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DATA SHEET

MitoProfile® Rapid Microplate Assay Kit for PDH Activity and Quantity (Combines Kit MSP18 & MSP19)

Cat. No.MSP20

DESCRIPTION

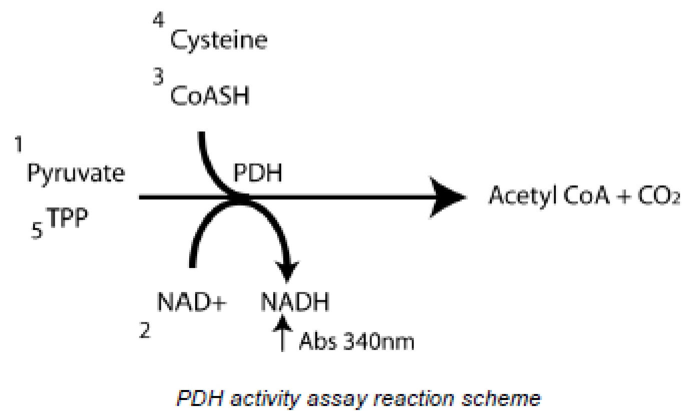
MitoProfile® Rapid Microplate Assay Kit PDH Activity (MSP18) and PDH Quantity (MSP19) Kit Contents: Included in this kit is the necessary buffer (Tube 1), Detergent for sample preparation, and Reagent Mix for the reaction. The kit contains a 96-well microplate with a monoclonal antibody pre-bound to the wells of the microplate. This plate can be broken into 12 separate 8-well strips for convenience; therefore the plate can be used for up to 12 separate experiments.

Kit Contents:

Item	MSP18
20X Buffer (Tube1)	10 mL
Detergent	1 mL
Reagent Mix	2 x 0.5 mL
96-well microplate (12 strips)	1

Storage: Tube 1, Detergent and the covered microplate should be stored at 4°C. For multiple experiments the two supplied Reagent Mix tubes must be further aliquoted and stored at -80°C. For more details on aliquoting and storage, see frequently asked questions.

INTRODUCTION The Rapid Microplate Assay for PDH Activity (MSP18) is used to determine the activity of pyruvate dehydrogenase (PDH) in a sample. The PDH enzyme is immunocaptured within the wells of the microplate and activity is determined by following the reduction of NAD+ to NADH with an increase in the absorbance at 340 nm.



PDH activity assay reaction scheme

The supplied activity Reagent Mix contains the 5 substrates as noted in the reaction scheme figure. While PDH activity can be regulated by PDH kinase and PDH phosphatase this kit does not include PDH kinase or PDH phosphatase inhibitors. These may be incorporated into the sample preparation and/or assay buffers at the researcher's discretion. This rapid PDH microplate has been developed for use with human samples. Bovine, mouse, or rat materials are also compatible. Other species have not been tested. It is recommended to prepare a crude mitochondrial sample; for details see frequently asked questions section. It is also possible to use whole tissue lysates but may require some sample optimization. The protein concentration of the sample should be measured. Once diluted to the specified concentration the sample is detergent solubilized and diluted to within the linear range of measurement. A control or normal sample should always be included in the assay as a reference measurement. In addition a buffer control should be used as a negative control. Table 1. Typical ranges of measurement:

Tissue mitochondria extract	20-100 µg / 200 µL
Cultured cell extracts†	100-1000 µg / 200 µL

† Mitochondrial PDH activity is controlled by enzyme amount and by post-translational phosphorylation at key specific regulatory serine residues. Cellular metabolism governs these two factors. Consequently, cells with different metabolic requirements, such as those derived from different tissues, vary widely in their PDH activity and amount. Cells of the same kind but cultured in different growth conditions show similar effects. For example, cells grown in glucose rich media derive most of their energy by glycolysis. Cells grown in carbon sources which promote oxidative phosphorylation (such as galactose/glutamine) upregulate mitochondrial enzymes including PDH activity. For an example see Figure 3. Ultimately the cell type and growth conditions must be chosen carefully to obtain PDH activity measurements.

