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1. Purpose and Scope of the Standard Operating Procedure

1.1. Purpose of the SOP
This Standard Operating Procedure (SOP) describes the timing and the procedures for the collection, processing and storage of biological samples (blood, breast milk, vaginal swabs and child stool), from women and their children. Venous blood and vaginal swab samples will be collected from the mother at specified visits (see Table 1.1) and blood and stool will be collected from the child at specified visits (see Table 1.2).

1.2. Scope of the SOP
This SOP covers the collection, processing, temporary and long-term storage of blood, vaginal swabs urine and stool. It does not cover how to draw blood or how to conduct any assays performed with any of the samples after processing.

Table 1.1 Overview of type of biological samples & the collection schedule for mothers

<table>
<thead>
<tr>
<th>Samples collected</th>
<th>DYAD Visit 1 (6 weeks to 6 months)</th>
<th>DYAD Visit 2 (12 months)</th>
<th>DYAD Visit 3 (24 months)</th>
<th>DYAD Visit 4 (36 months)</th>
<th>Total Collections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>16 ml</td>
<td>16 ml</td>
<td>16 ml</td>
<td>16 ml</td>
<td>4</td>
</tr>
<tr>
<td>Breast milk (Gambia only)</td>
<td>5 ml</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Vaginal swab</td>
<td>4 swabs</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Urine</td>
<td>20 ml (only if not collected during PRECISE 42-days post-partum visit)</td>
<td>20ml</td>
<td>20ml</td>
<td></td>
<td>2 (or 3)</td>
</tr>
</tbody>
</table>

Table 1.2 Overview of type of biological samples & the collection schedule for children

<table>
<thead>
<tr>
<th>Samples collected</th>
<th>DYAD Visit 1 (6 weeks to 6 months)</th>
<th>DYAD Visit 2 (12 months)</th>
<th>DYAD Visit 3 (24 months)</th>
<th>DYAD Visit 4 (36 months)</th>
<th>Total Collections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>2-3 drops from heel prick</td>
<td>2-3 drops finger or 5ml when venepuncture</td>
<td>2-3 drops from finger or 5ml when venepuncture</td>
<td>2-3 drops from finger or 5ml when venepuncture</td>
<td>4</td>
</tr>
<tr>
<td>Stool</td>
<td>2 swabs</td>
<td></td>
<td></td>
<td></td>
<td>2</td>
</tr>
</tbody>
</table>
1.3. Roles and Responsibilities
The site co-ordinator (or his/her designee) is responsible for the implementation of SOP documentation at the participating sites.

The site co-ordinator is responsible for ensuring that all appropriate personnel are trained on this SOP.

All health care providers and technicians who implement this SOP at study sites are responsible for reading and understanding this SOP.

1.4. Safety Procedures

- **Risks**
  - Biofluid exposure

- **Required Safety Equipment**
  - Lab coats
  - Closed toe shoes
  - Face shield/safety goggles (recommended)
  - Gloves

All research personnel and technicians are expected to be trained and follow universal safety precautions when handling biological or hazardous materials and when performing any of the procedures described in this SOP. *Good Clinical Laboratory Practice (GCLP) training* is available on The Global Health Network.

The standard SOP approach assumes that all biological material is infected and potentially carrying HIV virus or other virulent factors. Therefore, all possible precautions are to be adhered to – both those outlined in this SOP and those imposed by the affiliated institution.

2. Maternal Blood Sample Collection and Processing

**Time of collection**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Visit 1 (6 weeks to 6 months)</td>
</tr>
<tr>
<td>2.</td>
<td>Visit 2 (12 months)</td>
</tr>
<tr>
<td>3.</td>
<td>Visit 3 (24 months)</td>
</tr>
<tr>
<td>4.</td>
<td>Visit 4 (36 months)</td>
</tr>
</tbody>
</table>

2.1. Materials Needed
The equipment and designated containers are listed below:

Table 2 Overview of the maternal venous blood sample collection and processing

<table>
<thead>
<tr>
<th>Tubes</th>
<th>Processing and aliquot assignment</th>
<th></th>
</tr>
</thead>
</table>
### Kit Contents

