



# MitoCheck® Mitochondrial (Tissue) Isolation Kit

Item No. 701010

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# TABLE OF CONTENTS



# GENERAL INFORMATION

## Materials Supplied

Kit will arrive packaged as a 4°C kit. For best results, remove components and store as stated below.

| Item Number | Item | Quantity/ Size | Storage |
|---|---|---|---|
| 701011 | Mitochondrial (Tissue) Isolation Buffer (5X) | 1 vial/50 ml | 4°C |
| 701012 | Mitochondrial (Tissue) Homogenization Buffer (5X) | 1 vial/20 ml | 4°C |

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

**WARNING:** THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

## Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

## Precautions

**Please read these instructions carefully before beginning this assay.**

***NOTE: It is recommended that gloves be worn at all time when working with isolated mitochondria and mitochondrial inhibitors.***

## If You Have Problems

**Technical Service Contact Information**

**Phone:** 888-526-5351 (USA and Canada only) or 734-975-3888
**Fax:** 734-971-3641
**Email:** techserv@caymanchem.com
**Hours:** M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

## Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section on page 3 and used before the expiration date indicated on the outside of the box.

## Materials Needed But Not Supplied

1. Clean glassware that has been washed with 70% ethanol in place of soap (to ensure the removal of detergent)
2. A refrigerated centrifuge and rotor capable of 1,000 x g and 10,000 x g
3. A pH meter and solutions of HCl (1 M) and KOH (1 M)
4. Surgical scissors
5. A polytron or tissumiser (for heart and muscle tissue)
6. SpectraMesh nylon mesh filter (300 µm)
7. A 50 ml glass Dounce homogenizer with loose pestle (A) and a 2 ml glass or Teflon homogenizer with loose pestle
8. A glass rod
9. Polycarbonate centrifuge tubes (4x30 ml) capable of withstanding 10,000 x g
10. Protein assay (Lowry or BCA)
11. A source of 'UltraPure' water for specifications. *NOTE: UltraPure water is available for purchase from Cayman (Item No. 400000).*



# INTRODUCTION

## Background

When measuring mitochondrial function or the effects of an unknown on mitochondrial function, it is difficult to determine a mechanism using a whole organism or tissue. When this is the case, isolated mitochondria provide the simplest and most biochemically relevant solution. Using Cayman's MitoCheck® Mitochondrial (Tissue) Isolation Kit, the isolation of mitochondria from tissues can be easily achieved using differential centrifugation in isotonic buffers.[1,2]

## About This Kit

With Cayman's MitoCheck® Mitochondrial (Tissue) Isolation Kit, it is possible to isolate coupled mitochondria* from freshly harvested mammalian heart, liver, and kidney tissue as long as precautionary steps are taken. The presence of trace amounts of detergent can have detrimental effects on mitochondrial membrane integrity. Therefore, when using Cayman's MitoCheck® Mitochondrial (Tissue) Isolation Kit it is important to ensure that all glassware (and polycarbonate centrifuge tubes), that will come into contact with isolation buffers, have been rinsed free of detergents. To remove detergents from glassware, wash using 70% ethanol in place of soap, followed by several rinses with deionized water. Glassware should then be rinsed in UltraPure water. It is strongly recommended that plasticware be avoided. All steps of this preparation are carried out on ice or at 4°C. When handling glassware containing tissue homogenates or crudely isolated mitochondria, take care not to wrap hands around the glassware. Heat from hands can be easily transferred to mitochondria, potentially compromising membrane integrity. Should membrane integrity be compromised due to the presence of detergents or heat, mitochondrial protein can still be isolated; however the quality of the resulting prep may be poor (*i.e.*, uncoupled, poor respiration rate).

**Coupled mitochondria are defined as mitochondria capable of phosphorylating ADP at the $F_1F_O$-ATP synthase (Complex V) through the utilization of a proton ($H^+$) gradient, which is generated by the electron transport chain (ETC) in the presence of appropriate substrates (e.g., succinate) and in the absence of uncouplers and mitochondrial inhibitors.*



# PRE-ISOLATION PREPARATION

## Reagent Preparation

1. **Mitochondrial (Tissue) Isolation Buffer (5X) - (Item No. 701011)**

   This buffer is supplied as a 5X concentrate. Before using, dilute to 1X by adding 200 ml of cold, UltraPure water. Check pH at 4°C and ensure that it is at 7.4 before beginning. If pH is slightly high, adjust with HCl. If buffer becomes too acidic, adjust using KOH. Do not use NaOH. This component will make 250 ml of 1X Mitochondrial Isolation Buffer.

