

Measurement of PDH Endogenous Activity Relative to the Fully-Phosphorylated and Dephosphorylated States

Rev.2

INTRODUCTION

This immunocapture PDH activity protocol describes how to measure endogenous PDH activity corresponding to the enzyme's phosphorylation state at the time of sample collection. The key to obtain meaningful PDH activity measurements that correspond as closely as possible to the activity *in vivo*, is to inhibit endogenous specific and non-specific phosphatases and kinases that may modify PDH E₁α during sample preparation and immunocapture steps. It is essential that serine/threonine protein phosphatase inhibitors are used, as well as a system to deplete ATP (and thus block the phosphorylation reaction).

This protocol describes MitoSciences' assay kit MSP18 to first immunocapture PDH from extracts of human, bovine, rat or mouse cells, tissues or mitochondria, and to then measure the PDH enzyme activity. This protocol extends the MSP18 protocol by treating the immunocaptured PDH in parallel samples with PDK3 and PDP1 to determine, respectively, the background activity of fully-phosphorylated PDH and the maximum activity of fully-dephosphorylated PDH.

Phosphorylation reaction conditions were optimized to obtain fully-phosphorylated serine²⁹³ and correspondingly fully-inactivated PDH. Similarly, dephosphorylation conditions were optimized to obtain fully-active PDH and correspondingly fully-dephosphorylated serine²⁹³. The endogenous levels of PDH activity (that reflect endogenous phosphorylation) relative to the maximum activity of the fully dephosphorylated enzyme, on a scale of 0 to 100 percent, can then be determined (Figure 1).

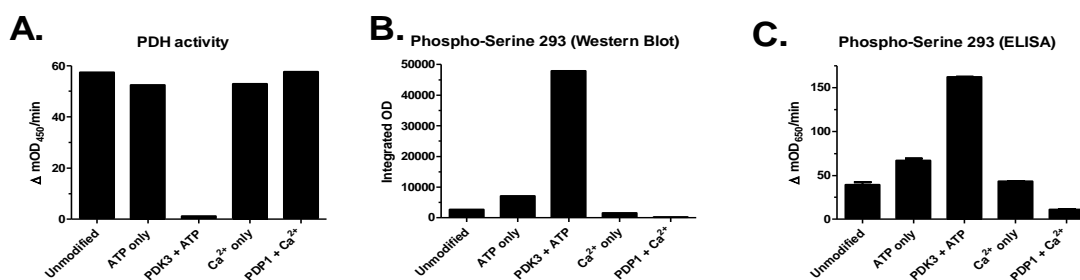


Figure 1. PDH activities and PDH E₁α Phospho-Serine²⁹³ levels of unmodified (endogenous), PDK3-phosphorylated and PDP1-dephosphorylated bovine heart PDH. As described in this protocol, PDH was immunocaptured from 50 μg of bovine heart mitochondria and unmodified, fully phosphorylated (PDK3 + ATP) and fully dephosphorylated (PDP1 + Ca²⁺) PDH activities (n=3) were measured with the use of MSP18 (A). PDH E₁α phospho-Serine²⁹³ 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 E₁α phospho-Serine²⁹³ levels, measured in this experiment with the use of Phospho-PDH Ser²⁹³ 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-Serine²⁹³ levels of the fully phosphorylated enzyme, respectively.

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

PDH Protocol #1 – Measurement of PDH Endogenous Activity

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. The PDH is a large complex of three enzymes, pyruvate dehydrogenase (E_1), dihydrolipoyl 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. The 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.

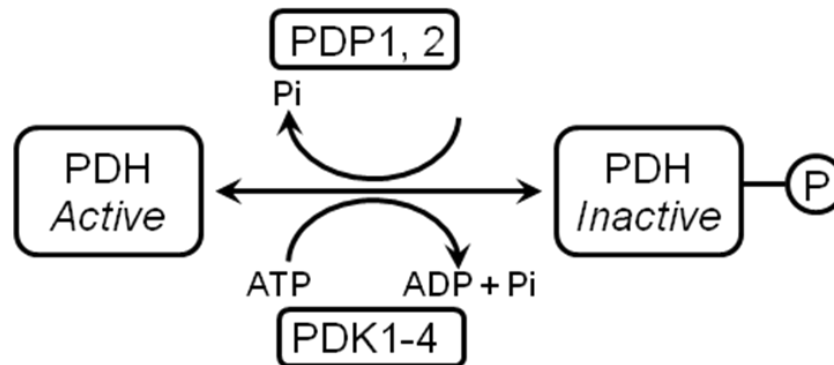


Figure 2. Schematic representation of the PDK-dependent phosphorylation and PDP-dependent dephosphorylation of PDH. Phosphorylation occurs at Serines 232, 293, and 300 of the human $E_1\alpha$ subunits. Phosphorylation results in inactivation of PDH, while dephosphorylation results in activation.

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.

