PROTOCOL

MitoSciences
1850 Millrace Drive, Suite 3A
Eugene, Oregon 97403
www.mitosciences.com

Complex IV Human Protein Quantity Dipstick Assay Kit

MS431

Rev.1

DESCRIPTION

Complex IV Human Protein Quantity Dipstick Assay Kit

Sufficient materials for 30 (MS431-30) or 90 (MS431-90) measurements.

Kit Contents:

Item	MS431-30	MS431-90
Dipsticks	30	90
Gold-conjugated antibody (dried in microplate wells)	30 wells	90 wells
Buffer A* (Extraction buffer)	15 mL	45 mL
Buffer B* (Blocking buffer)	1 mL	3 mL
Buffer C* (Wash buffer)	1 mL	3 mL

Storage:

Store dipsticks and gold-conjugated secondary antibody at room temperature out of direct sunlight in their provided containers. Both are stable for 6 months. High humidity conditions should be avoided. Store Buffers A, B, and C at 4°C, or at -20°C for long-term storage. **Buffers A, B, and C are interchangeable with Buffers A, B, and C from other Quantity Dipstick Assay Kits (MS131, MS133, MSF31, MSP31).*

INTRODUCTION

The Complex IV Human Protein Quantity Dipstick Assay Kit (MS431) is used to rapidly quantify the levels of the fully assembled cytochrome *c* oxidase (COX) enzyme complex (EC 1.9.3.1) from human tissue. Bovine samples can also be used with this assay. Based on the immunologic sandwich assay, this kit utilizes two monoclonal antibodies (mAbs) specific to different antigens present on the intact COX enzyme complex. One antibody is immobilized on nitrocellulose membrane in a thin line perpendicular to the length of the dipstick while the other is gold-conjugated for a visual signal (Figure 1B). When assembled COX is present in the sample, a red line appears on the dipstick at the site of the COX antibody line. The signal intensity is directly related to the level of COX enzyme complex in the sample. The signal intensity is best measured by a dipstick reader (MitoSciences' MS1000 is recommended) or may be analyzed by another imaging system.

A) B)

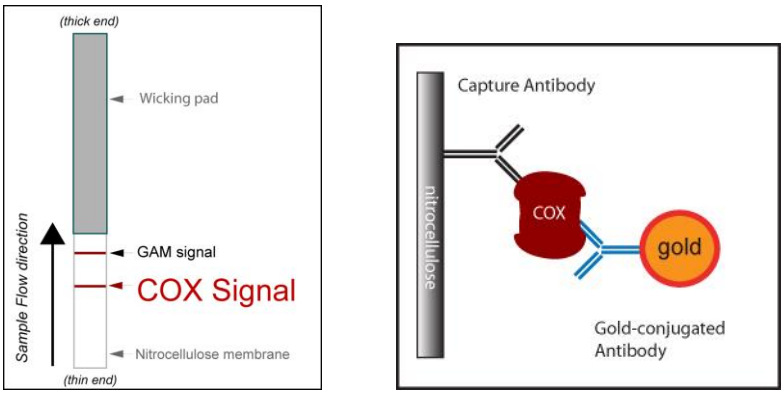


Figure 1. Schematics of a developed COX dipstick and antibody-COX sandwich diagram: ***(A)*** *A finished dipstick showing a strong signal for COX (bottom band) and the positive control (Goat anti-mouse antibody (GAM)) which is used to verify completion of the reaction (i.e. the flow of sample contents and gold-conjugated mAb past the capture antibody line). The dipstick is added to the sample with membrane end (thin end) down.* ***(B)*** *The antibody sandwich diagram showing the interaction between COX and the two antibodies.*

ADDITIONAL MATERIALS REQUIRED

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

DIPSTICK ASSAY PROTOCOL

A. Sample Preparation

Note: *Samples must be kept on ice. Protease inhibitors are not provided.*

1. Tissue Sample Preparation

    a. Start with approximately 25 mg of tissue sample. Add 10 volumes of Buffer A per microgram of sample (e.g. if the sample weighs 50 mg, add 500 µL of Buffer A).

    b. Homogenize the sample.

    c. Keep on ice for 20 minutes, mixing intermittently.

    d. Spin the cell extract in a microcentrifuge at 13,000-16,000 rpm at 4°C for 20 minutes to pellet cellular debris.

    e. Transfer supernatant to a new tube and determine protein concentration (e.g. BCA assays are recommended as the detergent in Buffer A does not affect the reading in the assay).

    f. Proceed directly to Part B of the Protocol or freeze samples at -80°C.

