Morogoro Regional Micronutrient Household and Biomarker Survey, Tanzania, 2019

Primary Investigators (PI):
Dr. Vincent Assey, Director of the Tanzania Food and Nutrition Center
Dr. Janneth Mghamba, Assistant Director of Epidemiology and Disease Control Section and Program Director, Tanzania Field Epidemiology and Laboratory Training Program (TFELTP), Ministry of Health (MOH)
Co-Primary Investigators (Co-PI):
Dr. Rogath Kishimba, Tanzania Ministry of Health, TFELTP
Dr. Elifatio Towo, Director, Department of Food and Nutrition, TNFC
Organizations: Tanzania Ministry of Health, Tanzania Food and Nutrition Center (TFNC); Tanzania Field Epidemiology and Laboratory Training Program (TFELTP)
Phone: 255 22 211 8137
Email Address: info@tfnc.go.tz and rogatkishimba@gmail.com

Table of Contents:

1 Summary

Micronutrient deficiencies are a major contributing factor to morbidity and mortality in the Tanzanian population. As a means to reduce the prevalence of micronutrient deficiencies a national food fortification program was established in 2013. This food fortification program only

includes the fortification of centrally produce wheat and maize flour. However, many regions in the country consume flour mostly produced by small and medium size millers. Before establishing a food fortification program that will cover these flour producers the Tanzania government wants to understand the current coverage and extension of their food fortification program in the Morogoro region because it is a microcosms of the country. The main objective of the Morogoro Region Micronutrient Household and Biomarker Study is to assess the nutritional status of anemia and other micronutrients, namely iron, vitamins 9 (folic acid) and B12, vitamin A and other indicators among women of reproductive age (15 – 49 years of age) in urban and rural areas within the Morogoro region.

2 Background and Rationale

Mandatory fortification of food products has been an effective global strategy to ensure adequate micronutrients in women of reproductive age (WRA) and reduce various micronutrient deficiencies, including that of folic acid and anemia. Maternal micronutrient deficiencies are important to address because they can lead to birth defects and other adverse birth outcomes. (Butha et al, 2008, Black et al. 2008). For example, folic acid is a water-soluble B9 vitamin needed for cell growth and development. Folate deficiency due to inadequate daily consumption of folic acid during the periconceptional period increases the risk for neural tube defects (NTDs, such as anencephaly, spina bifida, and encephalocele), and anemia (CDC 1992; IOM 1998; Bailey et al, 2015; Garret et al., 2018).

Micronutrient deficiencies continue to be a significant public health problem in Tanzania among WRA. According to the 2015-16 Tanzania Demographic and Health Survey (Table 1), a high proportion of WRA suffer from inflammation (27.8%), anemia (45%), vitamin A deficiency (42%), and iron deficiency (30%) (Tanzania MOH, 2016). The rates are slightly higher in Morogoro, in the eastern part of the country.

Table 1. Prevalence of Inflammation, Anemia and selected Micronutrients Deficiencies among women of reproductive age, Tanzania 2010, 2015

Micronutrient	**National[1] (%)**		**Morogoro[2] (%)**	**Dar es Salaam[3] (%)**
	2010	*2015*		
CRP (>3 mg/L)		27.8	23.5	
Anemia[1,2]	40.0	45.0	47.3	
Vitamin A		42.0	43.0	
Iron deficiency (sTfR>8.3µg/ml)		29.9	30.3	
Serum Folate		UNK	UNK	26.9
Red Blood Cell (RBC) folate deficiency (< 305 nmol/L)		UNK	UNK	
RBC Insufficiency (<748 nmol/L)		UNK	UNK	
Vitamin B12 Deficiency (< 148 pmol/L)		UNK	UNK	
Vitamin B12 Marginal deficiency (148 pmol/L<=B12<=221 pmol/L)		**UNK**	**UNK**	

[1] Ministry of Health, Community Development, Gender, Elderly and Children 2016
[2] National Bureau of Statistics (NBS) and ICF Macro. 2011.
[3] Noor RA et al., 2017.

To address the ongoing micronutrient deficiencies in the Tanzanian population, the government passed food fortification legislation in 2011 that requires all industrially milled wheat and maize flour to be fortified with zinc, iron, folic acid, and other B vitamins. However, serious obstacles in the pathway of the implementation, such as decentralized milling of maize flour, have impeded the coverage of appropriately fortified food from reaching vulnerable populations in the country. Most wheat flour that is consumed in Tanzania is industrially produced but only about half of the population consumes it and most of the consumers are from urban areas. On the other hand, maize flour is consumed by 93% of the population (FACT, 2015). However, coverage of fortifiable (packaged) maize flour is a challenge because of the consumption of unpackaged flour and the use of toll mills throughout the country; the current maize flour fortification strategies exclusively target mills who produce packaged maize flour.

Maternal deficiency of folic acid has been reported to contribute to a significant proportion of birth defects in Tanzania. A study in Dar es Salaam, Tanzania found the prevalence of major external birth defects to be 28.3 per 10,000 live births and prevalence of NTDs to be 9.9 per 10,000 live births. Among stillbirths and infants who died within 5 days, NTDs were the most frequently detected birth defect (Kishimba RS, et al., 2015). The NTD prevalence reports were based on hospital information, and these reports might underestimate the true NTD prevalence. Moreover, this report probably provides a glimpse of the NTD prevalence in the country.

To address the ongoing micronutrient deficiencies in the Tanzanian population, the government passed food fortification legislation in 2011 that requires all industrially milled wheat and maize flour to be fortified with zinc, iron, folic acid, and other B vitamins (TFDA, 2011). However, serious obstacles in the pathway of the implementation, such as decentralized milling of maize flour, have impeded the coverage of appropriately fortified food from reaching vulnerable populations in the country. In Tanzania's Morogoro region, 70% of the population lives in rural areas and a high proportion could be considered vulnerable. The vulnerable population has inadequate access to fortified wheat or maize flour because: 1) Only 50% of the population, mostly in urban areas, consumes wheat flour, and 2) Maize flour, consumed by 93% of the population (FACT, 2016). Furthermore, Assey et al. (2018) reported in a recent study that in Morogoro region less than 32% of the population access package maize flour (susceptible to be fortified) and over 40% never purchase package maize flour. This maize flour is produced by small, medium, and toll flour-mills that are not subject to the mandatory fortification program rules that apply to mills centrally producing flour.

The government intentionally excluded small and medium scale mills from the mandatory program so that it could first establish the baseline micronutrient status among non-pregnant WRA. To establish this baseline the Tanzania Food and Nutrition Center (TFNC) in collaboration with the Ministry of Health (MOH) will conduct the Morogoro Regional Micronutrient Survey. The survey will establish the baseline prevalence of folate status (including red blood cell folate insufficiency), vitamin B12 status, and the prevalence of anemia, iron deficiency, vitamin A deficiency and inflammation among non-pregnant WRA.

3 Objectives

a. Primary Objective

i. To assess the deficiency status of non-pregnant WRA (15-49 years of age) using assessment indicators related to anemia, iron, folate, vitamin A, homocysteine, and vitamin B12 (i.e., hemoglobin, hematocrit, serum ferritin, soluble transferrin receptor, serum folate, red blood cell folate, homocysteine, and serum vitamin B12)

b. Secondary Objectives

i. To determine the degree of sub-clinical inflammation of non-pregnant WRA by measuring concentrations of C-reactive protein (CRP) and α-1-acid glycoprotein (AGP)

ii. To determine the methylenetetrahydrofolate reductase (MTHFR) gene mutation status of women

iii. To determine the consumption of key foods, specifically wheat and maize flour, among households and non-pregnant WRA

iv. To assess the iron, folic acid, and vitamin B12 supplementation among non-pregnant WRA

v. To assess the factors contributing to micronutrient deficiency among non-pregnant WRA respondents

vi. Assess the proportion of household with fortified wheat and/or maize flour for iron content by taking small samples of flour from half of the selected households

4 Methodology

4.1 Data Collection

Morogoro Region Micronutrient Household and Biomarker Survey

The survey will be conducted on a sample of households selected from all seven districts in the Morogoro region. Data will be collected on electronic tablets.

Criteria for inclusion/exclusion of subjects include:

i. Inclusion criteria for study participants

a. Household residents who are present in their households at the time of the interview (or during 2 additional visits the same day)
b. Household has resided in current dwelling for at least 3 months
c. Individuals who consent to participate in the survey and prepare or purchase food for the household
d. At least one non-pregnant WRA (15-49 years) residing in the household

ii. Exclusion criteria for study participants

a. Any household that does not meet inclusion criteria will be excluded.

4.2 Sample Size

The sample size was determined based on the estimated prevalence, the desire precision as well as funds available, taking into account an expected maximum non-response of 70% (including refusals) at both the household and individual levels. Table 1 provides prevalence estimates for different biomarkers in Tanzania. For those biomarkers for which we do not have information, we have assumed a prevalence of 50% (most conservative approach). The sample size required for this survey is ~1170 women. This sample size takes into account urban and rural representation with 80% power, a precision of 95%, a margin of error of 5%, a design effect of 2, and a response rate of 68% (Henderson, 1982; Lemeshow, 1985; Rosenthal et al. 2017)).

4.3 Sampling Frame

The sampling frame for this survey will be based on the 2012 Tanzania Population and Housing Census for the Morogoro region. A list of census enumeration areas (EAs) is available with basic housing and population information. Table 2 shows the distribution of households and number of EAs from the sampling frame.

4.4 Sampling Procedure and Sample Allocation

This is a cross-sectional household-based survey with a sample size of 1170 non-pregnant women of reproductive age. The Morogoro region encompasses seven districts, and 45 clusters will be selected from all districts (20 from urban strata and 25 from rural strata).

Data from the 2012 Tanzania Population and Housing Census for the Morogoro region (Table 2) will provide the sampling frame for this survey. Because regional differences in the distribution of rural compared with urban populations in the region, a stratified, multistage probability sample will be used for urban and rural areas. This survey will allow for independent estimates for WRA (Henderson & Sundaresan, 1982). The first stage of the sample design will be the selection of enumeration areas (EA) with probability proportional to size (PPS) design (20 urban and 25 rural areas) (Lemeshow & Robinson, 1985). The Probability Proportional to Size (PPS) design will ensure proper representation by district and population distribution. In the second stage, 26 households will be selected from each EA (totaling 1170 women). To select the households, household listing and maps of their location will be conducted in all the selected EAs prior to the initiation of survey data collection. A household listing form will be used to identify and select eligible households. A two-member team, comprising one mapper and one lister, will visit each EA to locate and mark its physical boundaries. The mapping team will do a census of all households and prepare a list of households with the name of the head and address or location of each household. During household listing, a list of household members, including the individual who prepares and/or purchases food for the household and WRA between 15 – 49 years of age. In order to ensure that the household listing is current and to minimize non-response, the time period between household listing and the start of data collection will not exceed one month. Only one WRA participant per household will be selected.

The survey findings will be generalizable to the Morogoro region.

Table 2: Distribution of Morogoro región households, number of EAs by district and by type of residence (Census 2012).

District	Number of Households			Number of EAs		
	Rural	***Urban***	***Total***	***Rural***	***Urban***	***Total***
Kilosa	71836	30079	101915	762	358	1120
Morogoro Municipal	2328	73019	75345	24	688	712
Morogoro Rural	70369	3021	73390	756	30	786
Kilombero	64095	22553	86648	606	189	795
Mvomero	63178	8471	71649	428	78	506
Gairo	32019	4988	37007	307	44	351
Ulanga	45628	7599	53227	684	71	755
Total (%)	349453 (70%)	149730 (30%)	499183 (100%)	3567 (70.1%	1458 (29.9%)	5025

The survey manager will number the houses where WRA reside and then divide the number of houses in the EA by the number of households that are to be interviewed in the cluster in order to systematically select the households within the cluster (26 households to be selected per EA). The survey team will use a random number generator to select a random starting point of the sampling and then will select every nth household. If the household meets the eligibility criteria, an eligible woman from the household will be interviewed. If a household includes multiple eligible women, then one woman will be selected randomly to participate.

The survey findings will be reflective of the Morogoro region. A spreadsheet containing all sampling parameters and selection probabilities will be constructed to facilitate the calculation of sampling weights. Sampling weights will be further adjusted for household non-response and as well as for individual non-response. Several sets of weight will be calculated: one set for the households, one set for women individual interview and one for women blood drawing. The final weights will be normalized in order to give the total number of un-weighted cases equal to the total number of weighted cases at regional level, for both household weights and individual weights.

4.5 Data Collection Procedures

Prior to data collection, all field workers (supervisors, team leaders, interviewers, and phlebotomist and lab technicians) will participate in a one-week training on proper data and specimen collection. The training will consist of two days of theoretical training and 3 days on data collection procedures, instruments, and equipment. As part of the role-play, phlebotomist will draw blood specimens from field workers, and laboratory technicians will practice processing and labelling samples. At the end of the training, a written test will be administered to team members. As more trainees will be recruited than those needed, only the best performing individuals will be selected for actual data collection, and the best performing and most experienced interviewers will be hired as team leads. Following the classroom training a week of field pilot testing in 2 to 4 EAs (2 urban and 2 urban and rural EAs) not included in our selected EAs will be conducted to familiarize teams with logistical procedures, field dynamics, and real world scenarios.

- Household and Non-Pregnant Women of Reproductive Age Interviews

The Morogoro Biomarker Survey will collect information from individuals who prepare OR purchase food for household to gather information on general characteristics of the population and their household. Also, information will be collected on maize and wheat flour consumption. The household roster information will collect information on all household residents (including WRA) who share a common kitchen. Information about pregnancy status will be collected for each listed WRA house resident. Small amount of sample wheat and/or maize flour consumed by the household will be collected for analysis at TFNC laboratory as part of fortification assessment. One non-pregnant WRA will be selected to participate in the women questionnaire and blood drawing. The WRA selected will be asked about her medical and pregnancy status and history, and micronutrient supplementation and prescription drug intake. The information collected by the Morogoro Regional Biomarker Survey will follow the Aday and Andersen model (Aday, 1997; 2006). Information on characteristics include: 1) enabling factors (usual source of care, health insurance, wealth index, prenatal care, periconception care, enrollment in government programs, etc.); and 2) predisposing factors, such as woman's age, respondent's education.

Women selected to participate in the sample will be asked to provide informed consent (prior to the blood collection for measurement of biomarkers. For women of reproductive age, blood samples (5-6 mL and 5-6 mL EDTA whole blood) will be collected via venipuncture, and blood tubes will be transported in a cool box to a regional laboratory that will process the specimens within 4 hours of collection.

- Blood collection and processing

Trained phlebotomist will collect 10-12 mL of blood by antecubital venipuncture in certified tubes containing anticoagulant. Due to the nature of the survey, blood samples will be taken in non-fasting conditions. Whole blood with EDTA anticoagulant and non-anti-coagulated whole blood vacutainers well be maintained at 4─8oC for a number of hours and sent to the field laboratory. Upon arrival in the field laboratory whole blood with EDTA anticoagulant will be analyzed by hematology analyzer for determination of hemoglobin and hematocrit. A portion of this blood samples will be separated into serum, plasma, and whole blood and for preparation of aliquots for laboratory processing. A small portion of the EDTA blood will be diluted with ascorbic acid to make red blood cell lysate (1:11 dilution) for determination of RBC folate. The non-anti-coagulated whole blood will be centrifuged after allowing to clot at room temperature for about 30 minutes. After centrifugation, serum will be aliquoted for retinol, Vitamin B12, serum folate, ferritin, soluble transferrin receptor, C - reactive protein, alpha 1 glycoprotein, retinol binding protein. Serum will be then stored at -20oC until shipped to the central lab at TFNC. After samples are received at the central lab, they should be stored at -80 oC until TFNC and/or other labs will conduct biochemical analyses. Detailed procedures for sample collection, shipment, processing and storage are provided in field and laboratory protocol provided in **Appendix 8.3.**

4.6 Data management and analysis

Data from the Morogoro Region Micronutrient Household and Biomarker Survey will be analyzed in SAS 9.4. Sampling weights will be applied and prevalence estimates will be

calculated. Free text data will be translated into English and coded into categories before being analyzed.

Analysis and Statistical methods

The basic analysis is aimed at identifying the number of selected women of reproductive age with anemia, folate or vitamin deficiency, ferritin and retinol deficiency. Participants will be classified deficient based on each biomarker cut-off (Table 3).

Outcome Measures and Analysis

Descriptive analysis will include frequencies, mean and geometric mean values for each biomarker. In addition, for each biomarker prevalence will be determined with 95% confidence intervals. Risk factor analysis will be carried out to determine prevalence risk ratios (PRR), and 95% confidence intervals (CI) for single and combined deficiencies. Significance will be determined for contingency tables by Wald's X2 test. In addition, bivariate and stratified analyses will be performed to examine the association between independent variables and deficiency status and potential confounding and interaction effects. Independent variables that are significantly associated with deficiency status in bivariate analyses will be included in logistic regression models to determine the relative contribution of health services and access factors controlling for other factors. Independent variables will be analyzed as categorical variables. Potential multi-co-linearity among variables will be examined. Continuous outcome variables analysis will be also carried out.

Biomarker indicators

Biomarkers are biochemical, functional or clinical indices of an individual's nutrient intake, status or functional effects. They are required to support evidence-based clinical guidance for health programs and policies for nutrition and health of a population. These indices can reveal information about biological or physiological responses to dietary and behavior or pathogenic processes, as well as can be used to monitor responses to therapeutic interventions and to provide information on inter-individual differences in response to diet and nutrition.

Laboratory assessment of these biomarkers is not only used to detect subclinical deficiency states, but also to confirm clinical diagnosis. Biomarkers have become important with the growing importance of preventive medicine because they provide an objective means of assessment of nutritional status of an individual. These procedures can supplement other methods of nutritional assessment resulting in identification of specific nutritional problems and deficiencies. A nutritional biomarker can be any biological specimen that is an indicator of nutritional status with respect to intake or metabolism of dietary constituents. The biomarkers to be assessed in this study include a full blood count for hemoglobin and hematocrit as well as serum B12, serum and red blood cell folate, Ferritin, Vitamin A, sTfR, CRP and AGP, homocysteine and MTHFR. Table 3 provides summary of the biochemical indicators and analyses methodology. The TFNC laboratory and other laboratories will conduct biochemical analyses for these biomarkers. Detailed laboratory procedures (SOP) for analyses done under TFNC laboratory are provided in Field and laboratory protocol in **Appendix 8.3.** Any sample

shipment to the laboratories outside the country will be done as per signed material transfer agreement (MTA).

Table 3. Biochemical indicators and analyses methodology

Indicator Group	Indicator	Methodology	Cut-off
Anemia	Hemoglobin		Total: < 12.0 g/dL Mild: 11.0-11.9 g/dL Moderate: 8.0-10.9 g/dL Severe: <8.0 g/dL
Inflammatory biomarkers	C-reactive protein	C-reactive protein and α–1 acid glycoprotein-ELISA[1]	>5 mg/L
	Alpha-1 Glycoprotein	C-reactive protein and α–1 acid glycoprotein-ELISA[1]	>1 mg/L
Micronutrients	Ferritin	Ferritin and soluble transferrin receptor-ELISA[1]	Bond method (normal under 10 percentile)
	Serum transferrin receptor	Ferritin and soluble transferrin receptor-ELISA[1]	Bond method (normal under 10 percentile)
	Serum retinol	Modified relative dose response (MRDR)-HPLC [2]	<20 ug/dL (≤70 µmol/l)
	Retinol binding protein	Retinol binding protein-ELISA[1]	
	Serum Folate	Microbiologic Assay[3]	<7 nmol/L
	Red blood cell folate	Microbiologic Assay	Deficiency: <305 nmol/L Insufficiency: < 748 nmol/L
	Serum B-12	Immunoasay based on electrochemiluminescence	Deficiency: < 148 pmol/L Marginal deficiency: 148-221 pmol/L
Homocysteine	Vitamin B12 and folate	Immunoassay based on fluorescence polarization	>10 nmol/L
Methylene Tetra Hydrofolate Reductase (MTHFR)	Folate/one-carbon		NA

[1] Erhard et al., 2004
[2] Tanumihardjo et al., 2016
[3] Bailey et al., 2015

5 Ethical Considerations

Potential risk and benefit

The participants might experience temporary discomfort during the blood drawn. This should not affect how they feel. Trained staff will be drawing blood using sterile techniques and personal protective equipment to protect both the participants and staff. Sometimes a person will bleed or have a bruise where the blood is drawn. There is always a very slight chance of infection but this is unlikely to happen given the precautions that will be in place. Some people may feel dizzy or faint. If the participants are found to be severely anemic, we will notify the participants to the nearest heath center in the community. At any time during the survey, the participants can decide to stop or refuse to continue during both the questionnaire or blood drawn.

The participants' opinions and blood samples will help to inform programmers and decision makers about future nutrition programs that will be used to improve the health of the community and the findings will help inform public health strategies to improve the future nutrition in Tanzania. If the participants are found to be severely anemic, we will notify the participants to the nearest heath center in the community.

Confidentiality

All data collected in the study will be linked by identifiers and will be treated with strict confidentially throughout and beyond the duration of the trial, with identifier-linked data available only to study investigator, Names will be collected in the study only to ensure accountability of the survey team and non-duplication in the data/interviewing; for the purpose of analysis, names will be disposed of study participation, and all associated potential risks and benefits. Data collectors will emphasize the unconditional voluntariness of study participation, the right to discontinue at any point during the study, and the confidential nature of all information that will be collected. Consent forms will specify that participants have the option to deny consent for any or all parts of the study procedures without any objections.

Consent process

A consent document in English or Kiswahili will be provided to potential participants and used by the interviewer as a guide for the verbal explanation of the project. The interviewer will give participants adequate information concerning the study, provide adequate opportunity for participants to consider all options, respond to the participants' questions, ensure that participants have comprehended this information, obtain participants' voluntary agreement to participate, provide a facts sheet with information about the study with a contact number, and continue to provide information as participants or situation requires.

An additional informed consent will be asked to the selected non-pregnant WRA participating in the Women's questionnaire and for the blood assessments for full blood count including hemoglobin and hematocrit as well as B12, folate, Ferritin, Vitamin A, sTfR, CRP and AGP, homocysteine and MTHFR. The results for full blood count including hemoglobin will be reviewed as soon as possible after analysis. Individuals found with hemoglobin levels below designated cut-off points and who are severely anemic will be contacted and referred to nearest health facility for further assessment and treatment.

Potential participants will be informed that their participation is voluntary. Potential benefit and minimum risk of participation in this project will be explained to potential participants with clear

language. Households participating in providing wheat and maize flour (50g each) will be compensated in cash for the flour provided. Choosing not to participate or withdrawal from the participation will in no way impact on individual's relationship with any affiliated institution.

If participants agree to participate in the project, participants will be asked to sign a written consent form before the interviews or surveys.

6 Budget

Household and Biomarker Survey Budget

Budget Category	Amount
Transport	$ 78,307
Per diem	$ 131,544
Venue	$ 3,913
Communications	$ 5,043
Stationary	$ 2,748
Disposable Supplies	$ 43,106
Total	**$264,661**

Budget Justification

Transport: Vehicles, drivers, and maintenance associated with transporting existing lab equipment and materials from the central lab to the temporary regional labs for the study, transporting the field teams to the selected Enumeration Areas in the Morogoro Region, and transporting the blood samples between the field work and the labs (regional and central labs).

Per diem: Reimbursements for the field teams participating in the survey; team members include supervisors for the field teams and lab teams, interviewers, phlebotomists, community members involved in introducing the survey to the targeted Enumeration Areas.

Venue: Training facilities and meeting spaces

Communications: Tablets, data cards & data usage for tablets and phones for study staff

Stationary: Office materials such as paper, external battery for tablets, power strips, and other office supplies; locked storage cabinet.

Disposable Supplies: Supplies for the lab, such as disposable pipette, gloves, biohazard bags, vacutainer blood collection needles, sterile gauze pads, 96-Well microplate, PCR plate, vials, dry ice, cardboard storage box, Styrofoam insulated shippers and other lab supplies.

7 References

Ada LA, Awe WC. (1997). Health Services Utilization Models. In D. S.Gochman (Ed.), Handbook of Health Behavior Research: Personal and Social Determinants. New York: Plenum Press. pp 153-172.

Andersen RM. (2008). National health surveys and the behavioral model of health services use. Med Care 46:647-53.

Assey VD, Peterson S, Kimboka S, Ngemera D, Mgoba C, Ruhiye DM, Tylleskär T. (2009). Tanzania national survey on iodine deficiency: impact after twelve years of salt iodation. BMC Public Health 9(1):319.

Assey V: Personal communication, 2019

Bailey LB, Stover PJ, McNulty H, Fenech MF, Gregory JF, Mills JL, et al. (2015). Biomarkers of Nutrition for Development-Folate Review. J Nutr 145(7):1636S-80S.

Bailey RL, West KP, Black RE. (2015). The epidemiology of global micronutrient deficiencies. Annals of Nutrition and Metabolism 66(2):22-33.

Black RE, Allen LH, Bhutta ZA. Caulfield LE, de Onis M, Ezzati M, Mathers C, Rivera J, for the Maternal and Child Undernutrition Study Group. (2008). Maternal and Child Undernutrition: Global and Regional Exposures and Health Consequences. Lancet 371:243-60.

BhuttaZ, Ahmed T, Black R, Cousens S, Dewey K, Guigliani E, Haider B, Kirkwood B, Morris S, Sachdev H, Shekar M, for the Maternal and Child Undernutrition Study Group. (2008). What works? Interventions for maternal and child undernutrition and survival. Lancet 371:417-40.

CDC. (1992). Recommendations for the use of folic acid to reduce the number of cases of spina bifida and other neural tube defects. MMWR Recomm Rep 41(No. RR-14):1-7.