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

PDH Protocol #1 – Measurement of PDH Endogenous Activity

MATERIALS

Most of the components required to perform 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 following this protocol using 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 |
| 20X Reagent Mix (component of MSP18) | 2 x 0.6 mL | -80°C |
| 100X Coupler (component of MSP18) | 0.25 mL | -80°C |
| 100X Reagent Dye (component of MSP18) | 0.25 mL | -20°C |
| Phosphatase Inhibitor (PI) | 1.5 mL | 4°C |
| 100X ATP Depletion System | 65 µL | -20°C |
| 5X Stabilizer | 13 mL | 4°C |
| 200X Calcium | 0.1 mL | 4°C |
| 50X ATP | 0.2 mL | -80°C |
| PDH Kinase 3 – PDK3 (MSP43) | 3.5 µg | -80°C |
| PDH Phosphatase 1 – PDP1 (MSP45) | 70 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.

PDH Protocol #1 – Measurement of PDH Endogenous Activity

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 with immunocaptured PDH is left untreated to determine endogenous PDH activity, the second well is treated with PDK3 to determine the background (fully-phosphorylated) PDH activity and the third well is treated with PDP1 to determine the maximum (fully-dephosphorylated) PDH activity.

D. Activity Measurement.

Below are recommended amounts of materials to use. These amounts were carefully chosen to fit within the linear range of the PDH activity signal (Figure 6).

| 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) |

Note – Be completely familiar with the 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 the 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 |

PDH Protocol #1 – Measurement of PDH Endogenous Activity

3. Prepare the extraction according to the table below. Mix components immediately. *It is imperative to keep the ratio of components for extraction as specified below. Below are suggested volumes for preparation of sufficient amounts for loading of six wells, allowing analysis of each of the samples for the PDH activity of endogenous, phosphorylated and dephosphorylated enzyme in duplicates. See Notes 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 ₂ O (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 the 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) |

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B. PDH Immunocapture

1. Plate loading: Add 200 μ L 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 each 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.

C. Modification Reactions

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

| 1X Reaction/Wash Buffer | | | | |
|-------------------------|------------------------|-----------------|--------------------|------------|
| No. of Plate Strips | dH ₂ O (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 ₂ O (mL) | 20X Buffer (μ L) | 5X Stabilizer (μ L) | 50X ATP (μ L) | 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 Solutions is needed only for one third of wells.

| 2X Calcium Solution | | | |
|---------------------|------------------------------|-------------------------|------------|
| No. of Plate Strips | 1X Reaction/Wash Buffer (mL) | 200X Calcium (μ L) | 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 #1 – Measurement of PDH Endogenous Activity

4. Prepare the PDK3 Working Stock according to the table below at 1 $\mu\text{g}/\text{mL}$. 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) | PDK3 at 0.15 $\mu\text{g}/\mu\text{L}$ (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 $\mu\text{g}/\text{mL}$. 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) | PDP1 at 1.5 $\mu\text{g}/\mu\text{L}$ (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. Promptly add 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 in a layout below.

PDH Protocol #1 – Measurement of PDH Endogenous Activity

| | 1X Reaction/Wash Buffer (200 µL/well) | | | | PDK3 Working Stock (100 µL/well) | | | | PDP1 Working Stock (100 µL/well) | | | |
|----------|--|---|----|----|-------------------------------------|---|----|----|--------------------------------------|----|----|----|
| | | | | | 2X ATP Solution (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) PDH activity. 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. 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 ₂ 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. 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.

PDH Protocol #1 – Measurement of PDH Endogenous Activity

- Carefully add to each well 200 μ L of PDH Assay Solution. Avoid bubbles. Any bubble should be popped with a fine needle as rapidly as possible.
- 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 |

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

PDH Protocol #1 – Measurement of PDH Endogenous Activity

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. Control mitochondria prepared from a variety of animal tissues are available from MitoSciences (see http://www.mitosciences.com/immunocapture_controls.html).
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 endogenous PDH activity due to intrinsic phosphorylation. The PDH E₁α phospho-Serine levels can be determined in parallel by following MitoSciences' PDH Protocol #2. However, be aware that not every change of PDH activity is due to the changes in the PDH E₁α phospho-Serine level. Oxidative damage of PDH is also an important factor.

