ab136809 – Complex I Rodent ELISA Profiling Kit

Instructions for Use

For the measurement of complex I levels in rodent tissue extracts.

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

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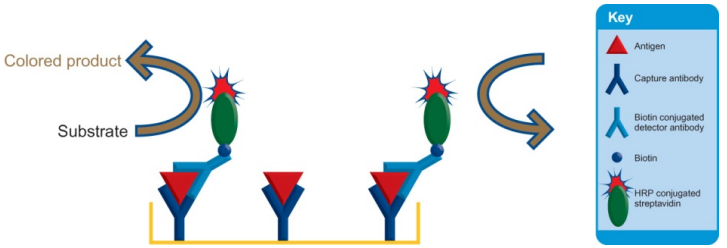
1. Introduction

Principle: ab136809 NADH dehydrogenase (Complex I) rodent profiling kit is an in-vitro enzyme-linked immunosorbent assay (ELISA) for the comparison of NADH dehydrogenase levels or profile in tissue lysates. The assay employs an NADH dehydrogenase specific antibody coated onto microplate well strips. Samples are pipetted into the wells and NADH dehydrogenase present in the sample is bound to the wells by the immobilized antibody. The wells are washed and a biotin labeled anti-NADH dehydrogenase detector antibody is added. After washing away unbound detector antibody, biotin affinity HRP conjugated streptavidin is pipetted into the wells. The wells are again washed, an HRP substrate solution (TMB) is added to the wells and color develops in proportion to the amount of NADH dehydrogenase (Complex I) bound. The developing blue color is measured at 600 nm. Optionally the reaction can be stopped by adding hydrochloric acid which changes the color from blue to yellow and the intensity can be measured at 450 nm.



Background:

NADH dehydrogenase (Complex I) is the first enzyme of the oxidative phosphorylation (OXPHOS) system within the mitochondrial inner membrane. NADH dehydrogenase is a large protein complex of 950,000 MW made up of 45-46 different subunits. Seven of the subunits of the complex are encoded on mitochondrial DNA (mtDNA), the remaining subunits are nuclear encoded, made in the cytosol and translocated into the organelle for assembly at the inner membrane. The enzyme complex catalyses electron entry from NADH via a flavin (FMN) and several non-heme iron centers. Mutations in mtDNA, or nuclear DNA genes encoding NADH dehydrogenase subunits or assembly factors are a common cause of genetic OXPHOS defects. Mutations or loss of mtDNA may cause enzymatic dysfunction by disrupting enzyme assembly or alternatively by specifically affecting enzymatic activity with no effect on enzyme assembly.

NADH dehydrogenase (like Complex III) has been proposed as a site of superoxide ‘leak’ from the mitochondrial OXPHOS system. Altered functioning and increased superoxide production by this complex has been proposed to contribute to several neurological disorders including Parkinson's disease. Also there is evidence of NADH Dehydrogenase involvement in diabetes.

2. Assay Summary

Equilibrate all reagents to room temperature. Prepare all the reagents, samples, and standards as instructed.

Add 50 µL sample to each well used. Incubate 2 hours at room temperature.

Aspirate and wash each well three times. Add 50 µL detector antibody to each well. Incubate 1 hour at room temperature.

Aspirate and wash each well three times. Add 50 µL prepared HRP label. Incubate 1 hour at room temperature.

Aspirate and wash each well three times. Add 50 µL HRP Development Solution to each well. Immediately begin recording the color development with elapsed time at 600 nm for 15 minutes. Alternatively add a Stop solution at a user-defined time and read at 450 nm.

3. Kit Contents

Item	Quantity
20X Buffer	20 mL
Extraction Buffer	15 mL
10X Blocking Buffer	6 mL
HRP Development Solution	12 mL
10X NADH Dehydrogenase Detector Antibody	1 mL
10X HRP Label	1 mL
NADH Dehydrogenase Microplate (12 x 8 coated microplate well strips)	96 Wells

4. Storage and Handling

Store all components at 4°C. This kit is stable for 6 months from receipt. After reconstitution the standard should be aliquoted and stored at -80°C. Unused microplate strips should be returned to the pouch containing the desiccant and resealed.