Erhardt JG, Estes JE, Pfeiffer CM, Biesalski HK, Craft NE. (2004). Combined measurement of ferritin, soluble transferrin receptor, retinol binding protein, and C-reactive protein by an inexpensive, sensitive, and simple sandwich enzyme-linked immunosorbent assay technique. J Nutr 134(11):3127-32.

Garret GS, Bailey LB. (2018). A public health approach for preventing neural tube defects: folic acid fortification and beyond. Annals of the NY Academy of Sciences 1444:47-58.

Global Alliance for Improved Nutrition (GAIN), United States Centers for Disease Control and Prevention (CDC), the Africa Academy of Public Health (AAPH), Ifakara Health Institute, and the National Bureau of Statistics (NBS). (2016). National Fortification Assessment Coverage Tool (FACT) Survey in Tanzania, 2015. Access at: https://www.nbs.go.tz/nbstz/index.php/english/statistics-by-subject/health-statistics/fortification-assessment-coverage-tool-fact-survey/749-fortification-assessment-coverage-tool-fact-survey-in-tanzania-2015

Henderson RH, Sundarsen T. (1982). Cluster sampling to assess immunization coverage: a review of experience with a simplified sampling method. Bull World Health Organization 60(2):253-260.

Lemeshow S. (1985). A computer simulation of the EPI survey strategy. Int J Epi 14(3):473-481.

Lemeshow S, Robinson D. (1985). Surveys to measure programme coverage and impact: a review of the methodology used by the expanded programme on immunization. Worl Hlth Q 38:65-75.

Kishimba RS, Mpembeni R, Mghamba JM, Goodamn D, Valencia D. (2015). Birth prevalence of selected external structural birth defects at four hospitals in Dar es Salaam, Tanzania, 2011–2012. J Global Hlth 5(2).

Noor RA, Abioye AI, Ulenga N, Msham S, Kaishozi G, Gunaratns NS, Mwiru R, Smith E, et al. (2017). Large -scale wheat flour folic acid fortification program increases plasma folate levels among women of reproductive age in urban Tanzania. Plos One 12(8):e0182099.

Rosenthal J, Largaespada N, Bailey LB, Cannon M, Alverson CJ, Ortiz D, Kauwell GP, Sniezek J, Figueroa R, Daly R, Allen P. (2017). Folate deficiency is prevalent among of childbearing women and is negatively affected by coexisting vitamin B-12 deficiency. Belize National Micronutrient Survey 2011. J Nutr 147(6):1183-1193.

Tanzania National Bureau of Statistics (NBS). (2013). 2012 population and housing census. Population distribution by administrative areas. Dar es Salaam: National Bureau of Statistics.

Tanzania National Bureau of Statistics (NBS) and ICF Macro. 2011. Tanzania Demographic and Health Survey 2010. Dar es Salaam, Tanzania: NBS and ICF Macro. Accessed at https://www.dhsprogram.com/pubs/pdf/FR243/FR243[24June2011].pdf.

Tanzania Ministry of Health, Community Development, Gender, Elderly, Children (MoHCDGEC), Tanzania National Bureau of Statistics (NBS), Office of Chief Government Statistician - OCGS/Zanzibar, & ICF. (2016). Tanzania Demographic and Health Survey and Malaria Indicator Survey (TDHS-MIS) 2015-2016. Accessed at http://dhsprogram.com/pubs/pdf/FR321/FR321.pdf.

Tanumihardjo SA, Russell RM, Stephensen CB, Gannon BM, Craft NE, Haskell MJ, et al. (2016). Biomarkers of Nutrition for Development (BOND)-Vitamin A Review. J Nutr 146(9):1816S-48S.

Tanzania Food, Drugs, and Cosmetics Act (TFDAd). (2011). Food Fortification Regulations. Government Notice No. 205, July 22, 2011. Access at: https://www.tfda.go.tz/sites/default/files/Food%20Fortification%20Regulations.pdf

8 Appendices

8.1 Appendix A: Terms and Definitions

Anemia: The condition of having a hemoglobin concentration below a specified cut-off point, which can change according to age, gender, physiological status, smoking habits, and altitude at which the population being assessed lives. The World Health Organization (WHO) defines anemia in women of reproductive age as a hemoglobin concentration <120 g/l at sea level. Although the primary cause of anemia worldwide is iron deficiency, it often coexists with a number of other anemia causes, including malaria and other parasitic infections; acute and chronic infections that result in inflammation and hemorrhages; deficiencies in other vitamins and minerals, especially folate, vitamin B12 and vitamin A

Food fortification: The addition of one or more micronutrients (vitamins and minerals) to a food during processing. Ideally, food fortification provides a public health benefit with minimal risks to health in the population

Iodine deficiency: The condition resulting when iodine intake falls below recommended levels, tested through median urinary iodine concentration (normal range 100-199 μg/l).

Iron deficiency: The most common nutritional deficiency in the world, resulting from insufficient iron in the body due to inadequate consumption of bioavailable iron, blood loss, or unmet increased iron requirements due to infection, pregnancy, rapid growth, dietary habits, or any combination of these.

Iron Deficiency Anemia (IDA): The condition in which the body does not have enough healthy red blood cells due to a deficiency in iron. Iron deficiency (above) and iron deficiency anemia are associated with fetal and child growth failure, compromised cognitive development in young children, lowered physical activity and labor productivity in adults, and increased maternal morbidity and mortality. Women and young children are the most vulnerable to IDA, which increases the risk of hemorrhage and sepsis during childbirth, and is implicated in 20 percent of maternal deaths. Furthermore, children with IDA suffer from infections, weakened immunity, learning disabilities, impaired physical development, and in severe cases, death.

Micronutrient(s): Vitamins and minerals are needed in small amounts by the body to produce enzymes, hormones, and other substances essential for proper growth and development. Iodine, vitamin A, iron, folate, vitamin B are the most important in terms of prevalence and severity; deficiencies are a major threat to the health and development of populations worldwide, particularly children and pregnant women in low-income countries

Micronutrient deficiency (ies): Deficiencies in one or more essential vitamin or mineral, often caused by disease and/or lack of access and/or consumption of micronutrient-rich foods such as fruit, vegetables, animal products, and fortified foods. Micronutrient deficiencies increase the severity and risk of dying from infectious disease such as diarrhea, measles, malaria, and pneumonia. More than two billion people in the world are estimated to be deficient in iodine, vitamin A, iron, folate, vitamin B12.

Vitamin A Deficiency: The condition resulting when vitamin A intake falls below recommended levels. Vitamin A deficiency may be exacerbated by high rates of infection, and greatly increases the risk that a child may die from diseases such as measles, diarrhea, and acute

respiratory infections, and is the leading cause of childhood blindness. Vitamin A deficiency compromises the immune systems of approximately 40 percent of the developing world's children under five and leads to the deaths of as many as one million young children each year.

Vitamin B9 Insufficiency (Folate Insufficiency): Vitamin B9 is a water-soluble B vitamin. This vitamin is essential for normal cell growth and replication. Deficiencies of folate is associate with inadequate maternal folate status and has been linked to pre-eclampsia, spontaneous abortion, congenital heart defects, stillbirths, low birth weight and serious congenital anomalies of the brain and spine such as NTDs. In order to prevent neural tube defects it has shown that women need to consume 400 μg of folic acid daily, to reduce the risk of having an NTD-affected pregnancy. Recently, WHO has established a red blood cell folate threshold as an indicator of folate insufficiency in women of reproductive age. At the population level, red blood cell folate concentrations should be above 400 ng/mL (906 nmol/L) in women of reproductive age to achieve the greatest reduction in NTDs. Women of reproductive age with folate concentrations below this threshold are folate insufficient.

Vitamin B12 (B12): Vitamin B12 deficiency is also associated with anemia and adverse birth outcomes, such as NTDs. B12 is necessary for methionine synthase-based activation of folate and subsequent entry into one-carbon metabolism. A low concentration of B12 can contribute to the reduce formation of metabolically active folate, reduce folate retention in developing red blood cell folate, and indirect reduction of intracellular folate deficiency.

8.2 Appendix B: Elaborated Recruitment Procedure

Recruitment Procedure

A total of 26 households will be selected from each of the 45 selected enumeration areas, totaling 1170 women. To select the households, the survey team will re-map the enumeration area based on the 2012 census maps. The survey team will number the houses and then divide the number of houses in the area by the number of households that are to be interviewed in the cluster in order to systematically select the households within the cluster. The survey team will use a random number generator to select a random starting point of the sampling and then will survey every n^{th} household.

When the enumerator reaches the household, they will introduce themselves and give a brief overview of the purpose of the study. The enumerator will then ask a series of screening questions to verify the eligibility of the household. These screening questions will be asked to one household member that is at least 18 years of age. If the household meets the eligibility criteria, an eligible individual aged 18 years or older will be interviewed for the household survey, and one non-pregnant woman of reproductive age will be interviewed for the Women's questionnaire and blood draw (see eligibility criteria below). If we encounter a household with multiple eligible and willing participants, one will be selected randomly to participate. The WRA selected will be asked about her medical and pregnancy status and history, and micronutrient supplementation and prescription drug intake.

Before beginning the survey, the enumerator will walk through a consent process with the eligible participants and will ensure they understand what is written. The enumerator will answer any questions that the participants may have before beginning the study. Women selected to participate in the sample will be asked to provide informed consent prior to the blood collection for measurement of biomarkers. For women of reproductive age, blood samples (5-6 mL and 5-6 mL EDTA whole blood) will be collected via venipuncture.

A household roster portion of the household questionnaire will collect information on all household residents (including WRA) who share a common kitchen. Information about pregnancy status will be collected for each listed WRA house resident. A small sample of wheat and/or maize flour consumed by the household will be collected for analysis at TFNC laboratory as part of fortification assessment. One non-pregnant WRA will be selected to participate in the women questionnaire and blood drawing. The information collected by the Morogoro Regional Biomarker Survey will follow the Aday and Andersen model (Aday, 1997; 2006). Information on characteristics include: 1) enabling factors (usual source of care, health insurance, wealth index, prenatal care, periconception care, enrollment in government programs, etc.); and 2) predisposing factors, such as woman's age, respondent's education.

Criteria for inclusion/exclusion of subjects include:

i. Inclusion criteria for study participants

a. Household residents who are present in their households at the time of the interview (or during 2 additional visits the same day)
b. Household has resided in current dwelling for at least 3 months

c. Individuals who consent to participate in the survey and prepare or purchase food for the household
d. At least one non-pregnant WRA (15-49 years) residing in the household

ii. Exclusion criteria for study participants

Any household that does not meet inclusion criteria will be excluded.

8.3 Field and laboratory protocol

Protocol for Specimen Collection, Processing, Storage, Shipment and laboratory analysis

Morogoro Regional Micronutrient Household and Biomarker Survey, Tanzania

2019

Acronyms

TFNC	**Tanzania Food and Nutrition Centre**
AHL	**Average House Listing**
ANM	**Auxiliary Nurse Midwife**
APL	**Above Poverty Line**
ASHAs	**Accredited Social Health Activists**
BPL	**Below Poverty Line**
CDC	**Centers for Disease Control and Prevention**
CHC	**Community Health Centre**
DBC	**Designing for Behaviour Change**
DNA	**Deoxyribonucleic Acid**
EB	**Enumeration Block**
FFI	**Food Fortification Initiative**
FGD	**Focus Group Discussion**
GAIN	**Global Alliance for Improved Nutrition**
ICDS	**Integrated Child Development Services**
IDD	**Iodine Deficiency Disorders**
IRB	**Institutional Review Board**
IT	**Information Technology**
MDD	**Micronutrient Deficiency Disorders**
MI	**Micronutrient Initiative**
MTHFR	**Methylene tetrahydrofolate reductase**
NFHS	**National Family Health Survey**
NGO	**Non-Government Organization**
NHM	**National Health Mission**
MPL	**Morogoro Processing Lab**
NPWRA	**Non-Pregnant Woman of Reproductive Ag (18-49 years)**
NTDs	**Neural Tube Defects**
OBC	**Other Backward Classes**
OPH	**Other Priority Households**
PDS	**Public Distribution System**
PGIMER	**Post Graduate Institute of Medical Education and Research**
PHC	**Primary Health Centre**
PPS	**Probability proportional to Size sampling**
PSU	**Primary Sampling Unit**
RBC	**Red Blood Cell**
SC	**Scheduled Class**
WCD	**Women and Child Development**
WHO	**World Health Organization**
WFP	**World Food Program**

Lab Specific Acronyms:

AA	**Ascorbic acid**
CRP	**C - reactive protein**
HCY	**Homocysteine**
HPLC	**High performance Liquid Chromatography**
sTfR	**Serum Transferrin Receptor**
MPL	**Morogoro Processing Lab**
S. B12	**Serum B 12**
S.Folate	**Serum Folate**
SOP	**Standard Operating Procedures**
WBL	**Whole Blood Lysate**

PART I – Specimen collection and Processing

INTRODUCTION

This manual covers the field and laboratory components of the Tanzania Food and Nutrition Survey. It describes field procedures such as specimen collection and processing and sample storage by aliquot as per test requirement at Morogoro lab, as well as laboratory analysis by the Tanzania Food and Nutrition Centre, Dar-Es-Salaam, Tanzania.

This training manual is to serve as a detailed guide for the survey laboratory personnel who will be recruited for sample collection, processing and laboratory analysis. The manual provides detailed methodology for field procedures, including universal precautions, labeling, specimen collection and processing, sample storage and transportation, and management of the cold chain. This guide should be read carefully prior to the field work, and any questions should be discussed with the survey coordinator, principal investigators, laboratory coordinator and CDC technical support team.

There will be one survey team, consisting of one supervisor, four interviewers, and four lab technicians. The study will consist of 55 villages (clusters) and 21 households will be visited per village to recruit one non-pregnant woman aged 18-49 years per household for a total of 790 participants. Each survey team will visit one village per day to interview 21 women each. After

interviewers complete the questionnaire, a lab technician will collect blood after informed consent. Two lab technicians will carry out blood collection into pre - labelled vacutainers (two purple-top and one yellow-top) after verification of the participant. After completion of blood collection from about 12 participants (approximately 3-4 hours), blood specimens will be transported to Morogoro processing laboratory. There, specimens will be processed, aliquoted and stored according to the details given below on the same afternoon. Twice a week the specimens both refrigerated and frozen, will be transported maintaining the appropriate cold chain requirement to the TFNC in Dar-Es-Salaam for laboratory analysis by various biochemical tests. Arrangements for sample collection for the next day should be completed the previous afternoon by the laboratory personnel.

Table 1: Baseline Biomarker Assessment: Laboratory Analyses and Indicators

Biomarker	Condition or nutrient
Hemoglobin	Anemia
Erythrocyte folate	Folate
Serum folate	Folate
Serum vitamin B12	Vitamin B12
Serum ferritin	Iron
Soluble transferrin receptor	Iron
Alpha 1 acid Glycoprotein	Inflammation
C-reactive protein	Inflammation
Retinol Binding Protein	Vitamin A
MTHFR	Folate/one-carbon metabolism
Homocysteine	Vitamin B12 and folate

9 Overall Universal Precautions and Considerations

- Collection, testing, and processing of biological specimens are critical parts of the Micronutrient Survey.

- Specimen collection, processing, transport, and storage must be done with great care to obtain valid laboratory results that accurately reflect the micronutrient status of the participant.

- **Universal precautions** are procedures that must be followed by all team members to prevent exposure to HIV, hepatitis, and other infectious agents that are encountered during all collection, processing, and handling of biological specimens.

- ALL specimens should be considered as POTENTIALLY INFECTIOUS. Practice of universal precautions (**Annexure 1**) is essential throughout specimen collection, transportation, processing, storage, and shipment.

- In case of any potential exposure to blood borne pathogens, stop all work and **immediately** notify your team supervisor.

10 Overview: Cold Chain Logistics in the field laboratory

In each of the 55 villages,blood collection will be carried out at the household after conducting the interview. Lab technicians will ensure that all necessary supplies for the collection of blood are kept handy so that there is no delay in sample collection. Four lab technicians should be able to finish 12 blood draws in about 4 hours; samples will be transported midday to MPL. After a short lunch break, the remaining blood draws should be completed and transported to MPL

It is essential that proper cold chain logistics (**Annex 2**) are followed throughout the survey. Cold chain follows biological specimen from initial collection until specimen analysis. **All** team members should be aware of cold chain logistics and assist the lab technicians in maintaining the cold chain during field work.

Portable Freezers and Cold Boxes

- Portable freezers and cold boxes (Figure 1) will be used in the field to ensure the proper storage of the specimens in the field.
- The blood draw team will have one portable freezer which will be used to store frozen gel packs to be placed in cold boxes.

Figure 1. Portable Freezer and Cold Box

- A cold box with a handle will be provided to the lab technicians and for ease of storage and transport of specimens in the field.
- Additional Styrofoam cold boxes can also be made available to the lab technicians if extra cold boxes are needed.

- Portable freezers are used in the field to keep gel packs frozen so that the thawed gel packs can be easily replaced by the frozen ones in the field.

- Frozen gel packs will be used in the field to keep specimens cool after specimen collection in the field lab.
    - Note that it can take up to 48 hours to initially and completely freeze gel packs. Be sure to place enough gel packs into a ≤-20°C freezer prior to the start of the survey.

    - Extra frozen gel packs will be available in a -20°C located at Morogoro Processing Lab so that enough frozen gel packs are available each day.
        - Be sure to refreeze gel packs which are used during the day so that there will be a constant supply of frozen gel packs available each day.

- Cold boxes can be used in the field to store specimens after specimen collection in the field lab and prior to specimen processing:
    - Each cold box should contain a minimum of (4-5) frozen gel packs, one (1) digital thermometer, (1) vacutainer rack for collected specimens, and a small strip of bubble wrap, which should be placed <u>between</u> the frozen gel packs and the vacutainer rack to prevent freezing of the collected specimens.

Essentials of Cold Chain Logistics

- **<u>BLOOD SPECIMENS SHOULD NEVER BE FROZEN OR PLACED INTO THE PORTABLE FREEZERS UNTIL THEY ARE PROCESSED</u>**.

- Prior to being centrifuged and transferred to cryovials, blood specimens in the Vacutainer should **ONLY** be stored in a cold box containing a few frozen gel packs. Do not allow specimens to directly touch frozen gel packs because they might freeze.

- Only open lid to cold box when placing new specimens into the cold box or when replacing thawing gel packs.
  - When you open the cold box, record the temperature of the inside of the cold box on the "Transmittal sheet/Biological Specimen Control Form" (**Annex 8**).
  - The temperature inside the cold box should always remain <8°C.
  - The Laboratory Technician should replace thawing gel packs with frozen gel packs when temperature is ~6°C.
    - The Laboratory Technician will have the frozen gel packs close to them in one of the portable freezers. The laboratory technicians can exchange thawing gel packs with frozen gel packs at the same time as the delivery of specimens to the Laboratory Technician for processing.
- At the end of each day, the Laboratory Technician should ensure that all gel packs be placed into the ≤-20ºC freezer at MPL every evening so that they are frozen until hard and available for use in the cold boxes during the next day's field use.
  - Processed specimens must be in a ≤20°C freezer for storage until transported to TFNC.
  - A designated Laboratory Technician will ensure the temperature of the freezer is monitored each day and record the temperature on their temperature logs temperature of the freezer each day using the "Freezer Temperature Monitoring Form" (**Annex 4 and 5)**.

- The survey coordinator should designate a Laboratory Technician each week to monitor the temperature of the refrigerator/cold box each day using the "Refrigerator Temperature Monitoring Form" (**Annex 4 and 5)**.

- To ensure the proper transport of biological specimens, procedures for cold chain logistics need to be maintained to avoid adverse effects on specimen results (see **Table 2** below).

Table 2: Cold Chain Logistics

	Field Venous Blood collection	Packaging for Transport-ation to Morogoro	Laboratory Processing & Storage Morogoro Laboratory	Packaging for Transportation to TFNC	Laboratory Analysis & Storage at TFNC, Dar-Es-Salaam, TZ
Procedure	Two EDTA lavender capped vacutainers One yellow capped vacutainer per subject	Cold box with frozen gel packs	Storage of serum, plasma and RBC lysate aliquots at -20^{0}C in freezer.	All boxes will be transported frozen in a portable freezer	Serum ferritin, sTfR, CRP, AGP, RBP, ferritin in PCR vial Plasma homocysteine MTHFR RBC & serum folate Serum B12
Temperature	< 8ºC (Replace thawing gel packs at ~6°C)	< 8ºC (Replace thawing gel packs at ~6°C) for cold box and -10º to -20ºC for Portable Freezer which will be used to keep the gel packs frozen.	1-6°C (refrigerator) and -20ºC (freezer)	-10^{0}C portable freezer.	<-20ºC and <-70ºC (freezer)
Cold Chain	Store blood specimens in cold boxes that are maintained with frozen gel packs (replace thawing gel packs with frozen gel packs as needed)		1-6°C (refrigerator) -20ºC Deep freezer	Cryovials and PCR vials – portable freezer.	All cryovial and PCR vial boxes <-70ºC freezer and purple top vacutainer in 1-6°C refirgerator.
Verification method	Digital Thermometer	Digital Thermometer	Thermometer	Thermo-meter	Thermometer

11 Overview: Responsibilities of Laboratory Personnel

- There are several personnel involved in the survey including, four interviewers, four laboratory personnel, and one supervisor.
- The interviewer is responsible for:
    - Conducting the HH interview.
    - Assigning a lab ID and label the questionnaire columns for those eligible in the HH.
    - Requesting informed consent to participate in blood collection.
- The laboratory personnel are responsible for:
    - Organization of supplies needed for specimen collection in the field every day (**Annex 3**).
        - Each day enough supplies need to be available for a total of 21 blood draws per day (plus 10% extra supplies).
    - Cleaning working area prior to specimen collection.
    - Performing venipuncture on selected participants.
    - Maintenance of cold chain in the field (**Annex 2**)
    - Specimen collection at the houshold and transporting samples to Morogoro Processing lab. (**Annex 8: Flow Chart for Specimen collection and processing at Morogoro Processing Lab**)
    - Conducting complete blood count testing using the automated hematology analyzer at Morogoro Processing Lab.
    - Alerting participants with Hemoglobin less than < 8 gms/dl to go to the nearest PHC for evaluation and treatment by a clinician.
    - Making whole blood lysates and aliquoting them into 2 cryovials.
    - Centrifuge yellow top serum separator and aliquot them into 6 cryovials.
    - Maintenance of cold chain in the field and at Morogoro.
    - Recording participant label and specimen information on transmittal sheet. (**Annex 3a**)
    - Ensure labels are appropriately used and forms completed correctly
    - Understand all aspects of data collection and conduct field testing.
    - Ensure the Hematology analyzer, centrifuge and other equipment are calibrated and functioning properly.
    - Keep in daily contact with the regional coordinator and team supervisor during field work.
    - Make sure the necessary supplies and equipment are ready the night before each day of field work.
    - Manage the work flow in the processing lab and maintain all documentation regarding the completion of the cluster.
    - Ensure labels are appropriately used and forms completed correctly.

- Review all data collection forms for consistency and completeness,
- Serve as back-up and assist team members if they need help.
- Ensure any difficulties with equipment/supplies are communicated to the team supervisor /coordinator as soon as possible.
- Troubleshoot any problems with fieldwork, request support if needed.
- Remaining flexible for accepting any reasonable tasks assigned that are not listed above
- A flowchart of responsibilities for the Lab technicians is located in Annex 15.

12 Overview: Labelling Procedures

Always read labels carefully before affixing them to anything. Match the survey participant to 1) the label and to 2) the correct specimen.

Labelling for Household Questionnaire

The field interviewer will be responsible for labelling all wrist ID bracelets and putting them on the participants who will be selected for the blood draw the next day. However, it is important that all team members are familiar with the different labels:

- HH Questionnaire (1)
- Wrist ID label (1)
- Transmittal form (1)
- Extra label (1)

Labelling of Biological Specimens

The lab technicians will be responsible for labelling the biological specimens in the field and at Morogoro Processing Lab.

Each **"Women of Reproductive Age (WRA)"** will have 18 labels (Figure 3)

- **Questionnaire**
- **Bracelet**
- **Biological specimen control form**
- **Purple Vacutainer-1**
- **Purple Vacutainer-2**
- **Yellow vacutainer**
- **Cryovial-WBL - Folate**
- **Cryovial-WBL - Extra**
- **Cryovial-plasma - HCY**
- **Cryovial-plasma - Extra**
- **Cryovial-serum - Folate**

- **Cryovial-serum - B12**
- **Cryovial-serum - Retinol**
- **PCR vial-serum - FER, sTfR, CRP, AGP, and RBP**
- **Cryovial-serum - Extra**
- **Extra labels**

- The Interviewer is responsible for:
    - Putting the "**Questionnaire**" label on the participant's questionnaire at the start of the interview.
    - Putting the "ID bracelet" label on the participant's ID bracelet
- The Phlebotomist is responsible for:
    - Placing the "**Biological specimen control**" labels onto the corresponding form.
    - Placing the "Purple Top vacutainer 1", "Purple Top vacutainer 2", and "Yellow Top vacutainer" labels onto corresponding vacutainers.

- The Laboratory Technician is responsible for:
    - Placing the "WBL - Folate", "WBL - Extra", "Plasma - HCY", "Plasma -Extra", "Serum - Folate", "Serum - B12", "Serum - FER", "Serum - sTfR", "Serum - CRP", and "Serum - Extra" labels onto corresponding cryovials.
    - Placing appropriate labels on the Boxes that to be transported from the regional lab to TFNC (Figure 3).

Extra Labels

- Each set of labels will have some additional "Extra" labels. These can be used in cases where labels may have been torn or damaged.

- Write the sample description on the label using a marker pen. Cryovial boxes will have to be appropriately labeled (Figure 4).

