

ab115345
Phospho S300 PDH E1
alpha protein (PDHA1)
ELISA Kit

Instructions for Use

For the measurement of phospho S300 PDHA1 protein in multiple species (Human, Mouse, Rat, Cow)

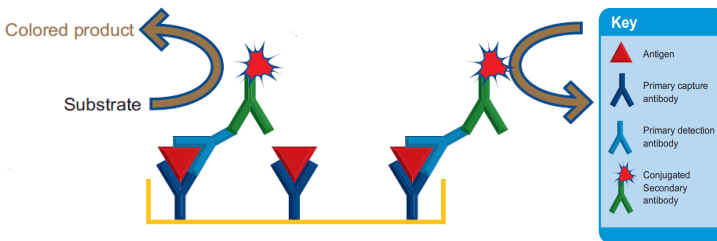
This product is for research use only and is not intended for diagnostic use.

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1. Introduction

Principle: ab115345 phospho S300 PDHA1 protein ELISA kit is an in vitro enzyme-linked immunosorbent assay to determine the levels of phospho S300 PDHA1 protein in cell and tissue lysates. The assay employs a mouse antibody specific for PDHA1 protein coated on a 96-well plate. Samples are pipetted into the wells and PDHA1 protein present in the sample is bound to the wells by the immobilized antibody. The wells are washed and a rabbit anti-phospho S300 PDHA1 protein detector antibody is added. After washing away unbound detector antibody, HRP-conjugated anti-rabbit antibody is pipetted into the wells. The wells are again washed, an HRP substrate solution (TMB) is added to the wells and color develops in proportion to the amount of phospho S300 PDHA1 protein bound. The developing blue color is measured at 600 nm. Optionally the reaction can be stopped by adding hydrochloric acid which changes the color from blue to yellow and the intensity can be measured at 450 nm.



Background: The pyruvate dehydrogenase complex performs the decarboxylation of pyruvate into acetyl CoA, a critical function in metabolism, linking glycolysis and oxidative phosphorylation in mitochondria. The enzyme is composed of multiple copies of three enzymes: pyruvate dehydrogenase (E1), dihydrolipoamide transacetylase (E2) and dihydrolipoamide dehydrogenase (E3). The E1 enzyme is a tetramer of two alpha (PDHA1) and two beta (PDHB) subunits and is present in 30 copies in the PDH complex.

The activity of PDH is regulated by reversible phosphorylation of three serine residues on the PDHA1 subunit at phospho S232, phospho S293, and phospho S300. ELISA assays to measure total PDHA1, phospho 232, phospho S293, and phospho S300 are available from Abcam (ab115342, ab115343, ab115344, ab115345). The phosphorylation of these sites is catalyzed by PDH kinases (PDK). There are four known PDK isoforms, distributed differently in tissues. Their expressions are regulated differently by factors such as starvation, hypoxia and utilization of glucose and fatty acids in various tissues. Dephosphorylation, to restore the activity of PDH, is catalyzed by PDH phosphatases (PDP). There are two known isoforms of PDP; PDP1 is present in high levels in skeletal muscle and PDP2 in liver and adipocytes. Functional PDH kinases and phosphatases are available from Abcam (ab110359, ab110354, ab110355, ab110356). A common feature of cancer cell aerobic glycolytic metabolism is the down-regulation of mitochondrial respiration and inactivation of the PDH complex as a result of

increased PDK1 expression and PDHA1 phosphorylation. One proposed therapeutic strategy is the inhibition of PDK enzymes returning cells to an aerobic metabolic state dependent upon PDH and mitochondria respiration.

Defects in PDHA1 are a cause of pyruvate decarboxylase E1 component deficiency (PDHE1 deficiency) [MIM:312170]. PDHE1 deficiency is the most common enzyme defect in patients with primary lactic acidosis. It is associated with variable clinical phenotypes ranging from neonatal death to prolonged survival complicated by developmental delay, seizures, ataxia, apnea, and in some cases to an X-linked form of Leigh syndrome (X-LS). Defects in PDHA1 are the cause of X-linked Leigh syndrome (X-LS) [MIM:308930]. X-LS is an early onset progressive neurodegenerative disorder with a characteristic neuropathology consisting of focal, bilateral lesions in one or more areas of the central nervous system, including the brainstem, thalamus, basal ganglia, cerebellum, and spinal cord. The lesions are areas of demyelination, gliosis, necrosis, spongiosis, or capillary proliferation. Clinical symptoms depend on which areas of the central nervous system are involved.

