INSTRUCTIONS

Mitochondria Isolation Kit for Tissue

PIERCE
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89801

1741.0

Number	Description
89801	**Mitochondria Isolation Kit for Tissue,** contains sufficient reagents to perform 50 isolations of intact mitochondria from soft and hard tissues

Kit Contents:

Mitochondria Isolation Reagent A, 50 ml

Mitochondria Isolation Reagent B, 500 µl

Mitochondria Isolation Reagent C, 65 ml

Bovine Serum Albumin, 230 mg

BupH™ Phosphate Buffered Saline, 1 pack, results in 0.1 M sodium phosphate, 0.15 M sodium chloride; pH 7.2 when reconstituted with 500 ml of ultrapure water

Storage: Upon receipt store kit at 4°C. Product is shipped at ambient temperature.

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Introduction

As new discoveries of human disorders are being linked to mitochondrial dysfunction, there is an increasing need for an effective method to isolate intact mitochondria from tissues. The Mitochondria Isolation Kit for Tissue offers two methods for isolation of intact mitochondria from both soft and hard tissues. The first method uses a unique reagent-based procedure that enables simultaneous multi-sample processing. The second method relies on traditional Dounce homogenization for tissue disruption and subsequent isolation of the organelle. Both procedures rely on differential centrifugation to separate the intact mitochondria using a bench-top microcentrifuge and are completed in less than 60 minutes. In addition, both procedures have been optimized for maximum yield of mitochondria with minimal damage to its integrity. The isolated mitochondria may be used for a number of downstream applications, including 2D/MS for proteomics research, disease profiling and metabolic studies.

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Important Product Information

- This kit allows two options for isolating mitochondria: a reagent-based method and a Dounce homogenization method. The kit contains sufficient reagents for 50 isolations from 50-200 mg samples of soft or hard tissues and provides > 50% reduction in organelle (i.e., lysosomes and peroxisomes) contamination from the heavy mitochondria fraction when performing the centrifugation at $3{,}000 \times g$.
- Up to six samples can be processed concurrently using the reagent-based method, whereas only one sample can be processed at a time with the Dounce method. Because of the time delay in processing multiple samples with the Dounce protocol, samples processed initially may yield a higher quantity of mitochondria compared to samples incubated on ice for subsequent isolation.
- The number of Dounce strokes required for optimal cell lysis with minimal damage to mitochondria for each tissue type must be determined empirically. Mammalian tissues can be classified under two categories: soft tissue, such as the liver and intestinal mucosa, or hard tissue, such as the heart and skeletal muscle. Soft tissue can be effectively homogenized more readily than hard tissue (see Table 1 in the Additional Information Section).

Additional Materials Required

- Variable-speed bench-top microcentrifuge refrigerated at 2-8°C
- 2 ml microcentrifuge tubes
- Vortex mixer
- Protease inhibitors, EDTA-free such as Halt™ Protease Inhibitor Cocktail Kit (Product No. 78415)
- Dounce tissue grinder, such as 2 ml Kontes or Wheaton Dounce Tissue Grinder
- (Optional) TPCK Trypsin (Product No. 20233)

  Notes: Pre-treating hard tissue with 0.3 mg/ml of trypsin facilitates cell structural breakdown and eases sample homogenization. Comparable mitochondrial yield can be obtained with or without trypsin treatment providing that the hard tissue is sufficiently minced before homogenization. Adding bovine serum albumin (BSA) to the sample quenches proteolytic activity of trypsin, removes free fatty acids and maintains mitochondrial respiration.

Material Preparation

Phosphate Buffered Saline (PBS)	Dissolve the dry-blend buffer with 500 ml of ultrapure water. For long-term storage of excess buffer, sterile-filter the solution and store at 4ºC.
BSA/Reagent A Solution	Add sufficient BSA to the Mitochondria Isolation Reagent A to make a 4 mg/ml solution. Each sample requires 1 ml of BSA/Reagent A Solution.
Wash Buffer	Just before use, dilute Mitochondria Isolation Reagent C with an equal volume of ultrapure water. Each sample requires 1 ml of Wash Buffer.