Figure (3) Example labels used in the Field and at Morogoro

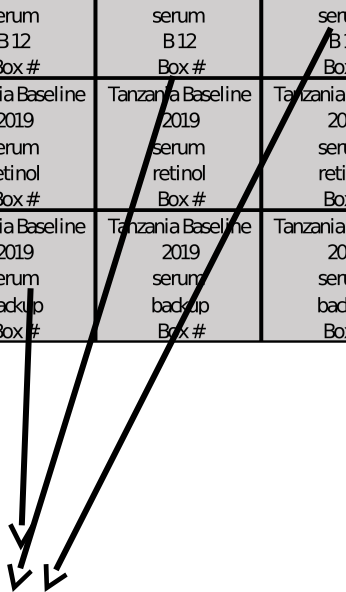
Tanzania Baseline 2019 WRA 15-49 Questionnaire IIIIIIIIIIIIIIIIIIIIIII I 0001	Tanzania Baseline 2019 WRA 15-49 Bracelet IIIIIIIIIIIIIIIIIIIIIII I 0001	Tanzania Baseline 2019 WRA 15-49 Specimen form IIIIIIIIIIIIIIIIIIIIIII I 0001
Tanzania Baseline 2019 WRA 15-49 Purple Vac 1 IIIIIIIIIIIIIIIIIIIIIII I 0001	Tanzania Baseline 2019 WRA 15-49 Purple Vac 2 IIIIIIIIIIIIIIIIIIIIIII I 0001	Tanzania Baseline 2019 WRA 15-49 Yellow Vac IIIIIIIIIIIIIIIIIIIIIII I 0001
Tanzania Baseline 2019 WRA 15-49 WBL Folate IIIIIIIIIIIIIIIIIIIIIII I 0001	Tanzania Baseline 2019 WRA 15-49 WBL backup IIIIIIIIIIIIIIIIIIIIIII I 0001	Tanzania Baseline 2019 WRA 15-49 plasma-HCY IIIIIIIIIIIIIIIIIIIIIII I 0001
Tanzania Baseline 2019 WRA 15-49 plasma-backup IIIIIIIIIIIIIIIIIIIIIII I 0001	Tanzania Baseline 2019 WRA 15-49 Serum-folate IIIIIIIIIIIIIIIIIIIIIII I 0001	Tanzania Baseline 2019 WRA 15-49 Serum-B 12 IIIIIIIIIIIIIIIIIIIIIII I 0001
Tanzania Baseline 2019 WRA 15-49	Tanzania Baseline 2019 WRA 15-49	Tanzania Baseline 2019 WRA 15-49

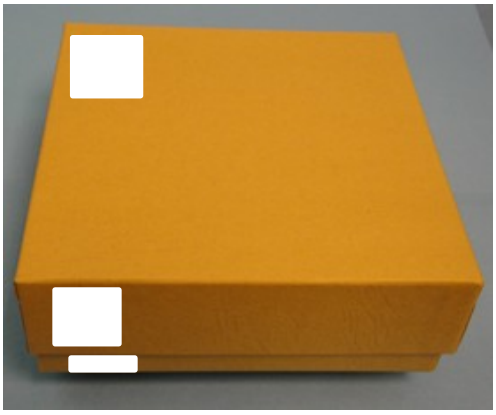
Serum-Retinol IIIIIIIIIIIIIIIIIIIIIIIII I 0001	Serum-backup IIIIIIIIIIIIIIIIIIIIIIIII I 0001	PCR vial IIIIIIIIIIIIIIIIIIIIIIIII I 0001	Enumerator
Tanzania Baseline 2019 WRA 15-49 Extra IIIIIIIIIIIIIIIIIIIIIIIII I 0001	Tanzania Baseline 2019 WRA 15-49 Extra IIIIIIIIIIIIIIIIIIIIIIIII I 0001	Tanzania Baseline 2019 WRA 15-49 Extra IIIIIIIIIIIIIIIIIIIIIIIII I 0001	Nurse Lab technician Extra labels

Note: The colors are only for learning purposes. The actual label strip is white with black print.

Figure 4: Labelling of Cryovial Boxes

Tanzania Baseline 2019 WB Lysate RBC Folate Box #	Tanzania Baseline 2019 WB Lysate RBC Folate Box #	Tanzania Baseline 2019 WB Lysate RBC Folate Box #
Tanzania Baseline 2019 WB Lysate RBC Folate backup Box #	Tanzania Baseline 2019 WB Lysate RBC Folate backup Box #	Tanzania Baseline 2019 WB Lysate RBC Folate backup Box #
Tanzania Baseline 2019 plasma Hcy backup Box #	Tanzania Baseline 2019 plasma Hcy Box #	Tanzania Baseline 2019 plasma Hcy Box #
Tanzania Baseline 2019 plasma Hcy backup Box #	Tanzania Baseline 2019 plasma Hcy backup Box #	Tanzania Baseline 2019 plasma Hcy backup Box #
Tanzania Baseline 2019 serum folate Box #	Tanzania Baseline 2019 serum folate Box #	Tanzania Baseline 2019 serum folate Box #
Tanzania Baseline 2019 serum B12 Box #	Tanzania Baseline 2019 serum B12 Box #	Tanzania Baseline 2019 serum B12 Box #
Tanzania Baseline 2019 serum retinol Box #	Tanzania Baseline 2019 serum retinol Box #	Tanzania Baseline 2019 serum retinol Box #
Tanzania Baseline 2019 serum backup Box #	Tanzania Baseline 2019 serum backup Box #	Tanzania Baseline 2019 serum backup Box #





- Each box will be labelled with 3 box labels:
    - One label goes on the top of the box lid, another goes on the side of the box lid, and the last goes on the side of the bottom of the box (Figure 4).
    - The team supervisors and laboratory coordinators are responsible for ensuring cryovial boxes are properly labelled and specimens properly sorted into cryovial boxes by the Laboratory Technicians.
        - Cryovial boxes will then be numbered numerically so that the specimen inside the box can be assigned to a specific cryovial box for the specimen inventory.
        - The regional laboratory coordinators and lab coordinator will work together to assign box numbers. The box numbers should not be duplicated (e.g., one box will receive "**Specimen type_cluster#_Box#1**", the second box will receive **Specimen type_cluster#_Box#2**, etc.). The box number must then be recorded on the "Transmittal Form "
        - At TFNC, the Laboratory coordinator and technicians will work together to receive and inventory box numbers:
        - A specimen inventory will also need to be generated by the laboratory coordinator at TFNC. The specimen inventory will include each specimen ID within a specific box.
        - All Boxes will be transported from regional to central lab in a portable freezer.

- All specimens will be in a -70°C freezer at TFNC laboratory until analysis, or stored (long-term) as backup specimens.

<u>REMEMBER:</u> Read the labels carefully before affixing them to anything.
Match the survey participant to the label and to the correct specimen.

Procedures for Specimen Collection in the field

Duties Prior to Specimen Collection:

- The Nurse should pack the backpack with all the supplies needed for daily field use (**Annex 3**).
- Check all equipment at MPL (centrifuges, freezers, etc.)

Specimen collection:

- The Nurse will be responsible for collecting blood in 3 vacutainers.
    - 6 ml of venous blood will be collected into the yellow top vacutainer.
    - 3 ml of blood will be collected for each one of the purple top vacutainer.
    - Each participant would have ~12 ml of venous blood collected.

Venous Blood Collection:

1. Obtain informed consent for blood collection
2. Set up all the supplies needed for the blood collection. This requires a comfortable location with a flat surface sufficiently large to lay out the absorbent pad and all equipment and supplies prior to collecting the venous sample from the participant.
3. Collect the venous blood specimen (**Annex 6: Procedure for Venous Blood Collection**). Sterile butterfly needles and vacutainers will be used for venous blood collection (Figure 2)

Yellow top	Purple top 1	Purple top 2
6 ml	3 ml	3 ml

4. Mix the specimens gently.
    - The nurse will have only two opportunities to collect blood from each participant.

5. The vacutainers should then be labelled with the correct corresponding participant's label. The nurse should also place the participant's label on the "**Transmittal Form/ specimen control**" form (**Annex 7**).

In each of the clusters, after identification of the participant who will be sampled, the samples will be collected at the Household.
A total of ~12 ml blood sample will be required per subject which will be collected in two K_2 EDTA (purple/lavender capped: 3 ml each) and one clotted blood samples for serum separation in yellow capped vacutainer (6 ml). Aseptic collection will be done using all products from Becton Dickinson BD Vacutainer® System disposable vacutainer blood collection needles
BD Vacutainer® Tubes, disposable Needles and Holders will be used together as a system for the collection of venous blood. BD Vacutainer® Blood Collection Needles are single-use, double-ended, medical grade stainless steel needles. They have a threaded hub that fits into the threads

of all BD Vacutainer® Holders. The venipuncture end of the needle has a point specially designed to enter the skin easily during venipuncture. The needle is lubricated with silicone. The needles to be used will be 1-1/2 inch lengths in 21 gauge. The BD Vacutainer® Luer Adapter which will be used is a male slip-luer fitting opposing a multiple sample non-patient (NP) needle. It is designed to be used with a BD Vacutainer® Holder. The BD Vacutainer® Luer-Lok™ Access Device is a holder with an integrated multiple sample NP needle and threaded male luer fitting.

Order of Draw: Venous blood sample has to be collected in the non-additive tube (yellow top: tube with Gel and Clot Activator) first before drawing blood in the two EDTA (purple top) vacutainers. Racks will be used to hold vacutainer tubes after sample collection during transit to MPL. The samples will be kept in the Styrofoam containers with frozen gel packs to keep samples cold during the day. Cold packs need to be kept in the -20^{0}C deep freezer before being put in the Styrofoam portable containers. Make sure that the vacutainers don't touch the frozen gel packs as that could lead to hemolysis.
(Note that it can take up to 48 hours to initially and completely freeze gel packs. Be sure to place enough gel packs into a ≤-20°C freezer prior to the start of the survey.)

Figure 5: Portable freezer/Cold Box for ease of transportation of samples to MPL and frozen samples to TFNC.

Items to be taken to the field for EACH survey teams. Complete list of supplies to be taken by each team for specimen collection of 21 women is given in Annexure 3

Specimen processing and storage at MPL:
Kindly ensure ACCURATE LABELLING of all samples.

After the samples are brought to the NPL, one of the EDTA vacutainers will be tested for automated blood cell counts in the Automated Blood Cell Counter. Any subject found to be anemic will be tested for the manual reticulocyte count.

The second EDTA tube will be used for red cell folate analysis for which 100 □l EDTA blood will be transferred after gently mixing to two plastic cryovials which contains 1 ml of 1% ascorbic acid solution. The pipette tip will be rinsed a few times in the ascorbic acid solution to ensure quantitative transfer of the EDTA blood. It is critical that the pipetting is done accurately. This vial should be labeled and stored at -20^{0}C.
The leftover sample will be spun for plasma separation at low speed and plasma will be transferred in two cryovials for plasma homocysteine. Samples will be stored at -20^{0}C at NPL.

Note: At room temperature there is an increase in HCY of about 10% per hour! Therefore it should be ensured that the sample processing is performed as soon as possible.

For serum separation, the clotted blood sample will be centrifuged at 1000 g for 10 minutes. Serum will be aliquoted (300□l each) into four plastic cryovials and one PCR vial: for Vitamin B12, retinol, folate and a backup vial. PCR vial for Juergen (Germany) needs and aliquot of ~100 ul serum for ferritin, sTfR, CRP, AGP, and RBP.

Table 3: Shows details of sample collection, parameters to be tested and storage conditions at NPL

Samples	**Biomarker**	**Storage Conditions**
K_2EDTA 1st vacutainer	Hemoglobin Reticulocyte count (if necessary) DNA extraction for MTHFR	4^0C
K_2EDTA 2nd vacutainer	1. RBC folate 2. Back up for whole blood folate 3. Plasma for homocysteine 4. Back – up vial for Plasma	-20^0C
Yellow capped vacutainer	1. Serum folate 2. Serum B 12 3. Serum Retinol 4. Serum backup 5. Serum Fe, sTfR, CRP, AGP, and RBP	-20^0C

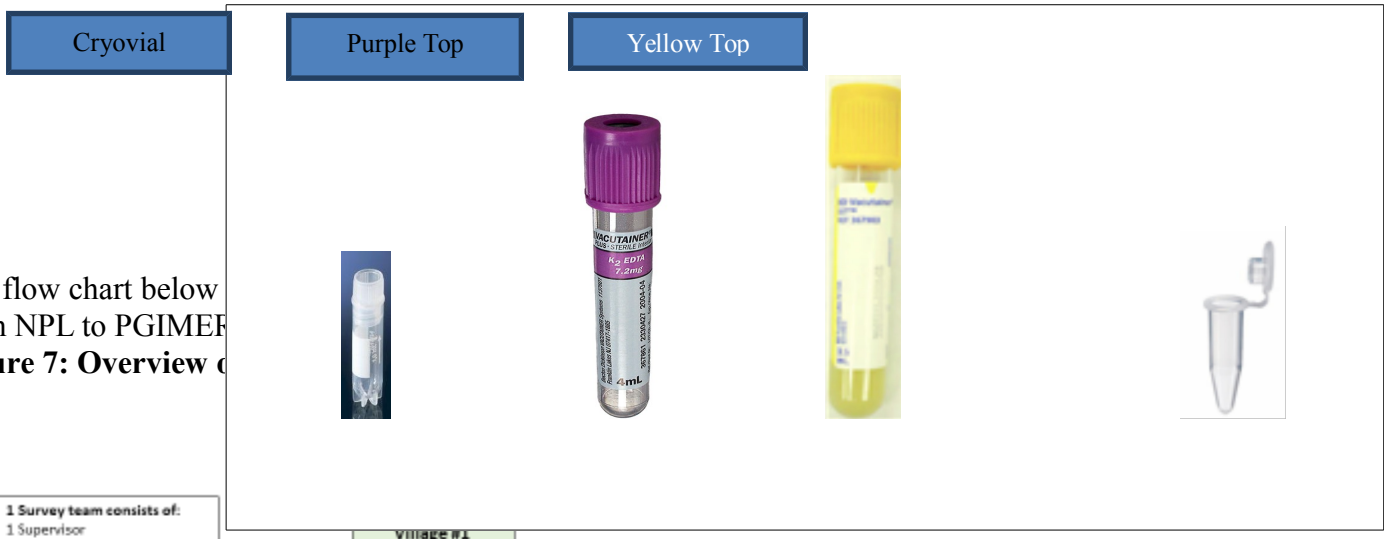
Note: All samples for tests to be performed in batches on frozen samples will be transferred to -70^0C deep freezers after the samples reach TFNC.

- At the end of each day, the Supervisor Laboratory Technician should ensure that all gel packs are placed into the -20ºC freezer in the MPL so that they are hard frozen and available for use in the cold boxes during the next day's field use.
    - Additional frozen gel packs will be available at the MPL facility.
    - Processed red cell hemolysate, EDTA sample for DNA extraction and serum aliquots should be placed into a ≤20°C freezer for temporary storage until transported to TFNC.
    - A designated Laboratory Technician of the district laboratory will ensure the temperature of the freezer is monitored each day and recorded using the "Freezer Temperature Monitoring Form" (**Annexure 4)**.

- The Laboratory regional coordinators should designate a Laboratory Technician in the field to monitor the temperature of the refrigerator/cold box each day using the "Refrigerator Temperature Monitoring Form" (**Annexure 5)**.

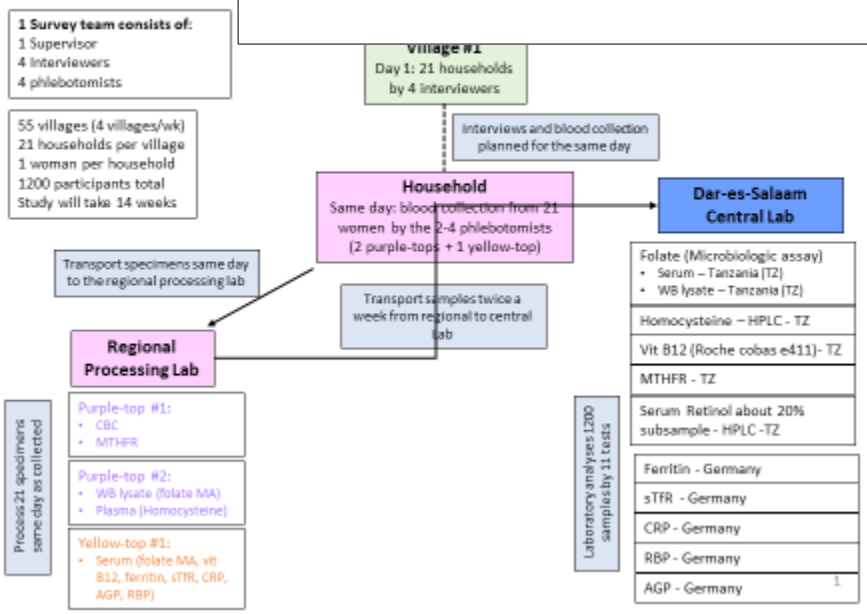
- To ensure the proper transport of biological specimens, procedures for cold chain logistics need to be maintained to avoid adverse effects on sample results (see **Table 2** above).

Figure 6: Cryovials and vacutainers



The flow chart below
from NPL to PGIMER

Figure 7: Overview



Samples will be arranged in vacutainer racks in the following order in the field (figure 8)
Figure 8: Arrangement of vacutainers in the field

Village:

- Collect 2 EDTA tubes and 1 serum-separator tube from each donor
- Label tubes appropriately (labels are organized by subject)
- Keep samples in portable cool boxes and maintain cold chain during transport

Purple EDTA tube 1	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6	Donor 7	Donor 8	Donor 9	Donor 10
Purple EDTA tube 2	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6	Donor 7	Donor 8	Donor 9	Donor 10
Yellow serum tube	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6	Donor 7	Donor 8	Donor 9	Donor 10

Purple EDTA tube 1	Donor 11	Donor 12	Donor 13	Donor 14	Donor 15	Donor 16	Donor 17	Donor 18	Donor 19	Donor 20
Purple EDTA tube 2	Donor 11	Donor 12	Donor 13	Donor 14	Donor 15	Donor 16	Donor 17	Donor 18	Donor 19	Donor 20
Yellow serum tube	Donor 11	Donor 12	Donor 13	Donor 14	Donor 15	Donor 16	Donor 17	Donor 18	Donor 19	Donor 20

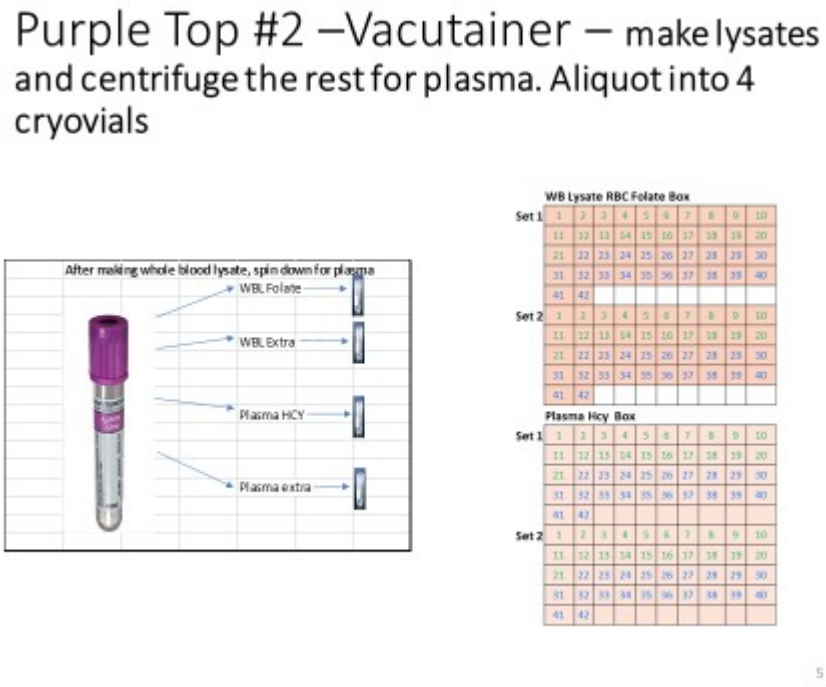
Purple EDTA tube 1	Donor 21									
Purple EDTA tube 2	Donor 21									
Yellow serum tube	Donor 21									

6

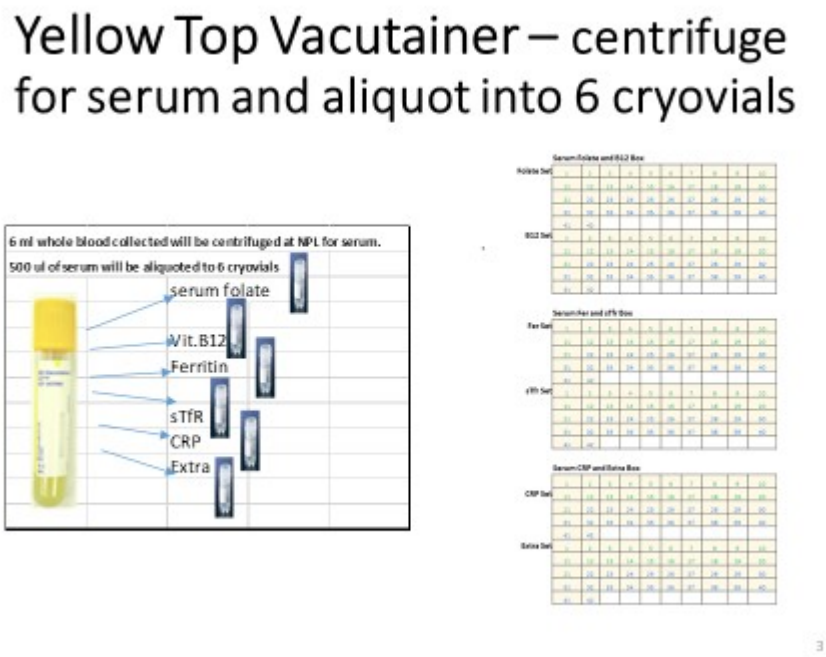
After reaching MPL, the vacutainers will be re-arranged for processing as shown below (Figure 9)

One lab tech will be assigned to run the hematology analyzer using purple top vacutainer#1 and the leftover sample will be refrigerated in the same vacutainer and transported to PGIMER. (Figure 10)

Two lab techs will be assigned to make whole blood lysates (see SOP for making lysates) first and then centrifuge the leftover sample for plasma. Aliquots will be made as shown below. (Figure 11)



The fourth lab tech will centrifuge the yellow top vacutainer for serum and aliquot into 6 cryovials as shown below (Figure 12)



After placing the samples in appropriate boxes, lab techs will place a check mark in appropriate places in the transmittal form. The boxes will have to be manually numbered and the box number has to be recorded in the transmittal form.

The leftover samples in one purple top vacutainer and the yellow top vacutainer should be kept refrigerated in a large Ziploc bag and transported to PGIMER.

13 Sorting Cryovials into Cryovial Boxes

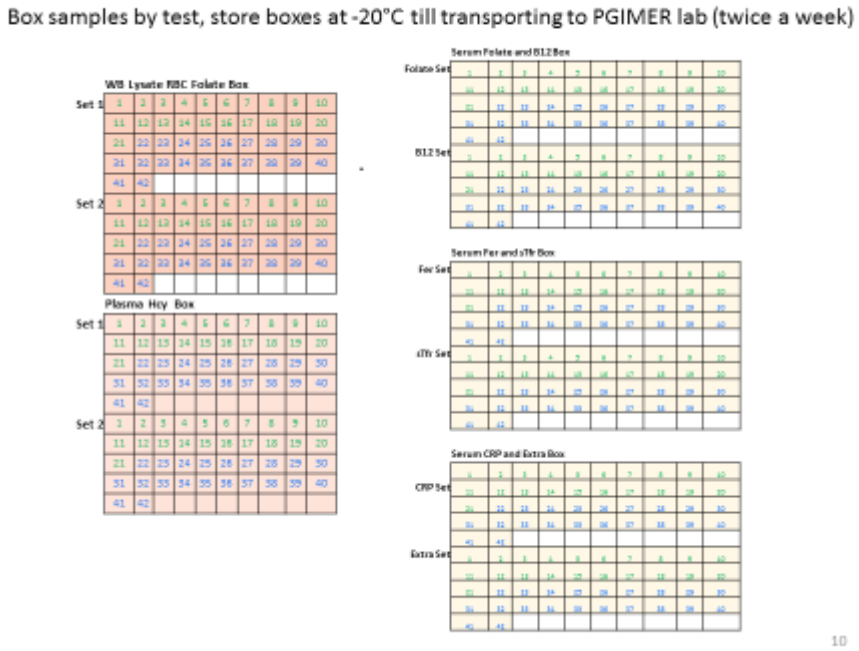
Cryovials must be sorted properly into cryovial boxes according to specimen type. This is essential to ensure the correct specimens are shipped to the correct laboratory for processing. Laboratory Technicians are responsible for sorting the cryovials into the correct cryovial boxes according to specimen type during the processing of specimens. Once the Hematology is completed, the lab tech will place the purple vac #1 in the fridge and place a check mark in the transmittal form.

All cryovials need to be sorted in the following order. Also see Figure 8.

- Serum Folate and Vitamin B12 will be arranged in one box.
- Serum Ferritin and sTfR in one box
- Serum CRP and extra serum vials in one box.
- WBL Folate and WBL Extra in one box.
- Plasma Homocysteine and Extra plasma vial in one box.

A total of 5 cryovial boxes will be transported in the portable freezer, and 1 Vacutainer rack will be transported in the cold box to PGIMER every 2 days.

Figure 13: Sorting of cryovials in boxes.



14 Transportation of Specimens from MPL to TFNC Lab

Every 2 days (cold chain logistics):

The supervisor, with the assistance of the Lab Technicians, is responsible for ensuring that all the specimens are consolidated and transferred to the laboratory for storage.
All processed specimens will be transported in the portable freezer except one purple top vacutainer rack which will be transported in the cold box. Sufficient gel packs need to be placed in the cold box to ensure the cold box is maintained at less than 8^0C until it reaches TFNC laboratory. Transmittal forms from 2 days of blood collection will be placed in file folder and be transported along with the specimens.

