

Cytochrome c Protein Quantity Microplate Assay Kit

MSA41

Rev.2

DESCRIPTION

Cytochrome c Protein Quantity Microplate Assay Kit

Kit Contents:

Included in this kit are the necessary buffers (Tubes 1-3), detergent for sample preparation, and substrate for the HRP reaction (Development Solution, Tubes A-B). The kit contains a 96-well microplate with a monoclonal antibody pre-bound to the wells of the microplate. This plate can be broken into 12 separate 8-well strips for convenience; therefore the plate can be used for up to 12 separate experiments.

Part Number	Item Description	Amount
8203028	20X Buffer (Tube 1)	15 mL
8203023	10X Blocking Solution (Tube 2)	15 mL
8209300	20X Wash Buffer (Tube 3)	15 mL
8203015	Development Solution	20 mL
8203024	Detergent	1 mL
8203025	20X Detector Antibody (Tube A)	1 mL
8203026	20X HRP Label (Tube B)	1 mL
8203027	96-well Microplate (12 strips)	1 EA

Storage:

Store the Detergent at room temperature. Store all other components at 4°C.

INTRODUCTION

The permeabilization of the mitochondrial outer membrane and the consequent release of cytochrome *c* and other apoptogenic proteins from the mitochondrial intermembrane space into cytoplasm is considered hallmark of many apoptotic pathways leading to cell death. Therefore assaying these proteins in mitochondrial and cytoplasmic fractions is a prime interest of many researchers. Most of the current biochemical methods of quantification of cytoplasmic and mitochondrial cytochrome *c* involve mechanical disruption of cells to obtain mitochondria-enriched and cytosolic fractions. These methods are time-consuming and limited to small number of samples.

The Cytochrome *c* Protein Quantity Microplate Assay Kit (MSA41) is designed to work with samples generated by the MS861 Cell Fractionation Kit - Standard and MS862 Cell Fractionation Kit - HT.

MSA41 is used to determine the amount of the cytochrome *c* in a sample. Cytochrome *c* is immunocaptured within the wells and the amount is determined by adding a cytochrome *c* specific antibody conjugated with horseradish peroxidase. This peroxidase changes the substrate from colorless to blue. This reaction takes place in a time dependent manner proportional to the amount of protein captured in the wells. The rate of development of blue color can be followed at 600 nm or the reaction

can be stopped, at a user defined time, by the addition of 100 μ L of 1.5 N HCl to each well (not supplied) and read at 450 nm.

This quantity microplate assay (MSA41) has been developed for use with human samples. Rat, mouse, and bovine samples can be used also. Other species have not been tested. Cytochrome *c* is highly conserved among species, therefore it is anticipated that this kit will work with a broad range of species.

This assay is designed for use with purified mitochondria, homogenized tissue and whole cells. Samples should be solubilized with the supplied detergent. The protein concentration should be measured and diluted within the linear range as described below. Control or normal samples 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	
Cultured cell extracts	0.5 - 50 μ g / mL (3×10^4 - 3×10^5 cells/mL)
Tissue homogenate	1-10 μ g / mL
Pure cytochrome <i>c</i>	1 ng - 100 ng / mL

Table 1. Typical intra-assay variations (same day, same sample) <10%.
NOTE: the range of the assay can be extended by using a dilution series of normal sample and a curve fit.

The protocol has 4 steps:

- A) Sample preparation
- B) Plate loading
- C) Addition of Antibodies and Measurement
- D) Data Analysis

This cytochrome *c* detection assay was developed in conjunction with MitoSciences' MS861 Cell Fractionation Kit - Standard and MS862 Cell Fractionation Kit - HT, and the results were validated with MSA12 ApoTrack™ Cytochrome *c* Apoptosis Cocktail and with MSA07 ApoTrack™ Apoptosis Detection Kit for Immunocytochemistry.

