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Fluorescence-based Heme Quantitation in *Toxoplasma Gondii*

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[Abstract] *Toxoplasma gondii* is a highly prevalent protozoan pathogen throughout the world. As a eukaryotic intracellular pathogen, *Toxoplasma* ingests nutrients from host cells to support its intracellular growth. The parasites also encode full or partial metabolic pathways for the biosynthesis of certain nutrients, such as heme. Heme is an essential nutrient in virtually all living organisms, acting as a co-factor for mitochondrial respiration complexes. Free heme is toxic to cells; therefore, it gets conjugated to proteins or other metabolites to form a "labile heme pool," which is readily available for the biosynthesis of hemoproteins. Previous literature has shown that *Toxoplasma gondii* carries a fully functional *de novo* heme biosynthesis pathway and principally depends on this pathway for intracellular survival. Our recent findings also showed that the parasite's intracellular replication is proportional to the total abundance of heme within the cells. Moreover, heme abundance is linked to mitochondrial oxygen consumption for ATP production in these parasites; thus, they may need to regulate their cellular heme levels for optimal infection when present in different environments. Therefore, quantitative measurement of heme abundance within *Toxoplasma* will help us to understand the roles of heme in subcellular activities such as mitochondrial respiration and other events related to energy metabolism.

Keywords: *Toxoplasma gondii*, Protozoan, Heme, Fluorescence assay, Biochemical quantitation, Protoporphyrin IX, Fluorescence plate reader

[Background] Heme, an organic molecule, plays a vital role in virtually all living organisms. For example, heme binds to hemoglobin and myoglobin for oxygen transport and serves as a co-factor for several enzymes within the electron transport chain for mitochondrial respiration (Ponka, 1999). Previous literature has shown that *Toxoplasma* has a fully functional *de novo* heme biosynthesis pathway (Bergmann *et al.*, 2020). Genetic deletion of *Toxoplasma* heme biosynthetic enzymes results in decreased replication *in vitro* (Bergmann *et al.*, 2020; Krishnan *et al.*, 2020; Tjhin *et al.*, 2020) and the loss of acute virulence in a murine model (Bergmann *et al.*, 2020); therefore, inhibition of *de novo* heme production sheds light on the development of novel therapeutic strategies for managing *Toxoplasma* infections.

Generally, there are four methods for the measurement of heme abundance in cells: 1) Pyridine hemochrome-based heme quantitation (Sinclair *et al.*, 2001). This strategy replaces the nitrogen ligands of protein-bound heme with pyridine under alkaline conditions. The resulting hemochrome is further reduced and oxidized before spectrophotometric quantitation; 2) Reversed-phase HPLC-based

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quantitation of heme and its intermediates (Sinclair *et al.*, 2001). An acetone/HCl/water solution is used to extract heme and its biosynthetic intermediates from intact cells or cell homogenates, followed by separation on a C18 HPLC column. The standards of pure heme and its intermediates are run on the column before sample measurement to help recognize their peaks and quantitate their abundances; 3) Protoporphyrin IX (PPIX)-based fluorescence assay (Sinclair *et al.*, 2001). PPIX is produced by the penultimate enzyme within the classic *de novo* heme biosynthesis pathway (Phillips, 2019). PPIX is further conjugated with a ferrous iron group to form a functional heme molecule. The non-conjugated PPIX molecule displays strong fluorescence, whereas heme is non-fluorescent; therefore, stripping ferrous ion from heme generates fluorescent PPIX, which can be quantitated by a fluorometer and represents heme abundance; 4) Biosensor-based heme quantitation by live-cell imaging or flow cytometry (Song *et al.*, 2015; Hanna *et al.*, 2016; Yuan *et al.*, 2016). Genetically encoded hemoproteins, such as horseradish peroxidase (HRP)- or ascorbate peroxidase (APX)-based biosensors, can be expressed in different organelles to detect their labile heme content (Yuan *et al.*, 2016). Additionally, the heme-binding proteins are genetically fused to heme-sensitive fluorescent proteins to quantitate labile heme within live cells by ratiometric fluorescence quantitation or fluorescence resonance energy transfer (FRET) (Song *et al.*, 2015; Hanna *et al.*, 2016).

