MANUAL OF PROCEDURES

Trans-omics integration of multi-omics studies for male osteoporosis

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STUDY SUMMARY

Title	Trans-omics integration of multi-omics studies for male osteoporosis
Sponsor	National Institute on Aging
NIH Grant Number	5U19AG055373
Study Design	Cross-sectional study
Study Duration	5 years
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Study Center	Tulane Center for Biomedical Informatics and Genomics (CBG) at Tulane University School of Medicine.
Objectives	1) gaining comprehensive insights into the fundamental molecular and environmental mechanisms underlying risk of osteoporosis. 2) discovering novel pathways and druggable targets for therapeutic cures. 3) identifying (epi-)genetically susceptible individuals, so that future preventions and interventions can be targeted to and based on individuals' specific (epi-)genotypes.
Number of Subjects	1,055
Inclusion Criteria	1) Caucasian or African American males 2) Aged 20-50 years

	3) Willing to visit the Center for Bioinformatics and Genomics' clinical recruitment sites for obtaining objective clinical measurements, donation of 120 ml blood, and collection of a small amount of stool sample. 4) Willing to release de-identified genetic information derived from this study into a scientific database, and share it with other researchers for use in future studies of health/medical/biomedical purposes. 5) No antibiotic usage in the past 3 months. 6) No gastroenteritis in the past 3 months. 7) No major surgeries involving hospitalization in the past 3 months. 8) No intercontinental travel in the past 3 months.

1. INTRODUCTION

Osteoporosis is mainly characterized by low bone mineral density (BMD) and its risk later in life can be most powerfully predicted by peak BMD achieved at ages 20-40[1, 2]. Although women have a higher osteoporosis risk, men suffer much higher morbidity and mortality rates following osteoporotic fractures[3, 4]. The (epi-)genetic factors underlying e majority of the BMD heritability (>85%) (especially those sex-specific ones) are largely unknown mainly due to the limitation of the technology and approaches used[5, 6]. Studies focusing on male osteoporosis are rare[7]. Our General Hypothesis is that: while individual omics studies (genome /transcriptome /epigenome) are useful, integrative trans-omics analyses of multi-omics data will be much more productive and more powerful, in not only identifying novel risk (epi-)genes/variants, but also most importantly illuminating their functions in vivo in humans. The Overall Goal of this U19 program project (PPG) is to most comprehensively identify/characterize (epi-) genes/environmental factors and their functional mechanisms for male osteoporosis risk. We will pioneer a comprehensive and novel approach by investigating osteoporosis risk factors simultaneously at the genome- (DNA), transcriptome- (mRNA and miRNA), and epigenome- (DNA methylation) levels in males, while considering environmental factors. We will assess sex/ethnicity/population specificity of the identified (epi-)genes. Furthermore, we will perform in-depth functional studies for detailed molecular mechanisms and functional confirmation of specific novel osteoporosis susceptibility genes/variants to be discovered. Particularly, in addition to the state-of-the-art analyses of individual omics data, we will go beyond the horizon to develop/apply innovative analytical approaches to characterize interactions within and across different omics (such as interactions for miRNA-methylation, and regulation of mRNA by DNA variants and by miRNA/methylation). We will innovatively integrate such interactions across various -omics to identify/characterize (epi-)genetic variants for male osteoporosis.

Identifying (epi)genes/environmental factors and their in vivo functional mechanisms for human BMD variation, especially for men, is necessary and important for 1) gaining comprehensive insights into the fundamental molecular and environmental mechanisms underlying risk of osteoporosis, 2) discovering novel pathways and druggable targets for therapeutic cures; 3) identifying (epi-)genetically susceptible individuals, so that future preventions and interventions can be targeted to and based on individuals' specific (epi-)genotypes. This PPG is expected to break new ground for its innovation, which serves as a pioneering example to stimulate similar studies of other complex diseases.

2. OBJECTIVES

a. Recruitment: In total, 700 Caucasian and 400 African-American males with age between 20-50 years, will be recruited from the subjects participating in the ongoing Louisiana Osteoporosis Study (LOS) in four years. The BMD value will be ascertained by Dual Energy X-ray Absorptiometry (DXA).

b. Phenotyping: includes measuring or ascertaining BMD and bone quality, body composition (body fat mass, lean mass, and percentage fat mass), blood pressure, and other related traits such as weight, height, age, race, lifestyle factors (e.g., exercise, alcohol consumption, smoking, etc.), and medical and family history that may be relevant to osteoporosis.

c. Collect 120 ml blood from each of the 1100 subjects, which will be used for the PBMs isolation (with 110ml of blood) and for measuring serum levels of vitamin D [25(OH)D] (with 10ml of blood).

d. Extract DNA and RNA from the PBMs. The extracted DNA and RNA will be stored in a -80°C freezer, which will be used for downstream methylation and gene expression analyses to attain the goal as specified above.

e. Collect a small amount of stool sample from each subject, which will be used for DNA extraction. The extracted DNAs will be stored in a -80°C freezer, which will be used for subsequent whole genome metagenomic short-gun sequencing.

3. STUDY POPULATION

We will recruit 700 Caucasian males and 400 African-American males with age between 20-50 years. Finally, we recruited 655 Caucasian males and 398 African-American males aged between 20-51 years old for this study.

3.1 The Recruitment Strategy

We have been implementing the Louisiana Osteoporosis Study (LOS) (largely supported by Tulane University) at our clinical site since 2011. The study aims to build a population-based cohort (NOT ascertained through targeted outreach to osteoporosis related clinics) to serve as a large sample pool and database supporting our NIH-funded projects (e.g., U19AG055373, P50AR055081, R01AR050496, R01AR059781) for aging research. Thus far, the LOS has accumulated >17,000 subjects.

Our database manager will first screen the LOS database based on the selection criteria of this study (see below). The clinical coordinators will then initiate the contact with the eligible subjects by direct conversation when the subject is visiting our clinical sites for other projects (e.g., LOS), or by mails, emails, text message, and/or phone calls. In addition, subjects who read about the study on our clinical recruitment website (http://tulane.edu/publichealth/bio/clinical-recruitment.cfm) may contact our clinical coordinator by phone or email for further information. Meanwhile, flyers will be sent to the potential participants in the surrounding area and posted on websites, such as Craigslist, social media, and our website. Postcards will be mail to the potential subjects. On average, $5/participant will be given to the referrer once he/she refers **at least two** participant who successfully enrolls and completes the MM study procedures. Since our clinic facility only has $10 and $25 gift cards, referrers will not receive a gift card until they refer at least two participants. They receive a $10 Walmart gift card for every 2 participants recruited or a $25 Walmart gift card for every 5 participants recruited. Each participant referred can only count once. Each participant referred can only count once. There is no limit to the compensation amount. During their conversation, phone call or email communication, the clinical coordinator will briefly introduce the study to the subjects and ask if they are interested in this study. If the subject is interested in participating in the study, a visit will be scheduled to our clinical recruitment site (11th floor, 1440 Canal Street, New Orleans, LA 70112).

Subjects who need more time for deciding on participation will be allowed to bring the consent form home and be asked for their willingness to be re-contacted by our research staff regarding their final decisions.

One subject is supposed to participate in this study once, and our staff will be well trained to avoid duplicative enrollment adopting the following strategies: 1) Current enrollment list of all previous participants are available to our front desk and we will check full name and birthdate to avoid duplicative enrollment. 2) We will check each subject's driver's license to make sure self-reported identification information (full name and birth date) are reliable. However, duplicative enrollment may still occur due to staff's negligence, mistakes, etc. For duplicative enrollment, we will delete all data and specimen samples collected from the second enrollment. The first enrollment data will be retained for research usage.

3.2 Inclusion and Exclusion Criteria

Individuals must meet the following inclusion criteria to be eligible to participate in the study:

1) Caucasian or African American males

2) Aged 20-50 years

3) Willing to visit the Center for Bioinformatics and Genomics' clinical recruitment sites for obtaining objective clinical measurements, donation of 120 ml blood, and collection of a small amount of stool sample.

4) Willing to release de-identified genetic information derived from this study into a scientific database and share it with other researchers for use in future studies of health/medical/biomedical purposes.

5) No antibiotic usage in the past 3 months.

6) No gastroenteritis in the past 3 months.

7) No major surgeries involving hospitalization in the past 3 months.

8) No intercontinental travel in the past 3 months.

We will adopt the following exclusion criteria to minimize non-genetic influence on bone mass variation, so as to empirically enhance the chance to detect individual genetic factors for bone mass. The same criteria are being used in our ongoing LOS study.

1) Serious residuals from cerebral vascular disease;

3) Diabetes mellitus, except for those controlled under medication;

4) Chronic renal failure;

5) Chronic liver failure;

6) Significant chronic lung disease;

7) Alcohol abuses as defined by those who drink alcohol regularly and cannot control themselves and get drunk at least once a week;

8) Chronic obstructive pulmonary disease (COPD);

9) Corticosteroid therapy at therapeutic levels for more than 6 months duration;

10) Treatment with anticonvulsant therapy for more than 6 months duration;

11) Other metabolic or inherited bone diseases including hyper- or hypoparathyroidism, Paget's disease, osteomalacia, osteogenisis imperfecta, and hypochondrogenesis;

12) Rheumatoid arthritis, except for minor cases that involve only hand joint and wrist;

13) Chronic gastrointestinal disease including celiac disease, postygastrectomy, Crohn's disease, ulcerative colitis, liver transplant, and cirrhosis;

14). Upper or lower limb loss or disability;

We will not exclude people with a diagnosis of idiopathic osteoporosis, or those on calcium and/or vitamin D supplements. However, information collected for calcium and/or vitamin D supplements may be used as co-variates in analyses.

Given that blood monocytes are also essential component of the immune system, we will adopt the following additional exclusion criteria in order to minimize the effect of diseases or conditions, which may potentially lead to monocyte gene/protein expression changes:

1) Autoimmune or autoimmune-related diseases: systemic lupus erythematosus, multiple sclerosis, Graves disease, Hashimoto's thyroiditis, myasthenia gravis, Addison's disease, dermatomyositis, Sjogren's syndrome, Reiter's syndrome.

2) Immune-deficiency conditions: HIV infection, severe malnutrition, splenectomy, ataxia-telangiectasia, DiGeorge syndrome, Chediak-Higashi syndrome, job syndrome, leukocyte adhesion defects, panhypogammaglobulinemia, selective deficiency of IgA, combined immunodeficiency disease, Wiscott-Aldrich syndrome.

