ab109902 Pyruvate Dehydrogenase (PDH) Enzyme Activity Microplate Assay Kit

Instructions for use:

For the analysis of PDH enzyme activity from human, rat, mouse and bovine cell and tissue extracts.

This product is for research use only and is not intended for diagnostic use.

Version 6c Last Updated 19 January 2024

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1. BACKGROUND

Pyruvate Dehydrogenase (PDH) Enzyme Activity Microplate Assay Kit (ab109902) is designed for the analysis of pyruvate dehydrogenase (PDH) activity from human, rat, mouse and bovine cell and tissue extracts.

This kit recognizes PDH in human, rat, mouse and bovine cell and tissue extracts and isolated mitochondria. Each of the 96 wells in the kit has been coated with an anti-PDH monoclonal antibody (mAb) which is able to capture fully-intact functionally-active enzyme complex. PDH activity is then determined by following the reduction of NAD+ to NADH, coupled to the reduction of a reporter dye to yield a colored (yellow) reaction product whose concentration can be monitored by measuring the increase in absorbance at OD450 nm (figure 1).

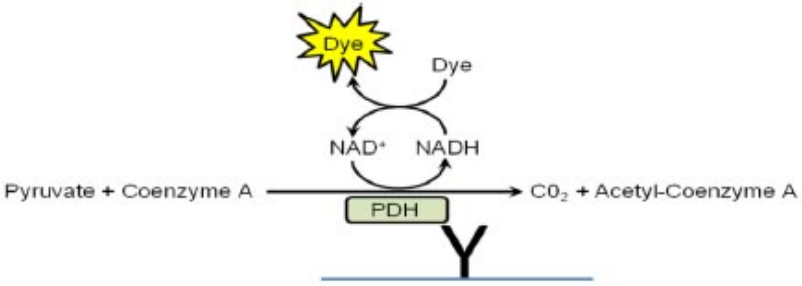
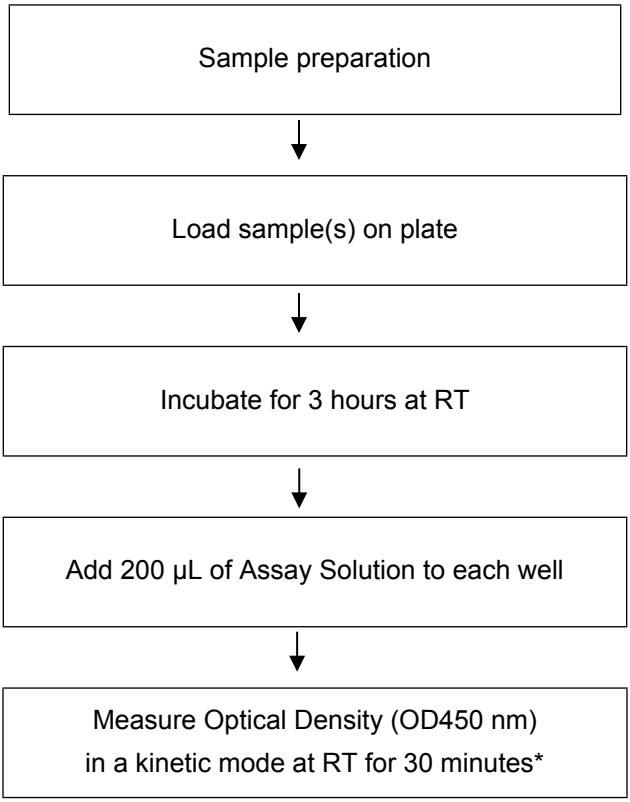


Figure 1: PDH activity assay reaction scheme.

By analyzing the enzyme's activity in an isolated context, outside of the cell and free from any other variables, an accurate measurement of the enzyme's functional state can be understood.

PDH is the key regulatory enzyme of cellular metabolism because it links the TCA cycle and subsequent oxidative phosphorylation with glycolysis and gluconeogenesis as well as with both lipid and amino acid metabolism. PDH activity is regulated by PDK-dependent phosphorylation and PDP-dependent dephosphorylation of PDH. Phosphorylation inactivates PDH whereas dephosphorylation activates PDH.

