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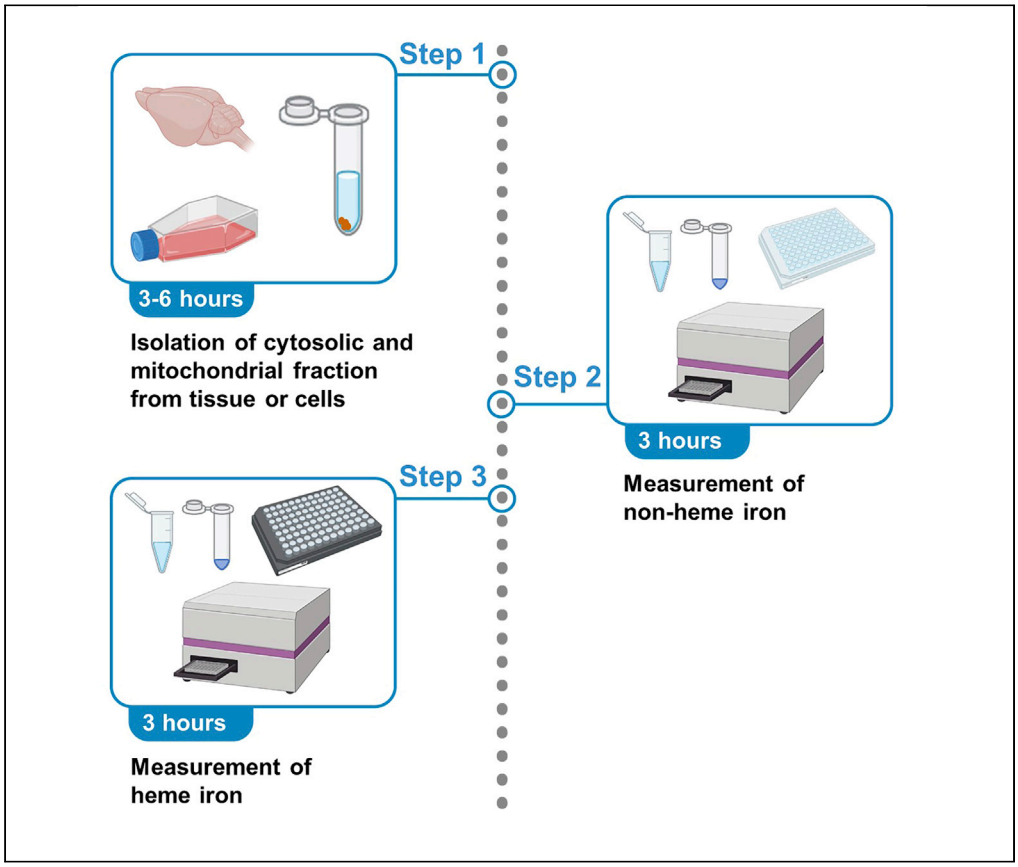
Optimized protocol for quantification of mitochondrial non-heme and heme iron content in mouse tissues and cultured cells



Impaired mitochondrial iron metabolism is associated with aging and a variety of diseases, and there is a growing need to accurately quantify mitochondrial iron levels. This protocol provides an optimized method for evaluating non-heme and heme iron in mitochondrial and cytosolic fractions of tissues and cultured cells. Our protocol consists of three steps: sample fractionation, non-heme iron measurement, and heme iron measurement.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for measuring iron content in mitochondrial and cytosolic fractions

Both non-heme iron and heme iron can be evaluated

Step-by-step guide for efficient sample preparation

Applicable to both tissues and cultured cells

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Optimized protocol for quantification of mitochondrial non-heme and heme iron content in mouse tissues and cultured cells

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SUMMARY

Impaired mitochondrial iron metabolism is associated with aging and a variety of diseases, and there is a growing need to accurately quantify mitochondrial iron levels. This protocol provides an optimized method for evaluating non-heme and heme iron in mitochondrial and cytosolic fractions of tissues and cultured cells. Our protocol consists of three steps: sample fractionation, non-heme iron measurement, and heme iron measurement.