- The team supervisor should ensure that the specimens reach TFNC laboratory and are stored appropriately.
- All of the gel packs used in the field should be frozen until they are hard every night.
- The freezer must be set to at least -20º C.
- It is critical the gel packs are completely frozen until hard before teams leave for the field each day.
- The leftover samples from one purple top and one yellow top vacutainer have to be transported to TFNC laboratory along in a cold box.

15 Lab Procedures for Specimen Storage

- All specimens except the vacutainer rack need to be stored properly in a -70°C freezer until they are analyzed.
- Cryovial boxes will be numbered numerically so that the specimen inside the box can be assigned to a specific cryovial box for the specimen inventory.
    - Box numbers will be assigned once specimens are processed at NPL.
- A specimen inventory should be created for all specimen boxes and divided according to specimen type.
- All cryovial boxes will be stored in a dedicated freezer and the vacutainer rack will be stored in the fridge.
- Once samples arrive in TFNC laboratory, a resident Lab tech will ensure that all samples stated in the transmittal form have arrived.

16 Annexure 1: Universal Precautions

1. Universal precautions are defined by CDC as a set of precautions designed to prevent transmission of human immunodeficiency virus (HIV), Hepatitis B virus (HBV), and other blood-borne pathogens.
2. Blood and other patients' body fluids are considered potentially infectious for HIV, HBV, and other blood-borne pathogens.
3. Therefore health-care workers who handle body fluids such as blood, mucus, sputum, urine, stool, etc. should observe the following precautions:
    - Prevent skin and mucous-membrane exposure when handling blood or other blood-borne pathogens.
    - Use personal protection barriers (e.g. gloves, lab coats and eye glasses).
    - Wash hands after removing the gloves.
    - Clean laboratory benches before and after procedures with an appropriate disinfectant.
    - Dispose needles in sharps containers to prevent injuries.
    - Dispose cuvettes and all other used materials in biohazard bags for incineration or appropriate disposal.
    - Immediately report all accidents or injuries to your supervisor and follow the below precautionary measures:
        - In case of injury, it is necessary to squeeze the blood out of the injury, thoroughly wash the injury with soap and running water, cleanse the skin with 70% alcohol.
        - In case of contamination of hands with the blood, immediately wash the hands with warm water and soap.
        - In case blood gets to face, it should be thoroughly washed with warm water and soap.
        - Test the specimen of the source individual for HIV and hepatitis as early as possible (within 24 hours of exposure).
        - Document the following data, related to the nature of exposed, status of source individual & status of exposed health worker

- Name and data of the source individual.
- Time & date of exposure.
- Nature of exposure.
- Body site exposed.
- Infective status of the source.
- Previous testing & Immune status of the exposed health worker.

- Seek medical assistance as soon as possible

17 Annexure 2: Cold Chain Logistics

Frozen Gel Packs	Fresh frozen gel packs MUST be used at the beginning of each day. The Lab Technicians must store the gel packs at -20º C in the portable freezer when not in use. Whenever possible, gel packs that have thawed while in the field must be replaced with fresh frozen gel packs (i.e., when temperature of the cold box reaches 5-6°C call the Supervisor to have a Driver deliver new frozen gel packs). At the end of the day, gel packs used in the field should be placed in the portable freezers and kept at least at -20ºC so that they will be frozen and ready for the next day. A Driver will deliver the portable freezer to the laboratory to be plugged into electricity overnight. The Driver will then transport the portable freezer with frozen gel packs back to the field at the beginning of the following day.
Vacutainers	After blood collection, the Lab Technicians should place the labelled vacutainers in the vacutainer rack and place in the cold box containing frozen gel packs. Bubble wrap should be placed between the vacutainers and the frozen gel packs so that they do not touch. There will be 2 purple top vacutainers and one yellow top vacutainer. All the specimens in the cold box and the portable freezer must be sent at the end of the day for processing and storage.
Cold Boxes	The Lab Technicians should keep the cold box closed at all times. Avoid leaving the lid open and exposure to direct sun. A digital thermometer will be included in each cold box. The temperature of the cold box should remain <8°C. When temperature of the cold box reaches ~6°C call the Supervisor to have a Driver deliver new frozen gel packs.
Purple Top Vacutainer#1	The purple top vacutainer #1 for measuring: 1) Hemoglobin/CBC using the automated hematology analyzer at MPL 2) Reticulocyte count at MPL if necessary. 3) DNA extraction for MTHFR. Extracted DNA will be stored in labeled Eppendorf tubes after completing the extraction procedure.
Purple Top Vacutainer#2	The purple top vacutainer #2 will be used to make 1) WBL for the Folate microbiological assay 2) Backup of WBL. Then the vacutainer will be spun

	down in the centrifuge for 3) Plasma HCY 4) Back up vial for Plasma.
Yellow Top Vacutainer	The yellow top vacutainer needs to be spun down for serum using the centrifuge and then aliquoted into 4 cryovials and 1 PCR vial for 1) Serum Folate, 2) Serum B12, 3) Serum Retinol, 4) Back-up vial for serum, 5) PCR vial for Ferritin, sTfR, CRP, AGP, RBP

18 Annexure 3: List of Supplies Needed Daily for Specimen Collection

19 Four teams will collect 21 samples from each village, n = 21

**Includes enough supplies for 21 households per village (plus extra supplies)	
Supply Item	Quantity Required
Backpack	4
Yellow Top Vacutainers	25 per team= 50 per day
Purple Top Vacutainers	50 per team= 100 per day
Tourniquet	2 per team= 4 (can be reused)
21G Needles	25 per team= 50 per day
Vacutainer Barrels	4 per team (can be reused)
Sharps container	1 Box each= 2
Alcohol pads	30 per team= 60 per day
Gauze pads	30 per team= 60 per day
Absorbent pads	2 (can be reused if clean)
Biohazard bags	2
Ziploc bags	15 small per team= 30 per day
Labels	3 sets pre-printed per subject= 25 sets
Cold box	1 per team= 2
Frozen Gel Packs	4-5 for each cold box= 10
Bubble Wrap	2Large Piece (to protect specimens from frozen gel packs inside each cold box)
Digital Thermometer	1 each for cold box =2
Vacutainer Rack for Cold Box	1 each per box
Kim wipes	1 Box each = 2 boxes
Band-Aids	30
Gloves S, M, L	1 Box each per team of correct size (with a minimum of 30 pairs)
Pens	2
Log note books	4 (1 each)

20

21 Annexure 4: Freezer Temperature Monitoring Form(-20^{0}C)

District/Region:
Survey Laboratory Coordinator:
Field Laboratory Technician:
Freezer Information (i.e., type)

1.) To be used to monitor freezers located in the field laboratory at Morogoro.
2.) Please monitor the temperature of the freezer daily and record the date, time, temperature, initials, and any observed issues with the operation of the freezer.
3.) Report any issues to the Survey Laboratory Coordinator.
4.) Freezers will only be used to store specimens: a) until they are transferred to TFNC, Dar-Es-Salaam.
5.) Do not store processed specimens in refrigerators!

Date	Time	Temp., ˚C	Initials	Observations

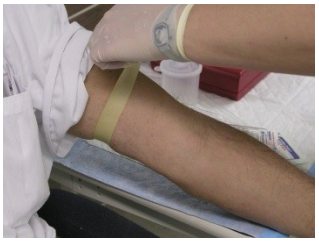
Annexure 5: Refrigerator Temperature Monitoring Form (1°- 6°C)

District/Region:
Survey Laboratory Coordinator:
Field Laboratory Technician:
Refrigerator Information (i.e., type)

1.) To be used to monitor refrigerators located in Field laboratory at Morogoro.
2.) Please monitor the temperature of the refrigerator daily and record the date, time, temperature, initials, and any observed issues with the operation of the refrigerator.
3.) Report any issues to the Survey Laboratory Coordinator.
4.) Refrigerators will only be used to keeping samples, reagents and quality control material.

Date	Time	Temp., ˚C	Initials	Observations

ANNEX 6: PROCEDURE FOR VENOUS BLOOD COLLECTION



The lab technician will be responsible for performing venipuncture on all participants at the household. After obtaining consent, the Nurse will be given only two chances to collect blood from each participant.

I. Always remember to practice Universal Precautions

1. Assume that all human blood is potentially infectious for HIV, HBV, and other infectious agents.
2. Use gloves and any additional personal protective equipment (PPE) if available (e.g., eye protection, lab coats)
3. Always use sterile, single-use disposable supplies for sample collection.

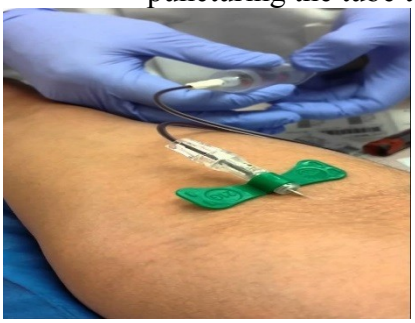
II. Preparing for venipuncture

1. Obtain informed consent for blood collection
2. Lay out a clean disposable absorbent pad on a table at the central field laboratory
3. Lay out all blood collection supplies and necessary labels. Assemble needle or butterfly needle into Vacutainer holder being sure that it is firmly seated into threads.
4. Loosely place Vacutainer tube into holder, but do not puncture top
5. For each participant, wear a new pair of clean gloves and conduct all procedures on a new, clean disposable absorbent pad

III. Venipuncture Procedure

1. Check the ID bracelet against remaining lab labels
2. Make sure the participant is sitting comfortably.
3. Examine both arms to find the best vein.
4. Locate the puncture site and apply the tourniquet
5. Cleanse the area with an alcohol wipe by wiping in a circular motion making sure the area is thoroughly cleaned
6. Repeat with a second wipe, if necessary

7. Dry with gauze
8. Fix the vein by pressing down on the vein about 1 inch below the proposed point of entry
9. Remove the butterfly needle shield
10. Approach the vein in the same direction the vein is running, holding the needle so that it is at an approximately 15º angle with the participant's arm.
11. Push the needle, with the bevel facing up, firmly and deliberately into the vein.
12. Activate the vacuum collection tube by pushing the tube onto the needle and puncturing the tube top.



13. If the needle is in the vein, blood will flow freely into the tube.
14. If no blood enters the tube, probe for the vein once or twice until entry is indicated by blood flowing into the tube.
15. After blood flow is established, loosen the tourniquet immediately and release entirely as the last tube fills.
16. For all participants, fill one yellow top vacutainer with 6 ml of blood and fill 2 Purple Top Vacutainer with 3 ml of blood

Yellow top	**Purple top 1**	**Purple top 2**
6 ml	3 ml	3 ml

17. Upon completion of blood collection, withdraw the needle. Applying heavy pressure as the needle is being withdrawn should be avoided.
18. Have the mother/caregiver continue to hold the gauze in place for several minutes. This will help prevent hematomas.
19. Invert the Purple Top Vacutainer 10 times after blood collection so that the blood will mix with the EDTA in the tube to prevent clotting. Gently invert the Blue Top Vacutainer 5 times after blood collection to mix with clot activator
20. Place an adhesive bandage on the participant's arm
21. Label all tubes with the correct and corresponding labels provided (i.e "**Purple Top Vac1**" or "**Purple top vac 2" or** "**yellow top vac**")
22. Place the correct corresponding "**Specimen Ctrl**" label on the "**Biological Specimen Control Form**" (Annexure 7)and complete the necessary information
23. The needle should be discarded into a sharps container.

24. Place **labeled tubes** into a rack inside a cold box with frozen gel packs and discard waste into a biohazard bag.
25. Report to the team leader/supervisor any reaction experienced by the participant during the venipuncture procedure

Annexure 7 - Specimen Control Form

BIOLOGICAL SPECIMEN CONTROL FORM - TANZANIA PROJECT- HH BIOMARKERS COLLECTION

Complete all sections. Be sure to sign control form each day

GENERAL INFORMATION

District: _____ Date _____ (Day) _____ (Month) _____ (Year)

Village: _____

Place labels for all women in blocks provided below

Total number of WRA samples collected:	Vacutainer (P)	Vacutainer (P)	Vacutainer (Y)

Phlebotomist Name _____ Phlebotomist Signature _____

SPECIMEN COLLECTION, FIELD TESTING, AND TEMPERATURE MONITORING FOR COLD BOX

Instructions for Phlebotomists:

1.) Record the time frozen gel packs are placed into the cold box in the morning

2.) Place each WRA's label in box below and mark specimen with "X" if specimen type was collected and/or test was performed.

3.) Record temperature of cold box each time the lid is opened. Be sure to only open lid when placing specimens inside the cold box.

4.) Replace thawing gel packs with frozen gel packs when the temperature is ~6 ∘C (temperature of cold box should remain <8 ∘C).

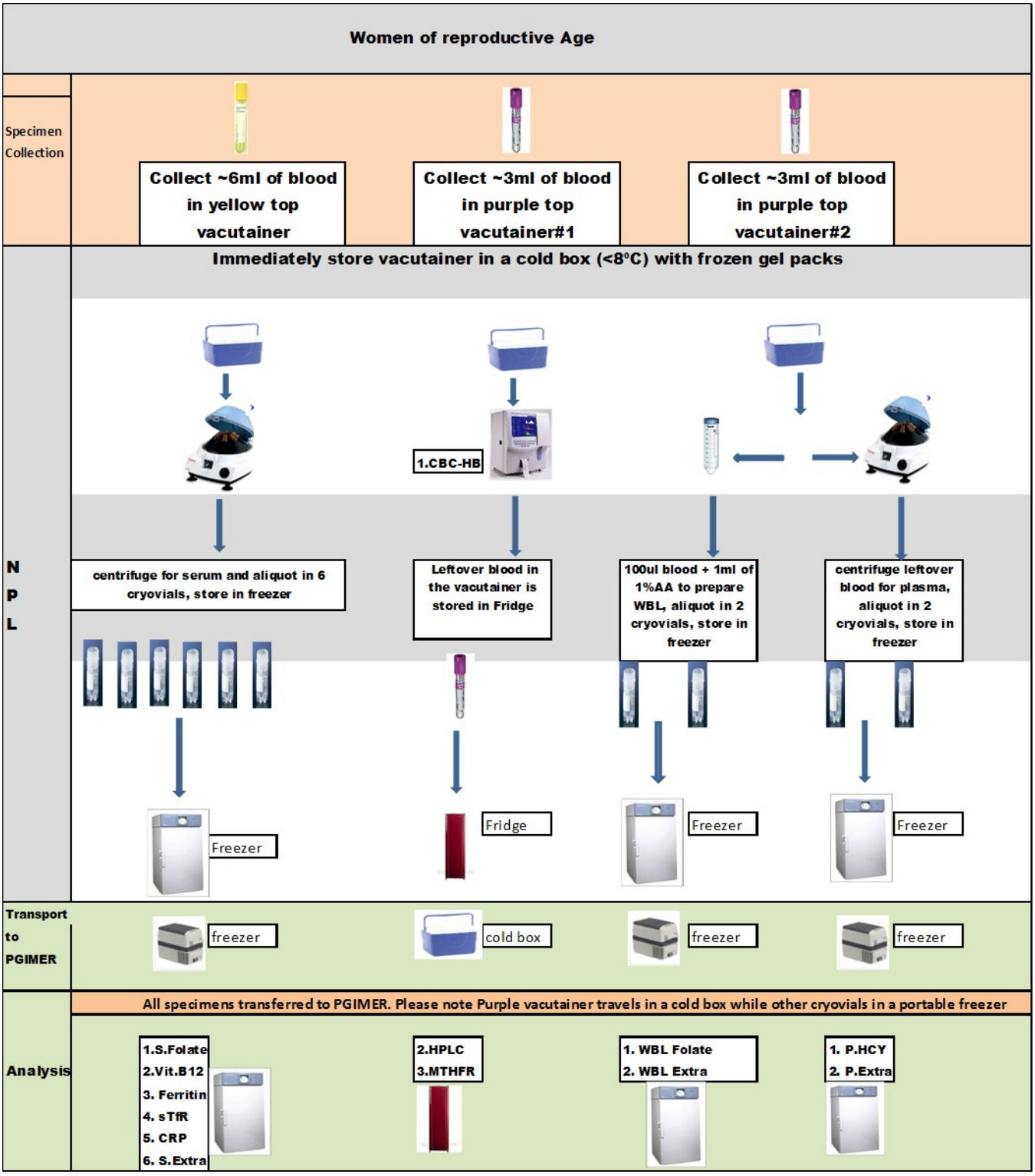
Time frozen gel packs placed into the cold box in the morning _____

	Specimen Type		NPL- processing		Specimen vials	check (x)	Volume	Time Processed	Box #
					Serum Folate *(500ul)*		-		
					Serum B12 *(500ul)*		-		
			CBC		Serum Ferritin *(500ul)*		-		
	Vacutainer (P)		WBL		Serum TfR *(500ul)*				
1.	Vacutainer (P)		PLASMA		Serum CRP *(write ul)*				
	Vacutainer (Y)		SERUM		Serum Extra *(write ul)*		-		
					Plasma HCY		-		
					Plasma Extra		-		
	Temp: _____		Temp _____		WBL Folate (500 ul)				
					WBL extra *(500 ul)*				

	Specimen Type		NPL- processing		Specimen vials	check (x)	Volume	Time Processed	Box #
					Serum Folate *(500ul)*		-		
					Serum B12 *(500ul)*		-		
			CBC		Serum Ferritin *(500ul)*		-		
2	Vacutainer (P)		WBL		Serum TfR *(500ul)*				
	Vacutainer (P)		PLASMA		Serum CRP *(write ul)*				
	Vacutainer (Y)		SERUM		Serum Extra *(write ul)*		-		
					Plasma HCY		-		
					Plasma Extra		-		
	Temp: _____		Temp _____		WBL Folate (500 ul)				
					WBL extra *(500 ul)*				

	Specimen Type		NPL- processing		Specimen vials	check (x)	Volume	Time Processed	Box #
					Serum Folate *(500ul)*		-		
					Serum B12 *(500ul)*		-		
			CBC		Serum Ferritin *(500ul)*		-		
3	Vacutainer (P)		WBL		Serum TfR *(500ul)*				
	Vacutainer (P)		PLASMA		Serum CRP *(write ul)*				
	Vacutainer (Y)		SERUM		Serum Extra *(write ul)*		-		
					Plasma HCY		-		
					Plasma Extra		-		
	Temp: _____		Temp _____		WBL Folate (500 ul)				
					WBL extra *(500 ul)*				

Annexure 8: Flow Chart for specimen field collection and processing at MPL



Part II – Standard Operating Procedures

	TANZANIA FOOD AND NUTRITION CENTRE **MICROBIOLOGY LABORATORY SECTION** **Study Specific Standard Operating Procedure (SOP) for Venous Blood Collection**		
Version No:		**Copy no.**	
Prepared by:			
Reviewed by:	1. 2.		
Authorized by:			

TANZANIA FOOD AND NUTRITION CENTRE

Changes:

……
……
……..

Reasons for changes:

……
……
……..

SCOPE

Applied during venous blood sample collection for survey of micronutrients biomarker study

PURPOSE

- To ensure proper technique during vein puncture and blood sample collection

RESPONSIBILITIES

1. The phlebotomies will be responsible for performing venipuncture on all participants at the field.
2. All personnel (Nurses, Laboratory technologist) involved in the study should be trained and follow this SOP during field sample collection at the households.

PRINCIPLE

Venous blood sample is collected from the vein by pressing down on the vein about 1 inch below the proposed point of entry. A tourniquet is applied after location the puncture site and the blood sample is filled into the vacutainer tubes.

ABBREVIATIONS

EDTA Ethylene diamine tetra-acetic acid
ID Identification

Materials needed:

- Vacutainers
- Specimen labels with barcode
- Marker pen
- Absorbent pad
- Gauze

PROCEDURES

I. Preparing for venipuncture

1. Obtain informed consent for blood collection
2. Lay out a clean disposable absorbent pad on a table/ chair available at the house hold
3. Lay out all blood collection supplies and necessary labels. Assemble needle or butterfly needle into Vacutainer holder being sure that it is firmly seated into threads.
4. Loosely place Vacutainer tube into holder, but do not puncture top
5. Wear a new pair of clean gloves and conduct all procedures on a new, clean disposable absorbent pad

II. Venipuncture Procedure

1. Check the participant ID against remaining lab labels
2. Make sure the participant is sitting comfortably.
3. Examine both arms to find the best vein.
4. Locate the puncture site and apply the tourniquet.
5. Cleanse the area with an alcohol wipe by wiping in a circular motion making sure the area is thoroughly cleaned.
6. Repeat with a second wipe, if necessary
7. Dry with gauze.
8. Fix the vein by pressing down on the vein about 1 inch below the proposed point of entry.
9. Remove the butterfly needle shield.
10. Approach the vein in the same direction the vein is running, holding the needle so that it is at an approximately 15º angle with the participant's arm.
11. Push the needle, with the bevel facing up, firmly and deliberately into the vein.

12. Activate the vacuum collection tube by pushing the tube onto the needle and puncturing the tube top.
13. If the needle is in the vein, blood will flow freely into the tube.
14. If no blood enters the tube, probe for the vein once or twice until entry is indicated by blood flowing into the tube.
15. After blood flow is established, loosen the tourniquet immediately and release entirely as the last tube fills.
16. Fill one yellow top vacutainer with 4 ml of blood and fill 2 Purple Top Vacutainer with 4 ml of blood.
17. Upon completion of blood collection, withdraw the needle. Applying heavy pressure as the needle is being withdrawn should be avoided.
18. Have the mother/caregiver continue to hold the gauze in place for several minutes. This will help prevent hematomas.
19. Invert the Purple Top Vacutainer 10 times after blood collection so that the blood will mix with the EDTA in the tube to prevent clotting. Gently invert the Blue Top Vacutainer 5 times after blood collection to mix with clot activator.
20. Place an adhesive bandage on the participant's arm.
21. Label all tubes with the correct and corresponding labels provided (i.e "Purple Top Vac1" or "Purple top vac 2" or "yellow top vac").
22. Place the correct corresponding "Specimen Ctrl" label on the "Biological Specimen Control Form" and complete the necessary information.
23. The needle should be discarded into a sharps container.
24. Place labeled tubes into a rack inside a cold box with frozen gel packs and discard waste into a biohazard bag.
25. Report to the team leader/supervisor any reaction experienced by the participant during the venipuncture procedure

I have read, understood and agreed to follow the procedure as documented

No.	Name	Signature	Date

Standard Operating Procedure (SOP) – for operating Sysmex XP 300 Hematology Analyzer

	TANZANIA FOOD AND NUTRITION CENTRE **BIOCHEMISTRY LABORATORY SECTION** **Standard Operating Procedure (SOP) for operating Sysmex XP 300 Hematology Analyzer**		
Version No:		**Copy no.**	
Prepared by:			
Reviewed by:	1. 2.		
Authorized by:			

TANZANIA FOOD AND NUTRITION CENTRE

Changes:
……
……
……..

Reasons for changes:
……
……
……..

SCOPE
This procedure describes the test method (automated) used for whole blood analysis (Full Blood Count - FBC) using the Sysmex XP 300 Hematology Analyzer. For the study titled: "Morogoro Region Micronutrient Household and Biomarker Survey, Tanzania, 2019"

PURPOSE
To ensure proper analysis and reporting of complete blood cell count with Sysmex XP 300 Hematology Analyzer

RESPONSIBILITIES

Laboratory Scientist/ technologist/ technicians involved in the study should be trained and follow this SOP during sample analysis

PRINCIPLE

The Sysmex XP 300 utilizes technology of fluorescence flow. The combination of side scatter (inner complexity of the cell), forward scatter (volume) and fluorescence intensity of nucleated cells gives a concise but precise image of each cell detected in the peripheral blood. A well-defined physical description of the different leucocyte populations (clusters) is obtained. Abnormal and immature cells, with their larger nuclear volume show much higher fluorescence intensity than normal cells, and are easily distinguishable in the DIFF scattergram.

Materials needed:

CellPack
Stromatolyser
Sulfolyser
CellClean (detergent

PROCEDURES

Equipment startup

1. Check priority to operation such as reagents check, printer paper, instrument, sampler and waste fluid
2. Turn on the instrument by pressing power button
3. Turn on the IPU power ON, window system will start automatic
4. Log on window will appears, log on with user name and password provided
5. After successful log in equipment will perform self-test and temperature stabilization for 30minutes
6. After temperature stabilization equipment will perform background check for maximum of 3 minutes

Quality control procedures:

1. At the beginning of each work shift, all parameters are tested with blood control.
2. The 3 levels include: Abnormal Low, Normal, Abnormal High
3. Controls are stored at 2-8ºC and brought to room temperature on a roller mixer before use.
4. Controls are gently inverted eight times according to the manufacturer's instruction before use.
5. From the RUN screen, press [SPECIMEN TYPE].
6. Use the arrow key on the keyboard to move the cursor to the appropriate QC file (i.e., low, normal or high) and press the [QC SPECIMEN] key.
9. Control values must be within three standard deviations, otherwise the measurement has to be repeated, if the control still out of range:

a. Check operation of the machine, ensuring it is clean and that all required supplies are present in sufficient quantities.

b. Check reagents for expiration dates and lot numbers. Ensure that all machine lines are in appropriate receptacle where applicable, If this does not solve the problem:
•Prepare new control(s) and try again.
•If the controls are still out, inform your supervisor to check the operator's manual, or recalibrate instrument and If controls are still out. Contact Medical Maintenance where applicable, or servicing engineer.
7. All control data are managed using software that provides graphical reports (Levey-Jennings graphs, and monthly cumulative histograms).