Typical intra-assay variations (same day, same sample) <10% The protocol has 4 steps: A) Sample preparation **Note - methods for mitochondria OR cultured cells are described** B) Plate loading C) Measurement. D) Data Analysis

ADDITIONAL MATERIALS REQUIRED

- Spectrophotometer measuring absorbance of 340 nm
- Deionized water

- Multichannel Pipetting devices
- Protein assay method
- Phosphate buffered saline solution (PBS)

1.4 mM KH_2PO_4
8 mM Na_2HPO_4
140 mM NaCl
2.7 mM KCl, pH 7.3

MICROPLATE ASSAY PROTOCOL

A. Sample Preparation Method #1 – Tissue Mitochondria

1. Determine the sample protein concentration using a standard method such as BCA method (Pierce). Adjust the concentration of the sample to 5 mg/mL using PBS.

For details of sample solubilization conditions see frequently asked questions section.
2. Extract the proteins by adding Detergent. To do this add 1/10 of a volume of Detergent (e.g. if the total sample volume is 500 µL, add 50 µL of Detergent). Mix well. Place the tube in a rack at room temperature for 10 minutes to allow solubilization.

3. Add contents of Tube 1 (10 mL) to 190 mL deionized H_2O. Label this mixture as Solution 1. Mix.

4. Dilute all samples to the desired concentration in Solution 1. Table 1 above shows a good linear range for the assay, the table below shows a recommended mid-range value at which to load an experimental sample.

A. Sample Preparation Method #2 – Cultured cell extract

1. Resuspend the cell pellet in 0.1 mL PBS per plate of confluent cells. Determine the sample protein concentration using a standard method such as BCA method (Thermo Fisher - Pierce). Adjust the concentration of the sample to 5 mg/mL using PBS. *Approximate numbers of cells per mg of protein are given in the frequently asked questions section.*

2. Extract the proteins by adding Detergent. To do this add 1/10 of a volume of Detergent (e.g. if the total sample volume is 200 µL, add 20 µL of Detergent). Mix well. Place the tube in a rack at room temperature for 10 minutes to allow solubilization.

3. Add contents of Tube 1 (10 mL) to 190 mL deionized H_2O. Label this mixture as Solution 1. Mix.

4. Dilute all samples to the desired concentration in Solution 1. Table 1 above shows a good linear range for the assay, the table below shows a recommended mid-range value at which to load an experimental sample.

Recommended sample dilutions:

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Tissue mitochondria extract and Cultured cell extracts e.g.	50 µg / 200 µL
HepG2 (glucose)	1000 µg / 200 µL
HepG2 (galactose)	500 µg / 200 µL

B. Plate Loading

1. Add 200 µL of sample prepared in Section A4 to each well of the microplate that will be used for this experiment. Be sure to include a normal or control sample in addition to a buffer control as described in the introduction.

2. Incubate microplate for 3 hours at room temperature.

C. Measurement ***For multiple experiments the Reagent Mix should be aliquoted and stored at -80°C according to frequently asked questions.***

1. Thaw Reagent Mix.

2. Empty or aspirate the wells of the microplate (this can be accomplished by turning the plate over, shaking out the liquid, and blotting on paper towel to complete) and to each well add 300 µL of Solution 1.

3. Make Assay Solution (1.75 mL/strip) according to the following table:

No. of Strips	Reagent Mix (µL)	Solution 1 (mL)
1	84	1.67
2	167	3.33
3	250	5.00
4	333	6.67
5	417	8.33
6	500	10.0
7	583	11.67
8	667	13.33
9	750	15.00
10	833	16.67
11	917	18.33
12	1000	20.00

4. Empty the wells of the microplate.

5. Rinse all wells once more with 300 µL Solution 1.

6. Empty the wells again.

7. Add 200 µL of Assay Solution to each well carefully, to avoid bubbles. Any bubbles should be popped with a fine needle as rapidly as possible.

8. Measure the absorbance of each well at 340 nm at room temperature using a kinetic program for at least 60 minutes. The interval between readings should be as short as your reader allows but not longer than 5 minutes between reads. Incorporate a shake step between reads if possible.

