PROTOCOL

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# Complex IV Rodent Enzyme Activity Dipstick Assay Kit

**MS432**

Rev.2

## DESCRIPTION

**Complex IV Rodent Enzyme Activity Dipstick Assay Kit**

Sufficient materials are provided for 30 (MS432-30) or 90 (MS432-60) measurements.

**Kit Contents:**

| Item | MS432-30 | MS432-90 |
|---|---|---|
| Dipsticks | 30 | 90 |
| Buffer A (Extraction buffer) | 15 mL | 45 mL |
| Buffer B (Blocking buffer) | 2 mL | 6 mL |
| Buffer C (Wash buffer) | 1 mL | 3 mL |
| Tube 1 (DAB – 100X stock) | 100 µL | 300 µL |
| Tube 2 (Reduced cytochrome *c* – 20X stock) | 500 µL | 1.5mL |
| Tube 3 (Buffer for activity assay) | 10 mL | 30 mL |
| 96-well microplate | 1 | 3 |

**Storage:**

Store dipsticks at room temperature in their provided container and out of direct sunlight. High humidity conditions should be avoided. Store Buffer A, B, and C at 4°C or at -20°C for long term storage. Store Tubes 1 and 2 at -20°C or at -80°C for long term storage; they can also be aliquoted upon receipt to prevent freeze/thaw cycles. Tube 3 can be stored at room temperature.

## INTRODUCTION

The Complex IV Rodent Enzyme Activity Dipstick Assay Kit (MS432) is used to quantify the activity of the Complex IV (COX) enzyme complex (EC 1.9.3.1) from rat and mouse samples; isolation of mitochondria is not necessary. Using an antibody immobilized on the dipstick that specifically recognizes COX, the enzyme complex is immno-captured (i.e immuno-precipitated in active form) and enzyme activity is determined directly on the dipstick. Using a traditional method for determining COX enzyme activity by histo-chemical methods and in-gel activity assays, Di-amino benzidinetetrachloride (DAB) serves as the reporter of COX activity (Figure 1A). The greater the signal of precipitated DAB, the greater the amount of COX enzyme complex (Figure 1B). In addition, the reaction is cyanide sensitive. The signal intensity is best measured by a dipstick reader (MS1000, MitoSciences dipstick reader) or may be analyzed by another imaging system.

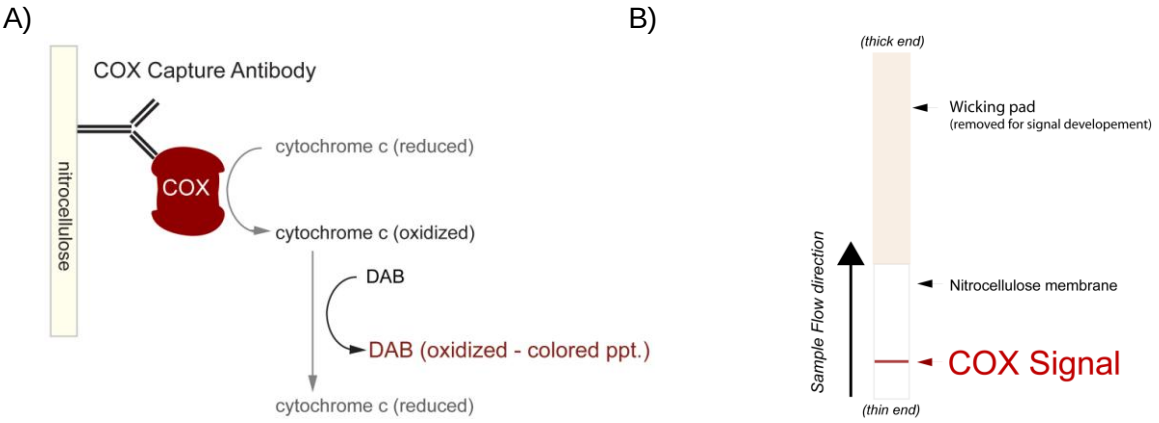


***Figure 1**: **Schematics of the COX Activity reaction and a fully developed COX activity dipstick. (A)** Mechanism of the COX activity assay; the immunocaptured COX dipstick is immersed in a solution containing reduced cytochrome c and DAB. COX then generates oxidized cytochrome c, which in turn oxidizes DAB to form a red-colored precipitate at the antibody line. **(B)** A COX activity dipstick fully developed (dipstick wick is removed before color development). The anti-COX mAb is stripped ~7mm from the bottom of the dipstick.*

