Mitochondrial Physiology Network 17.02: 1-9 (2013)

Version 3: 2013-11-22

PBI-Shredder HRR-Set: preparation of tissue homogenates for diagnosis of mitochondrial respiratory function

Anna Draxl,[1] Andrea Eigentler,[2] Erich Gnaiger[1,2]

[1]**OROBOROS INSTRUMENTS Corp**
high-resolution respirometry
Schöpfstr 18, A-6020 Innsbruck, Austria
Email: erich.gnaiger@oroboros.at
www.oroboros.at

[2]Medical University of Innsbruck
Department of Visceral, Transplant and Thoracic Surgery
D. Swarovski Research Laboratory, 6020 Innsbruck, Austria
http://wiki.oroboros.at/index.php/K-Regio_MitoCom_Tyrol

Section

Page

The PBI-Shredder HRR-Set is an auxiliary HRR-Tool providing a standardized approach to prepare homogenates of various tissues with high reproducibility of mitochondrial yield and mitochondrial function. In this guide to applications with high-resolution respirometry (HRR), we refer to

the PBI User Manual for safety information, product use limitations and warranty information, and to the Product Specification Sheet by Pressure BioSciences Inc. (PBI).

1. Introduction

Figure 1: The PBI-Shredder SG3 with handle (red) and torque driver (white) assembled with the force setting lever (metal) ready for application.

Application of high-resolution respirometry with gently prepared tissue homogenates offers a versatile tool to study mitochondrial function in small amounts of tissues. The **PBI-Shredder SG3** (**Figure 1**) is a low shear mechanical homogenization system, designed to apply reproducible force to the tissue with three positions of the force setting lever. This yields standardized, rapid and safe disruption of cells with preservation of intact, functional mitochondria. The laboratory-specific or even operator-specific protocols for tissue homogenization are thus standardized, providing reproducible and consistent results for quantitative and inter-laboratory comparison. The easy handling enables especially beginners to obtain reliable results.

The PBI-Shredder HRR-Set includes Shredder-Tubes for ambient pressure processing, without and with a metal insert to disrupt tough cellular structures. In our primary applications with mouse and fish myocard and liver, Shredder-Tubes with and without metal inserts gave comparable results. Optimization of homogenization with various tissues will be possible using either type of Shredder-Tubes, force settings, and duration of shredding.

2. Materials and Chemicals

2.1. Components of the PBI-Shredder HRR-Set

http://www.bioblast.at/index.php/PBI-Shredder_HRR-Set

- PBI-Shredder SG3, stored in the Shredder-Kit Box, with torque driver and convertible handle, metal SG3 base (use pre-chilled after storage in the fridge) with 3 position force setting lever (FSL), battery charger and two lithium ion batteries (**Figure 1**).
- Shredder-Tube Cap Tool (**Figure 2**).
- Shredder-Tube Ram Tool

- Box of 100 Shredder-Tubes FT500-PS with plastic lysis disk, with Shredder-Rams and Shredder-Screw Caps (use pre-chilled; **Figure 3**).
- Box of 100 Shredder-Tubes\Metal FT500-PMS with metal lysis disk, with Shredder-Rams and Shredder-Screw Caps (use pre-chilled; **Figure 3**).
- Pair of dissecting forceps, stainless steel, antimagnetic, sharp straight tips.
- 1 pair of dissecting scissors (straight tip, sharp front).

2.2. Other materials

- Microbalance Mettler-Toledo, 0.01 mg display; http://www.bioblast.at/index.php/Microbalance-Set
- Petri dish and 12-well tissue culture plate
- 50 ml Falcon tubes (1 per Shredder-Tube)
- 500 µl pipette with tips
- Cap of a 1.5 ml Eppendorf tube (cut off)
- Filter paper or soft tissues
- Timer (1-60 s)
- Ice

2.3. Media

- BIOPS: The relaxing and organ preservation solution BIOPS contains 10 mM Ca-EGTA buffer, 0.1 µM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM $MgCl_2$, 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1 (MiPNet03.02). BIOPS can be stored frozen at -20 °C.
- MiR05, MiR05Cr, MiR06 or MiR06Cr (MiPNet14.13).

3. Sample preparation

3.1. Organ harvest

Heart and liver are excised from the sacrificed animal and immediately separated into specific subsamples and added into Falcon tubes containing sufficient ice-cold BIOPS (30 ml for the entire mouse heart and trout heart) or respiration medium (trout liver) to cover the entire tissue sample. Keep on ice and minimize transportation and storage time as far as possible.