DATA ANALYSIS

1. The endogenous PDH activity is equal the activity of the unmodified sample. The endogenous PDH activity of a condition/treatment can be compared to a control/mock treatment.
2. The endogenous PDH activity that reflects the activity due to intrinsic enzyme phosphorylation, expressed as a percentage of the maximum activity of the fully dephosphorylated enzyme, can be determined as follows:

$$Act^{Endogenous} (\%) = 100 \times (Act^{unmodified\ sample} - Act^{PDK3-treated\ sample}) / (Act^{PDP1-treated\ sample} - Act^{PDK3-treated\ sample})$$

Abbreviation: *Act* = PDH activity

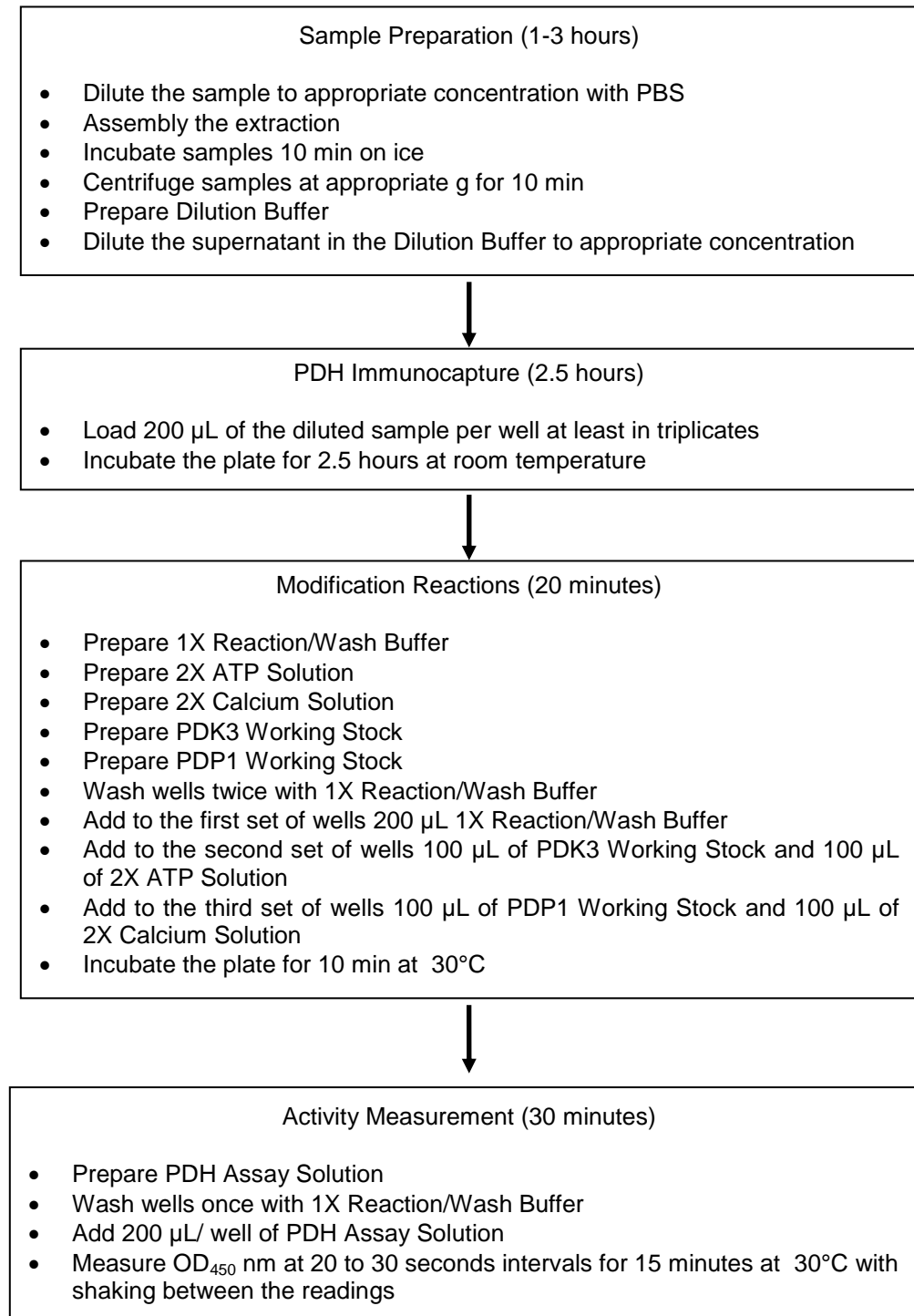
See Note 4.

3. If the amount of PDH protein, in addition to activity, under various conditions/treatment is of concern, we recommend measuring the amount of PDH in each sample with the use of our PDH Protein Quantity Microplate Assay Kit (Cat. # MSP19) and expressing the activity data as relative specific activities. Alternatively, phospho-specific antibodies are available against the three PDH phosphorylation sites, and all three antibodies are suitable for use as detectors with the MSP19 sandwich ELISA assay kit.