The pyridine hemochrome-based strategy is not very sensitive and requires large amounts of parasites as initial material. The HPLC-derived quantitation requires expensive equipment and columns. Additionally, it only can quantitate one sample per run. The biosensor-based method demands the implementation of biosensors in target organisms. Overexpression of biosensors may disturb labile heme pools in the cells and further impair their health status. The PPIX-based fluorescence heme quantitation is economical and sensitive. Moreover, it can be performed in 96-well plate format as a semi high-throughput strategy for multiple samples simultaneously.

In the procedure for the PPIX-based heme quantitation assay, the heme-bound ferrous ion can be chemically removed by oxalic acid via boiling before measurement. During the assay, non-boiled parallel samples must be included to detect background fluorescence signals within parasite strains, which will be subtracted from the corresponding boiled samples. Here, we modify the previously published PPIX-based fluorescence heme quantitation assay (Sinclair *et al.*, 2001). In this protocol, the parasites are mechanically liberated from host cells, filter-purified, and ruptured by sonication prior to heme quantitation. As an example, we measured and compared heme abundances in wildtype parasites and a heme-deficient transgenic *Toxoplasma* strain. This method employs a plate reader to quantitate heme in a 96-well plate format; it shows high sensitivity and only requires 5-10 × 10^7 *Toxoplasma* parasites for measurement, which can be harvested from 1-2 T25 flasks. This strategy can be modified for measuring heme abundances in other apicomplexan parasites.

Materials and Reagents

1. Nucleopore track-etched hydrophilic membrane filter, 3.0-μm pore size (VWR, catalog number:28158-624) with filter holder (Fisher Scientific, catalog number: SX0002500)

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2. 15 ml polystyrene conical tubes (VWR, catalog number: 10026-084)
3. 1.5 ml Eppendorf microcentrifuge tubes (VWR, catalog number: 89000-028)
4. Black 1.5 ml microcentrifuge tubes (VWR, catalog number: 47751-688)
5. 10 ml syringes (VWR, catalog number: BD309646)
6. Blunt-end needles (McMaster-Carr, catalog numbers: 75165A761 [20G]; 76165A759 [25G])
7. Cellstar® black 96-well plates without lids (VWR, catalog number: 82050-732)
8. 1,000 ml glass beaker (VWR, catalog number: 10754-960)
9. 5 ml and 10 ml serological pipettes (VWR, catalog numbers: 89130-896, 89130-898)
10. 20 µl, 200 µl, and 1,000 µl micropipette tips (VWR, catalog numbers: 76322-134, 76322-150, 76322-138)
11. *Toxoplasma gondii* RHΔ*ku80*Δ*hxg* strain lacking the *ku80* and hypoxanthine-xanthine-guanine phosphoribosyl transferase genes. This strain is provided by the Carruthers lab at the University of Michigan Medical School. Lack of the *ku80* gene boosts homology-dependent recombination in the parasites (Huynh and Carruthers, 2009). Loss of hypoxanthine-xanthine-guanine phosphoribosyl transferase (HXG) allows this strain to be genetically modified by exogenous DNA vectors carrying the *HXG* gene. This strain is widely used in the *Toxoplasma* research field as a wildtype parental strain.
12. RHΔ*ku80*Δ*hxg*Δ*cpox* strain (abbreviated to Δ*cpox*). A heme-deficient parasite strain was generated in a previous study (Bergmann *et al.*, 2020). This strain lacks coproporphyrinogen-III oxidase (TgCPOX) that catalyzes the antepenultimate reaction within the parasite's heme biosynthesis pathway.
13. RHΔ*ku80*Δ*hxg*Δ*cpoxCPOX* strain (abbreviated to Δ*cpoxCPOX*). A TgCPOX complementation strain was generated in a previous study (Bergmann *et al.*, 2020). Heme deficit is restored in this strain.
14. Human Foreskin Fibroblasts (HFFs) (American Type Culture Collection (ATCC), catalog number: SCRC-1041)
15. Corning® Dulbecco's Modified Eagle Medium (DMEM) (VWR, catalog number: 45000-304)
16. 100× Penicillin-Streptomycin (VWR, catalog number: 45000-652)
17. 200 mM L-glutamine (VWR, catalog number: 45000-676)
18. 1 M HEPES Free Acid (VWR, catalog number: 45000-696)
19. Hyclone® Cosmic Calf Serum (Cytiva, catalog number: SH30087.03)
20. HyClone® Dulbecco's Phosphate-Buffered Saline (DPBS) powder, without calcium and magnesium (Powder) (Cytiva, catalog number: SH30013.04)
21. Pig hemin powder (VWR, catalog number: BT138155-1G)
22. Oxalic acid dihydrate (VWR, catalog number: 97064-978)
23. Isopropanol (VWR, catalog number: BDH11744)
24. DPBS (see Recipes)
25. D10 medium (see Recipes)
26. 1 mM heme solution (see Recipes)

