

## PDH Kinase Inhibitor Screening Based on PDH Activity

Rev.3

### INTRODUCTION

This protocol describes high throughput screens for inhibitors (or activators) of pyruvate dehydrogenase kinase (PDK) isoforms PDK1, PDK2, PDK3 or PDK4 by measuring the activity of PDH after exposure of immunocaptured PDH to each kinase in the presence and absence of test compounds. Each set of measurements can be performed on 96 samples.

Although a variety of generic approaches can be used to measure protein kinase activity, the protocol described here is unique as it allows one to screen rapidly for inhibitors/activators of each of the 4 isoforms of PDK by monitoring the functional effects of PDK phosphorylation on the activity of PDH purified from any tissue or cell type of interest, including whole tissue/cell extracts of human, bovine, rat or mouse cells, tissues or mitochondria. The PDH of interest is first quickly purified and immobilized in microplate arrays by microscale immunocapture and then exposed to the PDK of choice in the presence or absence of PDK inhibitors/activators. Residual PDH activity in each microwell is then measured to determine whether or not test compounds inhibited, activated, or had no effect on PDK phosphorylation of PDH. Unaltered PDKs phosphorylate PDH at a defined rate, resulting in quantitative inactivation of PDH activity. In contrast, drug-induced inhibition of PDK activity blocks PDK-mediated inhibition of PDH activity. Thus, compounds of interest can be added to the PDK phosphorylation reaction and their effect on PDH phosphorylation can be analyzed, making this product an ideal screening tool for inhibitors or activators of PDKs. Optimized conditions of the PDK-mediated phosphorylation reactions are described that result in fully phosphorylated one (or more) of PDH E<sub>1</sub>α Serines (232, 293 and 300), and fully inactivated PDH (Figures 2, 3, 4 and 5) when unaltered PDK is used.

The main advantage of this protocol is that PDH is purified by immunocapture in individual microwells and the endogenous specific and non-specific kinases originally associated with the enzyme are washed away. Therefore, the subsequent PDK-mediated phosphorylation reaction is dependent only on the activity of exogenously added PDK. Further advantages are as follows: (1) a variety of PDH sources can be used and there is no need to biochemically purify PDH laboriously or to depend on a commercial enzyme, (2) the phosphorylation reaction is stopped rapidly by a simple wash, (3) the wash removes reactants of the phosphorylation reaction that would otherwise interfere with the PDH activity assay, (4) the wash also removes traces of test compounds (potential PDK inhibitors/activators) that might otherwise interfere directly with the PDH activity assay. Finally, if desired, the immunocaptured PDH can first be fully dephosphorylated after immunocapture, but prior to exposure to PDKs to maximize the PDK effect. Dephosphorylation can be accomplished with the use of specific recombinant PDH phosphatases (PDP1 or PDP2) available from MitoSciences (MSP45 and MSP46, respectively), and then the PDPs can be washed away prior the PDK-mediated phosphorylation of immunocaptured PDH.

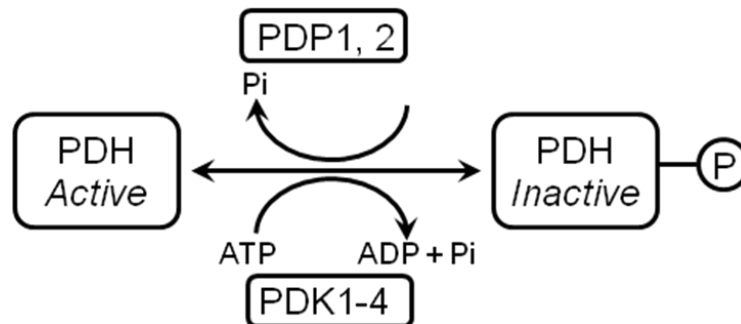
### REGULATION OF PDH ACTIVITY

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<sub>1</sub>), dihydrolipoyl acyltransferase (E<sub>2</sub>), and dihydrolipoyl dehydrogenase (E<sub>3</sub>), and one structural protein (E<sub>2</sub>/E<sub>3</sub> binding protein). In total the enzyme is composed of five different polypeptide chains, E<sub>1</sub>α, E<sub>1</sub>β, E<sub>2</sub>, E<sub>3</sub> and E<sub>2</sub>/E<sub>3</sub>BP 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<sub>1</sub>α 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 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<sub>2</sub> domain of PDH. The PDH kinases phosphorylate three specific sites of the E<sub>1</sub>α 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 (Figure 1). Each of the

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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<sub>2</sub> 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 1. 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<sub>1</sub>α subunits. Phosphorylation results in inactivation of PDH, dephosphorylation results in activation of PDH.

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.

See the MitoSciences PDH Playbook for an overview of applications possible using MitoSciences PDH assays and tools. This protocol (MitoSciences PDH Protocol #3) describes how to screen for inhibitors and/or activators of each of the four PDH kinases, using PDK-regulated PDH activity as a reporter. A complementary protocol (PDH Protocol #4) describes how to screen for inhibitors and/or activators of the 4 PDKs by measurement of PDK-mediated phosphorylation of PDH subunit E<sub>1</sub>α at serines 232, 293 and 300. The two protocols (PDH activity-based and PDH phosphorylation-based) can be performed in parallel to obtain a comprehensive analysis of the effects of potential inhibitors and/or activators of PDKs.

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### 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 Reagent Dye (component of MSP18)	0.25 mL	-20°C
100X Coupler (component of MSP18)	0.25 mL	-80°C
20X Reagent Mix (component of MSP18)	2 x 0.6 mL	-80°C
50X ATP	500 µL	-20°C
5X Stabilizer	13 mL	4°C
Phosphatase Inhibitor (PI)	1.5 mL	4°C
Choice of PDKs:		
PDK1 (MSP41)	50 µg	-80°C
PDK2 (MSP42)	50 µg	-80°C
PDK3 (MSP43)	50 µg	-80°C
PDK4 (MSP44)	50 µg	-80°C

Avoid repeated freeze/thaw cycles of 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 450 nm, preferably in a kinetic mode.
- Method for determining protein concentration
- Multichannel pipette
- Deionized water
- PBS (phosphate buffered saline) – for recipe see Appendix A

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### ASSAY PROTOCOL

This protocol has four steps:

A. Sample Preparation.

PDH-containing extracts are prepared.

B. PDH Immunocapture.

PDH from the sample of interest is immunocaptured to a desired number of wells.

