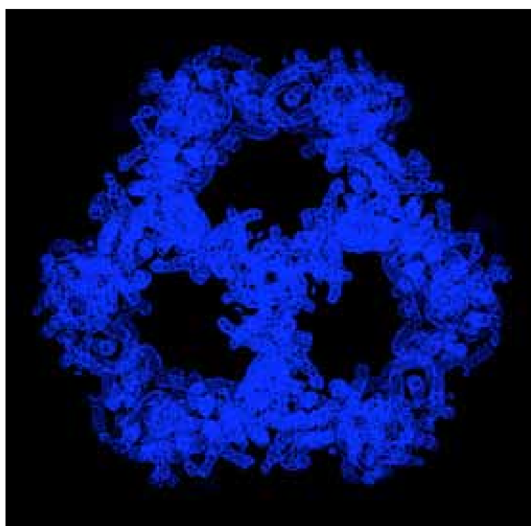
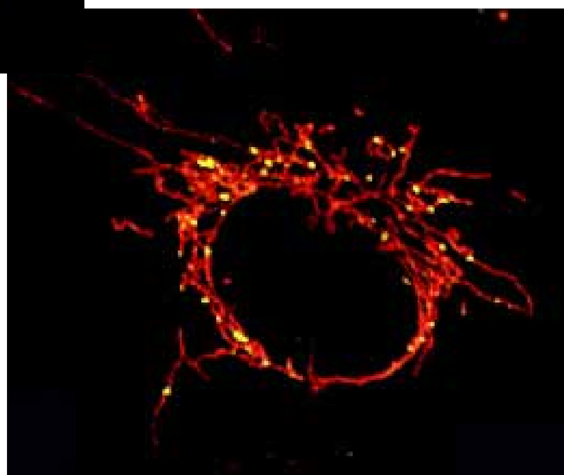
THE PYRUVATE DEHYDROGENASE COMPLEX PLAYBOOK



Tips and Techniques for Researching PDH Using Tools from MitoSciences



MitoSciences®
Advancing vital discoveries in mitochondrial research

I. Introduction

The pyruvate dehydrogenase complex (PDH or PDC) is the key regulatory site in cellular metabolism, in that it links the citric acid cycle and subsequent oxidative phosphorylation with glycolysis and gluconeogenesis, as well as with both lipid and amino acid metabolism. When carbohydrate stores are reduced in mammals, PDH activity is regulated downward to limit the use of glucose by oxidative phosphorylation in those tissues that can use fatty acids or ketone bodies, such as heart and skeletal muscle. The important exception is neuronal tissue, which processes glucose almost exclusively for ATP production.

Activation of PDH both facilitates use of carbohydrate to meet energy demands and also converts surplus dietary carbohydrates to fatty acids for longer term energy storage. Perturbation of the regulation of the choice of glucose or fatty acids as energy source is a key part of diabetes, metabolic syndrome and obesity, while metabolic substrate switching from oxidative phosphorylation to glycolysis defines the cancer phenotype, hence the recent renewed interest in PDH.

II. Structure and Activity

The pyruvate dehydrogenase complex is a 9.5 megadalton assembly of four proteins: pyruvate dehydrogenase (E_1), dihydrolipoamide acyltransferase (E_2), dihydrolipoyl dehydrogenase (E_3), and one structural protein (E_2/E_3 binding protein). The E_1 enzyme is a heterotetramer of two α and two β subunits. PDH component proteins are arranged as a core of 60 E_2 subunits around which are distributed 30 copies of the E_1 heterotetramer, 12 copies of E_3, and 12 copies of the E_2/E_3 binding protein. PDH catalyzes irreversible oxidative decarboxylation of pyruvate to acetyl Coenzyme A, as shown in Figure 1.

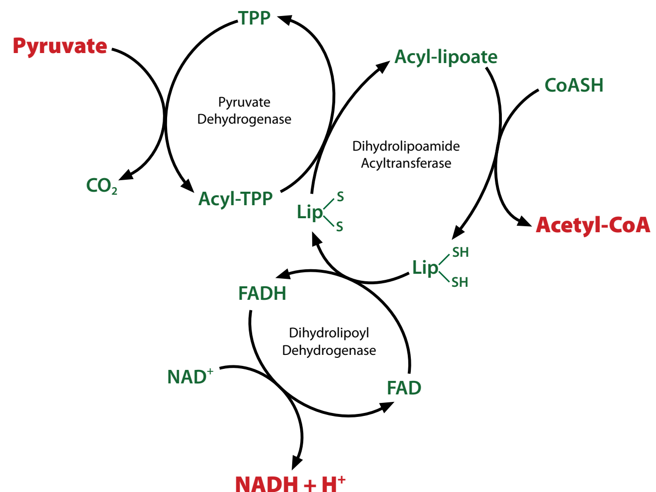


Figure 1. *Five sequential reactions of PDH-catalyzed oxidative decarboxylation of pyruvate to Acetyl Coenzyme A.*