For complete details on the use and execution of this protocol, please refer to Sato et al. (2022).[1]

BEFORE YOU BEGIN

Iron is an essential trace metal for living organisms. Cellular iron exists as 1) heme iron, a complex composed of a ferric ion (Fe^{2+}) and a porphyrin, or 2) non-heme iron, the majority of which is stored in ferritin or in the form of iron-sulfur (Fe/S) clusters. Excess free iron catalyzes the Fenton reaction which produces highly cytotoxic hydroxyl radicals ($OH^{\cdot}$) from hydrogen peroxide (H_2O_2). Therefore, intracellular iron homeostasis must be tightly regulated.[2]

Recently, impairments of mitochondrial iron homeostasis have been shown to be associated with normal physiological changes, such as aging, and pathological disorders, such as cardiovascular and neurodegenerative diseases. However, the mechanisms of dysregulated mitochondrial iron metabolism in many of these disorders are yet to be elucidated. We recently showed that increased mitochondrial non-heme iron is associated with degradation of the cellular iron exporter ferroportin (FPN1) in the cerebral cortex of aged mice as well as elevated mitochondrial oxidative stress.[1] Thus, it is important to accurately quantify cellular and mitochondrial non-heme and heme iron in both tissues and cells to better understand disease pathophysiology and to identify novel therapeutic targets.

The protocol below describes the specific steps for measuring mitochondrial and cytosolic iron contents in the murine brain cortex.[1] We have also used this protocol in the murine heart,[3–5] human heart,[6] murine liver,[1] murine gastrocnemius muscle[1] and cultured H9c2 rat cardiomyoblasts.[5]

Institutional permissions

This protocol uses harvested mouse tissue. All animal studies need to be performed in strictly accordance with Institutional Animal Care and Use Committee (IACUC) guidelines and must be approved by the local IACUC or equivalent oversight committee before you begin. Our protocol used the brain

cortex from 4-month-old and 22-month-old female UM-HET3 mice, which is a genetically heterogeneous mouse model that is the first-generation offspring of a CByB6F1 × C3D2F1 cross to produce a diverse heterogeneous population.[7]

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Mitochondria Isolation Kit for Tissue	Pierce	89801
ProteaseArrest Protease Inhibitor	G-Biosciences	786-437
BCA Protein Assay Kit	Pierce	23225
3-(2-Pyridyl)–5,6-di(2-furyl)–1,2,4-triazine-5′, 5″-disulfonic acid disodium salt (Ferrozine)	Sigma-Aldrich	82940
Trichloroacetic acid	Sigma-Aldrich	T6399
Thioglycolic acid	Sigma-Aldrich	T3758
HCl	Sigma-Aldrich	H1758
Sodium acetate	Sigma-Aldrich	S2889
$FeSO_4 \cdot 7H_2O$	Sigma-Aldrich	215422
Oxalic acid	Sigma-Aldrich	194131
Hemin	Sigma-Aldrich	H9039
Experimental models: Organisms/strains		
Mouse (Mus musculus, C57BL/6, female, 4 and 22 months of age)	Jackson Laboratories	NA
Mouse (Mus musculus, C57BL/6, female, 4 and 22 months of age)	Dr. Miller lab	NA
Software and algorithms		
GraphPad Prism	GraphPad	Version 9
Other		
Blade	NA	NA
Curved tip forceps	NA	NA
Ice bucket	NA	NA
1.5 mL microcentrifuge tubes	Fisherbrand	05-408-129
2.0 mL microcentrifuge tubes (flat bottom)	Axygen	14-222-180
Dry ice	NA	NA
Liquid nitrogen	NA	NA
70% ethanol	NA	NA
Polytron® Tissue Grinder	Polytron	PT-2100
PBS	NA	NA
Triton-X	Sigma-Aldrich	9036-19-5
Heating block	NA	NA
Falcon™ 15 mL Conical Centrifuge Tube	Falcon	14-959-49D
CAP LOCKS	Fisher Scientific	NC0341075
ddH_2O	NA	NA
DMSO	Sigma-Aldrich	D8418
Plate reader	Molecular Devices	SpectraMax M Series
96-well clear, flat bottom assay plate (black wall)	Thermo Scientific	165305
96-well clear, flat bottom assay plate (clear)	Thermo Scientific	456537

MATERIALS AND EQUIPMENT

Protein precipitation solution (PPS)		
Reagent	Final concentration	Amount
HCl	0.5 N	5 mL
Trichloroacetic acid	5%	5 mL
PPS can be stored at 4°C for a week.		