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27. 2 M oxalic acid solution (see Recipes)

Equipment

1. Eppendorf CellXpert® C170 cell culture incubator (Eppendorf)
2. Eppendorf refrigerated centrifuge (Eppendorf, model: 5810R)
3. Branson Analog Sonifier ultrasonic cell disrupter S-250A (VWR, catalog number: 33995-309) with a tapered 1/8-inch microtip (VWR, catalog number: 33996-163)
4. Biotek Synergy H1 Hybrid Multi-Mode microplate reader (Biotek Instruments)
5. Micropipette set (P-1000, P-200, and P-20) (VWR, catalog number: 75788-456)
6. Hemocytometer (VWR, catalog number: 15170-168). Instructions can be found in http://www.hausserscientific.com/products/reichert_bright_line.html
7. Hot plate (VWR, Advanced Hot Plate, catalog number: 97042-642)

Software

1. BioTek® Gen5 Data Analysis Software
2. Microsoft Excel
3. GraphPad Prism (Version 9)

Procedure

Please see **Figure 1** for a schematic description of the entire procedure.

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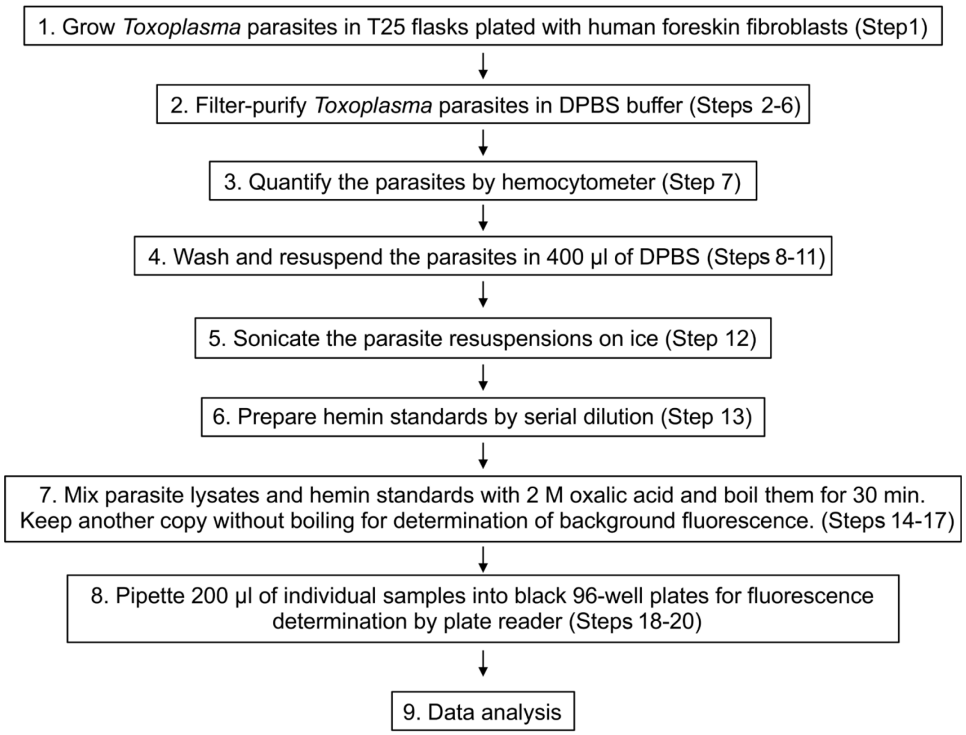

Figure 1. A schematic outline of the fluorescence-based heme quantitation in *Toxoplasma gondii*. Please refer to the text for more details.