## ADDITIONAL MATERIALS REQUIRED

- Dipstick reader (MitoSciences' MS1000) or other imaging system
- Method for determining protein concentration
- Pipetting devices
- Protease inhibitors



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# DIPSTICK ASSAY PROTOCOL

## A. Sample Preparation

The preparation of the sample is a critical step in this procedure. The effective solubilization of the mitochondrial membranes in the sample is necessary. Please choose the sample preparation method that best fits your sample type. Follow each of the protocol steps carefully.

***Note:*** *Samples must be kept on ice.*

1. Tissue samples:
    a. Begin with approximately 25 mg of sample.
    b. Add 5 volumes/ weight of iced Buffer A to the sample (e.g. if the total sample weight is 50 mg, add 250 µL of Buffer A).
    c. Homogenize the sample.
    d. Keep the sample on ice for 20 minutes and mix intermittently.
    e. Spin the cell extract in a micro-centrifuge at 13,000 to 16,000 rpm for 20 minutes at 4°C.
    f. Remove the supernatant and determine the protein concentration of the protein extract from the sample. Protein concentration should be a 1 mg/mL.
    g. Proceed directly to Part B of the Protocol or freeze samples at -80°C.
2. Cell culture samples:
    a. Add 5 volumes of Buffer A / volume of cell pellet (e.g. if the total sample is 50 µL of volume, add 250 µL of Buffer A).
    b. Keep the sample on ice for 20 minutes and mix intermittently.
    c. Spin the cell extract in a micro-centrifuge at 13,000 to 16,000 rpm for 20 minutes at 4°C.
    d. Remove the supernatant and determine the protein concentration of the protein extract from the sample. Protein concentration should be ≥ 1 mg/mL.
    e. Proceed directly to Part B of the protocol or freeze samples at -80°C.


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## B. Dipstick Procedure

The assay is most accurate with a user established standard curve for interpolation of signal intensity. Following the protein concentration ranges as defined in Table 1, generate a standard curve using a positive control sample (see example experiment on pg. 7 for reference).

| Sample Type | Working Range |
|---|---|
| Mouse tissue | 5 - 75 µg |
| Rat tissue | 5 - 75 µg |

**Table 1**: *Suggested working range for different sample types.*

1. Load the amount of protein that corresponds toward the high end of the user generated standard curve (~3/4 of the high end).
2. Dilute samples to 25 µL with Buffer A.
3. Add 25 µL of Sample in Buffer A and 25 µL of Buffer B to a microplate well and mix.

   *Note: If protein concentration is too low, 100 µL reaction volumes are possible. Be sure to keep the ratio of sample in Buffer A to Buffer B constant.*
4. Gently add a dipstick to the sample (place the thin/nitrocellulose end of dipstick down).
5. Allow the sample to wick up into the dipstick (this takes 15-45 minutes depending on sample viscosity and total volume).

   *Note: The entire sample volume has to be absorbed by the dipstick before proceeding to the next step, but do not allow the dipstick to dry at any time during this procedure.*
6. Wash by adding 30 µL of Buffer C to each well with a dipstick.
7. Allow the dipstick to wick up the buffer for 10 minutes.

   *Note: Do not allow the dipstick to dry out at any time.*
8. Prepare the Activity Buffer by combining Tube 1, 2, and 3 as per Table 2. Keep at room temperature.

| Amount of Activity Buffer produced | Tube 1 (µL) | Tube 2 (µL) | Tube 3 (mL) |
|---|---|---|---|
| 5 mL | 50 | 250 | 4.7 |
| 10 mL | 100 | 500 | 9.4 |

**Table 2.** *Preparation of Activity Buffer*

9. Add 300 µL of Activity Buffer to an empty microplate well for each dipstick used.
10. Remove the wicking pad from the dipstick. Make sure to remove the pad at the junction with the membrane.

