Systems analyses of the immune response to the seasonal influenza vaccine

NIH Protocol Number: 19-I-0126

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List of Abbreviations

AA	African American
AE	Adverse event
ATAC-seq	Assay for transposase-accessible chromatin using sequencing
BMI	Body mass index
BTRIS	Biomedical Translational Research Information System
CBC	Complete blood count
CC	Clinical Center
CD	Cluster of differentiation
CHI	Center for Human Immunology
CITE-seq	Cellular indexing of transcriptomes and epitopes by sequencing
CLIA	Clinical Laboratory Improvement Amendments
CMV	Cytomegalovirus
COVID-19	Coronavirus disease 2019
CRIMSON	Clinical Research Information Management System of the NIAID
CyTOF	Cytometry by time of flight (mass cytometry)
dbGaP	Database of Genotypes and Phenotypes
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
FDA	Food and Drug Administration
GBS	Guillain-Barre syndrome
GCP	Good Clinical Practice
GEE	Generalized estimating equations
GEO	Gene Expression Omnibus
HA	Hemagglutinin
HIV	Human immunodeficiency virus
HRPP	Human Research Protections Program
Ig	Immunoglobulin
IRB	Institutional review board
LISB	Laboratory of Immune System Biology
MDCK	Madin Darby Canine Kidney
NCBI	National Center for Biotechnology Information
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institutes of Health
PBMC	Peripheral blood mononuclear cell
PI	Principal investigator
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
SAE	Serious adverse event
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SRA	Sequence Read Archive
TBNK	T, B, and natural killer (cells)
TDaP	Tetanus, diphtheria, and pertussis (vaccine)
UP	Unanticipated problem
USPHS	United States Public Health Service

Protocol Summary

Short Title:	Immune response to flu vaccine
Sample Size:	N=170 (n=24 subjects enrolled in year 1; targeting up to n=120 new subjects in year 2 and n=7 new subjects per year for years 3-5)
Accrual Ceiling:	N=300
Study Population:	Healthy volunteers aged 18 years and older (no upper age limit)
Accrual Period:	5 years (enrollment open August through February each year)
Study Design:	This is an open-label, prospective, exploratory study to assess the baseline and post-vaccination immune responses of healthy volunteers to an approved seasonal influenza vaccine. Subjects will undergo baseline blood collections on day -7 and on day 0 before receiving the study vaccine. After vaccination, blood will be collected on days 1, 7, 14, 28, 70, and 100. Optionally, subjects may also give blood once a month, as requested, up until 1 year after vaccination. Research evaluations will include vaccine antibody titers. Additional evaluations may include peripheral immune cell phenotyping, RNA sequencing (RNA-seq) of whole blood and defined peripheral blood cell subsets, and measurement of serum proteins and antibodies. Subjects may optionally provide stool samples at some visits for exploratory microbiome assessments. Additionally, subjects may optionally continue study participation annually through the 2023-24 influenza season.
Study Agent Description:	An approved inactivated seasonal influenza vaccine administered once by intramuscular injection. The study vaccine will be the vaccine stocked by the NIH pharmacy, either Flucelvax Quadrivalent or Fluvirin (Seqirus Inc.) for subjects under 65 years, or Fluzone High-Dose (Sanofi Pasteur) for subjects 65 years and older.
Primary Objective:	Identify baseline correlates of immune response to the seasonal influenza vaccine in healthy individuals
Secondary Objectives:	Validate the findings of prior study 09-H-0239 (“Cellular and Molecular Characterization of the Immune Response in Healthy

	NIH Employees at Baseline, and After Immunization with the H1N1 or Seasonal Influenza Vaccines"), specifically the relationship between cell populations and/or gene transcripts at baseline and after vaccination that correlate with vaccine immune response
	Evaluate the effect of prior severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection on the antibody response to the seasonal influenza vaccine in healthy volunteers
Primary Endpoint:	Change in antibody titer response to vaccination, as measured by microneutralization titers at day 0 and day 70 and its relationship with novel baseline biomarkers
Secondary Endpoint:	Change in antibody titer response to vaccination, as measured by microneutralization titers at day 0 and day 70 and its relationship with established baseline biomarkers ($CD38^+$ $CD20^+$ B cell frequencies and/or transcriptomic signatures) and post-vaccination biomarkers (plasmablast cell frequencies and/or transcriptomic signatures)
	Difference in antibody titer response to vaccination between subjects with a history of symptomatic or asymptomatic SARS-CoV-2 infection and those with no evidence of SARS-CoV-2 infection

Précis

Certain functions of the immune system are revealed only when the immune system is challenged. When a person is vaccinated, a coordinated response results: activation and interaction of distinct innate and adaptive immune cell populations and pathways, culminating in the formation of germinal centers from which antibody-producing plasma cells and memory B cells derive. By taking measurements at various time points before and after vaccination, we can build a comprehensive picture of how the immune system responds to a vaccine challenge. The seasonal influenza vaccination provides an excellent model of coordinated immune activity involving innate and adaptive responses, as demonstrated in a past NIH study in 2009-2011; however, scientific advances and the possibility of multi-season responses in individuals warrant a new follow-up study with more comprehensive sampling.

This is an open-label, prospective, exploratory study to assess the baseline and post-vaccination immune responses of healthy volunteers to an approved seasonal influenza vaccine. Subjects will undergo baseline blood collections on day -7 and on day 0 before receiving the study vaccine. After vaccination, blood will be collected on days 1, 7, 14, 28, 70, and 100. Optionally, subjects may also give blood once a month, as requested, up until 1 year after vaccination. Blood samples will be used to assess short- and long-term immunological effects of immunization. Evaluations will include vaccine antibody titers. Additional evaluations may include peripheral immune cell phenotyping, RNA sequencing (RNA-seq) of whole blood and defined peripheral blood cell subsets, and measurement of serum proteins and antibodies. Subjects may optionally provide stool samples at some visits for exploratory microbiome assessment. Additionally, subjects may optionally continue study participation annually through the 2023-24 influenza season.

The goal of this protocol is to use the collective information gathered across all healthy volunteers to understand how the immune system works as a whole.