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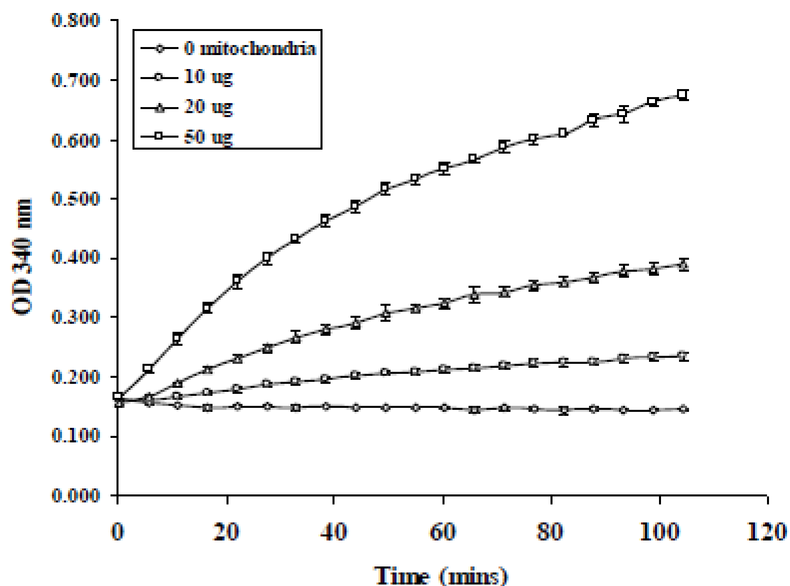
D. Data Analysis PDH activity is expressed as the initial rate of reaction, determined from the slopes of the curves generated (for example see Figure 1 below). Using the plate reader software or a spreadsheet application such as Microsoft Excel, the linear portion of the initial rate can be determined. Figure 1, an example of microplate reader recorded data:

Figure 1, an example of microplate reader recorded data:



This activity should always be related to a control or normal sample to obtain the relative activity of PDH in experimental samples. Figure 2 below shows an example of the activity of PDH captured from a bovine heart mitochondrial detergent extract. The sample was diluted to show that over this range of concentrations the signal is linear. Each sample was measured in triplicate and the low background signal was subtracted.

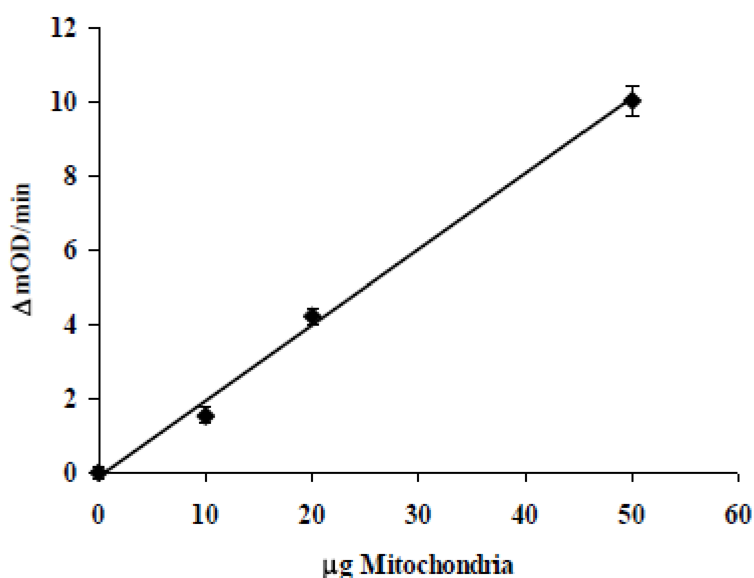


Figure 2. The rates of the initial slopes are determined as change in OD over time, and are best represented as change in milliOD per minute as shown here for heart mitochondria.

