Version 3 Last updated 9 February 2022

ab178011 Human NADH Dehydrogenase SimpleStep ELISA® Kit (Complex I)

For the semi-quantitative measurement of NADH Dehydrogenase in human cell and tissue extract samples.

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

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1. Overview

NADH Dehydrogenase *in vitro* SimpleStep ELISA® (Enzyme-Linked Immunosorbent Assay) kit is designed for the semi-quantitative measurement of NADH Dehydrogenase protein in human cell and tissue extract samples.

The SimpleStep ELISA® employs an affinity tag labeled capture antibody and a reporter conjugated detector antibody which immunocapture the sample analyte in solution. This entire complex (capture antibody/analyte/detector antibody) is in turn immobilized via immunoaffinity of an anti-tag antibody coating the well. To perform the assay, samples or controls are added to the wells, followed by the antibody mix. After incubation, the wells are washed to remove unbound material. TMB Development Solution is added and during incubation is catalyzed by HRP, generating blue coloration. This reaction is then stopped by addition of Stop Solution completing any color change from blue to yellow. Signal is generated proportionally to the amount of bound analyte and the intensity is measured at 450 nm. Optionally, instead of the endpoint reading, development of TMB can be recorded kinetically at 600 nm.

NADH dehydrogenase (NADH: ubiquinone reductase (H^+-translocating), 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. There is also evidence of NADH Dehydrogenase involvement in diabetes.

2. Protocol Summary

Prepare all reagents, samples, and controls as instructed

Add 50 µL control or sample to appropriate wells

Add 50 µL Antibody Cocktail to all wells

Incubate at room temperature for 1 hour

Aspirate and wash each well three times with 350 µL 1X Wash Buffer PT

Add 100 µL TMB Development Solution to each well and incubate for 10 minutes.

Add 100 µL Stop Solution and read OD at 450 nm

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

Store kit at +4°C immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components.

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	Quantity	Storage Condition
Human NADH Dehydrogenase Capture Antibody 10X	600 µL	+4°C
Human NADH Dehydrogenase Detector Antibody 10X	600 µL	+4°C
HeLa Lyophilized Lysate Control	2 Vials	+4°C
Antibody Diluent 5B	6 mL	+4°C
10X Wash Buffer LM	20 mL	+4°C
2X Cell Extraction Buffer LM	10 mL	+4°C
Sample Diluent NS*	12 mL	+4°C
TMB Development Solution	12 mL	+4°C
Stop Solution	12 mL	+4°C
SimpleStep Pre-Coated 96-Well Microplate	96 Wells	+4°C
Plate Seal	1	+4°C

*Sample Diluent NS is provided but not necessary for this product.

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 450 or 600 nm.
- Method for determining protein concentration (BCA assay recommended).
- Deionized water.
- PBS (1.4 mM KH2PO4, 8 mM Na2HPO4, 140 mM NaCl, 2.7 mM KCl, pH 7.4).
- Multi- and single-channel pipettes.
- Tubes for Control Lysate dilution.
- Plate shaker for all incubation steps.
- Optional: Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors).

8. Technical Hints

- Samples generating values higher than the highest Control Lysate should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, control and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Complete removal of all solutions and buffers during wash steps is necessary to minimize background.
- As a guide, typical ranges of sample concentration for commonly used sample types are shown below in Sample Preparation (section 11).
- All samples should be mixed thoroughly and gently.
- Avoid multiple freeze/thaw of samples.
- Incubate ELISA plates on a plate shaker during all incubation steps.

- When generating positive control samples, it is advisable to change pipette tips after each step.
- To avoid high background always add samples or controls to the well before the addition of the antibody cocktail.
- 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 or control will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.

9. Reagent Preparation

- Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for 96 wells. The sample volumes below are sufficient for 48 wells (6 x 8-well strips); adjust volumes as needed for the number of strips in your experiment.
- Prepare only as much reagent as is needed on the day of the experiment. Capture and Detector Antibodies have only been tested for stability in the provided 10X formulations.

