Sample Preservation for WWARN ARM Molecular Studies v1.5

Procedure

Molecular Module &
Artemisinin Resistance Markers Platform (ARM)
WorldWide Antimalarial Resistance Network (WWARN)

Suggested citation: Molecular Module. 2010. Sample Preservation for WWARN ARM Molecular Studies v1.5. WWARN Procedure.

Procedure ID: MOL01
This procedure developed by:

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Version History

Version number	Revision(s) & reason for amendment	Release date
1.5	Changes to template	27/10/2010
1.4	Addition of instructions to procedure	23/07/2010
1.3	Addition of instructions to procedure	19/05/2010
1.2	Addition of instructions to procedure	11/05/2010
1.1	Addition of instructions to procedure	10/04/2010
1.0	Creation of procedure	02/04/2010

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Contents

1. Purpose

This procedure describes the collection and preservation of samples for WWARN ARM molecular studies. Procedures outlined include steps to reduce the amount of human DNA in *Plasmodium*-infected whole blood samples for genomic studies by removing human white blood cells (WBC); collect filter paper blood spots for parasite DNA; and, optionally, to collect *Plasmodium falciparum* parasites for cryopreservation of viable parasites and RNA extraction.

2. Scope

The Worldwide Antimalarial Resistance Network Artemisinin Resistance Markers (WWARN ARM) Platform is being formed to pool clinical and molecular data for genome-wide association studies and other genomic studies aimed at identifying genetic loci responsible for artemisinin resistance with the ultimate aim of developing molecular resistance markers that will serve as surveillance tools to track and monitor resistance and to guide containment efforts. Participating sites are conducting clinical trials to measure *in vivo* responses to artesunate including parasite clearance time. Reduction of human DNA in blood from infected patients is required to meet criteria for next-generation sequencing, genotyping and gene expression platforms. This can be also be accomplished by culture-adapting parasite isolates, but analysis of samples taken directly from patient blood without a culture step is preferred to ensure that genotypes and expression profiles correspond to those associated with the original phenotype.

Sites with the ability to do so may also elect to preserve parasite RNA and cryopreserve viable parasites for further molecular and *in vitro* studies, as well as samples for pharmacokinetic and pharmacodynamic studies.

3. Abbreviations

WBC	White Blood Cell
mL	millilitre
mm	millimetre
PBS	Phosphate Buffered Saline
RPMI	Roswell Park Memorial Institute medium
RCF	Relative Centrifugal Force
RPM	Revolutions Per Minute
cm	centimetre

4. Duties and Responsibilities

N/A

5. Materials and Equipment

5.1 Materials

I. Blood collection for parasite DNA

- 4 mL or larger EDTA tubes

II. Lymphoprep WBC separation

- Lymphoprep solution (Axis-Shield, catalogue no. NYC-1114545 for 4*250 mL bottles)
- RPMI-1640 or PBS (phosphate buffered saline), without serum
- 15 mL or 50 mL conical centrifuge tubes
- Serological pipets (5 or 10 mL)

III. Plasmodipur filtration

- Plasmodipur Filters (EuroProxima B.V., catalogue no. 8011Filter25u)
- Syringes (minimum 5 mL)
- RPMI-1640 or PBS (phosphate buffered saline), without serum
- 50 mL centrifuge tubes
- Serological pipets (5 or 10 mL)
- Kwill filling tubes (5" or 127 mm, Avon Ref # E910) –OR– plastic transfer pipets (3-5 mL) –OR– large gauge needles (18-19g preferred; 21g acceptable)
- 1.8 mL cryotubes (450 pack, Fisher Scientific catalogue no. 12-565-298)
- Clamps and clamp stands (2 clamps and 1 stand each for as many samples as will be processed together at the same time)

