

Frataxin Protein Quantity Microplate Assay Kit

MSF41

Rev.2

DESCRIPTION

Frataxin Protein Quantity Microplate Assay Kit

Sufficient materials are provided for 96 measurements (one 96-well microplate).

Kit Contents:

Item	MSF41
20X Buffer (Tube 1)	20 mL
Detergent	1 mL
20X Detector Antibody (Tube A)	1 mL
20X HRP Label (Tube B)	1 mL
10X Blocking Buffer (Tube 2)	8 mL
Development Solution (Tube 3)	20 mL
96-well microplate (12 strips)	1

The 96-well microplate has a monoclonal antibody pre-bound to the wells. This plate can be separated into twelve 8-well strips and thus can be used for up to 12 individual experiments.

Storage:

Store all components at 4°C. The kit is stable for at least 6 months.

INTRODUCTION

Frataxin (Q16595) is encoded by the gene FXN on chromosome 9. Expression has been shown in many tissues where the protein is imported into the mitochondrial matrix and processed to a mature 17.3 kDa protein. The role of Frataxin is not clear, however, it has been proposed to function as an iron chaperone, an iron storage protein, as a repair enzyme of damaged FeS clusters in aconitase, as a defense against oxidative stress and activator of mitochondrial oxidative phosphorylation.

Partial frataxin deficiency in humans results in Friedrich ataxia (FRDA) an autosomal recessive disease characterized mainly by progressive neurodegeneration in the spinal cord, and frequently by hypertrophic cardiomyopathy and diabetes. FRDA is the most common form of ataxia (1:30,000-1:50,000 in US and Europe). In greater than 95% of cases the genetic basis of the deficiency is a triplet-nucleotide repeat of GAA in intron 1 of both Frataxin alleles. Normal individuals carry 'small number repeats' (6-12) while less than 20% of normal individuals contain 'large normal repeats' (14-34). The role of these repeats, if any, is unknown. Individuals with large number repeats are at increased risk of having offspring with expanded repeats (66-1500) which result in disease. Expanded repeats interfere with transcription of frataxin mRNA and result in a decrease in frataxin protein causing at the cellular level impairment of energy metabolism, increased oxidative stress, reduced heme biosynthesis, impaired iron metabolism and iron accumulation in the heart and nervous system at the tissue level.

The Frataxin Protein Quantity Microplate Assay Kit (Catalog # MSF41) is used to determine the quantity of this protein in a sample. Frataxin is immunocaptured within the wells. The quantity of protein is measured by adding a second frataxin specific antibody that is labeled with horseradish peroxidase. This peroxidase changes the substrate from colorless to blue. The rate of color development is proportional to

the amount of protein captured in the well and can be monitored at 600 nm. Alternatively the assay can be terminated, at a user defined time, by the addition of 1N HCl (not supplied) and the assay performed as an end point measurement at 450 nm.

Note – This protocol contains detailed steps for measuring frataxin quantity. Be completely familiar with the protocol and the FAQ section (page 8) before beginning the assay. Do not deviate from the specified protocol steps or optimal results may not be obtained.

The protocol has 4 steps:

- A. Sample preparation
- B. Plate loading
- C. Add detection antibodies
- D. Quantity measurement

This microplate assay (Catalog # MSF41) has been developed for use with human samples only. Mouse and rat samples are not appropriate for use with this kit. Other species have not been tested.

This assay is designed for use with homogenates from cultured cells. Isolated mitochondria and tissue lysates can also be but some sample optimization may be necessary. As described below, homogenized samples should be resuspended to 5.5 mg/mL protein. The proteins are detergent extracted and loaded to within the linear range of the assay (see below). A **control** or normal sample should always be included in the assay as a reference. Also, include a **null** or buffer control to act as a background reference measurement.

Typical linear ranges per well (200 µL) and per milliliter are listed below. The ranges may be extended by using a non-linear fit of the data from a normal sample.