9.1 1X Cell Extraction Buffer LM:

Prepare 1X Cell Extraction Buffer LM by diluting 2X Cell Extraction Buffer LM to 1X with deionized water. To make 10 mL 1X Cell Extraction Buffer LM combine 5 mL deionized water and 5 mL 2X Cell Extraction Buffer LM. Mix thoroughly and gently. If required protease inhibitors can be added.

9.2 1X Wash Buffer LM:

Prepare 1X Wash Buffer LM by diluting 10X Wash Buffer LM with deionized water. To make 50 mL 1X Wash Buffer LM combine 5 mL 10X Wash Buffer LM with 45 mL deionized water. Mix thoroughly and gently.

9.3 Antibody Cocktail:

Prepare Antibody Cocktail by diluting the capture and detector antibodies in Antibody Diluent 5B. To make 3 mL of the Antibody Cocktail combine 300 µL 10X Capture Antibody and 300 µL 10X Detector Antibody with 2.4 mL Antibody Diluent 5B. Mix thoroughly and gently.

10. Control Lysate Preparation

Kit Control lysates are provided at a concentration that give consistent signal between different lots. Lysates are produced and formulated by signal intensity to be consistent to within 30% of the previous lot. Control lysates are supplied as a control reagent – not an absolute quantitation measure.

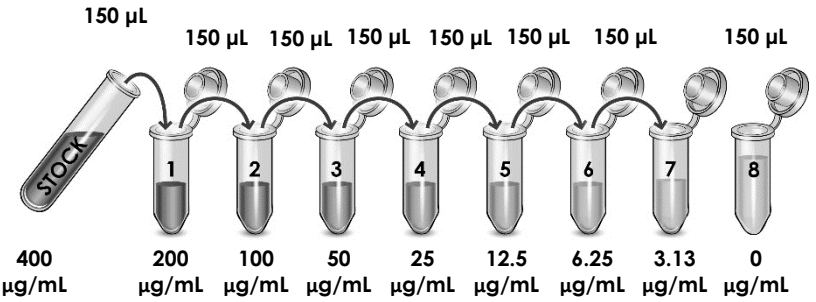
- Always prepare a fresh set of controls for every use.
- Discard working control dilutions after use as they do not store well.
- The following section describes the preparation of a control curve for duplicate measurements (recommended).

10.1 IMPORTANT: If the Hela Lyophilized Lysate Control vial has a volume identified on the label, reconstitute by adding that volume of Sample Diluent NS indicated on the label. Alternatively, if the vial has a mass identified, reconstitute the HeLa Lyophilized Lysate Control by adding 200 µL Sample Diluent NS. Hold at room temperature for 10 minutes and mix gently. This is the 400 µg/mL **Stock Lysate** Solution. Remaining stock material should be aliquoted and stored at -80°C.

10.1.1 Label eight tubes, Controls 1– 8.

10.1.2 Add 150 µL 1X Cell Extraction Buffer LM into tube numbers 1-8.

10.1.3 Use the Stock Lysate to prepare the following dilution series. Control #8 contains no protein and is the Blank control:



11. Sample Preparation

Typical Sample Dynamic Range	
Sample Type	Range
HeLa Cell Lysate	3 – 200 µg/mL
HepG2 Cell Lysate	1.5 – 75 µg/mL
143B Cell Lysate	3 – 200 µg/mL

11.1 Preparation of extracts from cell pellets:

11.1.1 Collect non-adherent cells by centrifugation or scrape to collect adherent cells from the culture flask. Typical centrifugation conditions for cells are 500 x g for 5 minutes at 4°C.

11.1.2 Rinse cells twice with PBS.

11.1.3 Solubilize pellet at $2x10^7$ cell/mL in chilled 1X Cell Extraction Buffer LM.

11.1.4 Incubate on ice for 20 minutes.

11.1.5 Centrifuge at 18,000 x g for 20 minutes at 4°C.

11.1.6 Transfer the supernatants into clean tubes and discard the pellets.

11.1.7 Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

11.1.8 Dilute samples to desired concentration in 1X Cell Extraction Buffer LM.

11.2 Preparation of extracts from adherent cells by direct lysis (alternative protocol):

11.2.1 Remove growth media and rinse adherent cells 2 times in PBS.

11.2.2 Solubilize the cells by addition of chilled 1X Cell Extraction Buffer LM directly to the plate (use 750 µL - 1.5 mL 1X Cell Extraction Buffer LM per confluent 15 cm diameter plate).