C. Phosphorylation Reactions.

The immunocaptured PDH is phosphorylated with PDK of choice in the presence (and in the absence) of compound being tested as a modulator of PDK activity.

D. Activity Measurement.

Below are recommended amounts of materials. These amounts were carefully chosen to fit within the linear range of PDH activity signal (Figure 6). For a control sample, purified bovine heart mitochondria (MS802) are available from MitoSciences.

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)

This protocol describes the use of sufficient PDK1, 2, 3 or 4 to perform 96 phosphorylation reactions with each enzyme, with each able to phosphorylate and fully inhibit PDH immunocaptured using recommended amounts of materials (50 µg of bovine heart mitochondria (available individually as MS802, Bovine Heart Mitochondria IP Control), corresponding to 1 milliunit of porcine PDH, Sigma P7032, 125 µg of rat or mouse heart tissues, or 500 µg of HepG2 cells).

The MSP18 immunocapture microplate can be broken into 12 separate 8-well strips, allowing 12 independent experiments. For convenience, considering the above, unless specified otherwise, the volumes in this protocol are given for analysis of multiples of three strips (24 wells). If different numbers of strips are used, simply adjust the volumes proportionally.

**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.**

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### A. Sample Preparation

- Determine the sample protein concentration using a standard method such as BCA method (Pierce).
- 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	5.3
Tissue homogenates	23.7
Cultured cells	15.0

- Prepare the extraction according to the table below. Mix components immediately after the addition of the detergent. *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 three strips (24 wells).*

Component	Purified mitochondria	Tissue homogenates	Cultured cells
Sample (µL)	285	237.5	1,350
Detergent (µL)	15	12.5	150
Total Volume (µL)	300	250	1,500
Final Protein Concentration (mg/mL)	5.0	22.5	13.5

- Incubate on ice for 10 minutes.
- 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

- Prepare the Dilution Buffer as specified below.

No. of Plate Strips	dH <sub>2</sub> O (mL)	20X Buffer (mL)	Total (mL)
3	5.7	0.3	6
6	11.4	0.6	12
9	17.1	0.9	18
12	22.8	1.2	24

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- 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

- Plate loading. Add 200  $\mu$ L/well of diluted sample prepared in Step 7 of Section A.
- Incubate microplate for 2.5 hours at room temperature.

### C. Phosphorylation Reactions

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

1X Reaction/Wash Buffer					
No. of Plate Strips	dH <sub>2</sub> O (mL)	20X Buffer (mL)	5X Stabilizer (mL)	PI (mL)	Total (mL)
3	29	2	8	0.8	40
6	58	4	16	1.6	80
9	88	6	24	2.4	120
12	117	8	32	3.2	160

- Prepare 2X Compound of Interest Working Stock. *Below are suggested volumes for preparation of sufficient amounts for loading of two wells, allowing analysis in duplicates.*

2X Compound of Interest Working Stock					
No. of wells	dH <sub>2</sub> O ( $\mu$ L)	20X Buffer ( $\mu$ L)	5X Stabilizer ( $\mu$ L)	Compound of Interest ( $\mu$ L)	Total ( $\mu$ L)
2	187.5-X	12.5	50	X	250

- Prepare PDK Working Stock following the appropriate table below. Prepare only the amount needed. NOTE – It is important to load the PDKs at sub-saturating levels so reductions in PDK activity (due to the action of a potential PDK inhibitor) can be detected easily. The formulation below yields final amounts of PDK1, 2 or 4 at 1  $\mu$ g/well, and PDK3 at 0.05  $\mu$ g/well, as these are rate-limiting amounts for many sample types (see Figures 2-5). It is recommended that PDK titration curves as shown in Figures 2-5 be run for any novel source of PDH used as a capture target in this assay.

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2X PDK1, 2 or 4 Working Stock							
No. of Plate Strips	dH <sub>2</sub> O (mL)	20X Buffer (mL)	5X Stabilizer (mL)	PI (μL)	PDK1, 2 or 4 at 1.5 μg/μL (μL)	50X ATP (μL)	Total (mL)
3	1.73	0.13	0.52	104	17	104	2.6
6	3.45	0.26	1.04	208	35	208	5.2
9	5.17	0.39	1.56	312	52	312	7.8
12	6.90	0.52	2.08	416	69	416	10.4

2X PDK3 Working Stock							
No. of Plate Strips	dH <sub>2</sub> O (mL)	20X Buffer (mL)	5X Stabilizer (mL)	PI (μL)	*PDK3 at 0.15 μg/μL (μL)	50X ATP (μL)	Total (mL)
3	1.72	0.13	0.52	104	9	104	2.6
6	3.44	0.26	1.04	208	17	208	5.2
9	5.15	0.39	1.56	312	26	312	7.8
12	6.86	0.52	2.08	416	35	416	10.4

\*Note – To ensure stability, PDK3 (MSP43) is shipped at 0.5 μg/μL and must first be diluted to 0.15 μg/μL before being diluted again to make a “2X PDK3 Working Stock” as described in the table. See Appendix A, Recipes for Required Components, page 11 “PDK of choice” for more details.

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 Reaction/Wash Buffer to wash the wells.
6. Repeat the steps 6. and 7. one more time for a total of 2 wash steps.
7. 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.
8. Add promptly 100 μL of 2X Compound of Interest Working Stock and 100 μL of 2X PDK Working Stock into each well. Mix immediately. See Note 2 for phosphorylation reaction controls.
9. Incubate the plate for 10 min at 30°C.

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### D. Activity measurement

1. Prepare the PDH Assay Solution, according to the table below. Warm up the PDH Assay Solution to 30°C.

PDH Assay Solution						
No. of Plate Strips	dH <sub>2</sub> O (mL)	20X Buffer (mL)	20X Reagent Mix (mL)	100X Coupler (μL)	100X Reagent Dye (μL)	Total Volume (mL)
3	4.75	0.25	0.25	50	50	5.35
6	9.50	0.50	0.50	100	100	10.70
9	14.25	0.75	0.75	150	150	16.05
12	19.00	1.000	1.00	200	200	21.40

2. Empty the wells of the microplate by turning it over and shaking out any remaining liquid. Blot the plate face down on paper towel.
3. Add 300 μL/well of 1X Reaction/Wash Buffer to wash the wells.
4. Repeat the steps 2. and 3. two more times for a total of 3 wash steps.
5. 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.
6. Carefully add to each well 200 μL of PDH Assay Solution. Avoid bubbles. Any bubble should be popped with a fine needle as rapidly as possible.
7. Promptly place the plate into a spectrophotometer and begin microplate reading using the following parameters. (Alternatively, an endpoint measurement can be made at a user defined time by recording absorbance at 450 nm.)