Chromogen solution (CS)		
Reagent	Final concentration	Amount
Ferrozine	0.5 mM	2.5 mg
Sodium acetate	1.5 M	1.2 g
Thioglycolic acid	0.1% (v/v)	10 μL
ddH_2O	N/A	10 mL
CS should be made fresh.		

Oxalic acid solution (OS)		
Reagent	Final concentration	Amount
Oxalic acid	2.0 M	1.8 g
ddH_2O	N/A	10 mL
OS can be stored at 4°C for a month.		

Note: Oxalic acid can precipitate at room temperature. If crystals appear, heat in the microwave for 5 s to dissolve the crystals before use.

STEP-BY-STEP METHOD DETAILS

Isolation of cytosolic and mitochondrial fraction from tissue

Timing: 3–6 h depending on the number of the samples

This step describes the fractionation of cytosol and mitochondria from murine cerebral cortex tissue using a mitochondrial isolation kit. A schematic flowchart for the fractionation method is shown in Figure 1.

1. Weigh frozen whole cerebral cortex tissues and cut into ~50 mg sections using a blade on a pre-chilled platform with dry ice.
   a. Place the cortex sections into pre-chilled 2.0 mL tubes (tubes A).
   b. Keep tubes A in liquid nitrogen or on dry ice until all samples to be analyzed have been processed.

⚠ CRITICAL: Do not thaw samples at this step.

2. Prepare Reagent A and Reagent C solutions in the mitochondria isolation kit and add 1/100 volume of protease inhibitors to each solution. Keep them on ice.

Note: After this step, a maximum of 6 samples may be processed simultaneously.

Note: Once the protease inhibitors have been added, reagents A and C cannot be stored and must be used on the day of the experiment.

3. Add ice cold 500 μL Reagent A solution into tubes A.
4. Perform homogenization using a Polytron Tissue Grinder (shaft size: 5.5 mm, speed: high) for 30 s while on ice.
   a. To minimize sample-to-sample contamination in the homogenization, clean the tip of the homogenizer with 70% EtOH once and ddH_2O twice between samples.

⚠ CRITICAL: Make sure the samples are completely homogenized by visually checking to see if there are any tissue fragments left or not. If tissues are not sufficiently homogenized, extend the homogenization time by 30 s and reassess homogenization.

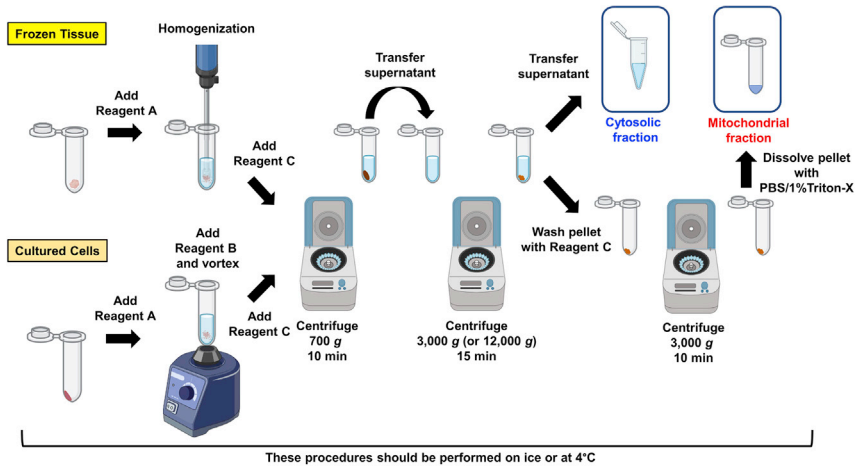

Figure 1. A schematic flowchart for the fractionation of cytosol and mitochondria from frozen tissue or cultured cells using a mitochondrial isolation kit

Note: Since this protocol is for measuring iron content rather than functional analysis, the addition of bovine serum albumin (BSA) is not required. Addition of BSA may complicate the process of measuring protein concentration in cytosolic and mitochondrial fractions. Alternatively, tissues may be homogenized using a Dounce homogenizer after mincing into small pieces.

5. After homogenizing all samples, add 500 μL of Reagent C into tubes A.
   a. Proceed to step 13 in the below section of "Isolation of cytoplasmic and mitochondrial fractions from cells".

Note: From this step, all samples may be processed simultaneously.

