# ACROSS - Laboratory Procedures

**Version 0.2**

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# Contents

**Samples collected:**

Hospital-based Surveys: - Venous blood (5.0-9.5 mL)

Cross-Sectional Surveys: - Capillary blood (200-500 µL)
- Venous blood (5.0-9.5 mL) from 1 volunteer/household

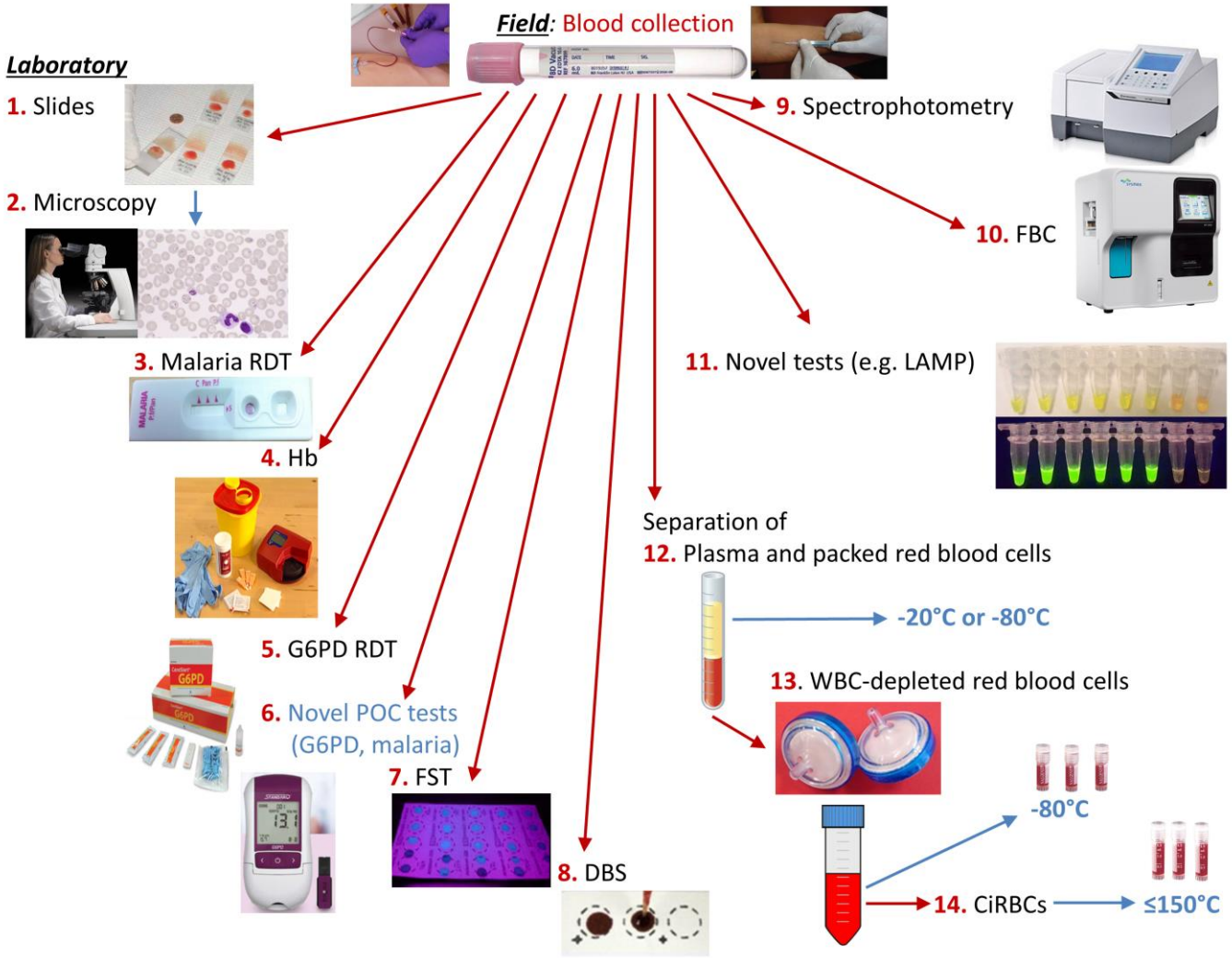
WHO guidelines on drawing blood: Best practices in phlebotomy (2010)
http://apps.who.int/iris/bitstream/10665/44294/1/9789241599221_eng.pdf

## OVERVIEW SAMPLE COLLECTION:

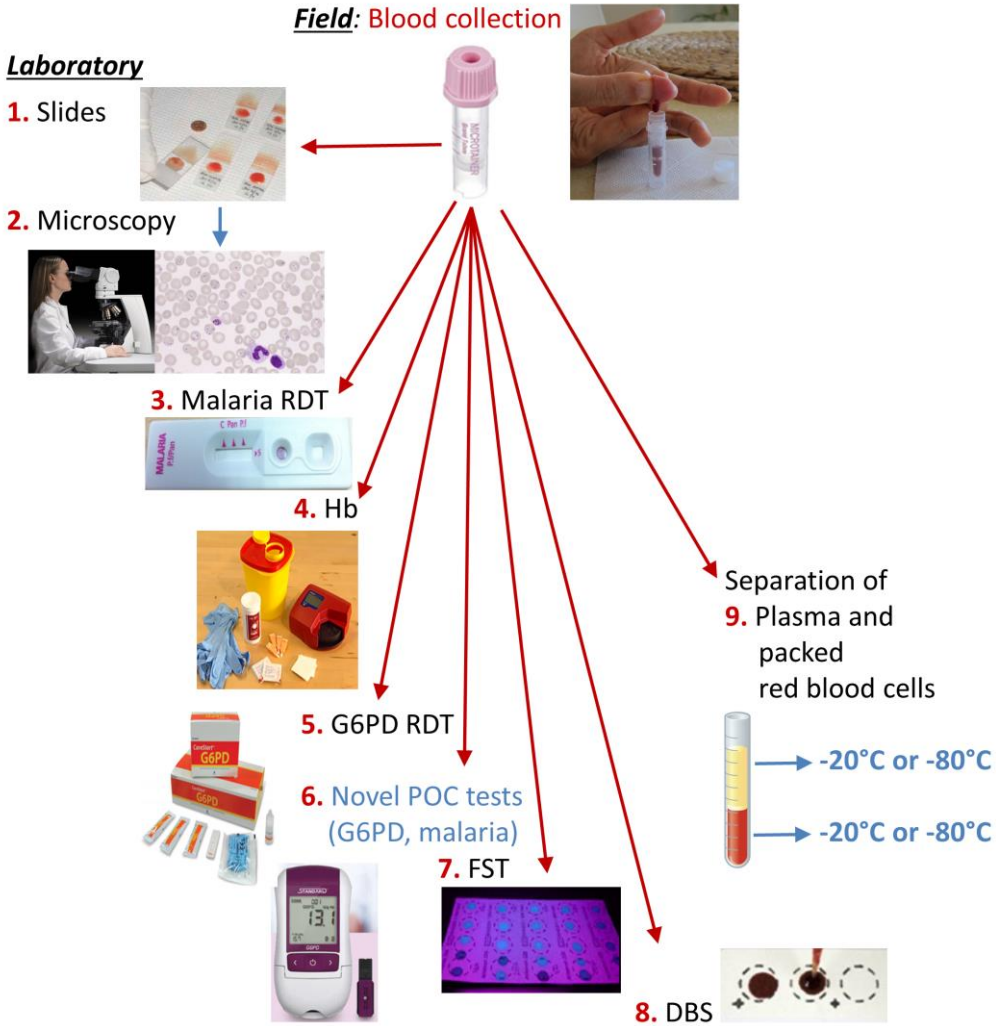
| **Venous blood** | **Capillary blood** |
|---|---|
| ***Field**:* | ***Field**:* |
| Blood collection | Blood collection |
| ***Local laboratory**:* | ***Local laboratory**:* |
| • Slides<br>• Microscopy<br>• Malaria RDT#<br>• Hb#<br>• G6PD RDT (*CareStart™*)<br>• Novel POC tests (G6PD, malaria) #<br>• FST#<br>• DBS | • Slides<br>• Microscopy<br>• Malaria RDT#<br>• Hb#<br>• G6PD RDT (*CareStart™*)<br>• Novel POC tests (G6PD, malaria) #<br>• FST#<br>DBS |
| ***Reference laboratory**:* | ***Reference laboratory**:* |
| • Spectrophotometry#<br>• FBC#<br>• Novel tests (e.g. LAMP) #<br>Separation of:<br>• Plasma and packed red blood cells<br>• WBC-depleted red blood cells<br>• CiRBCs<br>• Sample storage | • Spectrophotometry#<br>• Novel tests (e.g. LAMP) #<br>Separation of:<br>• Plasma and packed red blood cells*<br>• CiRBCs<br>• Sample storage |

