

CheKine™ Plus Micro Mitochondrial Complex IV Activity Assay Kit

Cat #: KTB1881

Size: 48 T/48 S 96 T/96 S

	Plus Micro Mitochondrial Complex IV Activity Assay Kit		
REF	Cat #: KTB1881	LOT	Lot #: Refer to product label
	Applicable samples: Animal and Plant Tissues, Cells		
	Storage: Stored at -20°C for 12 months, protected from light		

Assay Principle

Mitochondrial respiratory chain complex IV (EC 7.1.1.9), also known as cytochrome C oxidase, is also a common component of the main and branch pathways of the mitochondrial respiratory chain. It is responsible for catalyzing the oxidation of reduced cytochrome C, and ultimately transferring electrons to oxygen to produce water. CheKine™ Slow Micro Mitochondrial Complex IV Activity Assay Kit provides a convenient tool for detection of Mitochondrial complex IV Activity. The principle is Reduced cytochrome C has characteristic light absorption at 550 nm. Mitochondrial respiratory chain complex IV could catalyze reduced cytochrome C to oxidized cytochrome C. Therefore, the decreasing rate of light absorption at 550 nm can reflect the enzyme activity of mitochondrial respiratory chain complex IV. It can be used to determine animal, plant tissues and cell samples.

This kit effectively prolongs reaction duration by precisely regulating enzymatic reaction kinetics, eliminating result deviations caused by sample addition time differences, and providing ample buffer time for sample addition. Additionally, the pre-mixed working solution design further simplifies operational procedures. The kit is compatible with high-throughput batch sample testing and comprehensively addresses the detection challenges of mitochondrial respiratory chain complexes.

Materials Supplied and Storage Conditions

Kit components	Size		Storage conditions
	48 T	96 T	
Reagent I	60 mL	60 mL×2	4°C
Reagent II	30 mL	60mL	4°C
Reagent III	25 mL	40 mL	4°C
Reagent IV	Powder×1 vial	Powder×1 vial	-20°C, protected from light
Reagent V	Powder×1 vial	Powder×1 vial	-20°C, protected from light

Note: Before formal testing, it is recommended to select 2-3 samples with large expected differences for pre-experiment.

Materials Required but Not Supplied

- Microplate reader or visible spectrophotometer capable of measuring absorbance at 550 nm

- Incubator, ice maker, refrigerated centrifuge
- 96-well plate or microglass cuvette, precision pipettes, multi-channel pipette, disposable pipette tips
- Deionized water
- Homogenizer or mortar

Reagent Preparation

Reagent I : Ready to use as supplied. Placed on ice during the experiment. Store at 4°C.

Reagent II : Ready to use as supplied. Placed on ice during the experiment. Store at 4°C.

Reagent III : Ready to use as supplied. If the reagent has not been thawed, heat it in an 80°C water bath until complete dissolution and equilibrate to room temperature before use. Store at 4°C.

Working Reagent IV : Prepared before use. Add 1 mL deionized water for 48 T and 2 mL deionized water for 96 T to fully dissolve. The prepared Working Reagent IV should be stored at 4°C for 3 days or at -20°C for 1 month, protected from light. Avoid repeated freezing and thawing.

Working Reagent V : Prepared before use. Add 2 mL deionized water to fully dissolve. The prepared Working Reagent V should be stored at 4°C for 3 days or at -20°C for 1 month, protected from light. Avoid repeated freezing and thawing.

Working Solution: Prepared before use. Prepare the Working Solution by adding 201.9 µL per well (Reagent III 183 µL : Working Reagent IV 12.6 µL : Working Reagent V 6.3 µL, with a mixing ratio of 29 : 2 : 1), and mix thoroughly. **Incubate at 37°C for 5 min before use. Since high reagent viscosity may cause some loss, it is recommended to prepare about 20% more than the required amount.** The Working Solution should be prepared on demand and used immediately, stored in a sealed container and protect from light.

Note: Reagent III has high viscosity. To ensure accurate aspiration, the pipette tip should be rinsed once before using the pipette to accurately dispense the required amount of Reagent III. Working Solution should be gently inverted and mix thoroughly, avoiding vigorous shaking to prevent excessive foaming.