IV. Filter paper blood spot collection

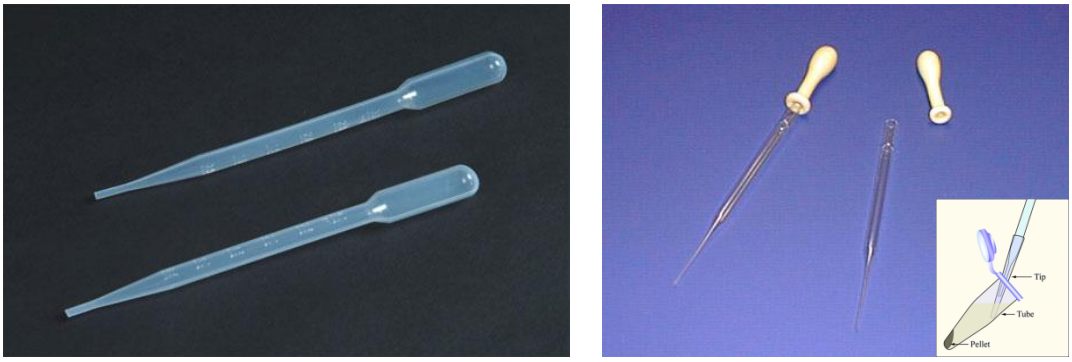
- FTA® Elute cards (Whatmann WB120410, 100 pack ; Fisher Scientific catalogue no. 09-301-231) or Whatmann 3MM filter paper, cut in 1.5 in x 3 in rectangles
- Zip-locking plastic bags
- Dessicant

V. Parasite cryopreservation

- Glycerolyte 57 solution (400 mL, Fenwal Blood Technologies catalogue no. 4A7831)
- 1.8 mL cryotubes (450 pack, Fisher Scientific catalogue no. 12-565-298)
- 15 mL centrifuge tubes

VI. RNA preservation

- PAXgene Blood RNA tubes (100 pack, PreAnalytix catalogue no. 762165)
- PAXgene Blood RNA Kit (50 pack, Qiagen catalogue no. 762164)
- 1.8 mL cryotubes (450 pack, Fisher Scientific catalogue no. 12-565-298)
- Ethanol (96-100%)
- Sterile, aerosol-barrier, RNase-free pipet tips (10 uL – 4 mL)
- Crushed ice



A) B)

Figure 1. Pipets for fluid transfer and removal. A) Plastic transfer pipets have volume markings on the side and can be used to transfer blood, remove supernantant above a pellet, etc. Image source: Medex International http://www.medexcompanies.com/shop/2006329165438.jpg B) Glass Pasteur pipets can be attached to a suction tube at the upper end and inserted into a P200 plastic pipet tip (right inset) and used to suction off supernatant to be discarded. Image source: Colorado University, Boulder http://orgchem.colorado.edu/equipment/glassware/pastpipet.html

5.2 Equipment

I. General equipment

- Pipet controller for 5/10 mL serological pipets
- Benchtop centrifuge for 15/50 mL tubes

II. For RNA preservation (in addition to general equipment)

- Vortex mixer
- Benchtop centrifuge capable of attaining 1000 – 8000 x *g* and equipped with a swingout rotor and buckets to hold PAXgene Blood RNA tubes
- Variable-speed microcentrifuge capable of attaining 1000–8000 x *g*, and equipped with a rotor for 2 mL microcentrifuge tubes, not exceeding 1400 RPM
- Shaker–incubator capable of incubating at 55° C and 65° C and shaking at 400 RPM, not exceeding 1400 RPM

- Pipet controllers (10 uL – 4 mL)
- Graduated cylinder

NOTE: If a vacuum pump and vacuum trap are available, use of a Pasteur pipet with clean P200 pipet tip and suction is an efficient and inexpensive option for removing supernatant to be discarded at several steps in the procedure.

6. Procedure

6.1 Leukocyte depletion

NOTE: Leukocyte depletion using CF11 columns (see WWARN procedure MOL02: Leukocyte Depletion of Whole Blood Using CF11 Columns) is a comparable alternative to Lymphoprep centrifugation and Plasmodipur filtration.

NOTE: For this procedure, DNA yield and purity sufficient for next-generation sequencing are obtained when both Lymphoprep and Plasmodipur steps (Steps 6.3 and 6.4) are performed, and both steps in this order are strongly recommended. At remote sites where centrifugation is not possible, CF11 columns are highly preferable.

