janus Green Stain	17 mL

4. Storage and Handling

Upon receipt spin down the contents of the IRDye®-labeled Secondary Antibody tube and protect from light. Store all components upright at 4°C. This kit is stable for at least 6 months from receipt.

5. Additional Materials Required

- 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.

6. Preparation of Reagents

1. Equilibrate all reagents to room temperature.

2. Prepare 1X PBS by diluting 45 mL of 10X PBS in 405 mL Nanopure water or equivalent. Mix well. Store at room temperature.

3. Prepare 1X Wash Buffer by diluting 0.625 mL of 400X Tween-20 in 250 mL of 1X PBS. Mix well. Store at room temperature.

4. Immediately prior to use prepare 8% Paraformaldehyde Solution by mixing 6.25 mL Nanopure water, 1.25 mL10X PBS and 5.0 mL 20% paraformaldehyde. Note – Paraformaldehyde is toxic and should be prepared and used in a fume hood. Dispose of paraformaldehyde according to local regulations.

5. Immediately prior to use prepare Blocking/Permeabilization Solution by diluting 5 mL 10X Blocking Solution and 0.75 mL of 33X Triton X-100 in 19.25 mL of Nanopure water or equivalent. The final blocking contents in this buffer should be 2X.

6. Immediately prior to use prepare 1X Incubation Buffer by diluting 2.5 mL 10X Blocking Solution and 0.75 mL of 33.3X Triton X-100 in 21.75 mL of Nanopure water or equivalent.

7. Sample Preparation

Note: The protocol below is described for a 96-well plate. If performing assay on a 384-well plate, adjust volumes accordingly. **This assay has been optimized for use on adherent cells. For suspension cells, assay optimization will be required.**

1. Seed adherent cells directly into an amine coated plate and allow them to attach for several hours to 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. For suggestions regarding the cell seeding see frequently asked questions. As an example, primary fibroblasts cells should be seeded at 30,000 – 40,000 cells per well.

2. The attached cells can be treated if desired with a drug of interest. If treatment is not required, proceed to the next step to measure endogenous FAO enzyme levels.

3. Fix cells by overlaying over the culture media 100 µL of 8% Paraformaldehyde Solution to the wells of the plate.

4. Incubate for additional 15 minutes.

5. Gently aspirate 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 300 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 provided seal. 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.

8. Assay Procedure

It is recommended to use a plate shaker (~300 rpm) during incubation steps and a humid box. Any step involving removal of buffer or solution should be followed by blotting the plate gently upside down on a paper towel. Use the perimeter wells of the plate as blank controls (stained only with secondary antibody) to prevent edge effects and improve on reproducibility. Unless otherwise noted, incubate at room temperature.

1. Remove PBS and add 200 µL Blocking/Permeabilization Solution to each well of the plate. Incubate 2 hours.

2. Prepare ACADVL 1X Primary Antibody Solution by diluting 100X Primary Antibody into appropriate volume of 1X Incubation Buffer (i.e. 35µL of antibody in 3.5mL of incubation buffer).

3. Repeat previous step for ACADM and HADHA antibodies. *Note – All antibodies are mouse in origin and should not be mixed*

4. Remove Blocking/Permeabilization Solution and add 100 µL 1X Primary Antibody Solution to each well of the plate. Record the location of the antibodies if using more than one in the same experiment. Incubate 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.*

5. 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 250 µL of 1X Wash Buffer. **Do not remove the last wash until step 8.7.**

6. Prepare 1X Secondary Antibody Solution by diluting 12 µL 1000X IRDye800-labeled Secondary Antibody into 12 mL 1X Incubation Buffer. Protect labeled antibodies from light

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

8. Remove Secondary Antibody Solution and wash 5 times briefly with 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.**

9. Wipe the bottom of the plate and the scanner surface with 70% ethanol and scan the plate on the LI-COR® Odyssey® system using 800 channel according to manufacturer's instructions. The optimal focus off-set for the provided plate is 3.9. *Note – The absolute value of the IR signal is dependent on the 800 channel intensity setting. Value 8.5 is recommended for initial scanning.*

10. Remove last 1X Wash and add 50 µL of Janus Green Stain per well. Incubate plate for 5 minutes at room temperature.

    Note – The IR signal must be normalized to the Janus Green staining intensity to account for differences in cell seeding density.

11. Remove dye, wash plate 5 times in deionized water or until excess dye is removed.

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

13. Measure using a LI-COR® Odyssey® scanner in the 700 nm channel or measure OD595 nm using a standard microplate spectrophotometer.