3.2. Tissue preparation

Place the tissue sample into a small Petri dish with fresh ice-cold BIOPS or respiration medium on a cooling plate. The tissue should be completely covered with liquid.

Heart: Open the left ventricle of the heart by using the dissecting scissors and forceps. Cut out muscle tissue and omit pericardium. Place small muscle pieces into a 12-well plate with ice-cold respiration medium.

3.3. Determination of wet weight, W_w

Prepare tissue samples of about 4 mg W_w of mouse heart muscle and about 16 mg W_w of trout heart muscle or trout liver for two O2k-Chambers (half the W_w if one Shredder Tube should be used for one O2k-Chamber).

Place the Eppendorf cap on the microbalance, add 100 µl of Biops or respiration medium and tare.
Transfer the samples with the pair of forceps onto a filter paper. During this time of a few seconds, wipe off any liquid from the sharp tip of the forceps with another filter paper. Then take the samples from the filter paper and touch it once more shortly onto a dry area of filter paper while holding it with the forceps. Afterwards, immediately place the samples into the Eppendorf cap and read the wet weight.

3.4. Quick protocol

1. Store the PBI-Shredder metal SG3 base and Shredder tubes at -20°C.
2. Take a Shredder tube and close the Cap side with a Shredder-Screw Cap using the Shredder-Tube Cap Tool
3. Add 500 µl of ice-cold respiration medium to the Ram side of the Shredder-Tube and pre-chill the Shredder-Tube on ice
4. After reading the W_W transfer the samples to the narrow Ram side of the pre-chilled Shredder-Tube
5. Cut the tissue samples into smaller pieces with a sharp pair of scissors
6. Evenly distribute the tissue pieces on the Lysis Disk at the narrow Ram side of the Shredder-Tube
7. Close the Shredder-Tube with a serrated Shredder-Ram
8. Place the filled Shredder –Tube into the pre-chilled Shredder Base with the Ram side down,
9. Twist the Shredder-Tube to set the Ram into the holder in the Shredder Base
10. When the tube is seated securely, place the SG3 Driver onto the Cap and turn on the SG3 Driver to seat the Driver bit into the crenellations of the Cap
11. With one hand press down the Driver and with the other hand set the lever into the appropriate position for the sample

12. Activate the Shredder for 10 seconds at position 1 (weakest) followed by 5 seconds at position 2 (stronger) – this accounts for mouse and trout heart as well as trout liver
13. Remove the homogenate by using the Shredder-Tube Cap Tool to unscrew the Shredder-Screw Cap from the Shredder-Tube by anticlockwise rotation
14. Transfer the sample into a 50 ml Falcon on ice using a 500 µl pipette
15. Rinse the tube with fresh cold respiration medium to recover any residual sample and add to the homogenate
16. Open the Shredder-Tube by using the Shredder-Tube Ram Tool to open the narrow side of the Shredder-Tube
17. Wash any residual tissue out of the tube into the 50 ml Falcon using fresh cold respiration medium
18. Rinse with 4.5 ml respiration medium in total to have 5 ml end volume which is intended for use with two O2k-Chambers and keep the sample on ice until used for HRR
19. Siphon off the respiration medium of the O2k-Chambers
20. Resuspend the homogenate thoroughly by pipetting 6 times up and down avoiding pipetting on the wall of the tube and any generation of foam
21. Insert 2.5 ml of homogenate into one O2k-Chamber
22. Repeat step 19 and 20 for the second O2k-Chamber
23. Insert the stoppers loosely into the O2k-Chambers and allow the homogenate to warm up to the experimental temperature for approximately 3 minutes
24. Close the chamber and siphon off the excess of respiration medium

3.5. Detailed protocol Tissue homogenization (shredding)

Immediately after reading the wet weight, the samples are transferred to the narrow Ram side of the pre-chilled Shredder-Tube, already capped with the Shredder-Screw Cap using the Shredder-Tube Cap Tool again (**Figure 3**) and containing 500 µl respiration medium (e.g. MiR06 or MiR06Cr), using the pair of straight dissection forceps, wetted with respiration medium. The tissue samples are then cut into smaller pieces with a sharp pair of scissors and evenly distributed on the Lysis Disk at the narrow Ram side of the Shredder-Tube.

The total volume of sample and respiration medium during shredding should not exceed 0.7 to 0.8 ml (this prevents medium from being forced into the threads of the cap where it might be lost during uncapping).

Figure 2: The Shredder-Tube Cap Tool.

After evenly distributing the small tissue pieces on the Lysis Disk at the narrow Ram side of the Shredder-Tube, a serrated Shredder-Ram is inserted with a twisting motion to press the sample between the serrated surface and the Lysis Disk by using the Shredder-Tube Cap Tool (**Figure 2**).