Option A: Isolation of Mitochondria from Soft Tissues

Protocol 1: Reagent-based Method for Soft Tissues

- A Dounce homogenizer or Polytron® Tissue Grinder is necessary for initial tissue disruption before using the reagent-based method. Pre-chill Dounce tissue grinder on ice before use.
- Immediately before use, add protease inhibitors to Mitochondria Isolation Reagents A and C; add inhibitors only to the reagent amount being used for the procedure and not to the stock solutions.
- Note that the required vortex speed changes during the protocol.

1. Wash 50-200 mg of tissue twice with 2-4 ml of PBS. Carefully remove and discard the PBS wash.
2. Cut tissue into small pieces and add 800 µl of PBS.
3. Disrupt tissue using a Polytron® Grinder or Dounce homogenizer while on ice. Perform sufficient number of Dounce strokes to obtain a homogeneous suspension but not to lyse cells; typically 3-5 Dounce strokes is sufficient.

4. Centrifuge tissue at 1,000 × *g* for 3 minutes at 4°C and discard the supernatant.
5. Suspend the pellet in 800 µl of BSA/Reagent A Solution.
6. Vortex at **medium** speed for 5 seconds and incubate tube on ice for exactly 2 minutes. Do not exceed the 2 minute incubation.
7. Add 10 µl of Mitochondria Isolation Reagent B. Vortex at **maximum** speed for 5 seconds.
8. Incubate tube on ice for 5 minutes, vortexing at **maximum** speed every minute.
9. Add 800 µl of Mitochondria Isolation Reagent C to the tube. Invert tube several times to mix (do not vortex).
10. Centrifuge tube at 700 × *g* for 10 minutes at 4°C. Discard pellet and transfer supernatant to a new 2 ml tube.
11. Centrifuge supernatant at 3,000 × *g* for 15 minutes at 4°C.

    Note: Centrifuging at 12,000 × *g* results in greater yield than centrifuging at 3,000 × *g*; however, lysosome and peroxisome contamination also will be greater.
12. Remove the supernatant from the mitochondrial pellet. If desired save the supernatant (cytosolic fraction) for analysis.
13. Add 500 µl of Wash Buffer to the mitochondrial pellet and perform a surface wash of the pellet. Centrifuge at 12,000 × *g* for 5 minutes and discard the supernatant.
14. Maintain the mitochondrial pellet on ice before downstream processing. Freezing and thawing may compromise mitochondrial integrity.

Protocol 2: Dounce Homogenization for Soft Tissues

- Pre-chill the Dounce tissue grinder on ice before use.
- Immediately before use, add protease inhibitors to Mitochondria Isolation Reagents A and C; add inhibitors only to the reagent amount being used for the procedure and not to the stock solutions.
- Empirically determine the number of Dounce strokes required for optimal cell lysis with minimal damage to mitochondria for each tissue type.

1. Wash 50-200 mg of tissue twice with 2-4 ml of PBS. Carefully remove and discard PBS wash.
2. Cut tissues into small pieces and add 800 µl of BSA/Reagent A Solution.
3. Perform Dounce homogenization on ice.
4. Add 800 µl of Mitochondria Isolation Reagent C. Invert tube several times to mix (do not vortex).
5. Centrifuge tube at 700 × *g* for 10 minutes at 4°C. Discard pellet and transfer supernatant to a new 2 ml tube.
6. Centrifuge supernatant at 3,000 × *g* for 15 minutes at 4°C.

    Note: Centrifuging at 12,000 × *g* results in greater yield than centrifuging at 3,000 × *g*; however, lysosome and peroxisome contamination also will be greater.
7. Remove the supernatant from the mitochondrial pellet. If desired save the supernatant (cytosolic fraction) for analysis.
8. Add 500 µl of Wash Buffer to the mitochondrial pellet and perform a surface wash of the pellet. Centrifuge at 12,000 × *g* for 5 minutes and discard the supernatant.
9. Maintain the mitochondrial pellet on ice before downstream processing. Freezing and thawing may compromise mitochondrial integrity.

Option B: Isolation of Mitochondria from Hard Tissues

Protocol 1: Reagent-based Method for Hard Tissues

- Immediately before use, add protease inhibitors to Mitochondria Isolation Reagents A and C; add inhibitors only to the reagent amount being used for the procedure and not to the stock solutions.
- Initial tissue disruption using a Dounce homogenizer or Polytron® Tissue Grinder is necessary before using the reagent-based approach. Pre-chill Dounce homogenizer on ice before use.

