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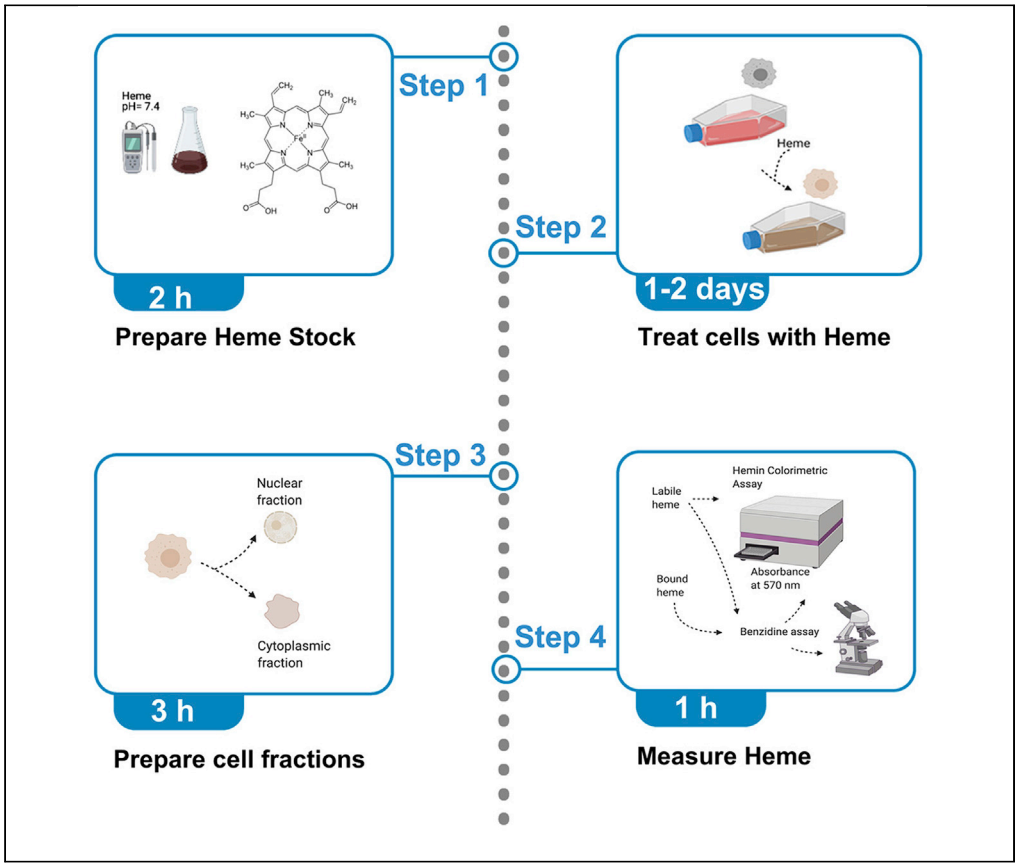

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Highlights

Preparation of heme and treatment of cells with heme

Detection of labile and total heme in prostate cancer cells

Measurement of heme in subcellular compartments in prostate cancer cells

Detection of heme in fixed cells

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Measurement of labile and protein-bound heme in fixed prostate cancer cells and in cellular fractions

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SUMMARY

Labile heme is present in the cells at very low concentrations, either unbound or loosely bound to molecules, and accessible for signaling as alarmin. Our recent work suggests that extracellular heme can be taken up and detected in the nuclei of cancer cells. Here, we describe the detailed protocol for detection of labile and total heme in prostate cancer cells and its measurement in subcellular compartments *in vitro*. The protocol can be adapted to be used for other cell types. For complete details on the use and execution of this protocol, please refer to Canesin et al. (2020).

BEFORE YOU BEGIN

Prepare heme stock solution

Timing: 2 h

Note: The final concentration of the heme stock can be determined by the user. We recommend a stock concentration of 1–10 mM and a final volume of 50 mL. The aliquots can be stored in the −80°C freezer until use. All tubes with heme should be kept in the dark.