1 Background Information and Scientific Rationale

1.1 Background Information

1.1.1 What is systems immunology?

Systems immunology is a field of research aimed to identify and understand how the different components of the immune system work together in a coordinated manner to achieve its functions, such as protecting against pathogens and mounting effective responses after vaccination. Although we have substantial information at the level of individual molecules and processes of the immune system that operate in isolation or as a part of smaller functional units, a significant gap in knowledge exists regarding how the different parts of the immune system work together as a whole.

Efforts to fully characterize the immune system have been limited due to 1) challenges in generating sufficient data to measure the diverse functions of the immune system, and 2) a lack of computational tools that combine all that data to create models of how the immune system works. Over the past decade, significant advances have been made in the volume and type of scientific data that can now be generated in both cell lines and living organisms, as well as advances in computational modeling techniques. By using these new modeling techniques to combine multiple types of data, we can build a more complete picture of the immune system than was previously possible.

1.1.2 Systems immunology applied to vaccines

Certain functions of the immune system are revealed only when the immune system is challenged. For example, in response to infection by Gram-negative bacteria, the Toll-like receptor 4 pathway is activated by bacterial lipopolysaccharide, a structural component of the Gram-negative bacterium outer wall, to produce inflammatory cytokines. A systems immunology approach has previously revealed important biological insights on the challenged immune system, such as the observation that chronic cytomegalovirus (CMV) infection is associated with both broad changes in numerous immune cell parameters in healthy individuals at baseline, as well as more robust flu vaccine responses in healthy young individuals.[1,2]

Similarly, specific immune system pathways are activated following vaccination. When a person is vaccinated, a coordinated response results: activation and interaction of distinct innate and adaptive immune cell populations and pathways, culminating in the formation of germinal centers from which antibody-producing plasma cells and memory B cells derive. By taking measurements at various time points before and after vaccination, we can build a comprehensive picture of how the immune system responds to a vaccine challenge.

The seasonal influenza vaccination provides an excellent model of coordinated immune activity involving innate and adaptive responses, as demonstrated previously in a study conducted at the Center for Human Immunology (CHI) at the NIH (protocol 09-H-0239). This study employed the sampling strategy described above to capture the immune response to seasonal influenza vaccine in 63 healthy NIH employees. Multiple types of data from the blood were collected over numerous time points, and these data were combined using computational modeling to create a detailed picture of how the immune system responds to influenza vaccine. Among the key findings in this study were: 1) baseline immune status and early responses (days 1 and 7 post-vaccination) were highly variable between healthy subjects, and 2) several parameters (such as certain B-cell subsets) were both stable over time and predictive of the magnitude of antibody response to the vaccine (Figure 1).[3]

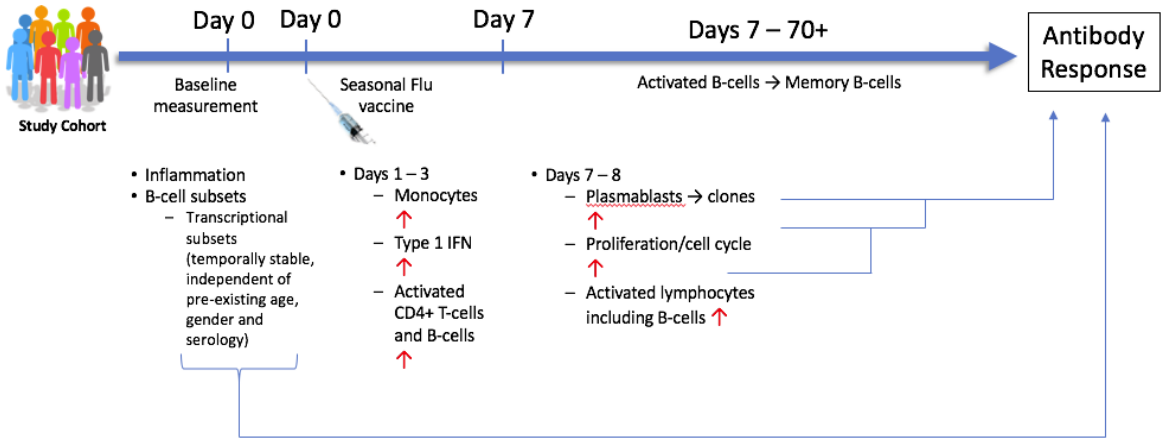

Figure 1. CHI study of influenza vaccination. Sixty-three healthy individuals had various blood measurements before and after vaccination with the seasonal influenza vaccine. Notable findings include: 1) an increase in innate immune cells (such as monocytes and activated $CD4^+$ T-cells) in the days immediately after vaccination, 2) an increase in antibody-producing B cells (plasmablasts) 7 days after vaccination, and 3) several cell subsets (such as certain B-cell subsets) that are both stable in the time period before and after vaccination and predictive of the magnitude of antibody response to the vaccine.

This systems immunology approach has revealed important biological insights about the response to influenza vaccination.[2,3] Additionally, this approach can illuminate interactions between acute infections and the acquired immunity that vaccines provide. Recently, it has been recognized that acute infections can have profound and persistent effects on the immune system. For example, measles infection frequently results in prolonged depletion of memory B cells and reduction in protective antibodies to previous infections and vaccinations, thus rendering patients at prolonged risk of infection from other organisms after measles recovery. [4,5] It is possible that other infections, including infection with SARS-CoV-2, may also affect immunological memory, response to past and future vaccinations, and other long-term immunological phenomena. In light of the recent SARS-CoV-2 pandemic, the long-term immunological effects of SARS-CoV-2 infection have become a question of public health importance.

1.2 Scientific Rationale

The previous CHI study was conducted between 2009-2011. Since that time, there have been numerous technological and scientific advances that merit a follow-up study with more comprehensive sampling. The specific motivations are the following:

1. The previous CHI study included a day 70 time point as the most distal from the time of vaccination. We will add additional time points to examine the resolution of immune response following vaccination and antibody durability (beyond day 70).
2. New technologies such as mass cytometry (cytometry by time of flight, CyTOF) and cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) will permit us to interrogate cellular responses in detail. For example, CyTOF can simultaneously characterize more than 40 intracellular and extracellular proteins.[6] CITE-seq is a technology that combines the power of extracellular marker detection with single-cell sequencing, allowing for single-cell gene expression to be linked to the specific cell (as defined by extracellular protein markers) from which the sequence originated.[7]
3. By allowing for sampling across several influenza seasons, we will be able to monitor for multi-season responses, ideally in the same individuals. Specific influenza vaccine strains are often repeated over sequential vaccine seasons; how this affects the immune response to subsequent vaccinations with the same influenza vaccine strain is not well understood.
4. Future studies of other vaccines (e.g., human papillomavirus vaccine) using the same systems immunology approaches would be able to use the data from this influenza vaccine study as a comparison.
5. This study is a unique opportunity to evaluate the potential effects of prior SARS-CoV-2 infection on the immune response to the seasonal influenza vaccine in healthy individuals.