- If trypsin pre-treatment is needed, prepare a 0.3 mg/ml solution of trypsin in PBS. Each sample requires 1 ml of tyrpsin/PBS Solution.
- Note that the required speed of vortex changes during the protocol.

1. Wash 50-200 mg of tissue twice with 2-4 ml of PBS. Carefully remove and discard PBS wash.
2. Cut tissue into small pieces. For trypsin pre-treatment, incubate tissue in trypsin/PBS solution on ice for 3 minutes. If trypsin pre-treatment is not necessary, add 800 µl of PBS containing 4 mg/ml of BSA to the tissue and skip to step 5.
3. Perform a quick centrifugation to pellet the sample and to remove the trypsin solution.
4. Add 800 µl of PBS containing 4 mg/ml of BSA to quench the proteolytic activity of trypsin.
5. Disrupt tissue using a Polytron® Grinder or Dounce homogenizer while on ice. Perform sufficient number of Dounce strokes to obtain a homogeneous suspension but not to lyse cells; typically, 3-5 Dounce strokes is sufficient.
6. Centrifuge tissue at 1,000 × *g* for 3 minutes at 4ºC. Carefully remove and discard the supernatant.
7. Suspend the pellet in 800 µl of BSA/Reagent A Solution.
8. Vortex at **medium** speed for 5 seconds and incubate tube on ice for exactly 2 minutes. Do not exceed the 2 minute incubation.
9. Add 10 µl of Mitochondria Isolation Reagent B. Vortex at **maximum** speed for 5 seconds.
10. Incubate tube on ice for 5 minutes, vortexing at **maximum** speed every minute.
11. Add 800 µl of Mitochondria Isolation Reagent C. Invert tube several times to mix (do not vortex).
12. Centrifuge tube at 700 × *g* for 10 minutes at 4°C. Discard pellet and transfer supernatant to a new 2 ml tube.
13. Centrifuge supernatant at 3,000 × *g* for 15 minutes at 4°C.

    Note: Centrifuging at 12,000 × *g* results in greater yield than centrifuging at 3,000 × *g*; however, lysosome and peroxisome contamination also will be greater.
14. Remove the supernatant from the mitochondrial pellet. If desired save the supernatant (cytosolic fraction) for analysis.
15. Add 500 µl of Wash Buffer to the mitochondrial pellet and perform a surface wash of the pellet. Centrifuge tube at 12,000 × *g* for 5 minutes and discard supernatant.
16. Maintain the mitochondrial pellet on ice before downstream processing. Freezing and thawing may compromise mitochondrial integrity.

Protocol 2: Dounce Homogenization for Hard Tissue

- Immediately before use, add protease inhibitors to Mitochondria Isolation Reagents A and C; add inhibitors only to the reagent amount being used for the procedure and not to the stock solutions.
- Pre-chill Dounce homogenizer on ice before use.
- Empirically determine the number of Dounce strokes required for optimal cell lysis with minimal damage to mitochondria for each tissue type.
- If trypsin pre-treatment is needed, prepare a 0.3 mg/ml of trypsin in PBS. Each sample requires 1 ml of tyrpsin/PBS Solution.
- Note that the required speed of vortex changes during the protocol.

1. Wash 50-200 mg of tissue twice with 2-4 ml of PBS. Carefully remove and discard PBS wash.
2. Cut tissues into small pieces. For trypsin pre-treatment, incubate tissue in trypsin/PBS solution on ice for 3 minutes. If trypsin pre-treatment is not necessary, add 800 µl of BSA/Reagent A Solution to the sample and then skip to step 5.
3. Perform a quick centrifugation to pellet and to remove the trypsin solution.
4. Add 800 µl of BSA/Reagent A Solution to the tube.
5. Perform Dounce homogenization on ice.
6. Add 800 µl of Mitochondria Isolation Reagent C. Invert tube several times to mix (do not vortex).

7. Centrifuge tube at 700 × *g* for 10 minutes at 4°C.
8. Discard the debris pellet and transfer the supernatant to a new 2 ml tube.
9. Centrifuge supernatant at 3,000 × *g* for 15 minutes at 4°C.