3) Haematopoietic and lymphoreticular malignancies: leukaemias, lymphomas (Hodgkin's disease, non-Hodgkin's disease), myeloma, Waldenström's macroglobulinaemia, heavy chain disease, leukemic reticuloendotheliosis, mastocytosis, malignant histiocytosis.

4) Other diseases: influenza (within one week of recruitment), active periods of asthma.

Subjects will only provide verbal confirmation of their HIV statuses for determination of study eligibility. HIV testing will NOT be performed on their blood samples.

4. PROCEDURES

The blood collection will be performed at our clinical recruitment site (11th floor, 1440 Canal Street, New Orleans, LA 70112). The clinical visits will be coordinated by the clinical coordinators for clinical measurements and experimental procedures. The clinical coordinators will also arrange for immediate transport of blood samples to the PI Dr. Deng's laboratories on the 3rd floor at Tulane JBJ building in New Orleans for downstream cell isolation experiments.

4.1 Questionnaire survey

For each study subject, information on age, gender, racial/ethnic background, medical history, family history, alcohol use, diet habits, smoking history, etc. will be assessed by a Louisiana Osteoporosis Study Questionnaire, Metagenomic Study Supplementary Questionnaire and a Food Frequency Questionnaire.

4.2 BMD and body composition measurement by DXA

If the subject had his previous DXA scan within 3 months, then no DXA scan will be administered to this subject in this visit. The subject's BMD and body composition data from the previous DXA scan will be retrieved from the LOS database.

If it has been greater than 3 months since the subject's last DXA scan, we will need to re-measure the subject's bone density.

We will measure BMD at the forearm of non-dominant hand, spine, left hip (or right hip if the left hip has metal implant), and whole body. Before obtaining DXA measurements, we will explain to the subject the position for each scan, referring to the guide photographs showing positions for accurate measuring. The subject will have learned before the visit to wear a non-metal, bone-scan-friendly outfit, without metal fasteners or zippers. The subject will need to remove any nylons/hosiery, as well as any metal fasteners. In the event a subject arrives without such clothing, disposable gowns and footwear will be available.

For spine BMD, the quantitative phenotype will be the combined BMD of the L_{1-4} vertebrae. For hip BMD, it will be the combined BMD of the femoral neck, trochanter, and intertrochanteric region. For each subject, BMD (g/cm^2) of the lumbar spine, hip, forearm, and total body will be measured by the DXA machine housed in our clinical recruitment sites. The DXA machine to be used in this study is the Hologic Discovery A system (Hologic Inc., Bedford, MA, USA). The machine will be calibrated daily. Software and hardware will be constantly kept up-to-date during the study. All DXA reports will be scrutinized for artifacts and confounding features such as significant scoliosis and osteophyte formation in the spine. Those reports with significant confounding features will be excluded from the analysis.

The Hologic Discovery A system DXA machine also performs body composition analysis, reporting body fat mass and lean mass in grams. We will calculate percentage fat mass as the ratio of fat mass divided by body weight (the sum of fat mass, lean mass, and bone mass). Similarly, percentage lean mass can be calculated as the ratio of lean mass divided by body weight.

4.3 Volumetric BMD and bone quality measurement by QCT

To have a more comprehensive bone health assessment, we will also measure volumetric BMD (vBMD), including cortical and trabecular bone components, at the proximal femur for the selected subjects, by QCT scan with GE Discovery* CT750 HD system at Tulane University Department of Radiology.

Furthermore, we will conduct finite element analysis (FEA) to estimate bone strength based the QCT data obtained. FEA is an engineering method that uses mathematical models to define how a structure or material will react to stress when loaded. FEA was recently applied to estimate bone strength by incorporating information about the geometry and density/distribution embedded in QCT bone scans and the loading conditions known to cause fractures.

4.4 Blood collection via phlebotomy for whole genome sequencing, RNA, DNA methylation, metabolomics data

Blood (120 ml) will be drawn by trained research staff. Samples will be temporarily stored in room temperature and then quickly transferred to Dr. Deng's laboratories at New Orleans for PBMs isolation.

If for some reason we are UNABLE to collect the full amount of 120ml blood from the subject, then the subject will still receive up to $100 Walmart/Amazon gift cards for the visit. At that time, we will ask the subject to return on a different day so that we may try again to collect his blood. The subject will receive another $50 Walmart/Amazon gift card on the returning visit.

4.5 Stool sample collection for metagenome data

The study subjects will be given a self-collection kit to collect a small amount (~0.5 gram, approximately the size of a green pea) of stool sample at our clinical site (if possible) or at home. If the subjects choose to collect the stool sample at home, we will also provide him a mailing package and ask the subjects to mail the stool samples collected in the kit back to us within 7 days after their clinical visit.

If a subject chooses to collect the stool samples at home, then he will receive up to $100 (for clinical measurements and 120 ml blood donation) Walmart/Amazon gift card after the completion of his visit to our clinical site. We will ask him to mail the stool samples collected in the self-collection kit back to us in 7 days (we will pay the mailing fee). The subject will receive another $50 Walmart/Amazon gift card after we receive the stool samples. If we are not able to extract DNA from a subject's stool sample

due to insufficient amount of stool sample, absence of reserving liquid, etc., we will ask the subject to donate an additional stool sample upon his agreement. The subject will receive another $25 Walmart/Amazon gift card once we receive another stool sample.

For subjects who choose to collect stool samples at home, we will remind them sending back their stool samples by sending emails and/or text messages to them on the 2nd and 4th (if not receiving their stool samples by then) day after their clinic visit. If we don't receive their stool sample by the 7th day after their clinic visit, we will send the reminder email and/or text message to the subjects once a week for additional 5 weeks (or until the stool sample are received).

4.6 Grip strength

To assess subjects' physical function, grip strength (kg) will be measured by a dynamometer for the dominant hand by a trained staff.

4.7 Wet Lab Experimental Procedures

The 10ml of collected blood from each subject will be used for measuring serum levels of vitamin D [25(OH)D].

The rest 110ml of blood samples from each subject will be used for PBM isolation using the LymphoprepTM (Greiner Bio-One, Monroe, NC) and Monocyte Isolation Kit II (Miltenyi Biotec Inc., Auburn, CA) following the manufacturer's instruction. DNA and total RNA will be extracted from PBMs by using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instruction. The extracted DNAs and total RNAs will be stored in a -80°C freezer, which will be used for downstream assays.

Whole genome DNA methylation profiles will be assessed by whole genome bisulfite sequencing or other comparable approaches. Targeted DNA methylation analysis for specific genomic regions will be assessed by Sequenom EpiTYPER methylation assays or other comparable approaches. The mRNA expression levels of selected candidate genes will be assessed by real-time RT-PCR. Molecular and cellular functional assays will be conducted on cultured monocyte cell lines.

DNA will be extracted from the stool samples using the MoBio PowerFecal DNA Isolation Kit (MoBio, Solana Beach, CA) following manufacturer's instructions. The extracted DNAs will be stored in a -80°C freezer, which will be used for subsequent whole genome metagenomic short-gun sequencing.

Note: More than one clinic visit/visits may be needed to complete the "Procedures". The additional visit/visits may result from the following reasons: 1) subjects would like to complete different procedures at different time, such as collecting stool samples at home, instead of in the clinic facility; 2) the quantitative computed tomography (QCT) scan will be conducted at Tulane University Department of Radiology. Based on the availability of study subjects and available spots of Tulane University Department of Radiology, the date for QCT scan might differ from the date of the visit to our clinical recruitment site; and/or 3) if we are UNABLE to collect the full amount of 120 ml blood from a subject, the subject will be asked to return on a different day so that we may try again to collect his blood. We have decided to collect additional information from our currently enrolled participant to consider the effect of external factors on the gut microbiota. Therefore, currently enrolled participants who agreed to be further re-contacted will be re-contacted by our research staff for collecting the information that our research needs. The additional information includes:

1. Whether the participant had taken antibiotics in 3 months of the enrollment date.

2. Whether the participant had gastroenteritis in 3 months of the enrollment date.

3. Whether the participant had major surgery involving hospitalization in 3 months of the enrollment date.

4. Whether the participant had traveled intercontinentally in 3 months of the enrollment date.

LIST OF SUPPLEMENTARY FILES

Supplementary file 1: Questionnaire survey

Metagenomic Study Supplementary Questionnaire

ID: _____ LOS ID: _____

SUBJECT INFORMATION

First Name _____Middle Name_____Last Name_____

Blood Type (Please circle): A / AB / B / O / Unknown

Occupation: _____

Marital Status: □ Single □ Married □ Widowed □ Separated □ Divorced

Do you have children? □ Yes □ No Age of children_____

Primary Physician _____Date of last physical exam _____

Have you travelled recently in the past 3 months? □ Yes □ No

If YES, where is your last travel destination? _____

Last travel dates (from MM/DD/YY to MM/DD/YY) _____

If you have changed address, phone number, or email address since your last LOS visit, please fill following information

Address

_____

__

City _____State_____ Zip:_____

Please circle your preferred method of contact: Home Work Cell Email

Home Phone (_____)_____-_____ Work Phone (_____)_____-

_____

Cell Phone (_____)_____-_____ Email

address:_____

Please circle your preferred time to contact: Morning (8–11am) Noon (12–1pm)
Afternoon (2-5pm) Evening (6-9pm)

CLINICAL INFORMATION

1. Have you used (oral or intravenous) antibiotics **in the past 12 months**? □ Yes □ No

   If yes, when was your last-time usage? (MM/YY):

   _____

2. Are you currently taking any probiotics supplements (e.g, Culturelle® Digestive Health Probiotic Capsules, Align® Probiotic Supplement, Nature's Bounty® Ultra Strength Probiotic 10, Schiff® Digestive Advantage Probiotic Capsules, etc)? □ Yes □ No

   If **yes**, please describe:

   Since when (MM/DD/YY): _____ (Approximate date is allowed, e.g., MM/YY)

How often? (select once)

□ once daily □ once every 2 days □ once a week □ Other_____

3. If you are not currently taking probiotics, have you taken any probiotics **in the past 12 months**?

   □ Yes □ No

4. Are you currently taking any gastric acid lowering medications (e.g, Prilosec, Nexium, Prevacid, Dexilant, Protonix, Zegerid, AcipHex, Vimovo, Prilosec OTC, Prevacid24, etc)? □ Yes □ No

   If **yes**, please describe:

   Since when (MM/DD/YY): _____ (Approximate date is allowed, e.g., MM/YY)

   How often? (select once)

   □ once a week □ More than once a week

5. If you are not currently taking acid lowering medications, have you taken any acid lowering medications **in the past 12 months**? □ Yes □ No

6. Please indicate your mode of birth (select one)

   a. How? □ Cesarean section □ Vaginal □ Don't know

   b. Where? □ Hospital/Clinic □ Home □ Don't know

7. Were you breastfed during your infancy?

   □ Yes □ No □ Don't know

If **yes**, can you indicate how long? (select one)

□ < 1 week □ > 1 week □ > 1 month □ > 1 years

8. Can you indicate your average stool frequency? (select one)

□ twice (or more) daily □ once daily □ once every 2 days □ <once every 2 days

9. Please classify the form of your stools (select one) on the Bristol Stool Scale:

□ Type 1

□ Type 2

□ Type 3

□ Type 4

□ Type 5

□ Type 6

□ Type 7

DIGESTIVE HISTORY

1. Do you associate any digestive symptoms with eating certain foods? □ Yes □ No

   If **yes**, please describe:

   _____

2. Have you taken laxatives in the **past 3 months**? □ Yes □ No

   If **yes**, what type/brand and how often?