These new goals are particularly important in light of the incompletely understood response to seasonal influenza vaccine and the importance of efforts aimed toward a universal influenza vaccine. The goal of this protocol is not to compare the immune response to the influenza vaccine between groups of healthy volunteers, but rather to measure and collectively evaluate numerous biological parameters (to include immune cell counts and whole blood gene expression) across all healthy volunteers in our study to understand how the immune system works as a whole. This systems immunology approach has previously revealed important biological insights, such as the previously mentioned observation that chronic CMV infection is associated with both broad immunological changes and response to the seasonal flu vaccine.[1] It has also helped shed light on the molecular drivers of immune aging,[8,9] and the immunological responses to infectious diseases such as malaria.[8] We aim to employ new technologies to study the immune response to the seasonal influenza vaccine over time (and seasons) in healthy volunteers. We hypothesize that these technologies, combined with a longer follow-up period

than previously employed, will allow us to better understand the molecular and cellular components of the immune system that together orchestrate protective vaccine responses.

2 Study Objectives

2.1 Primary Objective

Identify baseline correlates of immune response to the seasonal influenza vaccine in healthy individuals

2.2 Secondary Objectives

- Validate the findings of prior study 09-H-0239, specifically the relationship between cell populations and/or gene transcripts at baseline and after vaccination that correlate with vaccine immune response

- Evaluate the effect of prior SARS-CoV-2 infection on the antibody response to the seasonal influenza vaccine in healthy volunteers

2.3 Exploratory Objectives

Assess the immune responses over time and across seasons of healthy volunteers to influenza vaccination, including the relationship between this response and existing immunity to specific influenza vaccine strains, repeated immunization with the same vaccine strain, and African American (AA) race by evaluating a large number of biological parameters

3 Study Design

3.1 Description of the Study Design

This is a prospective, open-label, exploratory study to evaluate the baseline immune state and post-vaccination immune responses of healthy volunteers to the seasonal influenza vaccine. The study vaccine is approved and indicated by the US Food and Drug Administration (FDA) for active immunization for the prevention of influenza disease caused by influenza virus subtypes A and B. Blood samples will be collected at various timepoints before and up to 100 days after vaccination to explore short- and long-term immunological effects of immunization. Blood may also be collected at optional monthly study visits up until 1 year after vaccination. Subjects may optionally provide stool samples on days -7, 0, 28, and 100.

Subjects will remain on study and may optionally repeat study visits (including vaccination) annually through the 2023-24 influenza season, with final study follow-up up to 1 year after vaccination. Sampling individual subjects across several influenza seasons will allow us to monitor for multi-season responses.

Blood will be evaluated to characterize immune response parameters, primarily changes in antibody titers following vaccination. These evaluations will be used to assess expected response to the vaccination in order to identify baseline and early response correlates of vaccination responses (e.g., by searching among the many parameters we will measure, as listed in section 7.2). Other important immune response parameters include the frequency of peripheral monocytes and interferon transcriptomic signatures (past studies suggest both can be detected in the blood on day 1 following influenza vaccination), as well as the frequency of plasmablast and certain $CD4^+$ T-cell subsets (detectable around day 7 in past studies with influenza vaccination). In this study, these parameters should be captured exactly on days 1 and 7, as the earlier CHI study of influenza vaccination demonstrated that measuring these on the specified days will minimize variability and maximize detection of biologically important signals.[3] Additional research evaluations may include flow cytometry and transcriptome and genetic analyses.

In year 2 of this protocol, enrollment will be enriched according to history of SARS-CoV-2 infection. We will enroll up to 120 subjects by consecutive sampling in the following cohorts:

- Up to n=40 subjects with no history of SARS-CoV-2 infection, as determined by a negative antibody test at screening.
- Up to n=40 subjects with confirmed symptomatic SARS-CoV-2 infection, as determined by a documented positive polymerase chain reaction (PCR) test, and a positive antibody test at screening.
- Up to n=40 subjects with confirmed SARS-CoV-2 antibodies at screening or from an FDA-approved test as documented by the subject but with no history of symptoms.

This design will allow comparison between cohorts to explore the long-term immunological effects of SARS-CoV-2 infection. Recruitment in year 2 will be targeted toward these groups, and enrollment for each cohort will be stopped when its sample size is achieved for the year.

Because the vaccine response to the seasonal influenza vaccine in AA people has only started to be explored,[10] we will recruit up to 10 AA subjects (of the 40) for each group described above in order to evaluate the relationship between AA race and vaccine responses in each of the three cohorts above.

Because obesity is associated with decreased vaccine responsiveness,[11] we will exclude individuals with a body mass index (BMI) ≥ 30.

3.2 Study Endpoints

3.2.1 Primary endpoint

Change in antibody titer response to vaccination, as measured by microneutralization titers at day 0 and day 70 and its relationship with novel baseline biomarkers.

3.2.2 Secondary endpoints

- Change in antibody titer response to vaccination, as measured by microneutralization titers at day 0 and day 70 and its relationship with established baseline biomarkers ($CD38^+$ $CD20^+$ B cell frequencies and/or transcriptomic signatures) and post-vaccination biomarkers (plasmablast cell frequencies and/or transcriptomic signatures)
- Difference in antibody titer response to vaccination between subjects with a history of symptomatic or asymptomatic SARS-CoV-2 infection and those with no evidence of SARS-CoV-2 infection

3.2.3 Exploratory endpoints

Exploratory assessments may include, but are not limited to, pre- and post-vaccination evaluation of:

- Frequency of leukocytes (to include lymphocyte subsets) in peripheral blood as analyzed by complete blood count (CBC) with differential and clinical and research flow cytometry.
- Cellular transcriptional activity in immune cell subsets as analyzed by RNA-seq or microarrays.
- Whole blood transcriptome as analyzed by RNA-seq.
- Single-cell transcriptome as analyzed by RNA-seq or CITE-seq.
- Characterization of extracellular and intracellular proteins by CyTOF.
- Peripheral blood mononuclear cell (PBMC) transcriptome as analyzed by RNA-seq.
- Cellular chromatin accessibility in immune cell subsets as analyzed by the assay for transposase-accessible chromatin using sequencing (ATAC-seq).
- Relative abundance of serum proteins, such as that provided by the SomaLogic SOMAscan assay.
- Serum antibody titers.
- Additional “omics” assays for assessing immune receptor repertoires, profiling molecular states in cells or for quantifying the relative abundance of circulating molecules to assess the state of the immune system, or characterizing the microbiome in healthy volunteers.