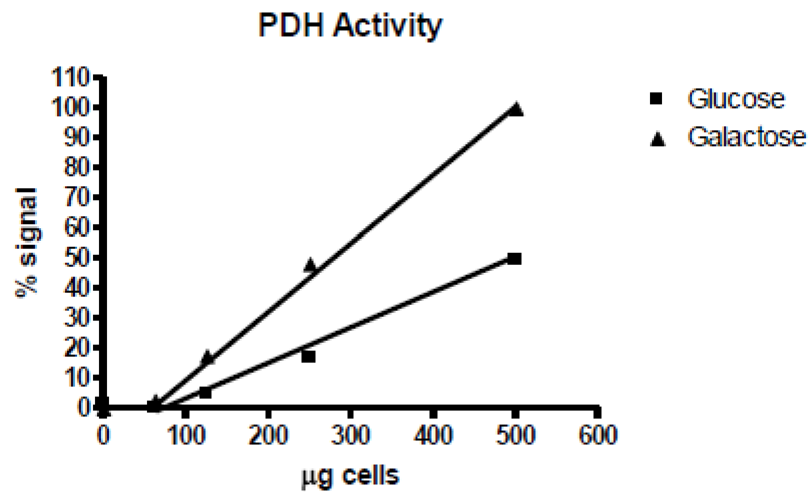
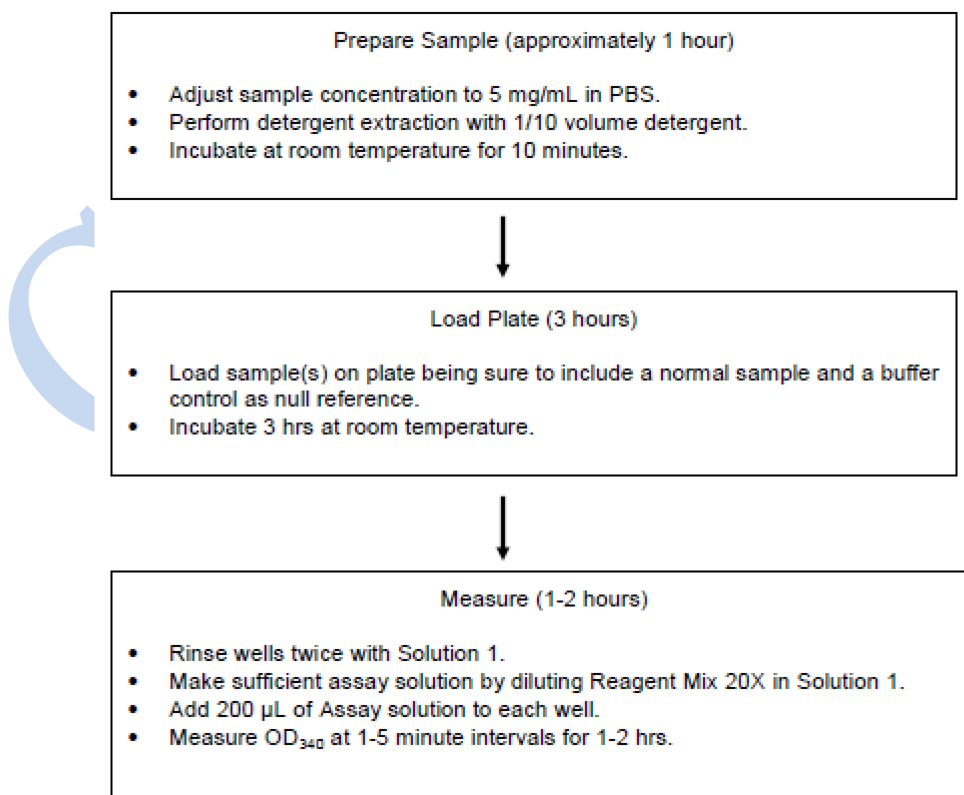


Figure 3. HepG2 cells cultured in galactose/glutamine have approximately twice as much PDH activity per mg of whole cell extract as the same cells cultured in glucose.

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



Frequently asked questions

How should I store my kit? Tube 1, the covered microplate and the detergent should be refrigerated and are stable for at least 6 months. Once Tube 1 is diluted 20X to make Solution 1 it must be used in the assay at room temperature. It can also be stored at room temperature, however for extended periods of time (>1 month) it should be stored in the refrigerator to prevent microbiological growth.

How should I store my reagent mix? Reagent mix is temperature sensitive. It must be stored at -80°C and is stable for at least six months from date of receipt. Because it is frozen it is also advisable to aliquot the reagent mix before freezing to prevent repeated freeze thaw cycles.

How should I aliquot my reagent mix? Divide the reagent mix into equal aliquots depending on how many experiments you wish to run (consult table step C3). The plate comes in 12 strips, therefore it is anticipated that up to 12 experiments on different days could be done. The reagent mix is supplied as 2 x 0.5 mL aliquots so for 12 independent experiments you could further aliquot each tube into 6 x 83 μL aliquots and immediately freeze at -80°C for storage until use.

How do I optimize sample solubilization? Mitochondria have two membrane bilayers which must be dissolved by detergent before PDH can be isolated. PDH is a huge, extremely complex, multisubunit enzyme which is sensitive to high detergent concentrations. Therefore the correct detergent:protein ratio must be used to efficiently solubilize the mitochondrial outer and inner membranes while maintaining PDH integrity. This kit contains an optimized ratio, however this ratio may need to be adjusted from sample to sample particularly when contaminated with excess extracellular proteins such as serum. Therefore, it may be necessary to adjust the concentration of detergent or isolate mitochondria.