Mode:	Kinetic
Wavelength:	450 nm
Time:	15 min
Interval:	20-30 sec
Shaking:	Shake between readings
Temperature:	30°C

8. 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.

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- Each experiment should include following two phosphorylation reaction controls using the PDH source of interest. Control 1 (mock-PDK phosphorylated sample), this is a sample treated in the absence of PDK and in the absence of compound of interest. Control 2 (mock-compound of interest phosphorylated sample), this is a sample treated with PDK in the absence of compound of interest.
- If a compound of interest (included in the phosphorylation reaction) increases the PDH activity and thus is suspected to be a PDK inhibitor, the direct toxic effect of the compound on PDH activity should be assayed. This can be accomplished by including the compound directly into the PDH assay solution.

## DATA ANALYSIS

- As explained in the Introduction, the PDK activity is inversely proportional to the PDH activity.
- The maximum PDH activity is equal the activity of the mock-PDK phosphorylated sample (Control 1). Control 1 is the sample treated in the absence of PDK and in the absence of compound of interest.
- The residual PDH activity is equal the activity of the mock-compound of interest phosphorylated sample (Control 2). Control 2 is a sample treated with PDK in the absence of compound of interest and its PDH activity is fully inhibited due to the phosphorylation of one (or more) PDH E<sub>1</sub>α Serines (232, 293 and 300).
- If a compound of interest (included in the phosphorylation reaction) does not inhibit the PDK-catalyzed phosphorylation of PDH E<sub>1</sub>α, the PDH activity of this sample is equal the activity of Control 2.
- If a compound of interest (included in the phosphorylation reaction) inhibits the PDK-catalyzed phosphorylation of PDH E<sub>1</sub>α, the PDH activity of this sample is higher than the activity of Control 2.
- If a compound of interest (included in the phosphorylation reaction) fully inhibits the PDK-catalyzed phosphorylation of PDH E<sub>1</sub>α, the PDH activity of this sample is equal the activity of Control 1.
- A direct inhibitory effect of a compound on PDH activity should be excluded. See *Note 3*.
- Then the inhibitory effect of a compound of interest on the PDK-catalyzed phosphorylation of PDH E<sub>1</sub>α is proportional to the PDH activity measured and can be expressed as follows:

$$\text{Effect of COI (\%)} = 100 \times (\text{Activity}^{\text{COI}} - \text{Activity}^{\text{Con2}}) / (\text{Activity}^{\text{Con1}} - \text{Activity}^{\text{Con2}})$$

Abbreviation are as follows:

COI = Compound of Interest  
Activity = PDH activity  
Con1 = Control 1  
Con2 = Control 2

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9. Thus, a compound of interest will have no inhibitory effect on PDK-catalyzed phosphorylation when the *Effect of COI* is 0 %. A compound of interest will have maximum inhibitory effect on PDK-catalyzed phosphorylation when the *Effect of COI* is 100 %.

**PDH Protocol #3 – PDH Kinase Inhibitor Screening Base on PDH Activity****APPENDIX A. Recipes for Required Components**

<b>Phosphatase Inhibitor (PI)</b>		
<ul style="list-style-type: none"><li>• NaF (EM Science SX-0550-3) FW 41.99</li><li>• Deionized Water</li></ul>		
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.		

<b>5X Stabilizer</b>		
<ul style="list-style-type: none"><li>• Albumin, from bovine serum (Sigma A4503)</li><li>• Deionized water</li></ul>		
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		

<b>50X ATP</b>		
<ul style="list-style-type: none"><li>• Adenosine 5'-triphosphate, disodium salt (Sigma A2383), FW 551.1</li><li>• Deionized water</li></ul>		
Component	Grams per 10 mL	Concentration (mM)
Adenosine 5'-triphosphate	0.551	100
Dissolve Adenosine 5'-triphosphate in deionized water. Adjust volume to 10 mL. Store aliquoted at -20°C.		

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### PDK of choice (MitoSciences)