6.2 Blood collection

- Suggested blood volume per sample is 3-5 mL for DNA preservation.

- Ideally, a minimum parasitemia of 0.2% (parasite density of 10,000/mm^3) is recommended. When possible, higher blood volumes should be collected for samples with lower parasitemia.

- Collect whole blood samples in blood tubes containing anticoagulant (e.g. EDTA or CPDA-1). Do NOT use heparin, which can inhibit Taq polymerase.

- Carefully label tubes at every step with study ID number or other unique identifier.

- Invert gently to mix well.

- Immediately store the blood samples at 4° C until they can be processed to remove WBCs.

  NOTE: Leaving the blood at room temperature will result in hemolysis and significant sample loss.

- Proceed to leukocyte depletion step within 6 hours to minimize lysis of WBCs. Do not leave samples at this stage for longer than 24 hours. Do **NOT** freeze samples at this stage—this will cause hemolysis and release of human DNA.

6.3 Lymphoprep density gradient centrifugation

I. Allow blood samples, Lymphoprep solution and PBS/RPMI to reach room temperature before beginning procedure. Cold solutions will negatively affect leukocyte separation.

II. Transfer blood to a 15 mL or 50 mL centrifuge tube (see example volumes below). Add 1 volume (same volume as original blood sample) of RPMI or PBS to the whole blood. Swirl or gently pipette up and down to mix.

III. Add 1 volume (same volume as original blood sample) of Lymphoprep to a new centrifuge tube.

Example volumes

3 mL blood sample:
- Add 3 mL RPMI or PBS to tube with blood
- Add 3 mL of Lymphoprep to a new 15 mL tube

5 mL blood sample:
- Add 5 mL RPMI or PBS to tube with blood
- Divide 5 mL of Lymphoprep into two new 15 mL tubes (2.5 mL in each tube, for layering of ½ of sample in each tube)

NOTE: It is recommended to use two 15 mL tubes rather than one 50 mL tubes for 5 mL samples to reduce mixing between blood and Lymphoprep layers.

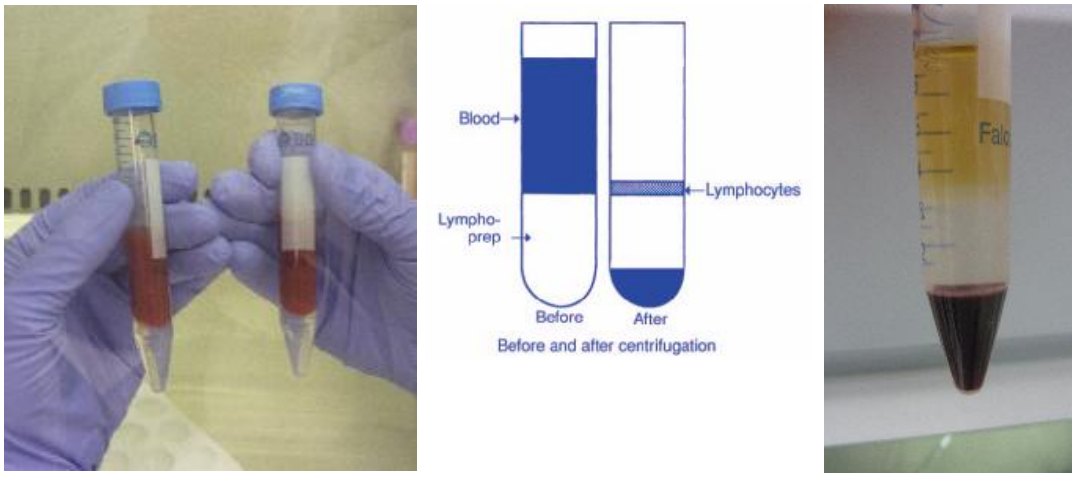
10 mL blood sample:
- Add 10 mL RPMI or PBS to tube with blood
- Add 10 mL Lymphoprep to a new 50 mL tube

IV. Pipet blood-media mixture extremely carefully to layer it on top of Lymphoprep, allowing the sample to slowly dribble down the side of the tube until it rests on top of Lymphoprep. Take care to avoid mixing of diluted blood and Lymphoprep fluid.

