Extraction of Drug Treated *P. falciparum* Trophozoites for LC-MS

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-Short version-

Summary: This protocol describes how to extract hydrophilic metabolites from *in vitro* cultures of *Plasmodium falciparum* using a methanol-based precipitation from a population of 90-95% pure mycoplasma-free trophozoite stage parasites following drug treatment. We describe how to use this method for preparing extracts following drug treatment or directly from drug-resistant parasite lines (1,2). We recommend that drug treatments and extractions be performed in technical triplicate to minimize errors in the downstream data analysis that may arise from inconsistent sample processing. Using this approach, one synchronous T75 flask containing 50mL of 2% hematocrit with 10% parasitemia culture should yield enough parasites for ~2 samples for analysis by liquid chromatography coupled with mass spectrometry (LC/MS). Each trial should be accompanied by a positive control treatment (*i.e.* atovaquone) and a negative untreated control (*i.e.* no drug or vehicle only) for determining parasite metabolic responses.

Culture requirements:

1) Synchronous cultures of trophozoites 24-28 hours post invasion (hpi) at ~10% parasitemia.
2) One synchronous 50 mL flask at 2% hematocrit and 10% parasitemia yields ~ 2x 20 **µ**L MS samples of purified trophozoites with this method.
   Note: Please ensure that cultures are mycoplasma-free before initiating your experiment.

Extraction of already purified and aliquoted parasites (equivalent to 1x10^8 parasites/sample):

Note: Everything from here on should be performed in a 4°C cold room or minimally with cold reagents, ice, and a chilled centrifuge (4°C). Additionally, all steps up to the MeOH quenching should be performed as quickly as possible to avoid non-drug related metabolic perturbation.

1) Remove one 6-well plate from the incubator.
2) Aspirate ~4mL of media from each well, changing pipettes between conditions, and resuspend cells in the remaining medium by rocking the plate side-to-side.
3) Angle the 6-well plate by propping on the lid to gather cells at the bottom edge of the well.
4) Using a filtered pipette tip transfer the remaining culture from a well (~1 mL) to a pre-chilled/pre-labeled 1.5 mL tube and place immediately on ice.
   Note: Cells may have settled at the bottom and along the edges of the well, gently resuspend with a pipette for transfer to the chilled microfuge tube.
5) Centrifuge all 6-1.5 mL tubes from one plate at 8,500 RPM for 0.5 minutes at 4°C.
6) Quickly aspirate RPMI and gently wash the iRBC pellet in 1 mL ice-cold PBS without vigorous mixing or pipetting.
7) Spin samples down at 8,500 rpm for 0.5 minutes at 4°C.
8) Aspirate PBS from a single tube and **QUENCH** metabolism by adding 1 mL of ice-cold 90% methanol (with isotope-labeled standard, see below) and quickly resuspend by vortexing for 10 seconds. **This step is the most crucial since it quenches parasite metabolism. Methanol and H_2O should be HPLC-grade.**
   Note: It is recommended to spike-in $^{13}C_4$, ^{15}N-Aspartate (Cambridge Isotope, Cat No. CNLM-544-H-PK) or a similar isotopically labeled compound (final concentration 0.5 µM) as an internal LC/MS standard to correct for technical variation due to sample processing in the data analysis phase.
9) Place quenched tube on ice and continue extraction of the remaining tubes and plates.
   Note: Extract 3 "blank" tubes by adding 1mL of ice-cold 90% methanol to a 1.5 mL tube and mix by vortexing.
10) Once extraction/quenching of all samples is complete, vortex tubes for an additional 10 seconds to ensure complete dissociation of the pellet and cell lysis.
11) Centrifuge all samples at maximum speed for 10 minutes at 4°C to pellet cell debris.
    a. Label a new set of tubes while the samples are spinning and place on ice.
12) Transfer supernatant to fresh pre-chilled tubes taking care to avoid transfer of **ANY** pelleted debris.
13) Store the samples at -80 until ready to dry-down under nitrogen gas (below) or lyophilize using a speed-vac.
    Note: It is preferable to dry down the samples ***immediately*** to prevent the decay of metabolites. Samples in MeOH should not be stored at -80°C for more than one month. However, dried metabolite extracts can be stored at -80°C for several months. For shipping, samples should be dried down and packaged in dry ice.

If shipping to the Llinás lab: Please provide a complete list of sample IDs with cell counts and label all samples clearly. For LC/MS, dried extracts are resuspended in HPLC-grade water to 1x10^6 parasites/µL (final), in a final volume of 100 µL.

References:

1. **Lu, W., Clasquin, M. F., Melamud, E., Amador-Noguez, D., Caudy, A. A., and Rabinowitz, J. D. (2010) *Anal Chem* 82, 3212-3221.**
2. **Allman, E. L., Painter, H. J., Samra, J., Carrasquilla, M., and Llinas, M. (2016) *Anti Agents & Chemo* Aug 29. pii: AAC.01224-16.**