ADDITIONAL MATERIALS REQUIRED

- Spectrophotometer measuring absorbance of 600 ± 1 nm in kinetic mode or 450 ± 1 nm in end-point mode (Molecular Devices' Spectramax readers are recommended)
- Deionized water
- Multichannel Pipetting devices
- Protein assay method
- 1.5 N HCl when measuring endpoint only

MICROPLATE ASSAY PROTOCOL

Note: *This protocol contains detailed steps for measuring cytochrome c quantity. Be completely familiar with the protocol before beginning the assay. Do not deviate from the specified protocol steps or optimal results may not be obtained.*

Sample Preparation for fractions generated by MitoSciences' MS861 (Cell Fractionation Kit - Standard). For samples prepared by MS862 see appropriate section, for all other samples see page 4.

A. Sample Preparation

1. Prepare a buffer solution by adding 15 mL 20X Buffer (Tube 1) to 285 mL deionized H₂O. Label this Solution 1 and set aside; store at 4°C for longer term.
2. Thoroughly mix each sample that was prepared as per MS861. To allow comparison between cell treatments, as per MS861, samples were re-suspended to the same concentration (3.3 x 10⁶ cells/mL, approximately 0.5-1 mg/mL depending on cell type). These samples were then separated into three fractions, cytosolic (C), mitochondrial (M) and nuclear (N) by the MS861 procedure.
3. Add 1/10 volume of Detergent to each sample (fraction). For example, if the total sample volume is 500 µL, add 50 µL of Detergent. If the sample becomes excessively viscous see FAQ section, page 10. Mix immediately. Keep at room temperature - do not chill. The solution may become cloudy.
4. Add 10 mL of 10X Blocking Solution (Tube 2) to 90 mL of Solution 1. Label this Solution 2.
5. Add to each sample 9 volumes of Solution 2. Store any remaining Solution 2 at 4°C.
6. Spin the samples in a tabletop microfuge at maximum speed (~20,000 g) for 20 minutes.
7. Carefully collect the supernatant and save as sample. Discard the pellet (note that there may be no observable pellet). Keep the samples at room temperature. Proceed to Section B.

Since samples (fractions) were diluted 10x in step A5, they are now derived from 3.3 x 10⁵ cells/mL (approx. 50 µg/mL).

Sample Preparation for fractions generated by MitoSciences' MS862 (Cell Fractionation Kit - HT). For samples prepared by MS861 see appropriate section, for all other samples see page 4.

A. Sample Preparation

1. Prepare a buffer solution by adding 15 mL 20X Buffer (Tube 1) to 285 mL deionized H₂O. Label this Solution 1 and set aside; store at 4°C for longer term.
2. Thoroughly mix each sample prepared as per MS862. To allow comparison between cell treatments, each cytosolic (C), mitochondrial (M) and nuclear (N) fractions were prepared from cells seeded at 15,000/well by the MS862 procedure. Thus the fractions are derived from 3 x 10⁵ cells/mL.

3. Prepare a buffer solution by adding 1 mL Detergent to 3 mL deionized H₂O. Label this Detergent Solution and set aside; store at room temperature for longer term.
4. Add 1/5 volume of Detergent Solution to the C and M fractions. Add 1/5 volume of deionized H₂O to the N fractions. (The N fractions already contain Detergent. The Detergent (from kit MSA41) is two times more concentrated than the Detergent HTIII (from kit MS862). For example, if the fraction sample volume is 40 µL, add 10 µL of Detergent Solution or water, as appropriate. If the sample becomes excessively viscous see FAQ section, page 10. Mix immediately. Keep at room temperature – do not chill. The solution may become cloudy.
5. Add 10 mL of 10X Blocking Solution (Tube 2) to 90 mL of Solution 1. Label this Solution 2.
6. Add to each sample 4 volumes of Solution 2. For example, if the sample volume (after the addition of Detergent Solution or water) is 50 µL, add 200 µL of Solution 2. Store any remaining Solution 2 at 4°C. Keep the samples at room temperature. Proceed to Section B.

Since samples (fractions) were diluted 5x in step A6, they are now derived from about 6×10^4 cells/mL (approx. 10 µg/mL).