2. Assay Summary

Bring all reagents to room temperature. Prepare all the reagents, and samples as instructed.



Add 50 μL sample to each well used. Incubate 2 hours at room temperature.



Aspirate and wash each well two times. Add 50 μL prepared detector antibody to each well. Incubate 1 hour at room temperature.



Aspirate and wash each well two times. Add 50 μL prepared HRP label. Incubate 1 hour at room temperature.



Aspirate and wash each well three times. Add 100 μL HRP Development Solution to each well. Record immediately the color development with time at 600 nm for 15 minutes. Alternatively add a Stop solution at a user-defined time and read at 450 nm.

3. Kit Contents

Item	Quantity
20X Buffer	20 mL
Extraction Buffer	15 mL
10X Blocking Buffer	6 mL
HRP Development Solution	12 mL
10X phospho S300 PDHA1 protein Detector Antibody	0.7 mL
10X HRP Label	1 mL
Microplate 96 antibody coated wells in 12 strips	1

4. Storage and Handling

Store all components at 4°C. This kit is stable for 6 months from receipt. Unused microplate strips should be returned to the pouch containing the desiccant and resealed.

5. Additional Materials Required

- Microplate reader capable of measuring absorbance at 600 nm (or 450 nm after addition of Stop solution - not supplied).
- Method for determining protein concentration (BCA assay recommended).
- Deionized water
- Multi and single channel pipettes
- PBS (1.4 mM KH_2PO_4 , 8 mM Na_2HPO_4 , 140 mM NaCl, 2.7 mM KCl, pH 7.3)
- Stop solution (optional) – 1N hydrochloric acid
- Optional plate shaker for all incubation steps

6. Preparation of Reagents

1. Bring all reagents and samples to room temperature (18-25°C) before use.
2. Prepare 1X Wash Buffer by adding 20 mL 20X Buffer to 380 mL nanopure water.

3. Prepare 1X Incubation Buffer by adding 6 mL 10X Blocking Buffer to 54 mL 1X Wash Buffer. After performing the ELISA freeze unused 1X Incubation buffer.
4. Dilute the phospho S300 PDHA1 protein detector antibody 10-fold with 1X Incubation Buffer immediately before use. Prepare 0.5 mL for each strip used.
5. Dilute the HRP label 10-fold with 1X Incubation Buffer immediately before use. Prepare 0.5 mL for each strip used.

7. Sample Preparation

Note on buffer supplements: Extraction buffer can be supplemented with protease inhibitors such as PMSF and a protease inhibitor cocktail prior to use. Additionally, if desired, extraction buffers can be supplemented with kinase inhibitors (e.g. DCA) or phosphatase inhibitors (e.g. NaF) however to ensure these supplements equally and completely inhibit kinases and phosphatases they may need to be optimized. The data shown in this protocol was generated without the use for extraction buffer supplementation. Supplements should be used according to manufacturer's instructions.

Cell lysates:

1. Collect non adherent cells by centrifugation or scrape to collect adherent cells from the culture flask. Typical centrifugation conditions for cells are 500 g for 10 min at 4°C.
2. Rinse cells twice with PBS.
3. Solubilize cell pellet at 2×10^7 /mL in Extraction Buffer.
4. Incubate on ice for 20 minutes. Centrifuge at 16000 x g 4°C for 20 minutes. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

Tissue lysates:

1. Tissue lysates are typically prepared by homogenization of tissue that is first minced and thoroughly rinsed in PBS to remove blood (dounce homogenizer recommended).
2. Suspend the homogenate to 25 mg/mL in PBS.
3. Solubilize the homogenate by adding 4 volumes of Extraction Buffer to a sample protein concentration of 5 mg/mL.

