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ab124537 –

Cyt C Reductase (Complex III) Human Profiling ELISA

Instructions for Use

For the measurement of Cyt C Reductase (Complex III) in human samples

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

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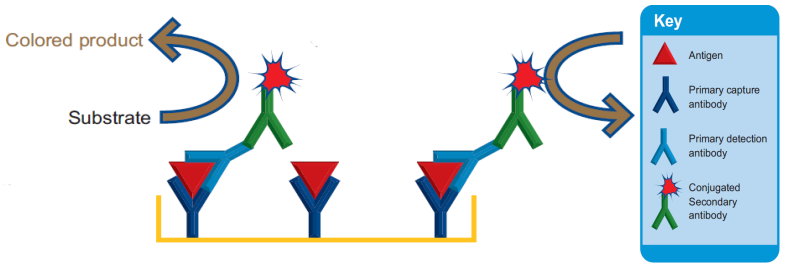
1. Introduction

Principle: ab124537 Cyt C Reductase (Complex III) human profiling kit is an in vitro enzyme-linked immunosorbent assay (ELISA) for the comparison of Complex III levels or profile in cell and tissue lysates. The assay employs a human Complex III specific antibody coated onto 96-well microplate strips. Samples are pipetted into the wells and Complex III present in the sample is bound to the wells by the immobilized antibody. The wells are washed and an anti-Complex III detector antibody is added. After washing away unbound detector antibody, an HRP-conjugated secondary antibody specific for the detector antibody 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 Complex III 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: Cyt C Reductase (Complex III) is the third enzyme of the oxidative phosphorylation (OXPHOS) system within the mitochondrial inner membrane. Cyt C Reductase is a large protein complex of approximately 250,000 MW made up of 11 different subunits. The enzyme forms a dimer in the mitochondrial inner membrane. The catalytic subunit cytochrome b is encoded on mitochondrial DNA (mtDNA). All other subunits are encoded by nuclear genomic DNA, made in the cytosol, and translocated into the organelle for assembly at the inner membrane. Complex III catalyses the pumping of protons across the inner membrane and transfer of electrons from ubiquinol to cytochrome c by a mechanism known as the Q cycle in which ubiquinol (Q) the electron carrier exists as a reduced, partially reduced or oxidized form.

Mutations in Complex III genes or assembly factors are a cause of genetic OXPHOS defects, though rare. It is believed that Complex III may be a major source of superoxides and reactive oxygen species contributing to multiple pathologies including neurodegenerative conditions and the free radical theory of aging itself. The partially reduced semi-quinone formed in the Q cycle may be the source of the electrons needed to make superoxide and the rate of electron leak may be increased during hypoxic conditions and in the presence of mitochondrial inhibitors.

2. Assay Summary

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

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

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

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

Aspirate and wash each well three times. Add 100 μL HRP Development Solution to each well. Immediately record 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 Complex III Detector Antibody	1 mL
10X HRP Label	1 mL
Complex III Microplate (12 x 8 antibody coated well strips)	1

4. Storage and Handling

Store all components at 4°C. This kit is stable for 6 months from receipt. 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
- Optional plate shaker for all incubation steps
- Plate cover or seals

6. Preparation of Reagents

6.1 Equilibrate all reagents to room temperature (18-25^{o}C) before use.

6.2 Prepare 1X Wash Buffer by adding 20 mL 20X Buffer to 380 mL nanopure water. Mix gently and thoroughly.

6.3 Prepare 1X Incubation Buffer by adding 6 mL 10X Blocking Buffer to 54 mL 1X Wash Buffer. Unused 1X Incubation buffer may be stored at -20°C for 6 months after performing assay. Mix gently and thoroughly.

6.4 Prepare the 1X Complex III Detector Antibody by diluting the 10X Complex III Detector Antibody 10-fold with 1X Incubation Buffer immediately prior to use. Prepare 0.5 mL for each 8 well strip used.

6.5 Prepare the 1X HRP label by diluting the stock 10X HRP Label 10-fold with 1X Incubation Buffer immediately prior to use. Prepare 0.5 mL for each 8 well strip used.