11.2.3 Scrape the cells into a microfuge tube and incubate the lysate on ice for 15 minutes.

11.2.4 Centrifuge at 18,000 x g for 20 minutes at 4°C.

11.2.5 Transfer the supernatants into clean tubes and discard the pellets.

11.2.6 Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

11.2.7 Dilute samples to desired concentration in 1X Cell Extraction Buffer LM.

11.3 Preparation of extracts from tissue homogenates:

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

11.3.2 Homogenize 100 to 200 mg of wet tissue in 500 µL – 1 mL of chilled 1X Cell Extraction Buffer LM. For lower amounts of tissue adjust volumes accordingly.

11.3.3 Incubate on ice for 20 minutes.

11.3.4 Centrifuge at 18,000 x g for 20 minutes at 4°C.

11.3.5 Transfer the supernatants into clean tubes and discard the pellets.

11.3.6 Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

11.3.7 Dilute samples to desired concentration in 1X Cell Extraction Buffer LM.

12. Plate Preparation

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
- Unused plate strips should be immediately returned to the foil pouch containing the desiccant pack, resealed and stored at 4°C.
- For each assay performed, a minimum of two wells must be used as the zero control.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Differences in well absorbance or "edge effects" have not been observed with this assay.

13. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all controls and samples in duplicate.

13.1 Prepare all reagents, working controls, and samples as directed in the previous sections.

13.2 Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal and return to 4°C storage.

13.3 Add 50 µL of all sample or control to appropriate wells.

13.4 Add 50 µL of the Antibody Cocktail to each well.

13.5 Seal the plate and incubate for 1 hour at room temperature on a plate shaker set to 400 rpm.

13.6 Wash each well with 3 x 350 µL 1X Wash Buffer LM. Wash by aspirating or decanting from wells then dispensing 350 µL 1X Wash Buffer LM into each well. Wash Buffer LM should remain in wells for at least 10 seconds. Complete removal of liquid at each step is essential for good performance. After the last wash invert the plate and tap gently against clean paper towels to remove excess liquid.

13.7 Add 100 µL of TMB Development Solution to each well and incubate for 10 minutes in the dark on a plate shaker set to 400 rpm.

Given variability in laboratory environmental conditions, optimal incubation time may vary between 5 and 20 minutes.

Note: The addition of Stop Solution will change the color from blue to yellow and enhance the signal intensity about 3X. To avoid signal saturation, proceed to the next step before the high concentration of the standard reaches a blue color of O.D.600 equal to 1.0.

13.8 Add 100 µL of Stop Solution to each well. Shake plate on a plate shaker for 1 minute to mix. Record the OD at 450 nm. This is an endpoint reading.

13.9 Alternative to 13.7 – 13.8: Instead of the endpoint reading at 450 nm, record the development of TMB Substrate kinetically. Immediately after addition of TMB Development Solution begin recording the blue color development with elapsed

time in the microplate reader prepared with the following settings:

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

Δ **Note**: that an endpoint reading can also be recorded at the completion of the kinetic read by adding 100 µL Stop Solution to each well and recording the OD at 450 nm.

13.10 Analyze the data as described below.

14. Calculations

14.1 Calculate the average absorbance value for the blank (zero) controls. Subtract the average blank (zero) control absorbance value from all other absorbance values.

14.2 **Create a control curve** by plotting the average blank control subtracted absorbance value for each control concentration (y-axis) against the target protein concentration (x-axis) of the control. Use graphing software to draw the best smooth curve through these points to construct the control curve.