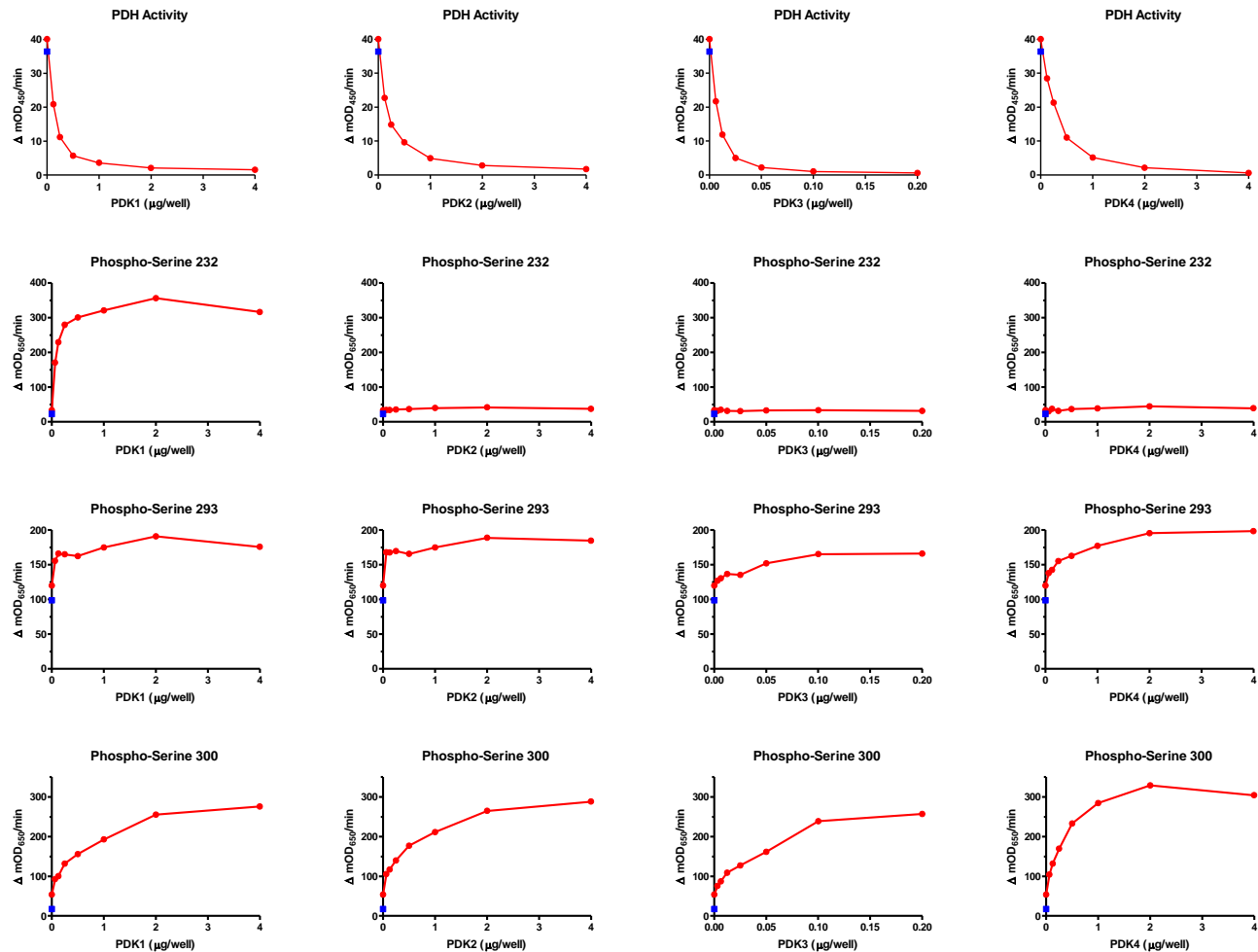
- PDK3, MitoSciences MSP43. **Note – This stock PDK3 is at 0.5 µg/µL and must be diluted to make the component PDK3 at 0.15 µg/µL**
- PDK1, MitoSciences MSP41. Note – This stock PDK1 is at 1.5 µg/µL and can be used as is for the component PDK1 at 1.5 µg/µL
- PDK2, MitoSciences MSP42. Note – This stock PDK2 is at 1.5 µg/µL and can be used as is for the component PDK2 at 1.5 µg/µL
- PDK4, MitoSciences MSP44. Note – This stock PDK4 is at 1.5 µg/µL and can be used as is for the component PDK4 at 1.5 µg/µL
- Dilution Buffer (10 mM Na-PO<sub>4</sub>, 250 mM NaCl, 30% glycerol, 5 mM DTT, 0.5 mM EDTA, 0.05% Triton X-100, pH 7.5 with HCl)

PDK of choice	Grams per mL	Concentration (µg/µL)
PDK1	NA	1.50
PDK2	NA	1.50
PDK3	NA	0.15
PDK4	NA	1.50

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

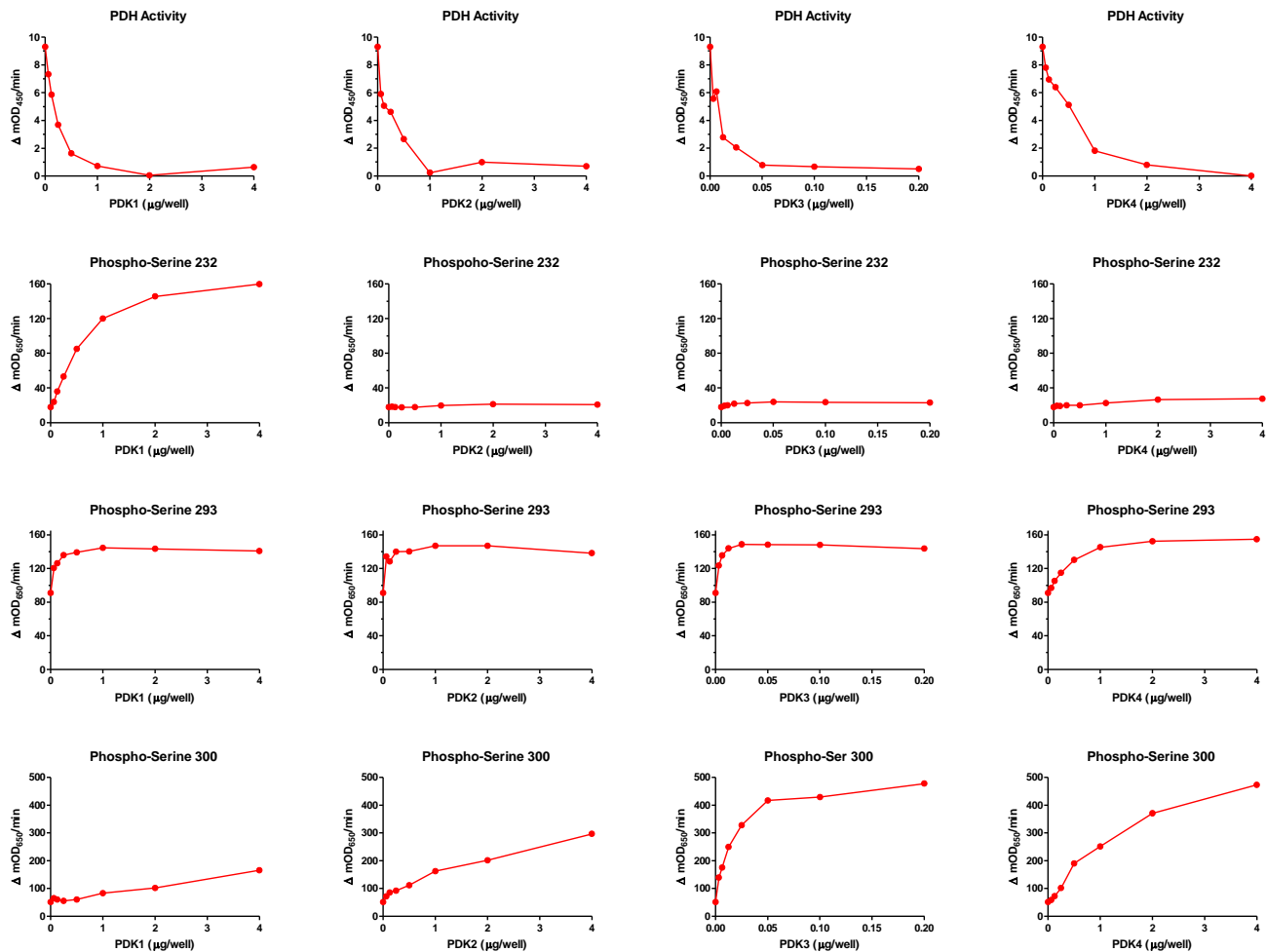
## PDH Protocol #3 – PDH Kinase Inhibitor Screening Base on PDH Activity