Sample Preparation

Note: Fresh samples are recommended to ensure enzyme activity. The samples should be extracted as fresh as possible. If immediate use is not required, store whole cells or tissues at -80°C for 1 month. After the extracted samples are resuspended with Reagent II, it should be detected as soon as possible. If testing cannot be done on the same day, store resuspension solution at -20°C for 1 day. The samples extracted by this kit can also be used for the determination of KTB1851, KTB1861, KTB1871 and KTB1891 (for detailed instructions, refer to the Precautions).

Extraction of Mitochondrial Respiratory Chain Complex IV:

1. Accurately weigh 0.1 g tissue or collect 5×10^6 cells, add 1 mL Reagent I, homogenize or mortar on ice. **Manual homogenization is recommended. If a large number of samples are prepared at one time, mechanical homogenization (in an ice bath) can be used to improve efficiency. The recommended parameters are 40 Hz, homogenization for 20 s, with a 10 s interval, repeated 3 times (2-3 mm steel beads). Direct mechanical homogenization of plant samples may result in enzyme activity decrease. It is recommended to conduct a pre-experiment to evaluate based on your own equipment conditions or use manual homogenization. Cells may also be processed by ice bath ultrasonic disruption for 5 min (power 20% or 200 W, ultrasonic 3 s, interval 7 s).**
2. Centrifuge the homogenate at 600 g for 5 min at 4°C, collect the supernatant to a new centrifuge tube and discard the pellet.
3. Centrifuge the supernatant again at 11,000 g for 10 min at 4°C. The pellet is the extracted mitochondria, which could be used to do step 5.
4. (Optional) The supernatant is cytoplasmic extract, which can be used as sample to determine mitochondrial respiratory chain complex IV leaking from mitochondria to judge the effect of mitochondrial extraction.
5. Add 400 µL Reagent II to the pellet, use a pipette to lift and gently shake the precipitate, and thoroughly resuspend it (avoid using a vortex mixer to mix vigorously as it may damage the complex; for subsequent steps, a gentle mixing with the vortex mixer can be used).
6. Dilution of the samples

Prior to formal testing, select 2-3 samples with anticipated significant differences and dilute them with Reagent II to different concentrations for preliminary experiments. Based on the results of these preliminary experiments, determine the appropriate dilution factor and follow the dilution protocol in the table below (for reference only):

Sample	Dilution Ratio	Sample	Dilution Ratio	Sample	Dilution Ratio	Sample	Dilution Ratio
Cells	undiluted	Mouse spleen	1-2	Rabbit heart	2-5	Rabbit lung	1-2
Mouse heart	8-10	Mouse lung	1-2	Rabbit brain	5-10	Rabbit kidney	5-10
Mouse brain	5-10	Mouse kidney	5-10	Rabbit liver	2-5	Rabbit muscle	undiluted
Mouse liver	8-10	Mouse muscle	1-2	Rabbit spleen	1-2	Plant tissues	undiluted

Assay Procedure

1. Preheat the microplate reader or visible spectrophotometer for more than 30 min, and adjust the wavelength to 550 nm. Visible spectrophotometer was returned to zero with deionized water.

2. Successively add 10 μ L sample and 200 μ L Working Solution into the 96-well plate or microglass cuvette. **Add the sample to the center of each well, and add the Working Solution vertically to the center of each well.** Read the initial absorbance value (0 min) at 550 nm as A_1 , then let it stand for 30 s, followed by shaking for 30 s, and then read the absorbance value (1 min) at 550 nm as A_2 . Finally calculate $\Delta A = A_1 - A_2$, $\Delta A_{\text{Supernatant}} = A_{1\text{Supernatant}} - A_{2\text{Supernatant}}$, $\Delta A_{\text{Pellet}} = A_{1\text{Pellet}} - A_{2\text{Pellet}}$.