Run Samples

1. Check to see that the reagents needed for the number of the samples to be processed for the day are available.
2. Turn ON the power switch on the right side of the unit. Self-check, auto rinse, and background check will be automatically performed, and the "Ready" (ready for analysis) will appear.
3. When auto rinse and background check are normally completed, "Ready" is displayed.
4. Perform quality control analysis on 3 levels of control blood material (low, normal and high) to verify that the instrument is performing within the specified ranges of the quality control material.
5. If the result of quality control in acceptable range input your blood samples.
6. Input from the panel keyboard.
7. Press [SAMPLE No.] key in the Ready status.
8. Entering patient ID, sample ID, Patient name, etc
9. Press [ENTER] key,This will fix the sample No. and the status becomes ready for analysis.
10. Mix the sample sufficiently before analysis.
11. Remove the plug while taking care not to allow blood scatter.
12. Set the tube to the sample probe, and in that condition, press the start switch.
13. when the LCD screen displays "Analyzing," remove the tube.
14. After that, the unit executes automatic analysis and displays the result on the LCD screen.
15. Then the unit turns to the ready status, becoming ready for analysis of the next sample

Preliminary blood report

Tanzania Food and Nutrition Centre

Biochemistry laboratory section

TANZANIA FOOD AND NUTRITION CENTRE

Name: Age/Sex: F Date:

Identification No.:

Lab. No.:

Address:

Mobile No.:

Investigation done: complete blood cell count

Report:

(attach results printout)

Interpretations:

Comments:

……

……..

Reported by Reviewed by

Reference

Sysmex XP300 System Operator's Manual.

I have read, understood and agreed to follow the procedure as documented.

No.	Name	Signature	Date

Standard Operating Procedure for specimen collection/processing/shipment for RBC and serum folate assay

	TANZANIA FOOD AND NUTRITION CENTRE **MICROBIOLOGY LABORATORY SECTION** **Standard Operating Procedure (SOP) for specimen collection/processing/shipment for RBC and serum folate assay**		
Version No:		**Copy no.**	
Prepared by:			
Reviewed by:	1. 2.		
Authorized by:			

TANZANIA FOOD AND NUTRITION CENTRE

Changes:

……
……
……..

Reasons for changes:

……
……
……..

SCOPE

This SOP describes the procedures for specimen collection/processing/shipment of red blood cell folate (Fol) for the study titled: Morogoro Regional Micronutrient Household and Biomarker Survey, Tanzania, 2019.

PURPOSE

- To ensure proper procedures for specimen collection/processing/shipment of red blood cell folate (Fol) for the study

RESPONSIBILITIES

Laboratory technologist/technicians involved in the study should be trained and follow this SOP during field sample collection and processing at the laboratory.

PRINCIPLE

Use of a consistently executed protocol for specimen collection and processing is essential, to eliminate data variability caused by erratic technique. Proper use of the pipette is a must as this ensures accuracy of the red blood cell (RBC) folate level. A hematocrit result is needed to calculate RBC folate from whole blood folate and needs to be obtained for each patient and communicated to the laboratory who analyzes the whole blood folate.

ABBREVIATIONS

EDTA Ethylene diamine tetra-acetic acid
ID Identification

Materials needed:

- Usual blood collection supplies
- 4-mL K2EDTA Vacutainers
- Specimen labels with barcode
- 2-mL cryovials containing 1 mL of 1 g/dL ascorbic acid diluent (1% w/v); prepared ahead of time and stored frozen at -70ºC for up to 6 months
- Alternatively: L-Ascorbic acid can be pre-weighed into 50-mL Falcon tubes (0.3 g/tube) and the tubes can be stored at room temperature, protected from light, for up to 1 year. The ascorbic acid solution (1% w/v) can be prepared freshly on a daily basis, as needed, by adding 30 mL of deionized water to the Falcon tube. Any left-over ascorbic acid solution should be discarded at the end of the day.
- 100-µL micropipet and disposable tips (to add EDTA whole blood to ascorbic acid vial for lysis of erythrocytes)
- White cardboard storage boxes, 9x9 array
- Marker pen
- Styrofoam insulated shippers
- Dry ice
- Cold packs
- Package sealing tape (clear type, such as 3M, preferred

PROCEDURES

I. Collection:

1. Follow standard venipuncture collection technique (SOP for Venous Blood Collection) to collect a 6 mL EDTA Vacutainer (spray-dried K2EDTA is preferred).

2. Mix the EDTA Vacutainer well by inversion at least 5 times after collection to ensure thorough mixing of the anticoagulant with the blood.

3. Place the EDTA Vacutainer into a coolbox that contains cold packs to keep it cool and protected from light during transport back to the laboratory.

4. Make sure though that you separate the Vacutainer from the cold packs through some paper to avoid unintentional hemolysis of red cells.

5. Discard the blood collection supplies properly in accordance with hospital regulations.

II. Processing:

1. Return to the laboratory promptly. Ideally, the whole blood should be lysed the same day as it was collected. However, if that is not possible, maintain the EDTA Vacutainer tube in the refrigerator protected from light for up to a maximum of 2 days before lysis.

2. When you are ready to lyse the whole blood, remove one vial with ascorbic acid diluent per subject from the laboratory freezer and allow it to thaw at room temperature.

3. Alternatively, prepare fresh 1% ascorbic acid solution and accurately pipet 1 mL into a 2-mL cryovial. Allow the EDTA Vacutainer to reach room temperature and re-mix its contents by inversion at least 8-10 times.

4. Remove the stopper of the EDTA Vacutainer and pipet 100 μL of well-mixed blood into the vial containing the ascorbic acid; mix well.

5. Determine the hematocrit by using a portion of the remainder EDTA whole blood.

6. If EDTA plasma is needed for analysis, re-stopper the EDTA Vacutainer carefully and centrifuge it for 10 minutes at 2500-2800 RPM to separate the plasma from the cells.

7. If not, discard the EDTA Vacutainer properly in accordance with hospital regulations.

III. Storage:

1. Place all RBF samples (lysed whole blood) in white 9x9 array cardboard storage boxes labeled appropriately for study, and freeze all samples at -70˚C.

2. When filling the box, mark the top left corner with a marker pen to indicate starting point.

3. Continue to fill each row, left to right, until box is completely filled.

4. As patient samples are collected and processed, create a logsheet for each box listing the subject id, date collected, location of the vials in the box, and any pertinent information

which may affect assay results such as short draw, fasting status if known, hemolysis, lipemia, etc. - and the patient hematocrit result for correct calculation of the RBF.

IV. Transport:

1. Wrap the pre-labeled white storage box with absorbent paper, place it into a Ziplock bag and seal the bag.
2. Place the Ziplock bag in the bottom of the shipping box. If necessary, use sheets of bubble wrap to ensure specimens remain in a vertical position.
3. Fill the Styrofoam-lined shipping box with dry ice. Allow one pound of dry ice for every 2 hours in transport.
4. Close the Styrofoam lid.
5. Place a copy of the study logsheet describing these samples between this lid and the outer cardboard lid.
6. Seal the outer carton lid.
7. Telephone the laboratory at your destination the day the shipment is mailed.

I have read, understood and agreed to follow the procedure as documented.

No.	Name	Signature	Date

Standard Operating Procedure (SOP) for lysing a whole blood samples for folate microbiological assay

	TANZANIA FOOD AND NUTRITION CENTRE **MICROBIOLOGY LABORATORY SECTION** **Standard Operating Procedure(SOP) for lysing a whole blood samples for folate microbiological assay**		
Version No:		**Copy no.**	
Prepared by:			
Reviewed by:	1. 2.		
Authorized by:			

TANZANIA FOOD AND NUTRITION CENTRE

Changes:

……
……
……..

Reasons for changes:

……
……
……..

SCOPE

Applicable during lysing whole blood sample for folate microbiological assay

PURPOSE

To ensure proper procedures for for lysing a whole blood samples for folate microbiological assay

RESPONSIBILITIES

1. Field laboratory supervisor and laboratory technicians are responsible with this SOP.
2. All Laboratory personnel involved in the study should be trained and follow this SOP during field sample processing at field laboratory.
3. All field lab technicians should be aware of this SOP and assist each other as a team.

PRINCIPLE

Hemolysing the RBC is to breakdown of the RBC, causing release of hemoglobin and resulting in the discoloration of the plasma. Abnormal hemolysis in an individual RBC unit may be caused by several factors including inappropriate handling during processing of blood, inappropriate storage conditions, bacterial hemolysis, antibodies that cause complement lysis, defects in the RBC membrane, or an abnormality in the blood donor. The degree of hemolysis is described as

the percent of free hemoglobin in relation to the total hemoglobin with appropriate correction for the hematocrit.

ABBREVIATIONS

EDTA	Ethylene diamine tetra-acetic acid
AA	Ascorbic acid
RBF	Red Blood Folate

PROCEDURES

Materials needed:

- 4 mL K2EDTA Vacutainers
- Specimen labels with barcode
- 100 µL micropipette and disposable tips (to add EDTA whole blood to ascorbic acid vial for lysis for RBF)
- Marker pen

Reagent preparation: 1% ascorbic acid (AA)

1. Ideally, the whole blood should be lysed the same day as it was collected. However, if that is not possible, the EDTA Vacutainer tube may be kept in the refrigerator of- (2°C-8°C) protected from light for a maximum of 4 days before lysis.
2. When ready to lyse the whole blood, prepare 1% ascorbic acid diluent as described. Weigh 5g of ascorbic acid dissolve in 500ml of autoclaved distilled water.
3. Transfer the ascorbic acid in 1ml vials and store in a refrigerator of -20°C.

Note: it should be freshly prepared each day of lysing.

Procedures for lysing a whole blood sample.

1. Allow the EDTA Vacutainer to reach room temperature and re-mix its contents by inversion at least 8–10 times.
2. Remove the stopper of the EDTA Vacutainer and pipet 100 µL of well-mixed blood into thawed 1ml tube of ascorbic acid (1 g/dL) solution (rinse the pipette tip 3–4 times with the RBC hemolysate), then mix well (avoid foaming) and allow to stand in the dark 30 min at room temperature.

Storage:

1. Place all RBF samples (lysed whole blood) in white 9x9 array cardboard storage boxes labeled appropriately for the study, and freeze all samples at -70°C.
2. When filling the box, mark the top left corner with a marker pen to indicate starting point. Continue to fill each row, left to right, until box is completely filled.

3. As household samples are collected and processed, create a log sheet for each box listing the following, the household ID, date collected, location of the vials in the box, and any information that may affect assay results, such as short draw, fasting status if known, hemolysis, or lipemia, and the household hematocrit result for correct calculation of the RBF.

I have read, understood and agreed to follow the procedure as documented

No.	Name	Signature	Date

Standard Operating Procedure (SOP) for Sample Separation, aliquoting and Storage

TANZANIA FOOD AND NUTRITION CENTRE

	TANZANIA FOOD AND NUTRITION CENTRE **BIOCHEMISTRY LABORATORY SECTION** **Standard Operating Procedure (SOP) for Sample Separation, aliquoting and Storage**		
Version No:		**Copy no.**	
Prepared by:			
Reviewed by:	1. 2.		
Authorized by:			

Changes:

……
……
……..

Reasons for changes:

……
……
……..

SCOPE

Procedure for sample separation, aliquoting and storage for the survey tittled: **Morogoro Regional Micronutrient Household and Biomarker Survey, Tanzania, 2019.**

PURPOSE

- To ensure proper technique in obtaining quality serum, plasma and hemolysate for laboratory testing is used.
- Ensure high quality serum ,plasma and hemolysate for laboratory testing is obtained
- Ensure proper specimen identification and labeling.
- Ensure separated samples are stored at appropriate temperature.

RESPONSIBILITIES

Laboratory technologist/technicians involved in the study should be trained and follow this SOP during field sample collection and processing at the laboratory.

PROCEDURE

For serum samples:

At local laboratory

- Centrifuge a red top vacutainer tubes that contains clot activator at 1800 RPM for 15 minutes, at room temperature and withdraw all (~ 4ml) serum into a separate labeled cryovial tube.
- Then, Transport both the clot and sera to regional lab at 4^{o}C

At regional laboratory

- Dispense serum into 325 µl aliquots in labeled tubes with color-coded caps and labels (orange) as indicated: 2 each for serum folate 1 each for ferritin, soluble transferin receptors, retinol, and CRP, 1 extra serum.
- These volumes are in excess of those required for each determination (250, 200, 300, and 75 µl, respectively).
- Any additional serum should be saved in more tubes for backup
- Transfer clot into a smaller labeled 2 ml cryotube
- All tubes should be logged, placed in storage boxes, and frozen at -70^{o}C.

For Plasma and RBC samples:

At local laboratory

- Mix the EDTA tube again, remove the top of tube, and do CBC using CBC analyzer and record results in the CBC analyzer results form, then transfer remaining blood as follows:
  - 100µl transferred into a previously thawed 1ml tube of ascorbic acid (1 g/dL) solution then mix, and allow to stand in the dark 30 min at room temperature (RBC hemolysate),
  - then transfer ~ 200 µl to a separate yellow color-coded whole blood tube.

After removal of the blood aliquots:

- Centrifuge the EDTA tubes at 1800 RPM for 15 minutes at room temperature and withdraw all (~ 2ml) plasma into a separate blue cryovial.
- Then transport whole blood, RBC hemolysate, plasma, and remaining cells in vacutainer to Regional lab at 4^{o}C.

At regional laboratory

- Dispense RBC hemolysate, 4 into aliquots each, containing 275 µl, RBC hemolysate for folate in labeled tubes with color-coded caps and labels.
- Dispense 250 µl of plasma to 2 cryovials each for vitamin B12 and 250 µl of plasma to 2 cryovials each for homocysteine or other analyses including backup of ferritin, retinol, or AGP.
- Transfer retained blood cells from the vacutainer into a 2 ml labeled cryovial.
- Store whole blood, RBC hemolysate,plasma and remaining cells in vacutainer to -80Ċ

24.1.1

TANZANIA FOOD AND NUTRITION CENTRE
SAMPLE PROCESSING LOGIN SHEET

s/n	Date of sample collection	Sample ID	Processing requested	Initial Storage location	Final Storage	Date and Initials
1						
2						
3						
5						
6						
7						
8						

COMMENTS……
……
……
……

I have read, understood and agreed to follow the procedure as documented.

No.	Name	Signature	Date

Sample Receipt Standard Operating Procedure (SOP) and Log

	TANZANIA FOOD AND NUTRITION CENTRE **UNIVERSAL LABORATORY SOP** **Laboratory standard operating procedure (SOP) for specimen receipt and handling procedures at TFNC laboratory**		
Version No:		**Copy no.**	
Prepared by:			
Reviewed by:			
Authorized by:			

TANZANIA FOOD AND NUTRITION CENTRE

Changes:
……
……
……..

Reasons for changes:
……
……
……..

SCOPE
This SOP applies to the receipt and handling of specimens for all laboratory personnel who have been trained and are competent in the receipt, handling and distribution of specimens for laboratory tests.

PURPOSE
To ensure proper steps are followed during receipt of blood samples for study, assessing acceptability, rejection criteria and storage.

RESPONSIBILITIES
Laboratory technologist/technicians involved in the study should be trained and follow this SOP

Standard precautions

Wear gloves when handling participant specimens to protect from exposure to blood borne pathogens.

Specimen receipt procedures
Study laboratory staff are responsible for receiving, storing and redirecting specimens when appropriate.

Check laboratory request form for the following information:

- Visit Date
- Participant's Gender
- Participant ID
- Date and time of specimen collection
- Initials and date of specimen collector
- Confirm collection column checked and initials

- If any data is missing, *CLARIFY ON REMARKS COLUMN OF SAMPLE RECEIVING LOG* and return to Site Coordinator.
- Process specimens that have study numbers so as not to compromise specimen integrity.
- Inspect all tubes/bottles for proper labeling with Study ID and Collection date (minimal) and initials of collector. Unlabeled specimens are not acceptable for testing.
- Determine if sufficient specimen of acceptable quality is available for all tests.
- Complete a DATA/SPECIMEN CLARIFICATION form if quantity or quality of specimen is inadequate. Describe tests that cannot be performed and request additional sample. Submit form to Site Coordinator.

Rejection Criteria

- If a sample is Mislabelled (Participant ID's on specimen blood and request form do not match. Unlabelled or No participant information on specimen. Specimen collected in incorrect tube type and inadequate volume of sample for testing, will be rejected and inform site supervisor.

TANZANIA FOOD AND NUTRITION CENTRE LABORATORY
SAMPLE RECEIVING LOG

TANZANIA FOOD AND NUTRITION CENTRE

Date	Site/Location	Received by (Initials)	Time Received	Sample ID	Sample type	Remarks

Comments……
……
……
……

I have read, understood and agreed to follow the procedure as documented.

No.	Name	Signature	Date

Standard Operating Procedure (SOP) Reagents Preparations for Folate Microbiological Assay

	TANZANIA FOOD AND NUTRITION CENTRE **MICROBIOLOGY LABORATORY SECTION** **Standard Operating Procedure (SOP) Reagents Preparations for Folate Microbiological Assay**		
Version No:		**Copy no.**	
Prepared by:			

TANZANIA FOOD AND NUTRITION CENTRE

Reviewed by:	1. 2.		
Authorized by:			

Changes:

……
……
……..

Reasons for changes:

……
……
……..

SCOPE

This SOP is applicable during reagent preparation for Folate Microbiological Assay

PURPOSE

This SOP describes the procedures and how to prepare reagent for the folate microbiologic assay

RESPONSIBILITIES

1. Laboratory supervisors and laboratory technicians are responsible with this SOP.
2. All Laboratory personnel involved in this analysis must be trained and competent to follow this SOP during laboratory analysis.

PRINCIPLE

Folate Microbiological Assay is very sensitive at detecting trace levels of folate and therefore in reagent preparation one need to work carefully to prevent folate contamination.

ABBREVIATIONS

AA Ascorbic acid
RBF Red Blood Folate
5-MeTHF 5- Methyl Tetra Hydro Folate

Reagents required are given below

1. Lactobacillus Casei/ Lactobacillus rhamnosus (NCIB 10463; or ATCC 27773 or MTCC 1408 from the Institute of Microbial Technology, Chandigarh) chloramphenicol resistant strain
2. Folic acid Lactobacillus casei medium without folate (Difco 082210, 100g)
3. 5-Methyltetrahydrofolate (Merck Eprova, Metafolin): used as a calibrator.
4. Folic acid (Sigma #7876, 1g) (to use as standard)
5. Ascorbic acid (Sigma A 5960, 100g)
6. Sodium ascorbate (Sigma A7631, 500g)
7. Tween-80 (Sigma P4780, 100mL)
8. Chloramphenicol (Sigma C-0378, 25g)
9. Manganese sulfate (Sigma M-7634, 100g)
10. Sodium Azid (Sigma S-8032, 25g)
11. Cysteine (Sigma C7352, 25g)
12. Glycerol (Sigma G-2025, 500 mL)

Other requirements:

- Pyrex beakers

- Foil
- Hot plate
- Analytical balance
- Stop watch
- Microplates
- Sealer
- Specimen labels with barcode
- 100 µL micropipette and disposable tips
- Marker pen

PROCEDURES
Preparation of Reagents for Folate Microbiologic Assay
All solutions must be prepared by autoclaved distilled water.

1) **Sodium ascorbate solution (0.5 g/dL) for diluting samples and standard**
   2.5 grams of sodium ascorbate is dissolved in 500 mL of deionized water.
   This solution should be freshly prepared before each assay**.**
2) **Assay growth medium (folate free)**

1. To prepare 200 mL of assay medium: dissolve 14.1 g of Folic Acid Casei Medium in 200 mL of distilled water, mix well and cover with foil.
2. Heat solution to boiling for 2-3 min while stirring, then cool down to ~37˚C.
3. Add 1 vial of Ascorbic acid stock solution (1ml per vial),1 vial of chloramphenicol stock solution(2 mL of chloramphenicol stock solution),1 vial of manganese sulfate stock solution(1ml per vial) finally 60 µL of Tween-80 then mix well until all the chemicals are dissolved for the few minutes.
4. Thaw 1 vial of frozen L. rhamnosus as quickly as possible (use running water) and add 700 µL (or amount specified in assay kit) into 200 mL of assay medium keep stirring gently**.**

3. Prepare 5-MeTHF calibration working solution

1. Calibrator dilution (to obtain working solution I and II)
2. Diluted 5MeTHF MA stock solution: Dilute 5MeTHF stock solution (1 µmol/L) at 1:5 (200 nmol/L) by adding 100 µL of stock solution (1 µmol/L) to 400 µL of 0.5 g/dL sodium ascorbate.
3. Working solution, I: dilute 50 µL of diluted 5MeTHF stock III with 0.5 g/dL sodium ascorbate in a 50 mL volumetric flask and make up to volume (1:1000 dilution; 200 pmol/L).
4. Working solution II: dilute 250 µL of diluted 5MeTHF stock III with 0.5 g/dL sodium ascorbate in a 50 mL volumetric flask and make up to volume (1:200 dilutions; 1 nmol/L).

I have read, understood and agreed to follow the procedure as documented.

No.	Name	Signature	Date

Standard Operating Procedure (SOP) for waste disposal after Folate Microbiologic Assay

	TANZANIA FOOD AND NUTRITION CENTRE **MICROBIOLOGY LABORATORY SECTION** **Standard Operating Procedure (SOP) for waste disposal after Folate Microbiologic Assay**		
Version No:		**Copy no.**	
Prepared by:			
Reviewed by:	1. 2.		
Authorized by:			

TANZANIA FOOD AND NUTRITION CENTRE

Changes:

……
……
……..

Reasons for changes:

……
……
……..

SCOPE

Applied during disposal of samples and clean up after analysis in Folate Microbiologic Assay

PURPOSE

This procedure describes how to dispose samples and how to handle all the materials used in Folate Microbiologic Assay.

RESPONSIBILITIES

1. Laboratory supervisors and laboratory technicians are responsible with this SOP.
2. All Laboratory personnel involved in this analysis must be trained and competent to follow this SOP

PRINCIPLE
Blood samples and assay medium containing bacterial should be disposed by autoclaving after analysis is completed. Place disposable plastic, glass, and paper (pipette tips, vials, tubes, gloves, microplates, etc.) that contact serum or blood in a biohazard autoclave bag and keep these bags in appropriate containers until sealed and autoclaved. Disposable bench diapers is used during sample preparation and serum/ blood handling and discard them in autoclave pan after use.

Materials Needed:
- 10% bleach solution
- Biohazard autoclaved bags
- Deionized water
- Disposable bench diapers
- Gauze

PROCEDURES
DAY 1 (experiment set- up)
1. Add a bleach to leftover growth medium containing the microorganism for a final concentration of approximately 10% and left it sit for 30 min to inactivate the microorganism. After that time, the growth medium turns brown and can be discarded into the drain.

2. Place disposable gloves and plastic glass and paper items (pipette tips, vials, glass tubes, gloves, microplates, disposable bleach liners etc.) that may have come in contact with serum or blood into a biohazard autoclaved bags and keep the bags in appropriate containers until they are autoclaved and properly disposed.

3. Discard leftover all the remaining solutions which used in the analysis into a drain.

4. Wash beakers and all apparatus used with deionized water.

5. Wipe all work surface with 10% bleach solution after the daily work is completed.

DAY 3 (after reading plates)
1. Discard the plates into a biohazard autoclaved bag after reading is completed and the plates have been visually inspected.

2. Wipe work surface with 10% bleach solution.

NOTE*: This assay is very sensitive at detecting trace levels of folate and therefore you need to work carefully to prevent folate contamination.*
- ***Use separate bench to prepare the calibrator working solutions***

- ***If possible, use separate sinks to clean up reusable glassware and to discard and to clean up the left over calibrator solutions.***
- ***Also remember to regularly decontaminate the bench surface and pipettes used for sample preparation.***

REFERENCE
CDC Folate Microbiological Assay Training Manual

I have read, understood and agreed to follow the procedure as documented.

No.	Name	Signature	Date

Standard Operating Procedure (SOP) for serum and red cell folate microbiologic assay

	TANZANIA FOOD AND NUTRITION CENTRE **MICROBIOLOGY LABORATORY SECTION** **Standard Operating Procedure (SOP) for serum and red cell folate microbiologic assay**		
Version No:		**Copy no.**	
Prepared by:			
Reviewed by:	1. 2.		
Authorized by:			

TANZANIA FOOD AND NUTRITION CENTRE

Changes:

……
……
……..

Reasons for changes:

……
……
……..

SCOPE

Procedure for conducting serum and red cell folate microbiologic assay at TFNC laboratory

PURPOSE

- To ensure proper technique in conducting serum and red cell folate microbiologic assay at TFNC laboratory
- Ensure proper analysis to obtain high quality results of RBC and serum folate

RESPONSIBILITIES

- 1. Laboratory supervisors and laboratory technicians are responsible with this SOP.

- 2. All Laboratory personnel involved in this analysis must be trained and competent to follow this SOP during laboratory analysis.

PRINCIPLE

Diluted serum or whole blood hemolysate is added to an assay medium contain Lactobacillus rhamnosus (formerly known as L. casei) (NCIB 10463) and all of the nutrients necessary for the growth of L. rhamnosas except for folate. The inoculated medium is incubated for 45 hours at 37 °C. Since the growth of L. rhamnosus is proportional to the amount of total folate present in serum or whole blood samples, the total folate level can be assessed by measuring the tubidity of the inoculated medium at 590 nm in a PowerWave microplate reader (Bio-Tek Instrument).

REAGENTS

1. Lactobacillus Casei/ Lactobacillus rhamnosus (NCIB 10463; or ATCC 27773 or MTCC 1408 from the Institute of Microbial Technology, Chandigarh) chloramphenicol resistant strain
2. Folic acid Lactobacillus casei medium without folate (Difco 082210, 100g)
3. 5-Methyltetrahydrofolate (Merck Eprova, Metafolin): used as a calibrator.
4. Folic acid (Sigma #7876, 1g) (to use as standard)
5. Ascorbic acid (Sigma A 5960, 100g)
6. Sodium ascorbate (Sigma A7631, 500g)
7. Tween-80 (Sigma P4780, 100mL)
8. Chloramphenicol (Sigma C-0378, 25g)
9. Manganese sulfate (Sigma M-7634, 100g)
10. Sodium Azid (Sigma S-8032, 25g)
11. Cysteine (Sigma C7352, 25g)
12. Glycerol (Sigma G-2025, 500 mL)

PROCEDURES
Preparation of Reagents for Folate Microbioloc Assay

All solutions will be prepared in autoclaved distilled water.

