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Measurement of Endogenous PDH-$E_1\alpha$ Phospho-Serine^{293} Levels Relative to the Fully-Phosphorylated and Dephosphorylated States

Rev.2

INTRODUCTION

This immunocapture PDH phosphorylation protocol describes how to measure endogenous levels of phosphorylated Serine^{293} of the PDH-$E_1\alpha$ subunit corresponding to the enzyme's phosphorylation state at the time of sample collection. The key to obtain meaningful measurements of a sample's endogenous PDH phosphorylation state is to inhibit endogenous specific and non-specific phosphatases and kinases that may modify PDH-$E_1\alpha$ during sample preparation and immunocapture steps. It is essential that serine/threonine phosphatase inhibitors are used, as well as a system to block the phosphorylation reaction (by depleting ATP).

MitoSciences' PDH enzyme activity assay kit MSP18 is used to first immunocapture PDH from extracts of human, bovine, rat or mouse cells, tissues or mitochondria, and then (instead of activity measurements) to measure the levels of phosphorylation at specific serine residues on the immunocaptured PDH-$E_1\alpha$ subunit. Endogenous phosphorylation levels are measured in parallel with samples treated post-capture with PDK3 and PDP1 to determine respectively, the signal generated by fully-phosphorylated PDH, and the "background" signal generated by fully-dephosphorylated PDH.

Phosphorylation reaction conditions were optimized to obtain fully-phosphorylated PDH-$E_1\alpha$ Serine^{293} (conditions that fully-inactivate PDH enzyme activity). Similarly, dephosphorylation conditions were optimized to obtain fully-dephosphorylated PDH-$E_1\alpha$ Serine^{293} (conditions that result in maximal PDH enzyme activity). The endogenous levels of PDH-$E_1\alpha$ Serine^{293} (that reflect endogenous enzyme activity) relative to the fully dephosphorylated and fully phosphorylated enzyme, on a scale of 0 to 100 percent, can then be determined (Figure 1).

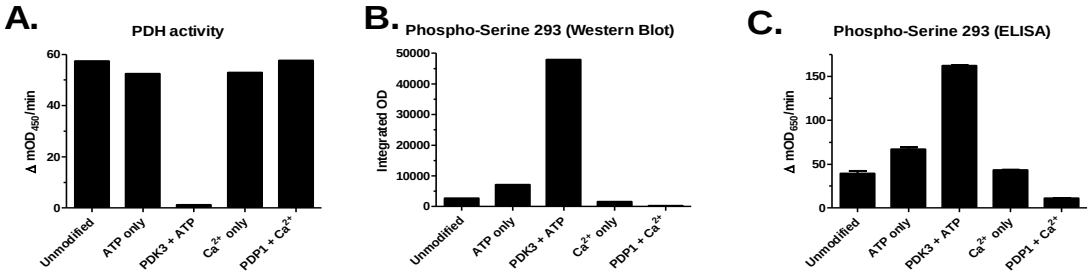

Figure 1. PDH activities and PDH-$E_1\alpha$ Phospho-Serine^{293} levels of unmodified (endogenous) PDK3-phosphorylated and PDP1-dephosphorylated bovine heart PDH. PDH was immunocaptured from 50 µg of bovine heart mitochondria and unmodified, fully phosphorylated (PDK3 + ATP) and fully dephosphorylated (PDP1 + Ca^{2+}) PDH activities (n=3) were measured with the use of MSP18 (A). 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 (n=2) as described in this protocol (C). Shown are mean values, error bars represent SEM. The endogenous bovine heart PDH activity, measured in this experiment, is 99 percent of the activity of the fully dephosphorylated enzyme. The endogenous bovine heart PDH $E_1\alpha$ phospho-Serine^{293} levels measured in this experiment with the use of Phospho-PDH Ser^{293} detector antibody (EMD Chemicals, catalog #AP1062) by Western blotting and by ELISA as described in this protocol are 5 and 19 percent of the Phospho-Serine^{293} levels of the fully phosphorylated enzyme, respectively.

Note #1 – Although this protocol describes conditions optimized for regulatory enzymes PDK3 and PDP1, similar experiments can be performed using PDK1, PDK2, PDK4 and PDP2.

PDH Protocol #2 – Measurement of Endogenous PDH-$E_1\alpha$ Phospho-Serine^{293} Levels

Note #2 – Although this protocol describes measurement of PDH-$E_1\alpha$ phospho-Ser^{293}, levels of PDH-$E_1\alpha$ phospho-Ser^{300} and/or PDH-$E_1\alpha$ phospho-Ser^{232} can also be measured by using the appropriate phosphosite-specific detector antibodies, i.e., PhosphoDetect™ Anti-PDH-$E_1\alpha$ (pSer^{300}) pAb (EMD Chemicals, cat. #AP1064) or PhosphoDetect™ Anti-PDH-$E_1\alpha$ (pSer^{232}) pAb (EMD Chemicals, cat. #AP1063).

REGULATION OF PDH ACTIVITY

The pyruvate dehydrogenase complex (PDH) (E.C.1.2.4.1) is the key regulatory enzyme of cellular metabolism, in that it links the TCA cycle and subsequent oxidative phosphorylation with glycolysis and gluconeogenesis as well as with both lipid and amino acid metabolism. PDH is a large complex of three enzymes, pyruvate dehydrogenase (E_1), dihyrolipoyl acyltransferase (E_2), and dihydrolipoyl dehydrogenase (E_3), and one structural protein (E_2/E_3 binding protein). In total the enzyme is composed of five different polypeptide chains, $E_1\alpha$, $E_1\beta$, E_2, E_3 and E_2/E_3BP with 30:30:60:12:12 stoichiometry. PDH catalyzes irreversible oxidative decarboxylation of pyruvate to acetyl coenzyme A. The activity of PDH is negatively regulated by reversible phosphorylation of the $E_1\alpha$ subunit. The phosphorylation is catalyzed by four PDH kinase isozymes (E.C.2.7.11.2), PDK 1, 2, 3 and 4. The dephosphorylation that activates the PDH is catalyzed by two PDH phosphatases (E.C. 3.1.3.43), PDP 1 and 2.

The PDH kinases are Serine/Threonine protein kinases. They are ATP-dependent enzymes that are bound to the E_2 domain of PDH. The PDH kinases phosphorylate three specific sites of the $E_1\alpha$ subunit (the phospho-Serine positions are given through this text with respect to the human protein): Site 1 (Ser^{293}), Site 2 (Ser^{300}) and Site 3 (Ser^{232}), thus inhibiting the enzyme activity (Fig. 2). Each of the kinases has different reactivity towards the three phosphorylation sites. While under normal dietary conditions PDK2 is the predominant isoform in skeletal muscle, the PDK4 is induced by exercise and diet as well as by starvation. This kinase is aberrantly upregulated in insulin resistant diabetes. The levels of PDK4 are sensitive to inhibitors of the PPAR transcription factors. In contrast, the levels of PDK1 are sensitive to O_2 levels and under regulation by the transcription factor HIF1. An increase in the level of PDK1 is a key part of Warburg effect, a switch from oxidative to glycolytic ATP production that characterizes cancer cells. Because of the manifold roles of PDKs in physiology and disease, there is considerable interest in identifying drugs that regulate PDK activities.