1. In this protocol, wildtype (RHΔ*ku80*Δ*hxg*), Δ*cpox* (a heme-deficient strain), and Δ*cpoxCPOX* (a *TgCPOX* complementation strain) strains are included as examples. If parasite strains are stored at -80°C, they must be thawed at 37°C and immediately passed to a T25 tissue culture flask containing fully confluent human foreskin fibroblast (HFF) cells. After the parasites lyse the host cells, ~300 µl fully lysed wildtype and Δ*cpoxCPOX* parasites were passed to one T25 tissue culture flask coated with HFF cells for a 2-day passage. For the Δ*cpox* strain, the inoculum was increased to 2 ml to compensate for its severe growth defects. 1-2 T25 flasks of parasites per strain are needed depending on their growth phenotype. Since the Δ*cpox* showed severe growth defects, two flasks of Δ*cpox* parasites were used in this assay. The wildtype and Δ*cpoxCPOX* strains were passed into one T25 flask.
2. Pre-cool DPBS on ice and keep the infected cells on ice. Scrape the infected HFFs and syringe them three times using a 20 G needle, followed by three times using a 25 G needle. The lysates were passed through a 3.0-µm filter to remove intact host cells and large host cell debris. Rinse the flasks with 5 ml ice-cold DPBS and pass through the filter to wash.
3. Fill a 1,000-ml glass beaker with water and keep it boiling on a hot plate for sample heating. The boiling water will be used in Step 16 to heat the samples.
4. Centrifuge parasites at 1,000 × *g* for 10 min at 4°C. After centrifugation, a parasite pellet is expected to be seen at the bottom of the centrifuge tube.
5. Carefully aspirate the supernatant and resuspend the parasite pellet in 10 ml ice-cold DPBS. Repeat Step 4. Check for the pellet before proceeding.

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6. Aspirate the supernatant carefully and resuspend the pellet in 10 ml ice-cold DPBS.
7. Quantitate the yield of purified parasites using a hemocytometer following the vendor's instructions.
8. Pellet the parasites at 1,000 × *g* for 10 min at 4°C.
9. Aspirate the supernatant and resuspend the parasites in 1 ml ice-cold DPBS and transfer to a 1.5-ml microcentrifuge tube.
10. Spin down the parasites at 5,000 × *g* at 4°C for 5 min.
11. Aspirate the supernatant and resuspend the remaining pellet in 400 µl ice-cold DPBS.
12. Sonicate each purified parasite strain inside a biosafety cabinet using a Branson Analog Sonifier ultrasonic cell disrupter S-250A with a tapered 1/8-inch microtip, using the following settings: output intensity = 3 and Duty% = 20%. Sonicate each purified parasite strain for 10 s and repeat 4 times. Wait for 30 s between each repeat and keep samples on ice to avoid overheating.
13. A hemin standard curve is needed for the measurement of absolute heme abundance per parasite. The hemin stock is initially diluted in DPBS to 1,200 nM, followed by a 3-fold serial dilution to generate 5 additional concentrations at 400, 133.3, 44.4, 14.8, and 4.9 nM. DPBS alone is also included as a blank for 0 nM hemin.
14. Add 100 µl each sample or hemin standard to black 1.5-ml centrifuge tubes. Two sets for each strain or hemin standard are prepared; one will be boiled and another one will remain at room temperature.
15. Add 900 µl 2 M oxalic acid to each sample and vortex for complete mixing.
16. Keep one set of samples and hemin dilutions in a rack at room temperature and place another set in a tube holder to boil for 30 min.
17. Place the boiled samples on ice for 5 min and then leave them at room temperature for 10 min. Mix all samples by vortexing.
18. Pipette 200 µl each parasite stain or hemin standard into each well of a black 96-well plate in triplicate.
19. Read the samples using a BioTek Synergy H1 Hybrid Multi-Mode Microplate Reader with the following settings: Excitation: 400 nm, Emission: 608 nm, Optics: Top, Gain: 135, Read Speed: Normal, Delay: 100 msec, Measurements/Data Point: 10, and Read Height: 7 mm. Export the acquired data to an Excel spreadsheet.
20. Repeat the assay in at least three biological replicates for statistical significance comparison.