**a.** 1 blood spot card *(only at DYAD visit 2)* (Fisher 09.923.344)

**b.** BD Vacutainer Safety-Lok blood collection set 21G x 3/4” (Fisher Scientific 02-683-20)

**c.** 14 FluidX microtubes 0.7 ml (Brooks 66-0700-00)

**d.** 6 external thread caps, orange (68-53100-Z6N)

**e.** 1 external thread cap, red (68-53100-Z2N)

**f.** 1 external thread cap, white (68-53100-Z1N)

**g.** 6 external thread caps, blue (68-53100-Z4N)

### Other equipment and supplies

**a.** BD Vacutainer Serum Tube 6 ml (Fisher Scientific 02-683-94)

**b.** BD Vacutainer EDTA 7 ml tube (Fisher Scientific 02-685-2B)

**c.** Extra Safety-Lok set (Fisher Scientific 02-683-20) or other safe blood collection device

**d.** Tourniquet

---

<table>
<thead>
<tr>
<th>BD Vacutainer EDTA 7 ml tube (purple top)</th>
<th>EDTA tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) approximately 3 drops (the amount that will cover the active zone of the card) of whole blood applied to each of the sample zones on the blood spot card. <strong>Only at the second DYAD visit.</strong></td>
<td></td>
</tr>
<tr>
<td>b) 1 x 500 μl of whole blood, to be stored in a 0.7 ml FluidX tube, closed by red caps</td>
<td></td>
</tr>
<tr>
<td>c) 6 x 500 μl plasma aliquots stored in 0.7 ml FluidX tube, to be closed by orange caps</td>
<td></td>
</tr>
<tr>
<td>d) 1 x buffy coat, to be stored in a single 0.7 ml FluidX tube, to be closed by a white cap</td>
<td></td>
</tr>
</tbody>
</table>

Remaining sediment (centrifuged erythrocytes) is discarded

<table>
<thead>
<tr>
<th>BD Vacutainer Serum Tube 6 ml (red top)</th>
<th>Serum tube (clotting for 30 minutes, then centrifugation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) 6 x 500 μl serum aliquots, stored in 0.7 FluidX tube, to be closed by blue screw caps</td>
<td></td>
</tr>
</tbody>
</table>

Remaining sediment (centrifuged erythrocytes) is discarded
2.2. Maternal Blood Sample Collection

Blood should be collected by a trained phlebotomist, midwife or research technician.

1. Attach a unique participant ID label to each of the collection tubes and to the label on the kit bag.

2. A certified phlebotomy technician should draw blood into each of the vacutainer tubes using the blood collection kit provided. The order of the draw is: 6 ml serum tube (red top) then 7 ml EDTA tube (purple top).

3. The serum tube should be inverted gently 5 times slowly (not shaken) to ensure that the reagents mix with the blood sample.

4. The EDTA tube should be inverted gently 8 to 10 times slowly (not shaken) to ensure that the reagents mix with the blood sample.

5. Record the time of sample collection, either by writing this on the kit bag or entering this into the database.

6. Set the timer for 30 minutes as soon as the blood draw has been completed as a reminder to centrifuge the tubes and place the serum tube in the rack to clot at room temperature.

7. Place the EDTA tube in a fridge/cool box until processing if not processing straight away.

8. Once the serum tube has been sitting at room temperature for 30 minutes, if it is not going to be processed immediately, store it in a fridge/cool box until it is processed.

2.3. Processing EDTA Tube (Purple Top)

2.3.1. Blood sport card – for DYAD visit 2 only

1. Flip over the card cover to expose the active zone (circle), without touching the active zone on the paper.
2. Remove the cap from the EDTA collection tube (purple top) and, using a pipette, withdraw 0.125 ml of blood. Carefully place drops of blood onto a pre-labelled zone, so the blood fills the designated circle (ideally just reaching the outer circular line).

3. Allow sufficient quantity of blood to soak through to completely fill the pre-printed active zone on the filter paper. Allow blood to dry in a clean place for 2-6 hours.

4. Once dried, flip the cover back over, align with the previous cards and store in the original cardboard box. Place the entire box in the sealable plastic bag and add silica gel pillows for storage.

5. Indicate on the box which sample ID range numbers are stored inside.

2.3.2 Whole blood

6. From the same EDTA tube (purple top), using a pipette, aliquot 500 μl of whole blood into the pre-barcoded FluidX tube with a red cap.

2.3.3. Plasma and buffy coat

1. Ensure the cap is securely fastened on the EDTA tube and place the tube in the centrifuge. Centrifuge at 4°C for 15 minutes at 2000 x g.

2. Record the start time of centrifugation in the database.

3. After centrifugation, three layers should be visible as the blood separates. Take care when removing tube from centrifuge so not to disturb layers.

   a. Top golden layer is plasma, usually semi-transparent in nature.
      o If it is pink or red, then it is haemolysed and this should be noted.
      o If it is opaque or white in colour, it is lipaemic and this should be noted.
      o Occasionally there is a lipid layer on the plasma surface. Avoid inclusion of the lipid layer at the top of the plasma when taking aliquots.

   b. Middle white layer is the buffy coat made up of white blood cells. Avoid disturbing this layer when taking plasma aliquots.

   c. Bottom dark red layer is the red blood cells. It is not collected and should be discarded according to the on-site laboratory SOPs.