III. Regulation of PDH Activity by Phosphorylation

Not surprisingly given its central role in metabolism, PDH is under tight and complex regulation, which includes regulation by reversible phosphorylation in response to the availability of glucose. In humans, PDH activity is inhibited by site-specific phosphorylation at three sites on the $E_1\alpha$ subunit (Ser^{232}, Ser^{293} and Ser^{300}), which is catalyzed by four different pyruvate dehydrogenase kinases (PDK1-4). Each of the four kinases has a different reactivity for these three sites. Interestingly, phosphorylation at any one site leads to the inhibition of the complex *in vitro*. Two pyruvate dehydrogenase phosphatases (PDP1 and PDP2) dephosphorylate the E1α and activate the enzyme. The phosphatases show little or no specificity. Both the kinases and phosphatases are differentially expressed in tissues. Each of the PDK's and PDP's is under transcriptional control in response to different cellular stress events as shown in Figure 2. In addition, the kinases are activated by acetyl Coenzyme A, NADH and ATP, meanwhile the availability of pyruvate and ADP leads to their inhibition.

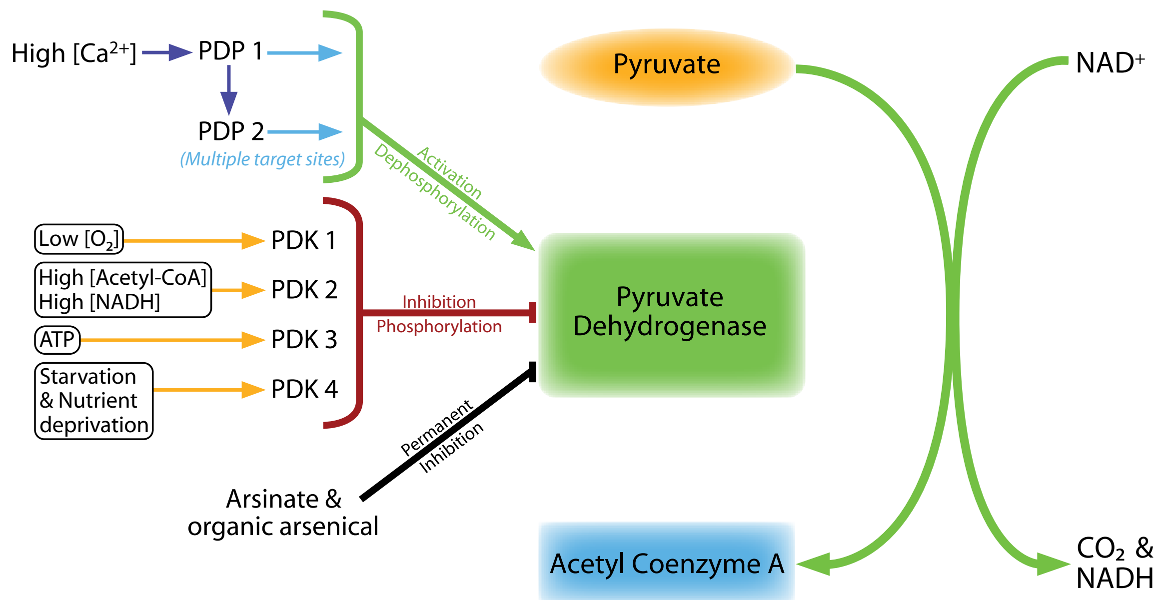
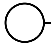


Figure 2. *A schematic of the reactions controlling pyruvate dehydrogenase.*



As an example of the transcriptional regulation, expression of PDK4 is suppressed under basal conditions in most tissues by maintaining relevant histones in deacetylated state but is induced, but its expression is increased during starvation by glucocorticoids that re-acetylate these histones, particularly in heart, skeletal and other muscle tissues, kidney, and liver. PDK4 is also up-regulated by a high fat diet and extended exercise. Insulin inhibits PDK4 expression via PI3K signaling that leads to lower histone acetylation. The levels of PDK4 are also regulated to PPAR transcription factors. Importantly, in diabetes caused by either insulin deficiency or insulin insensitivity, the uninhibited PDK4 overexpression prevents glucose oxidation.
In contrast, the levels of PDK1 are sensitive to O_2 levels and under regulation by the transcription factor HIF-1α. An increase in the level of PDK1 is a key part of the so-called Warburg effect, a switch from oxidative to glycolytic ATP production that characterizes cancer cells.