Typical Linear Ranges:		
Human liver mitochondria	0.3 -10 µg/well	1.5 - 50 µg/mL
Whole cultured cell extract	1 - 50 µg/well	5 - 250 µg/mL
Pure Frataxin (MSF42)	0.04-2.5 ng/well	0.2 -12.5 ng/mL

NOTE: Ranges for tissue extract may vary slightly. The lowest amount indicated is the lowest amount tested (>4x background). For sample loading use the recommended amount specified on page 3. Intra assay variation = 5.7% (n=96), inter assay variation = 12.5 % (n=48)

ADDITIONAL MATERIALS REQUIRED

- Spectrophotometer plate reader (Molecular Dynamics SpectraMax recommended) capable of measuring absorbance at 600 nm (or 450 nm after addition of 1N HCl (not supplied)).
- Method for determining protein concentration
- Deionized water
- Multichannel pipette
- PBS (phosphate buffered saline – for recipe see www.mitosciences.com/PDF/western.pdf)

MICROPLATE ASSAY PROTOCOL

A. Buffer and Sample Preparation

1. Prepare the buffer solution by adding 20 mL of 20X Buffer (Tube 1) to 380 mL deionized H₂O. Label this solution Solution 1.
2. For entire plate (12 strips) add 8 mL of 10X Blocking Buffer (Tube 2) to 72 mL of Solution 1. Label this Incubation Solution.

NOTE – When using fewer strips make proportionately less (i.e. 0.67 mL Tube 2 + 6 mL Solution 1 for each strip used).

3. Determine the sample protein concentration by a standard method and then adjust the protein concentration to 5.5 mg/mL in PBS.

NOTE – If the sample is less than 5.5 mg/mL, centrifuge to pellet again and take up in a smaller volume to concentrate the pellet and repeat protein concentration measurement. The optimal protein concentration for detergent extraction is 5.5 mg/mL.

4. Add 1/10 volume of Detergent to the sample (e.g. if the total sample volume is 500 µL, add 50 µL of Detergent). The final protein concentration is now 5 mg/mL.
5. Mix immediately and then incubate on ice for 30 minutes.
6. Spin in tabletop microfuge at maximum speed (~20,000 g, ~16,000 rpm) for 20 minutes. Carefully collect the supernatant and save as sample. Discard the pellet.
7. The microplate wells are designed for 200 µL sample volume per well, so dilute samples to the following recommended concentrations by adding Incubation Solution (Note: Do not use more than half the Incubation Solution prepared for sample dilution):

Sample Type	Recommended amount
Whole cultured cell extract	10 µg/200 µL (50 µg/mL)
Pure Frataxin	1 ng/200 µL (2.5 ng/mL)

8. Keep diluted samples on ice until ready to proceed.

B. Plate Loading

1. Add 200 µL of each diluted sample into individual wells on the plate. Include a normal sample as a positive control. Include a buffer control (200 µL Incubation Solution) as a null or background reference.
2. Incubate for 3 hours at room temperature.

C. Add Detection Antibodies

1. The bound monoclonal antibody has immobilized the protein in the wells. Empty the wells by turning the plate over and shaking out any remaining liquid.
2. Once emptied, add 300 μ L of Solution 1 to each well used.
3. Empty the wells again and add another 300 μ L of Solution 1 to each well used.
4. Separately, add 1 mL Detector Antibody (Tube A) to 20 mL of Incubation Solution. Label this Solution A.

When using fewer strips make proportionately less Solution A.

5. Empty the wells again by turning the plate over and shaking out the remaining liquid.
6. Add 200 μ L of Solution A to each well used.
7. Incubate at room temperature for 1 hour.
8. Separately, add 1 mL HRP Label (Tube B) to 20 mL of Incubation Solution. Label this Solution B.
When using fewer strips make proportionately less Solution B.
9. Empty the wells again quickly by turning the plate over and shaking out the remaining liquid.
10. Add 300 μ L of Solution 1 to each well used. Empty the wells again and add another 300 μ L of Solution 1 to each well used.
11. Empty the wells and add 200 μ L of Solution B to each well used.
12. Incubate at room temperature for 1 hour.
13. Empty the wells by turning the plate over and shaking out any remaining liquid.
14. Once emptied, add 300 μ L of Solution 1 to each well used.

D. Quantity Measurement

1. Empty the wells again and add another 300 μ L of Solution 1 to each well used. Repeat this step twice more for a total of 4 rinse steps.
2. Add 200 μ L of Development Solution to each well used. Any bubbles in the wells should be popped with a fine needle as rapidly as possible.
3. Place the plate in the reader and record with the following kinetic program (alternatively an endpoint measurement can be made by stopping the reaction at a user defined time by addition of 100 μ L of 1N HCl per well.