11. Place the dipstick in a well with Activity Buffer. The Complex IV (COX) capture mAb is ~7mm from the bottom of the dipstick.

12. Develop for 45 - 60 minutes.

    *Note: Since this is an end-point reaction, develop all dipsticks for the same time period.*

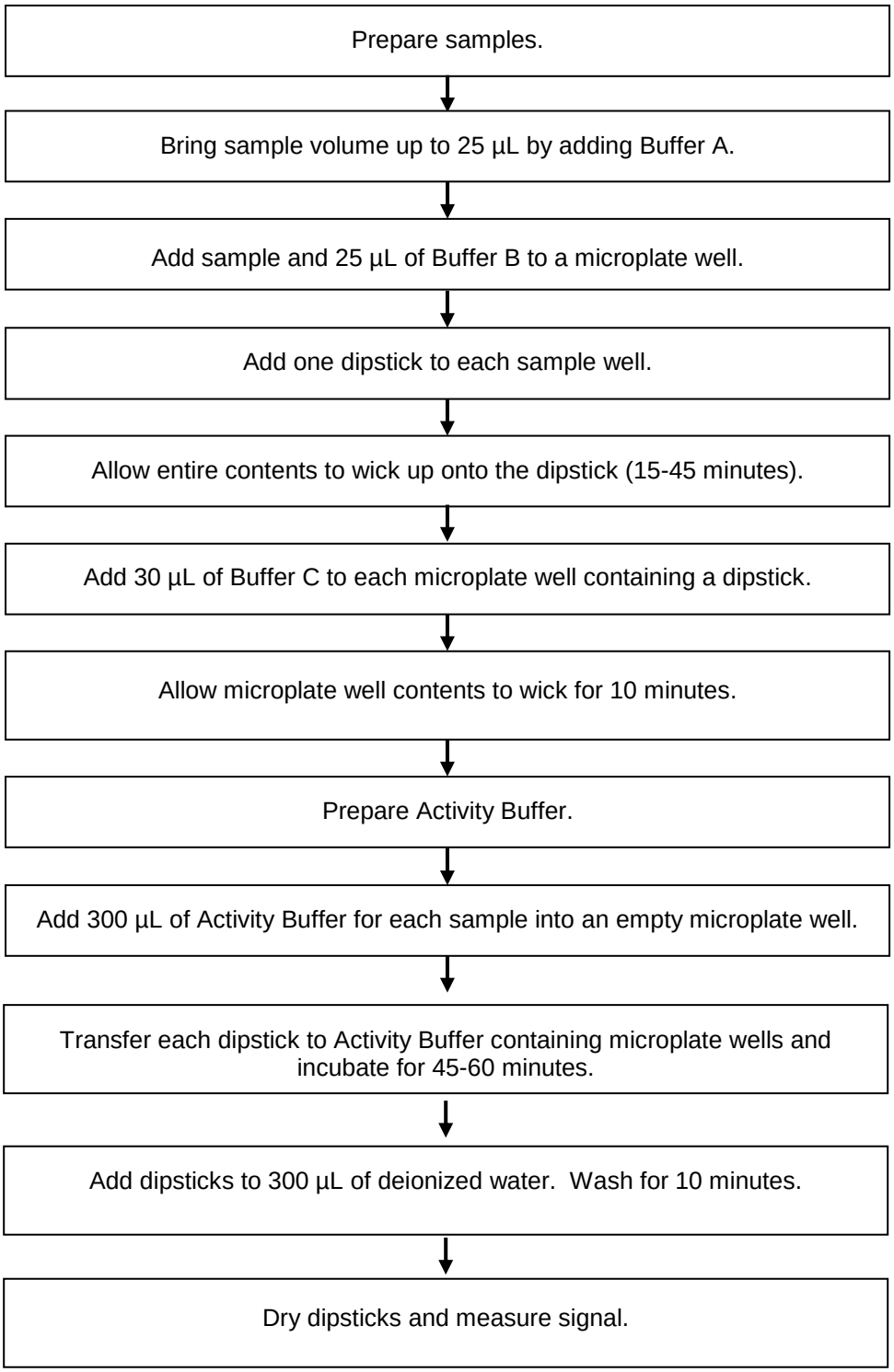
13. Add 300 µL deionized water to an empty well of the microplate.