Sample Preparation for all other samples – e.g. mitochondria, cultured cells and tissues.
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A. Sample Preparation

1. Prepare a buffer solution by adding 15 mL 20X Buffer (Tube 1) to 285 mL deionized H₂O. Label this Solution 1 and set aside, store at 4°C for longer term.
2. Measure protein concentration by BCA protein assay. Table 1 shows suggested final sample concentrations. Dilute the sample to 10x the desired concentration in Solution 1. For tissue samples – see the FAQ section, page 10.
3. Add 1/10 volume of Detergent. For example, if the total sample volume is 100 µL, add 10 µL of Detergent. Mix immediately. Keep at room temperature – do not chill. If the sample becomes excessively viscous see the FAQ section, page 10.
4. Add 10 mL of 10X Blocking Solution (Tube 2) to 90 mL of Solution 1. Label this Solution 2.
5. Add to the sample 9 volumes of Solution 2. *NOTE: The sample is now at the desired concentration. Keep sample at room temperature.*
6. Spin the sample in tabletop microfuge at maximum speed (~20,000 g) for 20 minutes.
7. Carefully collect supernatant and save as sample. Discard the pellet (note that there may be no observable pellet). Keep the samples at room temperature and store any excess Solution 2 at 4°C. Proceed to Section B.

B. Plate Loading

1. Add 200 µL of each sample into individual wells on the plate. Add replicates for accuracy. Also, include a buffer control (200 µL Solution 2) as a null or background reference.
2. Incubate the microplate for 3 hours at room temperature.

3. Meanwhile, prepare a buffer solution by adding 15 mL 20X Wash Buffer (Tube 3) to 285 mL deionized H₂O. Label this Wash Solution.
4. Measure 45 mL of Wash Solution into a clean bottle or tube. Add to the Wash Solution the remaining 5 mL of 10X Blocking Solution (Tube 2). Label this Incubation Solution. When not in use store Incubation Solution at 4°C. Proceed to Section C.

C. Addition of Antibodies and Measurement

1. The bound monoclonal antibody has immobilized the cytochrome c in the wells. Empty the wells by quickly turning the plate upside down and shaking out any remaining liquid.
2. Add 300 µL of Wash Solution to each well used.
3. When using all 12 strips add 1 mL of 20X Detector Antibody (Tube A) to 20 mL of Incubation Solution. Label this mixture as Solution A. When working with fewer strips make proportionately less.
4. Add 200 µL of Solution A to each well used.
5. Incubate the plate for 1 hour at room temperature.
6. Empty the wells of the plate and add 300 µL of Wash Solution to each well used.
7. When using 12 strips add 1 mL of 20X HRP Label (Tube B) to 20 mL of Incubation Solution. Label this mixture as Solution B. When working with fewer strips make proportionately less.
8. Empty the wells again and then add 200 µL of Solution B to each well used.
9. Incubate the plate for 1 hour at room temperature.
10. Empty the wells again and now add 300 µL of Wash Solution to each well. Repeat this step 4 more times.
11. Empty the wells and add 200 µL of Development Solution to each well used. Rapidly pop any bubbles that form with a needle.
12. Measure the absorbance of each well at 600 nm at room temperature using a kinetic program. Make a measurement every 1.5 minutes for 20 measurements for a total time of 30 minutes, with a 3 second auto-mixing step between reads.

NOTE: If necessary the user can stop the reaction at a desired time by the addition of 100 µL of 1.5 N HCl to each well and then measure the endpoint OD 450 nm.