### SUPPORTING DATA



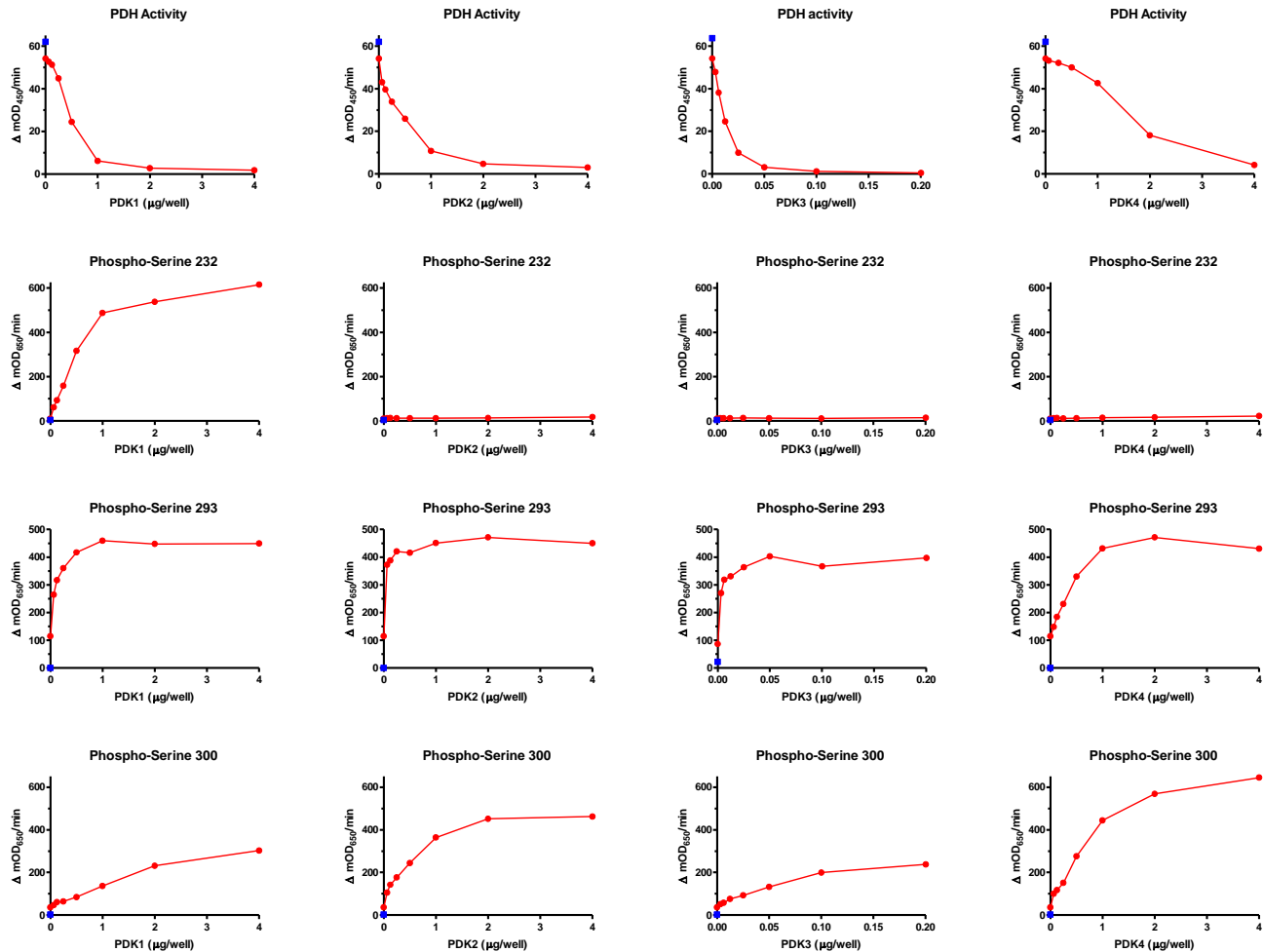
**Figure 2. The PDKs phosphorylate and fully inactivate bovine PDH.** As described in this protocol, PDH immunocaptured from 50  $\mu$ g of bovine heart mitochondria was treated with indicated amounts of recombinant PDKs in the presence of ATP (in red) or in the absence of ATP (in blue). PDH activities (first row) were measured following PDH Protocol #3. PDH E<sub>1</sub> $\alpha$  Phospho-Serine 232 (second row), 293 (third row), and 300 (fourth row) quantities were measured following PDH Protocol #4. Note that: (1) 2  $\mu$ g of PDK1 are sufficient to fully phosphorylate Serine 232, 293 and 300, and fully inactivate the PDH, (2) 2  $\mu$ g of PDK2 are sufficient to fully phosphorylate Serine 293 and 300, and fully inactivate the PDH, (3) 0.1  $\mu$ g of PDK3 is sufficient to fully phosphorylate Serine 293 and 300, and fully inactivate the PDH, and (4) 2  $\mu$ g of PDK4 are sufficient to fully phosphorylate Serine 293 and 300, and fully inactivate the PDH. Also note that only PDK1 can phosphorylate the Serine 232 of PDH E<sub>1</sub> $\alpha$ . In addition, note that the treatment with ATP alone (in the absence of PDKs) when compared to the treatment in the absence of ATP does not substantially reduce the PDH activity, indicating absence of endogenous kinases.