How do I make mitochondria? We have found that little or no optimization is necessary if crude mitochondria are made from samples. Mitochondria can be prepared by simple differential centrifugation of homogenized samples as described in our mitochondrial preparation kit handbook: http://www.mitosciences.com/isolation_kits.html

How do I grow and prepare cultured cell samples? PDH activity in cells from different origins differs greatly. Cells grown in glucose have a lower activity than those grown in galactose/glutamine. Consequently cell type and growth conditions are a large factor in PDH activity measured.

Approximately how much protein is yielded from my plate of cells? We find the following typical yield of cells from a single confluent 177 cm2 plate:

Human fibroblasts	1×10^7 cells	1.5mg total protein
Human HepG2	1.5×10^7 cells	3 mg total protein

It is recommended that you accurately determine from your first confluent plate the number of cells and the total protein yield.

DESCRIPTION

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MitoProfile® Rapid Microplate Assay Kit PDH Quantity (MSP19) Kit Contents: Included in this kit is the necessary buffer (Tube 1), a microplate pre-coated with a PDH binding antibody, detergent for sample preparation, antibodies for PDH detection (Tubes A & B), and substrates for an alkaline phosphatase reaction (Tubes 2 & 3). The kit contains enough materials to perform 96 tests. Since the plate is arranged as 12 strips of 8 wells it is suggested that up to 12 separate experiments can be performed.

Kit Contents:

Item	MSP19
20X Buffer (Tube 1)	15 mL
Detergent	1 mL
Detector Antibody (Tube A)	1 mL
AP Label (Tube B)	1 mL
Development Buffer (Tube 2)	20 mL
AP Development Solution (Tube 3)	0.4 mL
96-well microplate (12 strips)	1

Storage: Tube 1, Tube A, Tube B, Tube 2, Detergent and the covered microplate should be stored at 4°C. Tube 3 must be stored at -20°C or -80°C and for multiple experiments may first be aliquoted. For more details on aliquoting and storage see frequently asked questions. When stored correctly the kit is stable for 6 months.

INTRODUCTION The Rapid Microplate Assay Kit for PDH Quantity (MSP19) is used to determine the amount of pyruvate dehydrogenase in a sample. This assay is a 'sandwich' ELISA, where the PDH enzyme is captured by an antibody on the wells of the microplate. The amount of captured enzyme is determined by adding another PDH specific antibody which is then labeled with the enzyme alkaline phosphatase (AP). This phosphatase changes the substrate pNPP from colorless to yellow (OD 405 nm) in a time dependent manner which is proportional to the amount of protein captured in the wells. This rapid PDH microplate has been developed for use with human samples but bovine, mouse, and rat materials are also compatible. Other species have not been tested. It is also possible to use whole tissue or cell lysate without the need for mitochondrial isolation. The protein concentration of the sample should be measured. Once diluted to the specified concentration the sample is detergent solubilized and diluted to within the linear range of measurement. A control or normal sample should always be included in the assay as a reference measurement. In addition a buffer control should be used as a negative control. Typical ranges of measurement:

Tissue mitochondria extract	0-5 µg / 200 µL
Cultured cell extracts†	0-200 µg / 200 µL

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† Mitochondrial PDH quantity is controlled by cellular metabolism. Consequently, cells with different metabolic requirements, such as those derived from different tissues, vary widely in their PDH amount. Cells of the same kind but cultured in different growth conditions show similar effects. For example, cells grown in glucose rich media derive most of their energy by glycolysis. Cells grown in carbon sources which promote oxidative phosphorylation (such as galactose/glutamine) upregulate mitochondrial enzymes including PDH. Ultimately, the cell type and growth conditions must be chosen carefully to obtain PDH quantity measurements.

Typical intra-assay variations (same day, same sample) <15% The protocol has 4 steps: A) Sample preparation B) Plate loading C) Antibody addition D) Measurement and data analysis

ADDITIONAL MATERIALS REQUIRED

- ☐ Spectrophotometer measuring absorbance at 405 nm (alternatively 450 nm is acceptable)
- ☐ Deionized water
- ☐ Multichannel Pipetting devices
- ☐ Protein assay method
- ☐ Phosphate buffered saline solution (PBS)

1.4 mM KH2PO,4 8 mM Na2HPO4, 140 mM NaCl, 2.7 mM KCl, pH 7.3

MICROPLATE ASSAY PROTOCOL

A. Sample Preparation Method #1 – Tissue Mitochondria

1. Determine the sample protein concentration using a standard method such as BCA method (ThermoFisher - Pierce). Adjust the concentration of the sample to 5 mg/mL using PBS.