13. Analyze data as described in Section D.

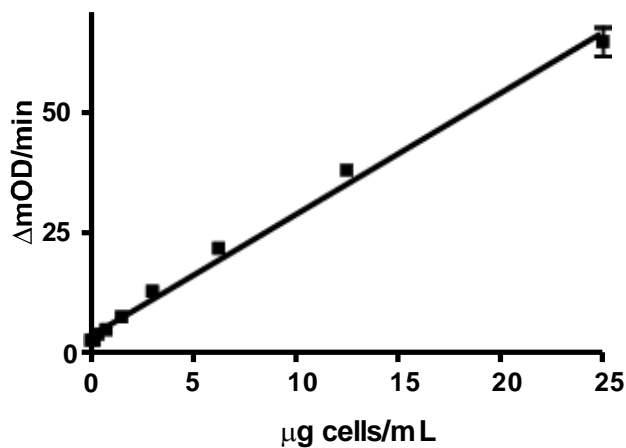
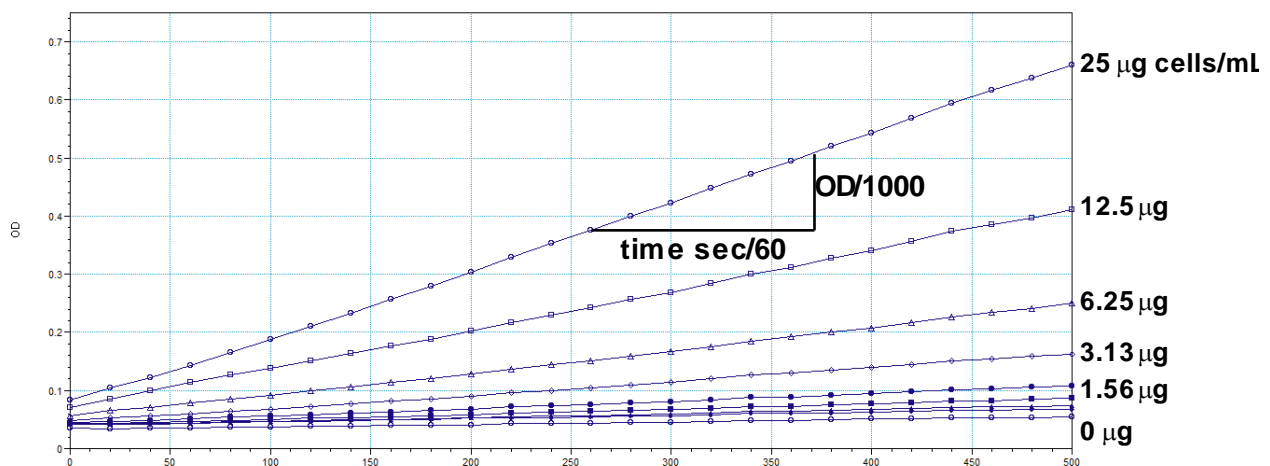
D. Data analysis

The quantity of cytochrome *c* is expressed as the amount relative to a normal or control sample.

When using the kinetic program at OD 600 nm, examine the color development and ensure that the rates are linear as shown below.