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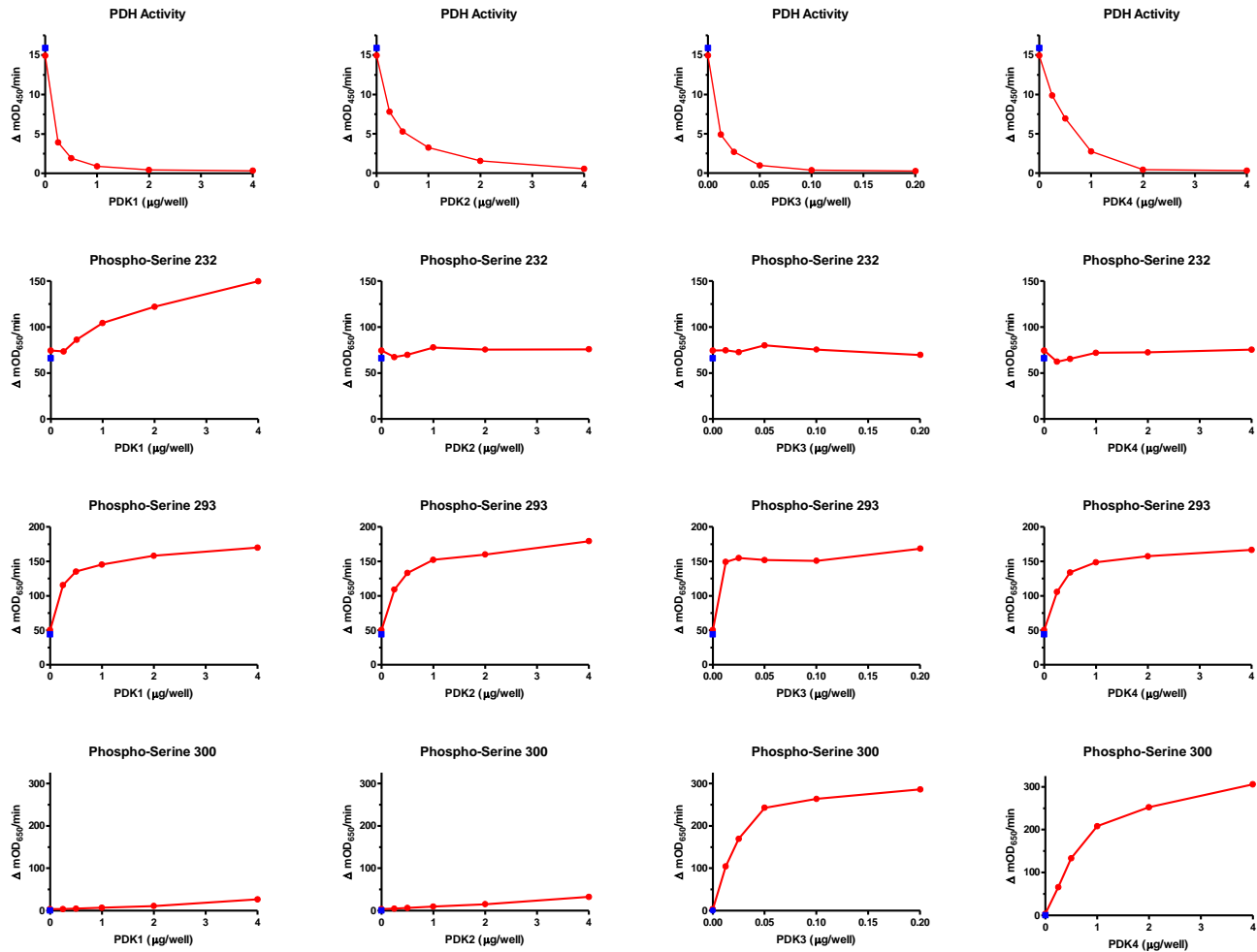
**Figure 3. The PDKs phosphorylate and fully inactivate rat PDH.** Extracts of rat heart homogenate were dephosphorylated with PDP1 to ensure maximum activity. Then, as described in this protocol, PDH was immunocaptured from 125 μg of the dephosphorylated rat heart homogenate and it was treated with indicated amounts of recombinant PDKs in the presence of ATP. PDH activities (first row) were measured following PDH Protocol #3. PDH E<sub>1</sub>α Phospho-Serine 232 (second row), 293 (third row), and 300 (fourth row) quantities were measured following PDH Protocol #4. Note that: (1) 2 μg of PDK1 are sufficient to fully phosphorylate Serine 232 and 293, and fully inactivate the PDH, (2) 2 μg of PDK2 are sufficient to fully phosphorylate Serine 293 and 300, and fully inactivate the PDH, (3) 0.1 μg of PDK3 is sufficient to fully phosphorylate Serine 293 and 300, and fully inactivate the PDH, and (4) 2 μg of PDK4 are sufficient to fully phosphorylate Serine 293 and 300, and fully inactivate the PDH. Also note that only PDK1 can phosphorylate the Serine 232 of PDH E<sub>1</sub>α.