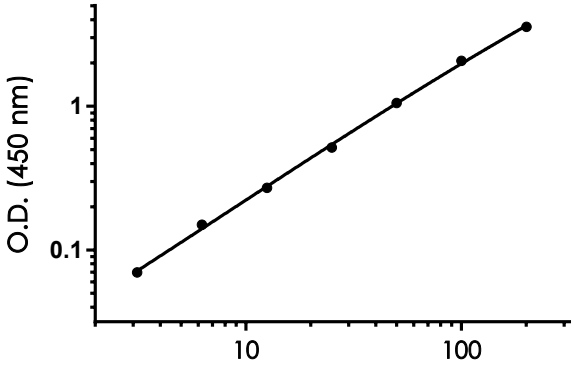
Δ **Note:** Most microplate reader software or graphing software will plot these values and fit a curve to the data. A four parameter curve fit (4PL) is often the best choice; however, other algorithms (e.g. linear, semi-log, log/log, 4 parameter logistic) can also be tested to determine if it provides a better curve fit to the control values.

14.3 Determine the concentration of the target protein in the sample by interpolating the blank control subtracted **absorbance values against the control curve**. Multiply the resulting value by the appropriate sample dilution factor, if used, to obtain the concentration of target protein in the sample.

14.4 Samples generating absorbance values greater than that of the highest control should be further diluted and reanalyzed. Similarly, samples which measure at an absorbance values less than that of the lowest control should be retested in a less dilute form.

15. Typical Data

Typical control curve – data provided **for demonstration purposes only**. A new control curve must be generated for each assay performed.



Control Curve Measurements			
Concentration (µg/mL)	O.D 450 nm		Mean O.D
	1	2	
0	0.059	0.067	0.064
3.13	0.134	0.132	0.133
6.25	0.211	0.214	0.213
12.5	0.346	0.323	0.335
25	0.595	0.566	0.581
50	1.125	1.111	1.119
100	2.137	2.125	2.132
200	3.601	3.649	3.625

Figure 1. Example of human NADH Dehydrogenase control curve in 1X Cell Extraction Buffer LM. The NADH Dehydrogenase control curve was prepared as described in Section 10. Raw data values are shown in the table. Background-subtracted data values (mean +/- SD) are graphed.

16. Typical Sample Values

SENSITIVITY –

The calculated minimal detectable dose (MDD) is 0.43 µg/mL. The MDD was determined by calculating the mean of blank (zero) control replicates (n=32) and adding 2 standard deviations then extrapolating the corresponding concentration.

RECOVERY –

Three concentrations of NADH Dehydrogenase HeLa Lyophilized Lysate Control were spiked in duplicate to the indicated biological matrix to evaluate signal recovery in the working range of the assay.

Sample Type	Average % Recovery	Range (%)
50% Cell Culture Media*	66	64-67
10% Fetal Bovine Serum	74	70-77
10% Normal Human Serum	110	108-111

*Media is DMEM containing 10% fetal bovine serum.

Linearity of Dilution

Linearity of dilution is determined based on interpolated values from the control curve. Linearity of dilution defines a sample concentration interval in which interpolated target concentrations are directly proportional to sample dilution.

Native NADH Dehydrogenase was measured in the following biological samples in a 2-fold dilution series. Sample dilutions are made in 1X Cell Extraction Buffer LM.

Dilution Factor	Interpolated value	75 µg/mL HepG2 Cell Extract
Undiluted	pg/mL	202
	% Expected value	100
2	pg/mL	103
	% Expected value	102
4	pg/mL	53.2
	% Expected value	105
8	pg/mL	27.0
	% Expected value	107
16	pg/mL	14.0
	% Expected value	111

PRECISION –

Mean coefficient of variations of interpolated values of NADH Dehydrogenase from three concentrations of HepG2 cell extract within the working range of the assay.