V. Centrifuge at 800 × *g* for 15 minutes at room temperature (approximately 22° C) To convert between RCF (× *g*) and RPM, see Appendix A. If ambient temperatures are high (e.g. a non-air-conditioned laboratory) and using a refrigerated centrifuge, do not set the temperature lower than 20° C.

VI. A layer of leukocytes (creamy white top layer) should separate from the red blood cell (RBC) pellet as illustrated in Figure 2. If a clear separation of layers is not visible, centrifuge for another 15 minutes. Carefully remove as much of the supernatant as possible, including leukocytes, without disturbing the RBC pellet at the bottom of the tube.

NOTE: It is preferable to leave up to ½ mL of clear supernatant on top of the RBC pellet than to disrupt the pellet. If the leukocyte layer (cloudy material at the serum-Lymphoprep interface) adheres to the wall of the tube, very gently dislodge it by pipetting up and down while ample Lymphoprep remains above the pellet. The remaining RBC pellet will be about 50% of the starting blood sample volume.



A) B) C)

Figure 2. Layering of blood and Lymphoprep. A) Blood samples layered on top of Lymphoprep solution before centrifugation. Image source: Susana Campino, 2010. B) Blood layers before and after centrifugation in Lymphoprep. Image source: Axis-shield (http://www.freewebs.com/eldri123/Package%20insert/Lymphoprep.pdf). C) RBC pellet and supernatant after centrifugation in Lymphoprep. Image source: Susana Campino. 2010.

VII. Add 10 mL RPMI or PBS to the tube. Pipette up and down gently to resuspend the pellet.

VIII. Centrifuge at 1000 × *g* for 10 min at room temperature (approximately 22° C).

IX. Carefully remove and discard the supernatant.

6.4 Plasmodipur filtration

I. Add an equal volume of RPMI or PBS to the RBC pellet. Pipette up and down gently to resuspend.

NOTE: Hematocrit of the cell suspension should not exceed 50%.

II. Set Plasmodipur filter on top of an uncapped 50 mL tube. Attach Kwill tube (or large gauge needle [18 or 19 g] if Kwills are unavailable) to syringe and use to draw up 3 - 5 mL RPMI or PBS. Mount the free end of the Kwill to the top of the Plasmodipur filter as illustrated in Figure 3, or remove the needle and mount syringe directly to Plasmodipur filter.

III. Apply gentle pressure to pass the RPMI or PBS through the filter such that fluid passes drop-wise through the bottom end of the filter. Discard filtered RPMI or PBS.

IV. Use the syringe plus Kwill to slowly draw up blood sample. Mount onto Plasmodipur filter.

V. If no Kwills are available, either transfer blood to a 50 mL tube so that you can use a syringe and large gauge needle (18 or 19 g) to draw up the blood (syringes will not fit inside 15 mL tubes); OR use a transfer pipet to transfer the blood into an open syringe attached to the Plasmodipur filter with the plunger removed. Remove needle and mount syringe directly on to Plasmodipur filter. If the plunger has been removed to pipet the blood into the syringe, use great care to re-insert the plunger very slowly and gently, to avoid forcing blood through the filter too quickly.

VI. Apply **very gentle** pressure, with intermittent pausing, to slowly pass the blood through the filter so that blood flows in a very slow, drop-by-drop fashion. After the syringe is empty of blood, air can be drawn into the empty syringe to apply gentle pressure to pass the remaining blood through the filter. Stop applying pressure as soon as the syringe and Kwill are empty of blood, being very careful to avoid introducing air into the filter. Allow the blood to drain until there is no residual blood in the Plasmodipur chamber above the filter.

VII. Draw 3-5 mL of RPMI or PBS into a fresh syringe or transfer pipet. To avoid introducing air between the blooded filter and the flush solution, use a Kwill, needle or transfer pipet to place flush solution direction onto the filter, filling filter chamber with solution from the bottom up. Applying **very gentle** pressure with intermittent pausing, wash filter to pass residual RBCs through. Stop when fluid exiting the bottom of the filter is mostly clear.