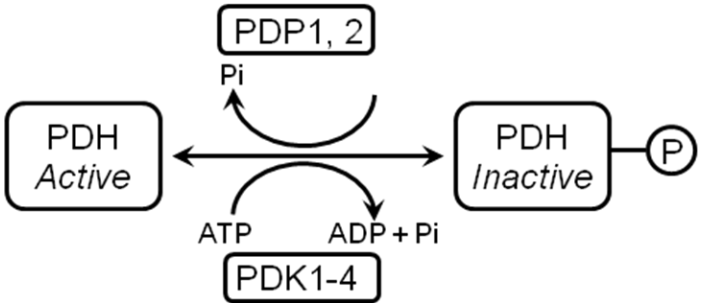

Figure 2. Schematic representation of the PDK-dependent phosphorylation and PDP-dependent dephosphorylation of PDH. Phosphorylation occurs at Serines 232, 293, and 300 of the human $E_1\alpha$ subunit. Phosphorylation results in inactivation of PDH, while dephosphorylation results in activation.

MitoSciences offers a comprehensive line of PDH-related assays and tools, including all four PDH kinases, both PDH phosphatases, PDH activity microplate assays and PDH protein quantity microplate assays. For convenience, these tools are available combined in kits and described in additional protocols.

PDH Protocol #2 – Measurement of Endogenous PDH-$E_1\alpha$ Phospho-Serine^{293} Levels

See the MitoSciences PDH Playbook for an overview of applications possible using MitoSciences PDH assays and tools. This protocol (MitoSciences PDH Protocol #2) describes how to measure endogenous levels of phosphorylated Serine^{293} of the PDH-$E_1\alpha$ subunit corresponding to the enzyme's phosphorylation state at the time of sample collection. A complementary protocol (PDH Protocol #1) describes how to measure endogenous levels of PDH activity. The two protocols (PDH activity and phosphorylation-based) can be performed in parallel for a comprehensive analysis of PDH functional and phosphorylation status at the time of sample collection.

MATERIALS

Most of the components required in this protocol are provided in the PDH Enzyme Activity Microplate Assay Kit (MitoSciences Cat. #MSP18). The recipes for the components not included in MSP18 are provided in Appendix A of this protocol. **Note – When using this protocol with MSP18, it REPLACES the standard MSP18 protocol.**

Item	Quantity	Storage
Detergent (component of MSP18)	2 x 1 mL	4°C
20X Buffer (component of MSP18)	15 mL	4°C
96-well Pre-coated Microplate (component of MSP18)	1 EA	4°C
100X ATP Depletion System	65 µL	-80°C
Phosphatase Inhibitor (PI)	1.5 mL	4°C
5X Stabilizer	13 mL	4°C
PDH Kinase 3 – PDK3 (MSP43)	50 µg	-80°C
50X ATP	0.2 mL	-20°C
PDH Phosphatase 1 – PDP1 (MSP45)	50 µg	-80°C
200X Calcium	0.1 mL	4°C
20X Wash Buffer	20 mL	4°C
10X Blocking Solution	5 mL	4°C
PhosphoDetect™ Anti-PDH-$E_1\alpha$ (pSer^{293}) Rabbit pAb (EMD Chemicals, catalog #AP1062)	50 µg	-20°C
20X HRP Label	1 mL	4°C
Development Solution	20 mL	4°C

Avoid repeated freeze/thaw cycles of the frozen components and keep them on ice when not in storage.

ADDITIONAL MATERIALS REQUIRED

- Spectrophotometer plate reader (Molecular Dynamics SpectraMax recommended) capable of measuring absorbance at 650 (or 600) nm, preferably in a kinetic mode.
- Method for determining protein concentration
- Multichannel pipette
- Deionized water
- PBS (phosphate buffered saline) – for recipe see Appendix A

ASSAY PROTOCOL

This protocol has four steps:

A. Sample Preparation.

PDH-containing extracts are prepared in the presence of phosphatase inhibitor and ATP-depleting system to prevent unwanted modifications of PDH during the sample preparation.

B. PDH Immunocapture.

PDH in each sample of interest is immunocaptured to three wells in parallel.

C. Modification Reactions.

One well is left untreated to determine endogenous phospho-Serine^{293} levels, the second well is treated with PDK3 to determine the maximum (fully phosphorylated) phospho-Serine 293 levels and the third well is treated with PDP1 to determine the background (fully dephosphorylated) phospho-Serine^{293} levels.

D. Quantity Measurement.

Below are recommended amounts of materials. These amounts were carefully chosen to fit within the linear range of phospho-Serine^{293} signal (Figure 7).

Sample type	Recommended amount
Purified mitochondria	50 µg/well (0.25 mg/mL)
Tissue homogenates	125 µg/well (0.625 mg/mL)
Cultured cells	500 µg/well (2.50 mg/mL)

PDK3 and PDP1 are used to perform, respectively, 32 phosphorylation and 32 dephosphorylation reactions, each able to fully phosphorylate (and fully inhibit) and fully dephosphorylate (and fully activate) PDH immunocaptured using the recommended amounts of materials (50 µg of bovine heart mitochondria, corresponding to 1 milliunit of porcine PDH, Sigma P7032, 125 µg of rat or mouse heart tissues, or 500 µg of HepG2 cells).

Since each sample is analyzed for endogenous, background and maximum PDH activity in three separate wells, the whole kit can be used for complete analysis of 32 samples in the 96 wells provided. The immunocapture microplate can be broken into 12 separate 8-well strips. For convenience, considering the above, the volumes in this protocol are given for analysis of multiples of three strips (24 wells) allowing complete analysis of 8 samples.

PDH Protocol #2 – Measurement of Endogenous PDH-$E_1\alpha$ Phospho-Serine^{293} Levels

Note – Be completely familiar with protocol and protocol notes before beginning the assay. Do not deviate from the specified protocol steps or optimal results may not be obtained.

A. Sample Preparation

NOTE *– It is critical to inhibit the endogenous PDH phosphatases and kinases during sample preparation and immunocapture to obtain values of the PDH activity of the unmodified sample that correspond to the endogenous PDH activity. Therefore, an inhibitor of endogenous phosphatases as well as a system that depletes ATP (which inhibits the kinase activity) is included in the extraction buffer. For the same reason the phosphatase inhibitor is also included in the immunocapture buffer. It is also recommended that the inhibitors are added to samples immediately upon homogenization, prior to detergent extraction.*

1. Determine the sample protein concentration using a standard method such as BCA method (Pierce).

2. Adjust the protein concentration of the sample, according to the sample type used, by dilution in PBS, as specified below. *See Note 1 for addition of protease inhibitors.*

Sample type	Protein concentration (mg/mL)
Purified mitochondria	5.5
Tissue homogenates	25.0
Cultured cells	15.9

3. Prepare the extraction according to the table below. Mix components immediately. *It is imperative to keep the ratio of components of extraction as specified below. Below are suggested volumes for preparation of sufficient amounts for loading of six wells, i.e. for the analysis of a single sample for endogenous phosphorylation, dephosphorylation and full phosphorylation in duplicate. See Note 2 and 3.*

Component	Purified mitochondria	Tissue homogenates	Cultured cells
Sample (µL)	90	45.0	297.5
100X ATP Depleting System (µL)	1	0.5	3.5
Phosphatase Inhibitor (PI) (µL)	4	2.0	14.0
Detergent (µL)	5	2.5	35.0
Total Volume (µL)	100	50	350
Final Protein Concentration (mg/mL)	5.0	22.5	13.5

4. Incubate on ice for 10 minutes.

5. Centrifuge in a tabletop centrifuge for 10 minutes at 4°C as specified below. Carefully collect and save the supernatant. Discard the pellet.