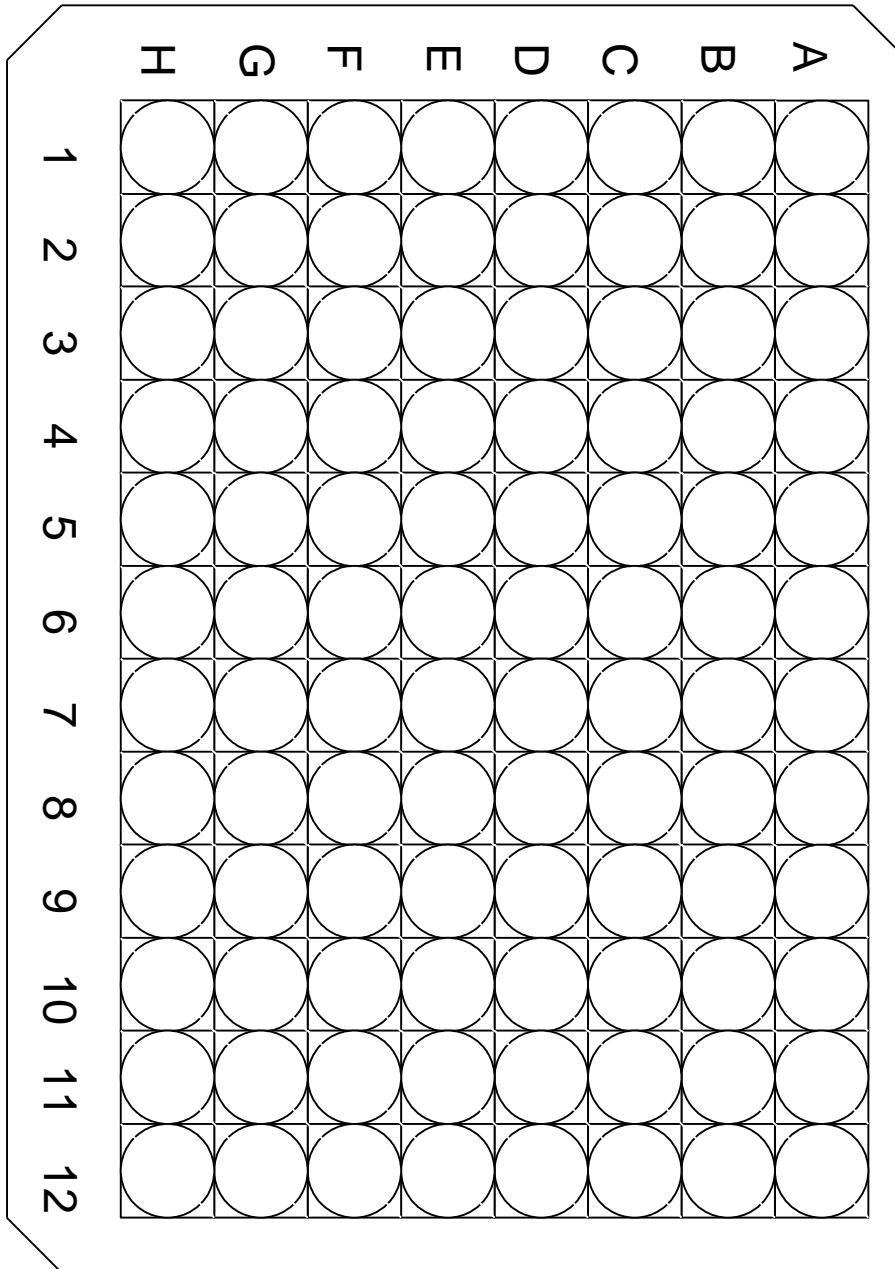
PDH Protocol #1 – Measurement of PDH Endogenous Activity

FLOW CHART

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



PDH Protocol #1 – Measurement of PDH Endogenous Activity



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PDH Protocol #1 – Measurement of PDH Endogenous Activity

APPENDIX A. Recipes for Required Components

| Phosphatase Inhibitor (PI) | | |
|---|------------------|-------------------|
| <ul style="list-style-type: none"> • 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 | | |
|--|------------------|--------------------------|
| <ul style="list-style-type: none"> • 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 setting the vial on a rotator. Add 0.25 g of Glycerol and mix on a rotator until dissolved. Store at -20°C. | | |

| 5X Stabilizer | | |
|---|------------------|-----------------------|
| <ul style="list-style-type: none"> • Albumin, from bovine serum (Sigma A4503) • Deionized water | | |
| Component | Grams per 100 mL | Concentration (mg/mL) |
| Albumin, from bovine serum | 5 | 50 |
| Dissolve Albumin in water. Adjust volume to 100 mL. Filter-sterilize and store at 4°C. | | |

| PDH Kinase 3 – PDK3 (MSP43) | | |
|--|--------------|-----------------------|
| <ul style="list-style-type: none"> • 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 Na-PO₄, 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 on ice when not in storage. Thaw PDK3 on ice. 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 #1 – Measurement of PDH Endogenous Activity

| 50X ATP | | |
|---|-----------------|--------------------|
| <ul style="list-style-type: none"> Adenosine 5'-triphosphate, disodium salt (Sigma A2383), FW 551.1 Deionized water | | |
| 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. | | |