NOTE: A second flush with 2-3 mL of PBS can be performed if the fluid still appears very bloody. Some blood will remain visible on the filter after washing. Discard the filter.

VIII. Centrifuge at 1000 × *g* for 10 min to pellet the RBCs. Remove supernatant.

IX. Store pellet in 1 or 2 Cryotubes at -20° C or colder until DNA extraction. If pellet is larger than 1.5 mL, split into two aliquots. Label carefully with unique identifier, study or site, and date. Pellets can remain frozen for long-term storage.

A) B)

Figure 3. Preparing a Plasmodipur filter. A) Plasmodipur filter mounted on top of an uncapped 50 mL centrifuge tube. B) Kwill attached to syringe mounted on top of Plasmodipur filter. Although not shown in the illustration, use of clamps and clamp stands to secure the filter and syringe is recommended. Image source: Susana Campino, 2010.

6.5 Filter paper samples

Filter paper samples will not produce sufficient DNA for most genome-wide genotyping platforms, but will be useful sources of back-up parasite DNA for examining candidate genes and markers identified in genome-wide studies. FTA cards are preferable since they are treated with agents that protect nucleic acids and prevent microbial growth, and can maintain stability of DNA for years. If FTA cards are unavailable, extra care should be taken to keep 3MM filter papers cool and dry to preserve integrity of the DNA.

- Label card or paper with unique identifier, study or site, date and post-treatment day, if applicable.

- Blot several drops of blood onto an FTA elute card to fill circles or on to edge of pre-cut filter paper (from finger prick or from EDTA blood sample collected at screening).

- Dry blood spot thoroughly at room temperature (do not apply excess heat) . Store at room temperature in a dark, dry place at room temperature in sealed plastic bag with desiccant pack.

6.6 Optional: parasite cryopreservation

NOTES*:* All steps are at room temperature unless otherwise stated. If available, all procedures should be performed under a sterile hood to avoid bacterial contamination. Aseptic techniques should be used to the extent possible.

- Collect at least 4 mL of blood in heparin or EDTA
- Preserve at least six 0.5 mL aliquots in Glycerolyte Cryoprotective solution as follows:
- Centrifuge blood for 10 min at 700 × *g*. To convert between RCF (× *g*) and RPM, see Appendix A.
- Remove the supernatant and estimate volume of packed cells, V.
- SLOWLY add 0.33 × V of glycerolyte through gentle mixing. Let the tube stand for 5 min.
- Add dropwise 1.33 × V of glycerolyte, mixing gently using a swirling motion.
- Distribute 1 mL of the preparation per sterile cryotube.
- Freeze at −70° C for at least 18 h.
- Transfer vials to liquid nitrogen for long-term storage.

6.7 Optional: RNA preservation

Ideally this should be performed on leukocyte-depleted blood.

- Collect 2.5 mL of blood into PAXgene Blood RNA tube.
- RNA in in collected blood will remain stable for 3 days at 18-25° C, 5 days at 2-8° C, or up to 6 months at -20° C or -70° C.
- Extract RNA using the PAXgene Blood RNA Kit (Qiagen) according to the manufacturer's protocol (PAXGene RNA Kit Handbook).
- Transfer final RNA eluate to a sterile cryotube.
- RNA should be stored at -20° C or -70° C.

7. References

Parasite cryopreservation:
Methods in Malaria Research, Malaria Research and Reference Reagent Resource Center (MR4) http://www.mr4.org/Portals/3/Methods_In_Malaria_Research-5theditionv5-2.pdf

Appendix A: Conversion between RCF (x *g*) and RPM

Relative centrifugal force, or RCF (measured in times gravity or x *g*), depends on both revolutions per minute (RPM) and the radius of the centrifuge's rotor. The relationship between RCF (x *g*) and RPM is as follows:

$$g = (1.118 \times 10^{-5}) * (\text{radius of rotor in cm}) * (\text{RPM})^2$$

A conversion calculator can be found at:
http://www.beckmancoulter.com/resourcecenter/labresources/centrifuges/rotorcalc.asp

A conversion table can be found at:
http://www.piercenet.com/files/TR0040-Centrifuge-speed.pdf