Note: 1. In order to guarantee the accuracy of experimental results, need to do a pre-experiment with 1-2 samples. If ΔA is too high (above 0.1), the dilution ratio can be appropriately adjusted, and then measured again. If the sample is excessively turbid or dark in color, which may interfere with the detection, it is recommended to dilute the sample appropriately with Reagent II, and then measured again. If ΔA is less than 0.01, consider increasing sample volume, enhancing tissue weight or cell count, or reducing extract volume of Reagent II. A negative ΔA value indicates the absence of mitochondrial respiratory chain complex IV or its degradation.

2. If multiple samples are detected simultaneously, use a multi-channel pipette can increase the speed of sample addition. Completing all sample addition procedures within 10 min and avoid shaking the 96-well plate or microglass cuvette before testing, which will not affect the test results.

Data Analysis

Note: We provide you with calculation formulae, including the derivation process and final formula. The two are exactly equal. It is suggested that the concise calculation formula in bold is final formula.

A. 96-well plates calculation formula as below

1. Calculated by fresh weight of samples

Unit definition: One enzyme activity unit defines as the consumption of 1 nmol reduced cytochrome C in 1 g tissue per min in the reaction system.

The activity of supernatant Complex IV (U/g) = $[\Delta A_{\text{Supernatant}} \times V_{\text{Total}} \div (\epsilon \times d) \times 10^9] \div (W \div V_{\text{Extraction}} \times V_{\text{Sample}}) \div T = 2199 \times \Delta A_{\text{Supernatant}} \div W$

The activity of pellet Complex IV (U/g) = $[\Delta A_{\text{Pellet}} \times V_{\text{Total}} \div (\epsilon \times d) \times 10^9] \div (W \div V_{\text{Resuspended}} \times V_{\text{Sample}}) \div T = 880 \times \Delta A_{\text{Pellet}} \div W$

Total activity (U/g) = $2199 \times \Delta A_{\text{Supernatant}} \div W + 880 \times \Delta A_{\text{Pellet}} \div W$

2. Calculated by cell density

Unit definition: Every 10^4 cells consume 1 nmol reduced cytochrome C per minute that is defined as one enzyme activity.

The activity of supernatant Complex IV (U/ 10^4) = $[\Delta A_{\text{Supernatant}} \times V_{\text{Total}} \div (\epsilon \times d) \times 10^9] \div (V_{\text{Sample}} \div V_{\text{Extraction}} \times N) \div T = 2199 \times \Delta A_{\text{Supernatant}} \div N$

The activity of pellet Complex IV (U/ 10^4) = $[\Delta A_{\text{Pellet}} \times V_{\text{Total}} \div (\epsilon \times d) \times 10^9] \div (V_{\text{Sample}} \div V_{\text{Resuspend}} \times N) \div T = 880 \times \Delta A_{\text{Pellet}} \div N$

Total activity (U/ 10^4) = $2199 \times \Delta A_{\text{Supernatant}} \div N + 880 \times \Delta A_{\text{Pellet}} \div N$

Where: V_{Total} : total reaction volume, 2.1×10^{-4} L; ϵ : cytochrome C molar extinction coefficient, 19.1×10^3 L/mol/cm; d: 96-well plate diameter, 0.5 cm; 10^9 : Unit conversion factor, 1 mol = 10^9 nmol; V_{Sample} : sample volume added, 0.01 mL; T: reaction time, 1 min; $\Delta A_{\text{Supernatant}}$: determination value of the supernatant; W: sample weight, g; $V_{\text{Extraction}}$: sample extract volume, 1 mL; ΔA_{Pellet} :

determination value of Pellet; $V_{\text{Resuspended}}$: Volume of the resuspend pellet 0.4 mL; N: total number of cells, use 10^4 as the unit (e.g., total number of cells: 5×10^6 , $N=500$).

B. Microglass cuvette calculation formula

The optical diameter d: 0.5 cm in the above calculation formula can be adjusted to d: 1 cm for calculation

Precautions

1. The Working Solution has high viscosity. When dispensing 200 μL of the Working Solution, first rinse the pipette tip with the Working Solution (residual will remain after rinsing, so no squeezing is needed during sample addition). Be careful to avoid bubbles that may affect the test results.