2. ASSAY SUMMARY



**For kinetic mode detection, incubation time given in this summary is for guidance only.*

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handle with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. STORAGE AND STABILITY

This product contains components that need to be stored at different temperatures. Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Aliquot components in working volumes before storing at the recommended temperature.

5. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)	Storage Condition (After Preparation)
20X Buffer	15 mL	4°C	4°C
Detergent	2 x 1 mL	4°C	4°C
20X Reagent Mix	2 x 600 µL	-80°C	-80°C
5X Stabilizer	13 mL	-80°C	-80°C
Coupler	250 µL	-80°C	-80°C
Reagent Dye	250 µL	-80°C	-80°C
96-well microplate (12 x 8 well strips)	1	4°C	4°C

7. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully perform this assay:

- Microplate reader capable of measuring absorbance at OD 450 nm.
- MilliQ water or other type of double distilled water (ddH_2O)
- PBS solution
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- 96 well plate with clear flat bottom
- Dounce homogenizer (if using tissue)
- Method for determining protein concentration: we recommend BCA Protein Quantification Kit (ab102536)
- (Optional) Protease inhibitors: we recommend Protease Inhibitor Cocktail (EDTA-free) (ab201111)
- (Optional) Phosphatase inhibitors: we recommend NaF (sodium fluoride)

For mitochondria isolation:

- Mitochondria Isolation Kit for Cultured Cells (ab110170)
- Mitochondria Isolation Kit for Tissue (ab110168) or Mitochondria Isolation Kit for Tissue (with Dounce Homogenizer) (ab110169)

8. TECHNICAL HINTS

- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.
- Samples which generate values that are greater than the most concentrated standard should be further diluted in the appropriate sample dilution buffer.
- Ensure plates are properly sealed or covered during incubation steps.
- Make sure you have the right type of plate for your detection method of choice.
- Make sure all necessary equipment is switched on and set at the appropriate temperature.

9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening

9.1. **20X Buffer:**

Prepare 1X Buffer by diluting 20X Buffer in ddH_2O: to make 200 mL 1X Buffer, combine 10 mL 20X Buffer with 190 mL ddH_2O. Mix thoroughly and gently. Label this mixture as "**Buffer**". Buffer can be stored at 4°C. Equilibrate to room temperature before use.

9.2. **Detergent:**

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C.

9.3. **20X Reagent Mix:**

Ready to use as supplied. Thaw immediately prior use. Aliquot reagent mix so that you enough volume to perform the desired number of assays. Avoid repeated freeze/thaw. Store at - 80°C.

9.4. **5X Stabilizer:**

Prepare 1X Stabilizer by mixing 1 volume of thawed 5X Stabilizer with 4 volumes of 1X Buffer.

Aliquot stabilizer so that you have enough volume to perform the desired number of assays. Avoid repeated freeze/thaw. Store at -80°C. Keep on ice while in use.

9.5. **Coupler:**

Ready to use as supplied. Aliquot coupler so that you have enough volume to performed the desired number of assays. Avoid repeated freeze/thaw, Store at -80°C. Keep on ice while in use.

9.6. **Reagent Dye:**

Ready to use as supplied. Keep on ice while in use. Store at -80°C.

9.7. **96-well microplate (12 x 8-well strips):**

Ready to use as supplied. This plate can be broken into 12 separate 8-well strips for convenience. Equilibrate to room temperature before use. Store at 4°C.

10. SAMPLE PREPARATION

General Sample Information

- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you snap freeze cells or tissue in liquid nitrogen upon extraction (once protein concentration has been determined) and store the samples immediately at -80°C. When you are ready to test your samples, thaw them on ice and continue with the detergent extraction procedure. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.
- Add protease and phosphatase inhibitors to sample preparation buffer immediately prior to use.
- Protein concentration of the sample must be measured prior to sample solubilization.
- PDH activity in cells or tissue from different origins differs greatly. Cell type and growth conditions are a large factor in PDH activity. For example, cells grown in glucose have lower activity than those grown in galactose/glutamine.
- Treat cells with PDH activators/inhibitors as per your experimental requirements: Dichloroacetate (PDK inhibitor) treatment leads to PDH activation.
- To preserve the phosphorylated status of endogenous PDH, add 10 mM NaF to sample buffer and/or sample extract.