5. Additional Materials Required

- Microplate reader capable of measuring absorbance at 600 nm (or 450 nm after addition of Stop solution - not supplied.
- Method for determining protein concentration (BCA assay recommended).
- Deionized water
- Multi- and single-channel pipettes
- PBS (1.4 mM KH_2PO4, 8 mM Na_2HPO_4, 140 mM NaCl, 2.7 mM KCl, pH 7.3)
- Tubes for standard dilution
- Stop solution (optional) – 1N hydrochloric acid
- Plate shaker (Optional) for all incubation steps
- Well plate cover or seals

6. Preparation of Reagents

6.1 Equilibrate all reagents to room temperature (18-25°C) prior to use.

6.2 Prepare 1X Wash Buffer by adding 20 mL 20X Buffer to 380 mL nanopure water.

6.3 Prepare 1X Incubation Buffer by adding 6 mL 10X Blocking Buffer to 54 mL 1X Wash Buffer. Excess unused 1X Incubation Buffer may be stored at -20°C for 6 months after performing the ELISA.

6.4 Prepare the 1X NADH Dehydrogenase Detector Antibody by diluting the 10X NADH Dehydrogenase Detector Antibody 1:10 with 1X Incubation Buffer immediately prior to use. Prepare 500 µL for each 8 well strip used.

6.5 Prepare the HRP labeled secondary antibody by diluting the 10X HRP Label 1:10 in 1X Incubation Buffer immediately before use. Prepare 500 µL for each 8 well strip used.

7. Sample Preparation

Note: The extraction buffer should be supplemented with either 10 mM Sodium Fluoride or a phosphatase inhibitor cocktail. It may also be supplemented with PMSF and/or protease inhibitor cocktail prior to use. Supplements should be used according to manufacturer's instructions.

7.1. Tissue lysates

7.1.1. Tissue lysates are typically prepared by homogenization of tissue that is first minced and thoroughly rinsed in PBS to remove blood (dounce homogenizer recommended).

7.1.2. The sample protein concentration in the homogenate may be quantified using a protein assay.

7.1.3. Suspend the homogenate to 25 mg/mL in PBS.

7.1.4. Solubilize the homogenate by adding 4 volumes of Extraction Buffer to one volume of sample to yield the final protein concentration of 5 mg/mL.

7.1.5. Incubate the sample mixture on ice for 20 minutes. Centrifuge at 16,000 x g for 20 minutes at 4°C. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C.

7.2. Sub-cellular organelle lysates e.g. mitochondria.

7.2.1. Prepare the organelle sample by, for example, subcellular fractionation.

7.2.2. Pellet the sample.

7.2.3. Solubilize the pellet by adding 9 volumes Extraction Buffer.

7.2.4. Incubate on ice for 20 minutes. Centrifuge at 16,000 x g for 20 minutes at 4°C. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C.

7.2.5. The sample protein concentration in the extract may be quantified using a protein assay.

Note: The sample should be diluted to within the working range of the assay in 1X Incubation Buffer. As a guide, typical ranges of sample concentration for commonly used sample types are shown below in Data Analysis.

8. Control Sample Dilution Series Preparation

Note: It is strongly recommended to prepare a dilution series of control (normal) material. The relative levels or profile of NADH dehydrogenase (Complex I) in unknown samples can be interpolated from within this control sample series.

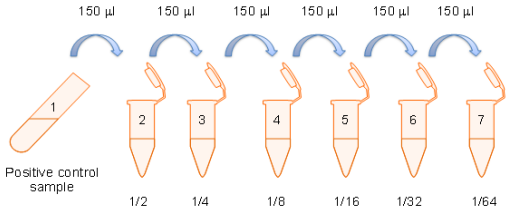
8.1. To create a dilution series of control sample, label a series of tubes #2-7. Add 150 µL 1X Incubation Buffer to each of tubes #2 through #7.