2. For the same sample, all reagent kits (KTB1851, KTB1861, KTB1871, KTB1881, and KTB1891) can perform detection. How should this be operated?

(1) Reagent I and Reagent II in the KTB1851, KTB1861, KTB1871, KTB1881, and KTB1891 kits are interchangeable; Reagent I and Reagent II from any of these kits may be used to prepare samples.

(2) Accurately weigh 0.1 g tissue or collect 5×10^6 cells, add 1 mL Reagent I, homogenize or mortar on ice. **Manual homogenization is recommended. If a large number of samples are prepared at one time, mechanical homogenization (in an ice bath) can be used to improve efficiency. The recommended parameters are 40 Hz, homogenization for 20 s, with a 10 s interval, repeated 3 times (2-3 3 mm steel beads). Direct mechanical homogenization of plant samples may result in enzyme activity decrease. It is recommended to conduct a pre-experiment to evaluate based on your own equipment conditions or use manual homogenization. Cells may also be processed by ice bath ultrasonic disruption for 5 min (power 20% or 200 W, ultrasonic 3 s, interval 7 s).**

(3) Centrifuge the homogenate at 600 g for 5 min at 4°C , collect the supernatant to a new centrifuge tube and discard the pellet.

(4) Centrifuge the supernatant again at 11,000 g for 10 min at 4°C . The pellet is the extracted mitochondria, which could be used to do step (6).

(5) (Optional) The supernatant is cytoplasmic extract, which can be used as sample to determine mitochondrial respiratory chain complex I - V leaking from mitochondria to judge the effect of mitochondrial extraction.

(6) Add 200 μL of Reagent II to the pellet, use a pipette to lift and gently shake the pellet, and thoroughly resuspend it (avoid using a vortex mixer to mix vigorously as it may damage the complex; for subsequent steps, a gentle mixing with the vortex mixer can be used). After completing step 7, place the sample on ice for testing.

For the detection of Complex I, Complex II, and Complex III, samples should be diluted with Reagent II according to the recommended dilution ratios specified in the instructions for KTB1851, KTB1861, and KTB1871, respectively. For Complex IV, samples must first be diluted 2-fold with Reagent II before further dilution using Reagent II according to the recommended ratio in the KTB1881 instructions. For Complex V, samples should be diluted 4-fold with Reagent II prior to direct use for detection.

Typical Data

Examples:

1. Test 0.1g mouse liver tissue, prepared the sample following the above protocol and measured with the 96-well plate:

$\Delta A_{\text{Supernatant}} = A_1 - A_2 = 0.462 - 0.450 = 0.012$, $\Delta A_{\text{Pellet}} (\text{Diluted } 10\times \text{ with Reagent II}) = A_1 - A_2 = 0.440 - 0.385 = 0.055$.

2. Calculated by fresh weight of samples:

Complex IV activity of the supernatant (U/g) = $2199 \times \Delta A_{\text{Supernatant}} \div W = 2199 \times 0.012 \div 0.1 = 263.88$ U/g

Complex IV activity of the pellet (Diluted $10\times$) (U/g) = $880 \times \Delta A_{\text{Pellet}} \div W = 880 \times 0.055 \div 0.1 = 484.00$ U/g

The total Complex IV activity (U/g) = $2199 \times \Delta A_{\text{Supernatant}} \div W + 880 \times \Delta A_{\text{Pellet}} \div W = 263.88 + 484.00 = 747.88$ U/g

Recommended Products

Catalog No.	Product Name
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KTB1851	CheKine™ Plus Micro Mitochondrial Complex I Activity Assay Kit
KTB1861	CheKine™ Plus Micro Mitochondrial Complex II Activity Assay Kit
KTB1871	CheKine™ Plus Micro Mitochondrial Complex III Activity Assay Kit
KTB1891	CheKine™ Plus Micro Mitochondrial Complex V Activity Assay Kit

Disclaimer

The reagent is only used in the field of scientific research, not suitable for clinical diagnosis or other purposes. For your safety and health, please wear a lab coat and disposable gloves.