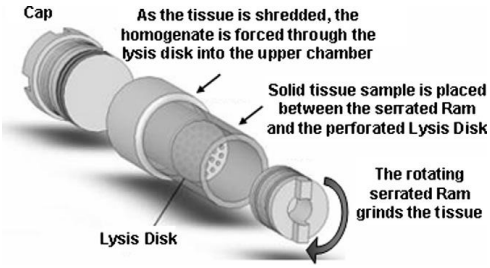

Figure 3: FT500-PS Shredder Pulse Tube for use with the PBI Shredder (reproduced from Gross et al, 2011).

Place the filled Shredder-Tube into the pre-chilled Shredder Base, Ram side down, and twist to set the Ram into the holder in the Shredder Base. When the tube is seated securely, place the SG3 Driver onto the Cap, and briefly turn the driver on in order to seat the Driver bit into the crenellations of the Cap.

While pressing down the Driver with one hand, set the lever into the appropriate position for the sample. For mouse and trout heart as well as trout liver, activation of the Shredder for **10 seconds at position 1 (weakest) followed by 5 seconds at position 2 (stronger)** was evaluated as optimum regime, with a maximum of the sample passing through the Lysis Disk into the upper chamber of the Shredder-Tube, containing functionally intact mitochondria. Position 3 (strongest) was not required in these samples. This short processing time does not significantly heat the sample. It is recommended to use a timer for application of the shredder.

3.6. Removing the homogenate

To remove the processed homogenate, use the Shredder-Tube Cap Tool to unscrew the Shredder-Screw Cap from the Shredder-Tube by anticlockwise rotation. Transfer the sample into a 50 ml Falcon on ice using a 500 µl pipette. To recover any residual sample, rinse the tube with fresh cold respiration medium and add to the homogenate. Use the Shredder-Tube Ram Tool to open the narrow side of the Shredder-Tube and wash the sample out of the tube with respiration medium. Rinse with 4.5 ml in total and at the end there should be 5 ml of homogenate in the Falcon tube on ice. This volume is intended for use with two O2k-Chambers. Keep the sample on ice until used for HRR.

3.7. Experimental setup with the Oxygraph-2k

For experiments with homogenate preparations, the medium of the O2k-chamber was siphoned off. The homogenate was resuspended thoroughly by pipetting 6 times up and down avoiding any generation of foam and 2.5 ml were inserted into one O2k-chamber. This was repeated for the second chamber. The homogenate was then allowed to warm up to the experimental temperature for 3 min without closing the chamber but with the stopper inserted loosely.

4. Conclusions

The PBI Shredder provides a standardized tissue preparation for obtaining disrupted cells with functional mitochondria that may be used directly for HRR, or the homogenization process may be followed by further isolation of mitochondria. In addition, the homogenate is suitable for optical measurements (e.g. O2k-Fluorometry with safranin for detection of mt-membrane potential) where a homogenous suspension is required. Furthermore, the oxygen diffusion gradients are reduced compared to permeabilized fibres.

The PBI Shredder combines a minimum processing time of 10 minutes and easy handling, that enables especially beginners to obtain reliable results and the closed Shredder-Tubes ensure safety throughout the entire sample preparation process. As with any other method, training of each individual person with the PBI Shredder improves the handling and the tissue preparation resulting in better results over time as shown with the cytrochrome *c* test. At the beginning of our experiments the cytochrome *c* effect was larger in cardiac mouse homogenate compared to permeabilized fibres, indicating a small degree of functional impairment of myocardial mitochondria is caused by the homogenization process. Over the time, our skills improved and we were able to diminish the cytochrome *c* effect in mouse heart homogenate and no cytochrome *c* effect occurred in mouse liver and mouse brain homogenate.

The homogenate obtained with this method may contain some tissue particles that are not homogenized, but complete cell permeabilization is obtained as evaluated with HRR. Therefore a potentially unequal distribution of the homogenate into different O2k-chambers can occur due to insufficient resuspension of the homogenate. Furthermore a fraction of mitochondria can potentially be lost when insufficient care is taken to retrieve the entire tissue. If not

all mitochondria are obtained from the tissue, it is difficult to evaluate if specific mitochondrial types are enriched or a representative subsample of all mitochondria is obtained. If not all mitochondria are obtained from the tissue, tissue mass-specific mitochondrial respiratory capacity can be measured only on the basis of additional measurements of a mitochondrial marker (e.g. CS activity) in the total tissue and in the homogenate, to quantify the mt-yield and refer respiration of the homogenate to W_w of tissue. The application of an additional tool to remove the serrated Shredder-Ram as well as the Shredder-Screw Cap after homogenization increased the mitochondrial yield by washing out the homogenate completely from both sides of the Lysis Disk.