   Type/brand_____

   □ 4+ days/week □ <4 days/week

3. Have you been diagnosed with a digestive disease/condition? □Yes

□No

If **yes**, please indicate your disease(s) from this following list (Multiple choice is possible):

1) Barrett's Esophagus □
2) Diverticular Disease □
3) Duodenal Ulcer □
4) Dyspepsia □
5) Gastric Ulcer □
6) Gastroesophageal Reflux Disease □
7) Hepatitis □
8) *H. Pylori* infection □
9) Lactose Intolerance □
10) Pancreatitis □
11) Others digestive diseases? (free text):_____

4. Please indicate how often you experience the following symptoms:

Heartburn	□Often	□Sometimes	□Rarel v
Gas	□Often	□Sometimes	□Rarel v
Bloating	□Often	□Sometimes	□Rarel v
Stomach Pain	□Often	□Sometimes	□Rarel v
Nausea/Vomiting	□Often	□Sometimes	□Rarel v
Diarrhea	□Often	□Sometimes	□Rarel v
Constipation	□Often	□Sometimes	□Rarel v

DIET HISTORY

1. Are you currently following any special diet or having diet restrictions or limitations for any reason (health, lose weight, cultural, religious or other)? □ Yes □ No

   If **yes**, check the following that apply:

   □ Low Fat □ Low Carb □ High Protein □ Low Sodium
   □ No Gluten □ Vegetarian □ Vegan □ Diabetic
   □ No Dairy □ No Wheat □ Weight Loss
   □ Other_____

2. Are you currently taking prescribed or over-the-counter medications and supplements to lose weight or control your current weight?

□ No

□ Yes. Please list all related medications and supplements

_____

_____

_____

3. In the **past 12 months**, have you tried to lose weight or control your weight by taking special diet, medications, or supplements?

□ No (skip to the question 5)

□ Yes, please describe all the methods that you tried

Diet Type _____, Duration (from MM/YY to MM/YY): _____

Diet Type _____, Duration (from MM/YY to MM/YY): _____

Diet Type _____, Duration (from MM/YY to MM/YY): _____

Medication/Supplement_____, Duration (from MM/YY to MM/YY):_____

Medication/Supplement_____, Duration (from MM/YY to MM/YY):_____

Medication/Supplement_____, Duration (from MM/YY to MM/YY):_____

Other _____, Duration (from MM/YY to MM/YY):_____

4. If **yes** to number 3, did you lose weight?

□ No

□ Yes. How much weight have you lost? _____Ibs.

How much of this weight, if any did you gain back? _____Ibs.

5. Which meals do you eat regularly, check all that apply:
   - □ Breakfast □
   - □ Lunch □
   - □ Dinner/Supper □
   - □ Snacks (times/day _____)

6. Check all that apply:
   - □ My family eats most meals together
   - □ Family meals are served at regular times on most days
   - □ Another member of my family is on a special diet or is trying to lose weight. Describe. _____

7. Please list any food allergies, sensitivities or intolerances _____

Supplementary file 2: Food Frequency Questionnaire

FOOD AND ACTIVITY QUESTIONNAIRE

RESPONDENT ID #

0 1 2 3 4 5 6 7 8 9

TODAY'S DATE

Month	DAY	YEAR
Jan		
Feb		
Mar	0 0	2014
Apr	1 1	2015
May	2 2	2016
Jun	3 3	2017
Jul	4	2018
Aug	5	2019
Sep	6	2020
Oct	7	2021
Nov	8	2022
Dec	9	2023

ABOUT YOU

SEX
- Male
- Female

If female, are you pregnant or breast feeding?
- No
- Yes
- Not female

AGE **WEIGHT** (pounds) **HEIGHT** (feet inches)

ABOUT THIS SURVEY

Please answer each question as best you can. Estimate if you aren't sure.

- **DETACH THE LAST PAGE OF THIS BOOKLET.** These are your portion pictures.
- **USE ONLY A NUMBER 2 PENCIL.**
- **FILL IN THE CIRCLES COMPLETELY** and erase completely if you make any changes.

INSTRUCTIONS

This form is about the foods you usually eat. Think about your usual intake over the last year. This includes all meals or snacks, at home, in a restaurant, or carry-out.

Please tell us...

1. **HOW OFTEN,** on average, did you eat the food? DO NOT SKIP any foods. Mark "Never" if you didn't eat any of the food.
2. **HOW MUCH** of the food did you usually eat on the days you ate it? Sometimes we ask "how much" as A, B, C or D. **LOOK AT THE PORTION PICTURES.** Pick the picture that looks the most like the serving size you usually eat. (If you don't have pictures: A=1/4 cup, B=1/2 cup, C=1 cup, D=2 cups.)
3. **WHAT TYPE?** For some foods we ask the type (low-fat, low-sugar...) near the end of the survey.

EXAMPLE: This person drank orange juice twice a week, and had one glass each time. Once a week this person ate a "C"-sized serving of cold cereal (about 1 cup).

	HOW OFTEN in the past year?									HOW MUCH on those days? SEE PORTION SIZE PICTURES FOR A-B-C-D				
	NEVER	A FEW TIMES per YEAR	ONCE per MONTH	2-3 TIMES per MONTH	ONCE per WEEK	2 TIMES per WEEK	3-4 TIMES per WEEK	5-6 TIMES per WEEK	EVERY DAY					
Orange juice	○	○	○	○	○	●	○	○	○	How many glasses	● 1	○ 2	○ 3	○ 4
Cold cereal	○	○	○	○	●	○	○	○	○	Which bowl	○ A	○ B	● C	○ D

PLEASE DO NOT WRITE IN THIS AREA

SERIAL #

Block 2014.1 ©www.NutritionQuest.com Phone 510-704-8514

EGGS and DAIRY FOODS	NEVER	A FEW TIMES per YEAR	ONCE per MONTH	2-3 TIMES per MONTH	ONCE per WEEK	2 TIMES per WEEK	3-4 TIMES per WEEK	5-6 TIMES per WEEK	EVERY DAY	HOW MUCH on those days? SEE PORTION SIZE PICTURES FOR A-B-C-D	
Breakfast sandwiches or breakfast burritos with eggs or meat										How many sandwiches in a day	1, 2
Other eggs like scrambled or boiled, or quiche (not egg substitutes)										How many eggs a day	1, 2, 3, 4
Yogurt (not frozen yogurt)										Which bowl	B, C, D
Cottage cheese, ricotta cheese										How much	A, B, C, D
Cream cheese, sour cream, dips										How many tablespoons	1, 2, 3, 4
Cheese, sliced cheese, cheese spread, including in sandwiches and quesadillas										How many slices	1, 2, 3, 4
CEREALS, GRAINS, BREADS											
Cold cereals, ANY KIND, like corn flakes, fiber cereals, sweetened cereals										Which bowl	B, C, D
Oatmeal, or whole grain cereal like Wheatena or Ralston										Which bowl	A, B, C, D
Grits, cream of wheat, cornmeal mush										Which bowl	A, B, C, D
Milk or milk substitutes on cereal											
Brown rice, or dishes made with brown rice										How much in a day	B, C, D
White rice, or dishes made with white rice, like rice and beans										How much in a day	B, C, D
Pancakes, waffles, French toast, crepes										How many	1, 2, 3, 4
Breakfast pastries, like muffins, scones, sweet rolls, Danish, Pop Tarts, pan dulce										How many pieces	1 sm, 1 med, 2, 3
Biscuits, not counting breakfast sandwiches										How many	1 sm, 1 med, 2, 3
Corn bread, corn muffins, hush puppies										How many pieces in a day	1/2, 1, 2, 3
Hamburger buns, hotdog buns, submarine or hoagie buns										How many buns in a day	1/2, 1, 2, 3
Bagels or English muffins, dinner rolls, pita, naan										How many	1/2, 1, 2, 3
Tortillas (not counting in tacos or burritos)										How many in a day	1, 2, 3, 4
Any other bread or toast, including white, dark, whole wheat, and what you have in sandwiches										How many slices in a day	1, 2, 3, 4
VEGETABLES											
Broccoli, Chinese broccoli, or Brussels sprouts										How much	A, B, C, D
Carrots and mixed vegetables containing carrots										How much	A, B, C, D
Corn										How much	A, B, C, D
Green beans, string beans, green peas										How much	A, B, C, D
Cooked greens like spinach, collards, turnip greens, kale, mustard greens										How much	A, B, C, D

	NEVER	A FEW TIMES per YEAR	ONCE per MONTH	2-3 TIMES per MONTH	ONCE per WEEK	2 TIMES per WEEK	3-4 TIMES per WEEK	5-6 TIMES per WEEK	EVERY DAY	HOW MUCH <u>on those days</u>? SEE PORTION SIZE PICTURES FOR A-B-C-D
Cabbage, cole slaw, Chinese cabbage										How much: A B C D
Green salad with lettuce or raw spinach										How much: 1/2 cup, 1 cup, 2 cups, 3+ cups
Raw tomatoes										How much: 1/4, 1/2, 1, 2
Salad dressing										How many tablespoons: 1, 2, 3, 4
Avocado, guacamole										How many tablespoons: 1, 2, 3, 4
Sweet potatoes, yams										How much: A B C D
French fries, home fries, hash browns, tater tots										How much: A B C D
Potatoes <u>not</u> fried, like baked, boiled, mashed, or in stew or potato salad										How much: A B C D
Any other vegetable, like squash, cauliflower, peppers, okra, nopales										How much: A B C D
FRUITS										
How often do you eat the following 2 items, <u>just during the summer months</u> when they are in season?										
Watermelon, cantaloupe, honeydew, other melons, <u>in season</u>										How much: A B C D
Strawberries or other berries, <u>in season</u>										How much: A B C D
How often do you eat the following fruits <u>all year round</u>? <u>Estimate your average for the whole year</u>. Include fresh or frozen fruits. Only include canned or dried fruit when mentioned.										
Bananas										How many in a day: 1/2, 1, 2
Apples or pears										How many in a day: 1/2, 1, 2
Oranges, tangerines, grapefruit										How many: 1/2, 1, 2
Peaches and nectarines										How many: 1/2, 1, 2
Any other fresh fruit, like grapes, plums, mango, fruit salad										How much: A B C D
Raisins, dates, other dried fruit										How much: A B C
<u>Canned</u> fruit, like applesauce, fruit cocktail, canned peaches or pineapple										How much: A B C D
BEANS, TOFU, and MEAT SUBSTITUTES **Include those eaten alone, or in mixed dishes like burritos, chili, stir-fry, salad**										
Refried beans, bean burritos, or hummus										How much: A B C D
Pinto beans, black beans, kidney beans, baked beans, lentils										How much: A B C D
Tofu or tempeh										How much: A B C D
Meat substitutes, like veggie burgers, veggie chicken, vegetarian hot dogs or vegetarian lunch meats										How much: A, B (1 patty or dog), C, D