8.2. Prepare all samples by detergent extraction as described.

8.3. Dilute the control sample lysate to 1 mg/mL in 1X Incubation Buffer, label this tube #1. Undiluted control sample can be frozen at -80°C.

8.4. Transfer 150 µL from tube #1 to tube #2 and mix thoroughly. With a fresh pipette tip transfer 150 µL from #2 to #3.

8.5. Repeat serial dilution as in step 8.4 for Tubes #3 through #7. Use 1X Incubation Buffer as the zero sample tube labeled #8. Use a fresh dilution series for each assay.



9. Assay Procedure

Equilibrate all reagents to room temperature before use. It is recommended all samples and standards be assayed in duplicate.

9.1 Prepare all reagents, working standards, and samples as directed in the previous sections.

9.2 Remove unused microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.

9.3 Load diluted samples at 50 µL per well.

9.4 Cover/seal the plate and incubate for 2 hours at room temperature. If available use a plate shaker for all incubation steps at 300 rpm.

9.5 Aspirate each well and wash, repeat this once more for a total of **two** washes. Wash by aspirating or decanting from wells then dispensing 300 µL 1X Wash Buffer into each well as described above. Complete removal of liquid at each step is essential to good performance. After the last wash, remove the remaining buffer by aspiration or decanting. Invert the plate and blot it against clean paper towels to remove excess liquid.

9.6 Immediately prior to use prepare sufficient (500 µL/8 well strip used) 1X NADH Dehydrogenase Detector Antibody (step 6.4) and add 50 µL to each well used.

Cover/seal the plate and incubate for 1 hour at room temperature. If available use a plate shaker for all incubation steps at 300 rpm.

9.7 Repeat the aspirate/wash procedure above.

9.8 Immediately before use, prepare sufficient (500 µL/8 well strip used) 1X HRP (step 6.5) and add 50 µL to each well used. Cover/seal the plate and incubate for 1 hour at room temperature. If available use a plate shaker for all incubation steps at 300 rpm.

9.9 Repeat the aspirate/wash procedure above. However, performing a total of three washes.

9.10 Add 50 µL of HRP Development Solution to each empty well and immediately record the blue color development with time in the microplate reader prepared with the following settings:

Mode:	Kinetic
Wavelength:	600 nm
Time:	up to 15 min.
Interval:	20 sec. - 1 min.
Shaking:	Shake between readings

Alternative– In place of a kinetic reading, at a user defined, time record the endpoint OD data at (i) 600 nm or (ii) stop the reaction by adding 50 µL stop solution (1N HCl) to each well and record the OD at 450 nm. Analyze the data as described below.

10. Data Analysis

Average the duplicate control sample dilution series readings and plot against their concentrations after subtracting the zero standard reading. Draw the best smooth curve through these points to construct a standard curve. Most plate reader software or graphing software can plot these values and curve fit. A four parameter algorithm (4PL) usually provides the best fit, though other equations can be examined to see which provides the most accurate (e.g. linear, semi-log, log/log, 4 parameter logistic). Read the relative NADH dehydrogenase (Complex I) concentrations for unknown samples from the control curve plotted. Samples producing signals greater than that of the highest control should be further diluted in 1X Incubation Buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.