2. **Mitochondrial (Tissue) Homogenization Buffer (5X) - (Item No. 701012)**

   This buffer is supplied as a 5X concentrate. Before using, dilute to 1X by adding 80 ml of cold, UltraPure water. Check pH at 4°C and ensure that it is at 7.4 before beginning. If pH is slightly high, adjust with HCl. If buffer becomes too acidic, adjust using KOH. Do not use NaOH. This component will make 100 ml of 1X Mitochondrial Homogenization Buffer.

# ISOLATION PROTOCOL

***Ensure all glassware, centrifuge tubes, centrifuge and rotor are pre-chilled prior to use. All steps are carried out at 4°C. NOTE: To ensure that enough Homogenization Buffer is left for homogenization, avoid excess washing of tissue.***

1. Starting with 1-20 g wet weight of freshly isolated tissue, place into a cold 100 ml beaker with ~10 ml of Mitochondrial Isolation Buffer. *NOTE: This protocol is designed for use with varying amounts of starting material. Mitochondrial protein yield will vary based on mitochondrial content and overall mass of starting material.*

## Tissue-Specific Preparations

**Liver and Kidney**

For Liver: chop finely. For Kidney: remove renal capsules and chop finely, allow tissue to settle, decant blood, and rinse with ~20 ml of Mitochondrial Isolation Buffer. A small amount of tissue loss during this step is expected.

a. Repeat wash/decant step two more times, until blood is removed.

b. Suspend tissue in Mitochondrial Homogenization Buffer and transfer to a pre-chilled 50 ml Dounce homogenizer.

c. Fill to just above the neck (~30 ml) with Mitochondrial Homogenization Buffer and homogenize using the loose fitting pestle (Pestle A) on ice for 10 passes (1 pass - 1 down stroke, 1 up stroke).

d. *Quickly* transfer the homogenate evenly into two 30 ml polycarbonate centrifuge tubes and fill with remaining Mitochondrial Homogenization Buffer.

e. Centrifuge for three minutes at 1,000 x g.

f. Taking care not to dislodge the pellet, carefully decant the supernatant into two clean 30 ml polycarbonate centrifuge tubes and fill with Mitochondrial Homogenization Buffer. It may be helpful to leave behind a few milliliters of supernatant so that excess blood is not transferred over.

g. Proceed to Step 2 on page 9.



**Heart and Muscle**

*NOTE: Lower mitochondrial yields are expected with these tissues due to intense homogenization.* Chop finely and allow tissue to settle, decant blood, and rinse with ~10 ml of Mitochondrial Isolation Buffer. A small amount of tissue loss during this step is expected.

a. Suspend tissue in 10 ml of Mitochondrial Homogenization Buffer and homogenize with tissuemiser for 20 seconds. Transfer homogenate to a 30 ml centrifuge tube.

b. Centrifuge for five minutes at 600 x g.

c. Transfer the supernatant through 300 µm mesh into a clean 30 ml centrifuge tube and set aside (on ice).

d. Homogenize pellet with tissuemizer as in step a.

e. Repeat steps b and c.

f. Distribute the supernatants evenly between the two tubes and fill each centrifuge tube with Mitochondrial Homogenization Buffer and proceed to step 2.

2. Centrifuge for 10 minutes at 10,000 x g (RCF).

3. Discard the supernatant (should appear cloudy) and add ~20 ml of Mitochondrial Isolation Buffer to the centrifuge tube. Use the glass rod to suspend the mitochondrial pellet and then fill tube with Mitochondrial Isolation Buffer.

4. Repeat Steps 2 and 3.

5. Supernatant should be now be clearer than that of the step 1. Add 10 ml of Mitochondrial Isolation Buffer to each centrifuge tube and suspend with the glass rod. Combine the suspension into one centrifuge tube. Fill with Mitochondrial Isolation Buffer and repeat steps 2, 3, and 4
6. Following completion of the 10 minute, 10,000 x g centrifugation step, discard the supernatant and suspend the pellet in ~300 µl of Mitochondrial Isolation Buffer. Transfer the suspension to a pre-chilled 2 ml Dounce homogenizer. Homogenize three strokes with the loose fitting pestle. Remove an aliquot for protein determination.
7. Freshly isolated mitochondria should be stored on ice in a 4 ml glass test tube. For coupled mitochondrial studies, use within four hours of isolation.

*NOTE: Freshly isolated mitochondria cannot be frozen and still maintain their coupled state. When using isolated mitochondria with the MitoCheck® ETC activity assays, mitochondria that have undergone a freeze thaw cycle are preferred.*

**Use with MitoCheck® Complex I, II, II/III, and IV Activity Assay Kits:** Using the appropriate assay kit buffer, dilute isolated mitochondrial protein to 5 mg/ml in a plastic microcentrifuge tube and store at -80°C until use. If immediate use is required, freeze/thaw using liquid $N_2$ or dry ice/ethanol. Use mitochondria as outlined in the MitoCheck® Activity Assay Kit booklet. To determine proper function of the MitoCheck® Activity Assay Kits, be sure to include the bovine heart mitochondria provided with each kit as a positive control.



