

MetaPath™ Fatty Acid Oxidation 4-Plex Dipstick Array

MSX32

Rev.1

DESCRIPTION

MetaPath™ Fatty Acid Oxidation 4-Plex Dipstick Array for Human MCAD, SCHAD, TFP and Frataxin Quantity

Sufficient materials for 30 or 90 dipstick reactions.

Kit Contents:

Item	MSX32-30	MSX32-90
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)	2 mL	6 mL

Storage:

Store dipsticks and gold-conjugated 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 and C at 4°C, and Buffer B at -20°C. **Buffers A, B, and C are interchangeable with other Quantity Dipstick Assay Kits (MSX31, MS131, MS133, MS431 MSF31, MSP31, MSA31). After use, be sure to remove any liquid from the plate before storing in foil bag with desiccant.*

INTRODUCTION

Fatty acid- or beta-oxidation is the process where fatty acids are sequentially shortened 2 carbons per round to yield acetyl-CoA, FADH₂ and NADH for the production of ATP in mitochondria. Fatty acids from outside the cell can readily enter the cytoplasm through the plasma membrane. After activation long, chain fatty acids are attached to carnitine by carnitine acyl transferase in the mitochondrial membrane and then transported into the mitochondrion where the carnitine is removed. Medium and short chain fatty acids are carnitine independent and freely enter the mitochondrion where they are also activated to form fatty-acyl-CoA.

The process of fatty acid catabolism requires four reactions and is shown schematically below (Figure 1).

Reaction 1: Dehydrogenation of the fatty-acyl-CoA to 2,3 Enoyl CoA and FADH₂ by acyl dehydrogenases. Since fatty acids vary in length there are several different acyl dehydrogenases: short chain (SCAD, 4-6 carbon), medium chain (MCAD, 6-12 carbon), and long chain (LCAD/VLCAD, 12+ carbon).

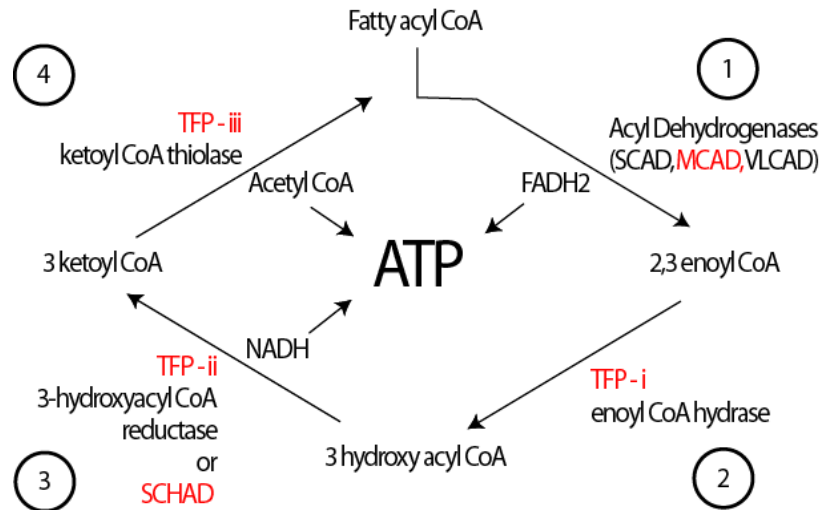
Reaction 2: the catalytic production of 3-hydroxyacyl-CoA by 2,3 Enoyl CoA hydratase (TFP).

Reaction 3: the production of 3-ketoacyl-CoA and NADH by 3-hydroxyacyl-CoA reductase, LCHAD (TFP)

Reaction 4: the production of acetyl CoA and a fatty acyl CoA which is now 2 carbons shorter by 3-ketoacyl-CoA thiolase (TFP).

For medium and long chain fatty acids reactions 2-4 are catalyzed by a single enzyme complex – the so called trifunctional protein associated with the inner membrane. However short chain fatty acids are processed by a separate series of soluble enzymes including SCHAD (see below).

Figure 1. Reactions in fatty acid oxidation.