# : According to the manufacturer's procedures (see Annexes)

# SAMPLE PROCESSING FLOW CHART – VENOUS BLOOD



# SAMPLE PROCESSING FLOW CHART – CAPILLARY BLOOD



# A) WHOLE BLOOD PROCESSING & ANALYSIS [LOCAL LABORATORY]

## 1. Microscopy Slides

Staff responsible for the preparation of the thick and thin smears and staining of the slides must be trained and must practice making smears according to the below SOP before the start of the study.

Up to two slides, each with both thick and thin smears can be prepared with each sample.

### *1.1 Slide preparation*

#### *1.1.1 Materials*

- Glass slides: new, clean, grease-free, with one frosted end
- Pencil or permanent marker
- Micropipette 0.5-20 µL
- Slide template
- 'Spreader' slide with bevelled corners and ground edges
- Methanol absolute (lab reagent grade or better)
- Slide warmer (if available)
- Slide drying rack

#### *1.1.2 Procedure*

- Write onto the frosted end of the slide the study code and date of blood collection, using a pencil or a permanent marker.
- Place the labelled slide on the template shown in Figure 1 below.
- Using a micropipette, place 6 µL of whole blood for the thick film and 2-5 µL for the thin film as shown on the template.

  ***** *Note*: Reverse pipetting is advisable when transferring blood with a micropipette to a slide to prevent the formation of bubbles in the thick film.
- Using the ground edge of the spreader slide, spread the blood for the thin film.
- Using the bevelled corner of the spreader slide, spread the blood for the thick film until the entire circle of 12 mm diameter is covered evenly.
- Dry the films on a flat surface, protected from dust and insects. Slides must be completely dry before staining by drying on a slide warmer at 37-40ºC for 1 hour, or overnight at ambient temperature.
- Fix the thin film by dipping in absolute methanol for a few seconds and then letting the slide air dry. The slide can be stained immediately after drying or can be stored up to a month in a sealed box with ample desiccant in a cool, dry place for staining at a later 1-2 months.

  ***** *Note*: Dry the thin film at an acute angle, with the film-side of the slide facing up and the thin film downwards. This protects the thick film from being fixed by methanol fumes and run-off.

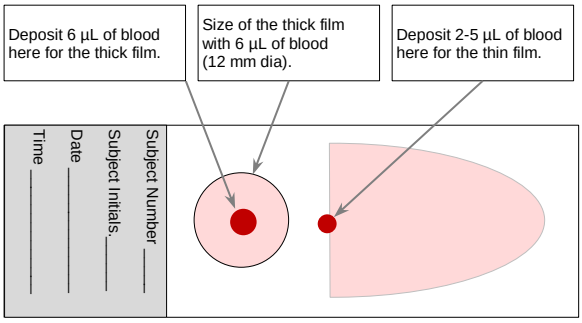



**Figure 1**: Template for preparation of slides with thick and thin blood smear

***** *Note*: Although it is possible to make thick and thin blood smears of acceptable quality without using a micropipette and template, using these tools helps ensure reproducibility and facilitates training of laboratory technicians.

## *1.2 Slide staining*

### *1.2.1 Materials*

- Volumetric flask or measuring cylinder, 1 L
- Distilled water
- Concentrated buffer solution or buffer tablets, pH 7.2
- 2% solutions of $Na_2HPO_4$ and $KH_2PO_4$
- pH meter or pH indicator paper
- Funnel
- Filter paper
- Dark or opaque bottle with stopper or cap
- Concentrated Giemsa solution
- Measuring cylinder, 50-100 mL
- Micropipette (100-1000 µL) with tips or 1-2 mL pipette with pipetting aid
- Buffered water, pH 7.2
- Slide staining rack
- Timer
- Forceps
- Wash bottle
- Slide drying rack

### ***1.2.2 Procedure***

#### ***1.2.2.1 Preparation of buffered water***

- Measure out 900 mL of distilled water into the volumetric flask or measuring cylinder.
- Adjust pH to 7.2.
- Using a calibrated pH meter or pH indicator paper, verify that the pH of the buffered water is within an acceptable range (pH 7.2 ± 0.1). Adjust the pH if needed using solutions of $Na_2HPO_4$ and $KH_2PO_4$ (2% w/v).

  ** Note*: If using commercially prepared buffer tablets, the pH is usually within acceptable ranges without adjustment. If needed, however, refer to the Basic Malaria Microscopy learner's guide (WHO, 2010, 2nd edition) for recipes of the solutions and further details on how to adjust the pH.

- Complete the volume of the buffer solution up to 1 L and store at 2 – 8 ºC until further use.

#### ***1.2.2.2 Preparation of Giemsa working solution***

- Using a funnel and filter paper, filter the stock Giemsa solution into a dry, dark or opaque bottle.

  ** Note*: Keep the bottle capped until the stain is used as moisture will cause the Giemsa stain to deteriorate. The concentrated stain solution can be filtered once daily. Any remainder may be poured back into the original container while taking care not to agitate the stain.

- Determine the volume of diluted stain (3% v/v) to be prepared from the below table:

| *Number of slides* | *Giemsa stock solution (mL)* | *Final volume, made up with buffered water pH7.2 (mL)* |
|---|---|---|
| 1 | 0.15 | 5 |
| 2-3 | 0.3 | 10 |
| 4-6 | 0.6 | 20 |
| N | 0.03 x N | 3 x N |

- Transfer buffered water (about half of the final volume) to a measuring cylinder and add the appropriate volume of concentrated stain solution. Complete the volume up to the level determined from the table above.

  ** Note*: Make sure that only dry pipets come in contact with the stock Giemsa stain as any introduced moisture will hasten the deterioration of the stock Giemsa stain. To ensure accurate dilutions, volumes of concentrated Giemsa stain should be measured using micropipettes or 1 – 2 mL serological pipettes with pipetting aids.

#### *1.2.2.3 Staining slides*

- Place the slides film-side up on a staining rack.
- Stain the slide for 40-50 minutes by covering them completely with freshly prepared 3% v/v Giemsa solution.

  ***** *Note*: If more than 10 slides are being stained at the same time, the timer should be started after the first slide has been flooded with stain.

- Rinse the slide by flooding it with a gentle flow of buffered water until the stain is removed.
- Allow the slide to dry completely, protected from dust. A drying box or a hair-dryer can be used for this purpose. Slides can be read immediately after they are dry or stored in cool, dry place within a sealed box with ample desiccant.

## *1.3 Quality control*

Blood films should be checked for obvious signs of poor quality and repeated if any such signs are seen:

- Dust on the films
- Films washed off during staining or rinsing

Before slides are read to detect or to estimate the density of malaria parasites, they must be assessed for quality with respect to the criteria below by scanning a few fields on the slide using immersion oil and the 100X objective. If the slide is not readable, it should be logged as such and the slide preparation must be repeated.