4 Study Population

4.1 Recruitment Plan

Healthy adult volunteers will be recruited through the NIH Clinical Research Volunteer Program, the Office of Patient Recruitment, or ResearchMatch. Volunteers will also be recruited from existing NIH protocols, including protocol 18-I-0101 (“Sample Collection From Healthy Volunteers For Assay Optimization”) and protocols that evaluate SARS-CoV-2 infection or

antibodies (e.g., protocol 20I0117, "Surveillance of individuals following SARS-CoV-2 exposure") to stratify enrollment for SARS-CoV-2 status as described in section 3.1. Recruitment in year 2 will also target AA volunteers for each cohort (section 3.1). Potential subjects will be identified by discussion between the study teams and review of medical and research records, including results of SARS-CoV-2 testing, if available. Race will be self-identified. A recruitment email will be approved by the IRB before distribution. Interested potential subjects may also contact the study team directly using the NIH email address: niaidflustudy2020@mail.nih.gov.

Local and regional recruitment will be done using internet ad campaigns, social media outlets, print ads, and from local clinics via the NIAID Office of Communications and the NIAID patient recruitment with Matthews Media Group, Inc.

Potential subjects may be contacted by email or telephone to set up a pre-screening phone call to discuss interest, eligibility (including any past SARS-CoV-2 testing), and scheduling prior to the screening visit.

For the convenience of the study (which involves questionnaires) and because this study does not offer benefit to subjects, only English speakers will be recruited.

4.2 Inclusion Criteria

Individuals must meet all of the following criteria to be eligible for study participation:

1. Aged 18 years and older (no upper age limit).
2. Able to provide informed consent.
3. Willing to have samples and data stored for future research.
4. Able to proficiently speak, read, and write English.

4.3 Exclusion Criteria

Individuals meeting any of the following criteria will be excluded from study participation:

1. CBC with differential, lymphocyte phenotyping with T, B, and natural killer cells (TBNK), acute care, mineral, and hepatic panels, anti-CMV immunoglobulin (Ig) G and IgM, and/or anti-Epstein-Barr virus (EBV) antibody panel values outside of the NIH Department of Laboratory Medicine normal reference ranges and deemed clinically significant by the PI at the time of screening.
2. Positive result for anti-HIV 1/2 antibody, antibody to hepatitis B surface antigen, or anti-hepatitis C virus antibody screening at the time of screening.
3. Prior receipt of a current seasonal influenza vaccine (for the season of participation).
4. History of allergy or hypersensitivity to any components of the study vaccine (e.g., egg protein, latex).

5. History of severe reactions to vaccines.
6. Use of an oral glucocorticoid within the past 30 days.
7. Receipt of a live-attenuated vaccine within the past 30 days.
8. Receipt of any experimental vaccine.
9. Receipt of any other type of vaccine (non-live and non-experimental, e.g., tetanus, diphtheria, and pertussis [TDaP]) within the past 14 days.
10. Planned vaccination before day 100 after study vaccination.
11. Current or recent use (within the past 90 days) of immunoglobulin therapy.
12. Surgery within the past 8 weeks, or planned surgery before day 28.
13. Current (within the past 30 days) treatment for active malignancy.
14. Cancer chemotherapy in the past 2 years.
15. Administration of any blood products within 90 days of the screening, or planned administration before day 100.
16. History of parasitic, amebic, fungal, or mycobacterial infections within the past 1 year, with the exception of tinea pedis and onychomycosis.
17. History of autoimmune or autoinflammatory disease.
18. History of a bleeding disorder.
19. Current use (within the past 30 days) of illicit drugs (per subject report), with the exception of marijuana.
20. Current alcohol use disorders (criteria per Diagnostic and Statistical Manual of Mental Disorders, fifth edition), within the past 30 days.
21. Serious, ongoing, uncontrolled infection within the past 30 days as per the judgement of the PI.
22. History of Guillain-Barre syndrome (GBS).
23. BMI $\geq$ 30.
24. Known or suspected immunodeficiency within 1 year, including documented HIV infection.
25. Pregnancy or planning to become pregnant during the study period. (Women of childbearing potential must have a negative urine or serum pregnancy test at screening.)
26. Presence of conditions that, in the judgment of the PI, may put the individual at undue risk or compromise the scientific objectives of the study.

Co-enrollment guidelines: Co-enrollment in other trials is restricted, other than enrollment on observational studies. Consideration for co-enrollment in trials evaluating the use of a licensed medication will require the approval of the PI. Study staff should be notified of co-enrollment on any other protocol as it may require the approval of the PI.

4.4 Justification for Inclusion/Exclusion of Special Populations

Pregnant women: Pregnant women are excluded from this study because pregnancy may cause changes in the immune system that could confound study results. During the course of the study, if a subject becomes pregnant or suspects she is pregnant, she should inform the study staff and her primary care physician immediately. Pregnancies occurring during study participation will be managed as outlined in section 12.3.

Children: Children are excluded from this study because there is no possibility of benefit to subjects from the research being conducted. The seasonal influenza vaccine is available as part of standard preventive care outside of this study.

NIH staff: NIH staff may be enrolled in this study if they meet the study entry criteria. Neither participation nor refusal to participate in the research will have an effect, either beneficial or adverse, on an individual's employment or position at NIH.

Every effort will be made to protect subject information, but such information may be available in medical records and may be available to authorized users outside of the study team in both an identifiable and unidentifiable manner.

The NIH Information Sheet on Employee Research Participation will be made available. Please see section 16.2 for consent of staff members.