4. Using a pipette, transfer as many 500 μl aliquots of plasma as possible into the pre-barcoded FluidX tubes from the collection kit. Be careful not to disturb the buffy coat.

5. If the last aliquot is not a full 500 μl, collect as much plasma as you can without disturbing the buffy coat and record the volume of plasma collect in the database.

6. If there is any plasma remaining once the 6 aliquots have been prepared, any leftover plasma should be added to the final aliquot. The final volume of the 6th aliquot must be recorded in the database.

7. Fasten the orange caps onto the FluidX tubes.
8. Using a sterile transfer pipette take the buffy coat layer. Aspirate slowly and carefully, using a circular motion, to pull all the visible buffy coat material. Some contamination of red blood cells with the underlying layer is expected. Store in the pre-barcoded FluidX tube and close with the white cap.

9. Check that all aliquot tube caps are secure and that each tube has a sample ID label.

2.4. Processing Serum Tube (Red Top)

1. The serum tube must be left for the blood to clot for 30 minutes after collection. As close to 30 minutes after collection as possible, the sample should be processed.

2. Ensure the cap is securely fastened on the collection tube and place the serum tube in the centrifuge. Centrifuge at 4°C for 15 minutes at 2000 x g.

3. Record the start time of centrifugation in the database.

4. After centrifugation, two layers will be visible, serum and sediment (erythrocytes). Normally the serum will be golden in colour and semi-transparent.
   a. If it is pink or red, then it is haemolysed and this should be noted.
   b. If it is opaque or white in colour, it is lipaemic and this should be noted.
   c. Avoid inclusion of the lipid layer at the top of the serum when taking aliquots.

5. Using a pipette, transfer as many 500 μl aliquots of serum as possible into the pre-barcoded FluidX tubes from the collection kit with blue caps.

6. If the last aliquot is not a full 500 μl, collect as much serum as you can and record the sample volume in the database.

7. If there is any serum remaining after the 6 aliquots have been prepared, any remaining serum should also be transferred to the 6th serum aliquot and the volume should be recorded in the database.

8. Fasten the blue caps onto the FluidX tubes and check that they are secure and that each tube has a sample ID label.

Converting RCF (g) to RPM:
2.5. Storage of Maternal Blood Samples (except blood spot card)

1. Once the aliquots have been prepared, the sample IDs, sample colour, time of centrifuge, time of freezing and storage location should be recorded in the OpenSpecimen database.

2. Place the samples in the -80°C freezer or liquid nitrogen (LN₂) storage container (where the LN₂ is in a gas phase) for freezing and storage. Do not flash freeze these samples or place them in liquid nitrogen, this is unnecessary for these sample types. The storage of these samples is site specific so refer to local SOPs for further sample storage instructions.

3. Mid-vaginal swab

**Time of collection**

1. Visit 1 (6 weeks to 6 months)

3.1. Materials Needed

The equipment and designated containers are listed below.

**Table 3** Overview of the vaginal swab processing

| Processing and aliquot assignment |
3.1.1. Kit Contents

| a. Sterile Dacron swabs (TSC SLS, SWA2046) |
| b. Sterile FLOQswabs (Copan Thermo Fisher Scientific 493CE02) |
| c. 7 FluidX microtubes 0.7 ml (Brooks 66-0700-00) |
| d. 7 external thread caps, light blue (Brooks 68-53100-Z5N) |
| e. 2 Fluid X tubes, 1.8ml (Brooks 64-7506) |
| f. 2 external thread caps, orange (Brooks 65-7552) |

3.1.2. Other equipment and supplies

| a. Mini Complete Protease inhibitor tablet (Roche Diagnostics 11697498001) |
| b. Sterile PBS (Sigma DB662) |
| c. Sterilised TE buffer (x1 molecular biology grade; Promega V6232) |
| d. Transfer pipettes (1.5 ml sterile; VWR 612-0965) |
| e. Sterile 30 ml universal tubes (Elkay 500-1000-301) |
| f. FluidX tubes 1.8 ml with light blue caps (68-53100-Z8N) |
| g. Clean scissors (clean with ethanol or cleaning fluid between samples to prevent contamination) |

3.2. Sample Preparation

3.2.1 Preparation of protease inhibitor solution for biochemistry:

1. Where possible in Class 2 hood, add 50 ml of sterile PBS + 1 Mini Complete protease inhibitor tablet to a 50 ml sterile falcon tube. Vortex and allow to sit at room temperature for about 20 min for tablet to dissolve. Vortex again.

