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ab109910 – Complex IV Human Specific Activity Microplate Assay Kit

For the quantitative analysis of Human Complex IV activity and quantity in Human samples.

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

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Table of Contents

1. Overview 1
2. Protocol Summary 2
3. Precautions 3
4. Storage and Stability 3
5. Limitations 4
6. Materials Supplied 4
7. Materials Required, Not Supplied 4
8. Reagent Preparation 5
9. Sample Preparation 6
10. Assay Procedure 7
11. Calculations 10
12. Typical Data 12
13. Assay Specificity 13
14. Notes 13

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1. Overview

Complex IV (EC 1.9.3.1), also called cytochrome c oxidase, is a complex of 13 different subunits, three of which (I, II and III) are encoded on mitochondrial DNA and the remainder in the nuclear DNA. The complex contains two heme groups (a and a3) and two copper atoms as prosthetic groups. Genetic alterations of this enzyme complex are a common cause of OXPHOS diseases and the enzyme is altered in patients with Alzheimer's disease. Also, there are reports of reduced amounts of this complex in hypoxic cancer cells.

ab109910 The Complex IV (cytochrome c oxidase) Human Specific Activity Microplate Assay Kit is used to determine the activity and quantity of this enzyme in a human sample. Complex IV is immunocaptured within the wells and activity is determined colorimetrically by following the oxidation of reduced cytochrome c as an absorbance decrease at 550 nm. The overall reaction is as follows:

4 cytochrome c- + $4H^{+}$ + $4H^{+(in)}$ + O_2 → 4 cytochrome c^{+} + $2H_2O$+$4H^{+(out)}$

reduced oxidized

↑Abs 550 nm ↓Abs 550 nm

Subsequently, in this assay kit, within the same wells the quantity of enzyme is measured by adding a Complex IV specific antibody conjugated with alkaline phosphatase. This phosphatase changes a substrate from colorless to yellow at 405 nm. This reaction takes place in a time dependent manner proportional to the amount of protein captured in the wells.

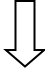
Homogenized tissue and whole cells can be used with this kit. Samples should be solubilized, the protein extracted and measured within the linear range. A control or normal sample should always be included in the assay as a reference. Also, include a null or buffer control to act as a background reference measurement.

The activity alone is available as an individual kit ab109909.

2. Protocol Summary

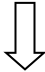
Prepare Samples (1 hour)

- Homogenize sample, pellet, and adjust sample to 5 mg/mL in Solution 1.
- Perform detergent extraction with 1/10 volume detergent followed by 16,000 rpm centrifugation for 20 minutes.
- Adjust concentration to recommended dilution for plate loading.



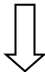
Load Plate (3 hours)

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



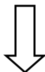
Measure (2 hours)

- Rinse wells three times with Solution 1.
- Prepare appropriate volume of assay solution and add to wells. Measure OD_{550} at 1-5 minute intervals for up to 2 hours.
- Proceed to Antibody binding immediately **or** store plate washed and covered overnight at 4°C.



Antibody Binding (2.5 hours)

- Empty wells.
- Add Solution A to each well and incubate 1 hour at room temperature.
- Rinse wells with Solution 1.
- Add Solution B to each well and incubate 1 hour at room temperature.



Measure (45 min)

- Rinse wells twice with Solution 1.
- Rinse wells with Solution 2.
- Add Development Solution to each well.
- Measure OD_{405} at 1.5 minute intervals for 30 min at room temperature with plate shake

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

Tube 1, 2, 3, A, B, Detergent and the covered microplate should be stored at 4°C. Tube 4 and Reagent C should be stored at -20°C or preferably -80°C.

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
20X Buffer (Tube 1)	15 mL	+4ºC
Wash Buffer (Tube 2)	2 mL	+4ºC
Development Buffer (Tube 3)	10 mL	+4ºC
AP Development Solution (Tube 4)	400 µL	-20/-80ºC
Detergent	1 mL	+4ºC
20X Complex IV Detector Antibody (Tube A)	1 mL	+4ºC
AP Label 2500X (Tube B)	12 µL	+4ºC
Human COX Microplate (12 strips)	1 EA	+4ºC
Reagent C (Reduced Cytochrome c)	1 mL	-20/-80ºC

7. Materials Required, Not Supplied

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

- Spectrophotometer that measures absorbance at 550±1nm and 405±1nm.
- Multichannel pipette (50 - 300 µL) and tips
- Deionized water
- Protein assay method

8. Reagent Preparation

– Prepare all solutions, immediately before use. Please read the Assay Procedure carefully before proceeding.

8.1 Solution 1:

Prepare immediately before use. Prepare the buffer solution by adding Tube 1 (15 mL) to 285 mL deionized H_2O. Label this solution as **Solution 1**.

8.2 Solution 2:

Prepare immediately before use. Add 2 mL of Tube 2 to 40 mL deionized H_2O. Label this as Solution 2.