9. Data Analysis

1. Correct the IR800 raw signals (Integrated Intensities) for the background signal by subtracting the mean IR800 of well(s) incubated in the absence of the Primary Antibody from all other IR800 readings.

2. Correct the Janus Green signal for the background signal by subtracting the mean Janus Green signal of wells that do not contain cells from all other Janus Green readings.

3. Normalize by dividing the IR800 background corrected signal by the background corrected Janus Green signal.

10. Assay Performance and Specificity

Assay specificity was demonstrated by using primary fibroblasts cell lines from patients with a well characterized enzymatic deficiency in one of the FAO enzymes. Figure 1 shows results after testing cell lines with well characterized mutations and deficiencies in these enzymes.

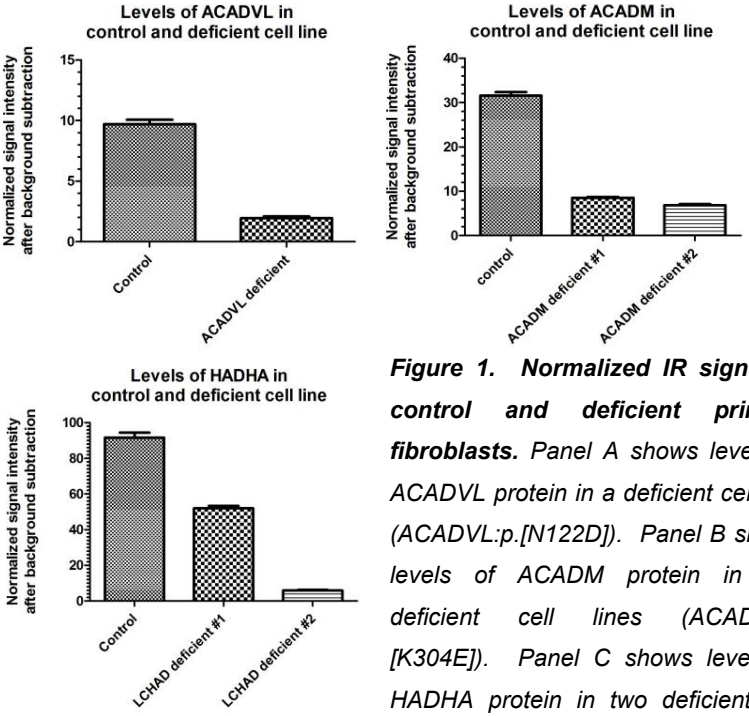

Figure 1. Normalized IR signal in control and deficient primary fibroblasts. *Panel A shows levels of ACADVL protein in a deficient cell line (ACADVL:p.[N122D]). Panel B shows levels of ACADM protein in two deficient cell lines (ACADM:p.[K304E]). Panel C shows levels of HADHA protein in two deficient cell lines: (1) HADHA:p.[E474Q] and (2) HADHB:p. [R61H];[R247H]. Note that although LCHAD#1 deficient has a partial deficiency as observed by ICC.*

Confidence in antibody specificity is critical to ICE data interpretation. Therefore, all antibodies in this kit were also tested for specificity by fluorescence immunocytochemistry testing the same deficient cell lines used for the ICE assay.

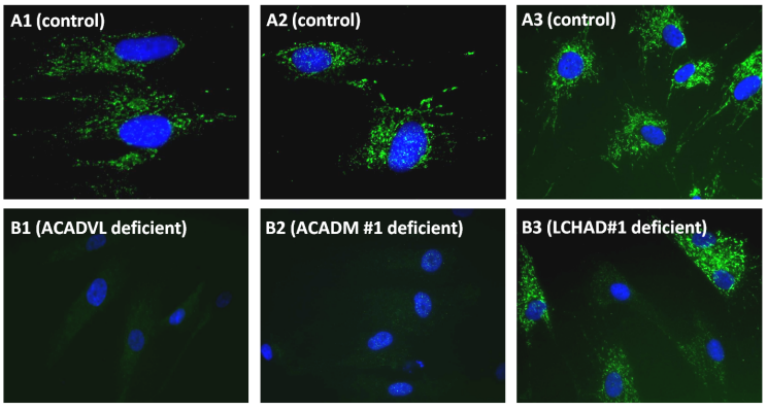

Figure 2. Antibody specificity demonstrated by immunocytochemistry. *Visualization under the microscope was carried out with a completely opened aperture and a very narrow field of visualization at 40x. Panel A shows control fibroblasts and panel B shows deficient fibroblasts. Left panel shows staining with anti-ACADVL ab, center panel with anti-ACADM ab and right panel with anti-HADHA ab. Note that although LCHAD#1 deficient had a characterized homozygous mutation, the antibody shows a mosaic pattern of staining with about 1/3 of the cells lacking HADHA staining and an overall 40% reduction of signal as observed by ICE.*

Furthermore levels of the proteins were independently validated by western blot in control and deficient cell lines. The antibody against ACADVL is only ICC, IP positive and therefore the levels of ACADVL in the deficient cell line were validated with Abcam antibody ab54698.