2. Cell Culture Sample Preparation

    a. Start with a cell pellet. Add 10 volumes of Buffer A to the cell pellet and mix (e.g. if the total sample volume displaces 50 µL of volume, then add 500 µL of Buffer A).

    b. Keep on ice for 20 minutes, mixing intermittently.

    c. Spin the cell extract in a microcentrifuge at 13,000-16,000 rpm at 4°C for 20 minutes to pellet cellular debris.

    d. Transfer supernatant to a new tube and determine protein concentration (e.g. BCA assays are recommended as the detergent in Buffer A does not affect the reading in the assay).

    e. Proceed directly to Part B of the protocol or freeze samples at -80°C.

B. Dipstick Procedure

The assay is most accurate with a user established standard curve for interpolation of the signal intensity. Following the protein concentration ranges as defined in Table 1, generate a standard curve using a positive control sample.

Sample Type	Working Range
Human fibroblasts	0.5 – 10 µg
PBMC	0.5 – 10 µg
Lymphoblastoid	0.1 – 6 µg
Fat	2 – 20 µg
Muscle	0.05 – 1.0 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. Add Buffer A to the sample to bring volume up to 25 µL.

3. Add the sample and 25 µL of Buffer B to a microplate well of dried gold-conjugated antibody, for a total sample volume of 50 µL. Incubate the sample for 5 minutes, allowing the gold-conjugate to hydrate.

4. Re-suspend the dried gold-conjugated secondary antibody using a pipette.

5. Gently add a dipstick to the microplate well (place the thin/nitrocellulose end of dipstick down). The dipstick must reach the bottom of the well.

6. Allow the sample wick up onto the dipstick until the entire sample volume has been absorbed (the positive control band typically appears within 2 minutes. A 50 µL sample should be wicked up completely in 13-20 minutes; more viscous extracts may take longer. **The dipstick must absorb the entire sample volume before Buffer C is added**.

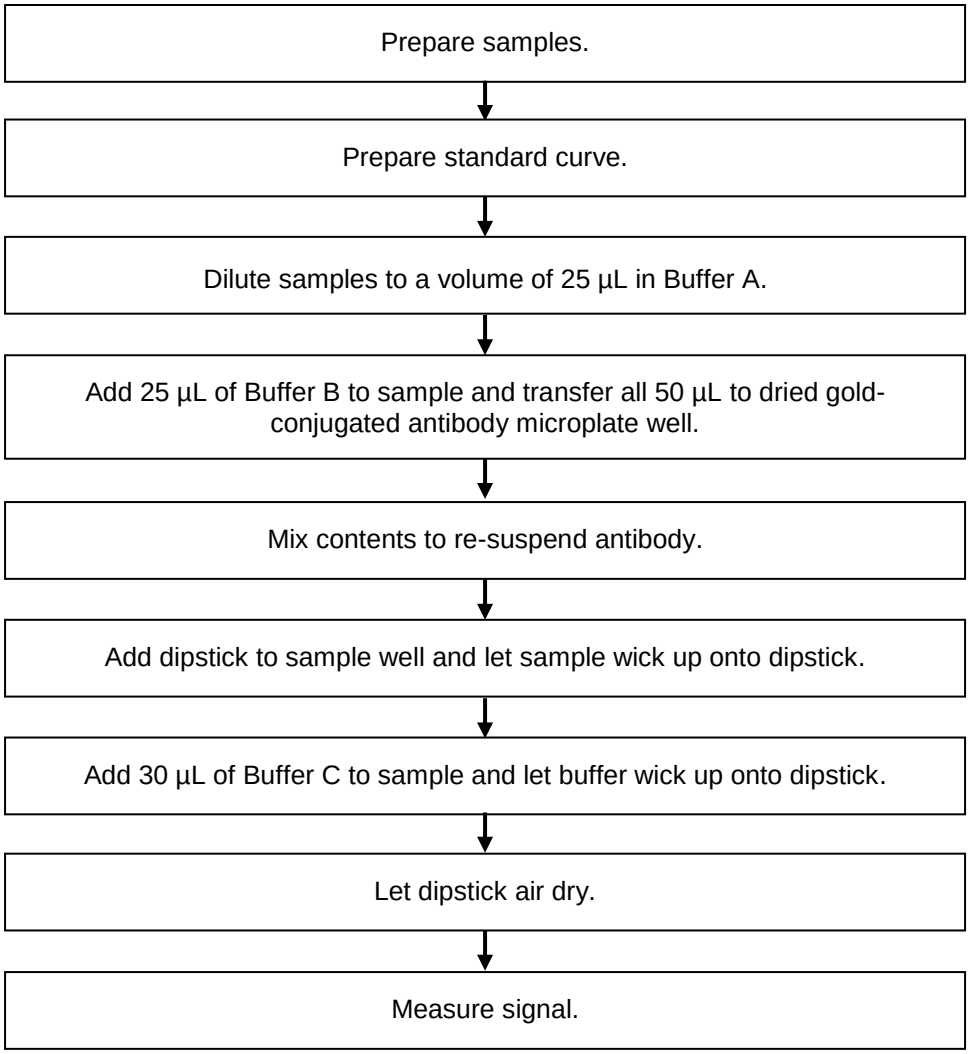
7. Add 30 µL of Buffer C and let the entire buffer volume wick up onto the dipstick.