⚠ CRITICAL: Heme is light-sensitive and therefore all work with heme should be performed in the dark.

1. Weigh 0.326 g Fe (III) heme (hemin chloride) in an Eppendorf tube and transfer to a 50 mL Falcon tube
2. Dissolve the powder in 0.1 M NaOH (freshly made) (20 mL).
   a. Assure that all the powder is well dissolved and no crystals are formed by mixing on the Vortexer.
3. Titrate the solution with 0.1 M HCl to the biological pH at 7.4.
4. Adjust the final volume (50 mL) with saline to obtain 10 mM stock.
5. Store the stock solution in 0.1–1 mL aliquots at −80°C in the foil until use. The tubes should not be repeatedly frozen and thawed. Preparing the aliquots in smaller volume may be beneficial.

Note: We recommend thawing each aliquot only once immediately before use and discard after using. If possible, preparing stock solution fresh is the best solution, however, we did

not see a major difference in the stocks that were frozen at −80°C for not longer than 6 months and thawed once just prior experimentation. We recommend a 4°C fridge for short term storage.

Higher concentrations of hemin in solution might aggregrate and form oligerimeric species in solution. If any precipitation is observed, we recommend preparing less concentrated stock (1–3 mM).

Prepare benzidine stock solution

Timing: 15 min

⚠ CRITICAL: O-dianisidine is light-sensitive and therefore all procedures using O-dianisidine should be performed in the dark or using an aluminum foil-wrapped tubes ensuring protection from light.

6. Weigh 60 mg O-dianisidine in Eppendorf tube and transfer to a 50 mL Falcon tube.
7. Add 0.2 mL glacial acetic acid to dissolve the powder completely.
8. Add distilled water to obtain a final volume of 30 mL.
9. Keep Benzidine Staining Stock Solution at 4°C until use or use immediately.

Note: Benzidine Staining Stock Solution is stable at 4°C for up to a week. After that, we recommend preparing a new stock solution.

Cell culture

Timing: 1–2 days

10. Plate 2 × 10^5 cells /well in a 6-well plate or on the cover slip in 2 mL of complete cell culture medium (for PC3 cells: RPMI medium supplemented with 10% FBS and 1% antibiotics).
11. Let the cells grow until 70% confluency is reached.
12. Proceed to "Heme treatment".

Note: Doubling time varies from cell line to cell line and optimal conditions must be optimized by the user.

Note: In order to perform staining on fixed cells, cells must be grown on glass coverslip following the same procedure.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Hemin chloride (heme)	Sigma-Aldrich	Cat# 51280
o-Dianisidine dihydrochloride	Sigma-Aldrich	Cat# D3252
RPMI medium	Wisent Inc	Cat # 350-000-CL
FBS	Gibco, Life Technologies	Cat # 10437-028
Antibiotics	Wisent Inc	Cat # 450-115-EL
Trypsin EDTA	Gibco, Life Technologies	Cat # 25200-072
Glacial acetic acid	Fisher Scientific	Cat # A38-212
Critical commercial assays		
Nuclear/cytoplasmic fractionation kit	BioVision	Cat# K270-50

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Hemin Colorimetric Assay Kit	BioVision	Cat# K672
BCA Protein Kit	Pierce	Cat# 23227
Experimental models: cell lines		
Prostate cancer PC3 cells	Dr. Steven Balk (BIDMC, Boston)	N/A
Other		
Spectrophotometer- plate reader, SpectraMax	Molecular Devices	MV05601

STEP-BY-STEP METHOD DETAILS

Labile heme is defined as heme loosely bound to acceptor molecules (i.e., proteins, DNA) and therefore present in metabolically active pool and accessible for signaling as alarmin in the various compartments of the cells (Gouveia et al., 2017). Treatment with exogenous heme contributes this additional pool of heme in the cell and it is measured here by means of benzidine staining (total heme: bound and labile heme) or labile heme detection kit (Biovision). Both methods provide ability to measure labile heme, with exception that benzidine staining may detect also heme bound to hemoproteins.

