PROTOCOL

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MitoTox™ MitoBiogenesis™ Dipstick Assay Kit MS631

Rev.2

DESCRIPTION

MitoTox™ MitoBiogenesis™ Dipstick Assay Kit

Sufficient materials are provided for 30 (MS631-30) or 90 (MS631-90) measurements.

Kit Contents:

Item	MS631-30	MS631-90
Dipsticks	30	90
Gold-conjugated antibody dried in a microplate	30 wells	90 wells
Extraction Buffer (Buffer A)	15 mL	45 mL
Blocking Buffer (Buffer B)	1 mL	3 mL
Wash Buffer (Buffer C)	1 mL	3 mL

Storage:

Store the dipsticks and the microplate at room temperature out of direct sunlight in their provided container. Both are stable for 6 months. High humidity conditions should be avoided. Store Extraction Buffer (Buffer A), Blocking Buffer (Buffer B), and Wash Buffer (Buffer C) at 4°C or at -20°C for long-term storage.

INTRODUCTION

The MitoTox™ MitoBiogenesis™ Dipstick Assay Kit (MS631) is a rapid and simple test for determining whether mitochondrial biogenesis has been up-regulated or down-regulated in human samples as a result of drug interactions or conditions such as oxidative stress and mitochondrial genetic disease. MS631 detects altered mitochondrial biogenesis whether caused by altered mtDNA levels or disfunctional protein synthesis by mitochondrial ribosomes. The assay works on very small amounts of sample material, including cheek swabs or single drops of blood, and results can be generated in under 30 minutes. The assay also works on cultured cells and is suitable for any situation in which speed and simplicity are required. MS631 is particularly suited for clinical evaluation of mitochondrial toxicity from antibiotic and antiviral drugs, and has been approved by the FDA for Investigational Use Only (IUO).

Each of the dipsticks in the kit contains a goat anti-mouse IgG-Fc antibody, an anti-frataxin monoclonal antibody (mAb) and an anti-Complex IV mAb deposited in a narrow upper zone, middle zone and lower zone, respectively, on a nitrocellulose membrane. Complex IV and frataxin are captured by their respective antibodies on the dipstick and visualized by gold-conjugated anti-Complex IV and anti-frataxin detector mAbs. The anti-Complex IV capture mAb and anti-Complex IV detector mAb identify different epitopes of Complex IV and form an antibody-Complex IV-antibody sandwich only with fully assembled Complex IV. Similarly, the anti-frataxin capture and detection mAbs identify different epitopes of frataxin and form an antibody-frataxin-antibody sandwich only with intact frataxin. The uppermost zone of goat anti-mouse IgG-Fc antibody on the dipsticks captures excess gold-detector mAbs regardless of the presence or absence of target antigen and, thus, verifies that the entire sample has wicked up through the nitrocellulose membrane (see Figure 1).

Standard curves of Complex IV and frataxin levels are generated by the user with a dilution series of control cells. The signal intensities of the Complex IV and frataxin bands on the dipsticks are measured

with a dipstick reader (MitoSciences' MS1000 is recommended) or may be analyzed with another imaging system. The levels of Complex IV and frataxin in drug-treated cells are then measured. The Complex IV: frataxin ratio of the drug-treated cells is then compared with that of the control cells by interpolating their signal intensities into their respective standard curves.