Sample type	RCF (x g)
Purified mitochondria	5,000
Tissue homogenates	1,000
Cultured cells	1,000

6. Prepare the Dilution Buffer as specified below. *See Note 3.*

No. of Plate Strips	dH_2O (mL)	20X Buffer (mL)	Phosphatase Inhibitor (PI) (mL)	Total (mL)
3	5.5	0.3	0.24	6
6	10.9	0.6	0.48	12
9	16.4	0.9	0.72	18
12	21.8	1.2	0.96	24

7. Dilute the samples' supernatants in the Dilution Buffer to recommended concentration according to the table below.

Sample type	Recommended concentration (mg/mL)
Purified mitochondria	0.25 (20X dilution)
Tissue homogenates	0.625 (36X dilution)
Cultured cells	2.50 (5.4X dilution)

B. PDH Immunocapture

1. Plate loading. Add 200 µL of diluted samples prepared in Step 7 of Section A. *It is recommended to load six wells with each sample to obtain duplicate values of PDH activity of unmodified, phosphorylated and dephosphorylated enzyme. For suggested sample loading layout, see Step 10 of Section C.*
2. Incubate microplate for 2.5 hours at room temperature.

PDH Protocol #2 – Measurement of Endogenous PDH-$E_1\alpha$ Phospho-Serine^{293} Levels

C. Modification Reactions

1. Prepare 1X Reaction/Wash Buffer according to the table below.

1X Reaction/Wash Buffer				
No. of Plate Strips	dH_2O (mL)	20X Buffer (mL)	5X Stabilizer (mL)	Total (mL)
3	28.1	1.88	7.5	37.5
6	56.2	3.75	15.0	75.0
9	84.4	5.63	22.5	112.5
12	112.5	7.50	30.0	150.0

2. Prepare 2X ATP Solution. The 2X ATP Solution is needed only for one third of wells. Prepare only the amount needed.

2X ATP Solution					
No. of Plate Strips	dH_2O (mL)	20X Buffer (µL)	5X Stabilizer (µL)	50X ATP (µL)	Total (mL)
1	0.71	50	200	40	1.00
2	1.42	100	400	80	2.00
3	2.13	150	600	120	3.00
4	2.84	200	800	160	4.00

3. Prepare 2X Calcium Solution according to the table below. The 2X Calcium Solution is needed only for one third of wells.

2X Calcium Solution			
No. of Plate Strips	1X Reaction/Wash Buffer (mL)	200X Calcium (µL)	Total (mL)
1	0.99	10	1.00
2	1.98	20	2.00
3	2.97	30	3.00
4	3.96	40	4.00

PDH Protocol #2 – Measurement of Endogenous PDH-$E_1\alpha$ Phospho-Serine^{293} Levels

4. Prepare the PDK3 Working Stock according to the table below at 1 µg/mL. The PDK3 Working Stock is needed only for one third of wells. Prepare only the amount needed.

PDK3 Working Stock			
No. of Plate Strips	1X Reaction/Wash Buffer (mL)	Enzyme (µL)	Total (mL)
1	0.84	5.7	0.85
2	1.69	11.3	1.70
3	2.53	17.0	2.55
4	3.38	22.7	3.40

5. Prepare the PDP1 Working Stock according to the table below at 20 µg/ mL. The PDP1 Working Stock is needed only for one third of wells. Prepare only the amount needed.

PDP1 Working Stock			
No. of Plate Strips	1X Reaction/Wash Buffer (mL)	Enzyme (µL)	Total (mL)
1	0.84	11.33	0.85
2	1.68	22.67	1.70
3	2.52	34.00	2.55
4	3.36	45.33	3.40

6. Empty the wells of the microplate by turning it over and shaking out any remaining liquid. Blot the plate face down on paper towel.

7. Add 300 µL/well of 1X Reaction/Wash Buffer to wash the wells.

8. Repeat steps 6. and 7. two more times for a total of 3 wash steps.

9. Again, empty the wells of the microplate by turning it over and shaking out any remaining liquid. Blot the plate face down on paper towel.

10. Add promptly 200 µL/well of 1X Reaction/Wash Buffer to the first set of wells, 100 µL/well of PDK Working Stock and 100 µL/well of 2X ATP Solution to the second set of wells, and 100 µL/well of PDP1 Working Stock and 100 µL/well of 2X Calcium Solution to the third set of wells, as shown.

PDH Protocol #2 – Measurement of Endogenous PDH-$E_1\alpha$ Phospho-Serine^{293} Levels

	1X Reaction/Wash Buffer (200 µL/well)				PDK3 Working Stock (100 µL/well) 2X ATP Solution (100 µL/well)				PDP1 Working Stock (100 µL/well) 2X Calcium Solution (100 µL/well)			
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	1	1	9	9	1	1	9	9	1	1	9	9
B	2	2	10	10	2	2	10	10	2	2	10	10
C	3	3	11	11	3	3	11	11	3	3	11	11
D	4	4	12	12	4	4	12	12	4	4	12	12
E	5	5	13	13	5	5	13	13	5	5	13	13
F	6	6	14	14	6	6	14	14	6	6	14	14
G	7	7	15	15	7	7	15	15	7	7	15	15
H	8	8	16	16	8	8	16	16	8	8	16	16

Recommended Microplate Layout for samples numbered 1-16, measured in duplicates for endogenous (purple), dephosphorylated (blue) and phosphorylated (red) levels of phospho-Serine^{293}. To make these modifications after the immunocapture, the first set of wells (columns 1-4) is left untreated, the second set of wells (columns 5-8) is treated with PDK3 in the presence of ATP, and the third set of wells (columns 9-12) is treated with PDP1 in the presence of calcium.

11. Incubate the plate for 10 min at 30°C.

D. Quantity Measurement

1. Prepare 1X Wash Buffer by adding 15 mL of 20X Wash Buffer to 185 mL of deionized water.

2. Prepare 1X Blocking Buffer according to the table below. Prepare only the amount needed.

1X Blocking Buffer			
No. of Plate Strips	1X Wash Buffer (mL)	10X Blocking Solution (mL)	Total Volume (mL)
3	10.8	1.2	12.00
6	21.6	2.4	24.00
9	32.4	3.6	36.00
12	43.2	4.8	48.00

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3. Prepare 1X Phospho-PDH Serine^{293} Detector Antibody according to the table below. Prepare only the amount needed.

1X Phospho-PDH Serine^{293} Detector Antibody			
No. of Plate Strips	1X Blocking Buffer (mL)	20X Phospho-PDH Ser^{293} Detector Antibody (mL)	Total Volume(mL)
3	5	0.25	5.25
6	10	0.50	10.50
9	15	0.75	15.75
12	20	1.00	21.00

4. Empty the wells of the microplate by turning it over and shaking out any remaining liquid. Blot the plate face down on paper towel.