The MetaPath™ Fatty Acid Oxidation 4-Plex Dipstick Array (MSX32) is a rapid and simple test for determining four mitochondrial protein parameters: Two key fatty acid oxidation acyl dehydrogenases SCHAD (short chain hydroxyacyl CoA dehydrogenase), and MCAD (medium chain acyl CoA dehydrogenase), and the TFP (trifunctional protein). The kit determines whether these enzymes have been up-regulated or down-regulated in human samples as a result of drug interactions or conditions such as oxidative stress and genetic disease. The kit also measures the levels of a key mitochondrial iron regulatory protein frataxin which is also the cause of Friedreich's ataxia (FA) the most prevalent inherited ataxia.

The method is based on an immunological sandwich assay (see Figure 2). Each enzyme is bound by two unique enzyme specific monoclonal antibodies. To accomplish this, one antibody is gold labeled and mixed with the sample. The other is embedded in the strip in one of four vertically separated zones. As the sample wicks past each zone the corresponding enzyme (now gold labeled) is trapped, this results a red colored band which is directly proportional in intensity to the amount of each enzyme captured.

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.

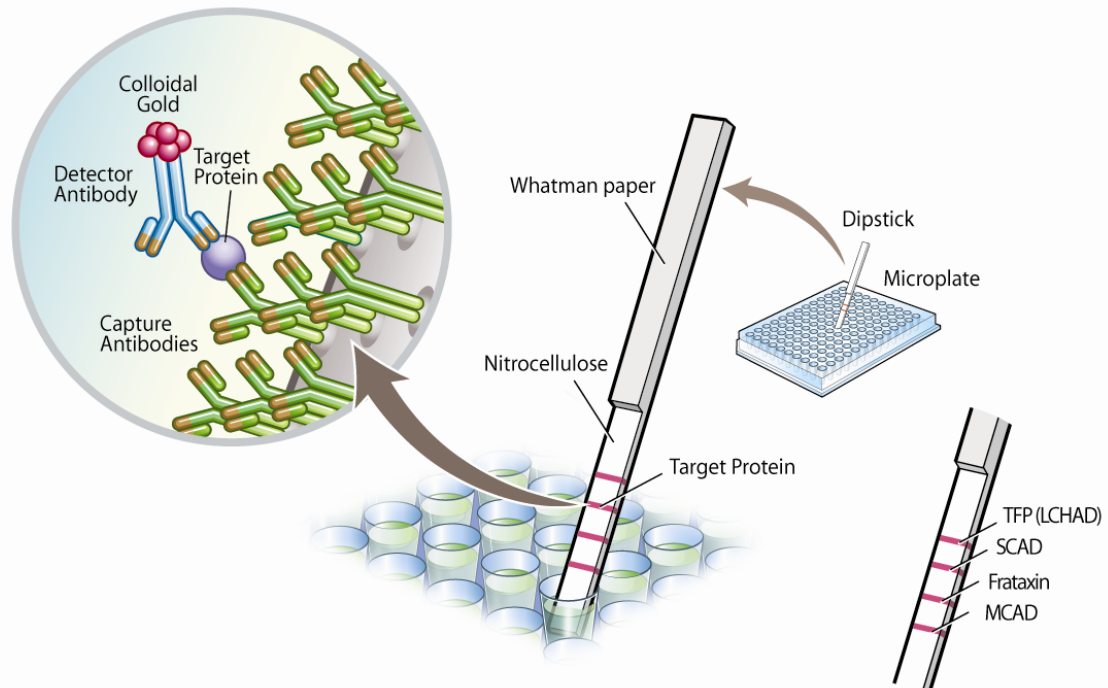


Figure 2. MSX32 is a lateral flow/dipstick device measuring the iron regulatory protein frataxin and the mitochondrial fatty acid oxidation enzymes SCHAD, MCAD and trifunctional protein TFP (including LCHAD).