	Intra-Assay	Inter-Assay
n =	5	3
CV(%)	2.7	5.9

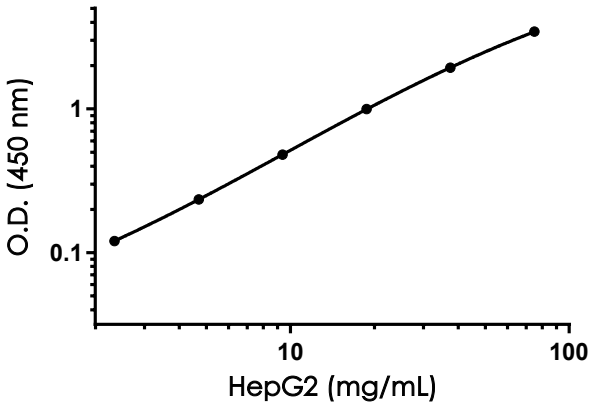


Figure 2. Titration of HepG2 cell lysate within the working range of the assay. Background-subtracted data values from triplicate measurements (mean +/- SD) are graphed.

17. Assay Specificity

This kit recognizes both native and recombinant human NADH Dehydrogenase protein in cell and tissue extract samples only.

Serum, plasma, urine, milk, saliva, and cell culture supernatant samples have not been tested with this kit.

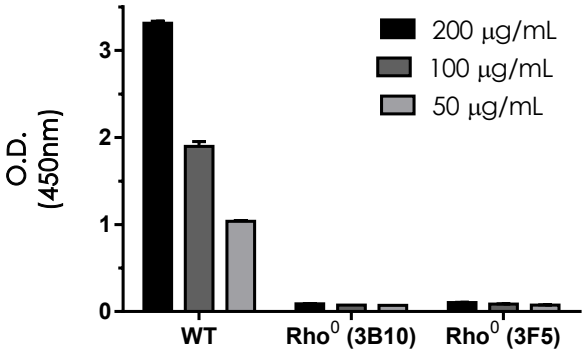


Figure 3. Comparison of NADH Dehydrogenase expression in 143B wildtype (WT) and two clones (3B10 and 3F5) of 143B-derived Rho^0 (mitochondrial DNA-depleted) cells. Background-subtracted data values from triplicate measurements of three lysate concentrations (200, 100 and 50 µg/mL) are graphed as mean +/- SD.

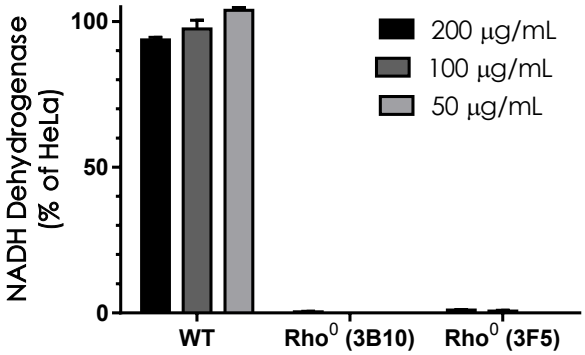


Figure 4. Quantification of NADH Dehydrogenase expression in 143B wildtype (WT) and two clones (3B10 and 3F5) of 143B-derived Rho^0 (mitochondrial DNA-depleted) cells. The concentrations of NADH Dehydrogenase were interpolated from data values shown in Figure 3 using NADH Dehydrogenase control curve of the HeLa Lyophilized Lysate Control, corrected for sample dilution, and graphed in percent relative to NADH Dehydrogenase expression in HeLa cell extract. The concentration of NADH Dehydrogenase in both Rho^0 cell lines was less than 1% of the concentration in the WT 143B cells.

18. Species Reactivity

This kit recognizes human NADH Dehydrogenase protein.

Other species reactivity not determined.

Please contact our Technical Support team for more information.

19. Troubleshooting

Problem	Reason	Solution
Poor Control Lysate curve	Inaccurate Pipetting	Check pipettes
	Improper control dilution	Prior to opening, briefly spin the stock Control Lysate tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; increase to 2 or 3 hour control/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
	Incubation times with TMB too brief	Ensure sufficient incubation time until blue color develops prior addition of Stop solution
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	Prepare fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	Store your reconstituted controls at -80°C, all other assay components 4°C. Keep TMB Development Solution protected from light.
Precipitate in Diluent	Precipitation and/or coagulation of components within the Diluent.	Precipitate can be removed by gently warming the Diluent to 37°C.

20. Notes

Technical Support

For all technical or commercial enquiries please go to:

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