SOUPS, MIXED DISHES, and NOODLES	NEVER	A FEW TIMES per YEAR	ONCE per MONTH	2-3 TIMES per MONTH	ONCE per WEEK	2 TIMES per WEEK	3-4 TIMES per WEEK	5-6 TIMES per WEEK	EVERY DAY	HOW MUCH on those days? SEE PORTION SIZE PICTURES FOR A-B-C-D
Split pea, bean, or lentil soup										Which bowl: B C D
Vegetable soup, vegetable beef soup, or tomato soup										Which bowl: B C D
Any other soup, including chicken noodle, cream soups, Cup-A-Soup, ramen										Which bowl: B C D
Pizza or pizza pockets										How many slices: 1 2 3 4
Macaroni and cheese										How much: B C D
Spaghetti, lasagna, other pasta with tomato sauce										How much: B C D
Other noodles like plain pasta, pasta salad, sopa seca										How much: B C D
Egg rolls, won tons, samosas, empanadas										How many pieces: 1 2 3 4
MEAT and CHICKEN										
Hamburgers, cheeseburgers, turkey burger, at home or from a restaurant										How many: 1 arc 1 kg 2 3
Hot dogs or dinner sausage like Polish, Italian, chicken apple										How many: 1 2 3 4
Bacon or breakfast sausage										How many pieces: 1 2 3 4
Lunch meats like bologna, sliced ham, sliced turkey, salami										How many slices: 1 2 3 4
Meat loaf, meat balls										How much: B C D
Steak, roast beef, pot roast, including in frozen dinners or sandwiches										How much: A B C D
Tacos, burritos, enchiladas, tamales, tostadas, with meat or chicken										How much: A B C D
Ribs, spareribs										How much: A B C D
Pork chops, pork roast, cooked ham (including for breakfast)										How much: A B C D
Any other beef or pork dish like stew, pot pie, corned beef hash, chili, Hamburger Helper, curry										How much: B C D
Liver, including chicken livers or liverwurst										How much: A B C
Pigs feet, neck bones, oxtails, tongue, chitlins										How much: A B C
Veal, lamb, goat, deer meat, other game										How much: A B C
Fried chicken, including chicken fingers, chicken nuggets, wings, chicken patty										How many median pieces: 1 2 pcs/6 nuggets 3 4
Roasted or broiled chicken or turkey										How much: A B medium piece C D half chicken
Any other chicken or turkey dish, like chicken stew or curry, chicken salad, stir-fry, Chinese chicken dishes										How much: B C D

FISH, SEAFOOD	NEVER	A FEW TIMES per YEAR	ONCE per MONTH	2-3 TIMES per MONTH	ONCE per WEEK	2 TIMES per WEEK	3-4 TIMES per WEEK	5-6 TIMES per WEEK	EVERY DAY	HOW MUCH on those days? SEE PORTION SIZE PICTURES FOR A-B-C-D
Oysters	○	○	○	○	○	○	○	○	○	How much: A ○ B ○ C ○
Shellfish like shrimp, scallops, crab	○	○	○	○	○	○	○	○	○	How much fish: A ○ B ○ C ○ D ○
Tuna, tuna salad, tuna casserole	○	○	○	○	○	○	○	○	○	How much of the tuna: A ○ B ○ C ○
Salmon, mackerel, sea bass, trout, sardines, herring, without breading	○	○	○	○	○	○	○	○	○	How much: A ○ B ○ C ○ D ○
Fried fish, fish sticks, fish sandwich, breaded fillets	○	○	○	○	○	○	○	○	○	How much: A ○ B ○ C ○ D ○
Any other fish	○	○	○	○	○	○	○	○	○	How much fish: A ○ B ○ C ○ D ○
NUTS, SEEDS, SNACKS										
Peanut butter or other nut butters	○	○	○	○	○	○	○	○	○	How many tablespoons: ½ ○ 1 ○ 2 ○ 3 ○
Walnuts or flax seeds (don't count flaxseed oil)	○	○	○	○	○	○	○	○	○	How much: 1 tbsp ○ 2 tbsp ○ ¼ cup ○ ½ cup ○
Peanuts, sunflower seeds, other nuts or seeds	○	○	○	○	○	○	○	○	○	How much: A ○ B ○ C ○ D ○
Energy or protein bars, like Power Bar, Clif, Balance, Luna, South Beach, Atkins	○	○	○	○	○	○	○	○	○	How much: Small ○ Medium ○ Large ○
Breakfast bars, cereal bars, granola bars (not energy or protein bars)	○	○	○	○	○	○	○	○	○	How many: 1 ○ 2 ○ 3 ○
Popcorn	○	○	○	○	○	○	○	○	○	How many cups: 1-2 ○ 3-4 ○ 5-9 ○ 10-12 ○
Whole grain crackers, like Wheat Thins, RyeKrisp, Ryvita, Wasa	○	○	○	○	○	○	○	○	○	How much: A ○ B ○ C ○ D ○
Any other crackers, like saltines, Ritz, Cheez-Its, cheese-filled pretzels	○	○	○	○	○	○	○	○	○	How much: A ○ B ○ C ○ D ○
Tortilla chips or corn chips, like Fritos, Doritos, corn nuts	○	○	○	○	○	○	○	○	○	How much: A ○ B ○ C ○ D ○
Any other snack chips, like potato chips, Cheetos, Chex mix	○	○	○	○	○	○	○	○	○	How much: A ○ B ○ C ○ D ○
SWEETS AND DESSERTS										
Donuts	○	○	○	○	○	○	○	○	○	How many: 1 mini ○ 1 med ○ 2 ○ 3 ○
Cake or snack cakes like cupcakes, Twinkies, pound cake, banana bread	○	○	○	○	○	○	○	○	○	How many pieces: 1 sm ○ 1 med ○ 2 ○ 3 ○
Cookies, brownies	○	○	○	○	○	○	○	○	○	How many: 1-2 ○ 3-4 ○ 5-6 ○ 7+ ○
Pumpkin pie, sweet potato pie	○	○	○	○	○	○	○	○	○	How many pieces: ½ ○ 1 ○ 2 ○ 3 ○
Any other pie or cobbler, including fast food pies, snack pies	○	○	○	○	○	○	○	○	○	How many pieces: ½ ○ 1 ○ 2 ○ 3 ○
Ice cream, ice cream bars, frozen yogurt, fast food milkshakes	○	○	○	○	○	○	○	○	○	How much: B ○ C ○ D ○
Pudding, custard, rice pudding, flan	○	○	○	○	○	○	○	○	○	How much: B ○ C ○ D ○
Chocolate or other flavored sauces or syrup, on ice cream	○	○	○	○	○	○	○	○	○	How much: 1-2 Tbsps ○ 3-4 Tbsps ○ ½ cup ○

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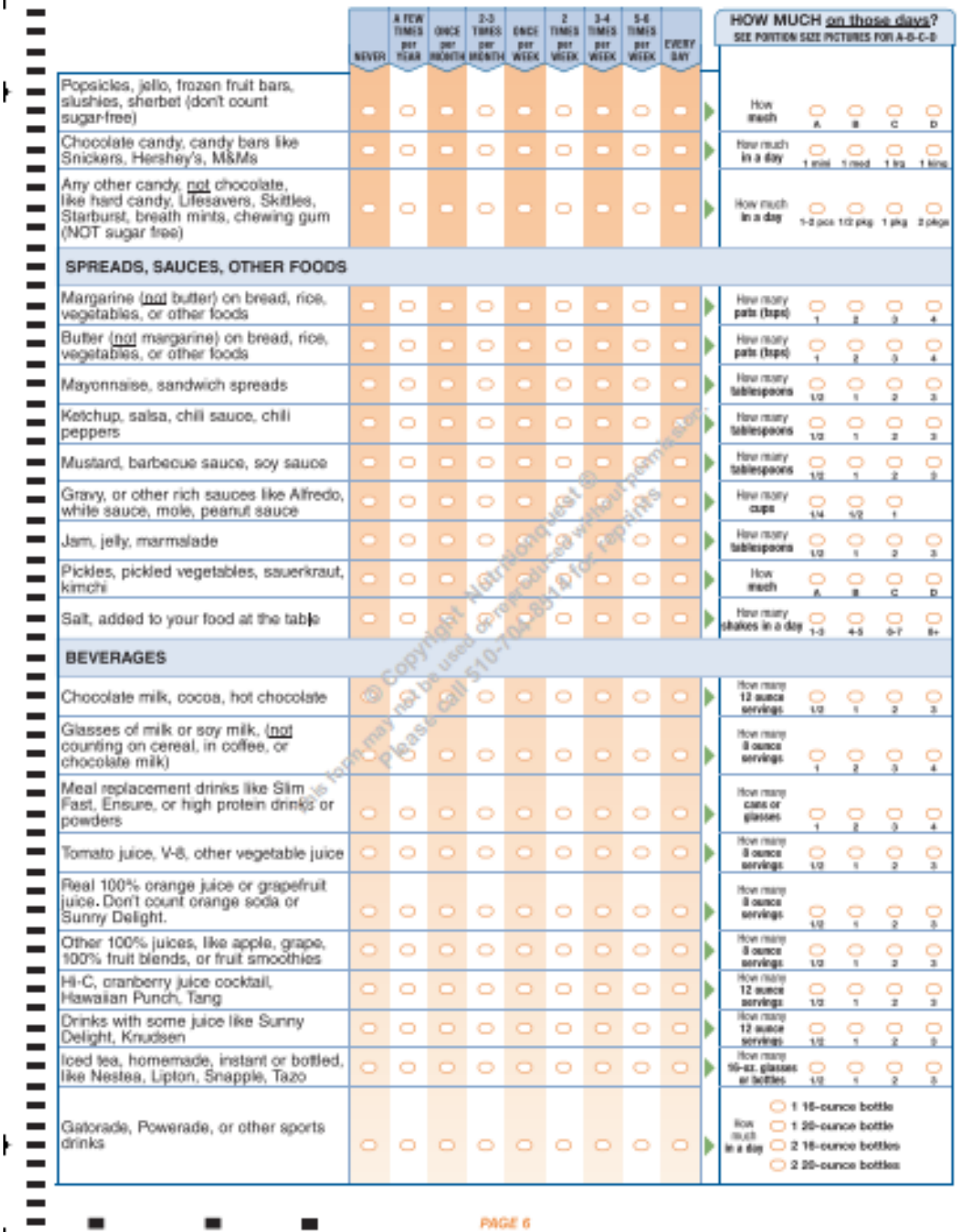