Slide quality assessment criteria for thick films:

- 6 to 14 WBC (white blood cells) per HPF (high power field) on average (with WBC count in the range of 6,400 – 12,800 per µL at 1,000 x total magnification and ocular lens Field Number 18)
- Pale blue-grey background (lysed RBC) free of dust and other artefacts
- WBC nuclei should be stained deep purple and platelets should be clearly visible and bright pink

Slide quality assessment criteria for thin films:

- Presence of a 'tail' or feathered edge with evenly distributed RBCs and no or very few overlapping cells
- RBCs stained grey-pink

## *1.4 Slide storage*

Before storage after microscopy, immersion oil must be removed from the slides by placing the slides smear-side downwards on soft tissue paper or newspaper for one night. Care must be taken while handling the slides not to wipe the slides on the tissue paper as this may cause the thick smear to come off. Slides must be stored in slide boxes with desiccant sachets and the boxes must be sealed once they are full to avoid degradation of the slides.

## 2. Microscopy

Microscopy reading of slides must be performed by individuals who have been trained to perform this task and in applying the relevant health and safety precautions.

### *2.1 Materials*

- Microscopy result log books
- Microscope:
    - Objective: 100X oil-immersion, plan achromatic lense
    - Ocular: 10X, Field Number 18
- Electrical light source
- Synthetic immersion oil, refractive index ≥ 1.5
- Single or multi-parameter tally counters
- Absorbent paper or Histoclear® reagent
- Slide boxes
- Adhesive tape

### *2.2 Procedure*

#### *2.2.2 Selection of thick or thin film for parasite density estimations*

- Place a small drop of immersion oil on the thick and thin films
- Scan 10 fields on the thick film using the 100X immersion oil objective
- Assess the slide quality according to the criteria outlined above and reject the slide if the quality is poor
- Count the numbers of parasites and WBCs seen
- If the number of asexual parasites is less than or equal to twice that of WBCs, continue with asexual parasite detection or parasite density estimation on the thick film
- If the number of parasites is greater than twice the number of WBCs, perform the asexual parasite density estimation on the thin film

#### *2.2.3 Detection and parasite density estimation on the thick film*

- Use tally counters set to zero to count the number of WBCs or fields and asexual/sexual parasites. Start counting from the first good quality field (i.e., fields with little or no debris/artefacts and 10-20 WBCs)

  ***** *Note*: Do not wait to find a parasite before starting to count WBCs/fields.

- If no asexual or sexual forms of the parasites are seen after 2,000 WBCs, declare the slide negative.
- Count all asexual stages in either single or mixed species infections. In mixed species infections, all asexual parasites are counted and multiple species are reported.
- Sexual stages (gametocytes) are not counted, but reported.
- Counting rules:
    - If ≥ 100 parasites were counted in 200 WBCs, counting is stopped and the result recorded as parasites per 200 WBCs.
    - If < 100 parasites were counted in 500 WBCs, counting is stopped and the result recorded as parasites per 500 WBCs.

- Record the result on the CRF as raw figure, i.e., species of the parasites, the number of asexual or sexual forms of the parasite over the number of WBCs counted.

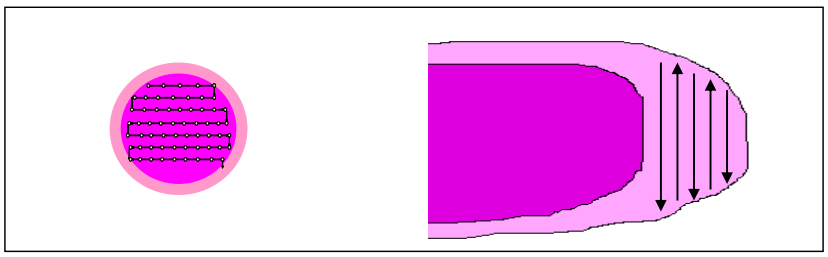


**Figure 2**: Diagrammatic representation of reading patterns on thick and thin blood films.

### ***2.2.4 Parasite density estimation from the thin film***

***** *Note*: If ≥ 100 parasites are present in each high power field (HPF, 100x objective) of a thick film, parasite counts have to be done on the thin film.

- Use tally counters set to zero to count the number of RBCs and asexual parasites. Start counting from the first good quality field (i.e., fields with little or no debris/artefacts and evenly spread, non-overlapping RBCs at the 'tail' or feathered edge of the thin film)

  ***** *Note*: Do not wait to find a parasite before starting to count RBCs.

- Count parasitized RBCs along with normal RBC's until a total of 5,000 RBCs have been counted. Count RBCs with more than one parasite or with multinucleate forms as 1 infected RBC. Parasites of different species need not be counted separately.

  ***** *Note*: Presence of *P. falciparum* schizonts, especially in large numbers, may indicate severe disease and clinicians must be informed accordingly.

- In the unlikely event that no asexual parasites are seen on the thin film after counting 5,000 RBCs, continue examining the slide on the thick film as described in the section above.

  ***** *Note*: If the selection of thin versus thick film for parasite density estimation has been done correctly, it is highly unlikely that no asexual parasites are seen on the thin film unless the thick film is uneven or the subject has an abnormally high RBC count.

- Count at least 500 WBCs on the thick film to check for the presence of other species and/or sexual forms of the parasite.
- Report the result in the laboratory journal as raw figure, i.e., the species of the parasite and the number of infected RBCs per total number of RBCs counted.

### ***2.2.5 Slide storage***

- Remove the immersion oil by leaving the slides overnight face down on absorbent paper (newspaper or tissue paper) or by dipping in Histoclear reagent and swirling gently.

  ***** *Note*: Histoclear® reagent is a solvent and is flammable. If using permanent markers to label the slides, protect the label from exposure to Histoclear® reagent. Use Histoclear® reagent in an open and airy space, away from sources of heat and/or open flames.

- Sort slides according to the date of collection, by patient, by study time-point, positive/negatives, etc. as convenient.
- Store the slides in slide boxes and log their positions. Protect the slides from humidity.

### *2.3 Quality control*

Proficiency of the microscopists in the ACROSS study should be tested regularly following national guidelines. Such guidelines should have been updated according to the WHO Malaria Microscopy Quality Assurance Manual (Version 2, 2016).

## 3. RDT (Rapid diagnostic test malaria)

Differing brands and types of malaria RDTs are used in different countries. Hence, malaria RDTs will be performed according to the manufacturer's standard operating procedures (SOP) that are provided with the RDT kits. The Quick Reference Guide for the SD BIOLINE Malaria Ag P.f/Pan RDT, as an example, is shown in Figure 3.