1) Sodium ascorbate solution (0.5 g/dL) for diluting samples and standard

   24.2 Two and half grams of sodium ascorbate is dissolved in 500 mL of deionized water. This solution should be freshly prepared before each assay.

2) *L. casei* assay medium inoculated with microorganism (folate free)

   To prepare 200 mL of assay medium: add 14.1 g of Folic Acid Casei Medium, 6 mg of chloramphenicol (or 2 mL of a 3 mg/mL chloramphenicol stock solution) and 60 µL of Tween-80 to 200 mL of ultrapure water, heat to boil for 2-3 min with stirring. Cool down to ~37°C, then add 30 mg of manganese sulfate (or 1 mL of a 30 mg/mL manganese sulfate stock solution) and 150 mg of ascorbic acid (or 1 mL of a 150 mg/mL ascorbic acid stock solution), and keep stirring until all the chemicals are dissolved. Thaw one vial of frozen *L. casei* as quickly as possible (use running water) and add 600-700 µL into 200 mL of assay medium, keep stirring slowly. About 25 mL of assay medium is needed per microplate.

3) Ascorbic acid stock solution (150 mg/mL)

   Dissolve 15 g of ascorbic acid in 100 mL of deionized water. Aliquot 1 mL/vial and store at -70°C. Add 1 mL into 200 mL of Folic Acid Casei medium. Make fresh stock solution every 6 months.

4) Manganese sulfate stock solution (30 mg/mL)

   Dissolve 3.6 g of manganese sulfate in 120 mL of deionized water, stir thoroughly (~20 min). Aliquot 1 mL/vial and store at -70°C. Add 1 mL into 200 mL of Folic Acid Casei medium. Make fresh stock solution every 6 months.

5) Chloramphenicol stock solution (3 mg/mL)

   Dissolve 600 mg of chloramphenicol in 4 mL of ethanol and then make up to 200 mL with deionized water, aliquot 2 mL into cryovials and keep at -70°C freezer. Add 2 mL of stock solution into 200 mL of Folic Acid Casei medium. Make fresh stock solution every 6 months.

6) *L. casei* growth medium (with folate to create new inoculum)

   To prepare 200 mL of growth medium: Add 9.4 g of Folic Acid Casei Medium, 40 mg of chloramphenicol, 40 µL of Tween-80 to 200 mL of deionized water. Heat to boil for 2-3 min. Cool down to ~37°C, add 100 mg of ascorbic acid, 300 µL of folic acid stock solution (100 ng/mL), stir to completely dissolve, sterilize the medium by either autoclaving or filtering through a 0.2 µm filter. Aliquot 20 mL into sterile 50-mL tubes and keep at -20°C.

7) Ascorbic acid (1 g/dL) for hemolysis of whole blood samples

Dissolve 1 g of ascorbic acid in 100 mL of deionized water. Prepare fresh solution before each use.

8) Blocking solution for color control

Sodium azide is used as a blocking solution. Five µL of a 3 g/dL solution are added to each control well. Alternatively, the disinfectant "Stericol" can be used at a 1/30 dilution with ultrapure water (10 µL /control well).

Folate MA Sample Dilution Table :

	Dilution	Final dilution	WBL (□L)	SA (□L)	Total (□L)
WBL (lysate at 1/11)	100	11x100	15	1485	1500
Serum	50	50	15	735	750

Standard Preparation

1) The concentrations of the folate stock solution is calculated using molar absorptivity. Information on the absorption maximum, absorption coefficient, and formula to calculate the concentration for the stock solution is provided in **Appendix 6.** Use Class A volumetric glassware where a volumetric flask is specified.

1) **5-MethylTHF primary stock solution (**< 200 µg/mL**):** Prepare a primary stock solution in a volumetric flask by dissolving an accurately known mass (±0.1 mg) of the pure solid compound in degassed and filtered 20 mM phosphate buffer (pH 7.2, containing 0.1% cysteine), targeting a final concentration of ~100 µg/mL (e.g., 5 mg in 50 mL). Vortex briefly to help dissolve the contents and make up to final volume.

   Remove a small aliquot (1 mL) of the primary stock solution to a microcentrifuge vial to determine its concentration by UV spectrophotometry. To the remaining stock solution add ascorbic acid powder to a final concentration of 1%. From the aliquot you removed, prepare two dilutions (e.g., 1/10 and 1/20), each in duplicate, measure the UV absorbance at the peak maximum using scan analysis against phosphate buffer as a blank, and calculate the primary stock solution concentration (**Appendix 1**). For 5-methylTHF, the ratio of absorbance at 290/245 nm can be monitored (simple reads analysis at each wave length) to ensure that no oxidation takes place. This ratio should exceed 3.3.

   Aliquot the remainder of the primary stock solution into cryovials that are stored at -70°C. The primary stock solution is stable for at least 2 years.

2) **5-MethylTHF microbiological assay stock solution (1 µmol/L):** Based on the exact concentration determined on UV spectrophotometry, dilute 5-MethylTHF primary stock solution with 0.5% degassed ascorbic acid in a 100-mL volumetric flask to get final concentration 1 µmol/L. Aliquot 500 µL into

labeled cryovial and freeze the aliquots at -70°C. Prepare a fresh MA stock solution from primary stock solution every 6 months.

d. Quality Control Materials have to be prepared:

Serum (400 µL) and whole blood lysate pools (500 uL) are aliquoted into 2.0 mL Nalgene cryovials, capped and frozen. The QC pools are stored at -70°C and are stable for at least 2 years. Means plus range limits for all pools are established by analyzing the QC pools in at least 20 runs.

e. Preparation of Cryopreserved Organism

1) Add one vial of freeze-dried *L. casei* into 20 mL of organism growth medium and incubate at 37°C for 24 hours.
2) Transfer 100–300 µL of the 24-hour culture into another 20 mL of organism growth medium and incubate at 37°C for 24 hours.
3) Repeat step 2 for the third 24-hour incubation.
4) Inoculate different amount of active culture in duplicates for optimization of response. Add 500 µL, 1 mL and 2 mL of Step 3 culture into 20 mL of fresh growth medium and incubate at 37°C. Measure OD at 590 nm from one of the duplicate cultures at different incubation time. Record the log growth phase (~18-20 hours). Mix the log phase culture by 50/50 with 80% glycerol (sterilized by autoclaving). Aliquot the mixture in sterile cyrovials (1 mL/vial) and store at -70°C.
5) Run a standard plate using the new inoculum and compare to a standard plate using the previous inoculum. The OD for the blank should be ~0.1, for the highest calibrator ~1, and the curve should have the expected polynomial 3rd degree shape.

Freshly prepare the following solutions for each assay

1. Prepare 200 mL of *Lactobacillus casei* assay medium before each experiment
Folic Acid Casei Medium (powder) 14.10 g from original bottle
Ultrapure water 200 mL
Chloramphenicol (antibiotic) 6 mg: add 2 mL of stock solution (3mg/mL)
Tween-80 (liquid): 60 □L
Heat the medium to boil for 2-3 minutes with stirring, cool down to ~37°C and then add the following chemicals
Manganese sulfate30 mg: add 1 mL of stock solution (30 mg/mL)
Ascorbic acid150 mg: add 1 mL of stock solution (150 mg/mL)
Keep slowly stirring until all chemicals are dissolved.
Lactobacillus casei~ 500 - 700 □L: Thaw 1 frozen vial quickly

2. 0.5% Sodium Ascorbate (SA, prepare before each experiment)
Before each experiment, add 2.5 g of sodium ascorbate in 500 mL of ultrapure water
3. Prepare 5-MeTHF calibration working solution

Calibrator dilution (to obtain working solution I and II)

- Diluted 5MeTHF MA stock solution: Dilute 5MeTHF stock solution (1 µmol/L) at 1:5 (200 nmol/L) by adding 100 µL of stock solution (1 µmol/L) to 400 µL of 0.5 g/dL sodium ascorbate.
- Working solution I: dilute 50 µL of diluted 5MeTHF stock III with 0.5 g/dL sodium ascorbate in a 50 mL volumetric flask and make up to volume (1:1000 dilution; 200 pmol/L).
- Working solution II: dilute 250 µL of diluted 5MeTHF stock III with 0.5 g/dL sodium ascorbate in a 50 mL volumetric flask and make up to volume (1:200 dilution; 1 nmol/L).

Plate layout

Calibration Plate -Table 1: Add 200 µL *L.casei* assay medium inoculated with microorganism

#1	1	2	3	4	5	6	7	8	9	10	11	12
A	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
B	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
C	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
D	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
E	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
F	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
G	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
H	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL

Calibration Plate - Table 2: Add 0.5 g/dL sodium ascorbate

#1	1	2	3	4	5	6	7	8	9	10	11	12
A	100ul	100ul	75ul	50ul	25ul	0ul	70ul	60ul	50ul	40ul	20ul	0ul
B	100ul	100ul	75ul	50ul	25ul	0ul	70ul	60ul	50ul	40ul	20ul	0ul
C	100ul	100ul	75ul	50ul	25ul	0ul	70ul	60ul	50ul	40ul	20ul	0ul
D	100ul	100ul	75ul	50ul	25ul	0ul	70ul	60ul	50ul	40ul	20ul	0ul
E	100ul	100ul	75ul	50ul	25ul	0ul	70ul	60ul	50ul	40ul	20ul	0ul
F	100ul	100ul	75ul	50ul	25ul	0ul	70ul	60ul	50ul	40ul	20ul	0ul
G	100ul	100ul	75ul	50ul	25ul	0ul	70ul	60ul	50ul	40ul	20ul	0ul
H	100ul	100ul	75ul	50ul	25ul	0ul	70ul	60ul	50ul	40ul	20ul	0ul

Calibration Plate - Table 3: Add 5-methylTHF working solution I and II

			working solution I				working solution II					
#1	1	2	3	4	5	6	7	8	9	10	11	12
A	0ul	0ul	25ul	50ul	75ul	100ul	30ul	40ul	50ul	60ul	80ul	100ul
B	0ul	0ul	25ul	50ul	75ul	100ul	30ul	40ul	50ul	60ul	80ul	100ul
C	0ul	0ul	25ul	50ul	75ul	100ul	30ul	40ul	50ul	60ul	80ul	100ul
D	0ul	0ul	25ul	50ul	75ul	100ul	30ul	40ul	50ul	60ul	80ul	100ul
E	0ul	0ul	25ul	50ul	75ul	100ul	30ul	40ul	50ul	60ul	80ul	100ul
F	0ul	0ul	25ul	50ul	75ul	100ul	30ul	40ul	50ul	60ul	80ul	100ul
G	0ul	0ul	25ul	50ul	75ul	100ul	30ul	40ul	50ul	60ul	80ul	100ul
H	0ul	0ul	25ul	50ul	75ul	100ul	30ul	40ul	50ul	60ul	80ul	100ul

Note: The total volume in each well is 300 □L.
About 22 mL of medium is needed for each plate.
The calibration curve is made up of 11 different concentration points. The total standard volume is 100 □L in each well.
If whole blood lysate samples have to be diluted at lower dilution than 1/40, color controls may be needed. Add 5 □L of 3 g/dL sodium azide to the third column of each sample (column 3, 6, 9, 12) to generate a color control (there won't be any growth in these wells). ODs from sample wells should be subtracted from color control wells.

Plate 2 Layout of Sample Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	**Low QC 1**	**Low QC 1**	Sample 4	Sample 4	Sample 8	Sample 8	Sample 12	Sample 12	Sample 16	Sample 16	Sample 20	Sample 20
B	**Low QC 1**	**Low QC 1**	Sample 4	Sample 4	Sample 8	Sample 8	Sample 12	Sample 12	Sample 16	Sample 16	Sample 20	Sample 20
C	Sample 1	Sample 1	Sample 5	Sample 5	Sample 9	Sample 9	Sample 13	Sample 13	Sample 17	Sample 17	Sample 21	Sample 21
D	Sample 1	Sample 1	Sample 5	Sample 5	Sample 9	Sample 9	Sample 13	Sample 13	Sample 17	Sample 17	Sample 21	Sample 21
E	Sample 2	Sample 2	Sample 6	Sample 6	Sample 10	Sample 10	Sample 14	Sample 14	Sample 18	Sample 18	Sample 22	Sample 22
F	Sample 2	Sample 2	Sample 6	Sample 6	Sample 10	Sample 10	Sample 14	Sample 14	Sample 18	Sample 18	Sample 22	Sample 22
G	Sample 3	Sample 3	Sample 7	Sample 7	Sample 11	Sample 11	Sample 15	Sample 15	Sample 19	Sample 19	Sample 23	Sample 23
H	Sample 3	Sample 3	Sample 7	Sample 7	Sample 11	Sample 11	Sample 15	Sample 15	Sample 19	Sample 19	Sample 23	Sample 23

Serum Plate – Table 4: Add 200 µL *L.casei* assay medium inoculated with microorganism

#2	1	2	3	4	5	6	7	8	9	10	11	12
	QC low 1		**Sample 2**		**Sample 6**		**Sample 10**		**Sample 14**		**Sample 18**	
A	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
B	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
	QC med 1		**Sample 3**		**Sample 7**		**Sample 11**		**Sample 15**		**Sample 19**	
C	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
D	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
	QC high 1		**Sample 4**		**Sample 8**		**Sample 12**		**Sample 16**		**Sample 20**	
E	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
F	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
	Sample 1		**Sample 5**		**Sample 9**		**Sample 13**		**Sample 17**		**Sample 21-blank**	
G	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL
H	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL	200uL

#2	1	2	3	4	5	6	7	8	9	10	11	12
	QC low 1		**Sample 2**		**Sample 6**		**Sample 10**		**Sample 14**		**Sample 18**	
A	50uL		50uL		50uL		50uL		50uL		50uL	
B	50uL		50uL		50uL		50uL		50uL		50uL	
	QC med 1		**Sample 3**		**Sample 7**		**Sample 11**		**Sample 15**		**Sample 19**	
C	50uL		50uL		50uL		50uL		50uL		50uL	
D	50uL		50uL		50uL		50uL		50uL		50uL	
	QC high 1		**Sample 4**		**Sample 8**		**Sample 12**		**Sample 16**		**Sample 20**	
E	50uL		50uL		50uL		50uL		50uL		50uL	
F	50uL		50uL		50uL		50uL		50uL		50uL	
	Sample 1		**Sample 5**		**Sample 9**		**Sample 13**		**Sample 17**		**Sample 21-blank**	

Sample Plate – Table 5: Add 0.5 g/dL sodium ascorbate

#2	1	2	3	4	5	6	7	8	9	10	11	12
	QC low 1		**Sample 2**		**Sample 6**		**Sample 10**		**Sample 14**		**Sample 18**	
A	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL
B	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL
	QC med 1		**Sample 3**		**Sample 7**		**Sample 11**		**Sample 15**		**Sample 19**	
C	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL
D	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL
	QC high 1		**Sample 4**		**Sample 8**		**Sample 12**		**Sample 16**		**Sample 20**	
E	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL
F	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL
	Sample 1		**Sample 5**		**Sample 9**		**Sample 13**		**Sample 17**		**Sample 21-blank**	
G	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL
H	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL	50uL	100uL

Sample Plate – Table 6: Add diluted serum QC and unknown samples

Volume in each well is 300 □L.
About 22 mL of medium is needed for each plate.

Similarly wells for Medium QC & High QC also need to be put up.

Seal the plate very tightly using Thermal Sealing membrane and Heat Plate Sealer (150°C for 5 seconds). Do not invert the plates.

Incubate the plates at 37°C for 40-46 hours depending on the lot of the medium.

Instrument & Software Setup for Microplate Reader

Folate MA protocols have to be created and saved as templates. After finishing pipetting and putting the plates in incubator, the experiment files needs to be generated: select an appropriate protocol based on the number of plates, enter sample IDs and dilution factors, and save the experiment by date in Experiment file.
Microplate reader parameter settings

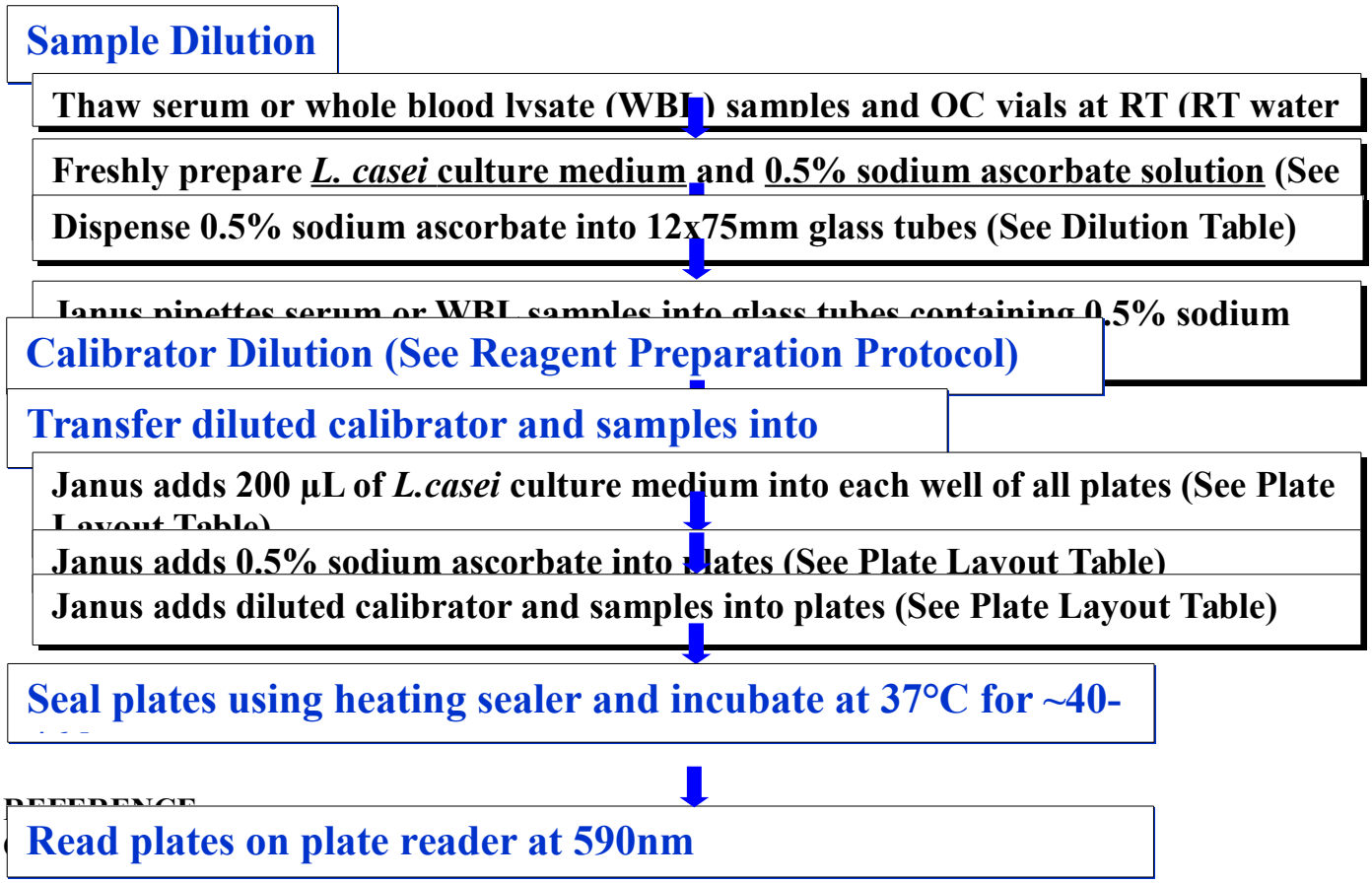
Reading Type	End Point
Wave Length	590 nm
Shaking Intensity	0
Shaking Duration	0
Temperature control	No

X Axis	Lin
Y Axis	Lin
Curve Fit	Polynomial Regression, Degree 3

For different plates, the layouts vary based on different samples analyzed. The calibration curve presents the following concentrations: 0, 0.05, 0.10, 0.15, 0.20, 0.30, 0.40, 0.5, 0.60, 0.8, 1.0 nmol/L.
Specific dilution factors have to be used for QCs and unknown samples
Take all the plates out of the incubator after incubating at 37°C for 40-42 hours, and let them cool down to room temperature while mixing them thoroughly by inverting for at least 1 min or using a rotator. If plates have been kept in the refrigerator before reading, they will have to reach room temperature first. Each plate should be inverted again just before taking off the sealing membrane and reading the OD.
Four replicates are run for each patient sample to assure to generate accurate results.
Whole blood lysate folate results are multiplied by 11, the dilution factor of the whole blood when preparing the lysate. The serum folate values (multiplied by 1.0 minus the hematocrit [Hct] expressed as a decimal) are subtracted, and the resulting value is divided by the Hct to yield RBC folate in nmol/L RBC. If an Hct value is not available for a patient sample or QC sample, an Hct of 40% is assumed for calculation. If a serum folate value is not available, a value of 18 nmol/L is assumed for calculation. We recommend use of the correction for serum folate level and Hct because it provides the most accurate reflection of folate body stores.
Calculation of RBC Folate Concentration:

$$\text{RBC folate, nmol/L} = \frac{(\text{Whole blood hemolysate folate} * 11) - \text{Serum folate}\,(1 - \text{Hct}/100)}{\text{Hct}/100}$$

Laboratory Protocol Flow Chart for Folate Microbiological Assay



I have read, understood and agreed to follow the procedure as documented.

No.	Name	Signature	Date

Standard Operating Procedure (SOP) for determination of Plasma Homocysteine levels using Cell Biolabs' Homocysteine ELISA Kits

	TANZANIA FOOD AND NUTRITION CENTRE **BIOCHEMISTRY LABORATORY SECTION** **Standard Operating Procedure (SOP) for determination of Plasma Homocysteine levels using Cell Biolabs' Homocysteine ELISA Kits**		
Version No:		**Copy no.**	
Prepared by:			
Reviewed by:	1. 2.		
Authorized by:			

TANZANIA FOOD AND NUTRITION CENTRE

Changes:

……
……
……..

Reasons for changes:

……
……
……..

SCOPE

Procedure for determination of Plasma Homocysteine levels using Cell Biolabs' Homocysteine ELISA Kits at TFNC laboratory

PURPOSE

- To ensure proper technique in determination of Plasma Homocysteine level
- Ensure high quality results during analysis of Plasma Homocysteine

RESPONSIBILITIES

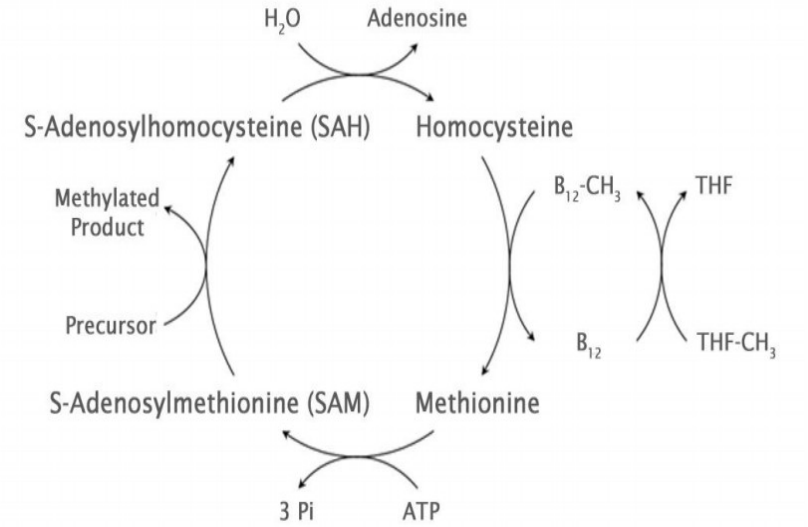
- Laboratory technologist/technicians involved in the study should be trained and follow this SOP

PRINCIPLE

Homocysteine is an amino acid intermediate formed during the production of the essential dietary amino acid methionine (Figure 1). Homocysteine is a homologue of cysteine, differing from cysteine only in that it contains an extra side chain methylene bridge. About 80% of homocysteine found in plasma is bound to protein. High levels of homocysteine in the blood

have been associated with premature incidences of vascular disease, and homocysteine is likely to be a risk factor for heart disease. Homocysteine initially stimulates the production of nitric oxide in endothelial cells but ultimately reduces nitric oxide bioavailability and increases oxidative stress by blocking glutathione peroxidase activity as well as causing cellular oxidative degradation (increasing free radical generation). In addition, elevated homocysteine levels leads to increased platelet and leukocyte adhesion and activation, increased vasoconstriction, and increased proliferation of smooth muscle (a hallmark of atherosclerosis)

Cell Biolabs' Homocysteine ELISA Kit is a competitive enzyme immunoassay developed for the detection and quantitation of homocysteine in plasma, serum, lysates, or other biological fluid samples. The kit has a detection sensitivity limit of 10 ng/mL Homocysteine-BSA. Each kit provides sufficient reagents to perform up to 96 assays including standard curve and unknown samples.



Metabolism of homocysteine.

PROCEDURE

Kit Components

Box 1 (shipped at room temperature) 1. 96 Well Protein Binding Plate (Part No. 231001): One strip well 96 well plate. 2. Anti-Homocysteine Antibody (500X) (Part No. 267002): One 15 µL vial. 3. Secondary Antibody, HRP Conjugate (1000X) (Part No. 231009): One 20 µL vial. 4. Assay Diluent (Part No. 310804): One 50 mL bottle. 5. 10X Wash Buffer (Part No. 310806): One 100 mL bottle. 6. Substrate Solution (Part No. 310807): One 12 mL amber bottle. 7. Stop Solution (Part. No. 310808): One 12 mL bottle.