5. References

Doerrier VC, Draxl A, Eigentler A, Gnaiger E (2013) Mitochondrial respiration in permeabilized fibres versus homogenate from trout heart and liver. Mitochondr Physiol Network 17.03.

Gross VS, Greenberg HK, Baranov SV, Carlson GM, Stavrovskaya IG, Lazarev AV, Kristal BS (2011) Isolation of functional mitochondria from rat kidney and skeletal muscle without manual homogenization. Analyt Biochem 418: 213-223.

Pressure BioSciences Inc. The Shredder SG3 and Shredder PULSE Tubes: Product Specification Sheet: 1-2.

Pressure BioSciences Inc. The Shredder SG3: User Manual: 1-16.

Mitochondr Physiol Network – MiPNet Manuals and Protocols

MiPNet03.02: Selected media and chemicals for respirometry with mitochondria and permeabilized cells. Mitochondr Physiol Network 3.2.

MiPNet11.05: Isolated mitochondria or permeabilized tissues and cells. Mitochondr Physiol Network 11.5.

MiPNet14.13: Mitochondrial respiration medium - MiR06. Mitochondr Physiol Network 14.13.

6. Author contributions and publication versions

Prepared by Draxl A, Eigentler A and Gnaiger E in February 2012. DA performed the experiments.

- Version 1: 2012-02-29 / 2012-03-14
- Version 2: 2013-01-15

Contribution to K-Regio project *MitoCom Tyrol*, funded in part by the Tyrolian Government and the European Regional Development Fund (ERDF).
www.oroboros.at/?MitoCom-Tyrol

http://www.bioblast.at/index.php/PBI-Shredder_HRR-Set

Description **PBI-Shredder HRR-Set**: Auxiliary HRR-Tool for tissue homogenate preparation; the Shredder-Kit Box contains the heavy duty high torque SG3 driver with convertible handle, SG3 base with 3 position force setting lever (FSL), battery charger, two lithium ion batteries, Shredder-Tube Cap Tool. The PBI-Shredder HRR-Set includes the Shredder-Kit Box with 100 Shredder-Tubes, 100 Shredder-Tubes\Metal, a pair of sharp forceps for tissue dissection and a pair of scissors.

Product ID 13200-02

Link PBI-Shredder @OROBOROS, O2k-Catalogue: PBI-Shredder, Purchase Order @OROBOROS

PBI-Shredder HRR-Set consists of

Title	Description	Product id	Product image
PBI-Shredder SG3	**PBI-Shredder SG3** for tissue homogenate preparation, heavy duty high torque SG3 driver with convertible handle, SG3 base with 3 position force setting lever (FSL), battery charger and two lithium ion batteries. The PBI-Shredder SG3 is included in the **PBI-Shredder HRR-Set**. Select 230 V or 120 V. OROBOROS INSTRUMENTS: world-wide distributor.	52100	
Shredder-Kit Box	**Shredder-Kit Box**: box for storage and shipping, for PBI-Shredder SG3	52101-01	
Shredder-Tube Cap Tool	**Shredder-Tube Cap Tool**: component of **PBI-Shredder_HRR-Set**.	52130-01	
Shredder-Accessory Box	**Shredder-Accessory Box**: 71x335x240 mm inner dimensions, for storage and shipping of Shredder accessories.	52201-01	
Shredder-Tubes	**Shredder-Tubes**: consisting of Shredder Tube FT500-PS with Lysis Disk, serrated Shredder-Ram and Shredder-Screw Cap, coral colour (Box of 100). 1 box is included in the **PBI-Shredder HRR-Set**.	52210-01	
Shredder-Tubes\Metal	**Shredder-Tubes\Metal**: consisting of Shredder Tube FT500-PMS with Metal Lysis Disk, serrated Shredder-Ram and Shredder-Screw Cap, coral colour (Box of 100). 1 box is included in the **PBI-Shredder HRR-Set**.	52220-01	
Forceps\stainless Steel\straight Tip\sharp	**Forceps\stainless Steel\straight Tip\sharp**: for tissue preparation, stainless steel, antimagnetic. One pair is recommended for insertion of the sample into the O2k-Chamber and for handling in combination with Forceps\stainless Steel\rounded Tip\sharp. Set: in HRR-Dissection Set and **PBI-Shredder HRR-Set**.	54210-01