IV. *Useful reading on the Structure and Function of PDH*

i. Sugden MC, Bulmer K, & Holness MJ. (2001) "Fuel Sensing Mechanisms Integrating Lipid and Carbohydrate Utilization." Biochem Soc Trans. 29, 272-278.

ii. Patel MS & Korotchkina LG. (2006) "Regulation of the Pyruvate Dehydrogenase Complex." Biochem Soc Trans. 34, 217-222.

iii. Roche TE & Hiromasa Y. (2007) "Pyruvate dehydrogenase kinase regulatory mechanisms and inhibition in diabetes, heart disease and cancer." Cell Mol Life. 64, 830-849.

iv. Degenhardt T, et al. (2007) "Three members of the human pyruvate dehydrogense kinase gene family are direct targets of peroxisome proliferator - activator receptor ß/delta. J Mol Biol. 372, 341-355.

V. *Monitoring PDH Activity in Cell and Tissue Extracts*

MitoSciences has provided a convenient way to assay PDH activity in complex biological samples. This involves separation of the active enzyme from other cellular components by immunocapture onto a solid surface (96 or 386 well plate or dipstick). NADH production can then be measured directly without interference from other NADH-utilizing or producing enzymes. We have recently introduced an improved version of the assay

in which the produced NADH is coupled to a colorimetric reaction, as illustrated in Figure 3. In addition, we have introduced changes in the sample preparation procedure when using cell culture material that have greatly increased the assay sensitivity. The MitoSciences approach is much simpler, faster, and safer than the classical method of using [^{14}C] pyruvate and measuring enzyme-catalyzed release of [^{14}C] CO_2.

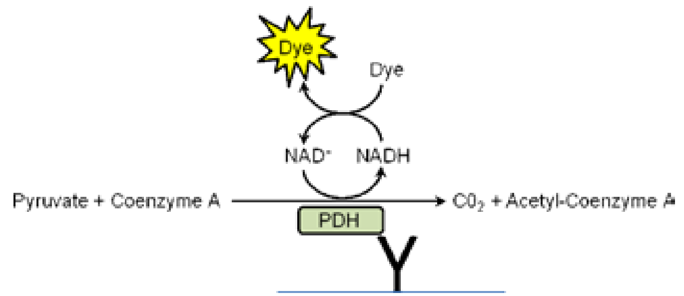


Figure 3. *MitoSciences' PDH activity assay reaction scheme. The production of NADH is coupled to the reduction of a dye which has an increased absorbance at 450 nm and so becomes yellow in color.*

Additionally, MitoSciences now offers a comprehensive line of PDH-related tools, including all four PDH kinases and both PDH phosphatases. These tools are available as add-ons to our standard microplate or dipstick kits. For convenience, detailed protocols are provided.

VI. *Assaying the Endogenous PDH Activity and Phosphorylation*

One of the most sought after measurements in monitoring glucose utilization is the determination of the activity of PDH *in vivo*. The key to such measurements is maintaining the enzyme in the *in vivo* state of phosphorylation throughout the isolation process. New protocols from MitoSciences now make this possible using human, bovine, rat and mouse cell or tissue extracts. The key is the use of MitoSciences extraction and immunocapture buffers formulated to inhibit endogenous specific and non-specific kinases and phosphatases, thus preventing unwanted PDH modifications during the sample preparation (see Figures 4 and 5).

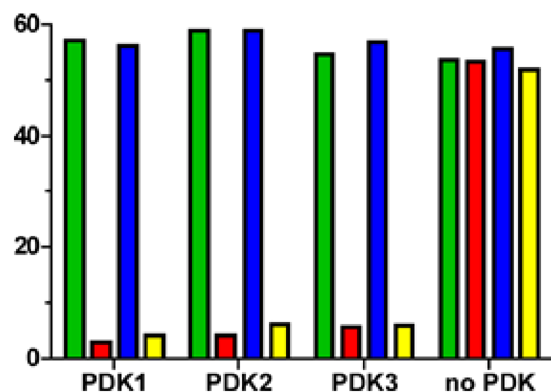


Figure 4. *Apyrase prevents PDK-catalyzed phosphorylation and inactivation of PDH. Porcine PDH was treated with indicated kinase (MSP41-MSP44) in the absence of ATP (in green), in the presence of ATP (in red), or in the presence of ATP and apyrase (in blue). In parallel, PDH was first treated in the presence of ATP with indicated kinase and then treated with apyrase (in yellow). PDH activity was measured.*