Box 2 (shipped on blue ice packs) 1. Homocysteine Conjugate (1000X) (Part No. 267001): One 20 µL vial. 2. Homocysteine-BSA Standard (Part No. 267003): One 20 µL vial of 4 mg/mL homocysteine conjugated to BSA in PBS.

Storage Upon receipt, store Homocysteine Conjugate (1000X) and Homocysteine-BSA Standard at -20ºC. Store the rest of the kit at 4ºC.

Preparation of Reagents • Homocysteine Conjugate Coated Plate: Determine the number of wells to be used, and dilute the Homocysteine Conjugate 1:1000 into PBS. Add 100 µL of 1X homocysteine conjugate to each well of the 96-well Protein Binding Plate. Incubate for 2 hrs at

37ºC or overnight at 4ºC. Remove the diluted homocysteine conjugate, blotting plate on paper towels to remove excess fluid. Wash wells 3 times with 200 µL of PBS and blot on paper towels to remove excess fluid. Add 200 µL of Assay Diluent to each well and block for 1 hour at room temperature. Transfer the plate to 4ºC until ready to begin the assay. Note: The Homocysteine Conjugate Coated Plate is not stable long-term. We recommend using it within 24 hours after coating. • 1X Wash Buffer: Dilute the 10X Wash Buffer to 1X with deionized water. Stir to homogeneity. • Anti-Homocysteine Antibody and Secondary Antibody, HRP Conjugate: Immediately before use dilute the Anti-Homocysteine Antibody 1:500 and the Secondary Antibody, HRP Conjugate 1:1000 with Assay Diluent. Do not store diluted solutions.

Preparation of Standard Curve Prepare a dilution series of Homocysteine-BSA standards in the concentration range of 0 to 40 µg/mL in Assay Diluent

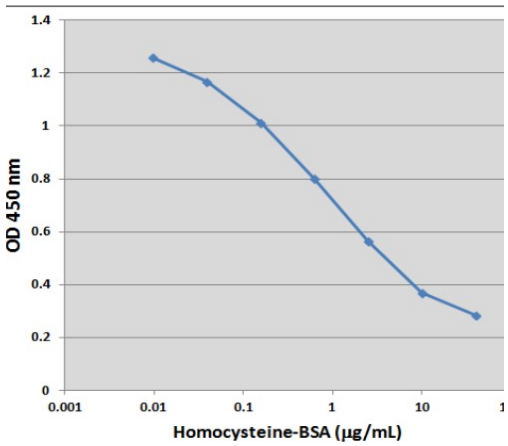
Preparation of Homocysteine-BSA Standards.

Standard Tubes	4 mg/mL Homocysteine-BSA Standard (µL)	Assay Diluent (µL)	Homocysteine-BSA (µg/mL)
1	4	396	40
2	100 of Tube #1	300	10
3	100 of Tube #2	300	2.5
4	100 of Tube #3	300	0.625
5	100 of Tube #4	300	0.156
6	100 of Tube #5	300	0.039
7	100 of Tube #6	300	0.010
8	0	300	0

Assay Protocol

1. Prepare and mix all reagents thoroughly before use.
2. Each unknown sample (see Preparation of Samples section), Homocysteine-BSA standard, and blank should be assayed in duplicate.
3. Remove the Assay Diluent from the plate and add 50 µL of unknown sample or standard to the Homocysteine Conjugate Coated Plate. Incubate at room temperature for 10 minutes on an orbital shaker.
4. Add 50 µL of diluted Anti-Homocysteine Antibody (see Preparation of Reagents section) to each well. Incubate at room temperature for 1 hour on an orbital shaker.
5. Wash microwell strips 3 times with 250 µL 1X Wash Buffer per well with thorough aspiration between each wash. After each wash, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess 1X Wash Buffer.
6. Add 100 µL of the diluted Secondary Antibody, HRP Conjugate to each well. Incubate at room temperature for 1 hour on an orbital shaker. During this incubation, warm Substrate Solution to room temperature.
7. Wash the strip wells 3 times according to step 5 above. Proceed immediately to the next step.
8. Add 100 µL of Substrate Solution to each well, including the blank wells. Incubate at room temperature on an orbital shaker. Actual incubation time may vary from 2-30 minutes. Note: Watch plate carefully; if color changes rapidly, the reaction may need to be stopped sooner to prevent saturation.

9. Stop the enzyme reaction by adding 100 µL of Stop Solution into each well, including the blank wells. Results should be read immediately (color will fade over time).
10. Read absorbance of each microwell on a spectrophotometer using 450 nm as the primary wave length.
Example of Results: The following figures demonstrate typical Homocysteine Competitive ELISA Kit results. One should use the data below for reference only. This data should not be used to interpret actual results.0



Homocysteine-BSA Standard Curve.
Normal plasma Hcy level in the adult population is usually in the range of 5-15 µmol/l. Hyperhomocysteinaemia is categorized as mild if the Hcy value ranges between 15-30 µmol/l, moderate when the value is between 30-100 µmol/l and severe when Hcy in plasma is higher than 100 µmol/l.

REFERENCE

ELISA kit operation Manual

I have read, understood and agreed to follow the procedure as documented.

No.	Name	Signature	Date

Standard Operating Procedure (SOP) for determination of serum Retinol using High Performance Liquid Chromatography (HPLC)

	TANZANIA FOOD AND NUTRITION CENTRE **BIOCHEMISTRY LABORATORY SECTION** **Standard Operating Procedure (SOP) for determination of serum Retinol using High Performance Liquid Chromatography (HPLC)**		
Version No:		**Copy no.**	
Prepared by:			
Reviewed by:	1. 2.		
Authorized by:			

TANZANIA FOOD AND NUTRITION CENTRE

Changes:

……
……
……..

Reasons for changes:

……
……
……..

SCOPE

This SOP describes determination of serum retinol using High Performance Liquid Chromatography at TFNC laboratory

PURPOSE

- To ensure proper technique followed during determination of serum retinol by HPLC
- Ensure high quality results of serum retinol

RESPONSIBILITIES

Laboratory technologist/technicians involved in the study should be trained and follow this SOP

PRINCIPLE

The test principle of this method utilizes high performance liquid chromatography with photodiode array detection to measure serum concentrations of vitamin A (retinol). A small volume (25 µL) of serum is mixed with twice the volume (50 µL) of an ethanol solution containing an internal standard - retinyl acetate. The retinol is extracted by adding additional acetonitrile, vigorously agitating, then centrifuging. The supernatant is collected for analysis. An

aliquot of the supernatant is injected onto a C18 normal phase column and isocratically eluted with a mobile phase consisting of 83% acetonitrile with 0.1% triethylamine and 17% deionized water. Absorbance of this substance in solution is linearly proportional to concentration (within limits), thus spectrophotometric methods are used for quantitative analysis. The wavelength of 325 nm, approximately corresponding to absorption maxima, is monitored and chromatograms are recorded. Quantitation is accomplished by comparing the peak height ratio of the analyte to internal standard in the unknown with the peak height ratio of a known amount of retinol to retinyl acetate in a calibrator solution.

MATERIALS REQUIRED

1 **REAGENTS**

a Autosampler 12x31mm widemouth vials (TCW224626, Wheaton, Millville, NJ) or equivalent

b 250-µL polypropylene inserts (TCW225259, Wheaton, Millville, NJ) or equivalent

c screw caps with teflon/silicone septa (TCW242762, Wheaton, Millville, NJ) or equivalent

d Assay Column: 10 cm x 4.6 mm Thermo Hypersil Gold aQ C18 3-µm particle size reversed phase column (Thermo Electron corporation, Bellefonte, PA)

e Serum extract filters: 0.45 µm syringe tip PVDF hydrophilic filter (4 mm diameter) (Millipore Corp, Medford, MA) or equivalent

f Solvent filters: 0.45 µm pore size, PVDF (HVHP04700, Millipore Corp, Medford, MA) or equivalent

g Plastic tuberculin syringes (obtained from various sources) or equivalent

h 2 mL polypropylene cryovials (Fisher Scientific, Inc, Fairlawn, NJ) or equivalent

12 x 75 mm disposable glass culture tubes (Fisher Scientific, Inc, Fairlawn, NJ) or equivalent 5¾ inch pasteur pipettes (Fisher Scientific, Inc, Fairlawn, NJ) or equivalent

k) Combitip Plus (0.5 mL) for Eppendorf repeater pipette (Eppendorf) or equivalent
l) Combitip Plus (2.5 mL) for Eppendorf repeater pipette (Eppendorf) or equivalent
m)Rainin tips for LTS pipette (Rainin, Woburn, MA) or equivalent
n) Gilson Microman positive displacement pipette tips (Gilson, Villiers-le, France) or equivalent
o) Various glass beakers, graduated cylinders and glass bottles, class A glassware and actinic glassware (obtained from various sources) or equivalent
p) 1.5-mL micro-centrifuge tubes (Fisher Scientific, Inc, Fairlawn, NJ) or equivalent
q) Waters Guard-Pak Module (cat. no. WAT 88141) with Guard Pak filters (cat. no. WAT032472) or equivalent

r) Aluminum foil (Fisher) or equivalent

(2) Purification Supplies

a) Column: 15 cm x 4.6 mm Burdick and Jackson OD5 C18 5µm particle size column (Burdick and Jackson Laboratories, Muskegan, MI)
b) Autosampler vials 12x31mm widemouth vials (cat. no. TCW224626; Wheaton) or equivalent
c) 250-µL glass inserts with spring foot (for any chloroform diluted materials) or equivalent
d) Screw caps with teflon/silicone septa (cat. no. TCW242762, Millville, NJ) or equivalent

(3) Chemicals

a) Acetonitrile HPLC grade (Sigma) or equivalent
b) Ethanol, absolute (Sigma) or equivalent) or equivalent
c) Polished Water 18 mΩ, (Aqua Solutions, Jasper, GA) or equivalent
e) Triethylamine, 'Baker' grade (Sigma) or equivalent
f) Sodium Chloride, ACS grade (Sigma) or equivalent
g) Methanol, HPLC grade, (Sigma) or equivalent
h) Bovine serum albumin, (Sigma) or equivalent
i) 0.01M PBS packets, (Sigma) or equivalent

(4) Standards

a) Retinol (Sigma) or equivalent
b) Retinyl acetate (Sigma) or equivalent

E. Instrumentation

In the case of simple laboratory instrumentation (e.g., pipettes, vortex mixer, analytical balance, etc.) a product listed herein may be substituted with equivalent product from a different manufacturer provided that it meets or exceeds the specifications of the product listed. In the case of analysis instrumentation (e.g., LC components, photodiode array) equivalent performance must be demonstrated experimentally in accordance with DLS Policies and Procedures Manual if a product substitution is made. Equivalent performance must also be demonstrated in accordance with DLS policies and procedures when multiple analysis systems are used, even if they are of the exact same type.

I. Waters HPLC system (Waters)
    a. Alliance HPLC model 2695
    b. Waters photodiode array detector (PDA) model 2996
    c. computer with the following specifications equivalent to: Microsoft Windows XP operating system, 1.7 Ghz, 524 MB RAM, 18 GB hard drive
II. Vortex mixer
III. Micro-centrifuge
IV. Magnetic stirrer
V. analytical balance (Mettler Toledo, Columbus, OH) or equivalent
VI. Cary 60 UV-visible spectrophotometer (agilent)

6. **Preparation of Reagents, Calibration (Standards), Controls, Equipment and Instrumentation**

A. Reagent Preparation

Though each reagent preparation step specifies a total volume of reagent to be prepared, these directions may be scaled up or down to prepare larger or smaller quantities, if desired.
Any water used to prepare reagents refers to deionized water with resistance of at least 17 megohms (MΩ). Reagent grade ethanol and HPLC-grade acetonitrile and methanol are used throughout.

Most reagent preparations may be conducted in non-volumetric glassware. Use class A volumetric glassware where a volumetric flask is specified.

1) Mobile Phase

a) Component 1: 100% acetonitrile (HPLC grade) with 0.1% trimethylamine (TEA). Acetonitrile is filtered using 0.45-µm pore-size membranes (Millipore HVHP04700 or similar product). TEA is added prior to filtering at 32 drops per liter using a glass Pasteur pipette to make a 0.1% TEA solution

b) Component 2: 100% unfiltered polished water.

The components are degassed by the system and automated solvent blending takes place in the Alliance 2695 HPLC system.

2 Vitamin A Standard Purification Diluent

The following solutions are filtered separately using 0.45-µm pore-size membranes (Millipore HVHP04700 or similar product). Triethylamine is added to each prior to filtering at 32 drops per liter using a glass Pasteur pipette to make a 0.1% TEA solution. Once the two solutions are prepared, mix them in equal parts in a single bottle for use to prepare the vitamin A standard (see 5B).

a) Component 1: 100% ethanol (200 proof) with 0.1% TEA

b) Component 2: 100% acetonitrile (HPLC grade) with 0.1% TEA

3) Extraction Solution

100% Acetonitrile is filtered using 0.45-µm pore-size membranes (Millipore HVHP04700 or similar product).

4) 10% Saline Solution

0.5 g NaCl is dissolved in 5.0 mL deionized water in a 12x75 mm test tube and stored at room temperature until used. This solution may be used when a serum specimen requires dilution for repeat analysis. Alternately, 4% albumin in 0.1M PBS may be used to dilute serum for repeat analysis.

5) 4% Albumin in 0.1 M PBS

2 g Albumin is dissolved in 50 mL of 0.1M phosphate saline. Vortex and stir on a magnetic stirrer until it is completely is dissolved. Store this solution in the refrigerator at 4 ^{o}C for up to two weeks. This solution may be used when a serum specimen requires dilution for repeat analysis. Alternately, 10% saline may be used to dilute serum for repeat analysis.

B. Standards Preparation

1) Purified Stock Solutions (retinol)

The stock solution of retinol is prepared by further purifying commercially purchased retinol. A small amount (non-quantitative amount) of concentrated retinol dissolved in chloroform is repeatedly injected on to column that is selective for retinol (Burdick and Jackson OD5 octadecylsilane 150 x 4.6 mm, 5 µm). A segment of the eluting peaks are collected and pooled then measured for concentration via a spectrophotometer at 325 nm. Dilutions to the purified material are done with the vitamin A standard diluent to prepare calibration materials to approximately the concentration shown in Table 1. The final working solution is aliquotted into pre-labeled amber vials. See 4022 SOP_Purification of VitA standard for detailed step-by-step instructions on the purification process.

3 Non-Purified Stock Internal Standard Solution

A small amount of retinyl acetate is dissolved in ethanol. No secondary purification is required. The solution is diluted with 100% filtered ethanol to obtain a solution with a UV absorbance of approximately 0.1300 AU for retinyl acetate at 325 nm (measured via a spectrophotometer).

4 Final Standard Solutions

The final retinol calibration standard solutions are stored in 1.8-mL aliquots in amber glass vials (National Scientific Co. part# C4012-2 12 x 32 mm) and the final internal standards are stored in larger glass bottles such as 20mL scintillation vials, both at 70 °C. These solutions are stable at -70 °C for at least 2 years.

7. Calibration and Calibration Verification Procedures

A. Method Calibration

Two calibration techniques may be used for calibration: a single point or a multipoint calibration curve. At the beginning of every run, each calibrator is prepared by combining 50 µL of the internal standard and 25 µL of the retinol standard solution using a positive displacement pipette. Thus, the final calibrator contains 1/3 as much retinol as the undiluted working solutions. However, since all samples will have the same proportion of internal standard added, the calibration concentrations entered for each calibrator are the undiluted concentration value (Table 2). The calibration process is based on the peak area ratios of retinol compared with the retinyl acetate internal standard.

(1) For single-point calibration: a single solvent based calibrator, generally at 50 µg/dL, is used to generate a one-point standard curve forced through zero for retinol. The concentrations of unknowns are calculated from the regression equation based on the height ratios between analyte and internal standard. The calibrator is injected again at the end of the run and treated as an unknown by the processing software. As an unknown, the value must agree within 15% of the calibrator value.

When using a single point calibration curve exclusively, additional calibration verification must be performed. Because the single point calibration procedure in this method does not include three or more levels of calibration material and does not includes a low calibrator near the LOD, mid, and high value, an additional requirement for calibration verification must be performed. However, periodically conducting runs using the multipoint calibration procedure eliminates the need to perform the semi-annual calibration verification. See 4022 Calibration Verification SOP for AECAR-VIA for detailed instructions regarding semi-annual calibration verification procedures.

(5) For multi-point calibration:

at least five solvent based calibrator points ranging from LOD to ≥ 100 µg/dL are used to generate a standard curve for retinol, not forced through zero. The concentrations of unknowns are calculated from the regression equation based on the height ratios between analyte and internal standard. One or more of the calibrators is injected again at the end of the run and treated as an unknown by the processing software. As an unknown, the value must agree within 15% of the calibrator value. This calibration procedure does not require the semi-annual calibration verification to be performed.

The instrument software (Empower 3) performs all calculations. Calibration curves are linear and based on a single injection analysis of the calibration points.

Table 1: Extinction Coefficients (EC) used to calculate concentrations in retinol and internal standard stock solutions.

Retinol EC provided by NIST from material dissolved in ethanol.

Analyte	Extinction Coefficient* (dL/g·cm)	CDC Wavelength (nm) (NIST)	Target Concentrations of Purified Stock Standards (µg/dL)	Column used for Stock Solution Purification	Solvent used to dilute purified stock to make standards

Standards					
Retinol	1850 CDC (1843 NIST)	325 (325)	0.52 5.0 10.0 20.0 30.0 50.0 100.0	Burdick and Jackson OD5 octadecylsilane 150 x 4.6 mm, 5 µm	50:50 ethanol:acetonitrile with 0.1% TEA
Internal Standard					
Retinyl acetate	1550 CDC (IS based on absorbance, conc for info only)	325	86 (IS based on absorbance, conc for info only)	NA	100% ethanol with 0.1% TEA

*A1%1cm is defined as the theoretical absorbance of a 1% solution (1 g/100 mL) in a cell of 1 cm pathlength

C. Preparation of Quality Control Materials

1) Bench Quality Control

Quality control materials for this assay are prepared in-house from blood products acquired from blood banks or from other volunteer blood donors.

Approximate QC target values for serum retinol are the lower-third of population distribution (< 54.1 µg/dL) and the upper-third of population distribution (>70.8 µg/dL). The most current distributions can be found in the latest Second National Report on Biochemical Indicators of Diet and Nutrition in the US Population [1]. This method will predominantly be used for populations that are severely deficient hence an ultra-low QC pool close to the deficiency cutoff value of 10 µg/dL should ideally be added to runs in this situation. This can be accomplished by extracting a diluted low QC pool as an ultra-low QC.

Serum units are pooled together then filtered through several layers of gauze to remove fibrin prior to aliquotting into sterile 1.8-mL vials. Typical aliquots volumes are 500 µL per vial. The vials are ideally blanketed with nitrogen or argon before sealing, but this is not required. The QC pools are stored at 70 °C for up to six years.

Pools can be prepared by selecting and blending sera that contain appropriate levels of the analyte or diluting the sera with a saline solution to the desired concentration. Spiking is generally successful for retinol. People who eat very large quantities of fresh fruits and vegetables and have high serum lipid concentrations are most likely to have high concentrations of fat-soluble micronutrients. In some instances dog serum, which typically has a high retinyl ester concentration, is added to the high pool. Sera from individuals taking vitamin supplements are also used. If the pool levels are unacceptably low and spiking needs to be done, it is essential that the spiked, pooled sera be mixed overnight before the filtration step.

Characterization limits are established by analyzing duplicates of each pool for at least 20 consecutive runs.

2) Blind Quality Control

All serum is filtered through sterile gauze before being stabilized with 6 g/dL MPA. A 1:5 dilution of serum in 6.0 g/dL MPA are aliquoted into sterile 2-mL Nalge cryovial, sealed, and vortexed. The blind QC pools are stored at -70 °C and are stable with little degradation for more than 10 years. Screen serum blood bank donors for endogenous fat-soluble micronutrients.

Donor sera are typically blended to achieve appropriate concentrations. Limits for all pools are established by analyzing duplicates for at least 20 runs.

D. Other Materials

With some exceptions, a material listed herein may be substituted with equivalent product from a different manufacturer provided that it meets or exceeds the specifications of the product listed. In the case of standards, internal standards, chemicals and reagents, the chemical and purity of the substitute must meet or exceed that of the listed product. In the case of the LC column, equivalent performance must be demonstrated experimentally in accordance with DLS policies and procedures.

Table 2: Calibrator concentrations for fat-soluble micronutrient assay

Analyte	**Retinol Stock Concentration** (Purified unmixed)	**Volume Standard/Internal Standard**	**True Final concentration** (when mixed with internal standard)	**Working Concentration** (Instrument Value)
Individual Standards				
Retinol (cal 1)	0.52 µg/dL ± 10%	25µL/50µL	0.17 µg/dL ± 10%	0.52 µg/dL ± 10%
Retinol (cal 2)	5 µg/dL ± 10%	25µL/50µL	1.67 µg/dL ± 10%	5.00 µg/dL ± 10%
Retinol (cal 3)	10 µg/dL ± 10%	25µL/50µL	3.33 µg/dL ± 10%	10.0 µg/dL ± 10%
Retinol (cal 4)	20 µg/dL ± 10%	25µL/50µL	6.67 µg/dL ± 10%	20.0 µg/dL ± 10%
Retinol (cal 5)	30 µg/dL ± 10%	25µL/50µL	10.0 µg/dL ± 10%	30.0 µg/dL ± 10%
Retinol (cal 6)	50 µg/dL ± 10%	25µL/50µL	16.7 µg/dL ± 10%	50.0 µg/dL ± 10%
Retinol (cal 7)	100 µg/dL ± 10%	25µL/50µL	33.3 µg/dL ± 10%	100 µg/dL ± 10%

This method uses ethanol or ethanol:acetonitrile as the matrix for the calibrators. It is well known information from CDC and NIST that spiking serum does not work well for this method.

For troubleshooting and accuracy verification, NIST SRM 968e (Level I, II, and III) is available.

The CDC laboratory participates in a proficiency testing (PT) program for retinol and other fat-soluble micronutrients, sponsored by the National Institute of Standards and Technology (NIST, Gaithersburg, MD). On a rotating basis, Round Robin materials are sent by NIST to assess laboratory performance. At least two PT challenges are performed annually by either participating in two NIST challenges or some combination of NIST round robins and/or in-house PT. Additionally, certified reference materials (SRM) for retinol, (currently NIST SRM 968e - certificate of values available on the network and at www.NIST.gov), are analyzed to determine the agreement between results obtained with the CDC laboratory method and the certified values. For general information on the handling, analysis, review, and reporting of proficiency testing materials see NBB_SOP Proficiency Testing Procedure and 4022_SOP In-House Proficiency Testing.

To provide adequate throughput for this method as well as backup instrumentation during times of repair and maintenance we may utilize multiple HPLC systems of the Waters Alliance type. In this case, equivalent performance (system verifications) must be demonstrated in accordance

with DLS Policies and Procedures when multiple analysis systems are used in parallel, even if they are of the exact same type. The comparisons involve analyzing several samples on each of the instruments and assessing the resulting Pearson correlation coefficients. Details about these procedures can be found in 4022 Calibration Verification SOP for AECAR-VIA.
Results from a series of in-house ruggedness testing experiments designed to assess accuracy when certain experimental parameters were varied are presented in Appendix B B. Pipettes (air displacement and positive displacement) Pipettes are calibrated or calibration is verified on a semi-annual basis.

C. Balances

Balances are calibrated annually and verified as used using calibrated weights.

D. Cary UV/vis spectrophotometer

Proficiency testing is done three times per year by participation in the CAP instrumentation survey. Additionally, every time the instrument is turned on there are internal diagnostics that are run. Calibration verification using certified filters is performed twice per year. Calibration verification of the certified filters is performed externally every other year.

8. Procedure Operating Instructions; Calculations; Interpretation of Results

A typical run consists of the following sequence of samples: calibrators, aqueous blank, first set of QC, up to 140 patient samples, and second set of QC, and reinject of calibrator (read as unknown). Three levels of serum QC are analyzed in duplicate in each run as bench quality control materials.

A. Preliminaries

(1) Remove all necessary QC, calibrators, unknown samples, and the internal standard solution from the -70 °C freezer. Allow them to reach ambient temperature then gently vortex. Visually inspect specimens for any unusual sample volume, specimen color or debris/precipitate

B. Calibrator Preparation

(1) Individually prepare calibrators by mixing 50 µL internal standard solution and 25 µL calibrator using a positive displacement pipette.
(2) Agitate the prepared calibrators and transfer to an HPLC vial using a pipette (any pipette including Pasteur). Cap immediately.

C. Unknowns and QC preparation

(1) All unknowns, blanks, and QCs will be prepared in micro-centrifuge tubes.
(2) Aliquot 50 µL of internal standard solution to each micro-centrifuge tube.
(3) Aliquot 25 µL of sample (patient serum, QC serum, or 18MΩ water blank) to each micro-centrifuge tube containing the internal standard solution.
(4) Aliquot 125 µL of filtered 100% acetonitrile (extraction solution) to each tube then snap close each cap.
(5) Vortex the mixture for 30 seconds by hand or 10 seconds by vortexer.
(6) Load the micro-centrifuge tubes into a centrifuge and spin for at least 3 minutes at 25 °C at 1,500 RPM/RCFgx10. Alternately, leave the sample on lab bench at 25 °C and allow to gravimetrically separate if a micro-centrifuge is not available.
(7) After carefully removing sample tubes from centrifuge, pipette 50 µL to 100 µL of the supernatant into a prepared autosampler vial (autosampler vial containing an insert). Use care not to disturb the pellet.
(8) Cap all the vials, tap the vials to remove any bubbles, and place them in the autosampler set at 20 °C for analysis

(9) Unused extracted samples may be stored in the micro-centrifuge tubes at 4 °C for several weeks in the event that reinjection repeats are required.