For details of sample solubilization conditions see frequently asked questions section.

2. Extract the proteins by adding Detergent. To do this add 1/10 of a volume of Detergent (e.g. if the total sample volume is 500 µL, add 50 µL of Detergent). Mix well. Place the tube in a rack at room temperature for 10 minutes to allow solubilization.

3. Add contents of Tube 1 (15 mL) to 285 mL deionized H2O. Label this mixture as Solution 1. Mix.

4. Dilute all samples to the desired concentration in Solution 1. The table above shows a good linear range for the assay, the table below shows a recommended mid-range value at which to load an experimental sample.

A. Sample Preparation Method #2 – Cultured cell extract

1. Resuspend the cell pellet in 0.1 mL PBS per plate of confluent cells. Determine the sample protein concentration using a standard method such as BCA method (ThermoFisher - Pierce).

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Adjust the concentration of the sample to 5 mg/mL using PBS. *Approximate numbers of cells per mg of protein are given in the frequently asked questions section.*

2. Extract the proteins by adding Detergent. To do this add 1/10 of a volume of Detergent (e.g. if the total sample volume is 200 µL, add 20 µL of Detergent). Mix well. Place the tube in a rack at room temperature for 10 minutes to allow solubilization.

3. Add contents of Tube 1 (15 mL) to 285 mL deionized H2O. Label this mixture as Solution 1. Mix.

4. Dilute all samples to the desired concentration in Solution 1. The table above shows a good linear range for the assay, the table below shows a recommended mid-range value at which to load an experimental sample.

Recommended sample dilutions:

Culture cell extracts	20 µg / 200 µL
	10 µg / 200 µL
	5 µg / 200 µL
Tissue mitochondria extract	1 µg / 200 µL
Tissue extract	5 µg / 200 µL

B. Plate Loading

3. Add 200 µL of sample prepared in Section A4 to each well of the microplate that will be used for this experiment. Be sure to include a normal or control sample in addition to a buffer control as described in the introduction.

4. Incubate microplate for 3 hours at room temperature.

C. Antibody Addition ***For multiple experiments the Tube 3 should be aliquoted and stored at -20°C/-80°C according to frequently asked questions.***

1. The bound monoclonal antibody has immobilized the enzyme in the wells. Empty/aspirate the wells (this can be accomplished by turning the plate over and shaking out the liquid. Blot on paper towel to complete aspiration).

2. Add 300 µL of Solution 1 to each well.

3. Add 1 mL antibody from Tube A to 20 mL of Solution 1. Label as Solution A.

If using only a portion of the strips make only a proportionate amount of solution A.
4. Empty/aspirate the wells and add 200 µL of Solution A to each well used. Incubate 1 hour at room temperature.

5. Empty/aspirate the wells and add 300 µL of Solution 1 to each well.

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6. Add 1 mL antibody Tube B to 20 mL of Solution 1. Label as Solution B.

Again, if using only a portion of the strips make only a proportionate amount of Solution B.
7. Empty/aspirate the wells and add 200 µL of Solution B to each well used. Incubate 1 hour at room temperature.