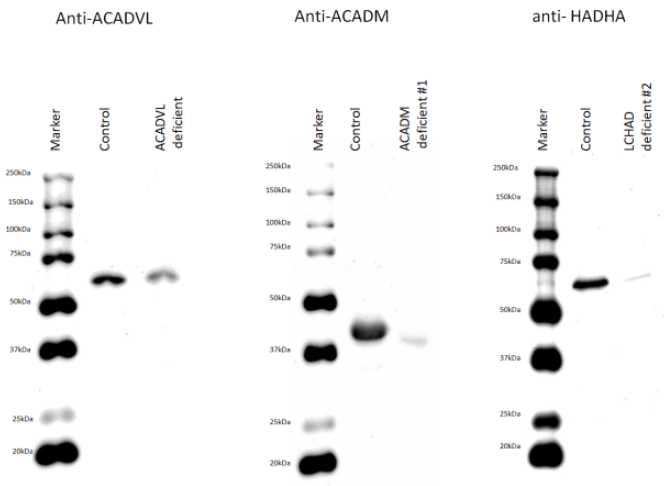

Figure 3. Validation of antibodies and cell lines by WB. *Western blot was run on a 10-20% gradient acrylamide gel. Samples were loaded at 40ug/lane for anti-ACADM and anti-HADHA antibodies and at 20ug/lane for the anti-ACADVL antibody. Blocking, primary and secondary antibodies (GAM-HRP) were carried out with 1X blocking solution (10x supplied with this panel) diluted in nanopure water.*

Reproducibility - ICE results provide accurate quantitative measurements of antibody binding and hence cellular antigen concentrations. The coefficient of the intra- and inter-assay of variation of this assay for primary fibroblast cell lines is less than 10% for all antibodies

11. Frequently Asked Questions

1. I want to use a cell line other than primary fibroblasts to perform this assay, how do I know how many cells to seed per well?

   The cell seeding density of the Assay Plate is cell type dependent. It depends on the cell size and the abundance of the target protein. The cell seeding can be determined experimentally by microscopic observation of cell density of serially diluted cells. For adherent cells, prepare serial dilution of the cells in a plate (of similar well dimensions) and allow them to attach prior to observation. Working on the high end of cell densities will generate stronger signals and allow small signal increases to be measured accurately. As an example, primary fibroblast cells should be seeded at 30,000 to 40,000 cells per well and HepG2 cells (or other transformed cell lines of similar size) should be seeded at 25,000 cells per well.

2. Can I do the assay in a cell culture plate with a different coating other than amine?

   We have only tested systematically amine and cell culture treated plates and found that amine coated plates improve reproducibility and specificity in comparison to standard plates. We cannot guarantee optimal performance of this assay using other coated surfaces.

3. Does the amine coating interfere with cell growth?

   The amine coating does not interfere with cell growth, but it does enhance cell attachment as well as even seeding.

4. 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.

5. 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.

6. I don't have access to a LI-COR® Odyssey® or Aerius® infrared imaging system, but I am interested in using this kit. Can I read in a different format?

   Yes, this assay works with colorimetric development. However the infra-red method gives superior specificity and reproducibility than the colorimetric method. To perform this assay in a colorimetric format, the IRDye800-labeled secondary antibody must be changed for a Goat-AntiMouse HRP labeled antibody (not included in the kit). It is also recommended to seed 10 – 20% more cells per well than what

is recommended for the IR method. The HRP labeled secondary antibody should be used in the same way as the supplied IR antibody; a dilution of 1:2,500 is recommended. After washing the secondary antibody, add 100 µL/well of TMB peroxidase substrate and incubate for 30 minutes at room temperature. Stop the reaction by overlaying on the peroxidase substrate 100 µL/well of 0.5M HCL. Incubate for another 10 minutes at RT and read end point at 450nm. Better reproducibility will be obtained by reading the peroxidase reaction as end point rather than kinetically.

12. Troubleshooting

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
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	Dirty bench or troughs used for multichannel pipetting could be dirty. Clean bench and troughs.
	Edge effects	Do not use the edges of the plate. Incubate in a humid box
	Variable cell seeding	Normalize with Janus green

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

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

China and Asia Pacific
Email: hk.technical@abcam.com
Tel: 108008523689 (中國聯通)
www.abcam.cn

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