10.1. **Preparation of extracts from cells (adherent or suspension):**

10.1.1. Harvest suspension cells by centrifugation or scrape to collect adherent cells from a confluent culture flask.

10.1.2. Wash cells twice with PBS.

10.1.3. Resuspend and dilute the cell pellet with 9 volumes of PBS (e.g. 50 µL pellet + 450 µL PBS to a total volume of 500 µL).

10.1.4. Determine the sample protein concentration (using a standard method such as BCA). Take a small aliquot of your unlysed cells and add a detergent, such as SDS (final 0.25%). Perform a BCA to determine sample protein concentration. Adjust the remaining amount with PBS to yield a final sample protein concentration of **15 mg/mL.**

Table 1 shows the typical protein yield of cells we obtain from a single confluent 177 cm^2 plate/T175 flask:

Cell type	Cell number	Total Protein	Suggested number plates
Human fibroblasts	1 x 10^7 cells	1.5 mg/plate	10
Human HepG2	2 x 10^7 cells	3 mg/plate	5

Table 1. Typical yield of cells and total protein from a confluent 177 cm^2 plate.

We recommend that you accurately determine from your first confluent plate the number of cells and the total protein yield.

10.1.5. Extract the proteins from the sample by adding Detergent solution to sample to a final dilution of 1/10 (e.g. if the total sample volume is 500 µL, add 50 µL of Detergent solution). Mix well.

10.1.6. Incubate the tube on ice for 10 minutes to allow solubilization.

10.1.7. Centrifuge the sample for 10 minutes at 4°C at 1,000 x *g* in a cold centrifuge.

10.1.8. Collect supernatant and transfer to a clean tube. Keep samples on ice. Please note the sample concentration now is **13.5 mg/mL**. This is the optimal concentration for intact PDH complex solubilization.

10.1.9. Dilute your samples to the desired concentration in 1X Buffer (from step 9.1). Table 2 indicates a typical linear range for the assay.

10.2. **Preparation of extracts from tissue:**

10.2.1. Harvest tissue for the assay (initial recommendation = 100 – 200 mg).

10.2.2. Wash tissue thoroughly in cold PBS to remove blood.

10.2.3. Resuspend tissue in 500 µL – 1 mL of ice cold PBS.

10.2.4. Homogenize tissue with a Dounce homogenizer sitting on ice, with 20 – 40 passes, or until sample is fully homogenized and is completely smooth. ***NOTE:*** *it is very important to achieve a thorough homogenization as sample must be completely homogenous.*

10.2.5. Determine the sample protein concentration (using a standard method such as BCA) by extracting a portion of your sample. Adjust concentration of the sample with PBS so that the final sample protein concentration is **23.7 mg/mL**.

10.2.6. Extract the proteins from the sample by adding Detergent solution to sample to a final dilution of 1/20 (e.g. if the total sample volume is 500 µL, add 25 µL of Detergent solution). Mix well.

10.2.7. Incubate the tube on ice for 10 minutes to allow solubilization.

10.2.8. Centrifuge the sample for 10 minutes at 4°C at 1,000 x *g* in a cold centrifuge.

10.2.9. Collect supernatant and transfer to a clean tube. Keep samples on ice. Please note the sample concentration now is **22.5 mg/mL**.

10.2.10. Dilute your samples to the desired concentration in 1X Buffer (from step 9.1). Table 2 indicates a typical range for the assay.

10.3. **Preparation of isolated mitochondria:**

You can isolate mitochondria using mitochondrial isolation kits such Mitochondria Isolation Kit for Cultured Cells (ab110170) or

Mitochondria Isolation Kit for Tissue (with Dounce Homogenizer) (ab110169).

Alternatively, mitochondria can be prepared by simple differential centrifugation of homogenized tissue samples – please see Section 17 for a general mitochondrial purification protocol.

10.3.1. Determine the sample protein concentration (using standard methods such as BCA) by extracting a portion of your sample. Adjust concentration of the sample with PBS so that the final sample protein concentration is **5.3 mg/mL**.