7. Sample Preparation

Note: Extraction buffer can be supplemented with phosphatase inhibitors, PMSF and protease inhibitor cocktail prior to use. Supplements should be used according to manufacturer's instructions.

7.1. Cell lysates.

7.1.1 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 10 min at 4°C.

7.1.2 Rinse cells twice with PBS.

7.1.3 Solubilize cell pellet at $2x10^7$/mL in Extraction Buffer.

7.1.4 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 for 6 months. The sample protein concentration in the extract may be quantified using a protein assay.

7.2. Tissue lysates.

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

7.2.2 Quantify protein concentration using a standard protein assay (BCA assay recommended).

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

7.2.4 Solubilize the homogenate by adding 4 volumes of Extraction Buffer to 1 volume of a sample to the final protein concentration of 5 mg/mL.

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

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

7.3.1 Prepare the organelle sample by, for example, using a sub-cellular fractionation kit.

7.3.2 Pellet the sample.

7.3.3 Solubilize the pellet by adding 9 volumes Extraction Buffer to 1 volume of sample pellet.

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

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

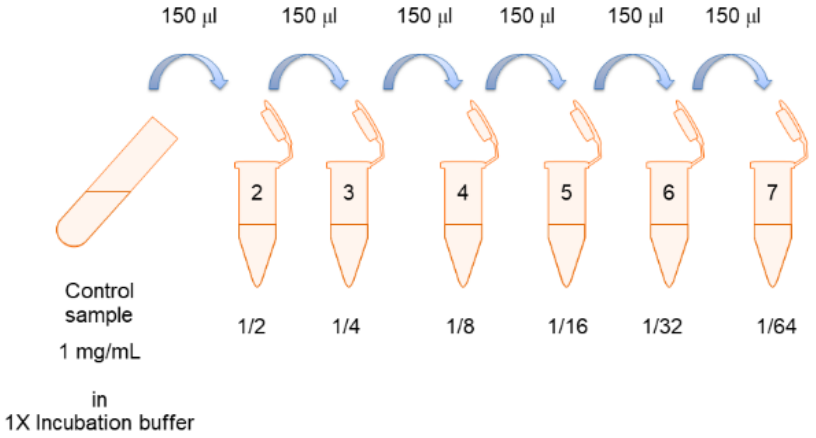
Note: It is strongly recommended to prepare a dilution series of control (normal) material. Signal of control material is heavily cell line dependent. The relative levels or profile of Complex III in unknown samples can be interpolated from within this control sample series. Here is an example on how to prepare a control sample dilution 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. 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.

8.3. Transfer 150 µL from tube #1 to tube #2. Mix thoroughly. With a fresh pipette tip transfer 150 µL from #2 to #3.

8.4. Repeat serial dilution as in step 8.3 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

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

9.1. Prepare all reagents, control and unknown samples as directed in the previous sections.

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

9.3. Add 50 µL of each diluted sample per well. It is recommended to include a dilution series of a control (normal) sample as a reference. Also include a 1X Incubation buffer as a zero standard.

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 (0.5 mL/strip used) 1X Complex III Detector Antibody in 1X Incubation buffer. Add 50 μL 1X Complex III Detector antibody 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 prior to use prepare sufficient (0.5 mL/strip used) 1X HRP label in 1X Incubation buffer. Add 50 μL 1X HRP label 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 400 rpm.

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

9.10. Add 100 μL HRP Development Solution to each empty well and immediately record the blue color development with elapsed 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 100 µL stop solution (1N HCl) to each well and record the OD at 450 nm.

9.11. 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 Complex III 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 SAMPLE RANGE - *For demonstration only.*

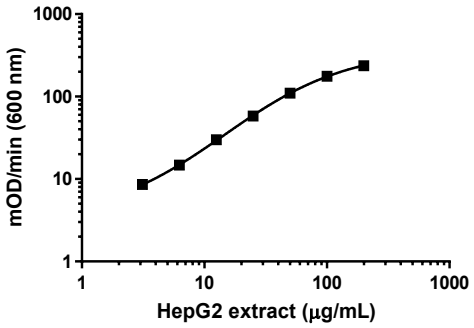


Figure 1. Example control sample curve.