Data analysis

1. Calculation of the normalized heme abundance in *Toxoplasma* parasites (**Figure 2**):

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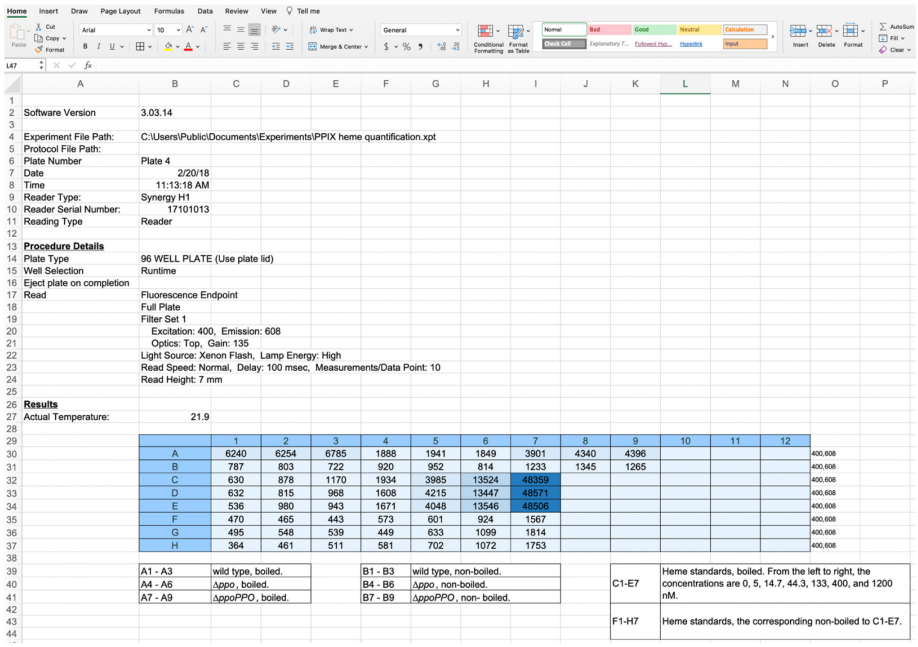
A



	1	2	3	4	5	6	7	8	9	10	11	12	
A	6240	6254	6785	1888	1941	1849	3901	4340	4396				400.608
B	787	803	722	920	952	814	1233	1345	1265				400.608
C	630	878	1170	1934	3985	13524	48359						400.608
D	632	815	968	1608	4215	13447	48571						400.608
E	536	980	943	1671	4048	13546	48506						400.608
F	470	465	443	573	601	924	1567						400.608
G	495	548	539	449	633	1099	1814						400.608
H	364	461	511	581	702	1072	1753						400.608

A1 - A3	wild type, boiled.
A4 - A6	Δ*ppo* , boiled.
A7 - A9	Δ*ppoPPO* , boiled.

B1 - B3	wild type, non-boiled.
B4 - B6	Δ*ppo* , non-boiled.
B7 - B9	Δ*ppoPPO* , non- boiled.

C1-E7	Heme standards, boiled. From the left to right, the concentrations are 0, 5, 14.7, 44.3, 133, 400, and 1200 nM.
F1-H7	Heme standards, the corresponding non-boiled to C1-E7.