2. Where possible in the class 2 hood, remove the lids from the 1.8 ml FluidX tube with clear/natural caps and aliquot 750 µl of the PBS+protease inhibitor into each tube. Replace caps.

3. Place the tubes in a freezer for storage until use. They can be stored for a maximum of 6 months.

3.2.2 Preparation of TE buffer in tubes for microbiome swabs:

1. Prior to sample collection, ensure the two 1.8 ml tubes with orange caps from the kit have 1 ml of TE buffer aliquoted into them in the laboratory. These should then be immediately transferred to the clinic for sample collection. If the tubes are not used, the whole kit should be stored in the fridge until a kit is required.

3.3. Vaginal Swab Sample Collection

1. Before samples are collected, collect a sample collection kit ensuring you have two tubes with orange caps with TE buffer (TE buffer stored in the fridge will need to be aliquoted into the tubes
in the kit prior to sample collection) and two tubes with natural/clear caps containing PBS + protease inhibitor (stored in the freezer, thawed before adding swab).

2. The samples in the natural/clear capped tubes must be kept on ice or in a fridge once removed from the freezer.

3. Participants will be asked to lie on a couch, having removed their underwear, with a sheet over their waist to maintain dignity. The participant will be asked to bend their legs with their feet together moved close to their bottom; knees moved outwards.

3.3.1 Biochemistry Swabs

1. The research nurse/midwife wearing gloves will part the labia and gently insert the two Dacron swabs into the mid-vagina (approximately 4-5 cm) and rotate gently 360 degrees for 15-20 seconds. The exposure is needed for the vaginal fluid to enter the swab stick. Failure to retain the stick for this duration might result in insufficient vaginal fluid collection, making the sample useless.

2. After removal of swabs from vagina, take the two pre-made tubes containing the PBS + protease inhibitor (natural/clear cap). Carefully open each of the two tubes and cut off the Dacron swab tips using scissors, placing one in each tube. Make sure that the tip is covered by the fluid.

3.3.2 Microbiome Swabs

1. Using the FLOQswabs, repeat the collection process as above, ensuring the swabs are gently rotated 360 degrees in the mid-vagina for 15-20 seconds to collect sufficient sample.

2. For the FLOQswabs, place one swab into a 1.8 ml FluidX tubes containing TE buffer with orange cap. Rotate against the side of the tube for 60 seconds to remove the bacteria. The swab should then be removed from the tube and discarded.

3. Repeat this process with the second FLOQswab in the second FluidX tube with orange cap.

4. Keep tubes on ice for transportation to the laboratory and kept in the fridge/cool box on arrival in the lab until the samples are processed. Please note that the sample must be processed within an hour of collection.

5. Record time of sample collection on the kit bag.

3.4. Swab Sample Processing

1. Record the time samples arrived in the lab in the database.

2. The orange capped tubes with the microbiome FLOQswab sample do not need any processing. Record the details in the database and store.

3. To process the samples taken with Dacron swabs for biochemistry, remove the two swab tips from the tubes with natural/clear caps and place into two clean tubes. Spin the tubes (that originally contained the swab) containing PBS + protease inhibitor and the clean tubes containing the tips for 10 minutes at 4°C at 2500 x g.

4. Remove the tips and pool all of the PBS and extra residual sample into one tube and spin the tube again for 10 mins at 4°C at 2500 x g.
5. Using a pipette, transfer as many 200 μl aliquots of the resultant supernatant as possible into in FluidX tubes with light blue caps for storage. If the final sample is less than 200 μl, record the volume in the database.

6. Enter data in the database.

7. Place the samples in the -80°C freezer or LN₂ storage container (where the LN₂ is in a gas phase) for freezing and storage. Do not flash freeze these samples or place them in liquid nitrogen, this is unnecessary for these sample types. The storage of these samples is site specific so refer to local SOPs for further sample storage instructions.

4. Breast milk (Gambia only)

**Time of collection**

1. Visit 1 (6 weeks to 6 months)

4.1. Materials Needed

The equipment and designated containers are listed below.