8.3 Solution A

Prepare immediately before use. For an entire plate, add entire contents of antibody Tube A to 20 mL of Solution 1. Label this solution as **Solution A**.

8.4 Solution B

Prepare immediately before use. For an entire plate, add 8.8 µl of Tube B (2500X AP Label) to 22 mL of Solution 1. Label this as Solution B.

8.5 Development Solution:

Prepare immediately before use. For an entire plate, add 400 µL of Tube 4 and 10 mL of Tube 3 to 10 mL of deionized H_2O. Label this as Development Solution.

9. Sample Preparation

9.1 Pellet the sample by centrifugation.

9.2 Resuspend the sample by adding 5 volumes of Solution 1. The sample must be homogenous before detergent extraction. Therefore, resuspend the sample thoroughly by pipetting (cultured cells), or homogenize with a microtissue grinder. Determine the protein concentration by a standard method and then adjust the concentration to 5.55 mg/mL.

9.3 Add 1 volume of Detergent to 9 volumes of sample (e.g. if the total sample volume is 450 µL, add 50 µL of Detergent). Mix immediately and then incubate the sample on ice for 30 minutes.
Note: The optimal protein concentration for detergent extraction is 5 mg/mL.

9.4 Spin in tabletop microfuge at maximum speed (~16,000 rpm) for 20 minutes.

9.5 Carefully collect the supernatant and save as sample. Discard the pellet.

9.6 The microplate wells are optimized for 200 µL sample volume, so dilute samples to the following recommended concentrations by adding Solution 1:

Typical Sample Dynamic Range	
Sample Type	**Range**
Cultured cell extracts	2.5-160 µg / 200 µL
Tissue extracts	1-10 µg / 200 µL
Tissue mitochondria	0.1-10 µg / 200 µL

9.7 Keep diluted samples on ice until ready to proceed to Assay Procedure.

10. Assay Procedure

- We recommend that you assay all controls and samples in duplicate.
- Prepare all reagents, working standards, and samples as directed in the previous sections.

10.1 Plate Loading

10.1.1 Add 200 µL of each diluted sample into individual wells on the plate. Include a normal sample as a positive control. Include a buffer control (200 µL Solution 1) as a null or background reference.

10.1.2 Incubate the plate for 3 hours at room temperature.

10.2 Measurement

10.2.1 The bound monoclonal antibody has immobilized the enzyme in the wells. Empty the wells by quickly turning the plate upside down and shaking out any remaining liquid.

10.2.2 Add 300 µL of Solution 1 to each well

10.2.3 In a sealable tube prepare an appropriate amount of Assay Solution using Reagent C and Solution 1. Mix gently by inversion. See table below for amounts required. Set Assay Solution aside.

No. of Strips	REAGENT C (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	10.00
7	583	11.67
8	667	13.33
9	750	15.00
10	833	16.67
11	917	18.33
12	1000	20.00

10.2.4 Set up the plate reader to a kinetic program to measure absorbance at 550 nm for up to 2 hours, with no shake and a measurement interval of approximately 1 minute (however a longer measurement interval may be used if necessary).

10.2.5 Empty wells and add 300 µL Solution 1 to each well used. Repeat this rinse.

10.2.6 Empty the wells again and now add 200 µL of Assay Solution to each well used, be careful to avoid the formation of

bubbles. Any bubbles should be popped with a fine needle as rapidly as possible.

10.2.7 Set plate in plate reader and begin recording immediately. For Activity Assay Data Analysis, Section 11.1.

10.2.8 After data recording proceed to Section C, Addition of Detection Antibodies, for enzyme quantitation. Alternatively, the plate can be washed 3X with 300 µL of Solution 1, covered, and stored overnight at 4°C before proceeding.

10.3 Addition of Detection Antibodies

Δ **Note**: The volume of A, B and Development Solution prepared below is for the analysis of 96 wells. For fewer reduce the volume of solution prepared proportionally.

10.3.1 The bound monoclonal antibody has immobilized the enzyme in the wells. Empty the wells by quickly turning the plate upside down to remove activity solution.

10.3.2 Add 200 µL of Solution A to each well used.

10.3.3 Incubate the plate for 1 hour at room temperature.

10.3.4 Empty the wells and add 300 µL of Solution 1 to each well. Repeat this step.

10.3.5 Empty the wells and add 200 µL of Solution B to each well used.

10.3.6 Incubate the plate for 1 hour at room temperature.

10.4 Quantity Measurement

10.4.1 Empty the wells and add 300 µL of Solution 1 to each well used. Repeat this step.

10.4.2 Empty the wells and add 300 µL of Solution 2 to each well used.

10.4.3 Empty the wells and add 200 µL of Development Solution to each well used. Rapidly pop any bubbles that form with a needle.

10.4.4 Measure the absorbance of each well at 405 nm at room temperature. Take a measurement every 1.5 minutes for 20 measurements for a total time of 30 minutes, shaking the plate between measurements.