| PDH Phosphatase 1 – PDP1 (MSP45) | | |
|---|--------------|-----------------------|
| <ul style="list-style-type: none"> PDP1, MitoSciences MSP45. Note – PDP1 is at 1.5 µg/µL and does not require dilution before use as component PDP1 at 1.5 µg/µL. Dilution Buffer (10 mM NaPO₄, 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. | | |

| 200X Calcium | | |
|--|------------------|--------------------|
| <ul style="list-style-type: none"> CaCl₂·2H₂O (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. | | |

| PBS (Phosphate Buffered Saline) | | |
|--|----------------------------------|----------|
| <ul style="list-style-type: none"> KH₂PO₄, Potassium Phosphate Monobasic (Sigma P0662), FW 136.09 Na₂HPO₄·7H₂O, Sodium Phosphate Dibasic Heptahydrate (Sigma S9390), FW 268.07 KCl, Potassium Chloride (Sigma P3111), FW 74.56 dH₂O NaCl, Sodium chloride (Sigma S3014), FW 58.44 | | |
| For 1 L PBS | | |
| 0.2 g | KH ₂ PO ₄ | (1.4 mM) |
| 2.2 g | Na ₂ HPO ₄ | (8 mM) |
| 0.2 g | KCl | (2.7 mM) |
| 8.2g | NaCl | (140 mM) |
| Add dry components to 0.9 L H ₂ O. The pH value should be 7.3, adjust if necessary. Add H ₂ O to 1 L. | | |