8. Empty/aspirate the wells and add 300 µL of Solution 1 to each well used. Repeat this twice more.

9. Add the 0.4 mL from Tube 3 to 20 mL from Tube 2. Label as Development Solution.

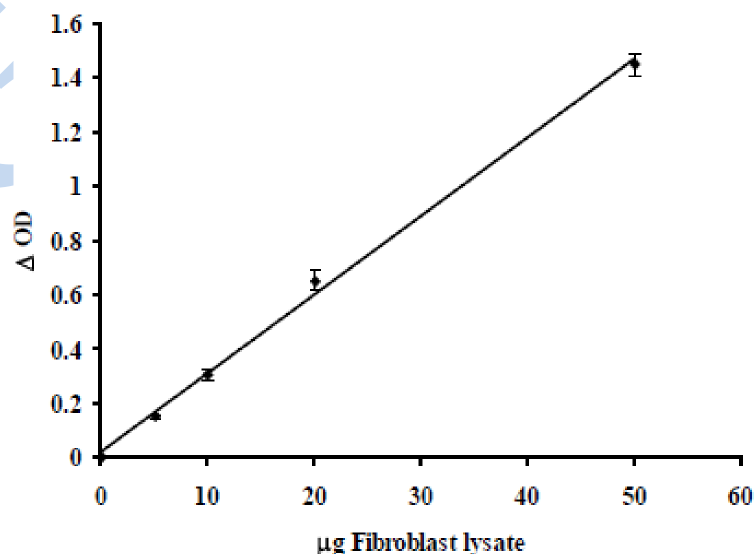
If using only a portion of the strips make only a proportionate amount of development solution. See frequently asked questions for examples.

10. Empty/aspirate the wells and add 200 µL of development solution to each well used.

11. Measure the absorbance of each well at 405 nm (or 450 nm) at room temperature using a kinetic program for at least 30 minutes. The interval between readings should be as short as your reader allows but not longer than 2 minutes between reads. Incorporate a shake step between reads if possible.

An endpoint measurement at 405 nm or 450 nm is also possible at a time defined by the user when a yellow color development in specific wells is acceptable.

D. Data Analysis Examine the color development over time in each well. Under the conditions stated above the color development should be linear over the 30 minute time period of measurement. Subtract the initial absorbance reading from the final absorbance reading to determine the quantity of PDH in each well. This quantity should always be related to a control or normal sample to obtain the relative quantity of PDH in experimental samples. Below is an example of the quantity of PDH capture from a fibroblast cell lysate. The sample was diluted to show that over this range of concentrations the signal is linear. Each sample was measured in triplicate and the low background signal was subtracted.



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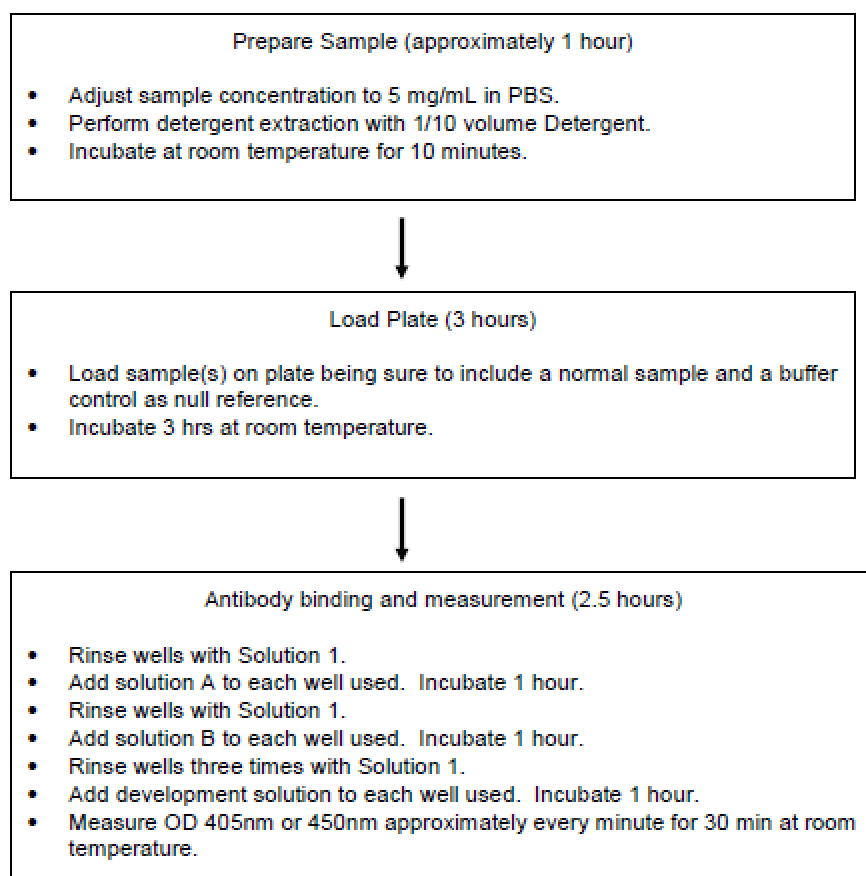

