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

## Llinás Lab 2016 (Updated 02/22/2017)

**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.

**EXAMPLES:**

- To test a single antimalarial compound, 250 mL of culture (2% hematocrit with 10% parasitemia) is required (9 samples in total – 3x positive control, 3x untreated control, 3x drug test).
- In our lab, we aim to test 5 compounds, atovaquone, and an untreated control per batch, which equates to roughly 600mL of parasite culture (21 samples in total – 3x positive control, 3x untreated control, 5 x 3x drug test).
- Alternatively, to test a drug resistant (or other) mutant parasite line against a parental line with and without drug, 150mL of culture is required per strain (6 samples in total per line - 3x untreated control, 3x drug treated).

**NOTE: If not testing the effect of drugs use the *alternative* approach (below) and exclude all drug treatment steps.**

Best results will be obtained by processing all samples carefully and quickly since the sensitivity of LC/MS will readily identify extraction execution errors in the output data, making interpretation difficult. **Most importantly, this analytical method is limited by final parasite concentration (ideally $3x10^8$ parasites/condition for triplicate extraction). If parasite numbers are less than the optimal concentration, data quality will be reduced.**

**NOTE: This procedure takes up the bulk of a workday so preparation is important for a successful outcome.**

## Materials:

### Magnetic Separation:

1) Synchronized cultures of trophozoite-stage parasites - 24-28 hours post invasion (hpi) - at ~10% parasitemia in 2% hematocrit (or equivalent to ensure $1x10^8$ parasites/sample)
   **Note:** Please ensure that cultures are mycoplasma-free before initiating your experiment.
2) VarioMacs magnet (or an analogous setup – see Appendix I below)
3) MACS CS column(s) with syringe, 3-way stopcock, and flow resistor (21G needle) attached (Miltenyi Biotech)
   **Note:** Each CS column will bind the contents of approximately one 50 mL culture with 10% parasitemia at 2% hematocrit.
4) Table-top centrifuge, chilled to 4°C
5) Complete RPMI warmed to 37°C
6) 1X PBS

### Biological Sample Preparation and Treatment Set-up:

1) Hemocytometer, light microscope (or automated cell counter)
2) Complete RPMI warmed to 37°C (with or without isotopic label)
3) Anti-malarial drugs to be tested (be sure to know the $IC_{50}$ values)
4) 6-well culture plates (see Appendix II to determine number of plates needed)

### Metabolite Extraction

7) One bucket of ice
8) Ice-cold 1x PBS in ddH20 (sterile-filtered)
9) Ice-cold 90% Methanol/$H_2O$ (both 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 standard for LC/MS analysis.
10) Chilled 1.5 mL microfuge tubes (one for each sample extracted + 3 blanks)
11) Microcentrifuge, chilled to 4°C
12) Vortex
13) Nitrogen evaporator with high purity gas OR Speed-vacuum concentrator

## Magnetic Separation:

**Note**: A single flask and column should take ~15 minutes, timing from step 4. Columns can be reused multiple times following elution, however the yield will slowly decrease.

1) Connect the column and the 3-way stopcock and place it on the magnet (See Appendix I for example set-up OR manufacturer's instructions for more detail)
2) Pre-equilibrate CS column(s)
    a. Fill the syringe with 10 mL 1X PBS and connect to port B of the stopcock. Turn to stopcock position to "fill". Fill column upright from the bottom with the 10 mL of 1X PBS by emptying the syringe.
    b. Turn the stopcock position to "run" and rinse column with four column reservoir volumes of RPMI (~ 30 mL) emptying into a waste container, without allowing the column to run dry.
    c. Fill the column with RPMI, turn the stopcock to "fill", and without running the column dry, draw one column reservoir volume (~6.5) of RPMI into the syringe.
    d. Carefully cut off the plastic tip of the flow resistor (21G needle) just below the tip of the needle and attach to port C of the 3-way stopcock (leave plastic sheath in place for safety).
    e. Run 1 more volume (~6.5 mL) of complete RPMI through into the waste container. Discard flow-through that has been collected in the waste container.