**Table 4** Overview of the breast milk processing

<table>
<thead>
<tr>
<th>Processing and aliquot assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) 5 x 1.0 ml of breast milk to be stored in 1.5 ml Starlab microtubes closed with a white/violet screw caps.</td>
</tr>
</tbody>
</table>

**4.1.1. Kit Contents**

a. 5 Starlab microtubes 1.5 ml (Starlab E1415-2240)
b. 5 x external thread caps, white/violet (Starlab E1480-0305)

**4.1.2. Other equipment and supplies**

a. 1 x 60 ml Narrow mouth Bio-Tite Specimen sterile (Samco 03 0007)
b. 1 x 5 ml transfer pipette
c. Cleaning wipes

**4.2. Breastmilk sample collection**

1. Breast Cleaning – put a glove on the hand that the participant (or you) will use to clean the ‘study breast’ (to be chosen by the participant) and clean the breast twice with provided wipes
using a newly-opened wipe each time. When appropriate, the participant may clean the breast with water and soap before cleaning with the wipes.

2. Milk collection - express by hand at least one tablespoon of milk, by dripping or squirting directly from the nipple into the sterile container. Ensure the samples are caught clean directly into the container (without touching the participant’s skin).

3. If, for some reason, not enough milk can be expressed from the ‘study breast’, it is acceptable to combine milk from both breasts. If this happens, just clean the “second” breast as described in Step 1 (with new glove and wipes) prior to collecting milk and include a note on the collection form as to what was done.

4. Swirl the expressed milk around to ensure the composition is consistent. Place it in an ice tray immediately after collection.

5. Record the following information on the form provided in the kit bag (Appendix 4)
   a. Time of sample collection - Record the time at which the sample collection was completed.
   b. Study breast: Left/Right - indicate from which breast the sample was collected. If not enough milk could be expressed from the ‘study breast’ and the milk from both breasts was combined, note that this was done.
   c. Was breast cleaned with water and/or soap before it was cleaned with wipe? Yes/no The breast should ideally be cleaned with water and soap before cleaning it with the cleaning wipe provided in the kit however some circumstances might make it difficult to complete this step before collection. If it was not possible to clean the breast with water and/or soap, indicate so.
   d. Time since breast was last suckled

Record the time since the breast was last suckled or pumped. You can prompt the participant by asking at what time the child was last fed / at what time she last pumped the breast.

4.2. Breast milk sample processing

1. Using the pipette, transfer 5 aliquots of 1 mL of breast milk into the pre-barcoded FluidX tubes from the collection kit with amber caps.

4.3. Storage of maternal breast milk samples

1. Once the aliquots have been collected, the sample IDs, time of freezing and storage location should be recorded in the database.

2. Place all the breast milk samples in the -80°C freezer or LN₂ storage container (where the LN₂ is in a gas phase) for freezing and storage. Do not flash freeze the samples or place them in liquid nitrogen, this is unnecessary for these sample types. The storage of these samples is site specific so refer to local SOPs for further sample storage instructions.

5. Maternal Urine

Time of collection

1. DYAD visit 1 (6 -weeks to 6 months) - only if not collected during PRECISE 42-days post-partum visit.
2. DYAD visit 2 (12 months)
3. DYAD visit 3 (24 months)

5.1. Materials Needed

The equipment and designated containers are listed below.

**Table 5** Overview of the maternal urine sample collection and processing

<table>
<thead>
<tr>
<th>Processing and aliquot assignment</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>a. <strong>2 x 2 ml of urine</strong> to be stored in 2ml tubes closed with screw cap (sourced locally, cap colour dependent on local availability)</td>
<td></td>
</tr>
</tbody>
</table>

5.1.1. Kit Contents

a. **2 x 2 ml tubes** (sourced locally)
b. **2 screw caps** (sourced locally)

5.1.2. Other equipment and supplies

a. 1 x 90 ml (or any volume) urine collection and transport container
b. 1 x 5 ml transfer pipette

5.2. Urine sample collection

1. Provide woman with the participant ID-labelled urine collection cup.

2. Ask the woman to provide a sample using the following instructions:
   
   o Remove the lid from the urine collection cup.
   o Begin to urinate into the toilet.
   o Pass the collection container into the urine stream, collecting at least 30 to 59 ml into the container.
   o Finish urinating into the toilet.
   o Screw the lid on the container and wipe any excess urine from the outside.
   o Wash hands
   o Return the container to the PRECISE lab technician for processing.

3. Collect the urine cup, ensure the lid is screwed on tightly. Record the time of sample collection, either write this on the kit bag or scan the participant ID into the database to record the sample collection time.

4. Store the urine in a fridge/cool box until it is processed.
5.3. Urine sample processing

Specimen processing should be completed and aliquots frozen within 2 hours of collecting urine from participants.