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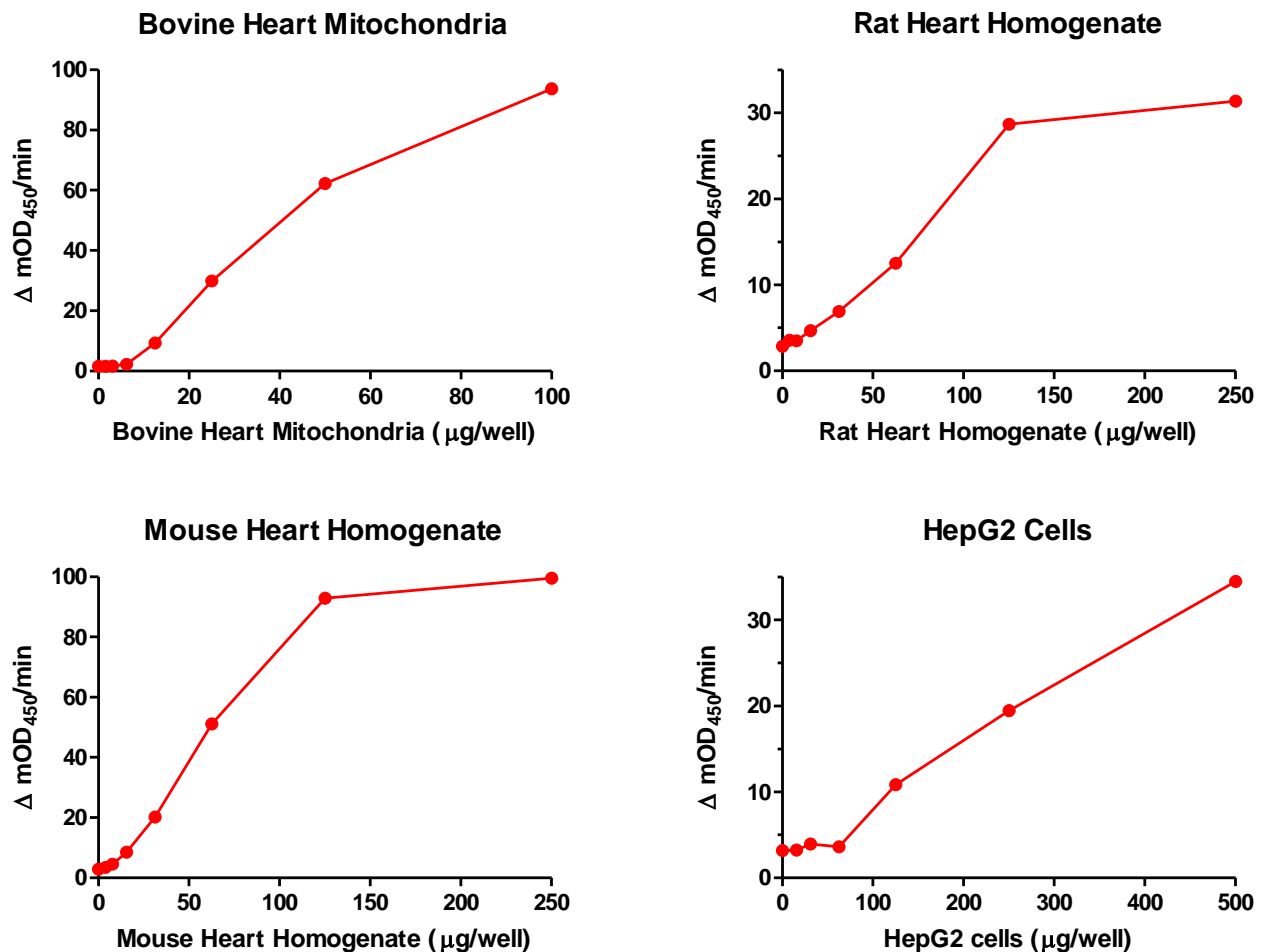
**Figure 4. The PDKs phosphorylate and fully inactivate mouse PDH.** Extracts of mouse heart homogenate were dephosphorylated with PDP1 to ensure maximum activity. Then, as described in this protocol, PDH was immunocaptured from 125 μg of the dephosphorylated mouse heart homogenate and it was treated with indicated amounts of recombinant PDKs in the presence of ATP (in red) or in the absence of ATP (in blue). PDH activities (first row) were measured following PDH Protocol #3. PDH E<sub>1</sub>α Phospho-Serine 232 (second row), 293 (third row), and 300 (fourth row) quantities were measured following PDH Protocol #4. Note that: (1) 2 μg of PDK1 are sufficient to fully phosphorylate Serine 232 and 293, and fully inactivate PDH, (2) 2 μg of PDK2 are sufficient to fully phosphorylate Serine 293 and 300, and fully inactivate PDH, (3) 0.1 μg of PDK3 is sufficient to fully phosphorylate Serine 293 and fully inactivate the PDH, and (4) 2 μg of PDK4 are sufficient to fully phosphorylate Serine 293 and 300, and fully inactivate the PDH. Also note that only PDK1 can phosphorylate the Serine 232 of PDH E<sub>1</sub>α. In addition, note that the treatment with ATP alone (in the absence of PDKs) when compared to the treatment in the absence of ATP does not substantially reduce the PDH activity, indicating absence of endogenous kinases.

## PDH Protocol #3 – PDH Kinase Inhibitor Screening Base on PDH Activity



**Figure 5. The PDKs phosphorylate and fully inactivate human PDH.** Extracts of HepG2 cells were dephosphorylated with PDP1 to ensure maximum activity. Then, as described in PDH Protocol #X, PDH was immunocaptured from 500 μg of the dephosphorylated HepG2 cells and it was treated with indicated amounts of recombinant PDKs in the presence of ATP (in red) or in the absence of ATP (in blue). PDH activities (first row) were measured following PDH Protocol #3. PDH E<sub>1</sub>α Phospho-Serine 232 (second row), 293 (third row), and 300 (fourth row) quantities were measured following PDH Protocol #4. Note that: (1) 2 μg of PDK1 are sufficient to fully phosphorylate Serine 232 and 293, and fully inactivate the PDH, (2) 2 μg of PDK2 are sufficient to fully phosphorylate Serine 293 and fully inactivate the PDH, (3) 0.1 μg of PDK3 is sufficient to fully phosphorylate Serine 293 and 300, and fully inactivate the PDH, and (4) 2 μg of PDK4 are sufficient to fully phosphorylate Serine 293 and 300, and fully inactivate the PDH. Also note that only PDK1 can phosphorylate the Serine 232 of PDH E<sub>1</sub>α. In addition, note that the treatment with ATP alone (in the absence of PDKs) when compared to the treatment in the absence of ATP does not substantially reduce the PDH activity, indicating absence of endogenous kinases.