Heme treatment

Timing: 30 min to 24 h

The goal of this step is to treat cells with exogenous heme and to study heme retention in different cellular compartments. We recommend treating cell lines with heme at concentrations ranging from 1 to 50 μM (optimal concentration may be determined dependently on the cell type). We recommend treating cell lines for short time points starting at 30 min and up to 24 h. The recommended treatment time with heme depends on the specific cell line. In prostate cancer cells the induction of HO-1 upon treatment with 50 μM heme is rapid (6–8 h) and heme levels intracellularly will decline thereafter, due to HO-1-mediated heme degradation (Canesin et al., 2020).

⚠ CRITICAL: Heme is light-sensitive and therefore this procedure should be performed in the dark with limited exposure of cells to the light.

1. Thaw an aliquot of heme (10 mM) and keep it in the dark (wrapping the tube in aluminum foil helps ensuring protection from light).
2. Prepare complete medium and add heme at the desired concentration. Note that the medium turns darker upon addition of heme (Figure 2A).
3. Take out the cell plate from the incubator, aspirate the cell culture medium and add fresh complete medium with heme (1–50 μM).
4. Immediately place the cell plate back into the humidified incubator at 37°C and incubate for appropriate time.

Note: We recommend including control group of cells receiving no heme treatment, in order to determine baseline heme levels in specific cellular compartments.

Prepare cell fractionation

Timing: 3 h

This step allows extraction of subcellular protein fractions (nuclear and cytoplasmic). The fractionation is performed using the FractionPREP Cell Fractionation kit (Biovision). Each buffer has

appropriate addition of the Protease inhibitor cocktail as suggested in the manufacturer's protocol. The following steps by the manufacturer's protocol are performed (https://www.biovision.com/fractionpreptm-cell-fractionation-kit.html). All aliquots of the cell fractionation kit need to be thawed on ice and therefore should be prepared 1–2 h prior start of the cell harvest.

⚠ CRITICAL: It is very important to remove all supernatant (Cytosolic Fraction) from the pellet (Nuclear Fraction) to avoid cross-contamination of the fractions.

5. Collect and wash cells:
   a. Take out the cell plate from the incubator and aspirate cell culture medium.
   b. Wash cells with 1× PBS.
   c. Add 2–3 mL of 0.05% Trypsin containing EDTA to detach cells and incubate at 37°C for 5 min. Collect each sample in a 15 mL conical tube upon blocking the trypsin with 8 mL of complete cell culture medium containing 10% FBS.
   d. Centrifuge at 700 × *g* for 5 min and discard the supernatant.
   e. Add 5 mL of ice-cold PBS, pipette several times to mix well. An aliquot of PBS should be placed on ice prior to cell harvesting.
   f. Centrifuge at 700 × *g* for 5 min and discard the supernatant.
   g. Resuspend the cell pellet in 1 mL of ice-cold PBS and transfer each sample to a clean microcentrifuge tube.
   h. Spin at 700 × *g* for 5 min and discard the supernatant.
6. Isolate Cytosolic Fraction:
   a. Resuspend the cell pellet in 400 μL of Cytosol Extraction Buffer, pipette several times to mix well on ice.
   b. Incubate samples on ice for 20 min and vortex every 5 min.
   c. Centrifuge the samples at 700 × *g* for 10 min.
   d. Collect the supernatant as the Cytosolic Fraction: place in a new Eppendorf tube and keep on ice.
7. Isolate Nuclear Fraction:
   a. Precool the centrifuge if necessary and place Membrane Extraction Buffer A and Eppendorf tubes on ice.
   b. Resuspend the pellet in 400 μL of ice-cold Membrane Extraction Buffer A, pipette up and down several times to mix well.
   c. Vortex for 15 sec.
   d. Add 22 μL of Membrane Extraction Buffer B, vortex for 5 sec and incubate on ice for 1 min.
   e. Vortex for 5 sec and centrifuge at 1,000 × *g* for 5 min at 4°C.
   f. The supernatant contains the Membrane Protein fraction: this fraction is NOT used for heme measurements, so it can be discarded. If needed for other purposes, transfer the supernatant to a clean pre-chilled Eppendorf tube and keep on ice.
   g. Resuspend the pellet in 200 μL of ice-cold Nuclear Extraction Buffer A and vortex for 15 sec to mix well.
   h. Incubate samples on ice for 40 min, vortexing for 15 sec every 10 min.
   i. Sonicate samples.
   j. Centrifuge samples at maximum speed for 10 min at 4°C.
   k. Collect the supernatant as the Nuclear Fraction: place in a new Eppendorf tube and keep on ice.
8. Determine protein concentration in Cytosolic and Nuclear fractions samples by using the standard BCA Protein Assay kit (Pierce, Cat# 23227).