Frequently asked questions *How should I store my kit?* Tube 3 should be stored frozen at -20°C or preferably -80°C and is stable for 6 months. Tube 3 can first be aliquoted if desired to prevent freeze thaw cycling. All other components in the kit should be stored at 4°C in a refrigerator. Once Tube 1 is diluted 20X to make Solution 1 it can be stored at room temperature, however for extended periods of time (>1 month) it should be stored in the refrigerator to prevent microbiological growth. *How should I aliquot my Tube 3?* Divide the Tube 3 into equal aliquots depending on how many experiments you wish to run (consult table below). The plate comes in 12 strips, therefore it is anticipated that up to 12 experiments on different days could be done. Tube 3 is supplied as 0.4 mL aliquots so for 12 independent experiments you could aliquot the tube into 12 x 33 µL aliquots and immediately freeze until use. *How much Development Buffer should I prepare?* Depending on how many strips you are using prepare the following :

No. of Strips	Tube 3 (µL)	Tube 2 (mL)
1	33	1.67
2	66	3.33
3	100	5.00
4	133	6.67
5	166	8.33
6	200	10.0
7	233	11.67
8	266	13.33
9	300	15.00
10	333	16.67
11	366	18.33
12	400	20.00

How do I grow and prepare cultured cell samples? The amount of PDH in cells from different origins differs greatly. Cells grown in glucose have a lower activity than those grown in galactose/glutamine. Consequently cell type and growth conditions are a large factor in PDH activity measured. *Approximately how much protein is yielded from my plate of cells?* We find the following typical yield of cells from a single confluent 177 cm2 plate:

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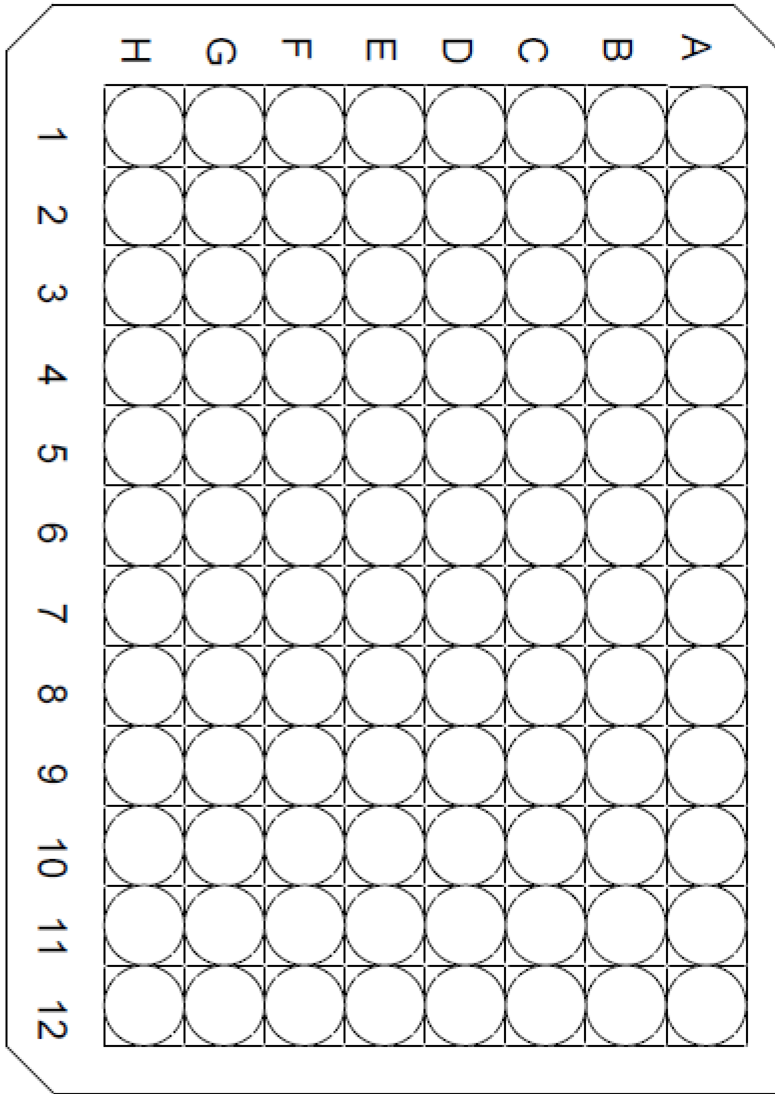

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