1. Using a single-use 5 ml transfer pipette, aliquot 2 ml of urine from the collection container into each of the 2 capped tubes.

2. Check each of the aliquot caps are secure and each aliquot has a specimen ID label.

5.4. Storage of maternal urine samples

1. Once the aliquots have been prepared, the sample IDs, time of freezing and storage location should be recorded in the database.

2. Place all the urine samples in the -80°C freezer or LN$_2$ storage container (where the LN$_2$ is in a gas phase) for freezing and storage. Do not flash freeze the samples or place them in liquid nitrogen, this is unnecessary for these sample types. The storage of these samples is site specific so refer to local SOPs for further sample storage instructions.

6. Child Blood

A sample of child blood is taken at each visit. We will either collect a blood spot using 2 or 3 drops of blood from heel (visit 1) or finger (for children older than 1 year), or collect blood through venepuncture (5 ml for children older than 1 year). The decision on how to collect blood will be up to the participant and the confidence of the staff in collecting the samples.

6.1. Child Heel Prick Sample Collection and Processing

<table>
<thead>
<tr>
<th>Time of collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Visit 1 (6 weeks to 6 months) – heel prick</td>
</tr>
<tr>
<td>2. Visit 2 (12 months) – finger prick</td>
</tr>
<tr>
<td>3. Visit 3 (24 months) – finger prick</td>
</tr>
<tr>
<td>4. Visit 4 (36 months) – finger prick</td>
</tr>
</tbody>
</table>

6.1.1. Materials Needed

The equipment and designated containers are listed below.

6.1.2. Kit Contents

| a. 1 Lancet (max depth allowed according WHO guidelines: heel-prick 2.4mm and finger-prick 1.5mm) |
| b. 1 Blood spot card (Fisher 09.923.344) |

6.1.3. Other equipment and supplies
6.1.4. Heel or Finger Prick Sample Collection

1. Clean the selected area of skin with a skin disinfectant and allow to dry for 30 seconds. Warming up of the foot/finger facilitates dilatation of blood vessels and ensures faster and easier protocol completion.

2. According to WHO guidelines, in heel-pricks, the depth should not go beyond 2.4 mm. The recommended depth for a finger-prick for a child over 6 months is 1.5 mm.

3. Position the foot/finger with the puncture site downwards. Take care to keep away from bony prominences. Press the loaded lancing device against the skin and push the white plunger.

4. While holding the foot/finger correctly, apply and release pressure to allow a drop of blood to form. Allow a large drop of blood to collect.

5. Once a drop of blood has formed, lightly touch the drop to the pre-printed circle on the blood spot card, allowing it to soak onto the circle. Allow sufficient quantity of blood to soak through to completely fill the pre-printed active zone on the filter paper. Do not layer successive drops of blood or apply blood more than once in the same collection circle.

6. Record the sample collection time in the database.

6.1.5. Sample Processing

1. Once the sample has arrived in the laboratory, allow blood to dry in a clean place for 2-6 hours.

2. Once dried, flip the cover back over, align with the previous cards and store in the original cardboard box. Place the entire box in the sealable plastic bag and add silica gel pillows for storage.

3. Record the sample ID and the samples storage location in the database.

6.2. Child Venous Blood Sample Collection and Processing

<table>
<thead>
<tr>
<th>Time of collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Visit 2 (12 months)</td>
</tr>
<tr>
<td>2. Visit 3 (24 months)</td>
</tr>
<tr>
<td>3. Visit 4 (36 months)</td>
</tr>
</tbody>
</table>

6.2.1. Materials Needed

The equipment and designated containers are listed below.

Table 6.2 Overview of venous blood sample processing at visit 2, visit 3 and visit 4.
<table>
<thead>
<tr>
<th>Tubes</th>
<th>Processing and aliquot assignment</th>
</tr>
</thead>
</table>
| EDTA 3 ml tube (lavender top) | 3 ml EDTA tube  
  a) 3 x 500 μl plasma aliquots stored in 0.7 ml FluidX tube, to be closed by black caps  
  b) 1 x buffy coat, to be stored in a single 0.7 ml FluidX tube, to be closed by a purple cap  
  Remaining sediment (centrifuged erythrocytes) is discarded |
| Serum 2 ml tube (red top with white ring) | 2 ml Serum tube (clotting for 30 minutes, then centrifugation)  
  c) 2 x 500 μl serum aliquots, stored in 0.7 FluidX tube, to be closed by amber caps  
  Remaining sediment (centrifuged erythrocytes) is discarded |