5 Study Vaccine

An investigational new drug application will not be submitted for the study vaccines being used in this protocol because the following exemption criteria are being met:

- The drug product is lawfully marketed in the United States.
- The investigation is not intended to be reported to the FDA as a well-controlled study in support of a new indication and there is no intent to use it to support any other significant change in the labeling of the drug.
- In the case of a prescription drug, the investigation is not intended to support a significant change in the advertising for the drug.
- The investigation does not involve a route of administration, dose, patient population, or other factor that significantly increases the risk (or decreases the acceptability of the risk) associated with the use of the drug product.
- The investigation is conducted in compliance with the requirements for review by an IRB and with the requirements for informed consent.
- The investigation is not intended to promote or commercialize the drug product.

5.1 Disposition, Dispensation, and Accountability

The study vaccine will be distributed and accounted for by the NIH pharmacy according to standard pharmacy procedures. Subjects will only receive one flu vaccine per year, which will be the vaccine provided by the NIH pharmacy.

5.1.1 Formulation and packaging

The study vaccine is a sterile suspension for intramuscular injection available in 0.5-mL prefilled syringes or a 5.0-mL multi-dose vial containing 10 doses of 0.5 mL each.

5.2 Study Agent Storage and Stability

The study vaccine should be stored in refrigeration at 2°C to 8°C (36°F to 46°F) and protected from light. It should not be frozen. The vaccine will not be used if it has been frozen or is past the expiration date. If the multi-dose vial is used, the vial will be returned to recommended storage conditions between uses.

5.3 Preparation, Administration, and Dosage of Study Agent/Intervention

5.3.1 Description

The specific study vaccine will be the vaccine stocked by the NIH pharmacy, either Flucelvax Quadrivalent or Fluvirin for subjects under 65 years (only one vaccine will be used during a given influenza season), or Fluzone High-Dose for subjects aged 65 years and older.

Flucelvax Quadrivalent: Flucelvax Quadrivalent is a subunit influenza vaccine prepared from virus propagated in Madin Darby Canine Kidney (MDCK) cells, a continuous cell line.[12] These cells were adapted to grow freely in suspension in culture medium. The virus is inactivated with β-propiolactone, disrupted by the detergent cetyltrimethylammonium bromide and purified through several process steps. Each of the 4 virus strains is produced and purified separately, and then pooled to formulate the quadrivalent vaccine.

Flucelvax Quadrivalent is a sterile, slightly opalescent suspension in phosphate buffered saline. It is standardized according to United States Public Health Service (USPHS) requirements for the current influenza season and is formulated to contain a total of 60 mcg hemagglutinin (HA) per 0.5-mL dose in the recommended ratio of 15 mcg HA of each of the four selected influenza strains. Each dose of Flucelvax Quadrivalent may contain residual amounts of MDCK cell protein (≤ 8.4 mcg), protein other than HA (≤ 160 mcg), MDCK cell DNA (≤ 10 ng), polysorbate 80 (≤ 1500 mcg), cetyltrimethlyammonium bromide (≤ 18 mcg), and β-propiolactone (< 0.5 mcg), which are used in the manufacturing process.

Flucelvax Quadrivalent 0.5-mL pre-filled syringes contain no preservatives or antibiotics. Flucelvax Quadrivalent 5-mL multi-dose vial formulation contains thimerosal, a mercury

derivative, added as a preservative. Each 0.5-mL dose from the multi-dose vial contains 25 mcg mercury. Flucelvax Quadrivalent 5-mL multi-dose vial formulation contains no antibiotics.

Fluvirin: Fluvirin is a trivalent, sub-unit (purified surface antigen) influenza virus vaccine prepared from virus propagated in the allantoic cavity of embryonated hens' eggs inoculated with a specific type of influenza virus suspension containing neomycin and polymyxin.[13] Each of the influenza virus strains is harvested and clarified separately by centrifugation and filtration prior to inactivation with β-propiolactone. The inactivated virus is concentrated and purified by zonal centrifugation. The surface antigens, HA and neuraminidase, are obtained from the influenza virus particle by further centrifugation in the presence of nonylphenol ethoxylate, a process which removes most of the internal proteins. The nonylphenol ethoxylate is removed from the surface antigen preparation.

Fluvirin is a homogenized, sterile, slightly opalescent suspension in a phosphate buffered saline. Fluvirin has been standardized according to USPHS requirements for the current influenza season and is formulated to contain 45 mcg HA per 0.5-mL dose in the recommended ratio of 15 mcg HA of each of the three selected viruses.

Each dose of Fluvirin may contain residual amounts of egg proteins (≤ 1 mcg ovalbumin), polymyxin (≤ 3.75 mcg), neomycin (≤ 2.5 mcg), β-propiolactone (not more than 0.5 mcg) and nonylphenol ethoxylate (not more than 0.015% weight/volume).

The 0.5-mL prefilled syringe presentation is formulated without preservative. However, thimerosal, a mercury derivative used during manufacturing, is removed by subsequent purification steps to a trace amount (≤ 1 mcg mercury per 0.5-mL dose). Also, the tip caps of the prefilled syringes may contain natural rubber latex.

The 5-mL multidose vial formulation contains thimerosal, a mercury derivative, added as a preservative. Each 0.5-mL dose from the multidose vial contains 25 mcg mercury.

Fluzone High-Dose: Fluzone High-Dose is an inactivated influenza vaccine prepared from influenza viruses propagated in embryonated chicken eggs.[14] The virus-containing allantoic fluid is harvested and inactivated with formaldehyde. Influenza virus is concentrated and purified in a linear sucrose density gradient solution using a continuous flow centrifuge. The virus is then chemically disrupted using a non-ionic surfactant, octylphenol ethoxylate (Triton X-100), producing a “split virus.” The split virus is further purified and then suspended in sodium phosphate-buffered isotonic sodium chloride solution. The Fluzone High-Dose process uses an additional concentration factor after the ultrafiltration step in order to obtain a higher HA antigen concentration.

Fluzone High-Dose is standardized according to USPHS requirements and is formulated to contain 180 mcg HA total per 0.5-mL dose, with influenza strains recommended for the current flu season.

