

ab109721- Complex I Enzyme Activity Assay Kit (Colorimetric)

For the measurement of Complex I enzyme activity in human, rat, mouse and bovine mitochondria, cell and tissue extracts.

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

For overview, typical data and additional information please visit:

<http://www.abcam.com/ab109721>

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.

Detergent extract of prepared samples loaded onto plate



Incubate for 3 hours at room temperature. Wash plate wells with Buffer 3X



Add 200 µL of Assay Solution to each well



Measure Optical Density (OD_{450 nm}) in kinetic mode at room temperature for up to 30 minutes*

**For kinetic mode detection, assay measurement time is sample-specific and therefore suggestion is for guidance only.*

Materials Supplied and Storage:

Store kit at 4°C in the dark immediately on receipt. Kit can be stored for 1 year from receipt, if components have not been reconstituted

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

Avoid repeated freeze-thaws of reagents.

Item	Quantity	Storage temperature (before prep)	Storage temperature (after prep)
20X Wash Buffer	25 mL	4°C	4°C
10X Blocking Solution	10 mL	4°C	4°C
Detergent	1 mL	4°C	4°C
20X NADH (lyophilized) 40 mM after addition of H ₂ O	1 vial	4°C	-80°C
100X Dye (lyophilized)	1 vial	4°C	-80°C
96-well microplate (12 strips)	1 unit	4°C	4°C

Materials Required, Not Supplied

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

- Microplate reader capable of measuring absorbance at OD 450 nm.
- Ultra-pure water or double distilled water (ddH₂O)
- 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
- Dounce homogenizer (if using tissue)
- Method for determining protein concentration: we recommend BCA Protein Quantification Kit (ab102536)

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)

Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

1. 20X Wash Buffer

Prepare 1X buffer by diluting 20X Wash Buffer in ddH₂O: to make 500 mL 1X buffer, combine 25 mL 20X Wash Buffer with 475 mL ddH₂O. Mix thoroughly and gently. Label this mixture as "1X Wash Buffer". Aliquot 20mL of this buffer and label it as "Dilution Buffer" to make activity buffer in step 10.1. Store Buffer at 4°C. Equilibrate to room temperature before use.

2. 10X Blocking Solution

Dilute 10X Blocking Solution in 1X Wash Buffer to create Incubation Solution: to make 100 mL Incubation Buffer, combine 10 mL of 10X Blocking Solution with 90 mL of 1X Wash Buffer. Mix thoroughly and gently. Label this mixture as "Incubation Solution". Store Incubation Solution at 4°C. Equilibrate to room temperature before use.

Note: 10X Blocking solution is also available as a separate product (ab126587)

3. 20X NADH

Reconstitute the lyophilized NADH in 1.1 mL of ddH₂O and mix thoroughly until dissolved to make a 40 mM solution. Aliquot reconstituted NADH so that you have enough volume to perform the desired number of assays. Store at -80°C. Reconstituted NADH is stable for up to 6 months. Keep on ice while in use.

4. 100X Dye

Reconstitute the dye in 250 µL of ddH₂O and mix thoroughly until dissolved. Aliquot reconstituted Dye so that you have enough volume to perform the desired number of assays. Reconstituted Dye is stable for up to 6 months. Store at -80°C. Keep on ice while in use.

Sample Preparation

General sample information:

We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.

We recommend that you use fresh samples. However, if you cannot perform the assay at the same time as the sample dilution and detergent extraction, we suggest that you freeze samples before extraction. Alternatively, if that is not possible, we suggest that you snap freeze detergent extracted samples in liquid nitrogen and store immediately at -80°C. When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

Considering careful experimental design, inhibitors or activators of Complex I can be added to samples, for example cells in culture, before extraction or added directly to assay solution during enzyme activity analysis.

Sample preparation for three types of sample – cultured cells, tissue and mitochondria:

Note: The Detergent solution is provided as 1 mL vial, enough to prepare 100 samples when using 90 µL of sample volume.