PDH Protocol #1 – Measurement of PDH Endogenous Activity

SUPPORTING DATA

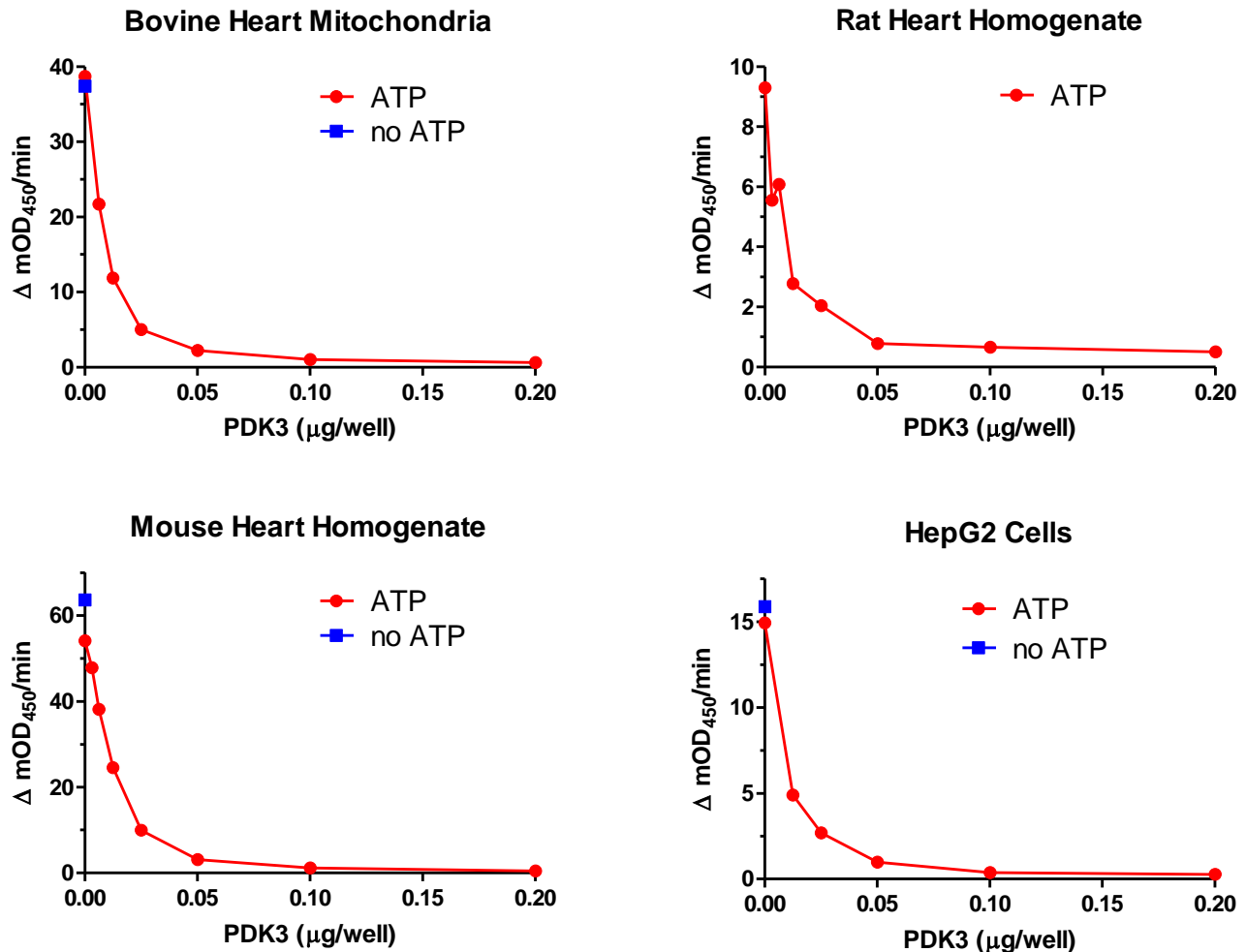


Figure 2. The PDH Kinase 3 (PDK3) fully inactivates immunocaptured bovine rat, mouse and human PDH. PDH immunocaptured from 50 μg of bovine heart mitochondria, 125 μg of rat heart homogenate, 125 μg of mouse heart homogenate, and 500 μg of HepG2 cells was treated with indicated amounts of recombinant PDK3 in the presence of ATP (in red) or in the absence of ATP (in blue), and PDH activity was measured as described in this protocol. 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 on the immunocaptured PDH.

PDH Protocol #1 – Measurement of PDH Endogenous Activity

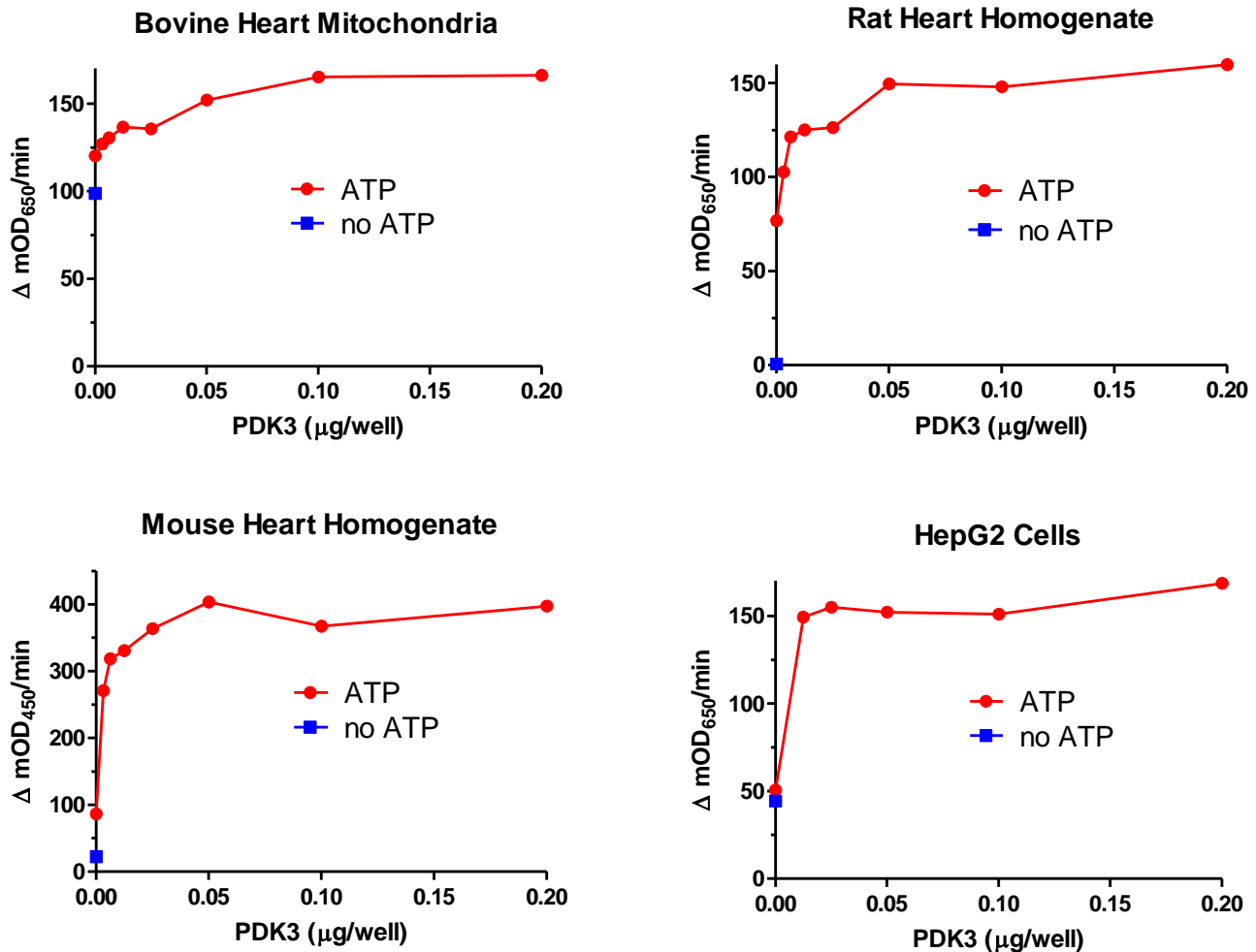


Figure 3. The PDH Kinase 3 (PDK3) fully phosphorylates immunocaptured bovine rat, mouse and human PDH at Serine²⁹³ of the E₁α subunit. PDH immunocaptured from 50 μg of bovine heart mitochondria, 125 μg of rat heart homogenate, 125 μg of mouse heart homogenate, or 500 μg of HepG2 cells was treated with indicated amounts of PDK3 in the presence of ATP (in red) or in the absence of ATP (in blue), and PDH E₁α pSer²⁹³ was measured. Note that 0.1 μg of PDK3 is sufficient to fully phosphorylate Ser²⁹³ 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 pSer²⁹³ signal, indicating absence of endogenous kinases associated with the immunocaptured PDH.