Fluzone High-Dose suspension is clear and slightly opalescent and is distributed in a 0.5-mL, single-dose prefilled syringe. Neither antibiotics nor preservative are used in the manufacture of Fluzone High-Dose. The prefilled syringe presentation is not made with natural rubber latex.

5.3.2 Dosing, administration, and duration of therapy

The study vaccine is administered as a single 0.5-mL intramuscular injection in the deltoid region of the upper arm. Each dose will be obtained from the NIH pharmacy and administered by qualified personnel according to standard procedures. The study vaccine should be shaken vigorously before use (for the multi-dose vial, shake the vial each time before withdrawing a dose). It should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit. After agitation, the study vaccine should be a slightly opalescent suspension. Use a separate sterile syringe and needle for each injection. It is recommended that small syringes (0.5 mL or 1 mL) should be used to minimize any product loss.

5.3.3 Dose delays

Vaccination will be delayed if a subject has evidence of acute infection (or another condition at the discretion of the PI) on the day of vaccine administration.

5.4 Concomitant Medications and Procedures

All concomitant prescription and nonprescription (including over-the-counter) medications taken during study participation will be recorded. For this protocol, a prescription medication is defined as a medication that can be prescribed only by a properly authorized/licensed clinician.

5.5 Prohibited Medications and Procedures

Receipt of additional vaccines, blood transfusion, and surgery will not be permitted prior to day 100 unless discussed with and approved by the PI. The individual's study schedule may be adjusted or ended early for that year of participation.

6 Study Schedule

All study visits will take place at the NIH CC. Subjects will be recruited as described in section 4.1 and will then sign the informed consent document before undergoing any research procedures. The study schedule is described below and in Appendix A. The subject may optionally repeat the schedule outlined below in each subsequent influenza season until the end of the study.

6.1 Screening (day -30 to -7)

In the first year of participation, screening will include an in-person health/medication history and physical exam (including height and weight measurement for BMI) as well as the evaluations listed below. For optional participation in subsequent influenza seasons, screening will have the same evaluations but an electronic REDCap health history and medication questionnaire instead of an in-person history/physical, and subjects will be asked their height and weight to estimate BMI. For individuals who report a history of SARS-CoV-2 infection, we will also review COVID-19 symptoms and/or documentation of SARS-CoV-2 testing (from the NIH or outside since December 2019).

The following evaluations will be conducted at the screening visit:

- Urine or serum pregnancy test for women of childbearing potential.
- Blood draw for the following screening tests:
    - CBC with differential.
    - Lymphocyte phenotyping with TBNK.
    - Acute care, mineral, and hepatic panels.
    - Anti-CMV IgG and IgM antibody and anti-EBV antibody panel.
    - Anti-HIV 1/2 antibody, antibody to hepatitis B surface antigen, and anti-hepatitis C virus antibody.
    - SARS-CoV-2 serum antibody test for individuals who do not provide documentation of a positive FDA-approved antibody test result (since December 2019).
        - If an individual with a documented positive SARS-CoV-2 PCR test result is tested for antibodies at screening and the result is negative, antibody testing will be repeated (possibly with a different FDA-approved test). If the second antibody test is negative, the individual will only be eligible to enroll in the cohort with no history of SARS-CoV-2 infection.

If any of these evaluations have been conducted on another protocol at the NIH within the previous 30 days, then they will not be repeated, and those results will be used to determine eligibility on this study. The exception is the pregnancy test, which must be done at the NIH within the previous 2 days. Screening labs conducted under this protocol may be repeated at the discretion of the PI within 30 days.

If the results of any screening tests are consistent with a previously unknown health condition or other incidental findings, then the subject will be informed of the results. The results will also be provided to the subject's primary physician if requested. If the subject does not have a primary

physician, then the study team will assist in referring them to an appropriate medical care provider for evaluation.

To reduce the number of visits and venipunctures, the screening visit may be combined with baseline (below) so all samples can be collected in one day. If a subject is determined to not be eligible for this study, then their samples will be appropriately discarded. The subject may also return for the baseline visit at a later date.

6.2 Baseline (day -7 ±2)

The following procedures and evaluations will be performed after eligibility is confirmed. Procedures will not be duplicated if baseline evaluations occur on the same day as screening.

- Electronic REDCap health history and medication questionnaire (if baseline is not the same day as screening).
- Blood draw for research testing (see section 7.2) and storage. (If baseline is not the same day as screening, CBC with differential, lymphocyte phenotyping with TBNK, and serum pregnancy test must be repeated.)
- Once at either day -7 or day 0, optional stool collection for research evaluations and storage.

6.3 Vaccination Day (day 0)

The following procedures will be performed prior to vaccination:

- REDCap health history and medication questionnaire.
- Blood draw for research testing and storage.
- Once at either day -7 or day 0, optional stool collection for research evaluations and storage.

The subject will also come to the clinic for a brief interview to review the completed health history and medication questionnaire. The subject will then proceed to receive a single dose of study vaccine as an intramuscular injection and will be observed for adverse events (AEs) for 15 minutes after administration. Appropriate medical treatment to manage possible anaphylactic reactions following administration will be available. If there are no concerns about AEs after this period, the subject will be discharged.

If the subject has evidence of acute infection (or another condition at the discretion of the PI) on day 0, the study vaccination will be rescheduled. If vaccination is delayed more than 2 days, women of childbearing potential must repeat the pregnancy test before vaccination. Based on the new vaccination date and PI discretion, other baseline and screening procedures may be repeated.

6.4 Follow-up Visits

The subject will return for follow-up visits on days 1 (±0), 7 (±0), 14 (±1), 28 (±2), 70 (±2), and 100 (±7). At these visits, the following procedures will be performed:

- Blood draw for research evaluations and storage.
- On days 28 and 100, optional stool collection for research evaluations and storage.

On days 1 and 7, the subject will be sent a REDCap questionnaire by email to assess AEs after vaccination.

Additionally, within 1 day of the scheduled visit, the subject will be emailed a separate REDCap questionnaire to assess for changes in health history or medications.

After the day 100 visit, study participation will be complete for the study year unless the subject agrees to return for optional follow-up as described below.

6.5 Optional Follow-up Visits

The subject will be asked to return for optional study visits monthly, defined as once per calendar month starting the calendar month after the day 100 visit, as requested, up until 1 year following vaccination (for a possible total of 8 monthly optional visits per year). At these visits, the following procedures will be performed:

- REDCap health history and medication questionnaire.
- Blood draw for research evaluations and storage.