	NEVER	A FEW TIMES per YEAR	ONCE per MONTH	2-3 TIMES per MONTH	ONCE per WEEK	2 TIMES per WEEK	3-4 TIMES per WEEK	5-6 TIMES per WEEK	EVERY DAY	HOW MUCH on those days? SEE PORTION SIZE PICTURES FOR A-B-C-D
Energy drinks like Red Bull, Rockstar, Monster	○	○	○	○	○	○	○	○	○	How much in a day: ○ 1 8-ounce can ○ 1 12-16 ounce can ○ 1 20-ounce can ○ 24 ounces or more
Kool-Aid, lemonade, fruit flavored drinks, like Crystal Light, atole, horchata (not iced tea)	○	○	○	○	○	○	○	○	○	How much in a day: ○ 1 8-ounce glass ○ 1 12-16-ounce glass or bottle ○ 1 20-ounce bottle ○ 30 ounces or more
Soft drinks, soda, pop, like cola, 7-Up, orange soda, regular or diet	○	○	○	○	○	○	○	○	○	How many in a day: ○ 1 can ○ 1 20-ounce bottle ○ 2 cans ○ Big Gulp or 3 cans
Beer or non-alcoholic beer	○	○	○	○	○	○	○	○	○	How much in a day: ○ 1 can ○ 2 cans ○ 3-4 cans or small pitcher ○ 5+ cans or large pitcher
Wine or wine coolers	○	○	○	○	○	○	○	○	○	How many glasses in a day: ○ 1/2 glass ○ 1 glass ○ 2 glasses, 1/2 bottle ○ 4+ glasses
Liquor or mixed drinks, cocktails	○	○	○	○	○	○	○	○	○	How many drinks: ○ 1 ○ 2 ○ 3 ○ 4
Water, bottled or tap	○	○	○	○	○	○	○	○	○	How many glasses: ○ 1 ○ 2 ○ 3-4 ○ 5+
Milky coffee drinks like latte, mocha, cappuccino, Frappuccino	○	○	○	○	○	○	○	○	○	How much in a day: ○ 12 oz ○ 16 oz ○ 20 oz ○ 24+ oz
Coffee (brewed or instant), regular or decaf	○	○	○	○	○	○	○	○	○	How many in a day: ○ 1 ○ 2 ○ 3 ○ 4+
Hot tea (not including herbal tea)	○	○	○	○	○	○	○	○	○	How many cups in a day: ○ 1 ○ 2 ○ 3 ○ 4+

MILKY COFFEE DRINKS: What kind do you usually drink? MARK ONLY ONE
○ Frappuccino ○ Mocha ○ Latte or cappuccino ○ Café con leche ○ Some of each ○ Don't drink them

What are your milky coffee drinks usually made with? **MARK ONLY ONE**
○ Whole milk ○ Skim milk or non-fat ○ Something else
○ 1 or 2% milk (reduced fat) ○ Soy milk ○ Don't drink

COFFEE: Is your coffee usually regular or decaf? ○ Decaf ○ Regular ○ Both kinds ○ Don't drink coffee

What do you usually add to your regular or decaf coffee? **MARK ONLY ONE**
○ Cream or half-n-half ○ Condensed milk ○ None of these
○ CoffeeMate, non-dairy creamer ○ Any other milk

Do you usually add sugar (or honey) to coffee? ○ No ○ Yes IF YES, how many teaspoons each cup? ○ 1 ○ 2 ○ 3 ○ 4

HOT TEA: Is your hot tea usually regular or decaf? ○ Decaf ○ Regular ○ I drink both kinds ○ Don't drink tea

What do you usually add to your hot tea? **MARK ONLY ONE**
○ Cream or half-n-half ○ Condensed milk ○ None of these
○ CoffeeMate, non-dairy creamer ○ Any other milk

Do you usually add sugar (or honey) to hot tea? ○ No ○ Yes IF YES, how many teaspoons each cup? ○ 1 ○ 2 ○ 3 ○ 4

If you eat the following foods, what type do you usually eat? MARK ONLY ONE ANSWER FOR EACH QUESTION

Milk	Whole milk	2% milk	1% milk (low-fat)	Skim milk, non-fat
	Soy milk	Rice milk	Almond milk, other	Don't drink
Slimfast, Ensure, or high protein drinks	Slimfast, Ensure, regular	Slimfast, Ensure, light or low-carb		
	High protein drinks, regular	High protein drinks, light or low-carb	Don't know/Don't drink	
Real 100% orange or grapefruit juice	Calcium-fortified	Not calcium-fortified	Don't know	Don't drink
Iced tea	Home-made, no sugar	Bottled, no-sugar	Don't drink	
	Home-made, with sugar	Bottled, pre-sweetened		
Drinks like Kool-Aid, lemonade, Crystal Light	Low-calorie, sugar-free	Regular		Don't drink
Energy drinks like Red Bull, Monster	Sugar-free	Regular		Don't drink
Soft drinks, soda, pop	Diet, low-calorie	Regular	Don't drink	
Do they usually have caffeine?	Has caffeine	No caffeine	Don't drink	
Beer	Regular	Light	Non-alcoholic	Don't drink
Wine or wine cooler	Red wine	White wine	Both red and white wine	Don't drink
Cheese	Low-fat	Regular-fat	Don't eat	
Yogurt	Plain (no sugar or fruit)	With fruit or other flavors		
Yogurt	Low-fat	Non-fat	Regular (whole milk)	Don't eat
Salad dressing	Low-fat, lite	Fat free	Regular	Oil & vinegar	Don't use
Spaghetti or lasagna	Meatless	With meat sauce or meatballs		Don't eat
Noodles, pasta	Rarely whole grain	Sometimes whole grain	Usually whole grain	Don't know/don't eat
Burgers	Hamburger	Cheeseburger	Turkey burger	Don't eat
Beef or pork	Avoid eating the fat	Sometimes eat the fat	Often eat the fat	Don't eat
Chicken or turkey	Avoid eating the skin	Sometimes eat the skin	Often eat the skin	Don't eat
Hot dogs, dinner sausage	Beef or pork	Chicken or turkey, low-fat		Don't eat
Lunch meats	Beef or pork	Chicken or turkey, low-fat		Don't eat
Cakes, snack cakes, cupcakes	Low-sugar, low-carb	Low-fat	Regular-fat	Don't eat
Cookies, brownies	Low-sugar, low-carb	Low-fat	Regular-fat	Don't eat
Ice cream, frozen yogurt	Low-sugar, low-carb	Low-fat or frozen yogurt	Regular	Don't eat
Energy or protein bars	High energy	High protein	Some of each	Don't know	Don't eat
Bagels, English muffins, rolls	White	Multi-grain	100% whole wheat	Eat all kinds	Don't eat
Burger, hot dog, submarine buns	White	Multi-grain	100% whole wheat	Eat all kinds	Don't eat
Bread	White (not whole grain)	100% whole wheat		Don't eat
	Multi-grain, rye, or other brown bread	Eat some of each		
Tortillas	Corn tortillas	Flour tortillas (wheat)	Eat all kinds or don't know	Don't eat
Popcorn	Air popped, fat-free	Low-fat or light	Regular	Caramel corn	Don't know	Don't eat
Crackers, pretzels	Low-fat, including RyeKrisp, rice cakes, or plain pretzels		Don't know	
	Regular-fat crackers or filled pretzels		Don't eat	
Mayonnaise or sandwich spreads	Low-fat, light	Regular	Don't eat	

If you eat **cold cereals,** what do you usually eat? Choose **ONE or TWO** that you eat most often. If you usually eat just one kind, only choose one.

All-Bran Original	Cinnamon Toast Crunch	Grape Nuts	Special K, plain
All-Bran Complete, Complete	Cocoa Krispies, Pebbles, Puffs	Honey Bunches of Oats	Special K, flavors
Apple Jacks, Cookie Crisp	Corn Flakes, Corn Puffs	Kashi GOLEAN, Heart to Heart	Total
Bran Flakes	Corn Pops	Life	Wheaties
Cap'n Crunch	Fiber-One, Bran Buds	Lucky Charms, Fruity Pebbles	*Other sweet cereal*
Cheerios, plain or Multi-Grain	Froot Loops	Oatmeal Squares, Oat Bran	*Other unsweetened cereal*
Cheerios, Honey Nut, flavors	Frosted Flakes	Raisin Bran	*Other whole grain cereal*
Chex, Wheat	Frosted Mini-Wheats	Rice Krispies, puffed rice	*Other bran or fiber cereal*
Chex, other	Granola	Shredded Wheat	*Don't eat cereal*

Which **fats or oils** are used most often for **cooking or frying** (not baking) in your home? **MARK ONLY ONE OR TWO**

Non-stick spray or none	Soft tub margarine	Corn oil, vegetable oil and blends	Other oil
Butter or ghee	Low-fat margarine	Peanut oil	Don't know
Butter/margarine blend	Olive oil	Lard, fatback, or bacon fat	
Stick margarine	Canola oil, safflower oil	Vegetable shortening, Crisco	

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What vitamin supplements do you take fairly regularly?