5. Add 300 µL/well of 1X Wash Buffer to wash the wells.

6. Again, empty the wells of the microplate by turning it over and shaking out any remaining liquid. Blot the plate face down on paper towel.

7. Add to each well 200 µL of 1X Phospho-PDH Serine^{293} Detector Antibody.

8. Incubate the plate for 1 hour at room temperature.

9. Prepare 1X HRP Label according to the table below. Prepare only the amount needed.

1X HRP Label			
No. of Plate Strips	1X Blocking Buffer (mL)	20X HRP Label (mL)	Total Volume (mL)
3	5	0.25	5.25
6	10	0.50	10.50
9	15	0.75	15.75
12	20	1.00	21.00

10. Empty the wells of the microplate by turning it over and shaking out any remaining liquid. Blot the plate face down on paper towel.

11. Add 300 µL/well of 1X Wash Buffer to wash the wells.

12. Again, empty the wells of the microplate by turning it over and shaking out any remaining liquid. Blot the plate face down on paper towel.

13. Add to each well 200 µL of 1X HRP Label.

14. Incubate the plate for 1 hour at room temperature.

15. Empty the wells of the microplate by turning it over and shaking out any remaining liquid. Blot the plate face down on paper towel.

16. Add 300 µL/well of 1X Wash Buffer to wash the wells.

17. Repeat steps 15. and 16. three more times for a total of 4 wash steps.

18. Again, empty the wells of the microplate by turning it over and shaking out any remaining liquid. Blot the plate face down on paper towel.

19. Add 200 µL/well of Development Solution. Avoid bubbles. Any bubbles in the wells should be popped with a fine needle as rapidly as possible.

20. Promptly place the plate into a spectrophotometer and begin reading using the following parameters. (Alternatively, an endpoint measurement can be made by stopping the reaction at a user defined time by addition of 100 µL/well of 1 N HCl and recording absorbance at 450 nm.)

Mode:	Kinetic
Wavelength:	650 nm
Time:	30 min
Interval:	20 sec - 1 min
Shaking:	Shake between readings

21. Save data and analyze them as described in the Data Analysis section.

PROTOCOL NOTES

1. If desired, samples can be supplemented with protease inhibitors, such as Protease Inhibitor Cocktail (Sigma P8340) to minimize nonspecific proteolysis during the sample preparation.

2. A control sample of bovine heart mitochondria at protein concentration 5.5 mg/mL is available for purchase as MS802.

3. It is critical to inhibit the endogenous PDH phosphatases and kinases during sample preparation and immunocapture to obtain values of the PDH activity of the unmodified sample that correspond to the endogenous PDH activity. Therefore, an inhibitor of endogenous phosphatases as well as a system that depletes ATP (which inhibits the kinase activity) is included in the extraction buffer. For the same reason the phosphatase inhibitor is also included in the immunocapture buffer. It is also recommended that the inhibitors are added to samples immediately upon homogenization, prior to detergent extraction.

4. This protocol describes determination of the extent of phosphorylation of Serine^{293}. The PDH activity can be determined in parallel with the use of MSP18 following the protocol *Measuring Endogenous Activity of PDH Relative to its Fully-Phosphorylated and Fully-De-Phosphorylated States*. However, be aware that not every change of PDH activity must be due to the changes in the PDH $E_1\alpha$ phospho-Serine levels. Oxidative damage of PDH is also an important factor.

DATA ANALYSIS

1. The endogenous PDH $E_1\alpha$ phospho-Serine^{293} level is equal to the phospho-Serine^{293} signal of the unmodified sample. The endogenous phospho-Serine^{293} level of a condition/treatment can be compared to a control/no treatment.

2. The endogenous PDH $E_1\alpha$ phospho-Serine^{293} level that reflects the activity due to intrinsic enzyme phosphorylation levels, expressed as a percentage of the phospho-Serine^{293} signal of the fully dephosphorylated enzyme, can be determined as follows:

   $$\text{P-Ser (\%)} = 100 \times (\text{P-Ser}^{\text{unmodified sample}} - \text{P-Ser}^{\text{PDK3-treated sample}}) / (\text{P-Ser}^{\text{PDP1-treated sample}} - \text{P-Ser}^{\text{PDK3-treated sample}})$$

   Abbreviation: P-Ser = Phospho-Serine^{293} quantity

   See Note 4.

3. If PDH amount under various conditions/treatment is of concern, we recommend measuring the amount of PDH in each sample with the use of MSP19 and expressing the activity data as (relative) specific activities. Alternatively, the phospho-Serine^{293} signal of the fully phosphorylated PDH $E_1\alpha$, obtained as discussed above can be used to determine relative amounts of immunocaptured PDH which also allows the activity data to be expressed as (relative) specific activities.

PDH Protocol #2 – Measurement of Endogenous PDH-$E_1\alpha$ Phospho-Serine^{293} Levels

FLOW CHART

For quick reference only. Be completely familiar with previous details of this document before performing the assay.

Sample Preparation (1-3 hours)

- Dilute the sample to appropriate concentration with PBS
- Assembly the extraction
- Incubate samples 10 min on ice
- Centrifuge samples at appropriate g for 10 min
- Prepare Dilution Buffer
- Dilute the supernatant in the Dilution Buffer to appropriate concentration

↓

PDH Immunocapture (2.5 hours)

- Load 200 µL of the diluted sample per well at least in triplicates
- Incubate the plate for 2.5 hours at room temperature

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Modification Reactions (20 minutes)