PDH Protocol #1 – Measurement of PDH Endogenous Activity

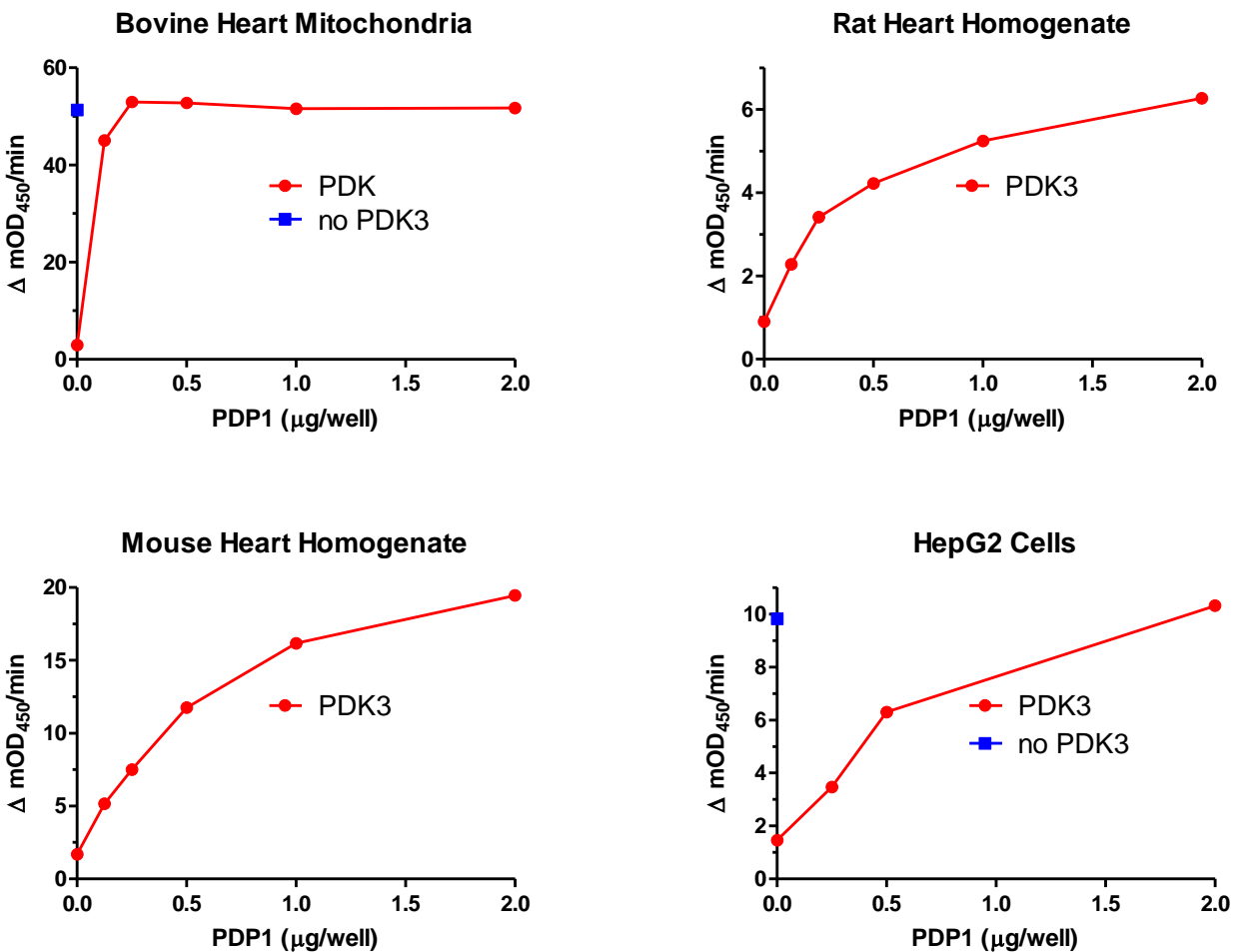


Figure 4. PDH Phosphatase 1 (PDP1) activates PDK3-phosphorylated immunocaptured bovine rat, mouse and human PDH. PDH was immunocaptured from 50 μg of bovine heart mitochondria, 250 μg of rat heart homogenate, 125 μg of mouse heart homogenate, or 500 μg of HepG2 cells and fully phosphorylated with 0.1 μg of PDK3 (in red) or mock-phosphorylated (no PDK3, in blue). The samples were then treated with indicated amounts of PDP1 in the presence of Ca²⁺, and PDH activity was measured as described in this protocol. 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 Ca²⁺ alone (in the absence of PDP1) does not substantially increase the PDH activity, indicating absence of endogenous phosphatases (data not shown, but see also Figure 1).