    **Note:** Make sure that the column does not run dry by turning the 3-way stopcock to the "closed" position when you are not passing liquid through the apparatus.
3) Prepare the parasite culture for magnetic separation.
    a. Collect the contents of one T75 flask into a 50 mL conical by centrifugation at 1,500 rpm for 5 minutes at room temperature. (Multiple flasks containing the same strain can be consolidated.)
    b. Remove supernatant.
    c. Resuspend to ~10% hematocrit with warm complete RPMI (*i.e.:* 10mL/1 mL of packed infected RBCs)
4) Turn the stopcock position to "run" and apply the culture suspension to the reservoir of the CS column(s) with a Pasteur pipette.
5) Once the equivalent of one culture volume (~10 mL) is loaded onto the column, wait for the meniscus to approach the matrix and start washing the column with warm RPMI.
6) Keep washing with warm RPMI until the eluent runs clear and then run one more reservoir volume (~6.5 mL) of warm RPMI through.
7) Remove column apparatus from magnet, remove needle, and elute trophozoite infected RBCs into fresh 50 mL conical tube by adding warm complete RPMI to the reservoir until the eluent runs clear (~30 mL).
8) Flush one reservoir volume (~6.5 mL) of warm complete RPMI up into the column using the syringe connected to the 3-way stopcock to ensure complete dissociation of trophozoite parasites.
9) Continue with all remaining cultures and columns.
10) Centrifuge the eluted material at 1,500 rpm for 5 min at room temperature.
11) Aspirate the supernatant.

    **Note:** At this point a Geimsa-stained smear of eluted material should be made to assess purity of trophozoite infected RBCs. With the 21 gauge needle this routinely results in ~95% parasitemia.
12) Gently resuspend the cell pellet in warm RPMI to a volume of 1.5X the number of target samples (*i.e.* 1 drug + 1 control = 6 samples or 6 x 1.5 = 9 mL RPMI) and remove 100µL aliquot to do a cell count by hemocytometer.
13) Place purified parasite culture tube(s) into a 37°C water bath until cell counts are completed.

## Cell Counts, Plate Setup, and Tube preparation:

**Note:** Perform cell counts quickly so parasites can recover during subsequent steps.

1) Dilute the 100 µL aliquot 1:100 in PBS or RPMI and obtain a cell count using a hemocytometer. Calculate the volume of media needed for a final concentration of $5x10^7$ – $1x10^8$ purified parasites/mL. Adjust the volume accordingly (step 12 above) and place into a flask in the incubator to recover for 1-2 hours.
2) Label 6-well plates based on conditions. (See sample layout based on experimental design in Appendix II.)

    **Note:** Samples are performed in triplicate, thus a single 6-well holds 2 conditions.
3) Prepare required volume of RPMI containing appropriate concentration of drug.

    **Note:** Drug concentration should be based on the FINAL concentration of drug in 5 mL (1/5 higher in 4 mL).
4) Aliquot 4 mL of RPMI, containing appropriate concentration of drug, into each well of the respective 6-well plate and place in the incubator.

    **Note:** We routinely use $10xIC_{50}$ concentration of drug (see Reference (2)).

***Alternative to steps 2-4 above (mutant parasite testing):*** Add 4mL of RPMI to each well of a 6-well plate, followed by 1mL ($1x10^8$ cells) of parasite culture (step 1, post adjustment). Place the plates in the incubator to recover for 1-2 hours before drug addition. During recovery, dilute drugs so that each well will receive 5µL of drug stock. This allows uniformity in solvent transfer (for DMSO this is 0.1% final) for each well. After recovery, aliquot the drugs into their respective wells and place the plates back into the incubator for 2.5 hours.

**Note:** If following this approach, complete step 5 and then proceed to ***Metabolite Extraction***.

5) Pre-label 1.5mL microfuge tubes according to the conditions that are being tested and place on ice.