## PDH Protocol #3 – PDH Kinase Inhibitor Screening Base on PDH Activity

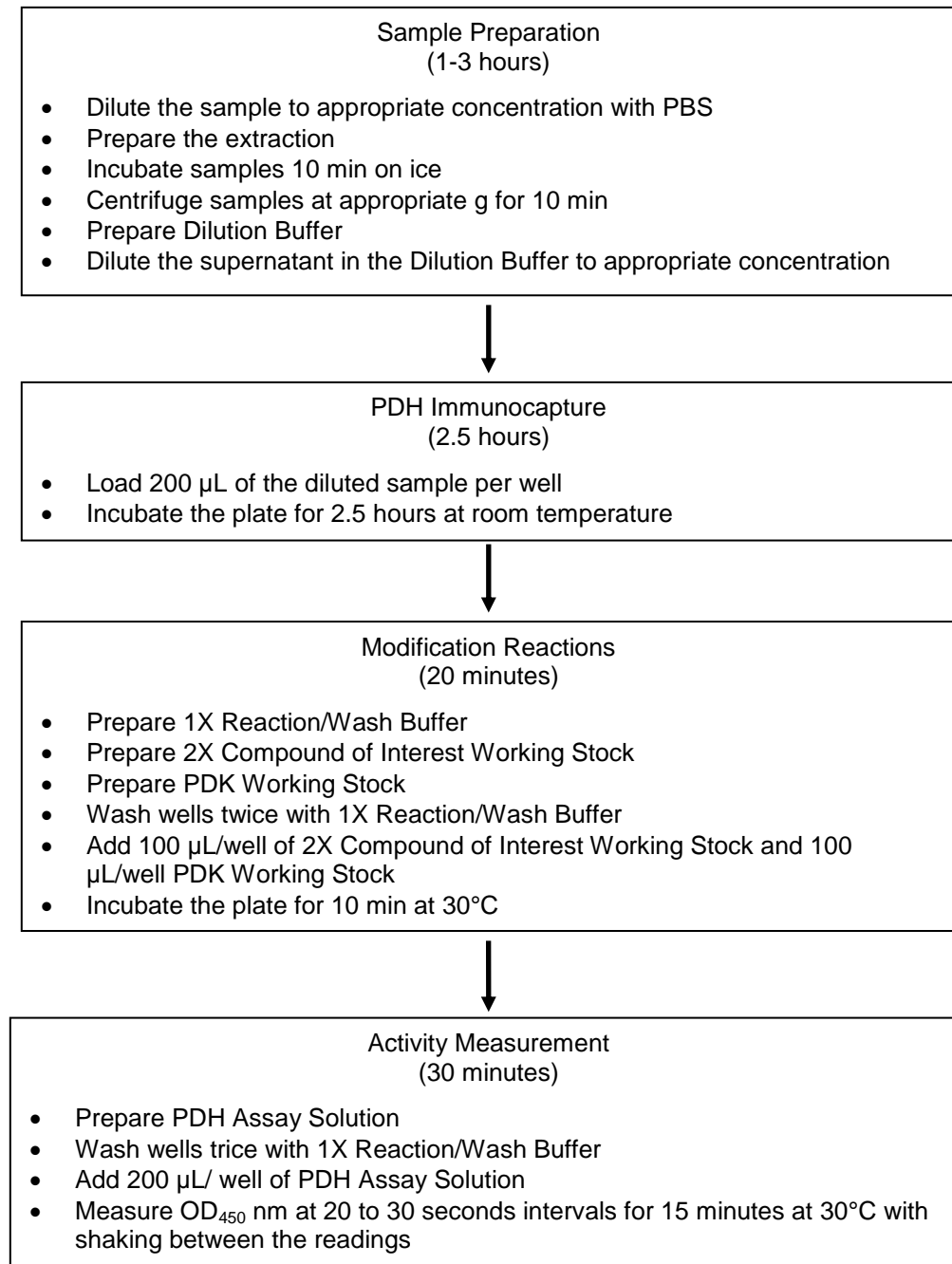


**Figure 6. Determination of a linear range of PDH activity signal of bovine, rat, mouse and human PDH.** Indicated amounts of extracts of bovine heart mitochondria, rat heart homogenate, mouse heart homogenate and human HepG2 cells were immunocaptured and PDH activities were measured following PDH Protocol #3. Prior the immunocapture, the extracts of rat heart homogenate, mouse heart homogenate and human HepG2 cells were dephosphorylated with PDP1 to ensure maximum activity.

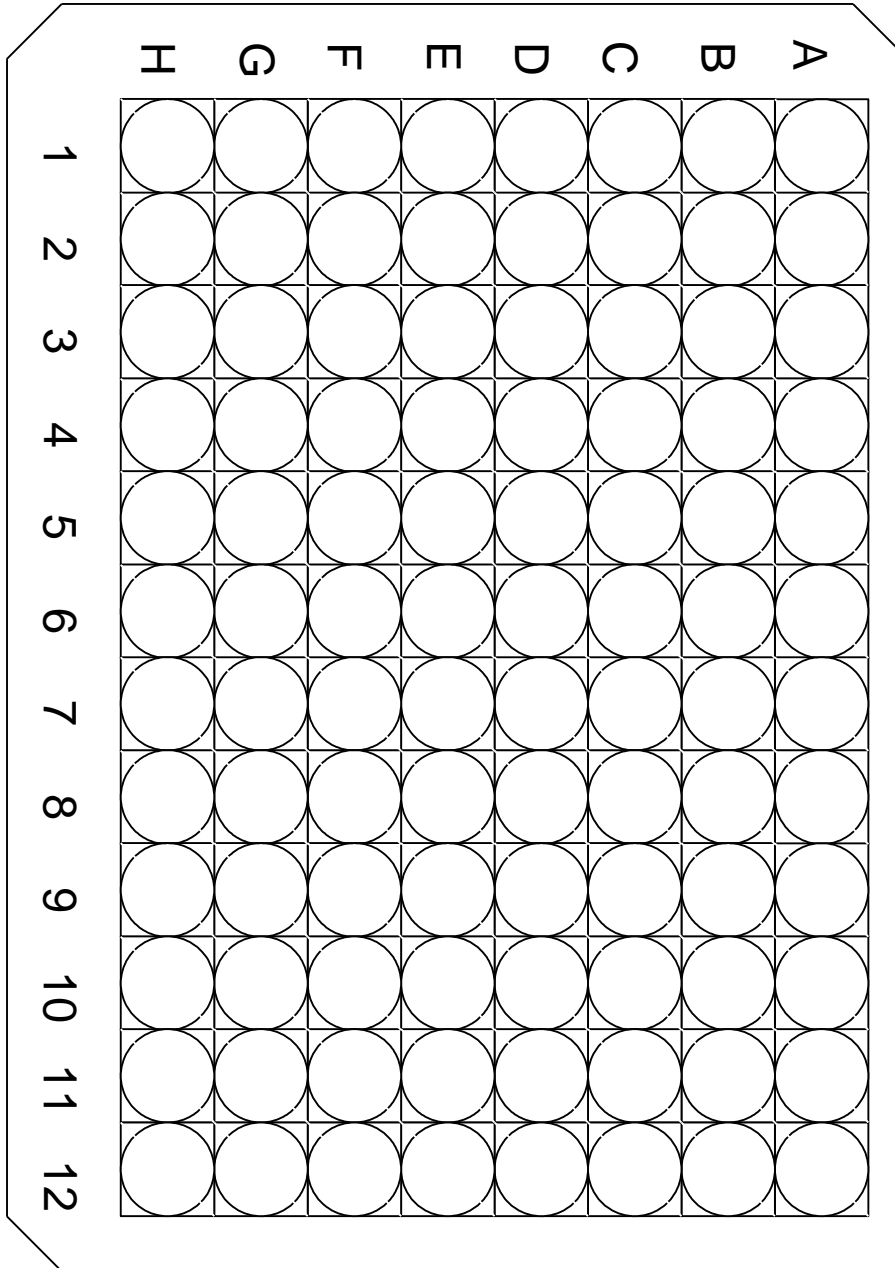
## PDH Protocol #3 – PDH Kinase Inhibitor Screening Base on PDH Activity

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



**PDH Protocol #3 – PDH Kinase Inhibitor Screening Base on PDH Activity**



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