B

Results

Actual Temperature: 21.9

	1	2	3	4	5	6	7	8	9	10	11	12	
A	6240	6254	6785	1888	1941	1849	3901	4340	4396				400.608
B	787	803	722	920	952	814	1233	1345	1265				400.608
C	630	878	1170	1934	3985	13524	48359						400.608
D	632	815	968	1608	4215	13447	48571						400.608
E	536	980	943	1671	4048	13546	48506						400.608
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G	495	548	539	449	633	1099	1814						400.608
H	364	461	511	581	702	1072	1753						400.608

Average fluorescence (A.U.) for each strain and blank from three replicates

	wildtype	Δ*cpox*	Δ*cpoxCPOX*	blank (0 nM)
Boiled	6426.3	1892.7	4212.3	599.3
Non-boiled	770.7	895.3	1281.0	443.0
Boiled minus non-boiled	5655.7	997.3	2931.3	156.3
Parasite strain minus blank	5499.4	841.0	2775.0	0.0

Parasite number calculated by hemocytometer

wildtype	Δ*cpox*	Δ*cpoxCPOX*
$9.8*10^7$	$4.8*10^7$	$6.2*10^7$

Average fluorescence (A.U.) per parasite for each strain

wildtype	Δ*cpox*	Δ*cpoxCPOX*
1.12E-03	3.50E-04	8.95E-04

Normalized heme abundance relative to wildtype parasites

wildtype	Δ*cpox*	Δ*cpoxCPOX*
100.0%	31.2%	79.8%

Figure 2. Example data and analysis for PPIX-based heme quantitation in *Toxoplasma* parasites. A. Excel table of the raw data from one biological replicate of heme quantitation in wildtype, Δ*cpox*, and Δ*cpoxCPOX* parasites. B. Data analysis of normalized heme abundances in *Toxoplasma* parasites. The heme abundance in wildtype parasites was set as 100% for normalization of heme amounts in other strains.

a. Calculate the average readings for each boiled and non-boiled sample.
b. Subtract the fluorescence of every non-boiled sample from the signal of the corresponding boiled sample.
c. Subtract the blank value (0 nM hemin sample) from each parasite strain.
d. Divide the mean values for the individual samples by the number of parasites calculated by the hemocytometer counts.

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Note: One-twentieth of the purified parasites are included in each well for quantitation of heme abundance (A quarter of the total purified parasites are mixed with oxalic acid before boiling, and one-fifth of the boiled mixture is pipetted into each well of a 96-well plate for fluorescence measurement).

e. Divide the average fluorescence value per parasite for each sample by that from the wildtype strain for normalization. The normalized value for wildtype parasites is set as 100% for comparison with other strains.

