Preparation of *Plasmodium vivax* field isolates for Whole Genome Sequencing

Procedure:

Cellulose Depletion of White Blood Cells from Whole Blood, and Short-term *ex vivo* Culture for *P. vivax* Field Isolates

This Procedure was developed by:

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Version History

Review Date	Modifications
19 April 2010	Adjustment of the *P. vivax in vitro* microtest (anti-malarial drug sensitivity assay) for *P. vivax ex vivo* schizont maturation
29 August 2012	Adjustment to include one cellulose column only

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Rationale

In order to yield good quality whole genome sequence data from patient samples of *Plasmodium*, it is essential to reduce 'contamination' from human DNA by depleting the white blood cell (WBC) content. The protocol described here includes two steps to deplete the WBCs – buffy coat separation (section 2), and cellulose filtration (section 3). Owing to frequently low parasite densities in *P. vivax* infections, we highly recommend an additional short-term culture step (section 4) where the facilities are available.

1. Blood Collection in the Field

Prior to patient treatment, collect whole blood samples in blood tubes containing anticoagulant (e.g. EDTA or CPDA-1). Ensure that the sample is inverted several times to mix the anticoagulant into the blood. Immediately store the sample at 4°C until it can be processed to remove the WBCs. **NOTE: Do not freeze the sample.** Immediate processing (<6 hours) is preferable in order to avoid substantial hemolysis of red blood cells (RBCs) and WBCs.

Suggested volumes

A minimum of 500 ng DNA is currently advised for standard (PCR-positive) whole genome sequencing using the Illumina platforms (note: thresholds are subject to change with technological developments). For PCR-free sequencing (which generally yields more uniform coverage), a minimum of 1μg DNA (preferably at ~20 ng/μL) is required. Higher DNA quantities reduce the probability of library preparation failure.
With consideration of DNA loss during WBC removal and DNA extraction, suggested volumes in order to yield at least 500 ng DNA are a minimum of 5 mL whole blood with moderate parasite density (≥4000 parasites/μL if short-term culture is not undertaken). For higher parasitaemias, lower blood volumes may be sufficient.

Suggested documentation to aid troubleshooting

It is useful to record the information below to allow the factor(s) responsible for poor WBC depletion efficacy to be identified, so that the procedure can be adjusted and optimized:

- Time/date of blood collection
- Time/date at start of WBC processing
- Time at end of WBC processing
- Volume of blood (pre-processing)
- Parasite density (pre and post cellulose filtration)
- Note any evidence of blood lysis or clotting

2. WBC Depletion by Buffy Coat Separation

EQUIPMENT/REAGENTS

- Plastic Pasteur (transfer) pipettes
- Centrifuge for blood tubes

PROCEDURE

1. Spin the EDTA/CPDA-1 blood tube in a centrifuge at 600-700g for 10-15 min at room temperature (RT: ~22°C). Use a low deceleration setting.
2. The blood will separate into 3 fractions – a plasma layer at the top, a concentrated RBC layer at the bottom, and a concentrated WBC layer (buffy coat) in between.
3. Remove and either discard or store the plasma layer for other studies.
4. Remove the buffy coat without disturbing the RBC layer - *P. vivax* schizont stages lie just below the buffy coat so care should be taken to avoid removing the top layer of RBCs. Either discard or store the buffy coat for other studies.
5. Add an equal volume of sterile PBS to the red cells and mix gently (i.e., if 2.5 mL RBC pellet, add 2.5 mL PBS).

3a. Preparation of the Cellulose Columns

EQUIPMENT

- 10 mL plastic syringes, centered not offset
- Glass wool and microscope lens cleaning tissue (Whatman, Cat. no. 2105-841)
- Cellulose powder (Sigma, Cat. no. C6288)

PROCEDURE

1. Remove plunger from syringe.
2. Fold Whatman filter paper twice and insert into the syringe to cover the tip of the syringe.
3. Add a small bundle of glass wool on top of the filter paper (if glass wool is not available, use an extra layer of filter paper).
4. Fill the syringe to the 5 mL mark with cellulose powder. Do not compress the powder; just tap the syringe to allow the powder to settle.
5. Cover the top and the tip of the cellulose column with aluminium foil and autoclave. After autoclaving, allow the columns to dry completely overnight and store in a cool, dry place until use.