Multiple Vitamins. Do you take..	HOW OFTEN: DIDN'T TAKE	A FEW DAYS per MONTH	1 DAY per WEEK	2 DAYS per WEEK	3-4 DAYS per WEEK	5-6 DAYS per WEEK	EVERY DAY	FOR HOW MANY YEARS? LESS THAN 1 YEAR	1-4 YEARS	5-9 YEARS	10+ YEARS
Prenatal vitamins	○	○	○	○	○	○	○	○	○	○	○
Regular One-A-Day, Centrum, "senior" vitamins or house brands of multiple vitamins	○	○	○	○	○	○	○	○	○	○	○
Stress-tabs or B-Complex type	○	○	○	○	○	○	○	○	○	○	○
Antioxidant combination, eye formula	○	○	○	○	○	○	○	○	○	○	○
Single Vitamins or Minerals, taken alone or in combination. Do not count what is in your multiple vitamins above.											
Vitamin A (not beta-carotene)	○	○	○	○	○	○	○	○	○	○	○
Vitamin B-6	○	○	○	○	○	○	○	○	○	○	○
Vitamin B-12	○	○	○	○	○	○	○	○	○	○	○
Vitamin C	○	○	○	○	○	○	○	○	○	○	○
Vitamin D	○	○	○	○	○	○	○	○	○	○	○
Vitamin E	○	○	○	○	○	○	○	○	○	○	○
Folic acid, folate	○	○	○	○	○	○	○	○	○	○	○
Calcium or antacids with calcium, like Tums	○	○	○	○	○	○	○	○	○	○	○
Iron	○	○	○	○	○	○	○	○	○	○	○
Zinc	○	○	○	○	○	○	○	○	○	○	○
Cod liver oil, other fish oils, omega-3, flax seed oil, algae	○	○	○	○	○	○	○	○	○	○	○
Fiber supplements like Benefiber, Metamucil	○	○	○	○	○	○	○	○	○	○	○

If you take One-A-Day, Centrum or other types of multiple vitamins, do you usually take types that
○ Contain minerals, iron, zinc, etc. ○ Do not contain minerals ○ Don't know

If you take vitamin C, how many milligrams of **vitamin C** do you usually take, on the days you take it? (Select the closest amount)
○ 100 ○ 250 ○ 500 ○ 750 ○ 1000 ○ 1500 ○ 2000 ○ 3000+ ○ Don't know

If you take vitamin E, how many IUs of **vitamin E** do you usually take, on the days you take it? (Select the closest amount)
○ 100 ○ 200 ○ 300 ○ 400 ○ 600 ○ 800 ○ 1000 ○ 2000+ ○ Don't know

If you take calcium, how many milligrams of **calcium** do you usually take, on the days you take it? (Select the closest amount)
○ 100 ○ 350 ○ 650 ○ 1250+ ○ Don't know

If you take vitamin D, how many IUs of **vitamin D** do you usually take, on the days you take it? (Select the closest amount)
○ 400 ○ 600 ○ 800 ○ 1000 ○ 2000 ○ 3000 ○ 4000 ○ 5000+ ○ Don't know

If you take omega-3 supplements, what type do you usually take? **MARK ALL THAT APPLY**
○ Fish oil ○ Flax oil, hemp oil, other seed oil ○ Krill oil ○ Algae ○ Don't know

SOME LAST QUESTIONS ABOUT YOU

	RARELY	1-2 per WEEK	3-4 per WEEK	5-6 per WEEK	1 per DAY	1 1/2 per DAY	2 per DAY	3 per DAY	4+ per DAY
About how many servings of vegetables do you eat, not counting salad or potatoes? 1 serving = 1/2 cup.	○	○	○	○	○	○	○	○	○
About how many servings of fruit do you eat, not counting juices? 1 serving = 1/2 cup or 1 medium fruit.	○	○	○	○	○	○	○	○	○
How often do you eat foods prepared at home that are cooked or fried in **fat or oil?**	○	○	○	○	○	○	○	○	○

During a regular day, how many **meals and snacks** do you usually eat?

Meals per day	○ 1	○ 2	○ 3	○ 4	○ 5+
Snacks per day	○ 1	○ 2	○ 3	○ 4	○ 5+

PHYSICAL ACTIVITY SURVEY

Think about the last 12 months. How often did you do the activities listed below?

	HOW OFTEN IN THE PAST YEAR						HOW MUCH TIME ON THOSE DAYS			
	RARELY OR NEVER	A FEW TIMES A MONTH	ONCE OR TWICE A WEEK	3-4 TIMES A WEEK	5-6 TIMES A WEEK	ALMOST EVERY DAY	LESS THAN 30 MINUTES	30-60 MINUTES	1-2 HOURS	2 OR MORE HOURS
Cooking, shopping, light cleaning like doing laundry or dusting, or running errands	○	○	○	○	○	○	○	○	○	○
Slow walking like walking the dog, or light work around the house like watering	○	○	○	○	○	○	○	○	○	○
Work on the job involving standing, like store clerk, or work involving driving (like truck driver)	○	○	○	○	○	○	○	○	○	○
Taking care of children (feeding, dressing), or moderate housework like sweeping, mopping, cleaning the tub	○	○	○	○	○	○	○	○	○	○
Weeding, raking, mowing the lawn, or light house repairs	○	○	○	○	○	○	○	○	○	○
Brisk walking, dancing, hunting or fishing, golf (NOT using a golf cart), or 'friendly' outdoor games like softball	○	○	○	○	○	○	○	○	○	○
Factory work, mechanic, restaurant work, or work involving walking, like mail carrier	○	○	○	○	○	○	○	○	○	○
Construction, painting, feeding livestock, or homecare like caring for an adult family member	○	○	○	○	○	○	○	○	○	○
Heavy work like moving boxes, heavy digging or shoveling snow, farm chores like baling hay, or other HARD labor	○	○	○	○	○	○	○	○	○	○
Exercising at the gym or at home, aerobics, weight training, jogging, or vigorous sports like basketball, soccer, tennis	○	○	○	○	○	○	○	○	○	○
Bicycling or swimming for exercise	○	○	○	○	○	○	○	○	○	○

Are you ○ Hispanic or Latino ○ Not Hispanic or Latino ○ Do not wish to provide this information

What race do you consider yourself to be? **MARK ALL THAT APPLY**

○ White
○ Black or African American
○ Asian
○ American Indian or Alaska Native
○ Native Hawaiian or Other Pacific Islander
○ Do not wish to provide this information

Thank you very much for filling out this questionnaire.
Please take a minute to go back and fill in anything you may have skipped.

PLEASE DO NOT WRITE IN THIS AREA

SERIAL #

Fold along this line, then carefully detach.

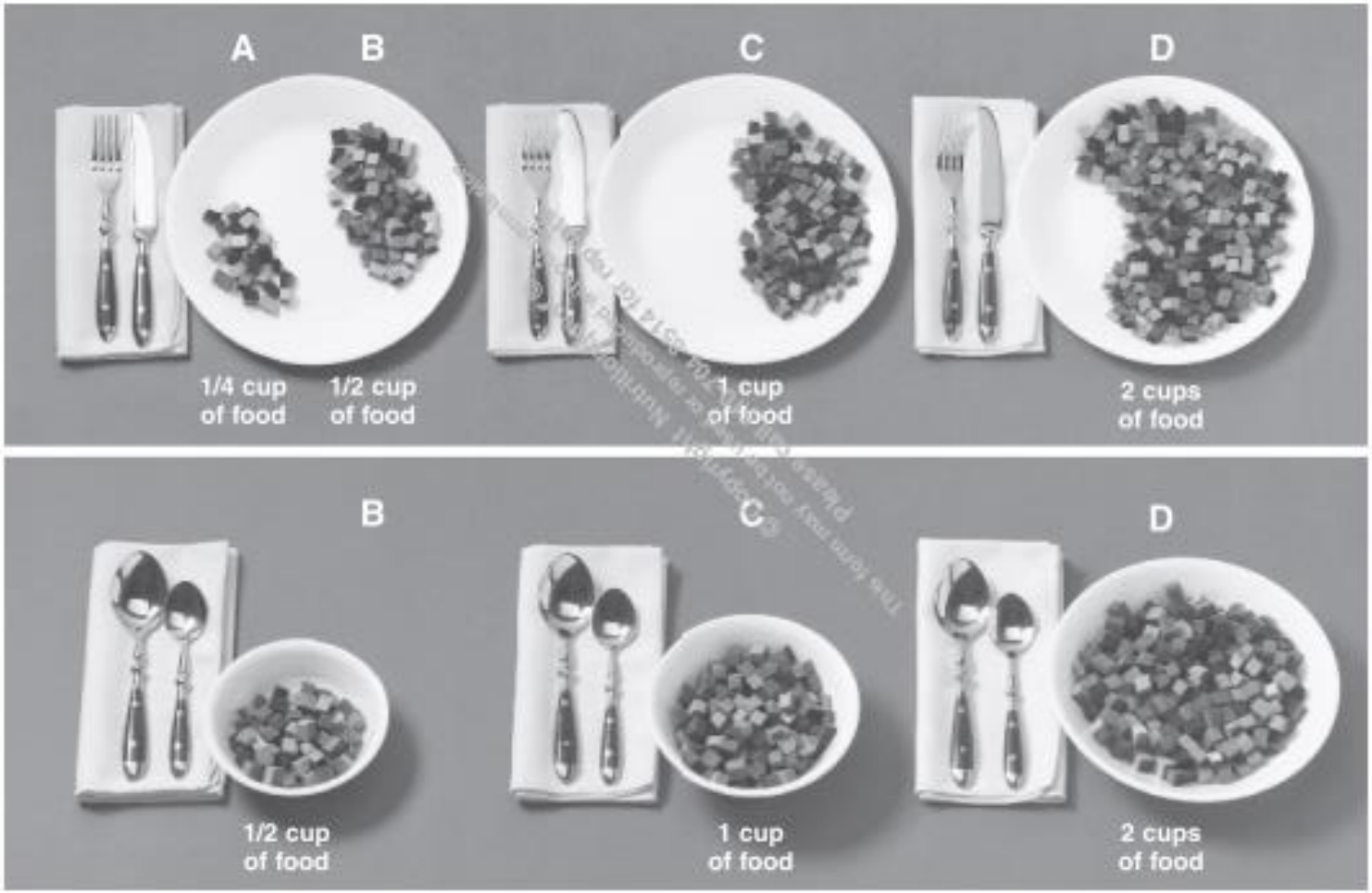
**Fold this page along the dotted line,
then CAREFULLY detach this page.
Your portion pictures are on the back.**

Fold along this line, then carefully detach

Portion Size Choices

Keep this in front of you while you are filling out The Food Questionnaire. You may use either the plates or the bowls to help you choose your usual portion size.