Isolation of cytosolic and mitochondrial fraction from cells

Timing: 3 h

6. Harvest cultured cells into a cell suspension using trypsin or a different cellular dissociation method.
   a. The number of cells required depends on the cell type; for example, the number of approximately 20 × 10^6 cells are needed for cultured H9c2 rat cardiomyoblasts.
   b. Collect cells in 15 mL conical tubes.
7. Gently wash the cell suspension in ice cold PBS twice, and transfer the suspension to a 1.5 mL microcentrifuge tube.
   a. Centrifuge at 1,000 *g* to pallet the cells (Tube A).
   b. Discard the supernatant.
8. Prepare Reagent A and Reagent C solutions in the mitochondria isolation kit and add 1/100 volume of protease inhibitors to each solution. Keep them on ice.

Note: Mitochondrial isolation kits are available for tissue and cultured cells. In our experience, it is possible to substitute tissue kits for cellular mitochondrial isolation.

9. Add ice cold 500 μL Reagent A solution into tubes A.
10. Add 6.25 μL Reagent B solution into tubes A.
11. Vortex vigorously for 5 s every minute for 5 min.

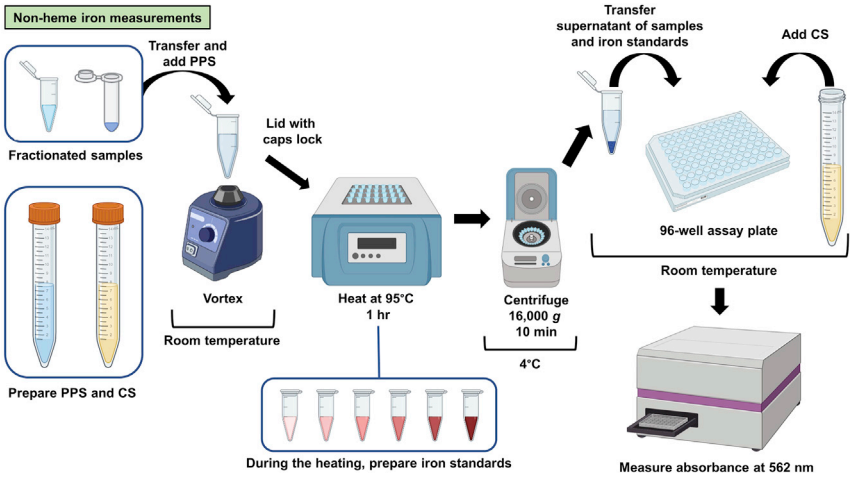

Figure 2. A schematic flowchart for non-heme iron measurement

12. Add 500 μL of Reagent C into tubes A and mix.
13. Centrifuge tubes A at 700 *g* for 10 min at 4°C.
14. Discard the pellet and transfer the supernatant to a new 2.0 mL tube (tubes B).
15. Centrifuge tubes B at 3,000 *g* for 15 min at 4°C.

Note: To collect additional mitochondria, centrifuge at 12,000 g instead 3,000 g; however, contamination of lysosomes and peroxisomes in the mitochondrial fraction will be greater.

16. Transfer the supernatant (cytosolic fraction) into new 1.5 mL tubes (tubes C).
17. Wash the pellet with 250 μL of Reagent C and centrifuge tubes at 3,000 *g* for 10 min at 4°C.
18. Add 200 μL of PBS containing 1% Triton X-100 and 1× Protease Arrest to the pellet of tubes B. This lysate contains the mitochondrial fraction.
19. Take 10 μL of each fraction for BCA protein assay to determine protein concentrations.
    a. Freeze lysates in −20°C for a month or use for subsequent measurements directly.

Measurement of non-heme iron

Timing: 3 h

This step describes the measurement of non-heme iron content in cytosolic and mitochondrial fractions. A schematic flowchart for the non-heme iron measurement is shown in Figure 2.