Standard curves of TFP, SCHAD, MCAD and frataxin levels are generated by the user with a control dilution series of normal sample material. The signal intensities of the four bands on the dipsticks are measured by a dipstick reader (MitoSciences' MS1000 is recommended) or may be analyzed by another imaging system. The levels of all 4 enzymes in an experimental sample are then measured by interpolating their signal intensities into the standard curves. The levels and ratios of the enzymes in experimental samples are then compared with the control dilution series.

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. Enough Buffer A is provided (Extraction Buffer) for preparation of 20 samples using 500 μ L /sample.*

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 μ L 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 5-10 volumes of Buffer A to the cell pellet and mix (e.g. if the total sample volume displaces 50 μ L of volume, then add 250-500 μ L 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

Generating a Standard Curve

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. We recommend performing a 1:2 serial dilution with 1 part Buffer A to 1 part Buffer B in a total volume of 50 μ L (See Example Experiment Section).

Sample Type	Suggested Working Range (μ g)
Fibroblasts	0.1 - 10
HeLa cells	0.1 - 10
HepG2 cells	0.05 - 5
Muscle extract (human)	0.1-1.5

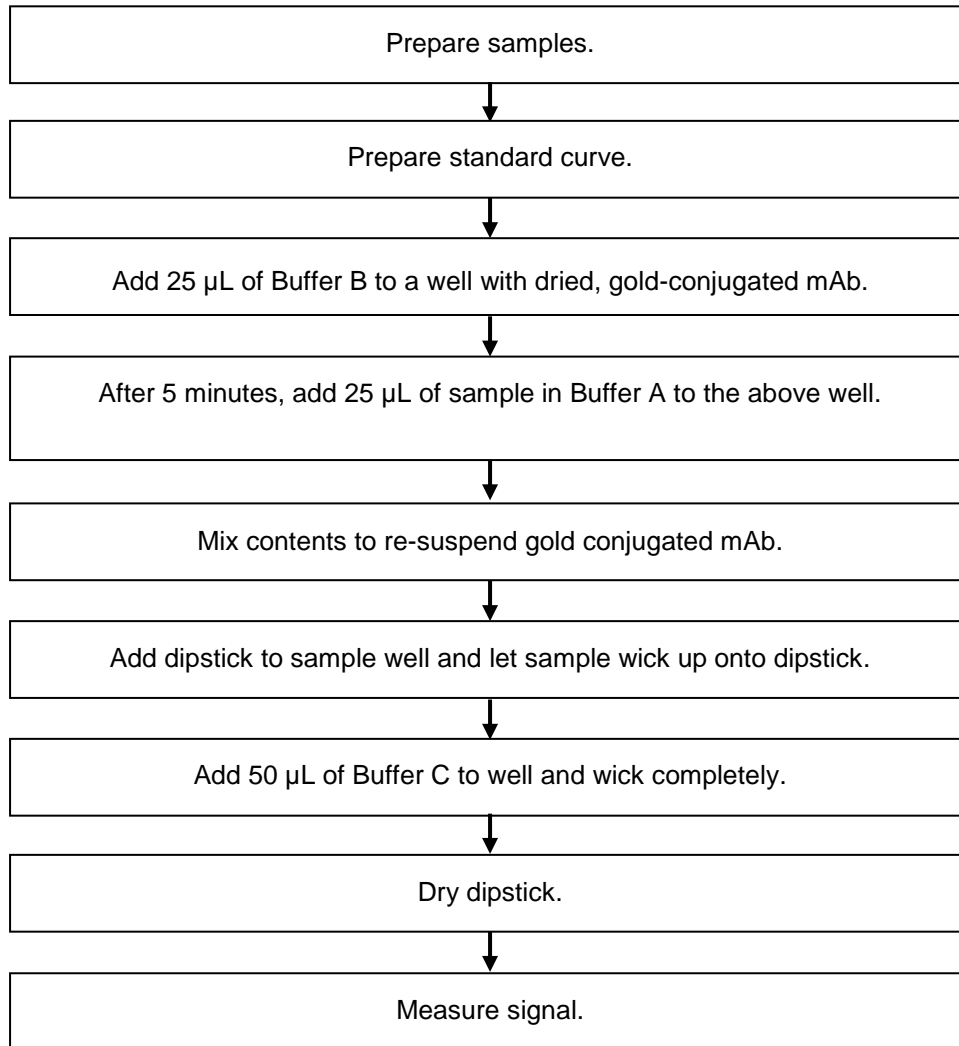
Table 1. *Suggested working range for different sample types*