Choose A, B, C or D: **A** = 1/4 Cup of Food **B** = 1/2 Cup of Food **C** = 1 Cup of Food **D** = 2 Cups of Food



Supplementary file 3: Whole Blood DNA Isolation

Protocol manual for handling of human whole blood

A) Clinical Section: (Complete this section within 2 hours)

1. Extract 20 ml human blood samples using 2 of 10 ml EDTA vacutainers (purple top).

Note: *Blood drawn in purple top vacutainer (EDTA as the anticoagulant).*

2. Mix the fresh whole blood by gentle inversion and centrifuge vacutainers for 10 min at 1500g, 4°C, to separate plasma from others.

Note: *1) Make sure tubes are balanced before centrifuging. 2) Make sure tubes are recapped tightly.*

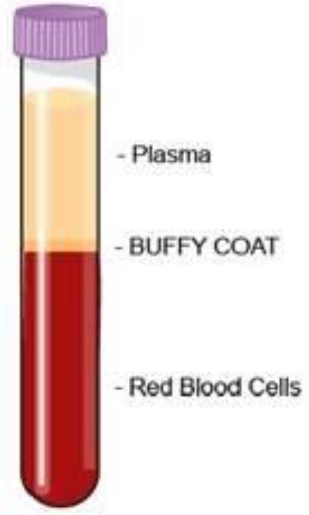
3. Carefully pipette off ~3-4 ml plasma (supernatant) into 9 of 2 ml collection tube (~300-400 ul/each tube) using transfer pipets of 1000ul capacity.

   Note: *The sample should have three distinct layers and* **Don't disturb buffy coat.**

   i, Plasma (yellowish hue)--Top layer.

   ii, Buffy coat (white)-- thin interface.

   iii, Red blood cells---bottom layer.



4. Put the 2 ml collection tubes (plasma) into -20 °C freezer immediately. Put the vacutainers (contains buffy coat and red blood cells) into 4 °C freezer immediately.

B) Lab Section:

Note: Pick up human blood samples every afternoon at 4:30 pm (including plasma collection tubes and vacutainers). Put the 2 ml collection tubes (plasma) into -80 °C freezer immediately. Put the vacutainers (contains buffy coat and red blood cells) into 4 °C freezer immediately.

1. Dispense 30 ml RBC Lysis Solution into a 50 ml centrifuge tube.

2. Add 10 ml whole blood, and mix by inverting 10 times.

3. Incubate 10 min at room temperature (15–25°C). Invert at least 3 times during the incubation to ensure complete red blood cell lysis.

4. Centrifuge for 5 min at 2000 x g to pellet the white blood cells.

5. Carefully discard the supernatant by pipetting or pouring, leaving approximately 200 µl of the residual liquid and the white blood cell pellet.

6. Vortex the tube vigorously to resuspend the pellet in the residual liquid. Vortexing greatly facilitates cell lysis in the next step. The pellet should be completely dispersed after vortexing.

7. Add 10 ml Cell Lysis Solution, and pipet up and down to lyse the cells or vortex vigorously for 10 s. Usually no incubation is required; however, if cell clumps are visible, incubate at 37°C until the solution is homogeneous. Samples are stable in Cell Lysis Solution for at least 2 years at room temperature.

8. Optional: If RNA-free DNA is required, add 50 µl RNase A Solution, and mix by inverting 25 times. Incubate for 15 min at 37°C. Then incubate for 3 min on ice to quickly cool the sample.

9. Add 3.33 ml Protein Precipitation Solution, and vortex vigorously for 20 s at high speed.

10. Centrifuge for 5 min at 2000 x g. The precipitated proteins should form a tight, dark brown pellet. If the protein pellet is not tight, incubate on ice for 5 min and repeat the centrifugation.

11. Pipet 10 ml isopropanol into a clean 50 ml tube and add the supernatant from the previous step by pouring carefully. Be sure the protein pellet is not dislodged during pouring.

12. Mix by inverting gently 50 times until the DNA is visible as threads or a clump.

13. Centrifuge for 3 min at 2000 x g. The DNA may be visible as a small white pellet.

14. Carefully discard the supernatant, and drain the tube by inverting on a clean piece of absorbent paper, taking care that the pellet remains in the tube.

15. Add 10 ml of 70% ethanol and invert several times to wash the DNA pellet.

16. Centrifuge for 1 min at 2000 x g.

17. Carefully discard the supernatant. Drain the tube on a clean piece of absorbent paper, taking care that the pellet remains in the tube. Air dry the pellet for 5 min. The pellet might be loose and easily dislodged. Avoid over-drying the DNA pellet, as the DNA will be difficult to dissolve.

18. Add 1 ml DNA Hydration Solution and vortex for 5 s at medium speed to mix.

19. Incubate at 65°C for 1 h to dissolve the DNA.

20. Incubate at room temperature overnight with gentle shaking. Ensure tube cap is tightly closed to avoid leakage. Samples can then be centrifuged briefly and transferred to a storage tube.

Supplementary file 4: Peripheral Blood Monocyte Isolation

Protocol for Peripheral Blood Monocyte Isolation (~3 – 4 hours)

A. Reagents Required

1. MACS Buffer (autoMACS Rinsing solution)
2. MACS BSA Stock solution
3. Monocyte Isolation Kit II
4. 10 X Dulbecco's PBS

B. Additional Materials Required

1. Refrigerate Centrifuge with 50ml and 15ml rotors
2. High-speed refrigerate microcentrifuge with 1.5 ml rotor
3. Pipette and Tips: 5mL, 1.0mL, 100 ul, 20ul, 10ul
4. Tubes:
    1) 50 mL Uni-Sep Lymphprep tube
    2) 50 ml centrifuge tube
    3) 1.5 ml conical tube
    4) 200 ul conical tube
    5) 2.0 ml conical tube
5. Ice Box

C. Reagent Preparation Before Experiment

1. Prepare the MACS (add 1 bottle of BSA solution to 1 bottle of MACS buffer) and store at 4 °C
2. Prepare 1 X PBS solution using the 10 X Dulbecco's PBS, store at 4 °C

D. Others Preparation Before Experiment

1. Clean your workbench with bleach before you start as well as before you leave for the day.
2. Please check the samples before use and always label new tubes before use
3. Always make sure the centrifuge is balanced or else it will cause damage to the instrument as well as create a hazardous atmosphere if the tubes leak or break.

Step 1: Procedures to isolate and wash mononuclear cells

1. Centrifuge blood samples (in Vacutainer) at 1000G for 15 min at 4^{o}C **(A-9; D3. Deceleration at a setting of 3 is Critical.)**

2. While blood is centrifuging-

   a) Label and prepare ten 2 ml tubes

   b) Prepare the barrier 50 ml tube (use two barrier 50 ml tubes per subject) – add 20 ml of Histopaque into each tube and centrifuge at 1000G for 1 min. Pour extra Histopaque back into the original bottle.

3. Carefully remove the Vacutainer tubes from centrifuge. Collect 20 ml Plasma and dispense into ten 2ml tubes (previously labeled). Make sure that the white Buffy Coat Layer is not disturbed. It is advisable to leave behind some plasma and try NOT to aspirate all of it.

4. Add 10 ml cold PBS to the required number of 50 ml Barrier tubes. Label all tubes with the subject number.

5. Carefully aspirate the Buffy Coat Layer (with a little overlying plasma and some underlying blood). Transfer to the barrier tubes containing PBS. Once the buffy coat is collected from all the Vacutainer tubes into the barrier tubes tighten the cap and mix by gently tilting the tubes 4-5 times.

6. Centrifuge at 1000 g 15 min at 4^{o}C **(A-9; D3. Deceleration at a setting of 3 is Critical.)**

7. CAREFULLY take the median white buffy layer into two new labeled 50 ml tubes. Typically we can recover ~10ml per barrier tube. Pour off the remaining buffer and PBS into a beaker. You may carefully wash the cells sticking to the wall to increase yield.

8. Add cold PBS to 45 ml mark. Re-suspend the cells well by tilting the tubes few times.

9. Centrifuge at 500g 10 min at 4^{o}C.

10. CAREFULLY and COMPLETELY remove the supernatant (platelets);

11. Repeat step 8, 9, 10 once more.

12. Suspend the cell pellet in 10 ml MACS buffer and combine the two cell pellets into one 50 ml tube.
13. Centrifuge at 300g 5 min at 4ºC.
14. COMPLETELY remove supernatant.

Step 2: Procedures of monocyte labeling

1. Re-suspend cell pellet in 1800µl MACS buffer. Make sure that the cell pellet is completely re-suspended or else the binding will be uneven.
2. Add 600µl FcR blocking reagent. Mix by pipetting in and out gently 2-3 times.
3. Add 600µl antibody cocktail. Mix by pipetting in and out gently 2-3 times.
4. Incubate at 4 °C for 15 min on a rotary shaker.
5. Add 1800µl MACS buffer. Mix by pipetting in and out gently 2-3 times.
6. Add 1200µl microbead. Mix by pipetting in and out gently 2-3 times.
7. Incubate at 4°C for 15 min on a rotary shaker.
8. Wash cells by adding MACS buffer, so that the total volume reaches to 30 ml.
9. Centrifuge at 500g at 4ºC for 5 min.
10. Remove supernatant completely.
11. Add 15 ml MACS buffer and re-suspend cells gently. Make sure there are no cell clumps.

Step 3: Procedures for magnetic separation of monocyte:

1. For LS column (10ml), use 3 columns per sample. Equilibrate the LS column with 10 ml MACS buffer.

   For XS columns (50ml), rinse it with 50 ml MASC buffer.

2. Place column into the magnetic holder on the MACS stand.

   - For LS column, apply the cell suspension onto the column (5-7 ml per

column); rinse each column with 5 ml MACS buffer.

- For XS column, apply all the cell suspension onto the column; rinse the column with 15 ml MACS buffer.

3. Collect the effluent into a single fresh 50 ml tube.
4. Make up the total volume to 30 ml with MACS buffer.
5. Centrifuge at 400g for 10 min, remove supernatant.
6. Add 30 ml PBS, re-suspend cells, centrifuge at 400g for 10 min, remove supernatant.
7. Re-Suspend the cell pellet with 1 ml PBS then add PBS to 10 ml, pipette 5ul cell sample to each of two 200ul tubes respectively, use one tube for cell counting, and immediately store the other tube at -80^{o}C (for cell purity testing).
8. Perform Cell Counting.
9. For the rest of the PBM samples,
    a. For DNA methylation and Multi-omics study, temporarily store PBMs in PBS at 4 ^{o}C for the following DNA/RNA extraction or PBM fixation.
    b. For MaleP study, centrifuge PBMs at 600g for 5 min, remove supernatant, and immediately store the PBM pellets at -80^{o}C.

Cell counting: using *Cellometer Mini*, dilute by adding 45ul of PBS to 5ul of cell sample.