Note: Cytosolic and nuclear Fraction samples can be immediately used for heme measurements or stored at −80°C until used.

Measurement of labile heme using a commercial kit

Timing: 1 h

This step allows measurements of labile heme in cells and/or cellular fractions by colorimetric assay. The measurement is performed using the Hemin Colorimetric Assay Kit (Biovision, Catalog #K672). The Hemin Colorimetric Assay kit measures the labile heme as per manufacturer's protocol (https://www.biovision.com/hemin-colorimetric-assay-kit.html): "The hemin assay kit detects free heme. Hemoglobin has heme in coordinated form with protein chains of alpha and beta globin. Heme bound in this form will not produce color in this assay." The reaction is based on the conversion of a colorless probe to a strongly colored ($\lambda = 570$) compound in the presence of hemin. Follow kit instructions for reagents preparation prior to heme measurement.

9. Prepare the Hemin Standard Curve:
   a. Dilute the 10 μM Hemin Standard (included in the kit) to 100 nM by adding 10 μl of the Heme Standard to 990 μl of Hemin Assay Buffer.
   b. Pipette up and down several times to mix well.
   c. Dilute Heme Standard further to 10 nM (= 10 fmol/μl) by adding 100 μl to 900 μl Hemin Assay buffer.
   d. In a 96 well plate, add 0, 4, 8, 12, 16, 20 μl of Hemin Standard (included in the kit) into a series of wells and adjust the volume of each well to 50 μl by adding Hemin Assay Buffer. The final concentration of Hemin Standard in each well will be 0, 40, 80, 120, 160, 200 fmol/well, respectively (Figures 1 and 3).
10. Prepare Samples:
   a. We suggest diluting samples 100-fold for prostate cancer cell lines but this will depend on the cell type that this protocol is applied for.
   b. Add 10 μl of diluted sample per well in duplicates in the 96 well plate.
   c. Adjust the volume of each well to 50 μl by adding Hemin Assay Buffer.

Note: Sample dilution depends on the heme content in the fractions of cell type. We recommend a dilution of at least 100-fold for prostate cancer cell lines, but some samples may require further dilution (up to 10,000-fold) dependently on what heme concentration was used for treatment. It is beneficial to determine the dilution factor before the experiment. Using several doses of sample can help to ensure that the readings are within the standard curve range. The dilution might be adjusted based on protein levels. In the case of cell treatment with heme, it is suggested to test the dilution before measurement of a larger sample set.

11. Add Reaction Mix:
   a. Following the kit instruction, prepare the Reaction Mix.
   b. Add 50 μl of the Reaction Mix to each well containing the Hemin Standard or the samples.
   c. Incubate for 10–30 min at RT (15°C–25°C), while protecting from light (cover the plate with aluminum foil).

Note: The color of the wells will gradually turn pink (Figure 1). While the 30 min incubation time gives the best results in terms of linearity and sensitivity, we recommend monitoring the color development or reading the assay in kinetic mode.