20. Mix 5 mL of 1 N HCl and 5 mL of 10% Trichloroacetic acid in a Falcon™ 15 mL conical centrifuge tube to prepare the protein precipitation solution (PPS). PPS can be stored at 4°C for a week.
21. Dissolve 2.5 mg of Ferrozine (final concentration: 0.5 mM) and 1.2 g of sodium acetate (final concentration: 1.5 M) in 10 mL ddH_2O and add 10 μL of thioglycolic acid (final concentration: 0.1% (v/v)) in another 15 mL conical centrifuge tube to prepare the chromogen solution (CS). CS should be made fresh for every assay.
22. Mix 80 μL of either the cytosolic or mitochondrial lysate fraction with 80 μL of PPS in 1.5 mL tubes (tubes D) and vortex well at room temperature.
23. Heat tubes in a heating block at 95°C for 1 h.
    a. Use cap locks to prevent the tubes from opening and solution from evaporating during heating.
24. During the heating step, prepare standards by diluting 1 mM $FeSO_4$ solution in PPS in 1.5 mL tubes (0 μM, 0.25 μM, 0.5 μM, 1.0 μM 2.5 μM, 5 μM, 10 μM, 25 μM).

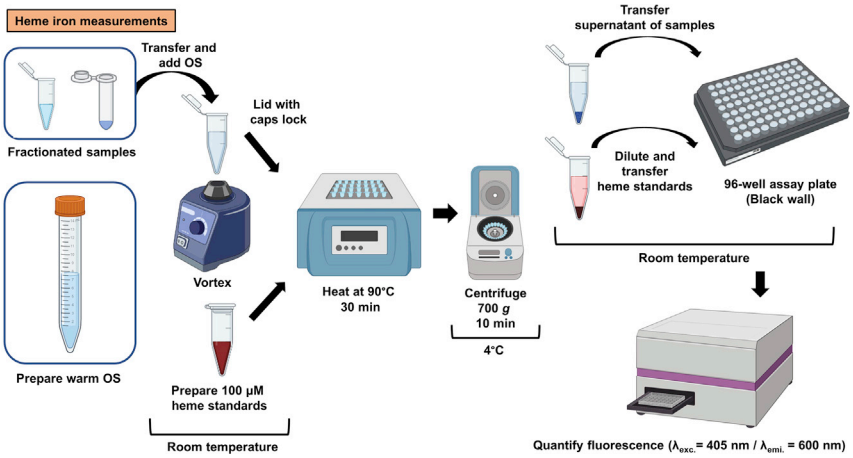

Figure 3. A schematic flowchart for heme iron measurement

25. Place standards on ice for 2 min.
26. Centrifuge standards and tubes D at 16,000 *g* at 4°C for 10 min.
27. After 1 h of heating from step 23, take 100 μL of standards or supernatant from tubes D and place into a single well of clear 96-well assay plates.

⚠ CRITICAL: Avoid contamination with precipitation. Contamination of precipitants into the samples may affect measurement results.

28. Add 100 μL of CS into the lysate per well of a 96-well assay plate.
29. Incubate at room temperature for 10 min.
30. Measure absorbance at 562 nm using a plate reader.
    a. Normalize values by dividing the amount of iron in 1 μL of sample by the amount of protein in 1 μL assessed by BCA method in section 19.

Measurement of heme iron

Timing: 3 h

This step describes the measurement of heme iron content in cytosolic and mitochondrial fraction. A schematic flowchart for the heme iron measurement is shown in Figure 3.

31. Dissolve 1.8 g of oxalic acid in 10 mL ddH_2O in a Falcon™ 15 mL conical centrifuge tube to prepare oxalic acid solution (OS, final concentration: 2.0 M). OS can be stored at 4°C.

Note: Oxalic acid can precipitate at room temperature. If crystals precipitate, heat in the microwave for 5 s to dissolve the crystal before use.

32. Mix 100 μL of lysates from the cytosolic or mitochondrial fraction with 100 μL of warm OS in 1.5 mL tubes (tubes E) and vigorously vortex at room temperature.
33. Prepare heme standards. Dissolve 6.5 mg of hemin in 1 mL of DMSO (10 mM hemin), and dilute 10 μL of 10 mM hemin in 990 μL warm OS in a 1.5 mL tube (100 μM hemin standard).
34. Heat heme-iron samples and heme standards in a heating block at 90°C for 30 min.
35. Centrifuge samples and standards at 700 *g* for 10 min at 4°C.
36. Dilute 100 μM heme standards in 1:1 mixture of OS and PBS with 1% Triton to desired concentration, and serve as measurement standards (for example, 0 μM, 0.25 μM, 0.5 μM, 1.0 μM 2.5 μM, 5 μM, 10 μM, 25 μM).