4. Incubate on ice for 20 minutes. Centrifuge at 16000 x g 4°C for 20 minutes. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

Sub-cellular organelle lysates e.g. mitochondria:

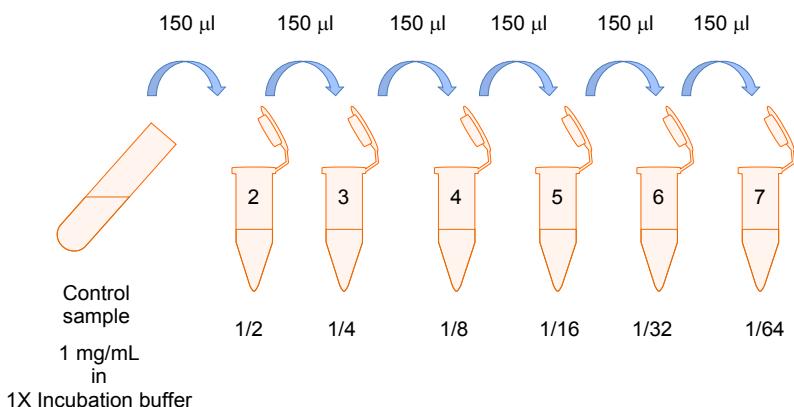
1. Prepare the organelle sample by, for example, sub-cellular fractionation.
2. Pellet the sample.
3. Solubilize the pellet by adding 9 volumes Extraction Buffer.
4. Incubate on ice for 20 minutes. Centrifuge at 16000 x g 4°C for 20 minutes. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

The sample should be diluted to within the working range of the assay in 1X Incubation Buffer. As a guide, typical ranges of sample concentration for commonly used sample types are shown below in Data Analysis.

8. Control Sample Dilution Series Preparation

Note: It is strongly recommended to prepare a dilution series of the control (untreated) material. The levels of phospho S300 PDHA1 in treated samples can be interpolated from within this control sample series. Also note to determine the amount of total PDHA1 protein in $\mu\text{g}/\text{mg}$ in all samples use total PDHA1 ELISA kit (ab115342).

1. To create a dilution series of control sample label tubes #1-7. Add 150 μL 1X Incubation buffer to each of tubes #2 through #7.
2. Prepare all samples by detergent extraction as described. Dilute the control sample lysate to 1 mg/mL in 1X Incubation buffer, label this tube #1. Undiluted control sample can be frozen at -80°C .
3. Transfer 150 μL from tube #1 to tube #2. Mix thoroughly. With a fresh pipette tip transfer 150 μL from #2 to #3.
4. Repeat for Tubes #4 through #7. Use 1X Incubation buffer as the zero sample tube labeled #8. Use a fresh dilution series for each assay.



9. Assay Procedure

Bring all reagents and samples to room temperature before use. It is recommended all samples be assayed in duplicate.

1. Prepare all reagents and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 50 μ L of each diluted sample per well. It is recommended to include a dilution series of a control (normal) sample as a reference. Also include a 1X Incubation buffer as a zero standard.

4. Cover/seal the plate and incubate for 2 hours at room temperature. If available use a plate shaker for all incubation steps at 300 rpm.
5. Aspirate each well and wash, repeat this once more for a total of **two** washes. Wash by aspirating or decanting from wells then dispensing 300 μ L 1X Wash buffer into each well as described above. Complete removal of liquid at each step is essential to good performance. After the last wash, remove the remaining buffer by aspiration or decanting. Invert the plate and blot it against clean paper towels to remove excess liquid.
6. Immediately before use prepare sufficient (0.5 mL/strip used) 1X Detector Antibody in 1X Incubation buffer. Add 50 μ L 1X Detector antibody to each well used. Cover/seal the plate and incubate for 1 hour at room temperature. If available use a plate shaker for all incubation steps at 300 rpm.
7. Repeat the aspirate/wash procedure above.
8. Immediately before use prepare sufficient (0.5 mL/strip used) 1X HRP label in 1X Incubation buffer. Add 50 μ L 1X HRP label to each well used. Cover/seal the plate and incubate for 1 hour at room temperature. If available use a plate shaker for all incubation steps at 300 rpm.

9. Repeat the aspirate/wash procedure above, however, performing a total of **three** washes.
10. Add 100 μ L HRP Development Solution to each empty well and immediately record the blue color development with time in the microplate reader prepared with the following settings:

Mode:	Kinetic
Wavelength:	600 nm
Time:	up to 15 min
Interval:	20 sec - 1 min
Shaking:	Shake between readings

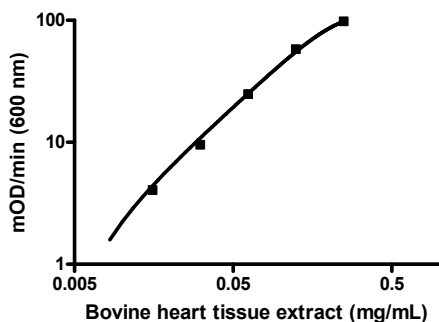
Alternative– In place of a kinetic reading, at a **user defined**, time record the endpoint OD data at (i) 600 nm or (ii) stop the reaction by adding 100 μ L stop solution (1N HCl) to each well and record the OD at 450 nm.