12. Read absorbance of the plate at 570 nm at spectrophotometer.

Measurement of total heme (labile and bound) by benzidine staining

Timing: 10 min

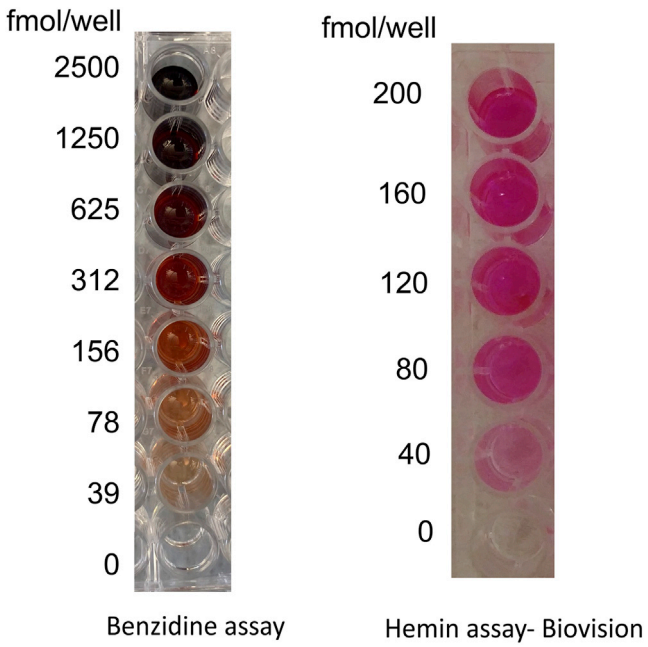

Figure 1. Illustration of 96-well microplates with hemin standards prepared using hemin chloride dilution in Benzidine assay (left panel) or according to the Hemin Colorimetric Assay kit (right panel).

In this step, protein lysates are incubated with benzidine and total heme concentration is then determined by reading the absorbance using a spectrophotometer. Benzidine staining has been previously optimized for MEL cells (Chen et al., 2013; Chung et al., 2015; Chung et al., 2017), however, has not been used for adherent cancer cells. This staining works for the labile heme as well as the protein-bound heme.

Note: If you run the Benzidine Staining in parallel with the Heme Commercial Kit measurement, you can use the same Standard Curve (see step 9) or prepare the standards from hemin chloride prepared as in step 1 (Figure 1).

13. Load each well of a 96 well plate with 10 µL of protein lysate at a concentration of 5 µg/µL
    a. Keep the plate on ice until you add the Benzidine Staining Solution 14. Prepare Benzidine Staining Working Solution:
        i. In a new Eppendorf tube, add 500 µL of Benzidine Stock Solution to 50 µL of 30% H_2O_2 Solution
        ii. In a new Eppendorf tube, dilute this solution 1:50 in PBS, to obtain the final Benzidine Staining Working Solution
14. Add 100 µL of Benzidine Staining Working Solution to each well containing the protein lysate
15. Incubate the plate at RT (15°C–25°C or 59°F–77°F) for 5 min, protect from light (cover the plate with aluminum foil).

Note: The wells will first turn green, then gradually change to a darker brown color