10.3.2. Extract the proteins from the sample by adding Detergent solution to sample to a final dilution of 1/20 (e.g. if the total sample volume is 500 µL. add 25 µL of Detergent solution). Mix well.

10.3.3. Incubate the tube on ice for 10 minutes to allow solubilization.

10.3.4. Centrifuge the sample for 10 minutes at 4°C at 5,000 x *g* in a cold centrifuge.

10.3.5. Collect supernatant and transfer to a clean tube. Keep samples on ice. Please note the sample concentration now is **5 mg/mL**.

10.3.6. Dilute your samples to the desired concentration in 1X Buffer (from step 9.1). Table 2 indicates a typical range for the assay.

Sample Type	Recommended Sample dilutions (µg/200 µL volume well)
Culture cell extracts	100 – 1000 µg
Whole tissue extracts	20 – 100 µg
Mitochondria extracts	10 – 100 µg

Table 2. Typical range of measurement per assay. Shows the linear working range for the assay using various samples. The working range for your sample should be confirmed by testing a representative reference control sample at a series of dilutions across the expected working range. Results from individual

experimental samples can then be compared directly when tested at concentrations within the working range.

11. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to correct temperature prior to use.**
- **We recommended to assay all controls and samples in duplicate.**
- **Prepare all reagents and samples as directed in the previous sections.**

11.1. **Plate Loading**:

Sample wells = 200 µL of sample (as prepared in Section 10).

Background wells = 200 µL sample buffer.

11.1.1. Cover plate and incubate for 3 hours at room temperature.

11.2. **Prepare Assay Solution:**

In a sealable tube, prepare only enough solution proportional to the number of microplate strips used according to the following table. Mix gently by inversion. ***NOTE:*** *thaw 20X Reagent mix immediately prior use as described in section 9.3.*

Number of strips	20X Reagent Mix (µL)	1X Buffer (mL)	Coupler (µL)	Reagent Dye (µL)
1	88	1.63	18	18
2	175	3.25	35	35
3	263	4.89	53	53
4	350	6.51	70	70
5	438	8.14	87	87
6	525	9.77	105	105
7	612	11.39	123	123
8	700	13.02	140	140
9	788	14.65	158	158
10	875	16.28	175	175
11	963	17.90	192	192
12	1050	19.53	210	210

11.3. **Measurement:**

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

11.3.2. Add 300 µL of 1X Stabilizer (Section 9.4) to each well used.

11.3.3. Empty the wells of the microplate by turning the plate over and shaking out any remaining liquid. Blot the plate face down on paper towel. Rinse all wells once more with 300 µL 1X Stabilizer.

11.3.4. Empty the wells again.

11.3.5. Add 200 µL of Assay solution to each well, carefully avoiding production of bubbles. Any bubbles in the wells should be popped with a fine needle as rapidly as possible.

11.3.6. Place the plate in the reader and record with the following kinetic program.

Mode	Kinetic
Wavelength:	450 nm
Time:	15 - 30 minutes
Interval:	20 sec - 1 min*
Shaking:	Shake between readings
Temperature	Room temperature

**The interval between readings should be as short as your reader allows but not longer than 1 minute between reads.*

***NOTE:** Sample incubation time can vary depending on enzyme activity in the samples.*

11.4. Save data and analyze as described in the “Data Analysis” section.

12. CALCULATIONS

- PDH activity is expressed as the initial rate of reaction, determined from the slopes of the curves generated at OD = 450 nm (see Figure 1). Monitor the rate of increase in absorbance at 450 nm over time. Calculate the rate between two time points for all the samples where the increase in absorbance is the most linear.

$$\text{Rate (mOD/min)} = \frac{\text{Absorbance 2} - \text{Absorbance 1}}{\text{Time (minutes)}}$$

- This assay is compatible with different sample types such as mitochondria, tissue or cell lysates and in multiple species including human, bovine and rodent samples. Typical linear range data are shown below in Figure 1.