11. Analyze the data as described below.

10. Data Analysis

Average the control sample dilution series readings and plot against their concentrations after subtracting the zero standard reading. Draw the best smooth curve through these points to construct a standard curve. Most plate reader software or graphing software can plot these values and curve fit. A four parameter algorithm (4PL) usually provides the best fit, though other equations can be examined to see which provides the most accurate (e.g. linear, semi-log, log/log, 4 parameter logistic). Read phospho S300 PDHA1 protein concentrations for unknown samples from the standard curve plotted. Samples producing signals greater than that of the highest sample standard should be further diluted in 1X Incubation buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.

TYPICAL SAMPLE RANGE - For demonstration only.



<u>Control sample</u> (mg/mL)	<u>Change</u> <u>mOD/min</u> (600nm)
0.0156	3.9,4.1
0.03125	9.1,9.8
0.0625	25.3,23.8
0.125	59,56
0.25	99,95
0.5	114,119

Figure 1. Example control sample curve. The phosphorylation state of PDHA1 can vary by treatment but also by cell culture conditions such as media supplements, nutrients and also cell density.

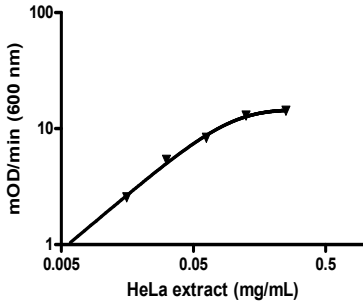


Figure 2. Example control sample curve. The phosphorylation state of PDHA1 can vary by treatment but also by cell culture conditions such as media supplements, nutrients and also cell density.

The phosphorylation signal of phospho S300 in cultured cells was determined to be very low. This signal could be manipulated by cellular treatment, kinases and phosphatases as shown below.

Typical Working Ranges:

Tissue extract (e.g. Heart muscle)	15 - 500 µg/mL
Whole cultured cell extract	15 - 250 µg/mL

SENSITIVITY

Determined minimum detectable dose = 15 µg/mL sample lysate.

REPRODUCIBILITY

	CV %
Intra (n= 8)	2.5
Inter (n=4 days)	7.8

11. Specificity

Species– human, rat, mouse, cow reactive. Others untested.

Alteration in the levels of phospho S300 in samples can be measured using this ELISA. Control compounds such as the specific PDK inhibitor dichloroacetate (DCA) decrease phosphorylation levels while the non specific serine/threonine phosphatase inhibitor sodium fluoride (NaF) increases phosphorylation levels, example data are shown in Figure 3. Another method of altering PDHA1 phosphorylation levels is treatment, after sample binding, in each well with Abcam purified recombinant PDH kinases and phosphatases. Details of how to perform the in-well treatment are described in the supplementary protocol below. An example of data generated using kinases PDK1 and 3 (ab110359, ab110355) and phosphatases PDP1 (ab110357) are shown in Figure 4.

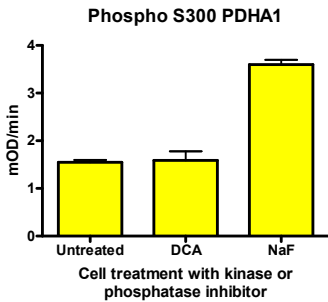


Figure 3. HeLa cells were cultured for 4 hours in media supplemented with DCA (20mM) to specifically inhibit mitochondrial PDH kinases, or NaF (20mM), a general inhibitor of serine/threonine protein phosphatases. The DCA treatment did not reduce the level of phospho S300 confirming that there is little endogenous phospho S300. Conversely NaF treatment, to inhibit cellular serine phosphatases, did increase the phosphorylation level of S300.