After the immunocapture, endogenous kinases and phosphatases are washed away and parallel samples can be phosphorylated with purified active recombinant PDK's and dephosphorylated with recombinant PDP's to determine, respectively, the residual activity of the fully-phosphorylated PDH, and the maximum activity of the fully dephosphorylated enzyme, in addition to the endogenous unmodified PDH activity. In parallel with the activity assay, the phospho-serine levels of the samples with unmodified, phosphorylated and dephosphorylated immunocaptured PDH can be measured to confirm that observed inhibition is related to phosphorylation and not a consequence of other modifications such as oxidative damage. Examples of such studies are shown in Figure 6. Importantly, the phosphorylation and dephosphorylation reactions were optimized to obtain, respectively, fully inactivated phosphorylated PDH, and fully activated dephosphorylated PDH, as shown in Figures 7, 8, and 9.

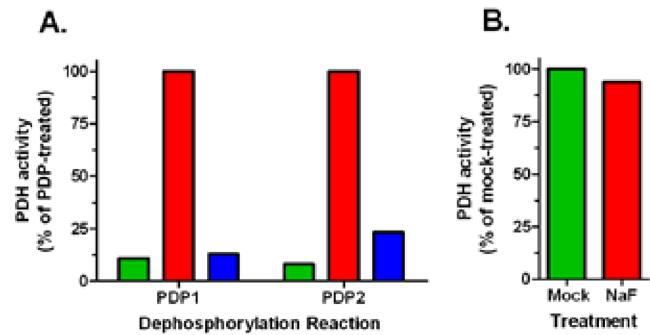


Figure 5. *Sodium fluoride inhibits PDP1-catalyzed dephosphorylation and activation of PDH, without having direct effect on the enzyme. (A) Porcine PDH was first fully phosphorylated with PDK3 (MSP43) and then mock-phosphatase-treated (in green), or treated with the indicated phosphatase (MSP45 or MSP46) in the absence (in red) or in the presence (in blue) of sodium fluoride. Subsequently, PDH activity was measured. (B) Addition of sodium fluoride into the PDH assay buffer has no direct effect on PDH activity.*

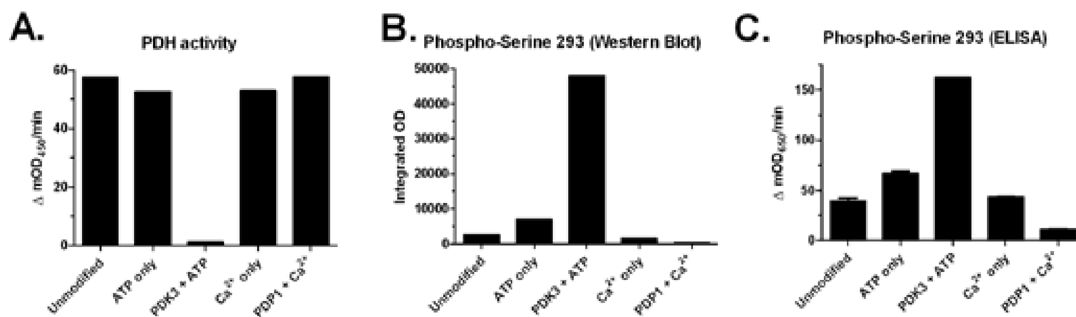


Figure 6. *PDH activities and $E_1\alpha$ Phospho-Serine 293 levels of unmodified (endogenous), PDK3-phosphorylated and PDP1-dephosphorylated bovine heart PDH. PDH was immunocaptured from 50 µg of bovine heart mitochondria and unmodified, fully phosphorylated (PDK3+ATP) and fully dephosphorylated (PDP1+Ca^{2+}) PDH activities (n=3) were measured with the use of MSP18. PDH $E_1\alpha$ Phospho-Serine 293 levels were assayed by Western blotting (n=1) on materials extracted from wells after the PDH activity measurement (B), or on parallel samples, prepared as in A., using 2-site ELISA with Phospho-Serine 293 detector antibody (MSP11)(n=2)(C).*