For Individual Dipstick Reactions

1. Add 25 μ L of Buffer B to each well of gold conjugated mAb as necessary.
2. Allow gold conjugated mAb to re-hydrate for 5-10 minutes.
3. Now, add 25 μ L of sample in Buffer A for at total volume of 50 μ L and mix to homogeneity.
4. Gently add a dipstick to the well with the thin end down (see Figure 1). The contents will immediately begin wicking up the dipstick.
5. Wick the entire contents before proceeding to the wash step (the positive control band typically appears within 1-2 minutes. A 50 μ L sample should wick completely in 12-20 minutes. More viscous extracts may take longer.
6. Add 50 μ L of Buffer C and wick completely.
7. Dry the dipstick(s) before analysis (~20 minutes to air dry, or ~10 minutes at 37°C).
8. 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.

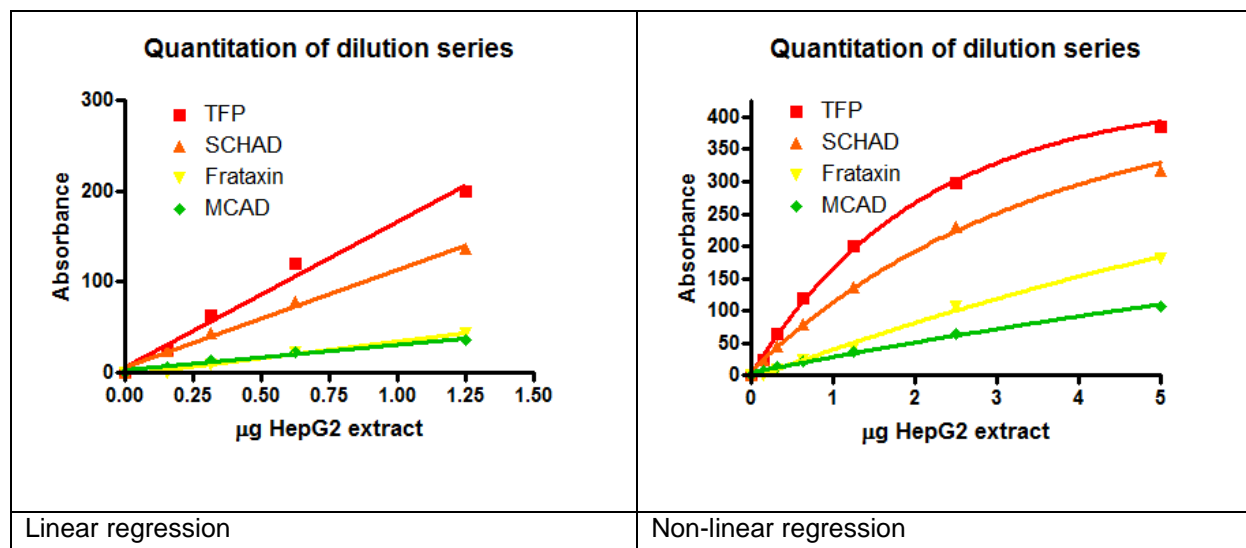


EXAMPLE EXPERIMENT

The experiment shown below shows a standard curve for each analyte generated from a dilution series of normal HepG2 cells. HepG2 whole cell extracts 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

Below we show a typical standard curve for the MSX32 Dipstick using HepG2 cell extract (see Table 1). We suggest using 7 to 8 dipsticks for covering the working range. In this example, the maximum sample load should be 1.25 ug linear regression above which non-linear regression should be used.