D. HPLC-PDA Instrument preparation

(1) Prepare the mobile phase components separately. See section 1A.

(2) Load fresh mobile phase into reservoirs or top-off mobile phase. Also, top-off methanol in seal and needle wash reservoirs.

(3) Turn on instrument as per HPLC-UV Instrument manual.

(4) Prime and purge lines as required by instrument: wet prime solvent and seal wash lines at 5 mL/min for 5 minutes

(6) Instrument method should contain the following parameters:

a) Run time: Typically 8.0 min (dependent upon column lot number and age)

b) Pump pressure limits: 20-4000 psi

c) Pump flow ramp: 3 min (time to accelerate to 10 mL/min/min)

d) Degas mode: normal or continuous

e) Pump Mode: Gradient mode, isocratic flow at 1.1 mL/min (see Table 3)

f) PDA sampling frequency: 1.0 points per sec

g) PDA resolution bandwidth: 4.8 nm

h) PDA wavelength: 325 nm

i) Column temperature: 25 °C (Room temperature without column heater/cooler)

j) Autosampler compartment temperature: 20 °C

Table 3: Isocratic conditions

Time	Flow	% Acetonitrile	% Water	Curve
	1.1	83	17	
20.0	1.1	83	17	6
60.0	0	83	17	6

(6)Collect baseline absorbance data at normal operating conditions to equilibrate with mobile phase (isocratic conditions 17:83 acetonitrile w/0.1% TEA, 1.1 mL/min) for at least 1 hour before collecting sample chromatography.

(7) Start the analytical run after equilibration is complete.

E. Processing/Integrating and Reporting a Run

I. The Waters Empower software is used to review/process a run. A LIMS database is used for additional levels of data review by the analyst, project lead, QA officer, and supervisor and for data reporting.

II. The integration parameters will vary with lamp age, column characteristics, column age, and other factors. Retinol is calibrated on the basis of height using a linear curve either forced through the origin (single point curve) or not forced through the origin with the lowest point at or near LOD. The parameters in Table 4 are acceptable for use as a starting point from which to optimize the analytical conditions. The retention times will vary with age of the column, from column to column, and from instrument to instrument so those in the table should be taken only as a guide. Actual retention times for a given column/instrument combination should be determined individually, monitored on a regular basis, and the component table updated when necessary.

(8) For a detailed step-by-step description of integrating and performing chromatography review, see 4022_SOP Processing and reporting a run.

a) Reviewing the chromatography

1.When the run is finished acquiring the data, the data is reviewed in Empower. Chromatograms for vitamins A are checked for retention times, peak shapes, peak separation, intensity and/or potential interferences.

b) Quantitation and integration of the completed data file

1. Generate results using auto integration.
2. Review integrations and make any necessary integration corrections either using the manual or auto integration option. Auto integration is preferred over manual integration.
3. Print the results for each sample as a PDF to allow future review and documentation (routine procedure) or print hardcopies (exception).
4. Save the results in an ASCII file to import into the LIMS database.
5. Import the results file into the LIMS database for further data review.

(9) Calculations

The Empower software performs all calculations. Retinol is calibrated on the basis of height using a linear curve either forced through the origin (single point curve) or not forced through the origin with the lowest point at or near LOD. The parameters in Table 4 are acceptable for use as a starting point from which to optimize the analytical conditions. The retention times will vary with age of the column, from column to column, and from instrument to instrument so those in the table should be taken only as a guide. Actual retention times for a given column/instrument combination should be determined individually, monitored on a regular basis, and the component table updated when necessary.

Table 4: Component Table Information

Component	Retention Time (min)	Window (min)	Channel (nm)	Metric	Quantitate	Internal Standard
Retinol (VIA)	4.10	0.20	325	Height	Yes	RAA
Retinyl Acetate (RAA)	6.10	0.20	325	Height	No (set to '1')	NA

F. System Maintenance (other than daily maintenance)

Waters HPLC-PDA – Preventative maintenance is performed on an annual basis by a qualified service engineer. Routine maintenance should be performed as indicated in the 4022 SOP VitA HPLC-UV Instrument preparation and in the Waters User Manuals as needed or due to sample delivery problems. Analysis may also set up a schedule for these operations.

10. Reportable Range of Results (AMR – Analytical Measurement Range)

This method is linear for retinol in the range 1-150 µg/dL. Results that are outside of the normal range (see Table 5 in section 12) are generally repeated. Values greater than 150 µg/dL should be diluted with a diluent (saline solution or 4% albumin in PBS) and confirmed by repeat testing. In

addition, results ≤ 10 μg/dL are indicative of severe vitamin A deficiency and should be verified by re-analysis. The differences (coefficients of variation (CV)) between repeat vitamin A values should be within 20% but is generally less than 5%. There is no known maximum acceptable dilution. Dilutions should be conducted in accordance to DLS Policies and Procedures; dilutions should not violate minimum volume requirements or violate serial dilutions limitations.The coefficients of variation (CV) for the vitamin A repeated analysis are generally less than 5%. When a repeat analysis with dilution is required due to either an elevated result or to improve internal standard recovery, mix a 50-μL aliquot of a diluent (saline or 4% albumin in PBS) plus a 50-μL aliquot of serum. The combined mixture should be used as the sample aliquot. All results from this aliquot must be multiplied by two.

11. Quality Control (QC) Procedures

A. Blind Quality Controls

Blind QC specimens can be inserted into the mix of patient specimens. Blind QC specimens are often prepared at two levels using serum pools that emulate different levels of serum fat-soluble micronutrients in patient samples. High levels may be achieved by spiking. Samples from these pools are prepared/extracted in the same manner as patient samples. One blind QC specimen randomly selected for concentration is included at a randomly selected location in every 20 specimens analyzed. Labels are identical to those used in the study. Alternately, an open label blind QC program can be used in which multiple pools (5-10 pools) are randomly packaged for the analyst to insert as blind QC at a rate of 1 per 20 samples. The blind QC pools are not physically blinded to the analyst but due to the number of pools in service and the randomization, the analyst is blind to the expected results for any given sample.

Blind QC are expected to be within 3 standard deviations (3S) of their characterization means, which are established during the course of 20 runs. If a blind QC result is out of control, all or part of the run is declared out of control

B. Bench Quality Controls

Bench QC specimens are prepared using three serum pools that represent low, intermediate, and high levels of serum or plasma retinol. These pools are prepared in the same manner as patient samples and analyzed in duplicate at the beginning and end of each run. The initial limits of each control are established by analyzing pool material in 20 consecutive runs. When necessary, the limits are re-evaluated periodically and updated to include more runs.

The results from the three pools are checked after each run. The system is declared “in control” if all three QC results are within 2S limits and the run is accepted (criteria B.2.a). If any one of the three QC results is outside the 2S limits then apply rules below (B.2.b and B.2.c) and reject if any condition is met - the run is then declared “out of control”:

(1) Multi-rule quality control system: quality control rules for two QC pools per run

a) One QC result per pool

1. If both QC run results are within 2 Si limits, accept the run

2. If one of the two QC run results is outside a 2 Si limit, reject run if:

(i) 1 3S Rule—Run result is outside a 3 Si limit or

(ii) 2 2S Rule—Both run results are outside the same 2 Si limit or

(iii) 10 X-bar Rule—Current and previous nine run results are on the same side of the characterization mean or;

(iv) R 4S Rule—Two consecutive standardized run results differ by more than 4 Si

d) Two QC results per pool

1.If both QC run means are within 2 Sm limits and individual results are within 2 Si limits, accept the run
2. If one of the two QC run means is outside a 2 Sm limit, reject run if:
(i) 1 3S Rule—Run mean is outside a 3 Sm limit or
(ii) 2 2S Rule—Both run means are outside the same 2 Sm limit or
(iii) 10 X-bar Rule—Current and previous nine run means are on the same side of the characterization mean
3. If one of the four QC individual results is outside a 2 Si limit, reject run if:
(i) Outlier—One individual result is beyond the characterization mean ±4 Si or
(i) R 4S Rule—Within-run ranges for both pools in the same run exceed 4 Sw (i.e. 95 percent range limit)
(2) Multi-rule quality control system: quality control rules for three QC pools per run
a) One QC result per pool (generally due to accidental loss of duplicate)
1. If all three QC run results are within 2 Si limits, accept the run
2. If one of the three QC run results is outside a 2 Si limit, reject run if:
(i) 1 3S Rule—Run result is outside a 3 Si limit or
(ii) 2 2S Rule—Two or more of the three run results are outside the same 2 Si limit or
(iii) 10 X-bar Rule—Current and previous nine run results are on the same side of the characterization mean or
(iv) R 4S Rule—Two consecutive standardized run results differ by more than 4 Si
e) Two QC results per pool
1. If all three QC run means are within 2 Sm limits and all six individual results are within 2 Si limits, accept the run
2. If one of the three QC run means is outside a 2 Sm limit, reject run if:
(i) 1 3S Rule—Run mean is outside a 3 Sm limit or
(ii) 2 2S Rule—Two or more of the three run means are outside the same 2 Sm limit or(iii) 10 X-bar Rule—Current and previous nine run means are on the same side of the characterization mean
3. If one of the six QC individual results is outside a 2 Si limit, reject run if:
(i) Outlier—One individual result is beyond the characterization mean ±4 Si or
(ii) R 4S Rule—Two or more of the within-run ranges in the same run exceed 4 Sw (i.e. 95 percent range limit)
Si = Standard deviation of individual results (the limits are not shown on the chart unless run results are actually single measurements)
Sm = Standard deviation of the run means (the limits are shown on the chart)
Sw = Within-run standard deviation (the limits are not shown on the chart)

C. Sample QC Criteria

Each individual sample result is checked against established sample criteria limits to assure data quality. The method uses the following sample criteria:

- Relative retention time (retention time VIA/retention time RAA)
- Internal standard peak height in each sample should be within ±80% of the run mean peak height (peak height RAA in each sample/mean peak height of RAA in all samples within run).

For details see 4022 SOP VitA HPLC-UV Sample QC Criteria.

12. Remedial Action if Calibration or QC Systems Fail to Meet Acceptable Criteria

(A) Sample Preparation

Look for sample preparation errors, e.g., vial spilled during preparation or analyst forgot or under-pipetted the internal standard. If analytes are uniformly high or low in the QC, check fluid dispensing devices (Digiflex, pipettes) for accuracy. Ensure pipettes are calibrated.
Look for extraction problems by assessing the recovery of the internal standards in the calibrators, QC samples and unknowns. Isolated poor recovery suggests a sample spill. Poor recovery for the entire run suggests analyte degradation. Retinol is generally stable under normal working conditions however, some degradation may be observed due to exposure to air, light and heat. Was the room too warm? Were the samples at room temperature for longer than necessary? Were the samples exposed to full-spectrum light for longer than necessary? Check any temperature logs available for the room and the current temperature. If the room was too warm when the samples were processed, and continues to be too warm, call the building manager.

(B) Calibration

Check the lot of calibrator used to quantitate the analyte that failed QC. Is it more than 2 years old? Check the chart showing peak height for that calibrator on the current column. Is there a trend showing decreasing peak height? You might need to schedule calibrator purification. Check the for the number of injections run on that column. Is it above average? Are low level peaks no longer recognized? If so, you probably need a new column. In addition, the column degradation may be confirmed when the retinol and retinol acetate begin to co-elute.

(C) Hardware

Check to make sure that the HPLC hardware is functioning properly. Ensure the PDA status light is solid green. Ensure the pump is operating at an appropriate pressure with steady delivery. Check the autosampler and the run sheet to make sure the injections are being made as programmed. Look at vial volumes and puncture marks. Make sure that the run sheet reflects the actual vial sequence by cross-checking sample numbers. Check the temperature of the autosampler. If you suspect that the injection volumes are too variable (check the internal standard peak height in the calibrators), you should run a test of the reproducibility of the injection volume using a standard. Check the Instrument Malfunction Log for past hardware/software problems. Check the Delta Pressure Log. When the ripple pressure (delta) exceeds 2.5% of the baseline system pressure, the check valves in the pump probably need to be replaced. If sensitivity is low, the detector lamp energy may be inadequate. Run lamp diagnostics. Replace lamp about every 6 months with constant use or replace when lamp hours exceed 1000 hours.
Many obscure problems can be corrected by re-booting the HPLC/detector system. Every time the system is re-booted, diagnostics checks are automatically made. The system should be re-booted at least once a week.

(D) Sample Integrity

Check the freezer logs for stable temperatures for assay materials requiring -70 °C storage. Obtain NISBI SRM material if you suspect that your QC pools or calibrators may have degraded (or after a significant change in the system).

(E) Chromatography

Check the chromatography for faulty integration or incorrect peak. Correct the integration if indicated. This is an uncommon event for controls, unless the column is new and the retention times/windows are not yet stable. If the steps outlined above do not result in correction of the "out of control" values for QC materials, consult the supervisor for other appropriate corrective actions. Do not report analytical results for runs not in statistical control.

13. Limitations of Method; Interfering Substances and Conditions

(A) The most common causes of imprecision are intermittently inaccurate micropipettors and pipetting errors.
(B) Calibrators, internal standards, quality control pools, and specimens should be mixed thoroughly via vortex prior to aliquotting.
(C) Handling calibrators and the internal standard in step-wise sequential manner will minimize the chances of cross-contaminations. column history.
(D) Changing of gloves after preparations of stock and working standards and internal standards is recommended to avoid any contamination.
(E) Ideally, the column cooler should be at 25 °C for 24 hours to allow the column to stabilize. The autosampler refrigeration unit needs approximately 45 minutes to stabilize. The lamp should have 1 hour to stabilize. The column should be under flow for at least 60 minutes before the first injection is made. In actual practice the system is only turned completely off if it will be idle for more than three days, except for the lamp, which is turned off when not in use.
(F) The following substitution may be made for the specified instrumentation: In the event that a centrifuge is not available, the extracts may be allowed to rest undisturbed for ≥ 30 minutes at room temperature. Following this the resulting supernatant may be analyzed as described within this document.
(G) All of the HPLC equipment is attached to line conditioners to minimize the effects of fluctuations of electrical current.
(H) This method has undergone a series of in-house ruggedness testing experiments designed to assess changes when experimental parameters are varied. Five parameters judged to most likely affect the accuracy of the method have been identified and tested. Testing generally consisted of performing replicate measurements on a test specimen with the selected parameter set at values substantially lower and higher than that specified in this method while holding other variables constant. Ruggedness testing findings for this method are presented in Appendix B. Refer to Chapter 20 of the 2017 DLS Policies and Procedures Manual for further information on ruggedness testing.

15. Critical Call Results ("Panic Values")

Any sample with a vitamin A result <10 µg/dL or greater than the 97.5% age-specific upper limits (Table 5) are repeated for confirmation and reported to the responsible party for the study (project officer) as soon as possible. Hypervitaminosis A with hepatotoxicity is suggested by a vitamin A/retinyl ester profile in a fasting serum with elevated retinol for age/sex and total retinyl esters > 40% of serum retinoids. Hypervitaminosis A cannot be diagnosed definitively using this method because this method does not measure retinyl esters. Emails sent concerning abnormal results are maintained by the supervisor for the duration of the study. Most of these studies are epidemiological in nature.

16.Specimen Storage and Handling during Testing

Specimens are allowed to reach room temperature during preparation. Once the samples are ready to run, they are placed in the autosampler at 20 °C. The unused portion of the patient specimen is returned to frozen storage (typically -70 □C) as soon as possible.

17. Alternate Methods for Performing Test of Storing Specimens if Test System Fails

Since the analysis of serum for fat-soluble vitamins is inherently complex and challenging, there are no acceptable alternative methods of analysis in the Nutrition Biomarkers laboratory. If the analytical system fails, then storage at ≤ 4 °C of the extracted specimens is recommended until the analytical system is restored to functionality.

18.Test Result Reporting System; Protocol for Reporting Critical Calls (If Applicable)
Test results that are reported to the collaborating agency at a frequency and using a method determined by the study coordinator.

19. Transfer or Referral of Specimens; Procedures for Specimen Accountability and Tracking
A LIMS database is used to maintain results and track specimens for this method.
CDC recommends that records, including related QA\QC data, be maintained for 5-10 years after completion of studies. Only numerical identifiers should be used (e.g., Sample ID); all personal identifiers should be available only to the medical supervisor or project coordinator

20. Method Performance Documentation
Method performance documentation for this method including accuracy, precision, sensitivity, specificity and stability is provided in Appendix A of this method documentation. The signatures of the branch chief and director of the Division of Laboratory Sciences on the first page of this procedure denote that the method performance is fit for the intended use of the method.

References
(1) U.S. Centers for Disease Control and Prevention. Second National Report on Biochemical Indicators of Diet and Nutrition in the U.S. Population 2012. Atlanta (GA): National Center for Environmental Health; April 2012.
(2) Chaudhary-Webb, M, Erhardt, JJ, Haynes MB, Schleicher, RL. Simplified HPLC/UV detection method for measuring serum retinol. Micronutrient Forum 2007.
(3) Caudill SP, Schleicher RL, Pirkle JL. 2008. Multi-rule quality control for the age-related eye disease study. Stat Med 27:4094-4106.
(4) "Quality Assurance of Clinical Measurements. JK Taylor, Lewis Publishers, Chelsea, Michigan. 1987. pp. 78-84."

I have read, understood and agreed to follow the procedure as documented

No.	Name	Signature	Date

	TANZANIA FOOD AND NUTRITION CENTRE **BIOCHEMISTRY LABORATORY SECTION** **Standard Operating Procedure (SOP) for determination of Serum Vitamin B12 by Electrochemiluminescence immunoassay "ECLIA"**		
Version No:		**Copy no.**	
Prepared by:			
Reviewed by:	1. 2.		
Authorized by:			

TANZANIA FOOD AND NUTRITION CENTRE

Changes:

……
……
……..

Reasons for changes:

……
……
……..

SCOPE

This SOP describes the procedure for determination of vitamin B 12 in serum by using Cobas e 411 machine for the study titled: "Morogoro Region Micronutrient Household and Biomarker Survey, Tanzania, 2019"

PURPOSE

To ensure proper procedures are followed during analysis for determination of vitamin B 12 in serum by using Cobas e 411

RESPONSIBILITIES

Laboratory technologist/Technician involved in the study should be trained and follow this SOP during sample analysis

PRINCIPLE

The Roche Diagnostic cobas e411 Immunoassay System is a fully automated, random access, softwarecontrolled system for immunoassay analysis. Three test principles are available on the system: competitive principle for extremely small analytes, sandwich principle (one or two steps) for larger analytes and a bridging principle to detect antibodies in the sample.
The cobas e411 automates the immunoassay reactions utilizing electrochemiluminescence (ECL). ECL is a process in which highly reactive species are generated from stable precursors at the surface of an electrode. These highly reactive species react with one another, producing light. The development of ECL immunoassays is based on the use of a ruthenium (II)-tris(bipyridyl) [Ru(bpy)] complex and tripropylamine (TPA). The final chemiluminescent product is formed during the detection step.
The chemiluminescent reactions that lead to the emission of light from the ruthenium complex are initiated electrically by applying voltage to the immunological complexes that are attached to the streptavidin-coated microparticles

Materials needed:
Cobas vitamin B12 (100 test), Cobas vitamin B12 calset ,Precicontrol universal (3ml , 2vials set),Cobas e procell 380 ml,Cobas blank cell 50ml,Cobas cleancell,Cobas cell check 380 ml,Cobas e sap test 250 tests,Cobas e sys wash,cobas sysclean,cobas e universal diluent,distilled/purified water,cobas 4 assay cups,Cobas e assay tips,Cobas e sample cups,Cobas sysclean adapter,Cobas e cleanliner(14bags pack),Cobas e Calset vials,cobas e control vials.

PROCEDURE

Preparation of Reagents, Calibration (Standards), Controls, and All Other Materials; Equipment and Instrumentation

Reagent Preparation

All reagents are supplied by Roche Diagnostics in a ready-for-use unit that cannot be separated. Store the reagent kit upright in order to ensure complete availability of the microparticles. Bring the cooled reagents to approximately 20 °C (45 minutes at room temp) and open the lids slightly before placing on the reagent disk of the analyzer. The reagent kit is stable until the expiration date or up to 12 weeks at 2-8 °C after opening, whichever comes first. The B12 reagent pack can only be stored on-board the e601 for a maximum of 5 weeks so the reagent pack is generally removed from the instrument and stored at 2-8 °C when all samples are completed. This extends the stability of the reagent pack to 60 days when stored alternatively in the refrigerator and on the analyzer.
B.Standards Preparation

Elecsys Vitamin B12 II CalSet is supplied by Roche Diagnostics [6] and is used to adapt the predefined master curve to the analyzer. The lyophilized standards are stable until the expiration date at 2-8°C. Use Class A volumetric glassware if volumetric glassware is specified in the package insert.
Dissolve carefully the contents of one bottle each of B12 II Cal1 and B12 II Cal2 by adding exactly 1.0 mL of distilled water to each. Allow to stand closed for 15 minutes to reconstitute.

Mix carefully, avoiding the formation of foam. Using the supplied labels, label 3-4 empty snap-cap vials for each calibrator. Transfer approximately 200 µL aliquots of the reconstituted calibrator into the appropriately labeled vials avoiding cross contamination. Perform only one calibration procedure per aliquot. Store the remaining CalSet vials at 2-8 °C for 3 days or at -20 °C for 3 months (freeze only once).

PROCEDURES

Turn front switch on analyzer to OFF position before doing maintenance procedures in the morning.

1. Remove reagent compartment lid and reagent wheel to check for condensation inside. Wipe out any moisture. Replace wheel, replace lid.
2. Clean S/R probe with distilled H2O on gauze, and then dry gauze. Use 70% Isopropanol when probe is visibly dirty.

Turn front switch to ON position. When analyzer has returned to standby, proceed with the following:

1. Check Inventory:

Lab-1599 |Cobas E411 Immunoassay System Routine Operation Page 1 of 3
Standard Operating Procedure

a. Each test reagent cartridge is displayed with # of tests and position #. Additional cartridges to be added should warm >45 minutes prior to loading.
b. Check for sample tips and cups. Do not add to partial racks.
c. Replace ProCell/Clean Cell if less than 20%. Move full bottles from left side to right and
load new bottles on left.
d. Empty waste containers.
e. Check volume of System Water.
f. Do a 'clear sample data' from the Overview screen.

2. Calibrate: Refer to "Calset calibration Instructions" for handling of calibrator. Confirm lot number by checking Calibration, Calibrator, and Test. If lot number is not on this screen, go to Install tab, insert card in slot and select BC Card Scan.

a. Allow calibrator vials to warm ~15 minutes before loading.
b. Load on ring aligning the slight extension on the rim of the calibrator vial with the notch
in the hole on the ring. Open caps to 90o angle.
c. Press START on keyboard. e411 will read barcodes on vials and perform calibration for
that test.

3. To Run QC:

a. (If running behind calibrators, leave a space open before inserting controls.) Allow calibrator vials to warm to room temperature before loading.
b. Allow control cups to warm 15 minutes before loading. Replace ring with sample disk.
c. Load control cups on ring. Manually program by selecting QC from Overview screen,

then Control, Position Assignment, and select control and position, then assign.
d. Controls are identified by letter and are poured into large Hitachi cups.
e. Press START on right side of screen and START on the START screen.
f. Be sure to unassign positions for QC after running QC, so other samples may be run in those same positions. From Overview screen, select QC, Control, and Position Assignment; then, select control, position, and remove.
4. Standby reagent packs:
a. Place new reagent pack on e411, do a reagent scan.
b. Calibrate all new packs. To program calibration, from Overview screen, touch Calibration, status, highlight standby, select manual, save. Check on system overview to see if done correctly. Allow controls to warm to room temperature before loading.
c. Replace ring with sample disk.
5. To run QC on standby pack, go to QC, status, standby bottle QC, check box, OK. Check on system overview to see if done correctly.
6. Place tube(s) in sample disk, barcodes facing out. Press START, and then START.
7. To run a STAT sample, if e411 disk is currently sampling other specimens, touch Work Place, Test Selection, Stat (E), type in position #, and test(s). Place tube in proper position (bar code facing out) and touch Save. Touch Stat mode on right side of screen, START and START. Return to home screen and Sample Tracking to make sure sample was programmed in as STAT.
8. If disk is in S. Stop, samples can be added by starting at the STOP tube. Place new ones in sample disk, bar codes facing out. Touch START and START.
9. Directions to print out selected reports when interface is down.
a. If the interface goes down, the instrument must be in STANDBY.
b. Go to Workplace, data review. Select data by clicking on what samples you want to print
or do a sample range start and end. Click on Print, highlight result report, click on print, printout.
10. Directions for Retransmission of cobas e411 results
a. To retransmit specimens when the interface was down once the interface comes back up:
11. Go to Workplace, Data review, select sample, sample range start, click on last sample, click on sample range end.
12. To delete samples one at a time, from data review screen select samples, delete record.
13. After transmitting the test to the host
Go to Utility, page 2, Documentation setup, upload, ok.

Reference

Roche Diagnostics cobas e411 System Operator’s Manual.

25 List of Attachments

25.1 Attachment 1: Questionnaire – English
25.2 Attachment 2: Questionnaire – Kiswahili
25.3 Attachment 3: Household Consent Form – English
25.4 Attachment 4: Household Consent Form - Kiswahili
25.5 Attachment 5: Non-pregnant WRA Interview and Blood Drawn Consent Form – English
25.6 Attachment 6: Non-pregnant WRA Interview and Blood Drawn Consent Form – Kiswahili
25.7 Attachment 7: CDC Human Subject Determination – Public Health Surveillance Approval
25.8 Attachment 8: CVs