# Simple Protocols for Isolation of Intact Mitochondria Using *The* SHREDDER SG3 and PBI Mitochondria Isolation Kits

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## Introduction

Isolation of intact mitochondria from human and animal tissue is crucial for studies that focus on the elucidation of their function and dysfunction in conditions such as aging, diabetes and cancer. As potential drug targets, high quality functional mitochondrial isolates are important for drug screening studies [1]. Mitochondria isolation from solid tissue is usually carried out using labor-intensive homogenizer-based methods [2] that require extensive operator experience. Here we describe a semi-automated method to release mitochondria from solid fibrous tissues, such as muscle and lung, using *The PCT Shredder* or *The* SHREDDER SG3 for tissue homogenization. This method can be combined with a subsequent Pressure Cycling Technology (PCT) step (Figure 1A) to further increase yield.

***The SHREDDER SG3***

*The* SHREDDER SG3 is similar in function to PBI's *The PCT Shredder*, and like *The PCT Shredder*, was developed to improve sample disruption and subsequent extraction. *The* SHREDDER SG3 differs from *The PCT Shredder* by the addition of a specially designed three position force locking mechanism that enables the operator to select and apply reproducible force to the sample during the shredding process. Further, the Force Setting Lever (FSL) eliminates the need for the operator to press down on the SG3 Driver for long periods when processing one or multiple samples. Both shredder models (Figure 1B) are designed to quickly yet gently grind tough samples such as plant leaves and fibrous animal tissues. Since shredding is performed in a disposable FT500-S sample container (Figure 1C), the likelihood of cross contamination with other samples is very low. In addition, shredding for 10-20 seconds generates relatively little heat and thus does not increase protein denaturation or damage delicate organelles.

***Pressure Cycling Technology (PCT)***

PCT destabilizes molecular interactions by rapidly and repeatedly raising and lowering pressure in the reaction vessel from ambient to high pressures (up to 45,000 psi [310 MPa, 3000 Atm]). High hydrostatic pressure acts preferentially on the more compressible constituents of the sample, leading to destabilization of molecular interactions, but not of covalent bonds. At lower pressures, e.g. 10,000 - 20,000 psi, PCT can lyse cells and release their intracellular contents, including intact organelles. This method has been shown to be relatively gentle, and it has already been used to isolate mitochondria from cell culture [3].

## Methods

**Isolation of Functional Muscle Mitochondria from Fresh Rat Muscle**

Muscle mitochondria-enriched fractions were prepared from freshly harvested adult rat gastrocnemius muscle as described in the **PBI Mitochondria Isolation Kit: Rat Muscle** product insert. Tissue was shredded using *The* SHREDDER SG3 at lever setting #2 (or for 10 seconds using *The PCT Shredder)*. Samples were either processed directly for mitochondria purification at this point ("Shredder alone"), or were subjected to pressure cycling for additional mitochondria extraction ("Shredder with PCT"). PCT was performed at 10,000, 20,000 or 30,000 psi for 5 cycles at 4˚C. Each cycle consisted of 20 seconds at high pressure and 5 seconds at atmospheric pressure.

Control skeletal muscle samples were transferred to a 50 mL glass homogenizer with a Teflon pestle, and homogenized gently by hand. All subsequent steps were carried out as described above. In order to generate consistent controls and eliminate user-to-user variability, all control samples produced by manual homogenization were prepared by the same experienced individual.

Mitochondrial respiration was measured using an Oroboros Oxygraph-2k system. The rates of respiration at state 2 (V2), state 3 (V3) and state 4 (V4) were determined, and a respiratory control ratio (RCR) was calculated as the ratio V3/V4 (RCR 3/4) or V3/V2 (RCR 3/2).

**Isolation of Lung Mitochondria for Proteomic Analyses**

Lung mitochondria-enriched fractions were prepared from frozen/thawed adult rat lung tissue essentially as described in the **PBI Mitochondria Isolation Kit: Rat Lung** product insert. Tissue was shredded using *The* SHREDDER SG3 at lever setting #2 (or for 10 seconds using *The PCT Shredder)*. The samples were either pressurized at 10,000, 20,000 or 30,000 psi for 5 cycles at 4˚C, or were not subjected to pressurization ("Shredder alone"). Each cycle consisted of 20 seconds at high pressure and 5 seconds at atmospheric pressure.

Control lung tissue samples were manually homogenized in a 50 mL glass homogenizer with a Teflon pestle. Mitochondria recovery by differential centrifugation was carried out as described above.