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

1. Harvest suspension cells by centrifugation or scrape to collect adherent cells from a confluent culture flask (initial recommendation = 1 – 2 x 10⁷ cells).
2. Wash cells twice with PBS.
3. Determine the sample protein concentration using a standard method such as BCA on a small portion of your sample. Then adjust concentration of the remaining sample with PBS so that the final sample protein concentration is 5.5 mg/mL.

Alternatively, resuspend and dilute the cell pellet to the approximately required concentration (5.5 mg/mL) by simply adding 9 volumes of PBS (e.g. 50 µL pellet + 450 µL PBS to a total volume of 500 µL).

- Using either method extract the proteins from the sample by adding 1/10 volume of Detergent solution to sample: to 90µL of sample volume add 10 µL of Detergent solution. Mix well by inversion.

Note: the sample concentration is now 5 mg/mL.

- Incubate the tube on ice for 30 minutes to allow protein extraction.
- Centrifuge the sample at 16,000 x g for 20 minutes at 4°C in a cold centrifuge.
- Collect supernatant and transfer to a clean tube on ice.
- Based on the protein concentration of sample extracted (5 mg/mL) dilute your samples to the desired concentration in Incubation Solution (from step 2). Table 1 indicates a typical linear range for the assay.

6. Preparation of extracts from tissue:

- Harvest tissue for the assay (initial recommendation - 100 to 200 mg).
- Wash tissue thoroughly in cold PBS.
- Resuspend tissue in 500 µL – 1 mL of ice cold PBS.
- Homogenize tissue with a Dounce homogenizer sitting on ice, with 20 – 40 passes, or until sample is fully homogenized and is completely smooth.
- Collect homogenate and transfer to a clean tube on ice.
- 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 5.5 mg/mL.
- Extract the proteins from the sample by adding 1/10 volume of Detergent solution to sample: to 90µL of sample volume add 10 µL of Detergent solution. Mix well.
- Incubate the tube on ice for 30 minutes to allow solubilization.
- Centrifuge the sample at 16,000 x g for 20 minutes at 4°C in a cold centrifuge.
- Collect supernatant and transfer to a clean tube on ice. Sample concentration: 5 mg/mL.
- Dilute your samples to the desired concentration in Incubation Solution (from step 2). Table 1 indicates a typical linear range for the assay.

7. Preparation of extracts from mitochondria:

You can isolate mitochondria using mitochondrial isolation kits such as ab110170 or ab110169.

- Determine the sample protein concentration accurately using a standard method such as BCA on a small portion of your sample. Then adjust the concentration of the remaining sample with PBS to a final sample protein concentration of 5.5 mg/mL.
- Extract the proteins from the sample by adding 1/10 volume of Detergent solution to the sample: to 90µL of sample volume add 10 µL of Detergent solution. Mix well by inversion.
Note: the sample concentration is now 5 mg/mL.
- Incubate the tube on ice for 30 minutes to allow protein extraction.
- Centrifuge the sample at 16,000 x g for 20 minutes at 4°C in a cold centrifuge.
- Collect supernatant and transfer to a clean tube on ice.
- Based on the protein concentration of sample extracted (5 mg/mL) dilute your samples to the desired concentration in Incubation Solution (from step 2). Table 1 indicates a typical linear range for the assay

Sample Type	Recommended sample concentration (µg/mL)
Cell culture extracts	125 – 1250
Fibroblast (MRC5) cell extracts	500
Heptoblastoma (HepG2) cell extracts	1000
Tissue extracts (mitochondria)	25 – 500
Heart extracts	100

Liver extracts	250
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Table 1. Typical ranges of measurement in µg/mL. 200 µL of sample is loaded per well.

Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature just prior to use and gently agitate.
- Assay positive control samples, background control samples and experimental samples in duplicate or more.