    **Note:** Be sure to also include 3 tubes for method blanks.

## Biological Sample preparation:

1) Remove 6-well plates and parasite flask from the incubator.
2) Gently resuspend the cell culture and aliquot 1 mL of parasites into each well of the respective 6-well plates.
   **Note:** This now gives you the required number of parasites needed to generate a quality LC/MS sample per 5 mL well, at the appropriate final drug concentration.
3) Place plates in incubator with the appropriate gas mixture for 2.5 hours.
   **Note:** Depending on the number of compounds being tested, plates should be staggered by 15 min (or less depending on the speed at which you are comfortable performing the next phase of the protocol) to avoid timing issues with subsequent metabolite extraction step.

## Metabolite Extraction:

**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.**
   **Note:** The methanol should contain an isotopically labeled internal standard, such as 0.5 µM [$^{13}C_4$,$^{15}N$]-Aspartate 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.
12) Label a new set of tubes while the samples are spinning and place on ice.
13) Transfer supernatant to fresh pre-chilled tubes taking care to avoid transfer of **ANY** pelleted debris.
14) 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.

## Nitrogen Drying:

**Note:** The Llinás lab uses a 27-port Reacti-Vap Evaporator (Thermo Fisher) attached to a tank of high purity dry $N_2$ gas.

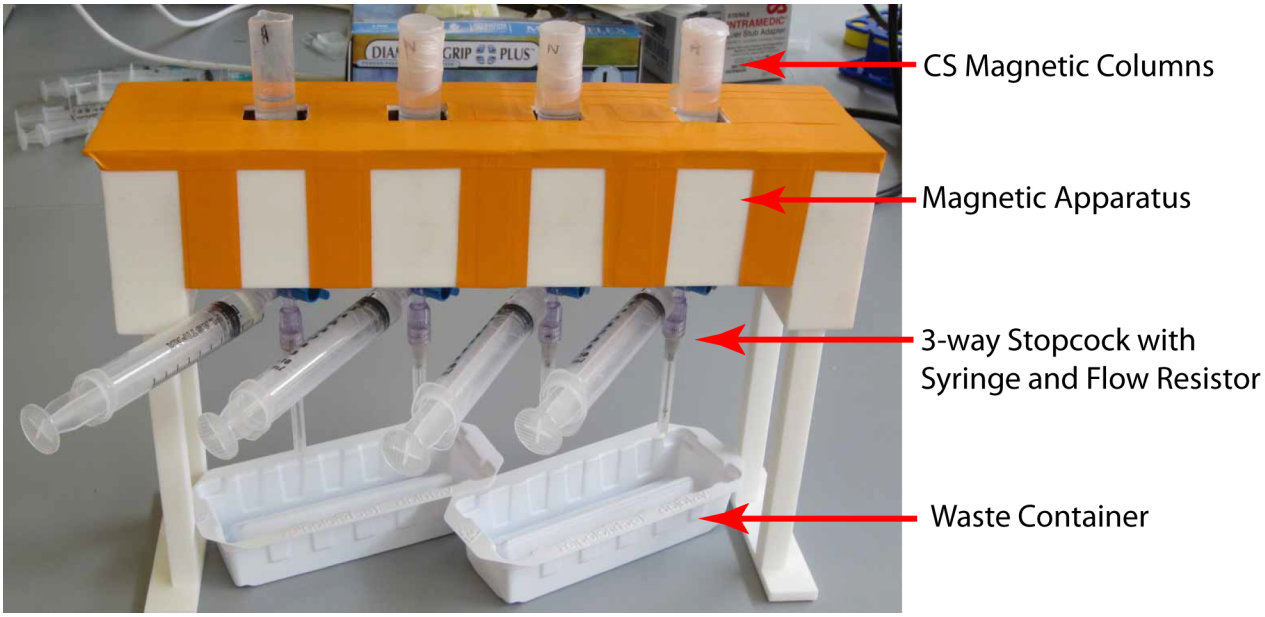
1) Rinse the needles with 100% MeOH, wipe clean, and insert into the appropriate positions on the gas manifold.
2) Gently place the sample tubes into the aluminum block with the lids open.
3) Carefully place the manifold above the tubes and lower it into position above the open tubes.
   **Note**: Ensure the needles are not touching the sample but are ~1/2 inch above the liquid surface.
4) With the manifold and needles in place SLOWLY being increasing the $N_2$ outflow pressure until you hear a slight hissing sound. At this point STOP and ensure the samples are not splashing.
5) Continue raising the outflow pressure until the sample surface is disrupted but not gurgling or splashing.
   **Note**: Never exceed 2 psi or you could damage the manifold.
6) Check on the samples every 10-15 minutes and carefully lower the manifold as the sample volume decreases, ensuring the needle never touches the sample and the sample does not splash.
7) Continue this process until the samples are dry, checking periodically by raising the manifold.
   **Note**: A dry sample will not look glassy and will not move when the tube is gently flicked.
8) Once dry, store samples at -80°C until ready for shipping or analysis.