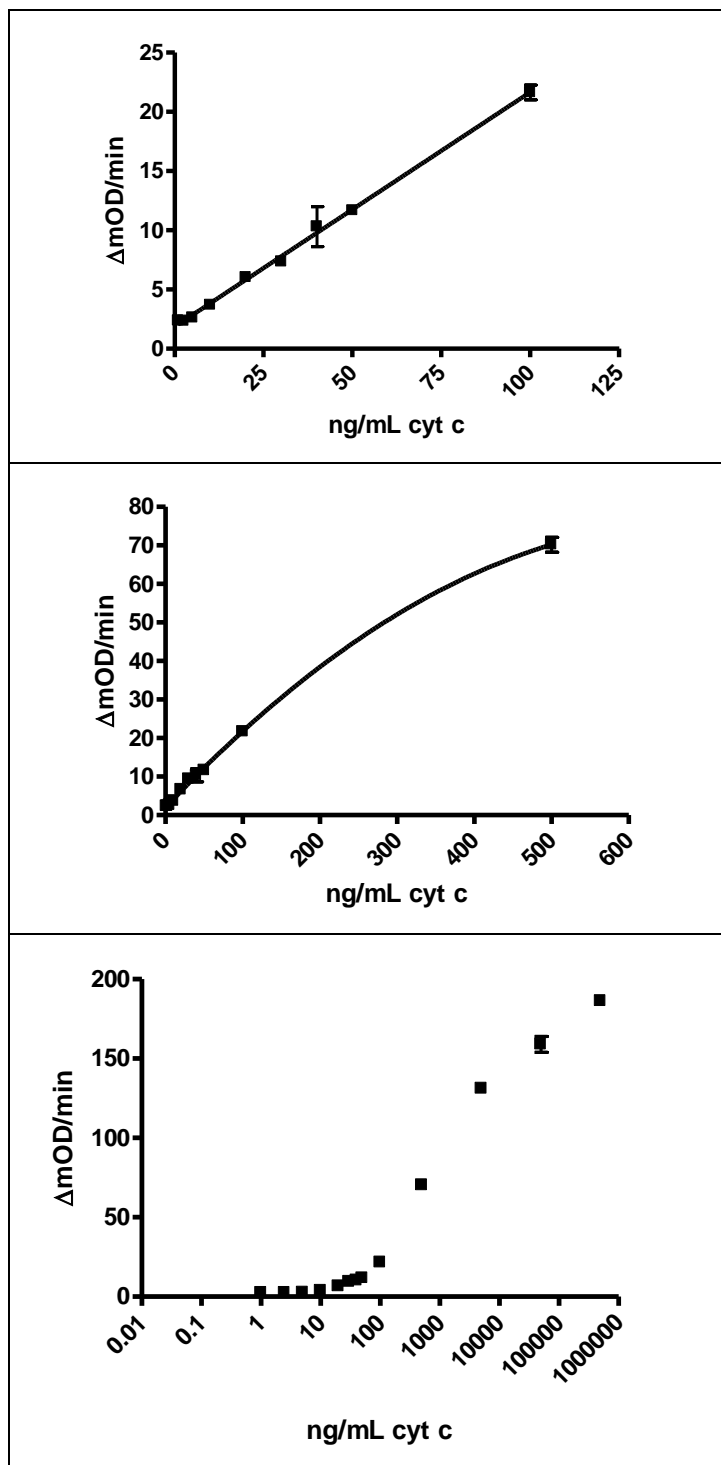
Subtract the initial absorbance reading from the final absorbance reading to determine the relative quantity of cytochrome *c* captured in each well.

Shown below are rates obtained with extracts from a dilution series of cultured cells (HepG2).



The change in absorbance is expressed as change in milliOD/min. In the example above the change OD is divided by 1000 and the time in seconds is divided by 60 to obtain minutes.

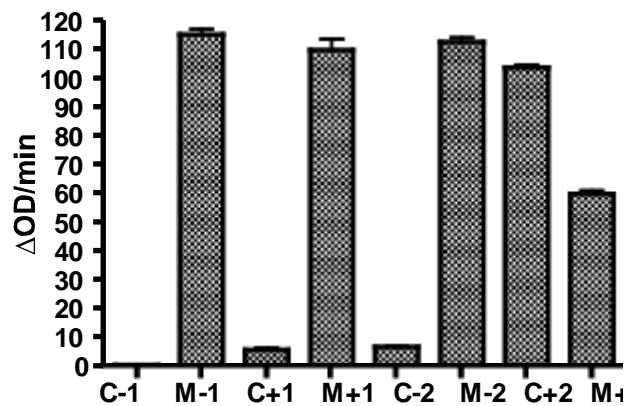
Cytochrome c can be used with this assay as a positive control or for calibration, as illustrated below.



EXAMPLE EXPERIMENT #1

Shown below are the relative amounts of cytochrome c in samples derived from the Mitosciences' MS861 Kit (see website for specific details of this kit).

Cytochrome c is detected in a sample only after apoptosis has been initiated and the cell outer membrane has been permeabilized using MS861 Cell Fractionation Kit Detergent I.