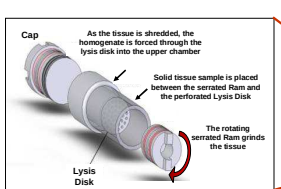


**Figure 1C. The FT500-S Shredder PULSE Tube.** As the tissue is disrupted, the serrated Ram of the PULSE Tube forces the homogenate through the holes of the Lysis Disk into the upper compartment, significantly reducing the risk of sample over-homogenization and mitochondrial damage.

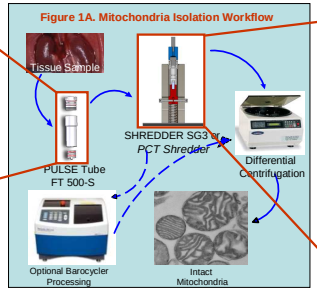

**Figure 1B. *The* SHREDDER SG3**

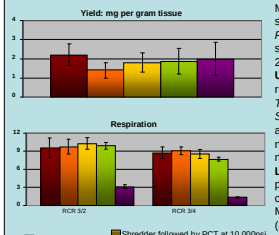


**Figure 2. Yield and Respiration of Skeletal Muscle Mitochondria.**

Mitochondria were extracted using a standard homogenizer method or *The PCT Shredder* with or without subsequent pressure cycling at 10,000, 20,000 or 30,000 psi.

**Upper Panel:** More mitochondria are released by PCT after disruption with *The PCT Shredder* than by *The PCT Shredder* alone, with yields that approach the control method (Control, n=15; Shredder alone, n=19; 10,000psi, n=18; 20,000psi, n=3; 30,000psi, n=2).

**Lower panel:** RCRs of mitochondria prepared using *The PCT Shredder* are comparable to homogenizer control. Mitochondria exposed to 30,000psi (n=2) are severely impaired, while those treated at 10,000 psi (n=7) and 20,000 psi (n=3) are comparable to controls (homogenizer; n=5, Shredder alone; n=5).

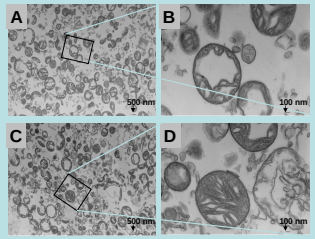


**Figure 3. Electron Micrographs of Muscle Mitochondria Preparations**

**A, B**: Muscle mitochondria, homogenizer control
**C, D**: Muscle mitochondria, Shredder with PCT at 10,000 psi, 5 cycles

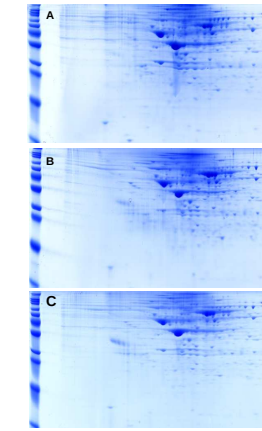


**Figure 4. Total Protein Profile of Rat Lung Mitochondria**

Mitochondria prepared using the control homogenizer method (A); *The PCT Shredder* alone (B); or *The PCT Shredder* followed by PCT at 10,000 psi (C) exhibited essentially the same protein pattern by 2D PAGE (125 µg per gel).
Molecular weight markers are shown on the left of each gel.

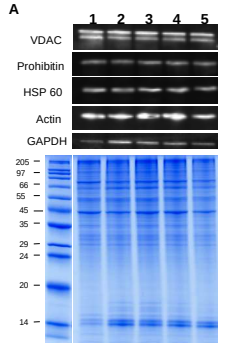


**Figure 5. Rat Lung Mitochondria Preparations.**

Western blots were probed with antibodies to VDAC (outer mitochondria membrane), Prohibitin (inner mitochondria membrane), HSP60 (mitochondrial matrix), actin and GAPDH (cytosolic protein). The presence of GAPDH in all the lung mitochondria preparations is likely due to carryover of cytoplasmic material and could be further reduced by additional washing of the mitochondrial pellet. The presence of actin in the mitochondrial pellet is expected, as it has been reported to be associated with mitochondria [4]. SDS-PAGE protein load was adjusted to 8 µg per lane. Molecular weight markers are shown on the left. Samples as follows:

1. Homogenizer control
2. Shredder alone
3. Shredder with PCT at 10,000psi for 5 cycles
4. Shredder with PCT at 20,000psi for 5 cycles
5. Shredder with PCT at 20,000psi for 15 cycles

Mitochondria were prepared either by manual homogenization, or *The PCT Shredder* with or without PCT at 10,000 or 20,000 psi for 5 cycles. Protein yield was measured by Bradford assay and normalized to mass of starting material.