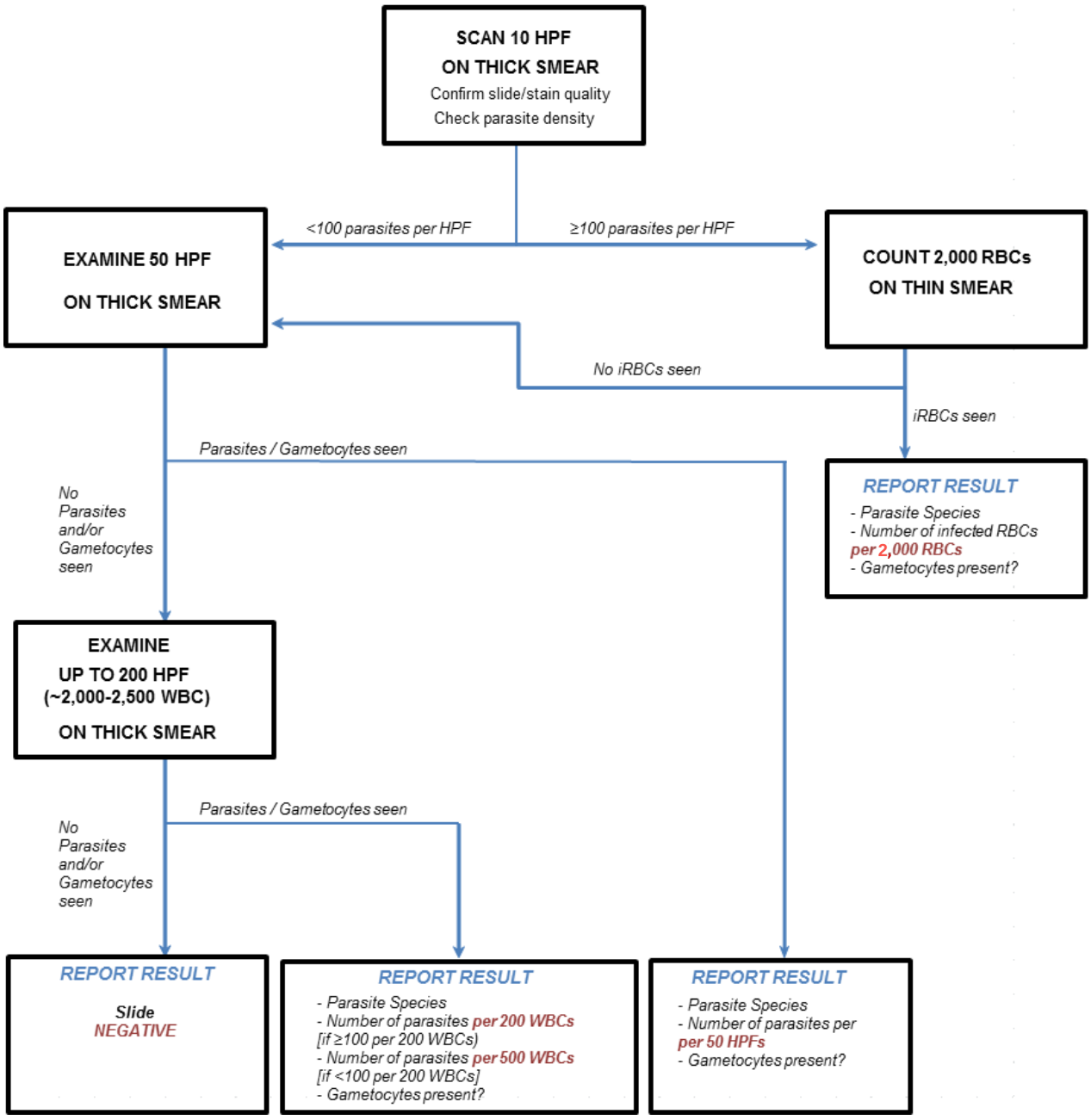



**Figure 3**: Counting and reporting microscopy results.


**Figure 4**: Quick Reference Guide for the SD BIOLINE Malaria Ag P.f/Pan RDT.

## 4. Hb (Haemoglobin measurement)

Haemoglobin measurements will be performed using Hemocue Hb301, or equivalent devices, according to the manufacturer's operating manual.

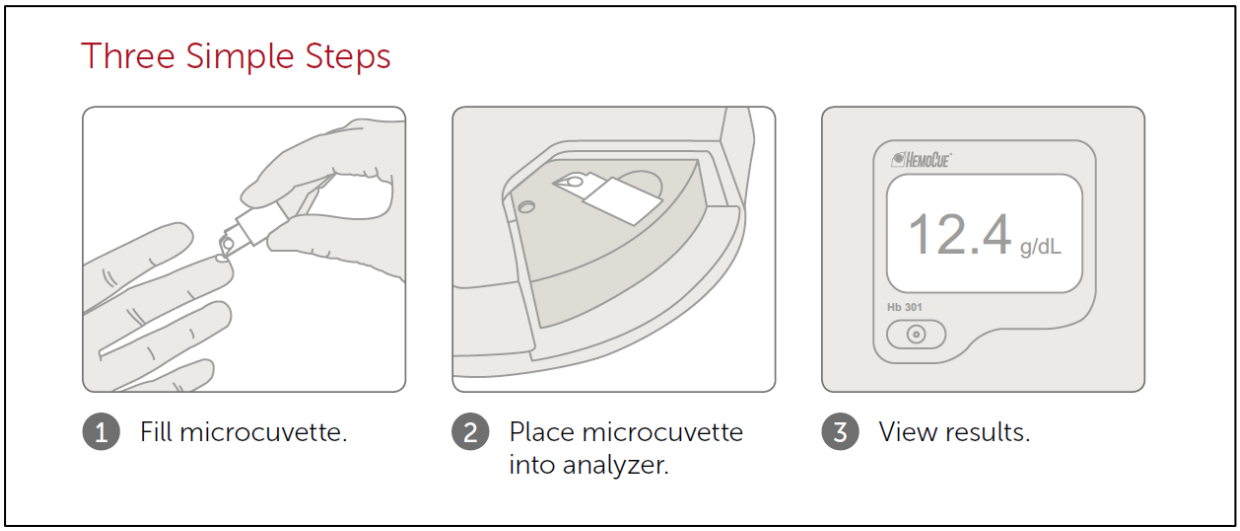



**Figure 5**: Short manual for Hemocue 301.

## 5. G6PD RDT (*CareStart™*)

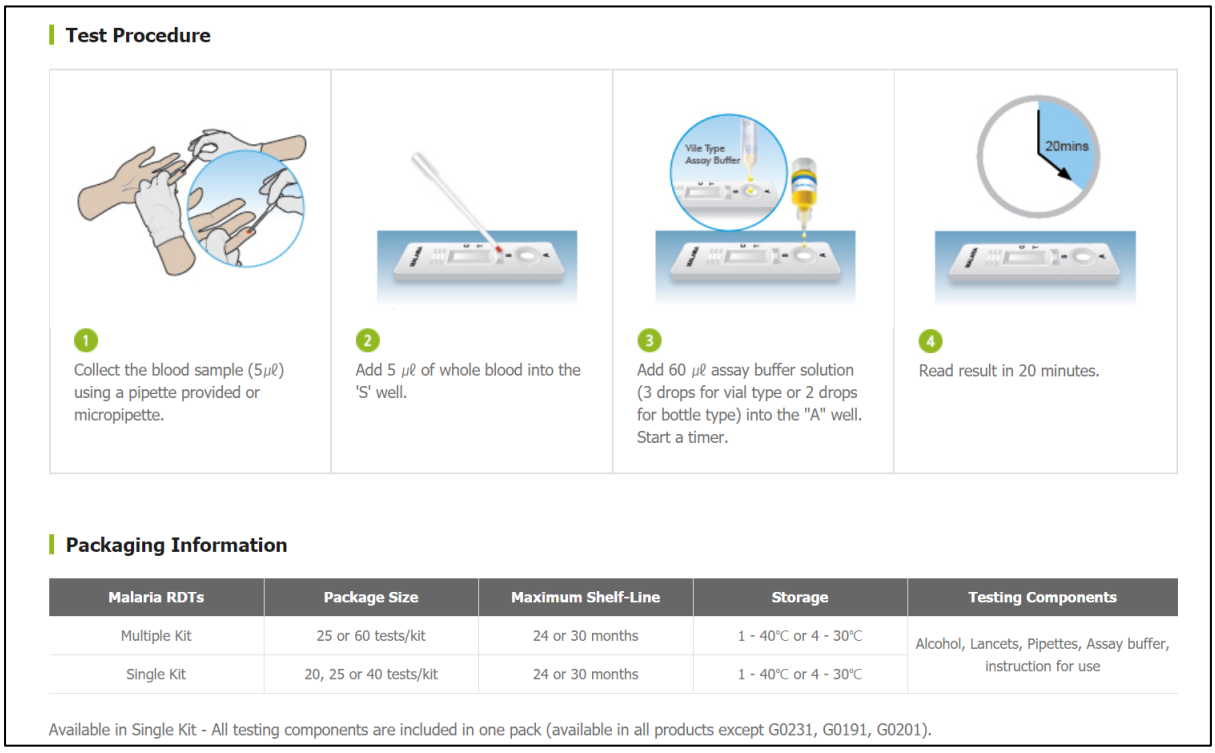



**Figure 6**: Quick guide to *Carestart™* G6PD RDT procedures.

# 6. Novel POC (point-of-care) tests (G6PD, malaria)

Novel POC tests will be performed according to the manufacturer's standard operating procedures (SOP).