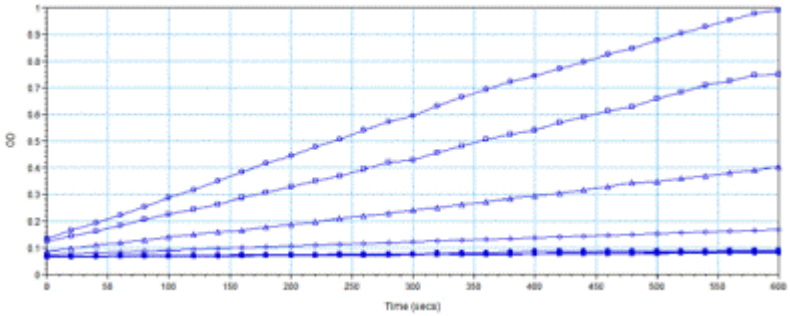


Figure 1: Example of raw data. Bovine heart mitochondria were loaded at 100 µg/well (top trace) and at 2-fold subsequent dilutions (stepwise lower traces). Activity should always be related to a control or normal sample to obtain the relative activity of PDH in experimental samples.

TYPICAL SAMPLE VALUES

REPRODUCIBILITY –

	Intra Assay
n=	60
CV (%)	<10

13. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.

This assay is compatible with different sample types such as mitochondria, tissue or cell lysates and in multiple species including human, bovine and rodent samples. Typical linear range data are shown below in Figure 2.

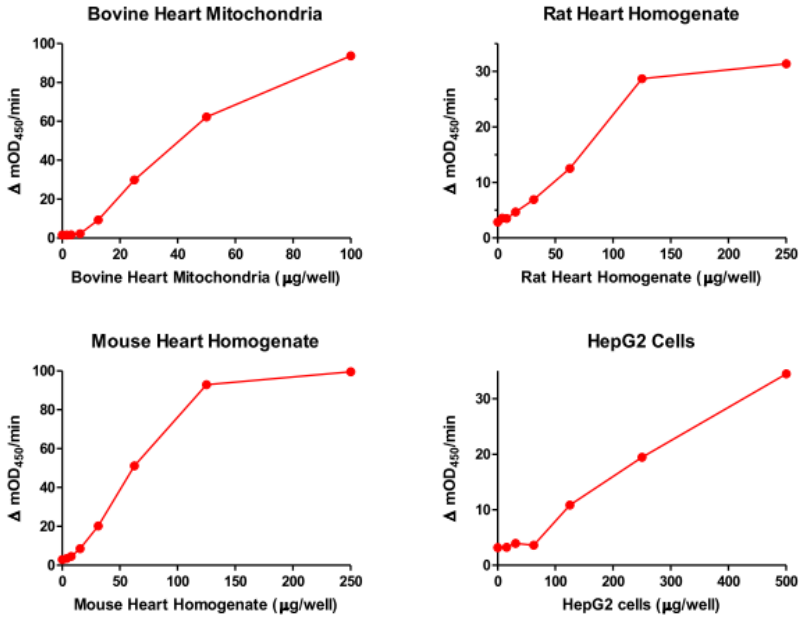


Figure 2: Mitochondria, tissue extracts and whole cultured cell extracts show linear relationships between signal and sample load at limiting concentrations. The rates shows were determined as change in OD over time, and these are best represented as change in milliOD per minute.

14. QUICK ASSAY PROCEDURE

NOTE: This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.

Prepare Sample (1 hours)

- Bring sample to appropriate concentration in PBS [15 mg/mL for culture cells; 23.7 mg/mL for whole tissues and 5.3 mg/mL for mitochondria].
- Perform detergent extraction with appropriate amount of Detergent [1/10 for cultured cells; 1/20 for whole tissues and purified mitochondria]

Load Plate (3 hours)

- Load sample(s) on plate being sure to include positive control sample and buffer control as a null reference.
- Incubate 3 hours at room temperature.

Measure (1 hour)

- Rinse wells twice with 1X stabilizer.
- Make sufficient Assay Solution to load 200 µL/well.
- Add 200 µL Assay Solution into each well.
- Measure OD 450 nm at 20 second intervals for up to 30 minutes.

