

MitoSciences Guide to Sample Preparation

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I. INTRODUCTION

A critical step in performing any biological assay is preparing the test sample. MitoSciences' antibody-based ELISA and Dipstick assays capture sample proteins and protein complexes in their native, non-denatured state. Many assays, notably our OXPHOS assays, capture membrane-bound multi-subunit enzymes. Sample proteins for these assays must be extracted by non-denaturing methods, not just for recognition by the capture antibodies, but also to retain their enzymatic function for activity assays. Therefore, we have developed assays using a non-denaturing detergent, n-Dodecyl-beta-D-maltopyranoside (CAS# 69227-93-6), which is commonly referred to as lauryl maltoside. Lauryl maltoside is a non-ionic detergent used for the stabilization and activation of enzymes in membrane research and is well suited for the analysis of mitochondrial membrane proteins and enzyme complexes¹⁻⁷.

This guide outlines the steps to perform lauryl-maltoside extractions of functional enzymes and proteins from tissues and cultured cells for use with MitoSciences' ELISA and Dipstick assays.

II. TISSUE PREPARATIONS

Many of our assays are reactive with rodent proteins and were developed with rodent tissues (mouse and rat). The data in this section of the guide was obtained with rodent tissues, but the procedure applies to human and other animal tissues as well. Fully functional enzymes can be extracted from tissues by following a few simple steps.

We use simple mechanical disruption with Dounce homogenizers to disrupt cells minimally. Tissue is homogenized sequentially with two different-sized pestles and processed until smooth enough to be pipetted. Sequential homogenization is started with a large-clearance pestle that provides a coarse disruption of the tissue and finished with a small-clearance pestle that provides a finer disruption of the tissue. Suitable Dounce homogenizers are available from MitoSciences (MS851).

For long-term storage, it is best to make tissue homogenates that are as concentrated as possible. We have found that tissue can be homogenized with Dounce homogenizers with relative ease up to 25 mg/mL. After homogenization, sample detergent lysates can be made immediately or tissue homogenates can be aliquoted and frozen at -80°C.

Table 1 (page 3) shows the amount of tissue homogenized per mL of buffer to make a homogenate that is approximately 25 mg/mL protein by BCA protein analysis.

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Tissue	Wet weight mg / mL Buffer	Expected protein concentration (by BCA)
Brain	500	25 mg / mL
Heart	375	25 mg / mL
Kidney	375	25 mg / mL
Liver	300	25 mg / mL

Materials for Tissue Homogenization:

- _Dounce homogenizer, 2mL (Kontes, 885300-0002 or MS851)
- _Homogenization buffer (MitoSciences MitoBuffer or PBS)
- _Protease inhibitor cocktail (Sigma, P8340)
- _BCA™ Protein Assay (Pierce, 23225)
- _Weighing balance
- _Scalpel
- _2.0 mL microtubes

Method for Tissue Homogenization:

1. Add 1 mL buffer (containing protease inhibitor) to chilled mortar on ice.
2. Wash tissue to remove any blood and trim to remove any connective tissue and fat. Weigh and mince the appropriate amount of tissue and add to 1 mL of buffer in the mortar.
3. Perform 20-40 strokes with the large-clearance Pestle A until smooth.
4. Perform 20-40 strokes with the small-clearance Pestle B until smooth.

5. Using a pipette, transfer homogenate to a 2.0 mL microtube on ice.
6. Perform a BCA™ Protein Assay to determine protein concentration.
7. Proceed to detergent extraction or aliquot and freeze the homogenate.

DETERGENT EXTRACTION OF PROTEINS FROM TISSUE PREPARATIONS

Protein extraction with lauryl maltoside is, in most cases, optimal with samples that contain a protein concentration of 5 mg/mL.

MitoSciences Kits involve one of two methods to extract protein from the prepared tissue homogenate. These two methods have shown no significant difference in protein yield or assay performance:

- I. 1X Extraction Buffer
(contains 1X lauryl maltoside detergent)
- II. Add 1/10 volume of 10X lauryl maltoside detergent
(catalog # MS910)

Exceptions:

ATP synthase – For the Complex V Microplate Activity Assay (MS541), it is recommended to thoroughly freeze/thaw the sample and wash the pelleted tissue homogenate to remove any soluble, non-membrane associated proteins. This is particularly important for liver preparations, which contain many non-membrane-associated proteins. Concentrating the membrane proteins before protein assay leads to an improved

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detergent solubilization step and maximizes enzyme stability and oligomycin sensitivity.

PDH – For the Pyruvate Dehydrogenase (PDH) Microplate Activity Assay (MSP18), it is recommended to add the supplied detergent to a protein concentration of 25 mg/mL (undiluted homogenate) in order to maintain enzyme stability. Extracts should be centrifuged at a low speed (1,000 x g).

Materials for Protein Extraction:

- _ 1X Extraction Buffer (1.5% lauryl maltoside)
or
- _ 10X Lauryl maltoside detergent (15% lauryl maltoside solution - catalog # MS911)
- _ PBS
- _ Microtubes
- _ High-speed benchtop microcentrifuge
- _ BCA™ Protein Assay (Pierce, 23225)
or
- _ NanoDrop™ 2000 Spectrophotometer (Thermo)

Protein Extraction Method I with 1X Extraction Buffer:

1. Resuspend tissue homogenate to 5 mg/mL in Extraction Buffer (e.g. dilute 100 µL of 25 mg/mL homogenate with 400 µL Extraction Buffer to a total volume of 500 µL). Add protease inhibitors.
2. Mix well and incubate on ice for 30 min.
3. Centrifuge at 16,000 x g for 20 min at 4° C.
4. Collect supernatant and discard the pellet.