## *6.1 Global Good Diagnostics, HRP-2 LFA*

### *6.1.1 Materials*

- GG/IVL LFA (Lateral Flow Assay)
- One micropipette (volume capacity 20–200 µL) per technician and two corresponding tips per test
- BD High-flow lancets for finger pricks when using capillary blood

### *6.1.2 Procedure (see also below picture)*

- Place 100 µL of blood onto the GG/IVL LFA in the well coloured red
- Place 100 µL of running buffer onto the GG/IVL LFA in the transparent well
- Let stand for 35 minutes
- Read and record result
- Immediately after recording of the 1st result, a second reader, blinded to the first result, must read and record the test result.

  ***** *Note*: **The test is not valid and need to be repeated if no control line appears. Please contact supervisor if this happens repeatedly**.

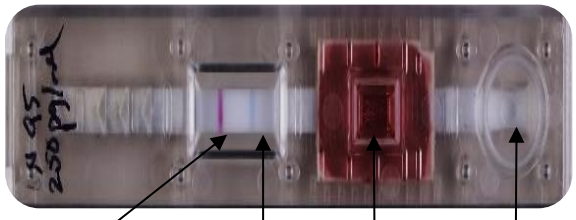
| **Details** | **Experimental RDT** |
|---|---|
| Blood transfer device | Pipette |
| Blood volume | 100 µL of blood |
| Buffer volume | 100 µL |
| Wait time | 35 minutes |
| Arrangement of test bands |  |

**Figure 7**: Job aid for the GG/IVL Experimental RDT.

## 7. FST (Fluorescent spot test)

FST for the qualitative assessment of G6PD activity will be performed according to the manufacturer's standard operating procedures (SOP).

The PATH guidebook and the Trinity Biotech SOP are appended to this document.

## 8. DBS (Dried blood spot)

DBS are prepared in the lab after blood for procedures 1-6 have been used. In order to standardize the volume per blood spot, it is recommended to us a micropipette to transfer the blood from the Vacutainer™ or Microtainer tube, respectively, onto the filter paper.

### *8.1 Materials*

- Filter paper (Whatman 2, 3 or 903)
- Micropipette (volume capacity 20–200 µL)
- Ziplock plastic bags
- Dessicant (e.g. left-over bags from recently opened RDTs)

### *8.2 Procedure*

- Cut Whatman filter paper into pieces measuring approximately 3.0 x 13 cm (Figure 4).
- Label paper with subject number, subject initials, and date and time of blood collection, using a pen.
- Designate and mark 3 distinct areas (i.e., 3 x 1.5 cm circles) on the Whatman 2, 3 or 903 filter with a pen.
- Pipette 30 µL whole blood from the collecting tube and drop it onto the designated spot area.
- Repeat this step for spot 2 and 3.
- Let the blood spots dry at room temperature for a minimum of 1 hour in an undisturbed area.

  ***** *Note*: Do not apply heat to dry the blood spots.

- Add a desiccant bag to each ziplock bag and place DBS from one patient in one bag only.
- Store samples
    - In a refrigerator at 4 °C when examination (e.g. genotyping and serology) is not to be performed immediately after collection of the sample, but within a 4-week period;
    - In a –20°C freezer when they are to be examined within 3-10 months;
    - In a –80°C deep freezer at –80°C when they are to be examined after a longer period (i.e. >1 year).

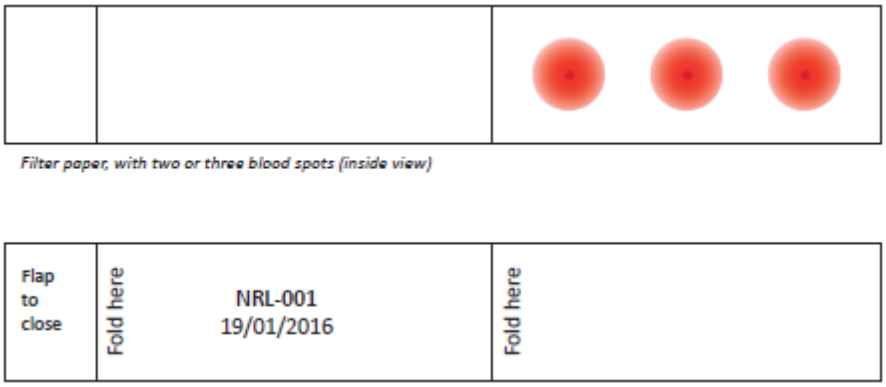



**Figure 8**: DBS layout using Whatman filter paper.
(MM-SOP-10, WHO, 2016)

## 9. Spectrophotometry (quantitative assessment of G6PD activity)[#]

The quantitative assessment of G6PD activity will be performed by using a commercial test according to the manufacturer's standard operating procedures (SOP).

The POINTE SCIENTIFIC kit manual is appended to this document.

### *9.1 Materials*

- POINTE SCIENTIFIC **Spectrophotometry Test Kit**:
  - **G6PD Reagent Set R1** (CATALOG No. G7583-180 PT)
  - **G6PD Lyse Reagent R2** (CATALOG No. G7583-LYS PT)
- POINTE SCIENTIFIC **G6PD Controls**:
  - G6PD Control **Normal** (CATALOG No. HC-108 ACS)
  - G6PD Control **Deficient** (CATALOG No. HC-108DE ACS)
- Spectrophotometer that allows measurement of **absorbance at 340 nM**; ideally, it also allows assessment of **fixed-time kinetics**.
- Timer
- Ice or cold box
- 3-5 mL reaction tubes with lid (alternatively, tubes can be sealed with Parafilm®)
- Pipettes that reliably deliver 100 µL, 200 µL, and 2.0 mL
- Buffered distilled $H_2O$, OR injectable $H_2O$ (for cleaning and BLANK reading)

### *9.2 Procedure*

#### *9.2.1 Before you start any measurement of control or patient samples*

- Prepare G6PD **R1** solution by reconstituting the G6PD **Reagent** vial with G6PD **Lyse Reagent R2**. Use the volume stated on vial R1.

  ***** *Note*: Do not use distilled $H_2O$ to reconstitute vial R1.

- Swirl gently and invert several times at room temperature (RT) to dissolve contents. Wait 2–3 minutes and mix again. Reconstituted G6PD **R1** solution is stable for 8 hours at room temperature (18–26°C), or 5 days refrigerated (2–8°C).

- G6PD **R1** solution can now be used as described below.

  *** Important:** Place fresh blood and controls, as well as R1 solution reagent R2 on ice, OR in a cold box (i.e., refrigerated at 2-8°C).

- Switch on the spectrophotometer and make sure that:
  - Cleaning procedures have been performed according to the manufacturer's recommendations.
  - Fixed-term kinetics program for absorbance at 340 nM at 37°C; 5 minutes (=300 seconds) lag phase plus 5minutes (=300 seconds) reading time, has been programmed.
  - Flow cell or block temperature of the spectrophotometer is at 37°C.
- Pre-label 3 reaction tubes per control (i.e., "NORMAL", "INTEMEDIATE", and "DEFICIENT") and patient (Patient ID, Date of Test, Time).

#### ***9.2.2 Measurement of control and patient samples***

- Process controls and samples in sequence (i.e., not in parallel).
- Add **1 mL** G6PD **R1** solution to tube 1 of control or sample.
- Add **10 µL control or fresh blood** and mix thoroughly to completely suspend erythrocytes.
- Let stand at room temperature (RT, 18–26°C) for 5–10 minutes.

****** *Note*: Make sure the spectrophotometer program is started and the **BLANK** (=aspiration of 750 µL buffered distilled $H_2O$) has been measured at this time point – Record the BLANK value in the lab book. The BLANK value should **not exceed 0.050**.

- Add **2.0 mL** reagent **R2** and mix gently by inverting several times.
- Aspirate sample immediately into the flow cell of the spectrophotometer.
- Read and record absorbance at **time point 0** (=after 5 minutes/300 seconds lag phase). This is the **INITIAL** measurement – Record the INITIAL value in the lab book.
- **Exactly 5 minutes later**, read and record absorbance 340 nm versus water again. This is the **FINAL** measurement – Record the FINAL value in the lab book.
- Enter INITIAL and FINAL test values of all 3 measurements into the Excel file "ACROSS_Spectrophotometry_results".
- Enter Hb value (gram/dL) into the Excel file "ACROSS_Spectrophotometry_results".
- The final values expressed as **U/gram Hb** are calculated automatically according to the "calculations" outlined in detail on the POINTE SCIENTIFIC specification sheet.