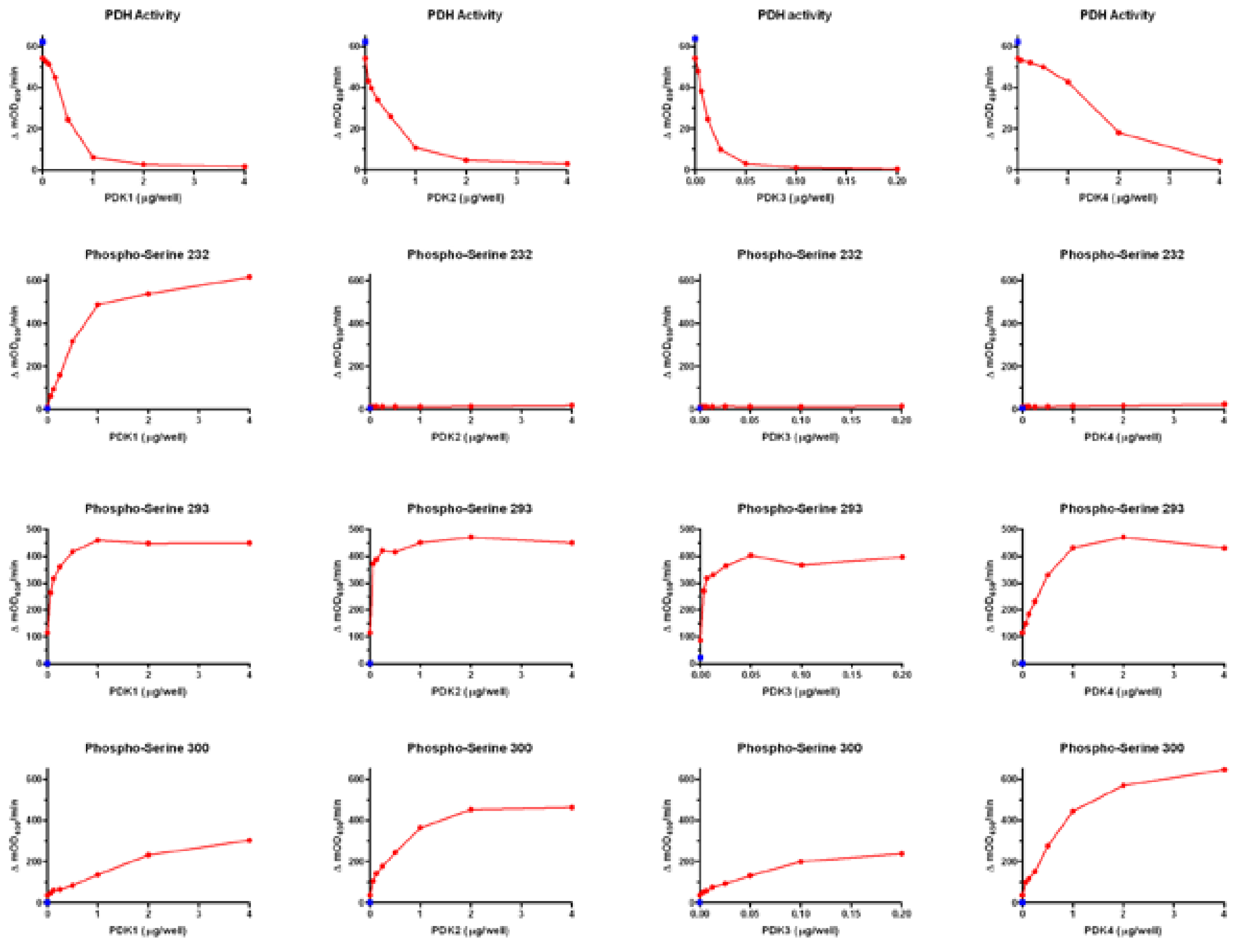


Figure 7. *The recombinant human PDKs phosphorylate and fully inactivate mouse PDH. Extracts of mouse heart homogenate were dephosphorylated with PDP1 to ensure maximum activity. Then PDH was immunocaptured from 125 µg of the material and it was treated with indicated amounts of recombinant PDKs (MSP41-MSP44) in the presence of ATP (in red) or in the absence of ATP (in blue). PDH activities (first row) were measured with the use of MSP18. PDH $E_1α$ Phospho-Serine 232 (second row), 293 (third row), and 300 (fourth row) quantities were measured with by 2-site ELISA with, respectively, PDH $E_1α$ Phospho-Serine 232, 293, and 300 detector antibodies.*

When required, the amounts of total PDH in each sample can be determined using MitoSciences PDH protein quantity assay kits, and this has the advantage of allowing the activity measurements to be expressed as relative specific activities. Alternatively, the phospho-serine signal of the fully phosphorylated PDH can be used to normalize the amounts of immunocaptured PDH. This is particularly important when multiple samples are being compared (i.e. from different tissues, or after several different treatments).

