Step-by-step guidance to assess population thiamine status with thiamine biomarkers: thiamine diphosphate (ThDP); and erythrocyte transketolase activity (ETKA)

1) Identify appropriate population group, calculate sample size, and select a representative sample of the population (more information in section 4.2)

2) Obtain ethical approval and informed consent

3) Data collection - as per the large-scale survey, but the minimum required data are:
   - Participant/survey ID; date of birth; sex; date
   - Biomarker to be analyzed
     - erythrocyte ThDP, or
     - whole blood ThDP (+ hematocrit or hemoglobin; explanation in step 8, below), or
     - ETKA

4) Sample collection (from skilled phlebotomists)
   - Clean subject's skin with alcohol at site of the antecubital vein
   - Restrict occlusion of subject's arm with tourniquet for < 1 minute
   - Draw blood and collect it into heparin or EDTA blood collection tubes

5) Sample preparation
   For analysis of erythrocytes ThDP and ETKA:
   - 3 cycles of washing with isotonic saline solution (0.9% NaCl), centrifugation, removal of supernatant and the top few mm of cells, resuspension in saline.
   - The washed cells, without supernatant, are frozen at -70^{o}C or colder and are osmotically lysed after thawing by re-suspending in water prior to analysis

   For analysis of whole blood ThDP: no additional steps are required

6) Sample storage
   - Protected from light and stored cold:
     - room temperature: a few hours
     - at -20°C: a few months
     - at -80°C: several months/years
   - If shipping is required, use dry ice or liquid nitrogen

7) Sample analysis
   For HPLC analysis of whole blood and erythrocyte ThDP:
   - Thaw samples in a dark room (or with amber light) and keep on ice until analyzed.
   - Precipitate proteins with TCA on ice
   - Spin down and transfer supernatant to fresh tube
   - Wash twice with water-saturated methyl-tert-butyl ether to remove lipid-soluble components
   - Aliquots of standards, blank and sample solutions are derivatized with methanol freshly prepared potassium ferricyanide and NaOH and filtered before analysis

- Samples are run on an HPLC with a fluorescence detector using an excitation wavelength of 375 nm and an emission wavelength of 435 nm.

For analysis of ETKA:

- Lyse washed erythrocytes with water;
- Pre-incubate lysate with ThDP ("activated") or buffer ("basal")
- Perform enzyme reaction with ribose-5-phosphate as substrate, coupled to triose phosphate isomerase (TPI) and glycerol dehydrogenase (GDH) with NADH as cofactor in a UV-transparent 96-well microplate and read using a UV-vis spectrophotometer at 37°C, at an absorbance of 340nm.
- Calculate ratio of activated to basal activity (linear reaction rate)

8) Data analysis and suggested* cut-offs
   - Whole blood ThDP: Data must be normalized to RBC volume or hemoglobin concentrations. Best practice would be to present both the measured ThDP (nmol/L whole blood) and the ThDP normalized to hematocrit (nmol/L RBCs) or hemoglobin (nmol per gram hemoglobin)
     - Healthy Range: 70-180 nmol/L
     - Deficiency: <70 nmol/L
   - Erythrocyte ThDP:
     - 120-150 nmol/L: mild deficiency
     - <120 nmol/L: deficiency
   - Erythrocyte transketolase assay
     - Erythrocyte transketolase activation coefficient (ETKAC), i.e. ETKA with added ThDP / baseline ETKA, should be reported, and is sometimes expressed as the percentage activation α:
       - Low risk of deficiency: ≤1.15 (α≤15%)
       - Moderate risk of deficiency: 1.15–1.25 (α15%-25%)
       - High risk of deficiency: >1.25 (% activation α>25%)
     - Alternatively, report basal ETKA per unit mass of hemoglobin. Due to the limited availability of reference ranges for basal ETK activity, the following cut-offs may be used to define thiamine deficiency:
       - Infants: ≤0.59 micromoles/min/gHb
       - Females aged 4–18y and 19–24y: ≤0.57 micromoles/min/gHb
       - Females aged 25–34y: ≤0.50 micromoles/min/gHb
       - Females aged 35–49y: ≤0.47 micromoles/min/gHb

**Different cut-offs have been proposed and there is no agreement on the most adequate values.*