16. Read absorbance of the plate at 570 nm at a spectrophotometer for both protocols.

Measurement of total heme by benzidine staining on fixed cells

Timing: 1 h

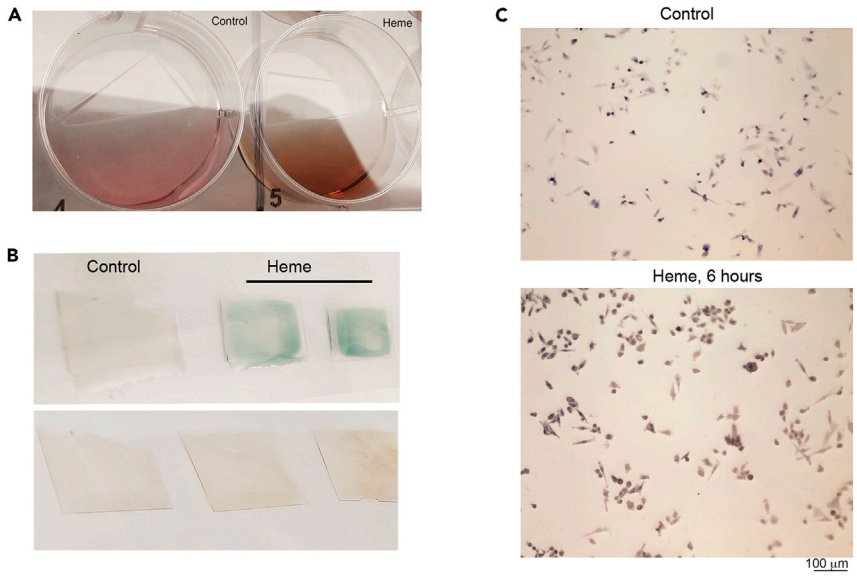

Figure 2. Measurement of heme in prostate cancer cell line

(A) PC3 cells were grown on the cover slips for 14–16 h and heme (50 μM) was added to the cultures for additional 6 h.

(B) Benzidine staining as in "Measurement of labile heme by benzidine staining on fixed cells". Two replicates of heme-treated PC3 cells on cover slips are shown. Progression of reaction from green (upper panel) to brown staining (lower panel) is shown.

(C) Representative pictures of PC3 cells treated with heme for 6 h and stained with benzidine solution for detection of labile heme. 100× magnification. Scale bar: 100 μm.

In this step, Benzidine solution is used to stain cells that are grown on a glass coverslip and treated with heme (Figure 2A). Benzidine solution stains labile and protein-bound heme in cells attached to cover slips.

17. Prepare a humidified chamber to perform the incubation.
18. Apply 100 μL of Benzidine Staining Working Solution to each glass coverslip (Figure 2B)
19. Incubate for 20 min at RT (15°C–25°C), while protecting from light (cover with aluminum foil).
20. Wash each glass coverslip at least 3 times with 1× PBS.
21. Proceed to fixation of the cells by adding 2% PFA to each glass coverslip.
21. Incubate for 20 min at RT (15°C–25°C).
23. Wash each glass coverslip at least 3 times with 1× PBS.
24. Wash each glass coverslip once in distilled water.
25. Proceed to hematoxylin staining for 15–30 seconds.
26. Proceed to de-hydration of the samples by incubating in ethanol 50% (3 min), ethanol 70% (3 min), ethanol 80% (3 min), ethanol 95% (3 min), ethanol 100% (5 min), xylol (5 min).
27. Mount each coverslip on a glass slide and let dry while protecting from light.
28. Observe the staining under the microscope (Figure 2C).

EXPECTED OUTCOMES

By applying Hemin Colorimetric Assay Kit or Benzidine staining we evaluated heme levels quantitatively by measuring the absorbances by spectrophotometer (Figure 1). In prostate cancer cells, the levels of nuclear heme are higher in the nucleus than in cytoplasm and highly elevated upon addition of exogenous heme to the cell cultures. The heme levels in response to heme treatment reach plateau after 6–10 h and decrease at later time point (24 h) due to activity of HO-1. For complete details and examples of analyses please refer to Canesin et al, 2020.

EXAMPLE CALCULATION

STANDARDS [fmol/well]	Absorbance	Heme [fmol/well]
200 fmol	2.03	200
160 fmol	1.71	160
120 fmol	1.18	120
80 fmol	0.78	80
40 fmol	0.23	40
0 fmol	0.08	0
SAMPLE 1	0.11	10.89108911
SAMPLE 2	2.02	200

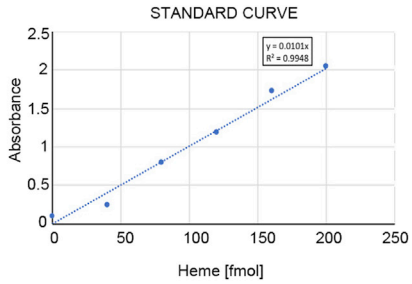

Figure 3. The example dataset with standard curve and calculation of heme in two samples using Hemin Colorimetric Assay Kit.

Detection of heme in the fixed cells allows for evaluation of heme in various cellular compartments, but only on the qualitative level. This method is also less sensitive than the above quantitative methods and allows for assessment of heme in the light microscope but only upon addition of extracellular heme (Figure 2C).