15. TROUBLESHOOTING

Problem	Cause	Solution
Assay not working	Use of ice-cold buffer	Buffers must be at room temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different 96-well plate	Colorimetric: Clear plates Fluorometric: black wells/clear bottom plate
Sample with erratic readings	Samples not deproteinized (if indicated on protocol)	Use deproteinization protocol provided
	Cells/tissue samples not homogenized completely	Use Dounce homogenizer, increase number of strokes
	Samples used after multiple free/ thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Use of old or inappropriately stored samples	Use fresh samples or store at -80°C (after snap freeze in liquid nitrogen) till use
	Presence of interfering substance in the sample	Check protocol for interfering substances; deproteinize samples
Standard readings do not follow a linear pattern	Pipetting errors in standard or reaction mix	Avoid pipetting small volumes (< 5 µL) and prepare a master mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the tubes
	Standard stock is at incorrect concentration	Always refer to dilutions on protocol
Unanticipated results	Measured at incorrect wavelength	Check equipment and filter setting
	Samples contain interfering substances	Troubleshoot if it interferes with the kit
	Sample readings above/ below the linear range	Concentrate/ Dilute sample so it is within the linear range

16. FAQs

Can I use frozen samples?

You can freeze cell pellets, but we would recommend working rapidly since metabolism is a function of media and supplements that feed the cells. Removal of those can change the metabolic state of the cell so harvesting and washing cells should be done rapidly and cell pellets should be transferred to colder temperatures as soon as possible.

When do I need to use phosphatases inhibitors?

Phosphatase inhibitors are only necessary if you want to measure true endogenous PDH activity. The PDH enzyme is typically dephosphorylated during the sample preparation step unless phosphatase inhibitors are added. A dephosphorylated PDH enzyme is highly active.

If you want to add phosphatase inhibitors, we recommend adding 10 mM NaF (sodium fluoride) to your sample buffer/extract.

Can you recommend any positive controls?

Any of the lysates mentioned below can be used as positive control in this assay:

ab110338 – Bovine Heart Mitochondrial lysate

ab110346 – Rat Liver Mitochondrial lysate

ab110347 – Rat Heart Mitochondrial lysate

ab113048 – Rat Brain Mitochondrial lysate

ab110349 – Mouse Liver Mitochondrial lysate

ab110350 – Mouse Heart Mitochondrial lysate

ab110351 – Mouse Brain Mitochondrial lysate

17. APPENDIX – MITOCHONDRIAL PURIFICATION PROTOCOL

Mitochondrial Purification Protocol

Reagents needed:

NKM buffer

- 1 mM Tris HCl, pH 7.4
- 0.13 M NaCl
- 5 mM KCl
- 7.5 mM MgCl2

Homogenization buffer

- 10 mM Tris-HCl
- 10 mM KCl
- 0.15 mM $MgCl_2$
- 1 mM PMSF
- 1 mM DTT

Always add PMSF and DTT immediately before use to NKM and homogenization buffer.

Mitochondrial suspension buffer

- 10 mM Tris HCl, pH 6.7
- 0.15 mM $MgCl_2$
- 0.25 mM sucrose
- 1 mM PMSF

Procedure

17.1. Collect cells by centrifugation at approximately 370 *x g* for 10 min. Decant supernatant and resuspend cells in 10 packed cell volumes of NKM buffer.

17.2. Pellet cells and decant supernatant, repeat this washing step two times. Resuspend cells in 6 packed cell volumes of homogenization buffer.

17.3. Transfer cells to a glass homogenizer and incubate for 10 min on ice. Using a tight pestle homogenize the cells. Check under the microscope for cell breakage, the optimum is around 60%. This may require 30 strokes or so of the pestle.

17.4. Pour homogenate into a conical centrifuge tube containing 1 packed cell volume of 2 M sucrose solution and mix gently.

17.5. Pellet unbroken cells, nuclei and large debris at 1,200 *x g* for 5 min and transfer the supernatant to another tube. This treatment is repeated twice, transferring the supernatant to a new tube each time, discarding the pellet.

17.6. Pellet the mitochondria by centrifuging at 7,000 *x g* for 10 min. Resuspend the mitochondrial pellet in 3 packed cell volumes of mitochondrial suspension buffer. Mitochondria are ready to use.

18. NOTES

For all technical or commercial enquiries please go to:

www.abcam.com/contactus
www.abcam.cn/contactus (China)
www.abcam.co.jp/contactus (Japan)