8. Leave dipstick to air-dry (requires approximately 20 minutes to air dry, or 10 minutes in an incubator at 37°C).

9. 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.

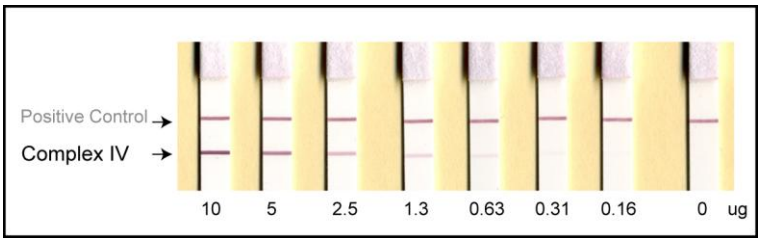


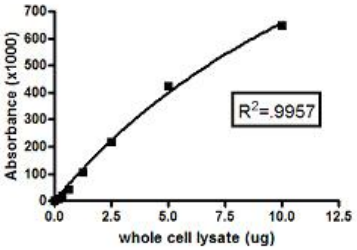
EXAMPLE EXPERIMENT

Illustrated is an experiment using the MS431 assay kit to quantify Complex IV levels using various concentration of human fibroblast extract. Samples were prepared as described in this protocol. All data were analyzed using MitoSciences Dipstick Reader (MS1000) and GraphPad software.

Step 1. Generating a standard curve

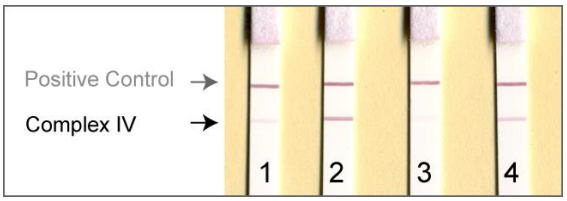
Shown is a 1:2 dilution series using a positive control sample (see Table 1 for recommended protein ranges). Approximately 7 to 8 dipsticks are suitable for covering the entire working range and the blank for background levels. In this example the dilution series starts with 10 µg of fibroblast extract. A one-site hyperbola line was generated for best-fit analysis using GraphPad.





Step 2. Analysis of samples

Based on the above standard curve values, 5 µg of protein extract were loaded to each dipstick for the four unknown samples (1-4).



unknown #	Signal (Absorbance x 1000)	% of Normal
normal	425.5	**100**
1	51.9	**10.5**
2	276.4	**63.9**
3	18.3	**3.6**
4	126.4	**26.7**

Using GraphPad software, the signal intensity from the standard curve was interpolated and the quantity of Complex I in samples 1-5 was determined. Based on the above analysis, the unknown samples had between 4% and 64% of Complex IV levels compared to the control.

TROUBLESHOOTING GUIDE

Signal is saturated

It is very important that the amount of sample used is within the working range of the assay (a best fit line for interpolation as generated with the GraphPad program). Therefore, it is crucial to know the working range for the sample type and avoid the region of signal saturation (see Table 1). Determination of the working range is recommended for the sample in case of signal saturation.

Signal is too weak

This occurs when the sample lacks measurable amounts of the protein. To increase signal add more sample to another dipstick.

Positive control band does not appear

Make sure that the wicking paper is in contact with the nitrocellulose membrane. Care should always be taken when handling the dipsticks to prevent disruption of this junction (see Figure 1).