If the subject opts in for the optional follow-up, then the final visit for the study year will be the last visit that he or she chooses to have.

6.6 Final Study Visit

The final study visit will be the last study visit the subject chooses to have in their last year of study participation.

6.7 Early Termination Visit

If a subject withdraws or is withdrawn from the study before the day 100 visit, they will be encouraged, but not required, to return to the CC for an early termination visit, where they will complete all of the study procedures for the day 100 study visit (section 6.4).

6.8 Recontact of Subjects After Trial Termination

Subjects may be recontacted after the end of study participation to be notified of potential eligibility for other NIH protocols.

6.9 Plan for the Return of Results

Results of this research will not regularly be returned to subjects; however, the study team will attempt to return any medically actionable incidental findings that are identified during the primary analyses. Results will be published as required on clinicaltrials.gov after study closure. Individual results will not be returned to subjects, but subjects will be instructed that they may contact the study team with any questions.

7 Study Procedures/Evaluations

7.1 Clinical Procedures/Evaluations

Medical and medication history and physical exam: At screening for the initial year of participation, subjects will have a complete review of medical and medication history, including review of influenza vaccine history and SARS-CoV-2 testing and symptom history, and a physical exam, including measurement of height, weight, and vital signs.

Blood draw: Blood will be drawn at each visit for laboratory evaluations and storage. The amount of blood drawn for research purposes will be within the limits allowed for adult research subjects by the NIH CC (Medical Administrative Series Policy M95-9, Guidelines for Limits of Blood Drawn for Research Purposes in the Clinical Center: http://cc-internal.cc.nih.gov/policies/PDF/M95-9.pdf).

Stool collection (optional): Stool samples will be self-collected at the subject's domicile 24 hours before or after a study visit. Sampling kits with verbal and written instructions for collection will be provided to each subject either on the day of or prior to selected visits (see Appendix C).

Electronic questionnaires: Two separate REDCap questionnaires will be completed electronically by subjects throughout the study. The questionnaires will be sent to the personal email address on file. One questionnaire will capture changes in health history or medications; the other will assess AEs following study vaccination (positive responses to the AE questionnaire will prompt a follow-up phone call from the study team). The questionnaires are provided in Appendix B.

7.2 Laboratory Evaluations

Pre- and post-vaccination whole blood samples will be processed to isolate serum, PBMCs, RNA, and TBNK cells, which will be used for the following research evaluations:

- Antibody titers on serum.
- CBC with differential.
- Lymphocyte phenotyping (TBNK flow cytometry performed by the NIH CC).
- Whole blood transcriptome as analyzed by RNA-seq from stabilized RNA (e.g., via Tempus or PAXgene tubes).

Additional research evaluations to evaluate a large number of parameters may include but are not limited to the following:

- Flow cytometry on whole blood and/or immune cell subsets.
- Cellular transcriptional activity in immune cell subsets as analyzed by RNA-seq and/or microarrays.
- Quantification of gene expression (transcriptome) of PBMCs with RNA-seq and/or microarrays.
- Characterization of chromatin accessibility and promoter/enhancer landscapes in immune cell subsets with ATAC-seq and/or other techniques.
- Targeted genetic analyses including genotyping of specific regions of the genome (e.g., human leukocyte antigen and single nucleotide polymorphism typing), if not already performed on another protocol.
- Measurement of relative abundance of serum proteins with SomaLogic's SOMAscan assay or other assays.
- Serum antibody titers.
- Serum SARS-CoV-2 antibody testing using the Roche Elecsys® Anti-SARS-CoV-2 antibody test (under Emergeny Use Authorization), which is classified as a Non-Significant Risk device because it does not meet any of the following definitions of a Significant Risk Device under 21 CFR 812.3(m):
    - Is intended as an implant and presents a potential for serious risk to the health, safety, or welfare of a subject;
    - Is purported or represented to be for use supporting or sustaining human life and presents a potential for serious risk to the health, safety, or welfare of a subject;
    - Is for a use of substantial importance in diagnosing, curing, mitigating, or treating disease, or otherwise preventing impairment of human health and presents a potential for serious risk to the health, safety, or welfare of a subject; or
    - Otherwise presents a potential for serious risk to the health, safety, or welfare of a subject.

  The use of the Roche Elecsys® Anti-SARS-CoV-2 antibody test as a Non-Significant Risk device will follow the abbreviated requirements under 21 CFR 812.2(b)(1).
- Additional “omics” assays for immune receptor repertoires (T-cell and B-cell receptor profiling), and for profiling molecular states in cells or for quantifying the relative abundance of circulating molecules (including but not limited to DNA, RNA, metabolites, and proteins), or characterizing the microbiome.
- Exploratory biomarker assays.

8 Potential Risks and Benefits

8.1 Potential Risks

8.1.1 Study vaccine

The specific seasonal influenza vaccine used in this study will be determined by the NIH pharmacy (see section 5.3.1). Thus, the risks of the study vaccine will be the risks of either Flucelvax Quadrivalent, Fluvirin, or Fluzone High-Dose, which are described below.

Flucelvax Quadrivalent: In clinical trials, the most common (occurring in ≥ 10% of subjects) local and systemic reactions in adults aged 18 through 64 years were injection site pain (45.4%), headache (18.7%), fatigue (17.8%) and myalgia (15.4%), injection site erythema (13.4%), and induration (11.6%). In adults 65 years and older, the most common reactions were injection site pain (21.6%) and injection site erythema (11.9%). In these populations, no reported serious AEs (SAEs) were assessed as being related to study vaccine.[12] Less common side effects include nausea, diarrhea, vomiting, loss of appetite, arthralgia, chills, malaise, ecchymosis, and fever.

There are insufficient data on the use of Flucelvax Quadrivalent in pregnant women to inform vaccine-associated risks in pregnancy. In a study using the trivalent formulation of Flucelvax in rabbits, there was no evidence of vaccine-related fetal malformations or variations and no AEs on pre-weaning development at a dose level about 11 times the human dose based on body weight.[12] Pregnant women are excluded from participation in this study.

Post-marketing reports include additional side effects such as extensive swelling of the injected limb.