## 10. FBC (Full blood count)

FBCs will be performed using automated blood cell counters, if available, according to the respective manufacturer's operating manual.

# 11. Novel diagnostic assays for malaria and G6PD activity (e.g. LAMP)

Novel diagnostic assays will be performed according to the manufacturer's standard operating procedures (SOP).

## *11.1 Meridian Bioscience illumigene® malaria and illumigene® malaria PLUS LAMP assays*

### *11.1.1 Materials*

- Meridian Bioscience *illumigene*® malaria and *illumigene*® malaria PLUS kits (provided)
- Meridian Bioscience *illumipro*-10™ assay reader (provided)
- Micropipette capable of dispensing 50 µL
- Micropipette capable of dispensing 250 µL
- DNase/RNase-free, aerosol resistant pipette tips
- Laboratory timer

***** *Note*:

  - Do not interchange kit reagents and Test Devices between lots.
  - The *illumigene* Malaria Test Devices contain lyophilized reagents. The protective pouches should not be opened until ready to perform the assay.
  - The *illumigene* Malaria Test Devices include a latch feature that is designed to prevent contamination of the test area with amplification product. Do not use Test Devices with broken latches.

### *11.1.2 Procedure*

Before the start of the assay, all the kit reagents and buffers need to be warmed to room temperature.

For the *illumigene*® malaria PLUS assay, the *M-prep* columns have to be visually inspected. Columns with disturbed resin beads or improperly placed filters shall not be used.

Samples (i.e., blood collected into EDTA containing tubes) should be tested as soon as possible. However, they may be stored for up to 7 days at room temperature (19-30 °C) or up to 14 days refrigerated (2-8 °C) prior to testing. Samples that will not be tested within this time frame should be frozen immediately at -20 °C for up to 30 days until tested.

#### *11.1.2.1 illumigene Malaria Specimen Preparation (Figure 9)*

- Invert EDTA whole blood sample 2-3 times to mix.
- Add 50 µL of the collected whole blood sample (with EDTA) to one tube of Buffer I. Mix by inversion 5 times or by vortexing for approximately 10 seconds. Hold the sample for 2 minutes.
- Mix by inversion 5 times or by vortexing for approximately 10 seconds and immediately transfer 50 µL of lysate into SMP PREP IV. Mix by inversion 5 times or by vortexing for 10 seconds.
- Gently squeeze the SMP PREP IV and slowly collect 5 to 10 drops into a clean Tube I. Visually verify that the eluate is tinted red to reddish brown. Label the tube with the specimen identification and proceed to the Test Procedure (11.1.2.3).

***11.1.2.2 illumigene Malaria PLUS Specimen Preparation (Figure 10)***

- Invert EDTA whole blood specimen 2-3 times to mix.
- Add 50 µL of the collected venous whole blood sample (with EDTA) to one tube of Buffer I. Mix by inversion 5 times or by vortexing for approximately 10 seconds. Hold the sample for 2 minutes.
- Mix by inversion 5 times or by vortexing for approximately 10 seconds and, using a micropipette, immediately transfer 250 µL of the prepared sample to the top of an appropriately labeled and prepared M-prep Column. Wait approximately 2 minutes, or until the sample has been absorbed by the column and flow stops. This step should take no longer than 30 minutes.
- Using a micropipette, add 250 µL of M-prep Buffer II to the top of the M-prep Column. Discard the pipette tip. The column will have a red appearance after the addition of M-prep Buffer II. Wait approximately 2 minutes, or until the red-colored buffer is absorbed by the column and flow stops. This step should take no longer than 30 minutes.
- Remove the last drop of liquid from the column tip with the ST Tube. Discard the tube.
- Place a clean ST Tube under the M-prep Column. Using a micropipette, add 250 µL of M-prep Buffer III to the top of the M-prep Column. Discard the pipette tip. Wait approximately 2 minutes or until flow stops. This step should take no longer than 30 minutes.
- Remove the last drop of liquid from the column tip with the ST Tube. Visually verify that the eluate is tinted red to reddish brown. Label the tube with sample identification information and proceed to the Test Procedure (11.1.2.3).

  ***** *Note*: Sample elution steps with M-prep Columns should take no longer than 30 minutes. Samples that take longer than 30 minutes to elute should be discarded and re-tested with the original patient sample.

***11.1.2.3 illumigene Malaria and Malaria PLUS Test Procedure***

- Remove 1 *illumigene* Malaria Test Device from its protective pouch per sample. Carefully open the device, holding the chambers such that the lyophilized reagents will not fall out upon opening. Place the device on a flat surface or in a rack that can accommodate the device.
- Using a micropipette, transfer 50 µL of the sample to both the TEST (White Bead) and CONTROL (Yellow Bead) chambers of the *illumigene* Malaria Test. Take care to not introduce air to the reaction mixture.
- Close the *illumigene* Test Device and fasten the latches securely.
- Tap device(s) on the bench top or mix to remove air bubbles. Carefully examine the Test Device(s) for rehydration of the Control/Test Bead, for air bubbles left in the chamber and liquid in the top of the device. If undissolved beads, air bubbles or liquid in the top of the device are noted, tap the device on the bench top and repeat visual inspection. Amplification and detection should be initiated within 15 minutes.
- Repeat Test Procedure Steps for all samples to be tested.
- Insert the *illumigene* Test Devices into the *illumipro*-10 and initiate run using the Malaria Program. Results will be displayed at the conclusion of the run.