QUANTIFICATION AND STATISTICAL ANALYSIS

Due to intrinsic variability of the assays, we recommend using 3-4 replicates repeated in 3-4 independent experiments for both Heme measurement Kit and Benzidine staining. The example dataset with standard curve and calculation of heme in two samples using Hemin Colorimetric Assay Kit is presented in Figure 3.

LIMITATIONS

Each of the protocols for heme measurement has limitations. Purity of the fractionation should be always confirmed by Western blotting using the following antibodies: GAPDH for cytoplasmic fraction and lamin A/C for nuclear fraction. The cross-contamination of nuclear fraction with a cytoplasmic fraction is common. A careful removal of the supernatant from the pellet in steps 4–5 is critical for high purity of nuclear fraction.

Benzidine staining does not distinguish between free heme and heme bound in hemoproteins. Therefore, we suggest supplementing benzidine staining with heme measurement by the Hemin Colorimetric Assay Kit. This commercial kit measures only labile heme.

These methods do not discriminate between endogenously synthesized heme and exogenously added heme. Optimizing and using a system where endogenous heme synthesis is blocked may help. The validity of the results depends on careful preparation of heme stock including prevention of crystal formation as well as exposure to light.

Sensitivity of colorimetric reaction is of concern and therefore other methods including heme sensors and fluorescence probes are good alternatives (Hanna et al., 2016; Yuan et al., 2016).

TROUBLESHOOTING

No major problems were faced by the lab personnel while running those protocols. We list some minor issues that users may encounter when applying these protocols.

Problem 1

Higher concentrations of hemin in solution might aggregate and form oligerimeric species in a solution (step 1).

Potential solution

If any precipitation is observed, we recommend preparing less concentrated stock (1–3 mM). See "Preparation of heme stock".

Problem 2

The levels of labile heme detected are higher than expected (step 3).

Potential solution

Cells produce and accumulate heme even in the absence of exogenous heme addition either due to low baseline level of HO and/or high ALAS. This is particularly true for cancer cell lines. Cancer cells have higher baseline level of detectable heme compared to non-cancerous cells. We recommend doing a thorough literature search to include a cell line without detectable heme levels and use these cells as a negative control. Further, blocking ALAS synthesis with selective inhibitors is an option. See "Heme treatment".

Problem 3

The levels of labile heme detected in non-cancerous cells are very low (step 3).

Potential solution

Dose-dependent treatment with exogenous heme is recommended to validate the presence of labile heme in specific cell type. See "Heme treatment".

Problem 4

Different cell lines treated with heme give different results (steps 3 and 4).

Potential solution

The recommended treatment time with heme depends on the specific cell line. In prostate cancer cells the induction of HO-1 upon treatment with 50 μM heme is rapid (6–8 h) and heme levels intracellularly will decline thereafter, due to HO-1-mediated heme degradation. See "Heme treatment".

Problem 5

The level of heme detected in samples in higher than the levels of standards provided as a Hemin Standard Curve (step 3).

Potential Solution

Sample dilution for measurement of labile heme depends on the heme content in the fractions of specific cell type. We recommend a dilution of at least 100-fold for prostate cancer cell lines, but some samples may require further dilution dependently on what heme concentration was used for treatment. It is beneficial to determine the dilution factor before the experiment. Using several doses of heme and sample dilutions can help to ensure that the readings are within the standard curve range. The dilution might be adjusted based on protein levels. In cells treated with heme, it is suggested to test the dilution before measurement of a larger sample set. See "Measurement of labile heme using commercial kit".

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. Barbara Wegiel (Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, bwegiel@bidmc.harvard.edu).

Materials availability

This study did not generate any unique reagents.

Data and code availability

The protocol includes all datasets generated or analyzed during this study that have not been reported elsewhere.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

G.C. and B.W. designed the study. L.J. performed experiments. G.C. and B.W wrote the paper with input from L.J. B.W. supervised the work.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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