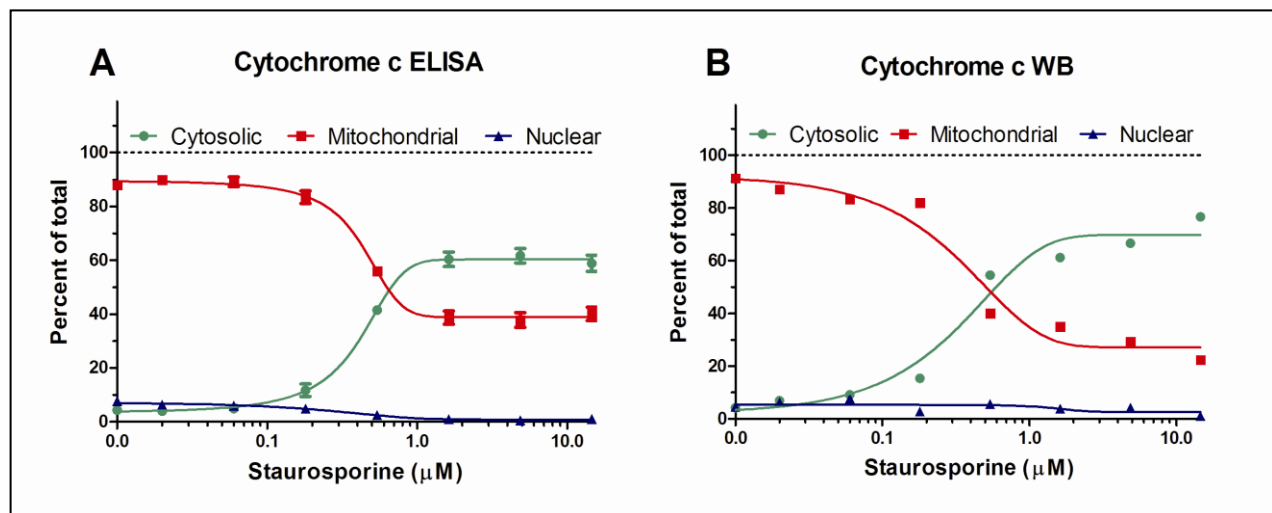


- C Cytoplasmic fraction,
- M Pellet containing the mitochondria
- No cell membrane semi-permeabilization
- + Cell membrane permeabilization by addition of MS861 Cell Fractionation Kit Detergent I.
- 1 No apoptotic induction
- 2 Apoptosis induced with 1 μ M staurosporine

EXAMPLE EXPERIMENT #2

Shown below are the relative amounts of cytochrome *c* in cytosolic, mitochondrial and nuclear fractions of HeLa cells induced to undergo apoptosis by Staurosporine treatment. The fractions, each derived from one well of a 96-well plate, were prepared with the use of MitoSciences' MS862 Kit. The amounts of cytochrome *c* were determined in each fraction by using the MSA41 Kit as described in this Protocol (A) or by Western blot analysis using MitoSciences' MSA12 ApoTrack™ Cytochrome *c* Apoptosis WB Antibody Cocktail (B). Data (in A) represent mean \pm standard error of the mean, $n=4$.

Staurosporine induces the release of cytochrome *c* from mitochondria into the cytosol with EC_{50} 0.41 μ M.



FREQUENTLY ASKED QUESTIONS

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

A signal of 2x background was measured when samples were diluted to 1 µg/mL of cell extract, approximately 3.3×10^3 cells (i.e. 0.2 µg/well or 660 cells/well). However, accuracy and reproducibility is not guaranteed when starting with samples of very low concentrations. For tissues and mitochondria it is anticipated that sensitivity will be even greater.

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, 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 this specified conditions, samples can be incubated for 1.5 hours and detector and label steps for 35 minutes each.

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

This plate can be used with mouse and rat samples. Cross reactivity with other species has not been tested with other species. However due to the very high level of sequence homologies of cytochrome c it is anticipated that this assay might function with a wide range of species.

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

Bovine heart cytochrome c.

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

Immunoprecipitations using these antibodies were performed on a large scale and analyzed by SDS-PAGE and mass spectrometry for purity.

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

The exact epitopes are unknown.