  ***** *Note*: A maximum of 10 samples can be processed in a single *illumipro*-10 run.

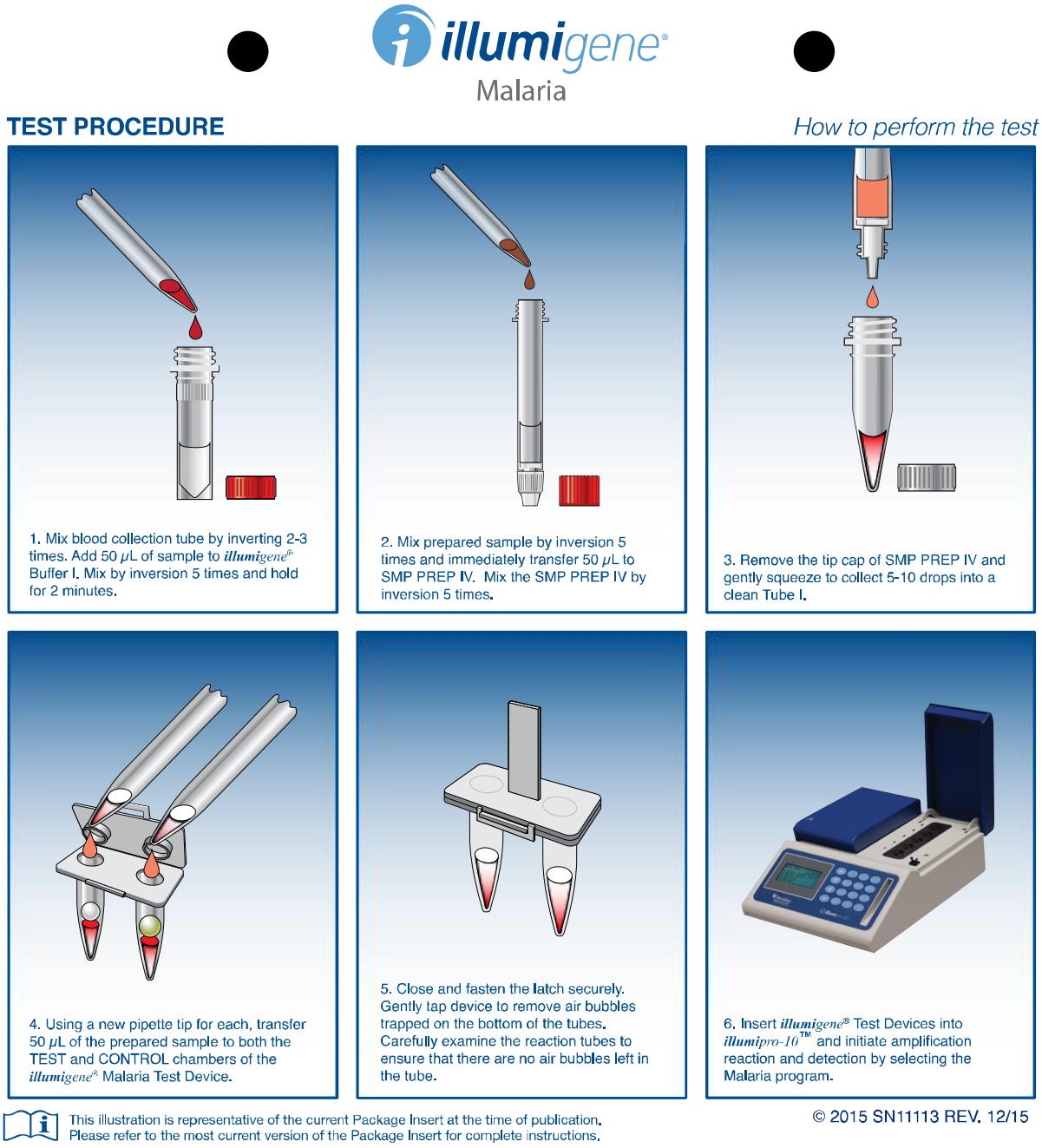



**Figure 9**: Meridian Bioscience *illumigene*® malaria Procedure Card.

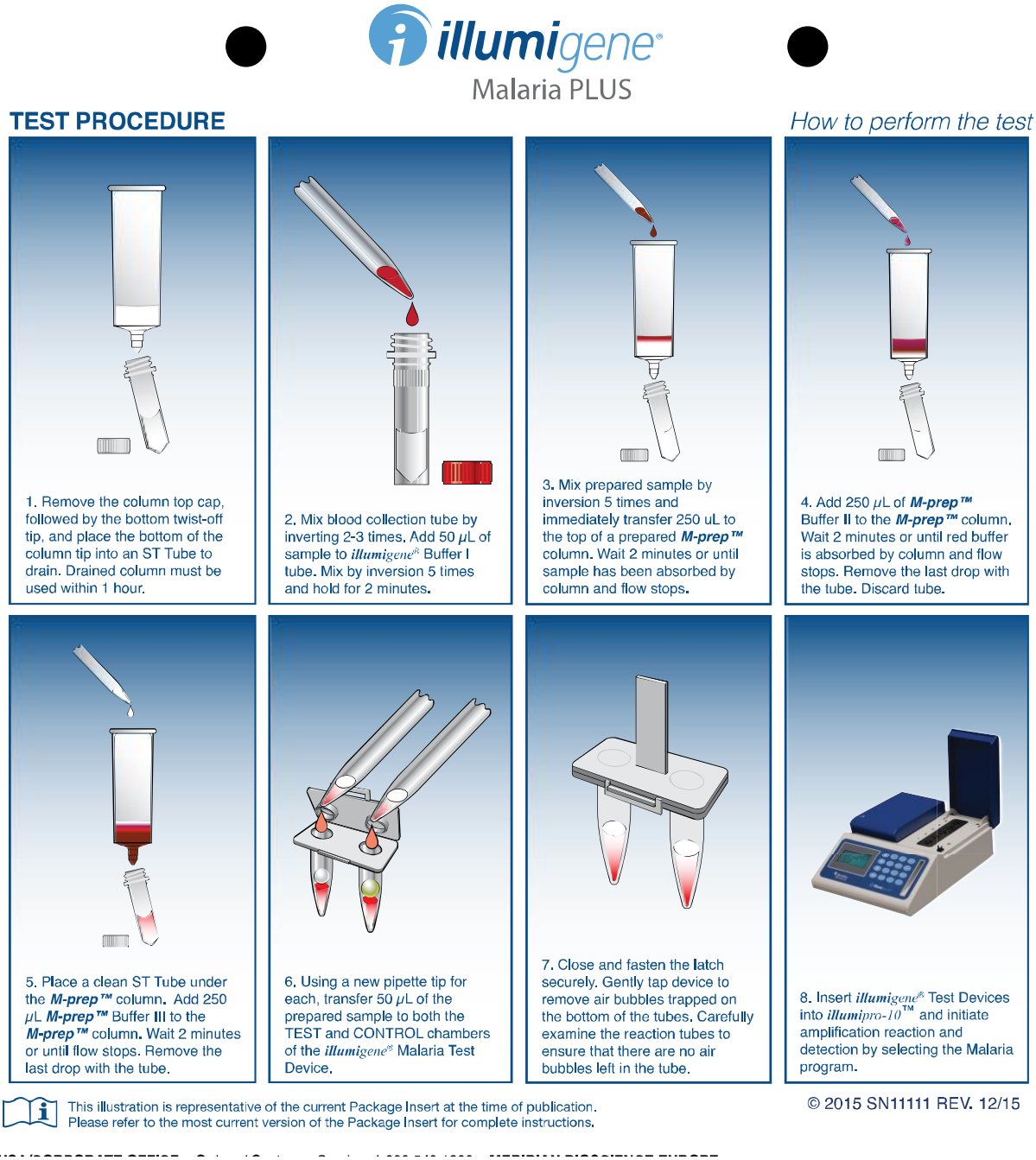
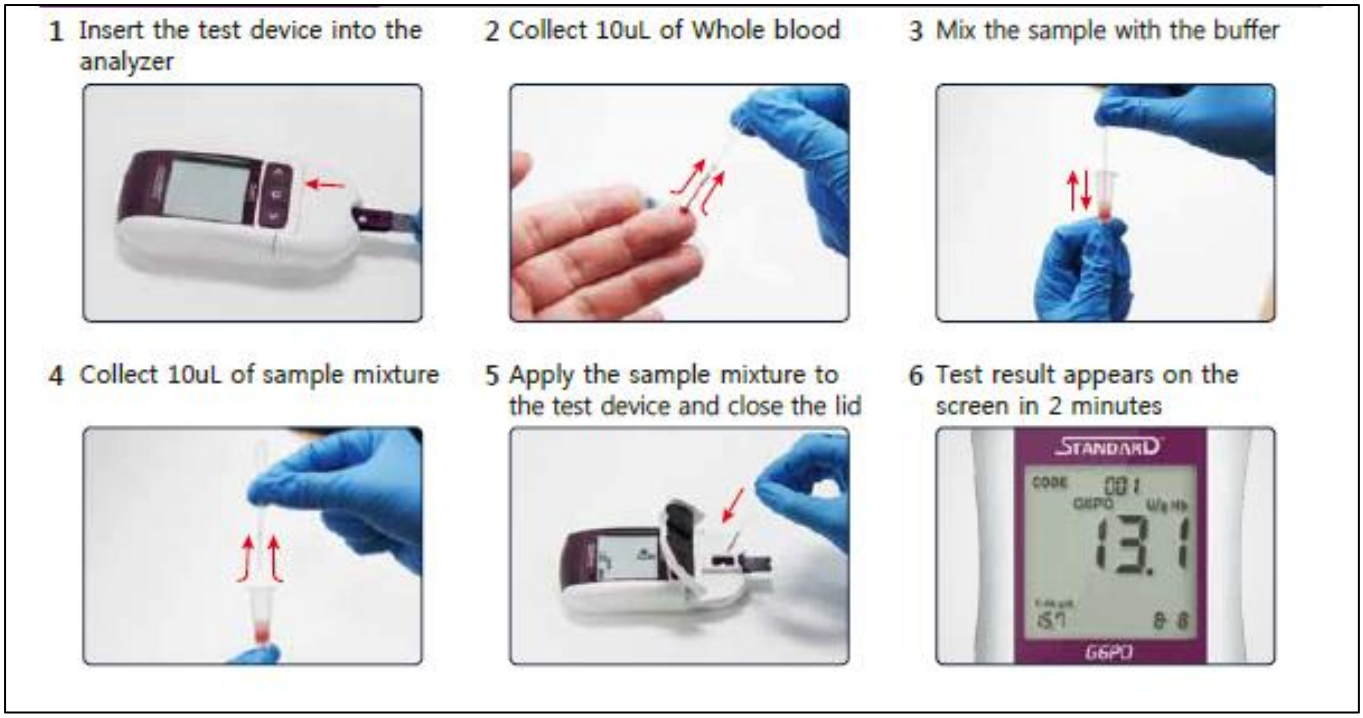