2. Calculation of the absolute heme abundance in *Toxoplasma* parasites (**Figure 3**):

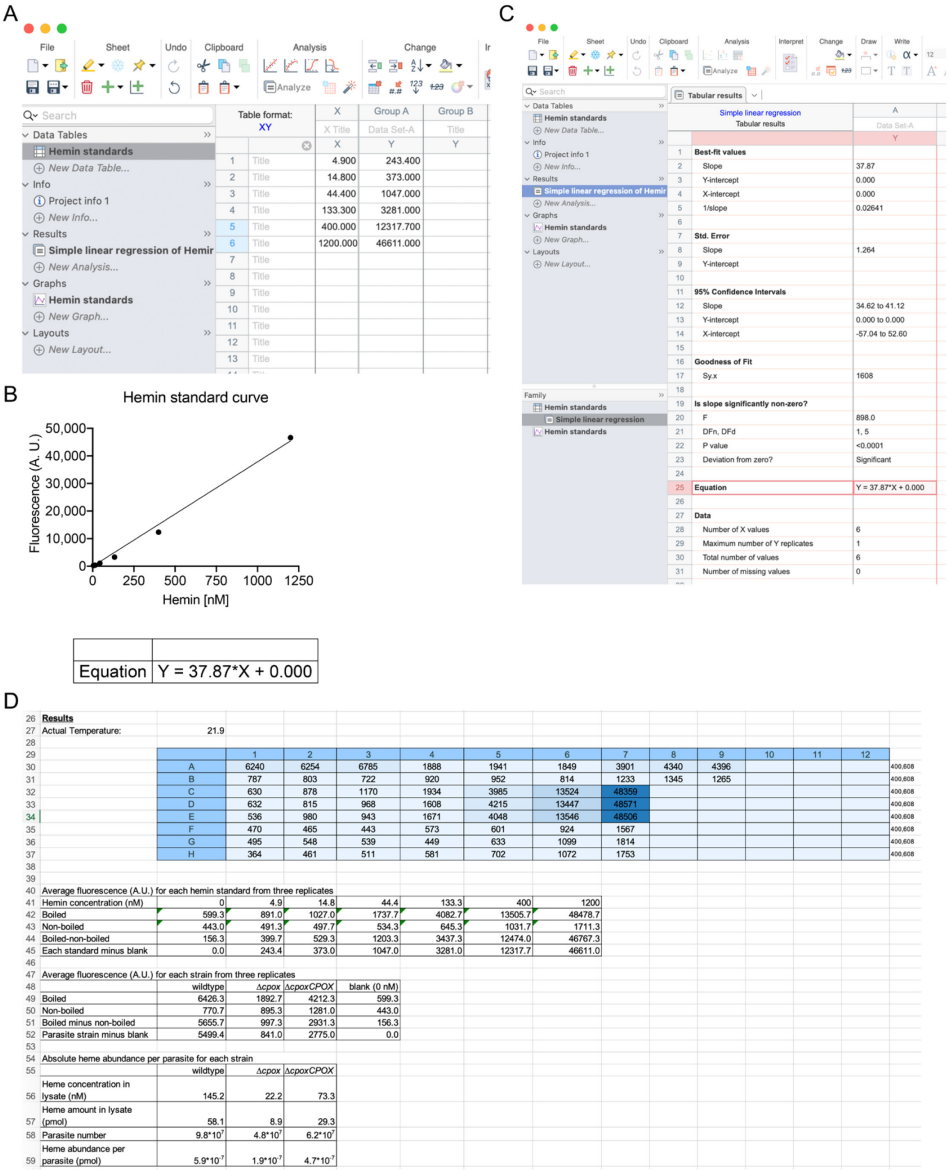

Figure 3. Calculation of the absolute heme abundance in *Toxoplasma* parasites. A. Table of fluorescence (A.U.) signals under each concentration of hemin standard. B. Graph of a linear regression of the plotted hemin standard curve. C. Linear regression analysis to generate the

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equation for calculating heme concentrations in lysates of purified parasites. D. Step-by-step data analysis for calculating heme amounts per parasite.

If the absolute heme abundance per parasite is desired, the standard heme curve is required to determine the heme concentration in sonicated parasite lysates by substituting fluorescence values from each strain in the heme standard curve equation. The total amount of heme in each parasite lysate is determined by multiplying the heme concentration of each sample by the volume (400 µl) and dividing by the total number of parasites to yield the absolute heme abundance per parasite.

To generate the hemin standard curve:

a. Average the fluorescence intensities of boiled and non-boiled samples for each concentration of hemin standard.
b. Subtract the average fluorescence values for the individual non-boiled samples from the corresponding boiled samples.
c. Subtract the blank value (0 nM hemin) from the individual hemin concentrations.
d. Plot the subtracted fluorescence values against the individual hemin concentrations at 4.9, 14.8, 44.4, 133.3, 400, and 1,200 nM.
e. Perform linear regression on the plotted data points for curve fitting to produce the heme standard curve equation, which will be used to calculate heme abundances in the tested parasite lysates.

Recipes

1. DPBS
   Add 9.6 g HyClone Dulbecco's phosphate-buffered saline powder in 1 L deionized water. The solution is autoclaved before use.
2. D10 medium
   Dulbecco’s Modified Eagle Medium (DMEM) is supplemented with 10 mM HEPES, 10% (v/v) Cosmic calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin.
3. 1 mM heme solution
   Dissolve 32.6 mg hemin in 50 ml 20 mM NaOH.
4. 2 M oxalic acid solution
   Add 126.07 g oxalic acid dihydrate to 500 ml deionized water. Heat water to ~50°C for solution preparation. The solution is oversaturated, and the solute will precipitate when the solution cools to room temperature. Use the supernatant in the assay.

Acknowledgments

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Competing interests

The authors declare no conflicts or competing interests.

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