7. My sample is extremely viscous. What should I do?

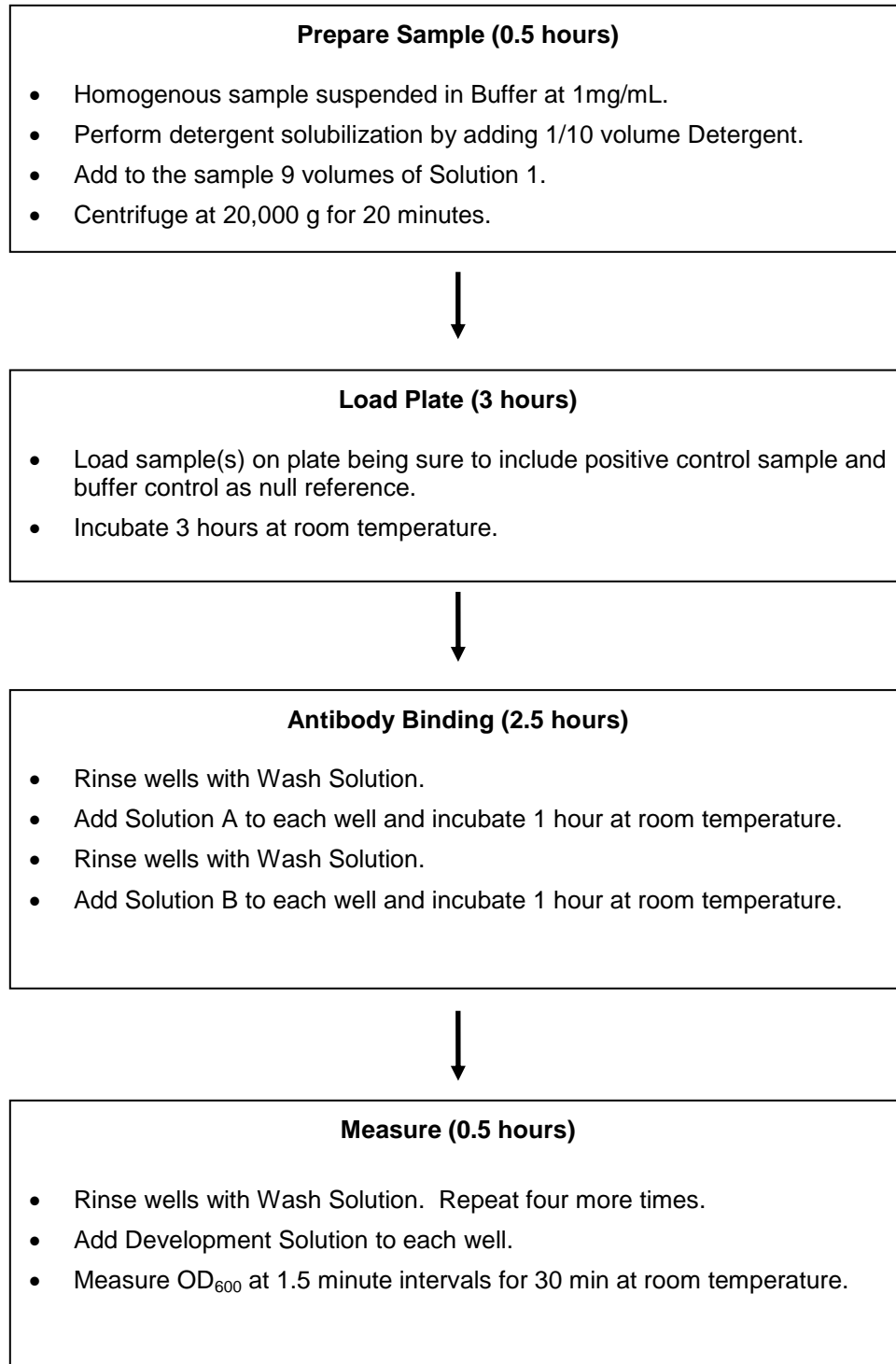
Samples extracted from high concentrations of cells may become very viscous due to the presence of DNA. To avoid pipetting errors it is important to mechanically shear the DNA or digest the DNA by a nuclease. The shearing is best done using a probe sonicator (Branson Sonifier 150). Alternatively the DNA may be sheared by very vigorous vortexing for about two minutes. The nuclease digestion can be done by addition of Benzonase Nuclease (Novagen cat # 70746) at 25 U/mL.