   Note: Centrifuging at 12,000 × *g* results in greater yield than centrifuging at 3,000 × *g*; however, lysosome and peroxisome contamination also will be greater.
10. Remove the supernatant from the mitochondrial pellet. If desired save the supernatant (cytosolic fraction) for analysis.
11. Add 500 µl of Wash Buffer to the mitochondrial pellet and perform a surface wash of the pellet. Centrifuge tube at 12,000 × *g* for 5 minutes and discard supernatant.
12. Maintain the mitochondrial pellet on ice before downstream processing. Freezing and thawing may compromise mitochondrial integrity.

Troubleshooting

Problem	Possible Cause	Solution
Small mitochondrial pellet recovered	Number of Dounce strokes were insufficient to achieve optimal lysis	Optimize the number of Dounce strokes for each tissue type
	Reagent-based method results in a two-fold lower yield than Dounce approach	Make sure tissue is adequately homogenized before using the reagent-based method
	Cells are difficult to lyse	Use Dounce method
Western blot revealed large amounts of cytochrome C in the cytosol	Exceeded the 2 minute incubation of the tissue in Reagent A	Adhere to the two minute incubation in Reagent A
	Used an excessive number of Dounce strokes to lyse the cells	Determine the optimal number of Dounce strokes to use for each tissue type
	Excessive vortexing damaged the mitochondria	Adjust the vortex to the appropriate setting as indicated in the protocol
Reduction of total mitochondrial protein with successive samples processed by the Dounce method	Samples processed last can deteriorate with time	If processing multiple samples, use the reagent-based method, which allows concurrent processing of up to six samples
Fats/lipids are present in the sample	Dietary intake contributes to fats/lipids in the system	Starve animal 24 hours before sacrificing and use BSA in the reagents to help remove free fatty acids

Additional Information

A. Cell Lysis

The number of Dounce homogenization strokes necessary for optimal cell lysis varies depending upon tissue type. Empirically determine the number of strokes required for optimal cell lysis with minimal damage to the mitochondria for each tissue type. Example tissue types are listed in Table 1.

Table 1. Number of Dounce homogenization strokes required for effective lysis of liver and heart tissue.

Tissue Type	Amount (mg)	Number of Strokes
Soft (liver)	50	20-30
	200	50-75
Hard (heart)	50	10
	200	30-50

B. Mitochondria Lysis

For analysis by Western blotting or gel electrophoresis, boil mitochondrial pellet with SDS-PAGE sample buffer and apply to the gel. For protein analysis using BCA™ Protein Assay Kit (Product No. 23225), lyse mitochondria with 2% CHAPS in Tris buffered saline (TBS; e.g., 25 mM Tris, 0.15 M NaCl; pH 7.2, Product No. 28379) as described below:

1. Depending on the mitochondrial pellet size or protein concentration, add 300-1,000 µl of 2% CHAPS in TBS to the mitochondrial pellet and vortex for 1 minute.
2. Centrifuge mitochondria at high speed for 2 minutes. The supernatant contains soluble mitochondrial protein that can be analyzed by BCA™ Protein Assay.

Related Pierce Products

23225	**BCA™ Protein Assay Kit**, sufficient reagents to perform 500 standard tube assays or 5,000 microplate assays
34080	**SuperSignal® West Pico Chemiluminescent Substrate**, 500 ml
34075	**SuperSignal® West Dura Extended Duration Substrate,** 100 ml
34095	**SuperSignal® West Femto Maximum Sensitivity Substrate,** 100 ml
78415	**Halt™ Protease Inhibitor Cocktail**, **EDTA-Free,** sufficient reagents for 100 ml of extract
78833	**NE-PER® Nuclear and Cytoplasmic Extraction Kit**, sufficient reagents for extracting 50 cell pellet fractions having packed cell volumes of 20 µl each
89826	**Mem-PER® Eukaryotic Membrane Protein Extraction Kit**, sufficient reagents for extracting 50 cell pellet fractions of 5×10^6 cells each
78501	**M-PER® Mammalian Protein Extraction Reagent,** 250 ml

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Patent pending on Mitochondria Isolation Kit technology.

BCA™ Technology is protected by U.S. Patent # 4,839,295.

SuperSignal® Technology is protected by U.S. Patent # 6,432,662.

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