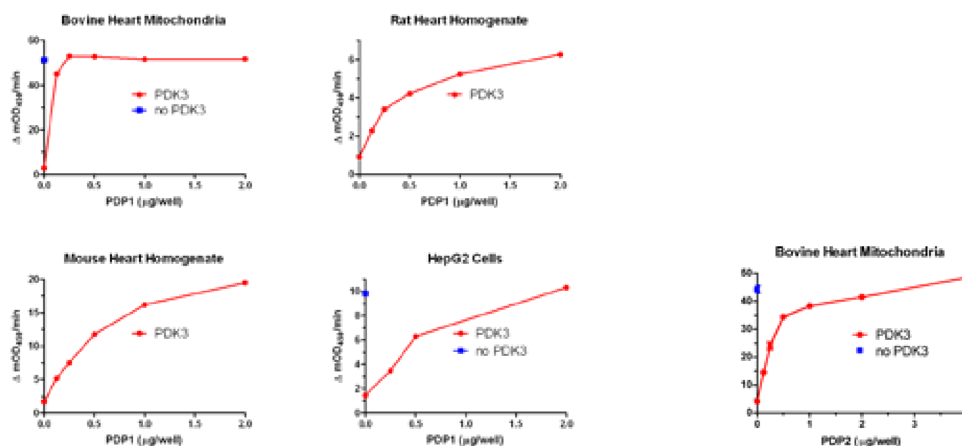


Figure 8. *Recombinant human PDP1 activates PDK3-phosphorylated immunocaptured bovine rat, mouse and human PDH. PDH was immunocaptured from 50 µg of bovine heart mitochondria, 250 µg of rat heart homogenate, 125 µg of mouse heart homogenate, or 500 µg of HepG2 cells and fully phosphorylated with 0.1 µg of recombinant human PDK3 (MSP43)(in red) or mock-phosphorylated (no PDK3, in blue). Then the samples were treated with indicated amounts of recombinant PDP1 (MSP45) in the presence of Ca^{2+}, and PDH activity was measured with the use of MSP18.*

Figure 9. *Recombinant human PDP2 activates PDK3-phosphorylated immunocaptured bovine PDH. PDH was immunocaptured from 50 µg of bovine heart mitochondria and fully phosphorylated with 0.1 µg of recombinant human PDK3 (MSP43) (in red) or mock-phosphorylated (no PDK3, in blue). Then the samples were treated with indicated amounts of recombinant PDP2 (MSP46), and PDH activity was measured with the use of MSP18.*

VII. *PDK Inhibitor Screen*

There is now a strong interest in developing specific inhibitors of the different PDH kinases as potential treatments for cancer, diabetes, etc. The MitoSciences assay tools allow a high throughput approach for conducting such drug screening studies. Thus, we provide PDK of choice which can be used to phosphorylate immunocaptured PDH in the presence of compound of interest. Having the enzyme attached to a solid support has many advantages over using the soluble enzyme: (1) a variety of PDH sources can be used (there is no need to biochemically purify PDH or depend on a commercial enzyme), (2) the endogenous specific and non-specific kinases are washed away and thus only the effect on exogenously added kinase of interest can be assayed, (3) the phosphorylation reaction is stopped rapidly by a simple wash, and (4) the wash removes reactants of the phosphorylation reaction, including the tested compound, that may interfere with the PDH activity assay. In addition, if the immunocapured PDH is not at the maximum activity due to intrinsic phosphorylation, a dephosphorylation reaction can be carried out with the use of recombinant PDPs. After the phosphorylation, PDH activity is measured.

VIII. *Detection and Quantification of PDH Levels in Tissues and Cells: Identification of PDH Deficiencies*

Defects in the pyruvate dehydrogenase complex are an important cause of lactic acidosis and can cause Leigh's Diseases in children. Mutations of E_2, E_3 and E_2/E_3 binding protein have been reported, but the majority of cases of PDH deficiency are due to mutations in the X-linked E1 alpha subunit. Traditionally, the disease has been diagnosed from activity assays followed by genetic studies. MitoSciences provides both plate-based and dipstick ELISA assays of PDH amount and a bead-based PDH immunocapture kit, which allows screening for subunit deficiencies in patients. Female carriers of $E_1\alpha$ are present mostly as mosaics, often with only a small percentage of defective cells. These can be screened by using our anti- $E_1\alpha$ and anti-E_2 mAbs in immunocytochemistry of patient cell lines or tissue as shown in Figure 10. These monoclonal antibodies are available already labeled with appropriate dyes for immunocytochemistry or flow cytometry analysis of the levels of defective cells, both in terms of reduction of, or elimination of, the $E_1\alpha$ subunit.