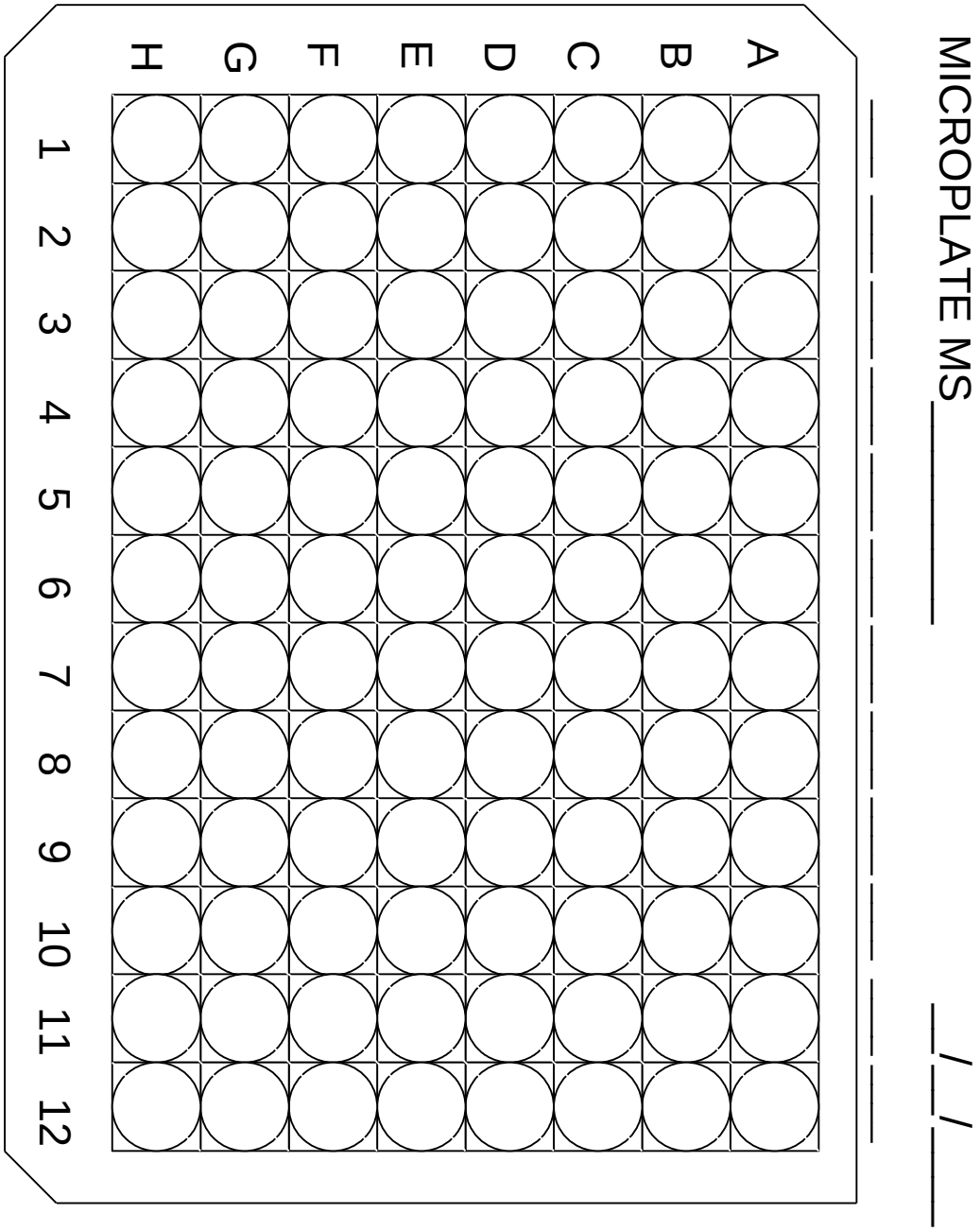
- Prepare 1X Reaction/Wash Buffer
- Prepare 2X ATP Solution
- Prepare 2X Calcium Solution
- Prepare PDK3 Working Stock
- Prepare PDP1 Working Stock
- Wash wells trice with 1X Reaction/Wash Buffer
- Add to the first set of wells 200 µL 1X Reaction/Wash Buffer
- Add to the second set of wells 100 µL of PDK3 Working Stock and 100 µL of 2X ATP Solution
- Add to the third set of wells 100 µL of PDP1 Working Stock and 100 µL of 2X Calcium Solution
- Incubate the plate for 10 min at 30°C

↓

Quantity Measurement (2.5 hours)

- Prepare 1X Wash Buffer
- Prepare 1X Blocking Buffer
- Prepare 1X 293 Detector Antibody
- Wash wells once with 1X Wash Buffer
- Add 200 µL/well of 1X Detector Antibody
- Incubate plate 1 hour at room temperature
- Prepare 1X HRP Label
- Wash wells once with 1X Wash Buffer
- Add 200 µL/well of 1X HRP Label
- Incubate plate 1 hour at room temperature
- Wash wells four times with 1X Wash Buffer
- Add 200 µL/well of Development Solution
- Measure OD_{650} at 20 sec - 1 min intervals for 30 minutes

PDH Protocol #2 – Measurement of Endogenous PDH-$E_1\alpha$ Phospho-Serine^{293} Levels



APPENDIX A. Recipes for components not included in MSP18

Phosphatase Inhibitor (PI)

- NaF (EM Science SX-0550-3), FW 41.99
- Deionized Water

Component	Grams per 100 mL	Concentration (M)
NaF	2.10	0.5

Dissolve NaF in deionized water. Adjust volume to 100 mL. Store at -20°C.

100X ATP Depletion System

- Apyrase, from potato (Sigma A6410)
- Deionized Water
- Glycerol (Sigma G8773), FW 92.09

Component	units per 0.5 mL	Concentration (units/mL)
Apyrase, from potato	200	400

Add to the vial with lyophilized Apyrase (200 units) 0.25 mL of deionized water. Dissolve the Apyrase by mixing on a rotator. Add 0.25 g of Glycerol. Mix on a rotator until dissolved. Store at -20°C.

5X Stabilizer

- Albumin, from bovine serum (Sigma A4503)
- Deionized water

Component	Grams per Liter	Concentration (mg/mL)
Albumin, from bovine serum	50	50

Dissolve Albumin in water. Adjust volume to 1000 mL. Filter Sterilize and store at 4°C.

PDK3

- PDK3, MitoSciences MSP43. Note – PDK3 is at 0.5 µg/µL and must be diluted to make the component PDK3 at 0.15 µg/µL.
- Dilution Buffer (10 mM $NaPO_4$, 250 mM NaCl, 30% glycerol, 5 mM DTT, 0.5 mM EDTA, 0.05% Triton X-100, pH 7.5 with HCl)

Component	Grams per mL	Concentration (µg/µL)
PDK3	NA	0.15

Avoid repeated freeze/thaw cycles of the enzyme and keep it on ice when not in storage. Thaw on ice the stock of PDK3. Equilibrate the Dilution Buffer to 4°C. Dilute PDK3 in the Dilution Buffer to the desired concentration. Aliquot to pre-chilled tubes. Store at -80°C.

PDH Protocol #2 – Measurement of Endogenous PDH-$E_1\alpha$ Phospho-Serine^{293} Levels

50X ATP

- Adenosine 5'-triphosphate, disodium salt (Sigma A2383), FW 551.1
- Deionized water (dH_2O)

Component	Grams per 10 mL	Concentration (mM)
Adenosine 5'-triphosphate	0.551	100

Dissolve Adenosine 5'-triphosphate in dH_2O water. Adjust volume to 10 mL. Store aliquoted at -20°C.

PDP1

- PDP1, MitoSciences MSP45. Note – This stock PDP1 is at 1.5 µg/µL and can be used as is for the component PDP1 at 1.5 µg/µL.
- Dilution Buffer (10 mM Na-PO_4, 250 mM NaCl, 30% glycerol, 5 mM DTT, 0.5 mM EDTA, 0.05% Triton X-100, pH 7.5 with HCl)

Component	Grams per mL	Concentration (µg/µL)
PDP1	NA	1.5

Avoid repeated freeze/thaw cycles of the enzyme and keep it on ice when not in storage. Store at -80°C.

200X Calcium

- $CaCl_2 \cdot 2H_2O$ (Mallinckrodt 4160), FW 147.02
- deionized water

Component	Grams per 100 mL	Concentration (mM)
$CaCl_2$	1.18	80

Dissolve $CaCl_2$ in deionized water. Adjust volume to 100 mL. Store at -20°C.

20X Wash Buffer

- Trizma Base (Sigma T6066), FW 121.14
- $MgCl_2$.$6H_2O$ (VWR 1483-01), FW 203.31
- NaF (EM Science SX-0550-3), FW 41.99
- n-Dodecyl-ß-D-Maltopyranoside (Anatrace D310S), FW 510.6

Component	Grams per Liter	Concentration
Trizma Base	121.14	1 M
$MgCl_2$	4.07	20 mM
NaF	8.4	200 mM
n-Dodecyl-ß-D-Maltopyranoside	3.00	0.3 %

Dissolve components in deionized water. Adjust pH with HCl to 7.5. Adjust volume to 1 L.
Store at -20°C.