6.2.2. Kit Contents

a. 6 FluidX microtubes 0.7 ml (Brooks 66-0700-00)

b. 3 external thread caps, black (Brooks 68-53100-Z13N)

c. 1 external thread cap, purple (68-53100-Z11N)
6.6.3. Other equipment and supplies

- Vacuette 2 ml Serum tube (Grainer 454096)
- Vacuette 3 ml K2EDTA tube (Grainer 454020)
- 23G or 25G butterfly needle
- 5 mL syringe
- Tourniquet
- Antiseptic wipes
- Gauze sponges
- Adhesive bandages
- Sharps container
- Gloves
- Pipette and 5 filter tips
- Transfer pipette
- Racks for FluidX tubes (Brooks Life Science Cat. # 66-51004)

6.6.4. Child Venous Blood Sample Collection

Blood should be collected by a trained phlebotomist, midwife, nurse or research technician.

1. Attach a unique participant ID label to each of the collection tubes and to the label on the kit bag.

2. A certified phlebotomy technician should draw blood into a 5 mL syringe using the 23G butterfly needle. Do not use the vacuette attached to the syringe. The pressure from the vacutainer system is too strong for smaller veins so will collapse them.

3. Remove the caps from the EDTA and serum tubes and transfer 2 mL of blood into the serum tube (red top with white ring) then 3 mL into the EDTA tube (lavender top).

   Note: If you are not able to draw 5 ml of blood, the priority should be given as follow:
   Total 5 ml blood = 2ml serum + 3ml EDTA
   Total 4 ml blood = 2ml serum + 2ml EDTA
   Total 3 ml blood = 1ml serum + 2ml EDTA
   Total 2 ml blood = 0ml serum + 2ml EDTA

4. The serum tube should be inverted gently 5 times slowly (not shaken) to ensure that the reagents mix with the blood sample.

5. The EDTA tube should be inverted gently 8 to 10 times slowly (not shaken) to ensure that the reagents mix with the blood sample.

6. Record the time of sample collection, either by writing this on the kit bag or entering this into the database.

7. Set the timer for 30 minutes as soon as the blood draw has been completed as a reminder to centrifuge the tubes and place the serum tube in the rack to clot at room temperature.

8. Place the EDTA tube in a fridge/cool box until processing if it is not being processed immediately.
9. Once the serum tube has been sitting at room temperature for 30 minutes, if it is not going to be processed immediately, store it in a fridge/cool box until it is processed.

6.6.5. Processing EDTA Tube (Lavender Top)

6.6.5.1 Plasma and buffy coat

1. Ensure the cap is securely fastened on the EDTA tube and place the tube in the centrifuge. Centrifuge at 4°C for 15 minutes at 2000 x g.

2. Record the start time of centrifugation in the database.

3. After centrifugation, three layers should be visible as the blood separates. Take care when removing tube from centrifuge so not to disturb layers.
   
   a. Top golden layer is plasma, usually semi-transparent in nature.
      
      i. If it is pink or red, then it is haemolysed and this should be noted.
      
      ii. If it is opaque or white in colour, it is lipaemic and this should be noted.
      
      iii. Occasionally there is a lipid layer on the plasma surface. Avoid inclusion of the lipid layer at the top of the plasma when taking aliquots.
   
   b. Middle white layer is the buffy coat made up of white blood cells. Avoid disturbing this layer when taking plasma aliquots.
   
   c. Bottom dark red layer is the red blood cells. It is not collected and should be discarded according to the on-site laboratory SOPs.

4. Using a pipette, transfer as many 500 μl aliquots of plasma as possible into the pre-barcoded FluidX tubes from the collection kit. Be careful not to disturb the buffy coat.

5. If the last aliquot is not a full 500 μl, collect as much plasma as you can without disturbing the buffy coat and record the volume of plasma collect in the database.

6. If there is any plasma remaining once three aliquots have been prepared, any leftover plasma should be added to the final aliquot. The final volume of the 3rd aliquot must be recorded in the database.

7. Fasten the black caps onto the FluidX tubes.

8. Using a sterile transfer pipette take the buffy coat layer. Aspirate slowly and carefully, using a circular motion, to pull all the visible buffy coat material. Some contamination of red blood cells with the underlying layer is expected. Store in the pre-barcoded FluidX tube and close with the purple cap.

9. Check that all aliquot tube caps are secure and that each tube has a sample ID label.

6.6.6. Processing Serum Tube (Red top with white ring)

1. The serum tube must be left for the blood to clot for 30 minutes after collection. As close to 30 minutes after collection as possible, the sample should be processed.