14. Once the dipstick(s) are developed add them to the well with deionized water for 10 minutes.

15. Dry the dipstick and measure the signal intensity with a dipstick reader (MitoSciences' MS1000 Dipstick Reader) or other imaging system, e.g. flat-bed scanner.

## FLOW CHART

For quick reference only. Be completely familiar with the previous details of this protocol before performing the assay.

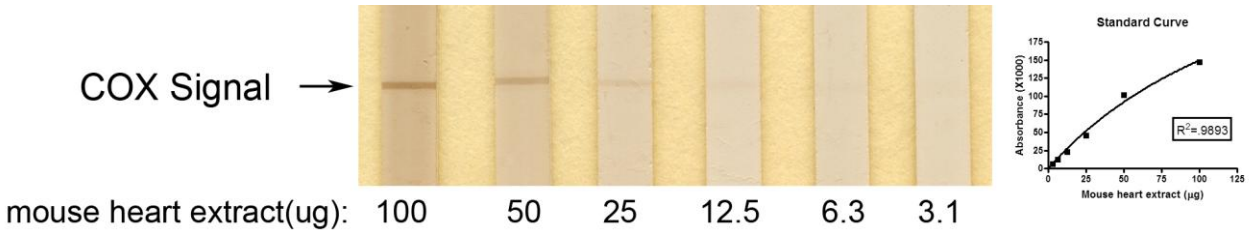


## EXAMPLE EXPERIMENT

Below is an example using the MS432 kit to quantify Complex IV enzyme activity in mouse protein extracts. Samples were prepared as described in the Sample Preparation section. All data were analyzed using MitoSciences' MS1000 Dipstick Reader and GraphPad software.

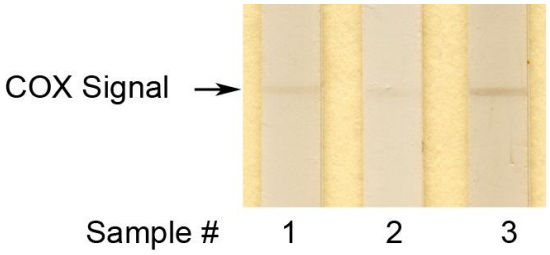
### Step 1. Generating a standard curve

Shown are developed dipsticks from a 1:2 dilution series using a positive control sample and the associated standard curve. Starting material was 100 µg of protein extract.




### Step 2. Analysis of samples

Based on the standard curve, 50 µg of protein extract were loaded onto a dipstick for each sample. The figure below shows three developed dipsticks from unknown samples (1-3). The analysis of the signal intensity and interpolation from the standard curve showed that the unknown samples have between 23-87% of normal Complex IV activity levels.




| Sample # | Abs.(X1000) | Extrapolated value(ug) | % control |
|---|---|---|---|
| 1 | 41.3 | 19.3 | 38.6 |
| 2 | 25.3 | 11.3 | 22.6 |
| 3 | 82.3 | 43.2 | 86.4 |



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## TROUBLESHOOTING GUIDE

### Signal is saturated

It is very important that the amount of sample used is within the working range of the assay (use a best fit line for interpolation). Therefore, it is crucial to determine the working range for your sample type and avoid the region of signal saturation.

### Signal is too weak

This occurs when the sample lacks measurable amounts of the protein. Increase the signal by adding more sample protein to another dipstick, or leave the dipstick in the activity solution for longer to maximize the signal

### Sample is not wicking up the dipstick

If the dipstick is not gently handled, the nitrocellulose membrane and wicking pad may become separated. Check this junction and simply pinch the dipstick at this point to reconnect the two. Check for proper wicking of the sample.



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