5. Measure protein concentration with BCA™ Assay or NanoDrop™ 2000 Spectrophotometer.

Protein Extraction Method II with 10X Lauryl Maltoside:

1. Dilute tissue homogenate to 5.5 mg/mL with MitoBuffer or PBS (e.g. dilute 100 µL of 25 mg/mL homogenate with 350 µL buffer to a total volume of 450 µL).
2. Add a 10-fold dilution of 15% lauryl maltoside (e.g. add 50 µL for a total volume of 500 µL).
3. Mix well and incubate on ice for 30 min.
4. Centrifuge at 16,000 x g for 20 min at 4°C.
5. Collect supernatant and discard the pellet.
6. Measure protein concentration with BCA™ Assay or NanoDrop™ spectrophotometer.

The protein concentration of the extract should now be ≤ 5 mg/mL depending on extraction efficiency. To measure protein concentration with a NanoDrop™ spectrophotometer, follow the manufacturer's instructions for measuring protein. In the pull-down menu for sample type, select "Other protein (E 1%)," then enter an extinction coefficient (Ext. Coeff, E 1% L/gm-cm) from Table 2 below.

Brain	8.7
Heart	13.0
Kidney	14.0
Liver	13.9

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It may be possible to freeze the extract at -80°C before running the assay without significant loss of activity.

III. CELL CULTURE PREPARATIONS

We have also developed assays for use with cultured cells (principally human cultured cells). The data in this section of the guide was obtained with HepG2, HeLa, and HL-60 cultures. Other cell types may vary significantly in yield. Cells were grown in 150 mm plates to confluency, then harvested, pelleted, and frozen prior to extraction with lauryl maltoside.

PROTEIN EXTRACTION FROM CELL CULTURES

Since cells vary greatly in size, the ratio of cells to detergent is cell-type dependant. However, as with tissue samples, lauryl maltoside extraction is, in most cases, optimal with samples that contain a protein concentration of 5 mg/mL. In our experience, this is typically approximately 10 million cells/mL.

No homogenization is necessary to prepare cultured cells for protein extraction. MitoSciences Kits involve one of two methods to extract protein from cultured cells. These two methods have shown no significant difference in protein yield or assay performance:

- I. 1X Extraction Buffer
(contains 1X lauryl maltoside detergent)
- II. Add 1/10 volume of 10X lauryl maltoside detergent
(catalog # MS910 or MS911)

Exception:

ATP synthase (MS541) – Freeze/thaw the cell pellet before protein assay and detergent extraction.

PDH (MSP18) – It is recommended to add the supplied detergent to a protein concentration of 15 mg/mL in order to maintain enzyme stability. Extracts should be centrifuged at only 1,000 x g.

Materials for Protein Extraction:

- _ 1X Extraction Buffer (1.5% lauryl maltoside)
or
- _ 10X Lauryl maltoside detergent (15% lauryl maltoside solution - catalog # MS911)
- _ PBS
- _ Microtubes
- _ High-speed benchtop microcentrigue
- _ BCA™ Protein Assay (Pierce, 23225)
or
- _ NanoDrop™ 2000 Spectrophotometer (Thermo)

Protein Extraction Method I with 1X Extraction Buffer:

1. Resuspend cell pellet with 9 volumes of MitoSciences Extraction Buffer (e.g. 50 μL pellet + 450 μL Extraction Buffer to a total volume of 500 μL). Add protease inhibitors.
2. Mix well and incubate on ice for 30 min.
3. Centrifuge at 16,000 x g for 20 min at 4°C .
4. Collect supernatant and discard the pellet.
5. Measure protein concentration with BCA™ Assay or NanoDrop™ Spectrophotometer.

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Protein Extraction Method II with 10X Lauryl Maltoside:

1. Resuspend and dilute the cell pellet with 8 volumes of PBS (e.g. 50 μ L pellet + 400 μ L PBS to a total volume of 450 μ L). Add protease inhibitors.
2. Add a 10-fold dilution of 10X lauryl maltoside (e.g. add 50 μ L to the cell suspension for a total volume of 500 μ L).
3. Mix well and incubate on ice for 30 min.
4. Centrifuge at 16,000 x g for 20 min at 4°C.
5. Collect supernatant and discard the pellet.
6. Measure protein concentration with BCA™ Assay or NanoDrop™ Spectrophotometer.

The protein concentration of the extract should now be \leq 5 mg/mL, depending on extraction efficiency, as measured by BCA™ Assay. To measure protein concentration with a NanoDrop™ Spectrophotometer, follow the manufacturer's instructions for measuring protein. In the pull-down menu for sample type, select "Other protein (E 1%)," then enter an extinction coefficient (Ext. Coeff, E 1% L/gm-cm) from Table 3 below.

HepG2	16.2
HeLa	14.4
HL-60	17.2

It may be possible to freeze the extract at -80°C before running the assay without significant loss of activity.

IV. REFERENCES

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