8. Plate Loading

- According to a sample map, load 200 µL of samples, positive controls and background/buffer only controls to the wells.
Sample wells: Sample prepared as described in the Sample Preparation section
Background/Buffer only control wells: 1X Incubation Solution
Positive control sample wells: customer defined positive sample
- Incubate microplate for 3 hours at room temperature on a plate shaker set to 400 rpm

9. Washing

- 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.
- Wash wells by adding 300 µL of 1X Wash Buffer solution to each well used.
- Repeat washing steps two more times. Do not aspirate the final wash until the assay solution is ready in 10.1.

10. Prepare Assay Solution

- Prepare 1.75 mL of Assay Solution per strip used according to the table below.
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Number of strips	1X Dilution Buffer (mL)	20X NADH (µL)	100X Dye (µL)	Total volume (mL)
1	1.645	87.5	17.5	1.75
2	3.29	175	35	3.5
3	4.935	262.5	52.5	5.25
4	6.58	350	70	7
5	8.225	437.5	87.5	8.75
6	9.87	525	105	10.5
7	11.515	612.5	122.5	12.25
8	13.16	700	140	14
9	14.805	787.5	157.5	15.75
10	16.45	875	175	17.5
11	18.095	962.5	192.5	19.25
12	19.74	1050	210	21

- Empty the wells from the last wash in 9.
- Add 200 µL of Assay Solution (from step 10.1) to each well carefully to avoid bubbles.
- Any bubbles created should be popped rapidly. Place the plate in the reader and record with the settings below.
- Save data, analyze as described in the "Data Analysis".

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

Δ Note: Measuring Time can vary depending upon the enzyme activity in the samples.

11. Data Analysis

- Extinction coefficient for dye (\square) = 25.9/mM/well
- Final concentration of NADH is 2 mM.
- Complex I activity in each well is proportional to the increase in absorbance at OD 450 nm within each well. The activity is expressed as the change in absorbance per minute per amount of sample loaded into the well.
- If the sample background control is significant, then subtract the sample background control from the sample reading.
- Examine the linear rate of increase in absorbance at OD 450 nm over time. An example is shown below where the rate/slope is calculated between these time points. Most microplate software of performing this function. Repeat this for all samples.
- Rate mOD/min = $\frac{\text{Abs 1} - \text{Abs 2 (mOD @ 450nm)}}{\text{Time 1} - \text{Time 2 (min)}}$

Precision

	Intra-Assay	Inter-Assay
n =	60	60
CV(%)	<10	<15

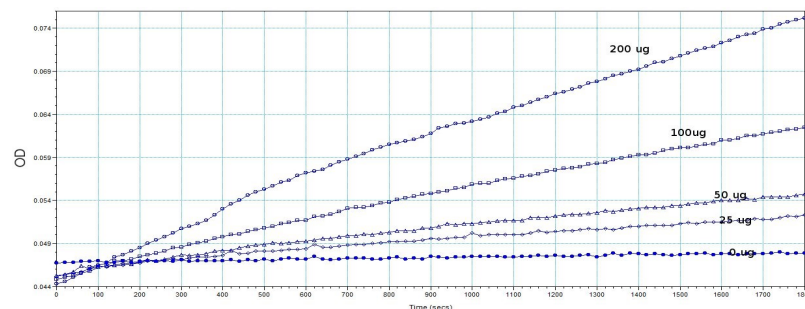


Figure 1. Example of raw data: change in OD 450 nm observed in cultured HepG2 cell lysate over time.

Technical Support

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FAQs / Troubleshooting

Problem	Reason	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 or no signal	Samples not efficiently extracted with detergent	Ensure sufficient protein and adequate detergent to protein ratio as detailed in the Sample Preparation section
	Cells/tissue samples not homogenized completely	Use Dounce homogenizer, increase number of strokes
	Samples used after multiple freeze/ 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) until use
Lower/ Higher readings in samples and Standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Allowing reagents to sit for extended times on ice	Always thaw and prepare fresh reaction mix before use
	Incorrect incubation times or temperatures	Verify correct incubation times and temperatures in protocol
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, rapidly pop any visible bubbles using a needle
	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