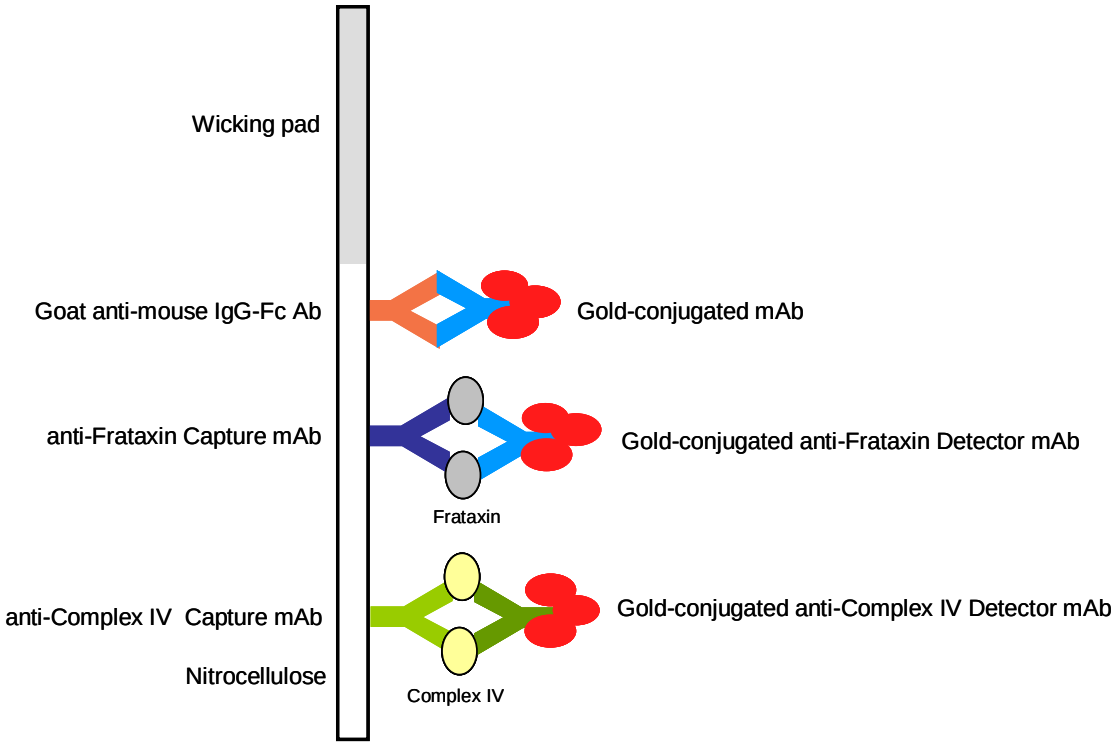

Figure 1. Schematic representation of the dipstick after the sample and detector mAbs have been added

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. Cell Culture Sample Preparation

    a. Start with a cell pellet. Add 10 volumes of Extraction Buffer (Buffer A) to the cell pellet and mix (e.g. if the total sample volume displaces 10 µL of volume, then add 100 µL of Extraction Buffer).

    b. Keep on ice for 20 minutes.

    c. Spin the cell extract in a microcentrifuge at 16,400 rpm at 4°C for 20 minutes to pellet cellular debris.

    d. Transfer the supernatant to a new tube and determine the protein concentration (e.g. BCA assays are recommended as the detergent in the Extraction Buffer 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

Carry out steps B1 – B7 with varying amounts of protein (e.g. 0, 0.5, 1, 2, 4, 8 µg protein) ***from the control (vehicle-treated) cells initially*** in order to determine the appropriate amount of protein to use for the drug-treated samples. The assay is most accurate with user-generated standard curves for interpolation of the signal intensities for Complex IV and frataxin. Table 1 shows a typical working range for generating standard curves with control cells.

Sample Type	Working Range
HepG cells	0.5 - 8 µg
Human fibroblasts	0.5 - 10 µg

Table 1. *Typical working range for different sample types*

1. Add Extraction Buffer (Buffer A) to each sample to bring the volume up to 25 µL.

2. Add 25 µL of each sample and 25 µL of Blocking Buffer (Buffer B) to a microplate well of dried gold-conjugated antibody. Incubate the sample for 5 minutes, ensuring that the dried gold-conjugated antibody is mixed well with the sample.

3. Gently add the dipsticks to the wells, placing the *thin/nitrocellulose* end of the dipsticks into the samples.

4. Allow the samples to wick up onto the dipsticks (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.

    Note: The dipstick must absorb the entire sample volume before the Wash Buffer (Buffer C) is added.

5. Add 30 µL of Wash Buffer (Buffer C) to each well and let *all* of it wick up onto the dipsticks.

6. Let the dipsticks dry either at room temperature for approximately 20 minutes or at 37°C for 10 minutes.

7. When the dipsticks have dried completely, measure the signal intensity of the Complex IV band and frataxin band on each dipstick with a dipstick reader (MitoSciences' MS1000 Dipstick Reader) or other imaging system, e.g. flat-bed scanner.

C. Generation of 2 standard curves using the signal intensities of Complex IV and frataxin

1. Using the signal intensities of the Complex IV band and frataxin band on each dipstick, generate 2 standard curves using a curve-fitting software program. Figure 2 shows a 2 fold dilution series using vehicle-treated HepG2 cells. Two dipsticks were used for each protein concentration. A one-site hyperbola line was generated for best-fit analysis using GraphPad software.

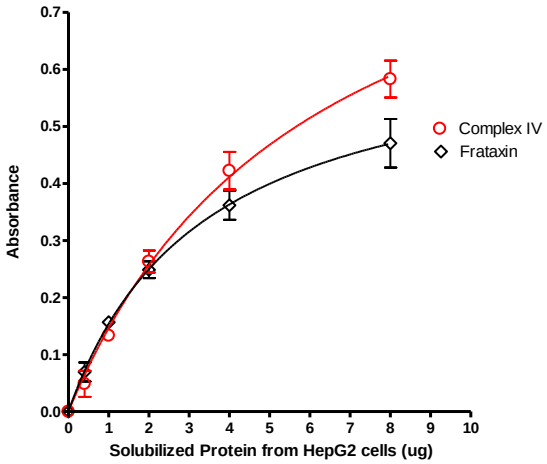

Figure 2. Example of standard curves.

2. Determine a suitable amount of protein for testing the drug-treated samples. This amount should correspond toward the middle of the standard curves. In this example it is approximately 2 µg of solubilized protein from HepG2 cells. Please note that the amount of protein required may vary.

D. Analysis of drug-treated samples and calculation of the Complex IV: frataxin ratio

Carry out steps B1 – B7 using the drug-treated samples.

By definition, the *control* (vehicle-treated) cells are assumed to contain 1 unit of each antigen per microgram total cell protein, i.e. a 1:1 ratio of Complex IV and frataxin. The ratio of these two proteins in the *drug-treated* samples in comparison to the 1:1 ratio in the control cells is then calculated by interpolating the Complex IV and frataxin signal intensities of the drug-treated samples into the respective standard curves generated from the control cells.

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 curve-fitting program). Therefore, it is crucial to know the working range for the sample type in order to avoid the region 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.

FLOW CHART

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