TYPICAL STANDARD CURVE - *For demonstration only.*

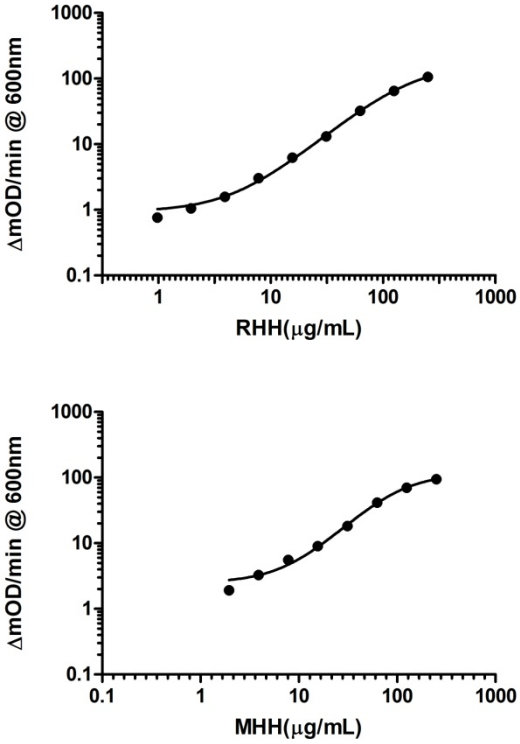


Figure 1. Example of positive control sample standard curve. A dilution series of extract in 1X Incubation Buffer in the working range of the assay. The extract was prepared with (upper) rat heart homogenate (RHH), or (lower) mouse heart homogenate (MHH).

TYPICAL SAMPLE RANGE

Typical working ranges	
Sample Type	Range
Rat heart homogenate (RHH)	4 – 500 µg/mL
Mouse heart homogenate (MHH)	4– 500 µg/mL

SENSITIVITY

Calculated minimum detectable dose = 0.4 µg/mL (zero dose n = 24 ± 2 standard deviations)

Determined minimum detectable dose for rodent heart tissues = 0.49 µg/mL

REPRODUCIBILITY (using rat heart homogenate as example material)

Parameter	CV%
Intra (n= 2-3, 4 repeat)	2.0%
Inter (n=3 days)	6.14%

RECOVERY

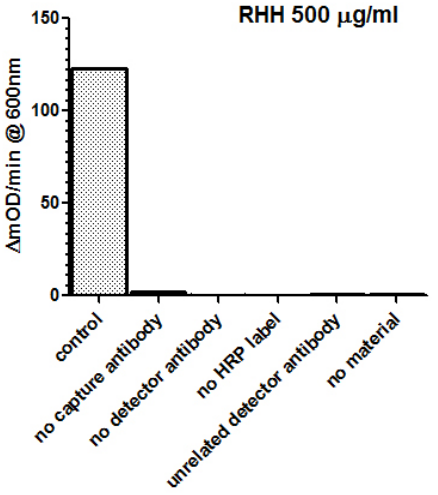
Sample Type	Average Recovery (%)	Range (%)
50% Culture Media	26%	17.4 - 27.6%
10% Goat Serum	10.2%	6.0 - 16.7%
50% Extraction buffer	49.4%	37.2 - 62.7%

Goat serum and culture media inhibit the assay significantly. High concentration of extraction buffer also affects the assay.

11. Specificity

ab136809 is suitable to quantify NADH dehydrogenase from rodent tissues, i.e. rat and mouse. This assay also works with human tissues. Other species have not been tested.

Components requirement and immune assay specificity were determined by skipping one critical component or replacing with unrelated antibodies. The sample was rat heart homogenate (RHH) at the concentration of 500 µg/mL.



12. Troubleshooting

Problem	Cause	Solution
Poor standard curve	Inaccurate Pipetting	Check pipettes
	Improper standard dilution	determine the protein concentration of control samples using a protein assay
Low Signal	Low NADH dehydrogenase concentration in sample	Use appropriate positive control. Rodent heart homogenates , for example, can be used as a positive control. Rodent mitochondrial fractions are even better positive controls which are available through Abcam website (ab110347 and ab110350)
	Incubation times too brief	Ensure sufficient incubation times; change to overnight incubation
	Inadequate reagent volumes or improper	Check pipettes and ensure correct preparation
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions
	Contaminated wash buffer	Make fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	Store assay components 4°C. Keep substrate solution protected from light

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