# ANALYSIS

## Calculations

Mitochondrial coupling is determined through the calculation the respiratory control ratio (RCR), which is determined by measuring the oxygen consumption rate (OCR) of actively phosphorylating mitochondria (State 3): non-phosphorylating mitochondria (State 4) (eq 1.).[3] Because mitochondria utilize the proton gradient generated by the electron transport chain to generate ATP, OCR will increase upon addition of ADP (*i.e.*, high State 3 rate). When all ADP has been phosphorylated, or the ATP synthesis is inhibited, OCR will slow as mitochondria enter State 4 (low State 4 rate). The State 4 OCR is determined by the integrity of the inner mitochondrial membrane. A leaky membrane would result in a high State 4 OCR and a low RCR.

$$\text{Respiratory Control Ratio (RCR)} = \frac{\text{Oxygen Consumption Rate (ADP - State 3)}}{\text{Oxygen Consumption Rate (no ADP - State 4)}}$$

## Performance Characteristics

### Sample Data

The data shown below are an example of data obtained with this kit. Your results will not be identical to these. Do not use these data to directly compare your samples as your results may vary substantially.

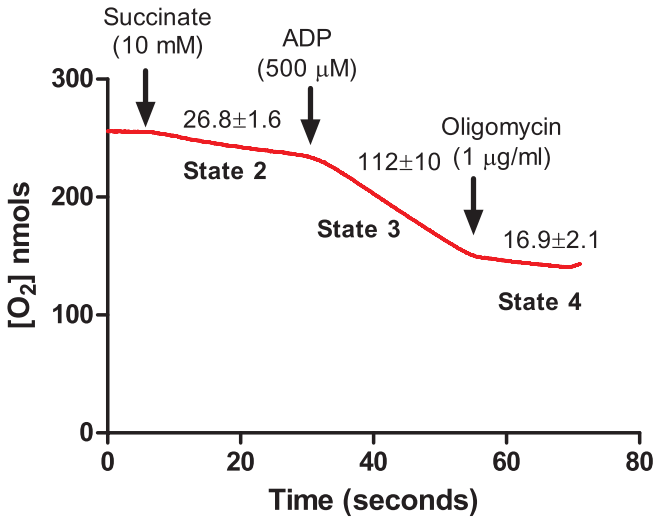


**Figure 1. Oxygen consumption rates for mouse liver mitochondria isolated using Cayman's MitoCheck® (Tissue) Mitochondrial Isolation Kit.** Oxygen consumption rate values indicated are means +/- standard error in nmols $O_2$/min/mg protein. RCR values for the trace were reported as 6.4. Oxygen consumption was measured using a Clark $O_2$ electrode in KCl-based medium containing 0.05% Fatty acid free BSA (not supplied) at 37°C. RCR value = 6.6.



# RESOURCES

## Troubleshooting

| Problem | Possible Causes | Recommended Solutions |
|---|---|---|
| Poor mitochondrial protein yield | Tissue not thoroughly homogenized | Ensure that no solid pieces of tissue are visible post homogenization as this can lead to decrease yield |
| Poor RCR or uncoupled mitochondria | A. Tissue homogenized too thoroughly<br>B. Detergent in glassware<br>C. Method of euthanasia | A. Be sure to use loose pestle and homogenize to the point where no tissue pieces are visible; excess homogenization/use of tight pestle can result in the shearing of mitochondria<br>B. Ensure that all glassware that comes into contact with buffers has been washed with ethanol and UltraPure water<br>C. Excess stress both prior to and during euthanasia can result in mitochondrial uncoupling due to the release of adrenaline |
| Excess blood in mitochondrial pellet | A. Tissue not washed thoroughly prior to homogenization<br>B. Some of the pellet from the low speed spin has been carried over | A. Ensure that all excess blood has been removed by washing tissue pieces with homogenization buffer until surrounding buffer is clear<br>B. Leave a small amount of supernatant behind to ensure that pellet is not disturbed |

## References


1. Smith, A.L. Preparation, properties, and conditions for assay of mitochondria: Slaughterhouse material, small-scale. *Method. Enzymol.* **10**, 81-86 (1967).
2. Fleischer, S., Rouser, G., Fleischer, B., *et al.* Lipid composition of mitochondria from bovine heart, liver, and kidney. *J. Lipid Res.* **8(3)**, 170-180 (1967).
3. Chance, B. and Williams, G.R. Respiratory enzymes in oxidative phosphorylation. III. The steady state. *J. Biol. Chem.* **217(1)**, 409-427 (1955).




## NOTES

## Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.