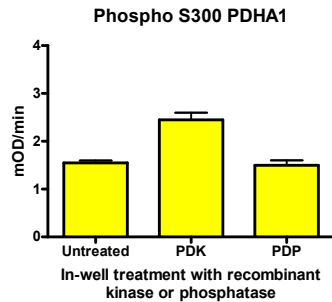
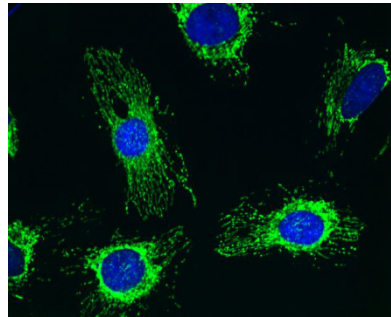
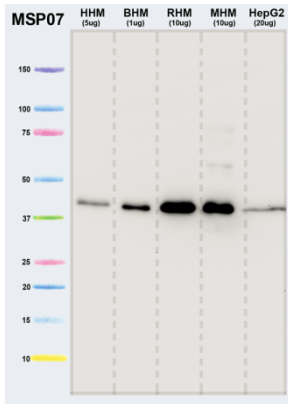


Figure 4. The PDHA1 bound from undosed HeLa cells was subject to in-well kinase treatment (PDK1&3) or in-well phosphatase treatment (PDP1) according to the supplementary protocol shown below. Untreated cells did not show a significant endogenous phosphorylation signal at S300 this could be increased by kinase treatment. Phosphatase treatment had no effect on the endogenous (low) level of phospho S300 signal.

The PDHA1 protein capture antibody in this ELISA is available as a standalone antibody ab110334.



Isolated mitochondria from human heart (lane 1), bovine heart (lane 2), rat heart (lane 3), mouse heart (lane 4), and HepG2 (lane 5) detected with (ab110334) anti-subunit E1 alpha antibody.

Mitochondrial localization of PDH in cultured, normal human HeLa cells labeled with anti-PDH-E1 alpha mAb 8D10E6 (ab110334) as the primary antibody (1 µg/ml) and an AlexaFluor® 488 goat anti-mouse IgG1 secondary antibody (2 µg/ml).

12. In-Well Kinase/Phosphatase Treatment

IN WELL KINASE TREATMENT

Between steps 5 and 6 the wells can be treated with kinase to phosphorylate the bound PDHA1

- I. Prepare phosphorylation solution by dispensing 22 mL 1X Wash buffer into a clean tube. Add MgCl_2 to 1mM and ATP to 2mM.
- II. After step 5 in the Assay protocol add 200 μL phosphorylation solution to each well to be treated by kinase.
- III. Add desired PDK to desired wells to phosphorylate the bound PDHA1. 2 $\mu\text{g}/\text{well}$ is recommended for PDK1,2, and 4 (ab110359, ab110354, ab110356). For PDK3 (ab110355) add 0.2 $\mu\text{g}/\text{well}$ is recommended. *Note our data indicate that PDK1 is the only kinase able to phosphorylate phospho S300.*
- IV. Incubate for 10 minutes at 30°C. If available use a plate shaker for all incubation steps at 300 rpm.
- V. Aspirate each well and wash, repeat this once more for a total of **two** washes. Resume from Step 6 (page 14).

IN WELL PHOSPHATASE TREATMENT

Between steps 5 and 6 the wells can be treated with phosphatase to dephosphorylate the bound PDHA1

- I. Prepare dephosphorylation solution by dispensing 22 mL 1X Wash buffer into a clean tube. Add $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ to 0.4mM.
- II. After step 5 in the Assay protocol add 200 μL dephosphorylation solution to each well to be treated by phosphatase.
- III. Add desired PDP (ab110357, ab110358) to desired wells to dephosphorylate the bound PDHA1. 4 $\mu\text{g}/\text{well}$ is recommended for PDP1 and 2.
- IV. Incubate for 10 minutes at 30°C. If available use a plate shaker for all incubation steps at 300 rpm.
- V. Aspirate each well and wash, repeat this once more for a total of **two** washes. Resume from Step 6 (page 14).

13. Troubleshooting

Problem	Cause	Solution
Poor curve	Inaccurate pipetting	Check pipettes
Low signal	Too brief incubation times	Ensure sufficient incubation time; change incubation to overnight
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
	Phosphatase activity	Consider optimization and use of phosphatases inhibitors

Large CV	Plate is insufficiently washed	Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed
	Contaminated wash buffer	Make fresh wash buffer
Sample-sample variation between similar samples	Growth conditions vary	<p>Metabolism tightly controls the phosphorylation state of PDHA1. Several factors affect the phosphorylation state including media, nutrition and cell density.</p> <p>Monitor cell culture conditions very carefully.</p>

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