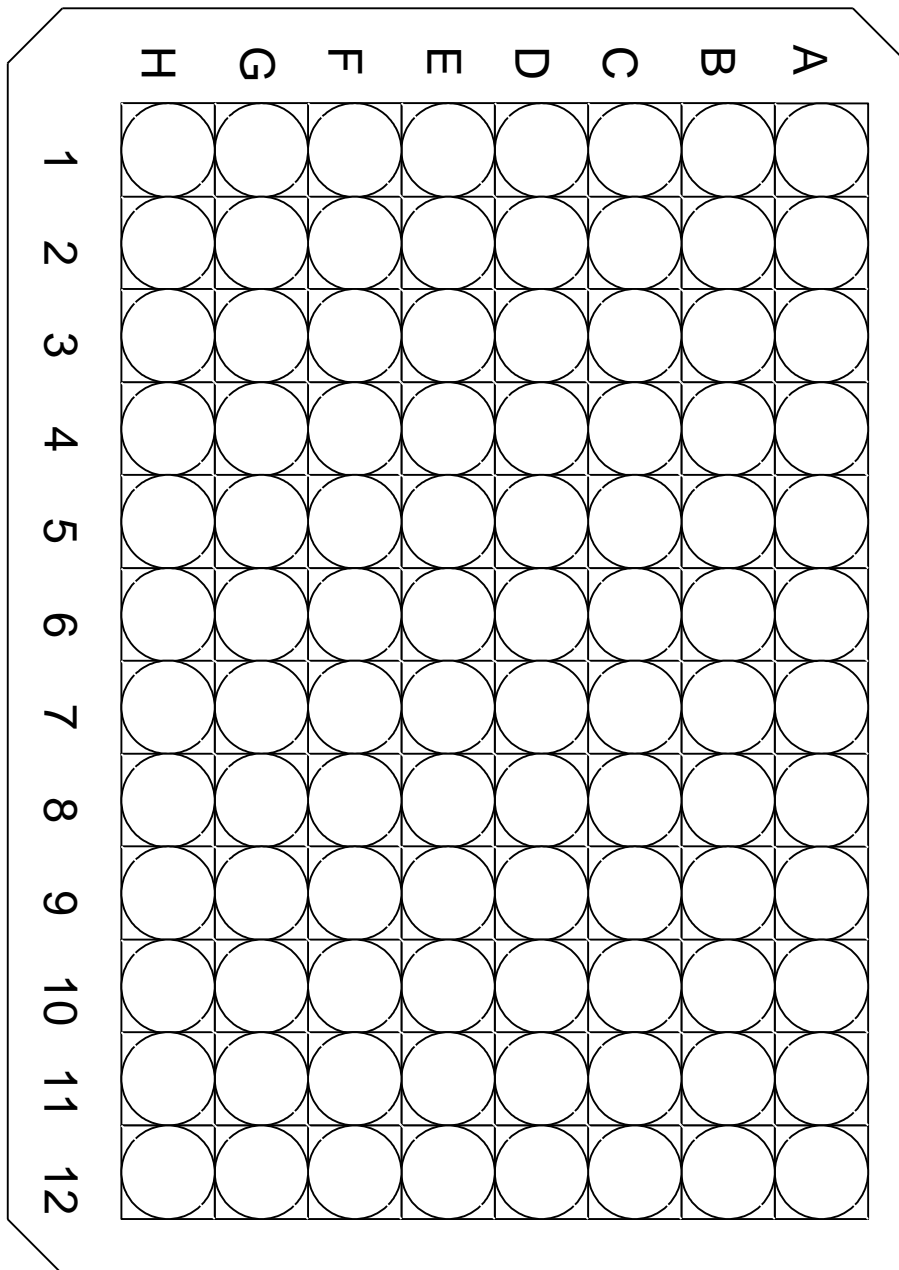
8. How do I analyze tissue samples?

Tissue samples must be homogenous before detergent extraction. Ensure homogenous tissue samples thoroughly by using a dounce homogenizer (see MitoSciences' MS851 Isolation Kit) or microtissue grinder/Ultraturrax T8. Measure protein concentration by BCA protein assay. Dilute to the desired protein concentration in Solution 1, then proceed with detergent extraction page 4 step A3.

FLOW CHART

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





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