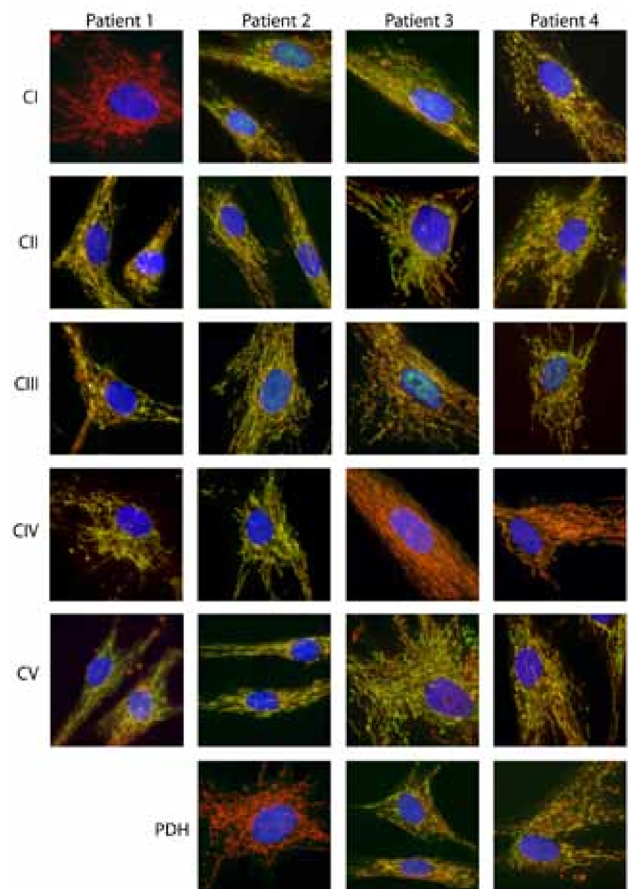


Figure 10. *Analysis of patients with Leigh's Dieases. Patient 2 has a mutation in the E1 alpha subunit as identified here by immunohistochemistry of a fibroblast sample. Other causes of Leigh's Disease are incomplete assembly of Complexes I or IV as show by Patients 1, 3, and 4 respectively.*

PDH levels are emerging as important biomarkers in acute injury and neurodegeneration. The MitoSciences PDH protein quantity dipsticks are being used to dissect the role of PDH in traumatic brain injury (Sharma, P et al. Journal of Emergencies, Trauma and Shock 2(2), 67-72, 2009). In a mouse Alzheimer's Disease model, mitochondrial bioenergetic deficit, including decrease in PDH amount and activity, precedes the pathological manifestation of the disease (Yao J., et al., Proc. Natl. Acad. Sci. USA, e-published ahead of print).