PDH Protocol #1 – Measurement of PDH Endogenous Activity

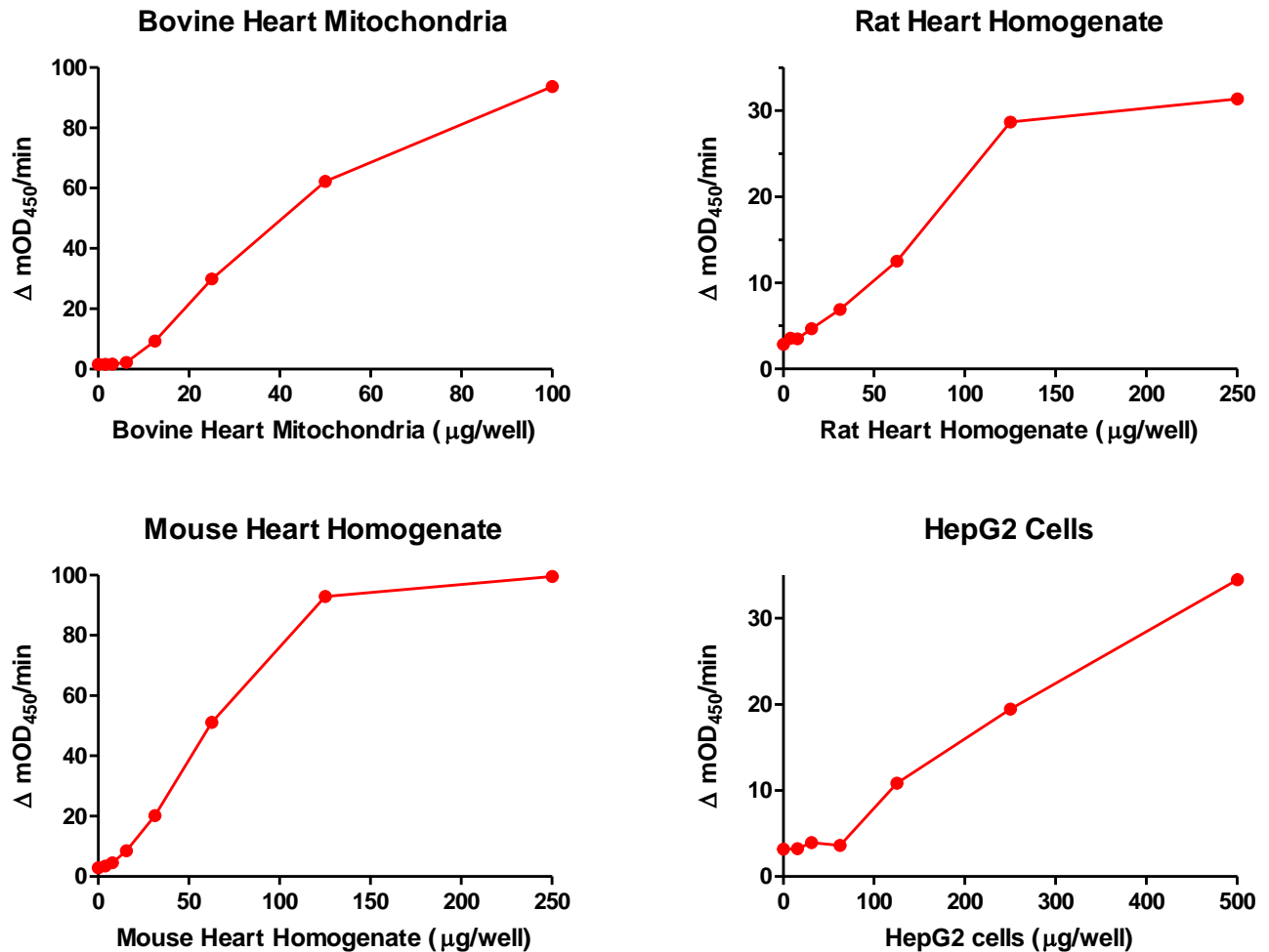


Figure 5. Determination of a linear range of PDH activity signal of bovine, rat, mouse and human PDH. Indicated amounts of extracts of bovine heart mitochondria, rat heart homogenate, mouse heart homogenate and human HepG2 cells were immunocaptured and PDH activity was measured as described in this protocol. Prior the immunocapture, the extracts of rat heart homogenate, mouse heart homogenate and human HepG2 cells were dephosphorylated with PDP1 to ensure maximum activity.