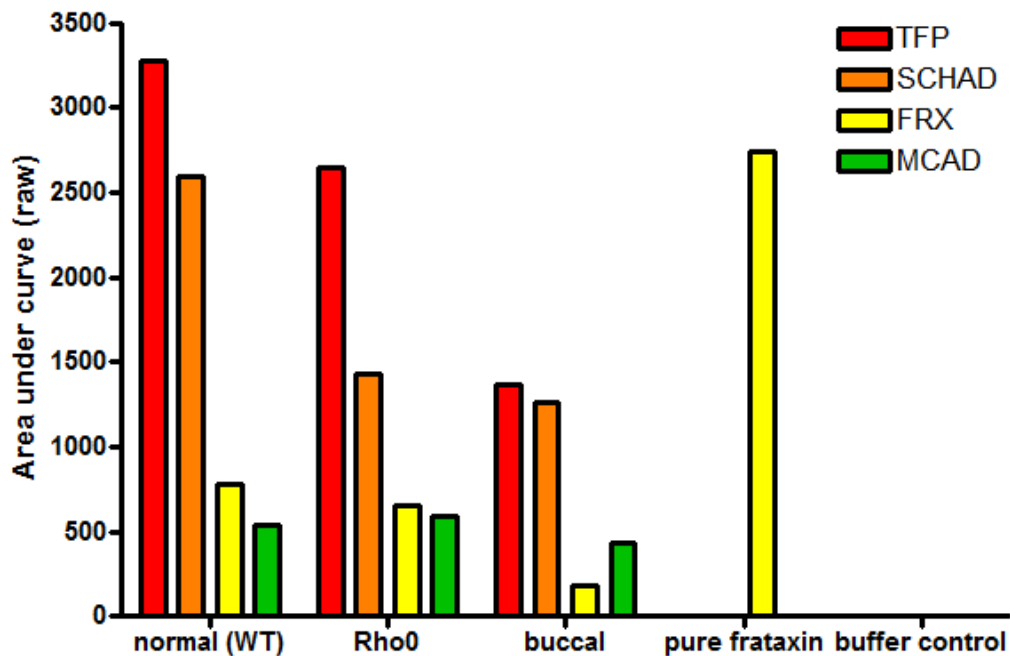
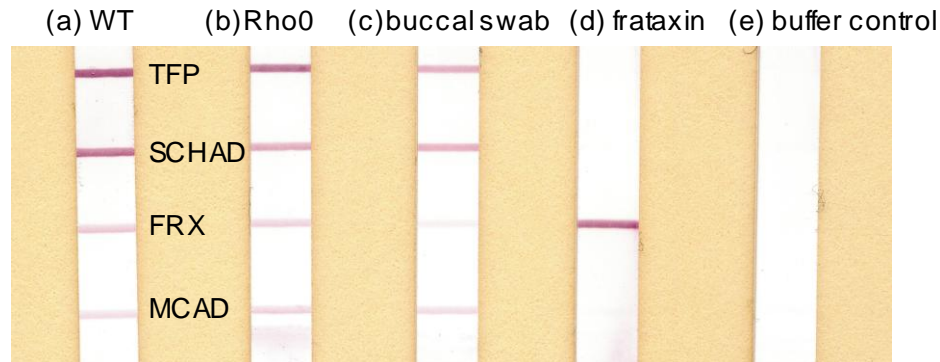
Intra-assay and inter-assay CVs are typically $\leq 10\%$.

Example: A 2.5 μg HepG2 sample was analyzed using MSX32 and bands quantified in absorbance units by MitoSciences' Dipstick Reader MS1000.

	Average	n	c.v.
TFP	308	4	2.6%
SCHAD	228	4	2.6%
Frataxin	98	4	7.4%
MCAD	58	4	6.5%

Measurement using desktop scanner: Analysis of samples with mitochondrial deficiencies show signal specificity.

Shown below are examples of dipsticks from (a) normal cells (b) rho0 cells which lack mtDNA and hence the respiratory chain but are otherwise well represented in these FAO enzymes (c) a sample collected by Mitosciences' buccal swab collection kit (d) pure frataxin and (e) buffer control with containing no sample. Data from these sticks was collected using a simple flatbed scanner and ImageJ software.



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.