## Results and Discussion

Here we demonstrate convenient extraction of intact mitochondria from skeletal muscle and lung tissue, using the new **PBI Mitochondria Isolation kits** with ***The* SHREDDER SG3** (or *The PCT Shredder*) and optional subsequent pressure cycling for increased yield. **The PBI Mitochondria Isolation Kit: Rat Muscle** was used to isolate intact and functional mitochondria from freshly harvested rat skeletal muscle tissue.

Protein assays of the mitochondria-enriched preparations demonstrate that sample processing by PCT after initial disruption with ***The* SHREDDER SG3** or *The PCT Shredder*, releases more mitochondria than shredding alone and that this yield approaches that obtained using the more traditional and labor-intensive manual homogenizer method (Figure 2, Upper panel). The results also demonstrate that for isolation of muscle mitochondria, a pressure of 10,000 psi is sufficient to increase yield and that little additional yield is recovered at higher pressures. No significant difference was observed in the respiratory control ratios (RCRs) of muscle mitochondria extracted by the standard manual homogenizer method, compared to those processed using the new kit. Respiration assays of mitochondria extracted with and without pressure cycling (Figure 2, Lower panel) confirm that muscle mitochondria exposed to hydrostatic pressure of 10,000 psi for five cycles, exhibit normal respiration kinetics. As expected, mitochondria exposed to 30,000 psi exhibited very low RCR values. It is likely that such high pressure results in isolation of fragments, rather than intact mitochondria. However, the mitochondria exposed to 20,000 psi appear almost as robust as controls, indicating that the range of pressures compatible with isolation of functional mitochondria, is wide. Electron micrographs (Figure 3) demonstrate the comparable morphologies of mitochondria extracted using the control manual method and those isolated using the new **PBI Mitochondria Isolation Kit: Rat Muscle** with pressure cycling at 10,000 psi.

**The PBI Mitochondria Isolation Kit: Rat Lung** was used to isolate intact mitochondria from frozen/thawed rat lung tissue for proteomic analyses. Two dimensional PAGE (Figure 4) and SDS-PAGE (Figure 5A) demonstrate that the protein profiles of control mitochondria samples isolated by manual homogenization, are essentially the same as the test samples prepared using the **PBI Mitochondria Isolation: Rat Lung kit** with ***The* SHREDDER SG3** (or *The PCT Shredder*) with or without subsequent pressure cycling. Western blots confirm that the lung mitochondria preparations are enriched for protein markers for all three mitochondrial compartments, providing further evidence that the mitochondria are intact (Figure 5A). The yield of lung mitochondria was significantly increased when shredding was followed by PCT treatment for 5 cycles at 20,000 psi (Figure 5B), and additional cycles of pressure (20,000 psi for 15 cycles) did not significantly improve yield.

## Conclusion

Traditional manual methods for isolation of intact mitochondria from solid tissues rely heavily on operator training, experience and skill. Considerable training is required in order to avoid common mistakes, such as tissue over-homogenization, that can result in damaged mitochondria and highly variable results. Here we describe a reproducible and convenient method for isolation of intact mitochondria from fresh rat skeletal muscle or frozen rat lung tissue. The new **PBI Mitochondria Isolation Kit: Rat Muscle** and **PBI Mitochondria Isolation Kit: Rat Lung** kits can be used either with ***The* SHREDDER SG3** or *The PCT Shredder*, with or without pressure cycling. During shredding, the design of the FT 500-S PULSE Tube forces the homogenate through the holes of the Lysis Disk into the upper compartment. This simple but effective design significantly reduces the likelihood of sample over-homogenization. In addition, we demonstrate that overall yield of mitochondria can be increased if initial shredder processing is followed by a brief PCT treatment at 10,000 psi (for muscle mitochondria) or 20,000 psi (for lung mitochondria). Mitochondria isolation with either kit, with or without subsequent PCT, is simple, easy to learn, and eliminates many of the problems of traditional manual mitochondria preparation methods.

## References

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[2] Rasmussen et al., (1997) Anal. Biochem. 252:153-159.
[3]Gross et al. (2008) Biotechniques 45(1):99-100
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