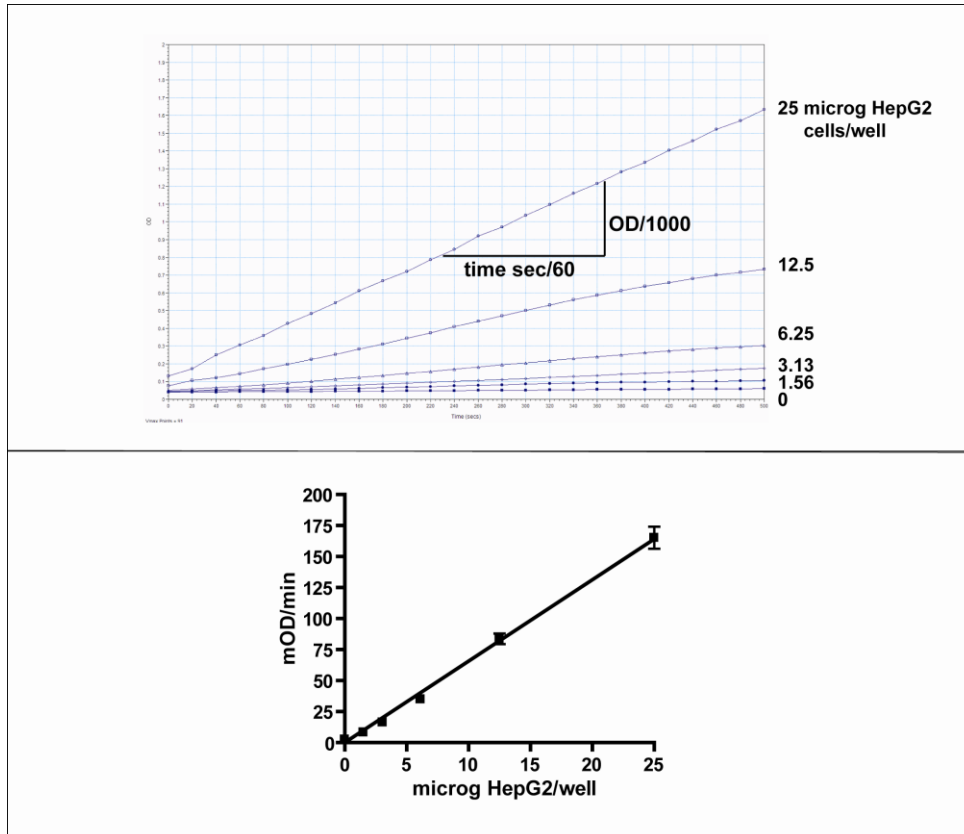
Kinetic measurement	Endpoint Measurement
OD 600 nm	Add 100 μ L 1 M HCl/well
Time: 30 mins	Autoshake before reading
Interval: 20 sec - 1 min	Measure OD 450 nm
Autoshake between readings	

4. Save data and analyze as described in the DATA ANALYSIS section.

DATA ANALYSIS

The quantity of frataxin captured in each well is proportional to the amount of horseradish peroxidase activity within each well. The quantity is the change in absorbance at 600 nm/minute/amount of sample loaded into the well.

Examine the linear rate of increase in absorbance at 600 nm with time. This is shown below where the rate/slope is calculated between these time points. Most microplate software is capable of performing this function. Repeat this for all samples.