PDH Protocol #2 – Measurement of Endogenous PDH-$E_1\alpha$ Phospho-Serine^{293} Levels

10X Blocking Solution		
• 10X Blocking Buffer (Sigma B6429)		
Component	Grams per L	Concentration (%)
10X Blocking Buffer	NA	100
Store at RT.		

20X Phospho-PDH Ser^{293} Detector Antibody		
• Phospho-PDH Serine^{293} Polyclonal Antibody (EMD Chemicals, catalog #AP1062. Note – This concentrated stock must be diluted to make the 20X component) • Antibody Diluent (2 mg/mL BSA, 50 mM Tris-HCl pH 7.5, 0.015% n-Dodecyl-ß-D-Maltopyranoside)		
Component	Grams per L	20X Concentration (µg/mL)
Phospho-PDH Serine^{293} rabbit Polyclonal Antibody	NA	10
Dilute the antibody in the Antibody Diluent. Store at 4°C.		

20X HRP Label		
• Goat-Anti-Rabbit-IgG (H+L)-HRP (Southern Biotech 4050-05) • Guardian Peroxidase Conjugate Stabilizer/Diluent, (Thermo Fisher 37548)		
Component	Grams per Liter	Concentration (µg/mL)
Goat-Anti-Rabbit-IgG (H+L)-HRP	NA	10
Dilute the label in Guardian Peroxidase Conjugate Stabilizer/Diluent. Store at 4°C.		

Development Solution		
• Sure Blue TMB Microwell Peroxidase Substrate (KPL 52-00-03)		
Component	Grams per L	Concentration (%)
Sure Blue	NA	100
Store at 4°C.		

PDH Protocol #2 – Measurement of Endogenous PDH-$E_1\alpha$ Phospho-Serine^{293} Levels

PBS (Phosphate Buffered Saline)
• KH_2PO_4, Potassium Phosphate Monobasic (Sigma P0662), FW 136.09 • $Na_2HPO_4 \cdot 7H_2O$, Sodium Phosphate Dibasic Heptahydrate (Sigma S9390), FW 268.07 • KCl, Potassium Chloride (Sigma P3111), FW 74.56 • dH_2O • NaCl, Sodium chloride (Sigma S3014), FW 58.44
For 1 L PBS
0.2 g KH_2PO_4 (1.4 mM) 2.2 g Na_2HPO_4 (8 mM) 0.2 g KCl (2.7 mM) 8.2 g NaCl (140 mM)
Add dry components to 0.9 L H_2O. The pH value should be 7.3, adjust if necessary. Add H_2O to 1 L.

SUPPORTING DATA

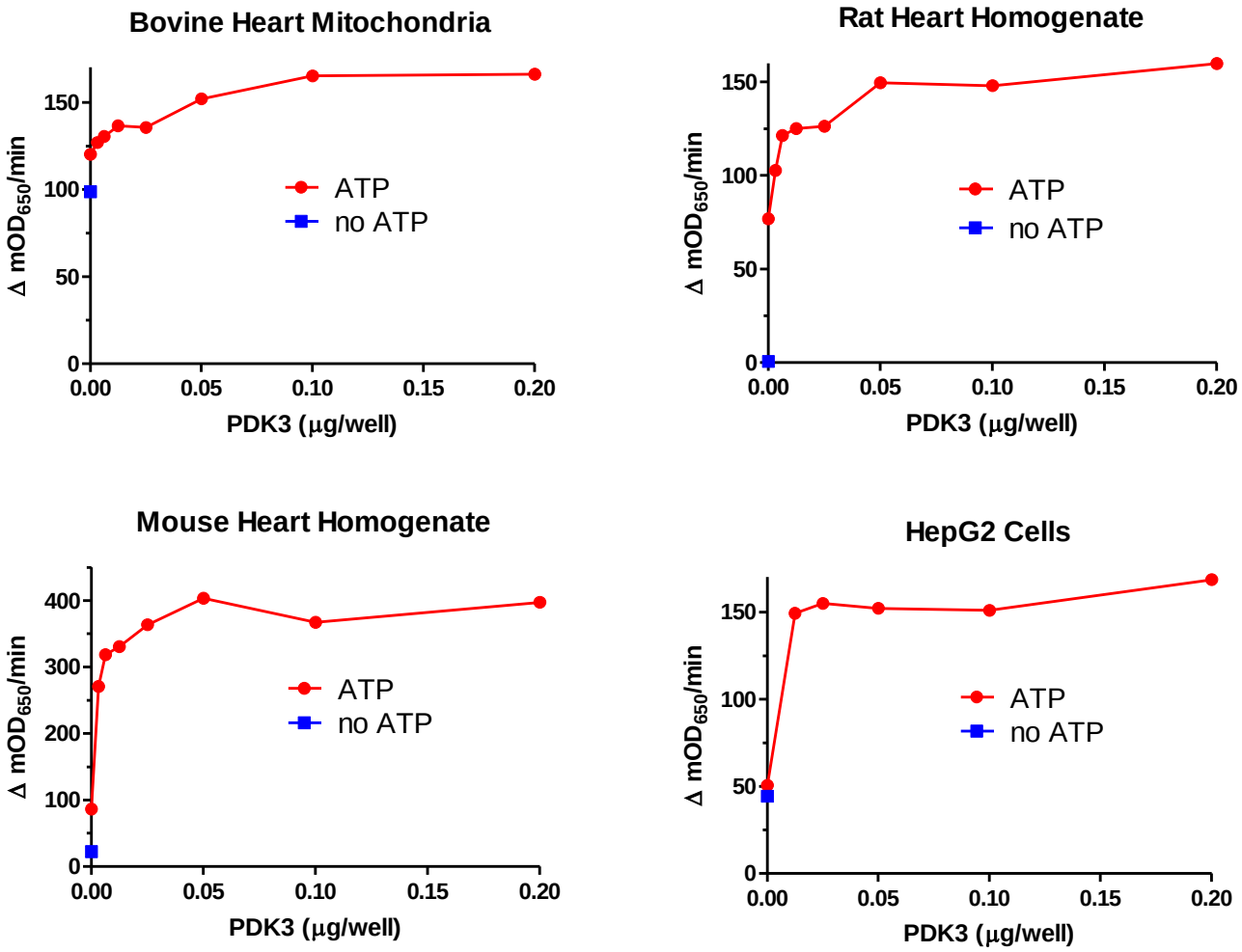


Figure 3. PDK3 fully phosphorylates immunocaptured bovine rat, mouse and human PDH at Serine^{293} of the $E_1\alpha$ subunit. PDH immunocaptured from 50 µg of bovine heart mitochondria, 125 µg of rat heart homogenate, 125 µg of mouse heart homogenate, or 500 µg of HepG2 cells was treated with indicated amounts of recombinant PDK3 in the presence of ATP (in red) or in the absence of ATP (in blue), and PDH $E_1\alpha$ Phospho-Serine^{293} was measured with the use of Phospho-PDH Serine^{293} detector antibody (EMD Chemicals, catalog #AP1062). Note that 0.1 µg of PDK3 is sufficient to fully phosphorylate the Serine^{293} of PDH immunocaptured from variety of materials tested. In addition, note that the treatment with ATP alone (in the absence of PDK), when compared to the treatment in the absence of ATP, does not substantially increase the PDH $E_1\alpha$ Phospho-Serine^{293} signal, indicating absence of endogenous kinases.