Experimental Flow Chart

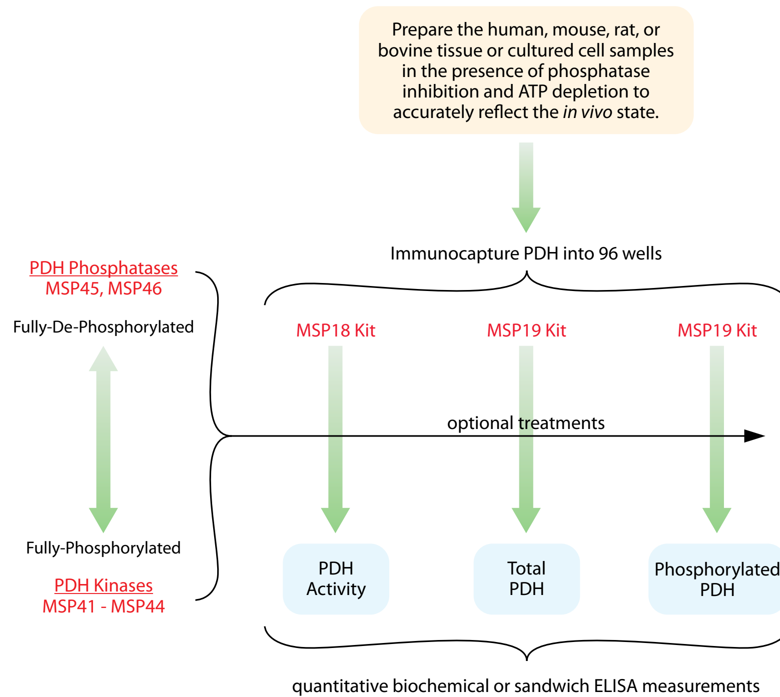


Figure 11. *Experimental Flow Chart Using MitoSciences' Products for Analyzing PDH. All experimental protocols recommend beginning by inhibiting the endogenous kinase and phosophatase activity during sample processing, ensuring that the sample maintains a state that is as close to the in vivo state as possible. Either Kit MSP18 or MSP19 is used to capture PDH depending on the desired measurement, and optional recombinant kinases and phosphatases are available to generate a subset of fully-phosphorylated or fully-de-phosphorylated samples.*

Ready-to-use kits and detailed protocols to investigate PDH as described in this PDH Playbook can be found at the following links:

- **MitoSciences PDH product page:** http://www.mitosciences.com/pyruvate_dehydrogenase.html
- **PDH Protocols:** http://www.mitosciences.com/pdh_protocols.html
  (This webpage includes protocols for high-throughput screening investigation of the phosphorylation status and phosphorylation-regulated activity of PDH.)

MitoSciences Products for PDH

Catalog #	Product Name	Applications*	Reactivity*	Quantity
Antibodies				
MSP01c	PDH capture antibody monoclonal antibody	IP	H, B	100 µg
MSP03	PDH subunit E1 alpha monoclonal antibody	WB, ICC	H, B, M	100 µg
MSP07	PDH subunit E1 alpha monoclonal antibody	WB, ICC	H, B, M, R, Dm, Ce	100 µg
MSP04	PDH subunit E1 beta monoclonal antibody	WB	H, B, M, R	100 µg
MSP05	PDH subunit E2 monoclonal antibody	WB, ICC	H, B	100 µg
MSP06	PDH subunit E2/E3bp monoclonal antibody	WB, ICC	H, B, M, R	100 µg
Antibody Cocktails				
MSP02	PDH Antibody Cocktail	WB	H, B	150 or 300 µg
In-Cell ELISA Kits				
MSP47	PhosphoPDH In-Cell ELISA Kit (IR)	ICE	H, B, M, R	2 x 96 tests
MSP48	PhosphoPDH In-Cell ELISA Kit (Colorimetric)	ICE	H, B, M, R	2 x 96 tests
Enzyme Activity Assays				
MSP18	PDH Enzyme Activity Microplate Assay Kit	IB	H, B, M, R	96 tests
MSP20	PDH Combo (Activity + Quantity) Microplate Assay Kit	IB, SE	H, B, M, R	96 tests
MSP30	PDH Enzyme Activity Dipstick Assay Kit	IB	H, B, M, R, Pn	30 or 90 tests
Protein Quantity Assays				
MSP19	PDH Protein Quantity Microplate Assay Kit	SE	H, B, M, R	96 tests
MSP20	PDH Combo (Activity + Quantity) Microplate Assay Kit	IB, SE	H, B, M, R	96 tests
MSP31	PDH Protein Quantity Dipstick Assay Kit	SE	H, B, M, R	30 or 90 tests
Enzyme Purification Kits				
MSP01	PDH Immunocapture Kit	IP	H, B	250, 500, or 750 µg
Recombinant Proteins				
MSP41	PDH Kinase 1 (PDK1)			50 µg
MSP42	PDH Kinase 2 (PDK2)			50 µg
MSP43	PDH Kinase 3 (PDK3)			50 µg
MSP44	PDH Kinase 4 (PDK4)			50 µg
MSP45	PDH Phosphatase 1 (PDP1)			50 µg
MSP46	PDH Phosphatase 2 (PDP2)			50 µg

** Reactivity Codes: Human (H), Bovine (B), Mouse (M), Rat (R), Drosophila melanogaster (Dm), C. elegans (Ce), Primate (Pt), Porcine (Pn)*
** Application Codes: Immunoprecipitation (IP), Western Blotting (WB), Immunocytochemistry (ICC), Sandwich ELISA (SE), Immunocapture Biochemical (IB)*

Front cover image (left) kindly provided by Dr. Peter Rosenthal, MRC National Institute for Medical Research, London.

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