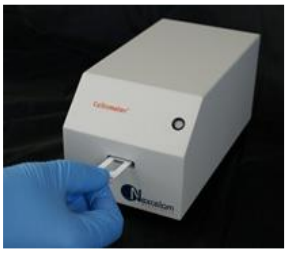
1) Disposable counting chamber accommodates 2 individual samples and can be loaded through either port. **Load 20µl of cell sample into one of the ports.**

2) Insert the loaded chamber into the Cellometer Mini sample slot and gently push the slide to the stop.

3) Select an Assay

4) Input Sample ID and Dilution Factor (DF=10.0)

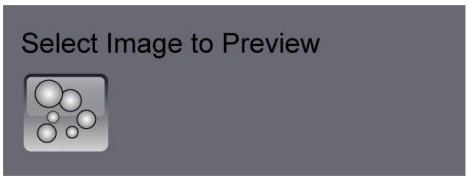
5) Prepare Sample, load disposable counting chamber and insert into instrument



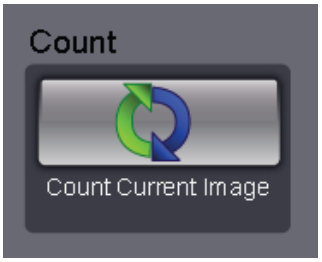
6) Click “Preview”

7) Adjust Focus

8) Click “Select image to preview” and adjust exposure if needed

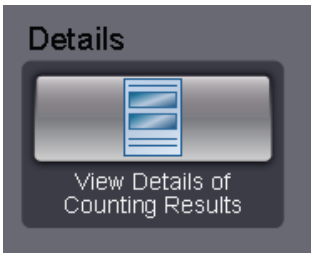


9) Click “Count” to begin counting process



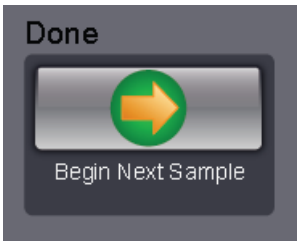
10) Review Counting Results

11) Click "Details" to review cell images and counted cell images.



12) Click "**Save Copy of Data"** to save the result. Record the cell count on the notebook.

13) Select Next Sample or Assay and Settings when done



Supplementary file 5: RNA/DNA Extraction

RNA/DNA Extraction (~1.5 – 2 hours)

- **Check for precipitates in all the reagents prior to use.**
- **If there are precipitates vortex lightly and thaw to room temperature (Except reagents that are to be kept on ice or @ -20 degrees)**

Prepare reagents and buffers prior to use

1. **Buffer RLT** – add 10µl β-mercaptoethanol (β-ME) per 1 ml Buffer RLT Plus. Mix well and tick the check box on the bottle label and write the date and initial. Mix the bottle by shaking before starting procedure.
2. **Buffer FRN** – add 42 ml Isopropanol to the bottle. Mix well and tick the check box on the bottle label and write the date and initial. Mix the bottle by shaking before starting procedure.
3. **Buffer RPE** – add 44 ml of Ethanol to the bottle. Mix well and tick the check box on the bottle label and write the date and initial. Mix the bottle by shaking before starting procedure.
4. **Buffer AW1** – add 25 ml ethanol to the bottle. Mix well and tick the check box on the bottle label and write the date and initial. Mix the bottle by shaking before starting procedure.
5. **Buffer AW2** – add 30 ml of Ethanol to the bottle. Mix well and tick the check box on the bottle label and write the date and initial. Mix the bottle by shaking before starting procedure.
6. **RNA-free DNAse I** – Inject (very important) 550 µl of RNAse free water into the vial. Mix by gently inverting the vial. DO NOT VORTEX and always keep on ice while working.

- **Do not overload the cartridges (Column)**
- **Do not exceed the required centrifuge speeds and time.**
- **Equilibrate all buffers to room temperature (15–25°C). Mix reconstituted Buffer RLT, FRN, Buffer RPE, Buffer AW1, and Buffer AW2 by shaking.**

Equipment and reagents needed

1. Ethanol
2. Isopropanol
3. 14.3M beta Mercaptoethanol
4. 1x PBS, sterile, Calcium and Magnesium free
5. 20G needle & syringe
6. 2 ml tubes, 1.5 ml tubes

Caution! **<u>For RNA/DNA/Small RNA extraction, all tubes to be kept on ice till Buffer RLT is added</u>**

1. Centrifuge to collect the pellet.
2. **Completely** loosen the cell pellet thoroughly by flicking the tube.
3. Add the appropriate volume of Buffer RLT Plus. (see Table)

Number of pelleted cells	Volume of Buffer RLT Plus
$<5 \times 10^6$	350 µl
$5 \times 10^6 - 1 \times 10^7$	600 µl

4. Vortex or pipet to mix.
5. Immediately disrupt and homogenize the tissue using a 20G needle and syringe until it uniformly homogeneous (usually 15 times). Work with one sample at a time. Combine all the lysates together before proceeding to next step.
6. Briefly centrifuge the tube to reduce foam.
7. Transfer the homogenized lysate to *an AllPrep DNA Mini spin column* placed in a 2 ml collection tube (supplied). Close the lid gently and centrifuge for 30 s at full speed (max 20,000 x *g*).
8. Place the *AllPrep DNA Mini spin column* in a new 2 ml collection tube (supplied) and store at 4°C for DNA purification later in steps. Transfer the flow-through to a new 2 ml micro-centrifuge tube (not supplied) for RNA purification (steps 9–25).

Total RNA (including miRNA) purification

9. Add 80 µl Proteinase K to the flow-through from step 5, and mix by pipetting.
10. Add 350 µl of 96–100% ethanol and mix well. Do not centrifuge.
11. Incubate for 10 min at room temperature.
12. Add 750 µl of 96–100% ethanol and mix well. Do not centrifuge.
13. Transfer up to 650 µl of sample, including any precipitate that may have formed, to an RNeasy Mini spin column placed in a 2 ml collection tube (supplied). Close the lid gently and centrifuge for 15s at full speed (max 20,000 x *g*). Discard the flow-through.
14. Reuse the collection tube in next step.
15. **Repeat until the entire sample has passed** through the RNeasy Mini spin column. **Do not add more than 650 µl to the tube at one time**
16. Reuse the collection tube in next step.
17. Add 500 µl Buffer RPE to the RNeasy Mini spin column. Close the lid gently and centrifuge for 15s at full speed (maximum speed of 20,000 x *g*). Discard the flow-through.
18. **Prepare fresh each time**. Add 10 µl DNase I stock solution to 70 µl Buffer RDD. Mix by gently inverting the tube and centrifuge briefly to collect residual liquid from the sides of the tube. **If you are doing more than one sample then prepare a master-mix**.
19. Add the DNase I incubation mix (80 µl) directly onto the RNeasy Mini-spin column membrane and incubate on the benchtop (20–30°C) for 15 min.
20. Add 500 µl Buffer FRN to the RNeasy Mini spin column. Close the lid gently and centrifuge for 15s at full speed (maximum speed of 20,000 x *g*). Save the flow-through for use in Next step.
21. **IMPORTANT: Do not discard the flow-through, as it contains small RNAs.**
22. Place the RNeasy Mini spin column in a new 2 ml collection tube (supplied). Apply the flow-through from step 17 to the spin column. Close the lid gently and centrifuge for

15s at full speed (maximum speed of 20,000 x *g*). Discard the flow-through.

23. Reuse the collection tube in step 24.

24. Add 500µl Buffer RPE to the RNeasy Mini spin column. Close the lid gently and centrifuge for 15s at full speed (maximum speed of 20,000 x *g*). Discard the flow-through.

25. Reuse the collection tube in step 26.

26. Add 500µl of 96–100% ethanol to the RNeasy Mini spin column. Close the lid gently and centrifuge for 2 min at full speed (maximum speed of 20000 x *g*) to wash the spin column membrane.

27. Place the RNeasy Mini spin column in a new 2 ml collection tube (supplied) and discard the old collection tube with the flow-through. Centrifuge at full speed for 2 min.

28. Place the RNeasy Mini spin column in a new 1.5 ml collection tube (supplied). Add 50µl RNase-free water directly to the spin column membrane. Close the lid gently, incubate for 1 min and centrifuge for 1 min at ≥8000 x *g* (≥10,000 rpm) to elute the RNA.

    Note: If RNA concentration < 100ng/ul, pipette eluted RNA solution to the spin column membrane which used in step 28, incubate for another 1 min and centrifuge for 1 min at ≥8000 x *g* (≥10,000 rpm) to elute the RNA.

29. Quantitate the RNA using the Nano Drop 2000, record the data on the notebook and save the results on the computer.

30. Pipette 3ul RNA sample to a 200ul tube for the RNA integrity analyses.

31. Immediately store the archive and integrity-analysis RNA samples at -80°C in the labeled boxes.

Genomic DNA purification

1. Add 350 µl Buffer AW1 to the AllPrep DNA Mini spin column from step 8. Close the lid gently and centrifuge for 15 s at full speed (maximum speed of 20,000 x *g*) to wash the spin column membrane. Discard the flow-through.

2. Reuse the collection tube in step 3.

3. Add 20 µl Proteinase K to 60 µl Buffer AW1, mix gently, and apply the mixture to the AllPrep DNA Mini spin column membrane. **If doing more than one sample prepare a master-mix.** (*Be sure to add the Proteinase K incubation mix directly to the membrane. Digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the spin column.*)

4. Incubate for 5 min at room temperature

5. Add 350 µl Buffer AW1 to the AllPrep DNA Mini spin column. Close the lid gently and centrifuge for 15 s at full speed (maximum speed of 20,000 x *g*) to wash the spin column membrane. Discard the flow through.

6. Reuse the collection tube in step 7.

7. Add 500 µl Buffer AW2 to the AllPrep DNA Mini spin column. Close the lid gently and centrifuge for 2 min at full speed to wash the spin column membrane

8. Place the AllPrep DNA Mini spin column in a new 1.5 ml collection tube (supplied). Add 100 µl Buffer EB directly to the spin column membrane and close the lid. Incubate at room temperature (15–25°C) for 1 min then centrifuge for 1 min at ≥8000 x g (10,000 rpm) to elute the DNA.

   Note: If DNA concentration < 100ng/ul, pipette eluted DNA solution to the spin column membrane which used in step 8, incubate for another 1 min and centrifuge for 1 min at ≥8000 x *g* (≥10,000 rpm) to elute the DNA.

9. Quantitate the DNA using the Nano Drop 2000. Record the data on the notebook and save the results on the computer.

10. Store the DNA samples at -80°C immediately in the labeled boxes.

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