**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 Llinás, M. (2016) *Anti. Agents & Chemo.* Aug. 29. pii: AAC.01224-16.**

## APPENDIX I: Example Apparatus Set-up for Magnetic Purification

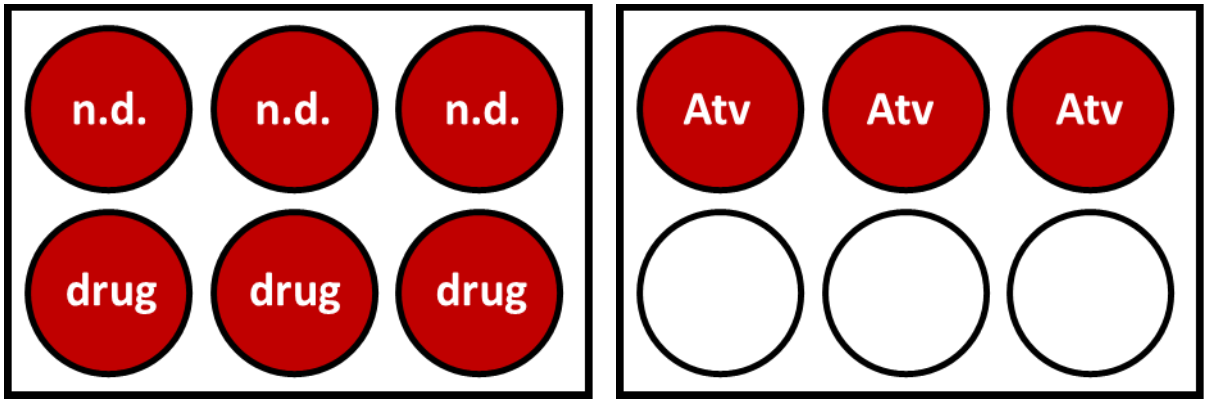



## APPENDIX II: Sample Experimental Designs

### Drug Treatment Response:

Follow the protocol above to obtain purified trophozoite stage parasites at the appropriate cell density.

1) Conditions and samples – Untreated (no drug = n.d.) x 3, Drug treated x 3, Atovaquone (Atv) x 3
2) Culture Requirements – 250 mL of culture (2% hematocrit with 10% parasitemia)




### Drug Resistant Metabolic Phenotyping:

Follow the protocol above to obtain purified trophozoite stage parasites at the appropriate cell density.

1) Conditions and Samples – Both sensitive and resistant lines should be grown, synchronized, and diluted as equally as possible. Untreated sensitive x 3, Treated sensitive x 3, Untreated resistant x 3, Treated resistant x 3
2) Culture Requirements – 300 mL of culture (2% hematocrit with 10% parasitemia)