2. Ensure the cap is securely fastened on the collection tube and place the serum tube in the centrifuge. Centrifuge at 4°C for 15 minutes at 2000 x g.
3. Record the start time of centrifugation in the database.

4. After centrifugation, two layers will be visible, serum and sediment (erythrocytes). Normally the serum will be golden in colour and semi-transparent.
   a. If it is pink or red, then it is haemolysed and this should be noted.
   b. If it is opaque or white in colour, it is lipaemic and this should be noted.
   c. Avoid inclusion of the lipid layer at the top of the serum when taking aliquots.

5. Using a pipette, transfer as many 500 μl aliquots of serum as possible into the pre-barcoded FluidX tubes from the collection kit with amber caps.

6. If the last aliquot is not a full 500 μl, collect as much serum as you can and record the sample volume in the database.

7. If there is any serum remaining after two aliquots have been prepared, any remaining serum should also be transferred to the 2nd serum aliquot and the volume should be recorded in the database.

8. Fasten the amber caps onto the FluidX tubes and check that they are secure and that each tube has a sample ID label.

Converting RCF (g) to RPM:

6.7. Storage of Child Blood Samples

1. Once the aliquots have been prepared, the sample IDs, centrifugation time, sample colour, time of freezing and storage location should be recorded.
2. Place the samples in the -80°C freezer or liquid nitrogen (LN$_2$) storage container (where the LN$_2$ is in a gas phase) for freezing and storage. Do not flash freeze these samples or place them in liquid nitrogen, this is unnecessary for these sample types. The storage of these samples is site specific so refer to local SOPs for further sample storage instructions.

7. Child Stool Swab

Time of collection

1. Visit 1 (6 weeks to 6 months)
2. Visit 3 (24 months)

7.1 Materials Needed

The equipment and designated containers are listed below.

Table 7 Overview of the stool sample processing

<table>
<thead>
<tr>
<th>Processing and aliquot assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. 2 x stool swabs to be stored in 2 ml lysis shield tube with green cap</td>
</tr>
</tbody>
</table>

6.1.1. Kit Contents

a. 2 x 2ml DNA/RNA Shield Lysis tube (Microbe) (Zymo Research R1103)
b. 2 x sterile flocked swab (Puritan PurFlock Ultra; VWR 10124-676)

6.2. Child Stool Swab Sample Collection

1. Using the sterile swab provided to swab the child’s stool to collect a small amount of stool. Roll the cotton swab in the stool to coat the tip.

2. Place the swab (covered in stool) into a green capped tube and snap off the swab head at the break point leaving the tip of the swab in the tube.

3. Close the tube tightly and shake for 30 seconds.

4. Repeat this process for the second swab placing the sample in the second green capped tube.

5. Record the time of sample collection and transport to the lab for processing.
6.3. Child Stool Sample Processing

1. Remove the swab and place the samples in the -80°C freezer or LN₂ storage container (where the LN₂ is in a gas phase) for freezing and storage. Do not flash freeze these samples or place them in liquid nitrogen, this is unnecessary for these sample types. The storage of these samples is site specific so refer to local SOPs for further sample storage instructions.

8. Colour coding scheme for the FluidX tubes

<table>
<thead>
<tr>
<th>Tube Type</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.7 ml whole blood tube (mother)</td>
<td>red cap</td>
</tr>
<tr>
<td>0.7 ml plasma tube (mother)</td>
<td>orange cap</td>
</tr>
<tr>
<td>0.7 ml buffy coat tube (mother)</td>
<td>white cap</td>
</tr>
<tr>
<td>0.7 ml serum tube (mother)</td>
<td>blue cap</td>
</tr>
<tr>
<td>0.7 ml vaginal swab –biochemistry tube (mother)</td>
<td>light blue cap</td>
</tr>
<tr>
<td>1.8 ml vaginal swab – microbiome tube (mother)</td>
<td>orange cap</td>
</tr>
<tr>
<td>1.5 ml breast milk (mother)</td>
<td>Natural cap</td>
</tr>
<tr>
<td>2.0 ml urine (mother)</td>
<td>Local procurement</td>
</tr>
<tr>
<td></td>
<td>Description</td>
</tr>
<tr>
<td>------------------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td><img src="image1.png" alt="Image" /></td>
<td>0.7 ml plasma tube (child) black cap</td>
</tr>
<tr>
<td><img src="image2.png" alt="Image" /></td>
<td>0.7 ml buffy coat tube (child) purple cap</td>
</tr>
<tr>
<td><img src="image3.png" alt="Image" /></td>
<td>0.7 ml serum tube (child) amber cap</td>
</tr>
</tbody>
</table>