TYPICAL SAMPLE RANGE

Typical working ranges for cell and tissue extracts	
Sample Type	Range
Cultured cell lysate	2.4 – 200 μg/mL
Tissue homogenate	0.6 – 50 μg/mL

SENSITIVITY

Determined minimum detectable dose:

0.2 µg/mL for cultured whole cell lysates e.g. HeLa and HL60.

REPRODUCIBILITY

Parameter	CV%
Intra (n=8)	4.7
Inter (n=6)	19.7

11. Specificity

SPECIFICITY

Species– human. Rat and mouse samples are not appropriate, other species are untested.

EXPERIMENAL DATA

Drug induced mitochondrial toxicity is a well-recognized phenomenon. Two examples of which are the inhibition of mitochondrial DNA replication by nucleoside analogs reverse transcriptase inhibitors (NARTI) and the inhibition of mitochondrial protein expression by inhibitors of the mitochondrial ribosome (e.g. antibiotics). The effects of a drug from each of these classes on the assembly of OXPHOS enzymes containing mtDNA encoded components (Complexes I, III, IV and V) versus the only entirely nuclear encoded enzyme SDH (Complex II) are shown below in Figure 2.

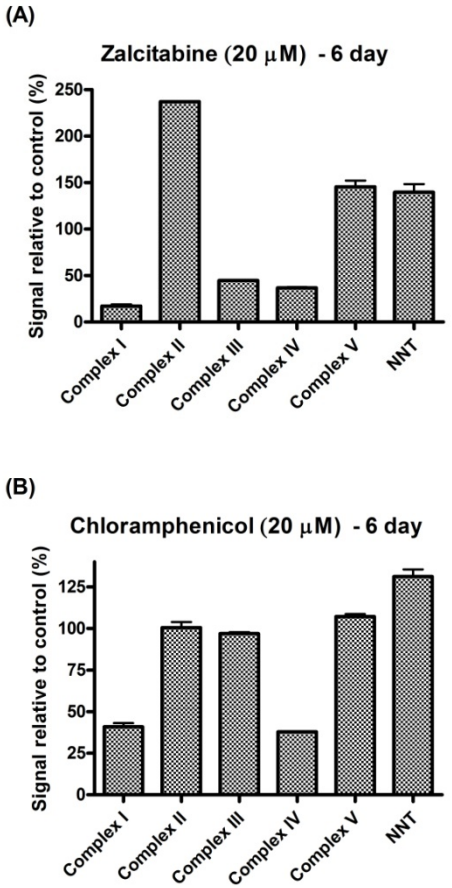


Figure 2. Human HepG2 cells were cultured in each drug for 6 days to ensure a significant effect on mitochondrial DNA replication and mitochondrial protein translation, respectively. (A) The NARTI Zalcitabine (ddC) reduced mitochondrial DNA levels and hence mitochondrial protein expression. As a consequence

the assembly of Complexes I, III and IV were severely affected. Note that loss of the two small mitochondrial DNA encoded subunits of Complex V (ATP synthase) does not affect overall assembly. Interestingly an increase in Complex II was induced as a consequence of I, III, IV loss possibly to up regulate mitochondrial citric acid cycle function. (B) The antibiotic chloramphenicol inhibited mitochondrial protein translation and assembly of Complexes I and IV but had no significant effect on Complex II, III or V.

12. Troubleshooting

Problem	Cause	Solution
Poor standard Curve	Inaccurate Pipetting	Check pipettes
Low Signal	Low SDH concentration in sample	Use appropriate positive control. Human cultured cells such a as HeLa or HepG2 are recommended.
	Incubation times too brief	Ensure sufficient incubation times; change to overnight standard/sample incubation
	Inadequate reagent volumes or improper dilution	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

13. Notes

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