PDH Protocol #2 – Measurement of Endogenous PDH-$E_1\alpha$ Phospho-Serine^{293} Levels

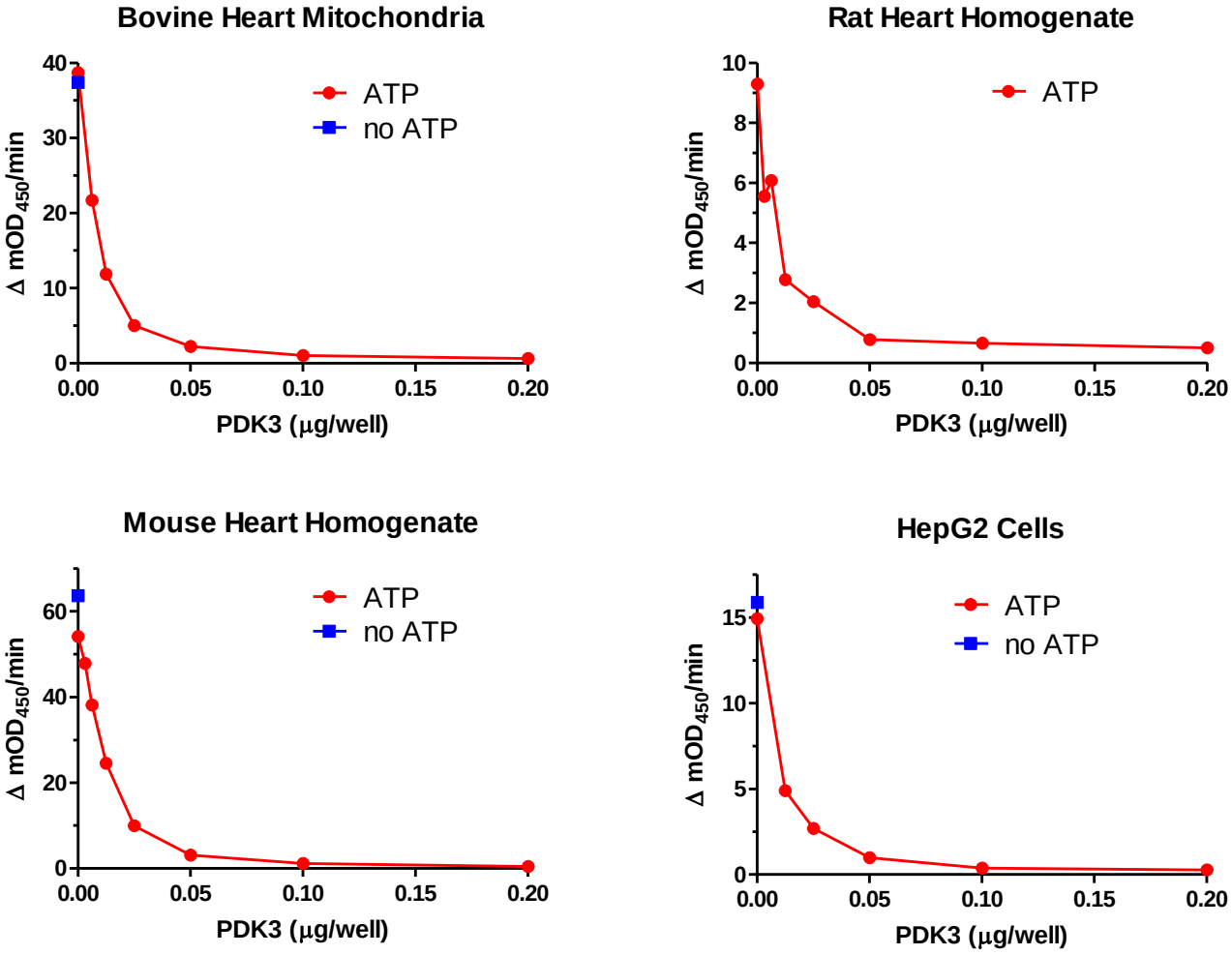


Figure 4. PDK3 fully inactivates immunocaptured bovine rat, mouse and human PDH. PDH immunocaptured from 50 µg of bovine heart mitochondria, 125 µg of rat heart homogenate, 125 µg of mouse heart homogenate, or 500 µg of HepG2 cells was treated with indicated amounts of recombinant PDK3 in the presence of ATP (in red) or in the absence of ATP (in blue), and PDH activity was measured with the use of MSP18. Note that 0.1 µg of PDK3 is sufficient to fully inactivate PDH immunocaptured from variety of materials tested. In addition, note that the treatment with ATP alone (in the absence of PDK3) when compared to the treatment in the absence of ATP does not substantially reduce the PDH activity, indicating absence of endogenous kinases.

PDH Protocol #2 – Measurement of Endogenous PDH-$E_1\alpha$ Phospho-Serine^{293} Levels