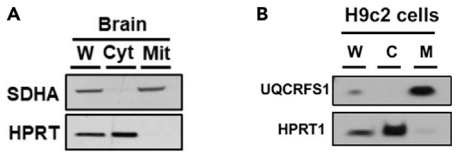

Figure 4. Representative images of immunoblots assessing the quality of mitochondrial isolation in tissue or cultured cells

(A) Cerebral cortex of the brain, reproduced from Figure 1G in Sato et al. eLife 2022.[1]

(B) H9c2 cells, reproduced from Figure S3D in Sato et al. PNAS 2018.[5] W = whole cell lysate, C or Cyt = cytosolic fraction, M or Mit = mitochondrial fraction, SDHA = Succinate Dehydrogenase Complex Flavoprotein Subunit A, HPRT = hypoxanthine phosphoribosyltransferase, UQCRFS1 = Ubiquinol-Cytochrome C Reductase, Rieske Iron-Sulfur Polypeptide 1.

37. Transfer 150 μL of supernatant or heme standards into a black-walled clear bottom 96-well plate and quantify fluorescence at an excitation wavelength of 405 nm and an emission wavelength of 600 nm.
    a. Normalize values by dividing the amount of iron in 1 μL of sample by the amount of protein in 1 μL assessed by BCA method in section 19.

⚠ CRITICAL: Black wall assay plates were used to minimize well-to-well interference.

EXPECTED OUTCOMES

In the case of successful mitochondrial and cytosolic fraction isolation, immunoblot results show that the mitochondrial fraction contains no detectable (or a negligibly small amount) cytosolic protein and the cytosolic fraction contains no detectable (or a negligibly small amount) mitochondrial protein. In our previous publications,[1,5] we presented representative immunoblots for the quality of mitochondrial isolation in tissue or cultured cells as shown in Figure 4. By measuring non-heme and heme iron in the lysates, the intracellular iron content can be quantified (e.g., mitochondrial iron deficiency or excess).

LIMITATIONS

While this mitochondrial iron isolation method allows us to separately quantify the heme- and non-heme iron contents of crude mitochondria, it does not allow for specific sub-mitochondrial iron quantification. Further isolation of crude mitochondria to pure mitochondria by Percoll gradient centrifugation[8] may allow for more detailed assessment of iron localization within mitochondria.

TROUBLESHOOTING

Problem 1

Contamination with red blood cells (at the time of organ harvesting).

Potential solution

Perfuse the tissue with PBS or saline to wash out blood before tissue removal from the animal, or wash with PBS or saline after cutting the tissue into small pieces. Note that red blood cell contamination can dramatically affect the results of heme iron measurements.

Problem 2

Contamination of the cytoplasmic and mitochondrial fractions (at the time of isolation of cytosolic and mitochondrial fraction).

Potential solution

Wash mitochondrial pellets thoroughly when isolating mitochondria (step 17). Since components of the cytoplasmic fraction adhere to the walls of tubes during the mitochondrial isolation, transferring the mitochondrial pellet to fresh new tubes minimizes further contamination of the cytosolic fraction.

Optimal conditions for mitochondrial isolation vary by the types of tissues and cells, so the quality of fractionation should be confirmed by immunoblotting (Figure 4).

Problem 3

Insufficient yield of mitochondrial fractions (at the time of isolation of cytosolic and mitochondrial fraction).

Potential solution

Mitochondrial content depends on the types of tissues and cells (steps 1 and 6). If the yield of the mitochondrial fraction is low, the tissue volume or the number of cells should be increased.

Problem 4

Contamination of iron metals from outside the sample of interest (throughout the protocol).

Potential solution

Since iron molecules, especially non-heme iron, can be present in any environment, instruments used in experiments should be sterilized. Avoid the use of rusty laboratory equipment, which may potentially affect the results of iron measurements.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Hossein Ardehali (h-ardehali@northwestern.edu).

Materials availability

Reagents are available from the lead contact upon request.

Data and code availability

This study did not generate or analyze any new datasets.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Experimental design and conceptualization, T.S., C.H.C., H.A.; Methodology, T.S., C.H.C., K.T.S., H.A.; Resources, H.A.; Writing-original draft, T.S., H.A.; Writing-review and editing, T.S., C.H.C., K.T.S., H.A.; Supervision, H.A.; Funding acquisition, H.A.

DECLARATION OF INTERESTS

H.A. has served as an expert witness.

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