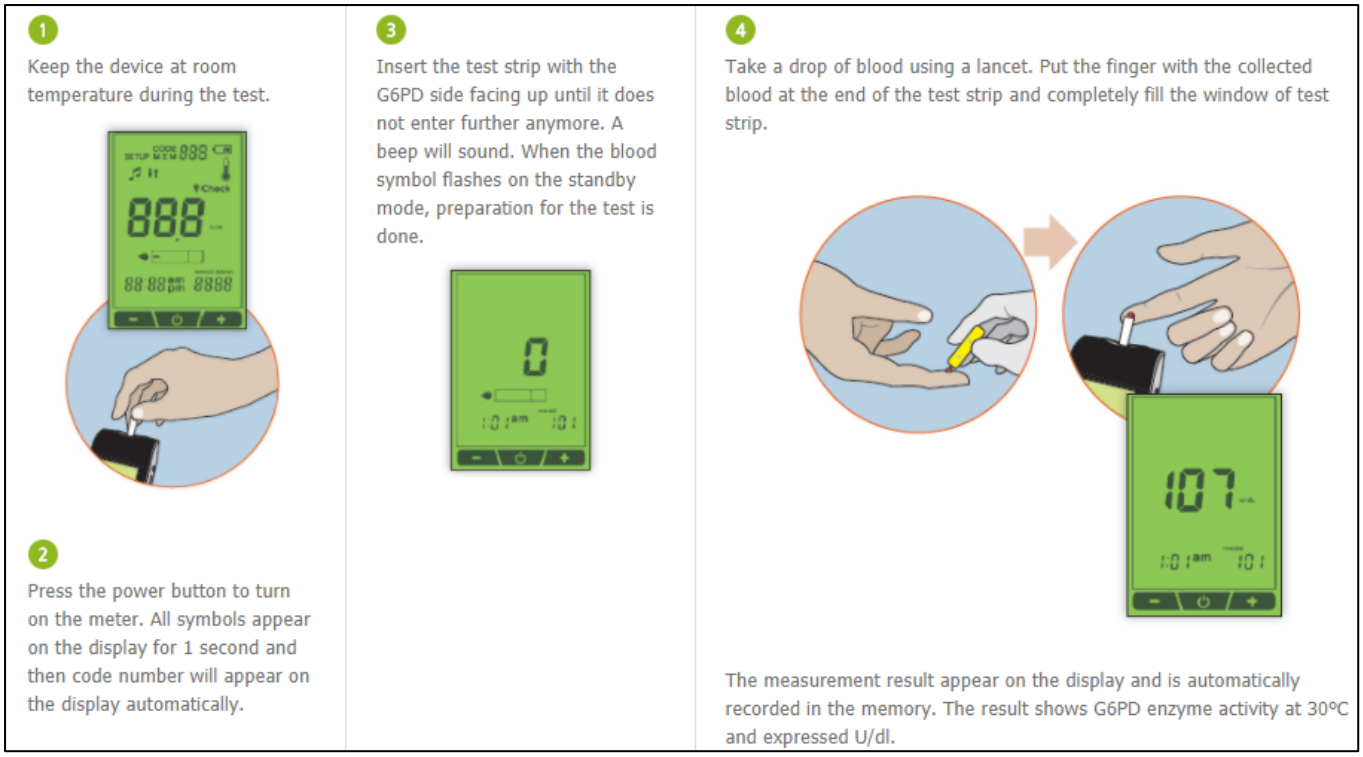

**Figure 10**: Meridian Bioscience *illumigene*® malaria PLUS Procedure Card.

## ***11.2 SD Biosensor SOP***




## ***11.3 Accessbio / Carestart Biosensor SOP***

*Please note that venous samples need to be inverted for 20 minutes prior to testing*



# B) PREPARATION OF OTHER SAMPLE TYPES FOR FURTHER LABORATORY ANALYSES [REFERENCE LABORATORY]

## 12. Separation of Plasma and packed iRBCs (infected red blood cells)

- Plasma is separated from the infected red blood cells (iRBC) by centrifugation at room temperature for 10 minutes at 800-900 g (=3,000-3,500 rpm, depending on centrifuge type and rotor).
- Transfer the plasma into 1.5 mL-2.0 mL cryovials and store the plasma at -20°C or -80°C.

## 13. W-iRBCs (WBC (white blood cell)-depleted infected red blood cells)

[required minimal whole blood volume: 5 mL]

### *13.1 Materials*

- Plasmodipur filters (EuroProxima BV, CAT. CODE: 8011Filter25U)
- Syringes (≥5 mL)
- Phosphate-buffered saline (PBS) pH 7.2-7.5
- 50 mL conical centrifuge tubes
- Sterile serological pipettes
- Plastic transfer pipettes
- 1.5 mL, OR 2.0 mL cryovials
- Clamp and clamp stands, OR adhesive tape

### *13.2 Procedure*

- After separation of the plasma, resuspend the iRBCs in an equal volume of PBS
- Set the Plasmodipur filter on top of an empty 50 mL centrifuge tube by using clamps or adhesive tape
- Humidification: Mount a 10 mL syringe without plunger on top of the Plasmodipur filter
- Fill the syringe with 7-9 mL PBS and let it slowly drop through the filter – do apply very gentle pressure with the plunger only.
- Filtration: Transfer the 50% iRBC-PBS mix into the syringe let it slowly drop through the filter – do apply very gentle pressure with the plunger only.
- Wash: Transfer 7-9 mL PBS into the syringe let it slowly drop through the filter – do apply very gentle pressure with the plunger only.
- Stop the wash step when the fluid dropping out the filter is almost clear. Note: Some blood will remain visible on the filter, even after extensive wash steps.
- Centrifuge the 50 mL tube at 800-1,000 g (=3,000-4,000 rpm, depending on centrifuge type and rotor) to pellet the iRBCs.
- Aliquot the pelleted iRBCs into 1.5-2.0 mL cryovials and store them at -20°C or -80°C.

## 14. CiRBCs (cryopreserved infected red blood cells)

[required minimal whole blood volume: 4 mL]

### *14.1 Materials*

- Glycerolyte® 57 solution (Baxter, code 4A7831)
- 1 mL insulin syringes
- 1.5-2.0 mL cryovials
- Pipettes and pipette aids

### *14.2 Procedure*

- After separation of the plasma, estimate the volume (V) of the pelleted iRBCs
- Slowly add dropwise 0.33 V of Glycerolyte® by using an insulin syringe
- Let the tube stand for 5 minutes
- Add dropwise 1.33 V of Glycerolyte® whilst mixing the tube by gentle swirling
- Aliquot the Glycerolyte®-iRBC mix into cryovials (1 mL each) and store them at the bottom of a -80°C for at least 4 hours or overnight.
- Remove the frozen CiRBC vials from the -80°C unit and place in a permanent, long-term storage freezer (-150°C) until eventual storage in liquid nitrogen $LN_2$.

# ANNEXES

- **Annex 1**_Hemocue Hb 301 Operating Manual
- **Annex 2**_Trinity qual (FST) G6PD test
- **Annex 3**_PATH - Fluorescent Spot Test (FST) Guidebook
- **Annex 4**_PointeScientific quant (spectrophotometry) G6PD test
- **Annex 5**_SOP P. falciparum GG-IVL LFA
- **Annex 6**_illumigene Malaria and Malaria PLUS LAMP assays