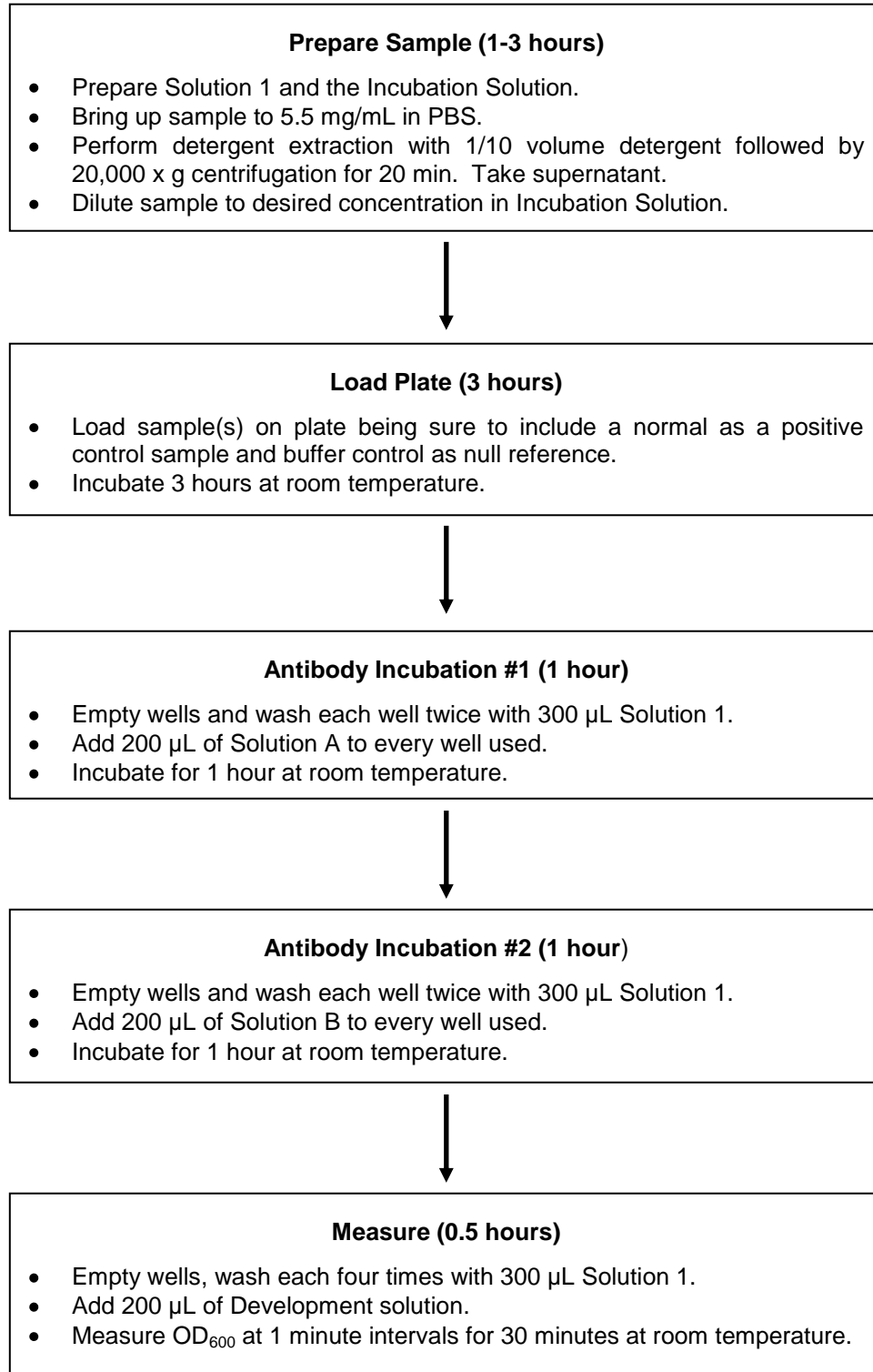


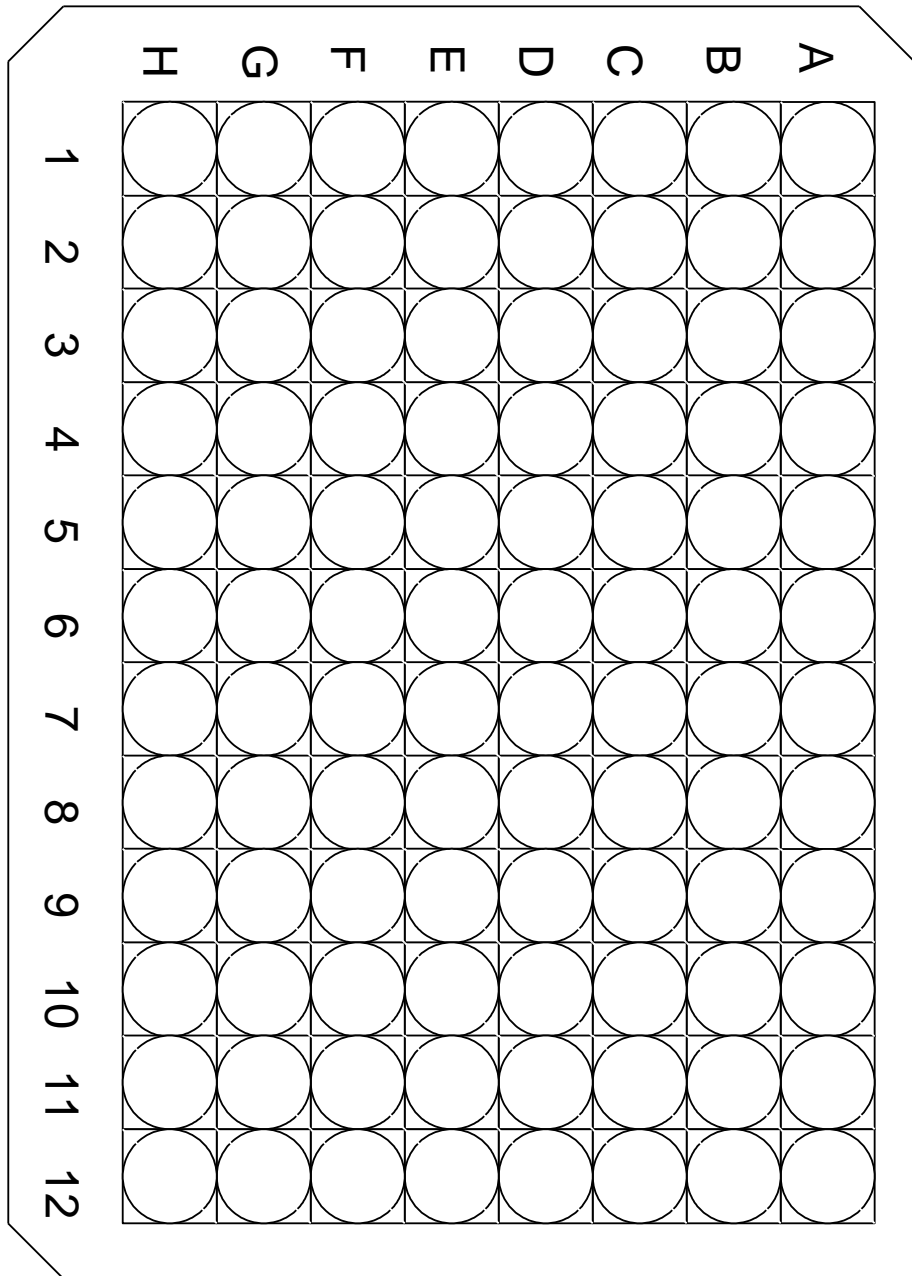
For the control or normal sample, the rate versus amount loaded is plotted as a straight line in the linear region of the assay as shown above.

Compare the rates of the **control** (normal) sample and with the rate of the **null** (background) and with your **unknowns**, **experimental** or **treated** samples to get the relative amount of frataxin. When using the pure recombinant frataxin MSF42 compare the rates of samples with the rates from diluted recombinant frataxin.

FLOW CHART

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





MICROPLATE MS _____ / /

Frequently Asked Questions

1. What is the minimum amount of cells or tissue needed to accurately measure Frataxin quantity?

A signal of 4x over background was seen with 1 μ g cell material per well. This corresponds to approximately 10,000 cells per well.

2. Is it possible to speed up this assay?

Antigen-antibody reactions are dependent on many conditions such as temperature and movement of molecules. The higher the temperature and the faster the movement of molecules and the sooner the saturation of binding sites occur. This assay can be performed in about half the time if sample, detector and label incubations are carried out at 37°C on a rotating platform. However, it is crucial to be consistent with all assays for cross-comparisons. Under these specified conditions, samples can be incubated for 1.5 hours and detector and label incubation times can be reduced to 35 minutes each.

3. Can I use this plate to determine frataxin quantity in tissues from rodents or other animal models?

No, the MSF41 microplate assay kit can not be used with mouse and rat samples.

4. Which immunogen was used to develop the antibodies used in this kit?

Recombinant human frataxin.

5. What evidence do you have that the captured protein is in fact pure frataxin?

Immunoprecipitations using these antibodies were performed on a large scale and analyzed by SDS-PAGE purity and Mass Spectrometry for identity.

6. Which is the exact epitope that binds to the capture antibody (attached to the plate) and the detector antibody provided with this kit?

The exact epitopes are unknown.

7. How do I analyze tissue samples?

Tissue samples must be homogenous before detergent extraction. Homogenize tissue samples thoroughly by using a Dounce homogenizer (see MitoSciences' product MS851) or microtissue grinder/Ultraturrax T8. Measure protein concentration by BCA protein assay (ThermoFisher-Pierce). Dilute to 5.5 mg/mL in PBS, then add 1/10 volume of detergent for extraction and proceed from page 3 step A5.