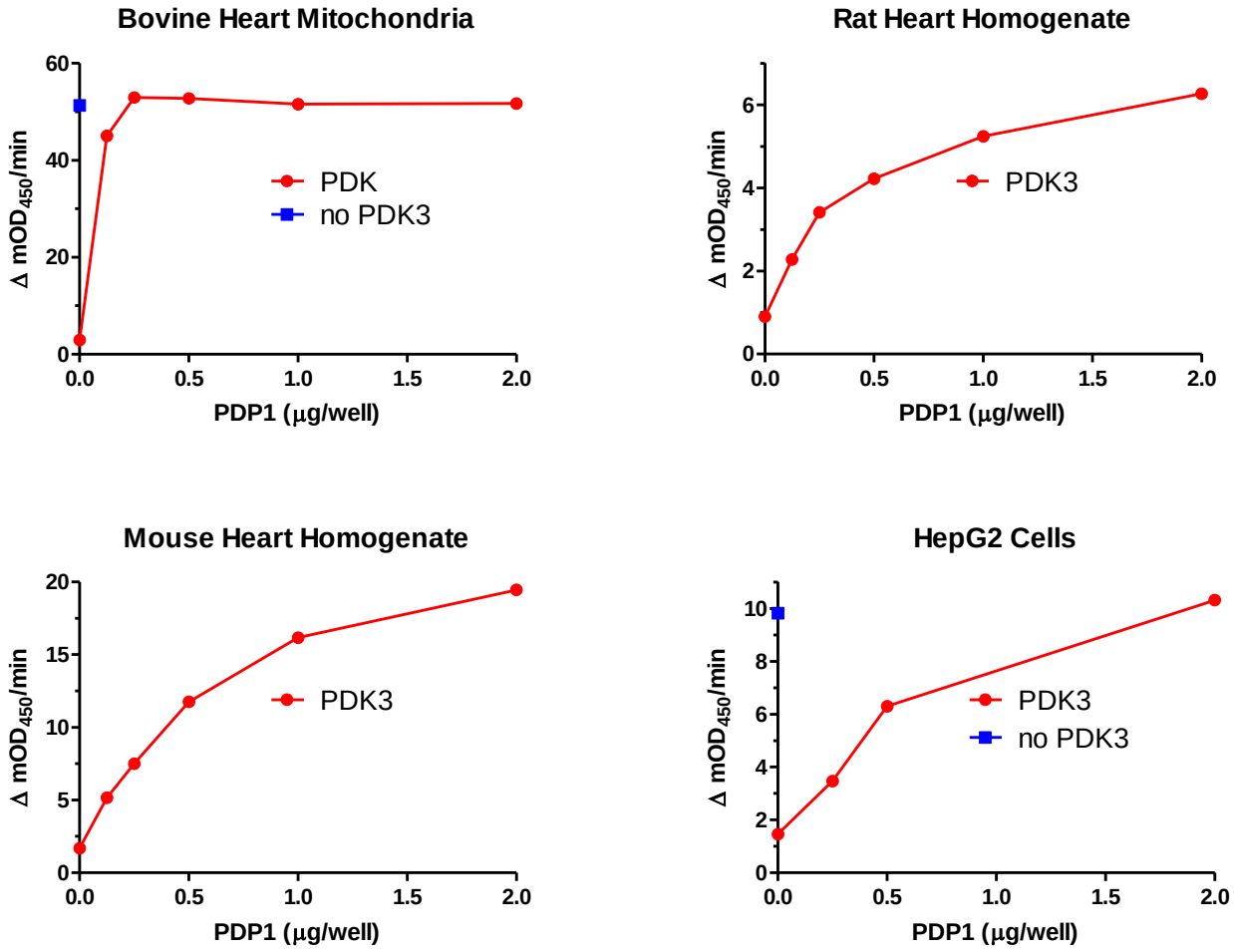


Figure 5. PDP1 activates PDK3-phosphorylated immunocaptured bovine rat, mouse and human PDH. PDH was immunocaptured from 50 µg of bovine heart mitochondria, 250 µg of rat heart homogenate, 125 µg of mouse heart homogenate, or 500 µg of HepG2 cells and fully phosphorylated with 0.1 µg of recombinant human PDK3 (in red) or mock-phosphorylated (no PDK3, in blue). The samples were then treated with the indicated amounts of PDP1 in the presence of Ca^{2+}, and PDH activity was measured with the use of MSP18. Note that 2 µg of PDP1 are sufficient to fully re-activate the PDK3-phosphorylated PDH immunocaptured from variety of materials tested. In addition, note that the treatment of PDK3-phosphorylated PDH with Ca^{2+} alone (in the absence of PDP1) does not substantially increase the PDH activity, indicating absence of endogenous phosphatases (data not shown).

PDH Protocol #2 – Measurement of Endogenous PDH-$E_1\alpha$ Phospho-Serine^{293} Levels

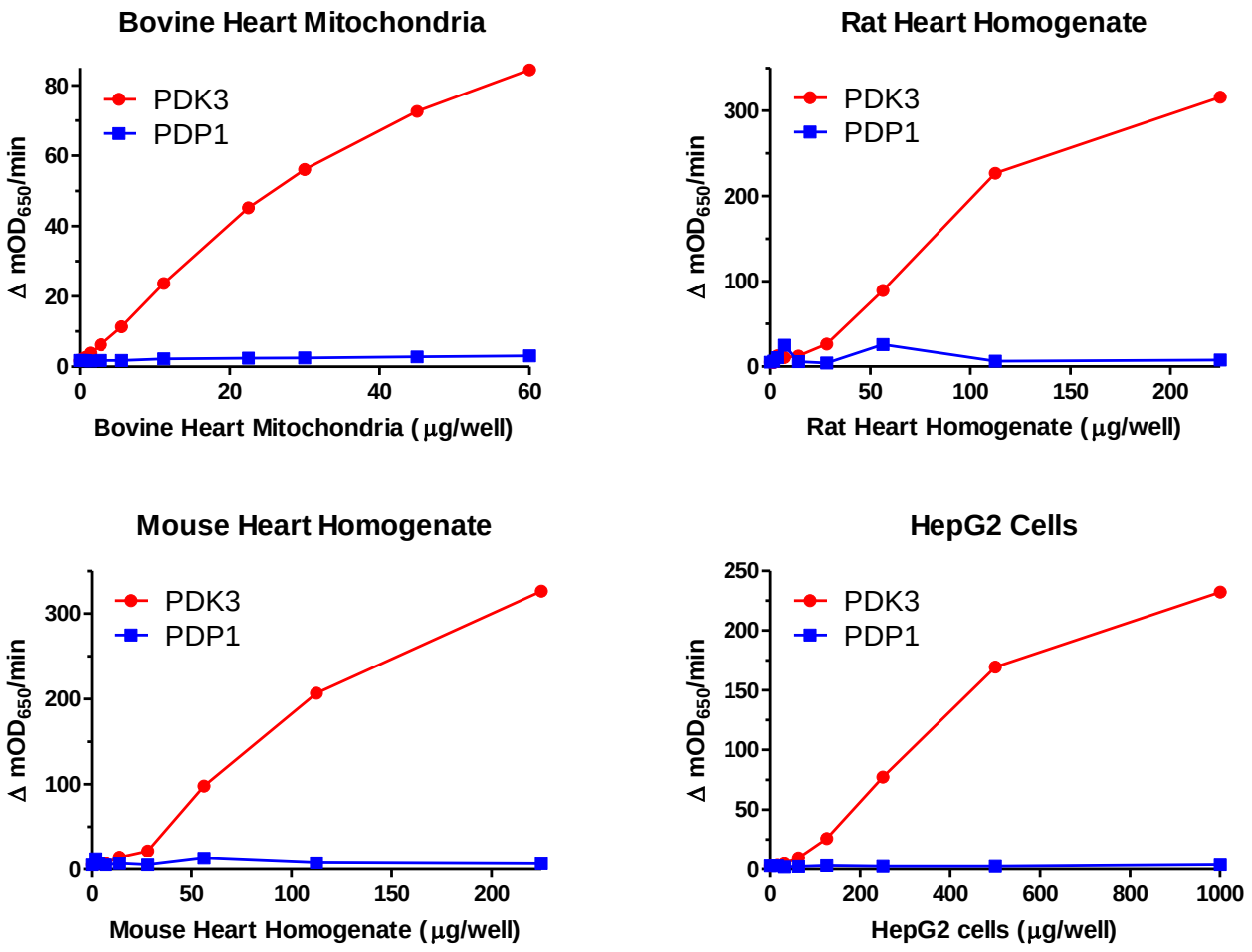


Figure 6. Determination of linear range of phospho-Serine^{293} signal of PDK3-phosphorylated bovine, rat, mouse and human PDH. Extracts of bovine heart mitochondria, rat heart homogenate, mouse heart homogenate and human HepG2 cells were fully phosphorylated with PDK3 or fully dephosphorylated with PDP1, and PDH from indicated amount of materials was immunocaptured and quantity of phospho-Serine^{293} were determined using Phospho-PDH Serine^{293} detector antibody (EMD Chemicals cat. #AP1062) as described in this protocol.