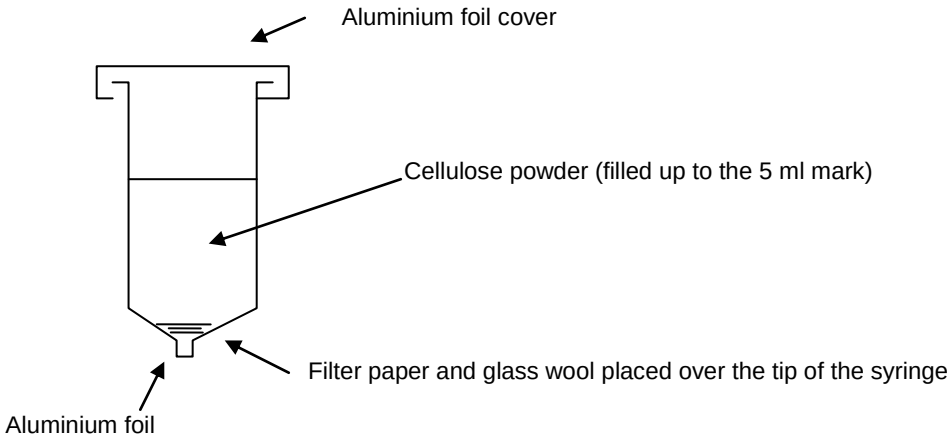

Figure 1. Preparation of Cellulose Columns

3b. WBC Depletion by Cellulose Filtration

EQUIPMENT/REAGENTS

- Cellulose columns
- 15 mL Falcon™ tubes
- Sterile 1 x PBS
- Plastic Pasteur (transfer) pipettes
- Centrifuge for 15 mL tubes

PROCEDURE

1. Remove the foil from the top and tip of a cellulose column and place the column over a 15 mL Falcon™ tube. Hold in place with sticky tape.
2. Wet the column by adding 5-10 mL PBS – wait until the PBS starts to drip through the tip of the syringe (when the column is fully wet) before proceeding to the next step.
3. Pipette the RBC suspension onto the cellulose column and allow the blood to pass through the column **without applying force**.
4. Wash the RBCs through the column by adding PBS until the liquid dripping into the 15 mL Falcon™ tube is no longer pink/red (you may need to use additional 15 mL Falcon™ tubes).
5. Centrifuge the blood at 1200 rpm for 5-10 min at RT.
6. Pipette off the supernatant and discard it.
    a. If short-term culture is possible, proceed immediately with the protocol outlined in section 4.

    OR

    b. If short-term culture is not possible, store the remaining RBC pellet at 4ºC for short-term storage (up to a week), or -20ºC or -80 ºC for long-term storage, until DNA extraction can be undertaken.

4. Short-term Culture

EQUIPMENT/REAGENTS

- McCoy's 5A medium (supplemented with HEPES [25 mM], L-glutamine [2 mM], and gentamicin [40 mg/L])
- AB^+ (or matched) human serum (heat-inactivated at 56°C for 30 min)
- D-Glucose solution (7.5% w/v)
- 15 mL and/or 50 mL Falcon™ tubes
- Culture flasks with filter cap (e.g. 75 cm^3)
- Serological pipette handler
- Serological pipettes (5 mL, 10 mL, 25 mL)
- P-20 and P-200 micropipettes
- P-20 and P-200 pipette tips
- Incubator (to maintain temperature at 37ºC)
- Candle jar and paraffin candles
- Centrifuge for 15 mL and/or 50 mL Falcon™ tubes
- Binocular microscope with 10x, 40x and 100x objectives
- Microscopy slides
- Standard reagents for malaria slide preparation (Giemsa stain, methanol, etc.)

PROCEDURE

1. Prepare **incomplete** McCoy's medium by adding D-Glucose (7.5% w/v) to a final concentration of 0.24%.
   - E.g. to 100 mL McCoy's medium (HEPES [25 mM], L-glutamine [2 mM], and gentamicin [40 mg/L]), add 3.2 mL D-glucose (7.5% w/v).
2. Prepare **complete** McCoy's medium by adding 20% AB^+ (or matched) human serum to the incomplete medium.
   - E.g. to 80 mL incomplete medium, add 20 mL AB^+ (or matched) human serum.
3. Add the post-cellulose filtered RBC pellet to the **complete** medium to prepare a **2% haematocrit** blood medium mixture (**BMM**).
   - E.g. add 2 mL packed RBCs to 98 mL complete medium.
4. Add the **BMM** to a culture flask (split accordingly into multiple flasks for large volumes) and leave in an incubator (for up to 48 hours) maintained at 37.0°C, using a candle jar to control O_2 and CO_2 levels.
5. Prepare occasional blood smears to assess the growth of the culture by microscopy (24 hours, then ~12 hour or ~6 hour intervals). If there are signs of extensive parasite death, stop the culture immediately. Otherwise, harvest the culture when ~40% of the rings and early trophozoites have matured into schizonts (i.e., before extensive schizont rupture) at ~40-48 hours.
6. To harvest the cells, remove the excess supernatant, transfer the culture to 15 or 50 mL Falcon™ tube(s) and centrifuge at 2,000 rpm for 10 min.
7. Discard the supernatant and store the RBC pellet at 4ºC for short-term storage (up to a week), or -20ºC or -80 ºC for long-term storage, until DNA extraction can be undertaken.

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