10.4.5 Analyze data as described in Quantity Assay Data Analysis Section 11.2.

11. Calculations

11.1 Activity Assay Data Analysis

Since the Complex IV reaction is product inhibited, the rate of activity is always expressed as the initial rate of oxidation of cytochrome c. This oxidation is measured as a **decrease** in absorbance at 550 nm. At higher activity levels the rate will slow down due to product inhibition and only the initial rate should be measured. At lower activity rates the linear range is extended over the entire duration of the assay.

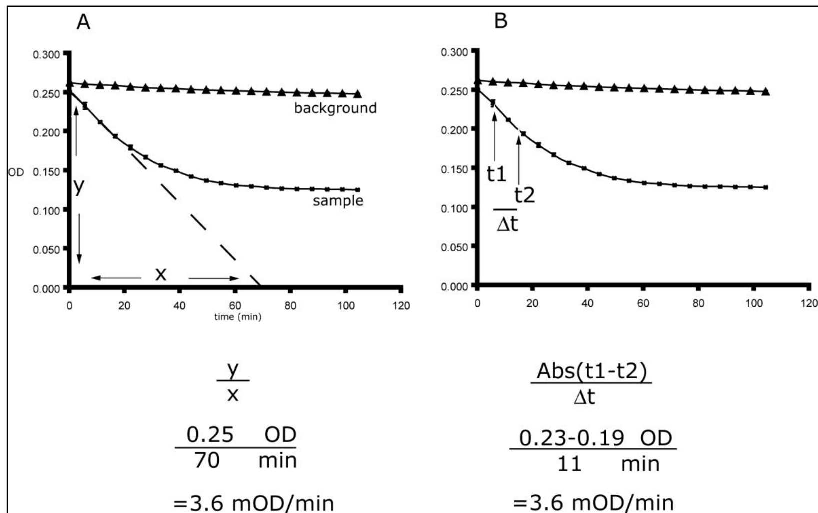
To determine the activity in the sample, calculate the slope by using microplate software.

Alternatively, the rate can be calculated manually as shown below.

Compare the sample rate with the rate of the control (normal) sample and with the rate of the null (background) to get the relative Complex IV activity

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

Example of manual calculation of activity rate:



A: The rate is determined by calculating the gradient of the initial slope over the linear region.
B: The rate is determined by calculating the slope between two points within the linear region.

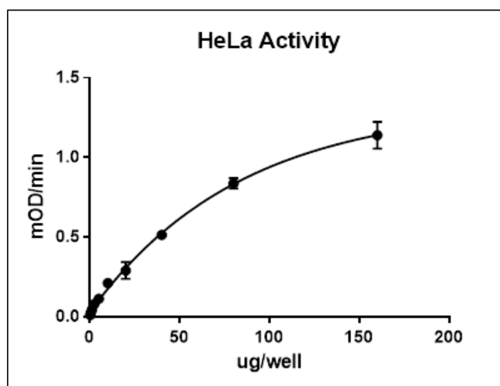
11.2 Quantity Assay Data Analysis

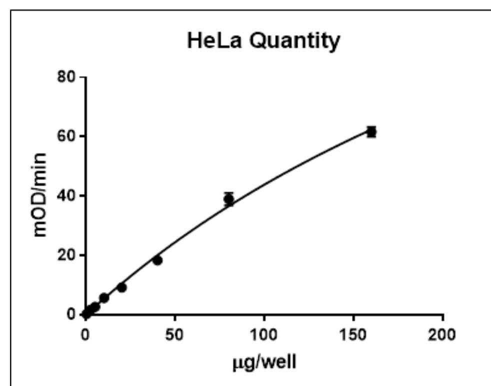
This quantity of Complex IV is proportional to an **increase** in absorbance at 405 nm and should be expressed as the amount relative to a normal or control sample.

To determine the quantity in the sample, calculate the slope by using microplate software.

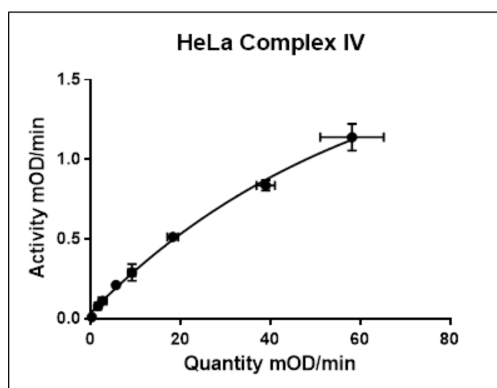
The rate can also be calculated manually in a manner similar to the activity manual calculation.

12. Typical Data





Combined the relationship between activity and quantity:



PRECISION –

	Intra-Assay	Inter-Assay
CV(%)	<7	<10

13. Assay Specificity

This activity/quantity multiplexing plate has been developed for use with human samples.

Mouse and rat samples are not suitable with this kit (separate microplate assay kits are available for these species). Other species have not been tested.

This assay is designed for use with purified mitochondria

Technical Support

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