Fluvirin: In combined clinical trial data from the 1998-1999 season through the 2004-2005 season, in adults aged 18 through 64 years, the most common (occurring in ≥ 10% of subjects) solicited local and systemic AEs within 96 hours after vaccination were associated with the injection site (e.g., pain, erythema, mass, induration, and swelling); the most common overall events were headache, fatigue, injection site reactions (pain, mass, erythema, and induration) and malaise. In adults 65 years and older in these same studies, the most common solicited local and systemic AEs were injection site pain and headache; the most common overall events were headache and fatigue.[13] Other less common events across these studies included ecchymosis or pruritis at the injection site, pharyngitis, chills, sweating, shivering, nausea, arthralgia or myalgia, chest tightness or other breathing difficulties, coughing or worsening of existing cough, fever, rhinitis, or rhinorrhea.

Fluvirin can cause allergic reaction to individuals who are hypersensitive to eggs or egg products or other components of Fluvirin. Additionally, if Fluvirin prefilled syringes are used, the tip caps may contain natural rubber latex, which may cause allergic reactions in sensitive individuals. If

Fluvirin is used as the study vaccine, individuals with a history of allergy or sensitivity to any components of Fluvirin (including egg protein and, if prefilled syringes are used, latex) are excluded from participation in this study.

In post-marketing experience, hypersensitivity reactions have rarely led to anaphylactic shock and death. Other post-marketing reports include paralysis (e.g., Bell's Palsy), and skin reactions such as Stevens-Johnson syndrome.

Fluvirin is classified in Pregnancy Category B. There was no evidence of impaired fertility or harm to the fetus due to Fluvirin in a study in rabbits at a dose level about 15 times the human dose based on body weight.[13] Pregnant women are excluded from participation in this study.

Fluzone High-Dose: In clinical trials of subjects aged 65 years and older, the most common side effects were pain at the injection site, myalgia, malaise, and headache. In one trial of over 2,500 subjects aged 65 years and older, the most common reported events were injection-site pain (35.6%) and erythema (14.9%), and the most common solicited systemic AEs were myalgia (21.4%), malaise (18.0%), and headache (16.8%). Other reported events included injection-site swelling and fever.[14]

Fluzone High-Dose can cause allergic reaction (including anaphylaxis) to individuals who are hypersensitive to any component of the vaccine, including egg protein, or to individuals who have had allergic reactions to previous doses of influenza vaccination. For subjects aged 65 years and older, because Fluzone High-Dose will be used as the study vaccine, individuals with a history of allergy or sensitivity to any components of this vaccine are excluded from participation in this study.

In post-marketing experience, individuals receiving Fluzone High-Dose have reported anaphylaxis and other hypersensitivity reactions (including urticaria and angioedema), facial palsy (Bell's Palsy), and skin reactions such as Stevens-Johnson syndrome.

Influenza vaccines in general: The following risks may be possible with either Flucelvax Quadrivalent, Fluvirin, or Fluzone High-Dose.

GBS is a theoretical risk of influenza vaccinations. Evidence for a causal relation of GBS with other influenza vaccines is inconclusive; if an excess risk exists, it is probably slightly more than 1 additional case per 1 million persons vaccinated.[12,13] Individuals with a history of GBS are excluded from participation in this study.

Allergic reactions to the influenza vaccine are possible, and during post-marketing surveillance, serious allergic reactions (e.g., anaphylactic shock) have been observed in individuals receiving

either study vaccine. Additionally, syncope is possible following injected vaccines and may be accompanied by transient neurological signs (e.g., visual disturbance, paresthesia, and tonic-clonic limb movements). Subjects will be monitored for these reactions as described in section 5.3.2.

8.1.2 Blood draw

The risks of drawing blood include pain, bruising, bleeding, fainting, and, rarely, infection.

8.1.3 Targeted genetic analyses

This study will not involve genetic tests intended to discover disease-determining genes; however, study analyses could potentially result in medically relevant incidental findings. Additionally, in the future, novel disease-associated phenotypes may be discovered that might be identified in samples stored under this study. Many research laboratory tests are not certified by the Clinical Laboratory Improvement Amendments (CLIA), so generated genetic data cannot be meaningfully interpreted outside the narrow focus of the study and will not be routinely returned to subjects or their physicians. If a clinically significant finding is discovered and a CLIA-certified test is available for confirmation, the PI (or designee) will contact the subject to inform them of the finding and counsel them on confirming the result through a clinical provider.

Genetic findings can have emotional and psychological consequences as well as implications for health, employability, and insurability for the subject and family members. Samples and the resulting data will be coded. Additionally, to protect confidentiality, results will be entered into a password-protected database restricted to the PI or appointed designees. Genetic information would only be divulged if a subject signs a waiver on an insurance application. Study analyses will not result in discoveries about identity or paternity.

8.2 Potential Benefits

Subjects will not receive direct health benefits from participation in this protocol, since the study vaccine is approved and widely available as part of standard preventive care. The results of this study may improve the investigators' understanding of immune response to vaccination and the immune system in general. In addition, it may help us understand how infection with SARS-CoV-2 influences the immune response to the seasonal influenza vaccine. Such information may also be useful in designing more effective vaccines to prevent the spread of influenza, which could indirectly benefit the participating healthy volunteers in the future.

9 Research Use of Stored Human Samples, Specimens, and Data

Intended Use: Samples and data collected under this protocol will be stored and used to study immune responses. Genetic testing will be performed within the scope of this protocol. Specimens may be used for genetic analyses of the microbiome, including metagenomic sequencing (section 7.2). Although human DNA sequences are generated as part of metagenomic

sequencing, human sequence data will not be evaluated and would be removed from datasets prior to sharing in a repository.

Storage: All of the stored study research samples are labeled by a code that only the investigators can link to the subject. Samples are stored in LISB laboratories at the NIH campus located in secure buildings with limited access. Data will be kept in password-protected computers. Only investigators or their designees will have access to the samples and data.

Tracking: Samples and data acquired under this protocol will be tracked using BSI Systems software.

Disposition of Samples and Data:

- In the future, other investigators (both at NIH and outside) may wish to study these samples and/or data. Before any sharing of samples, data, or clinical information, IRB approval will be obtained if the research is determined to be human subjects research.

Loss or Destruction:

- Any loss or unanticipated destruction of samples (for example, due to freezer malfunction) or data (for example, misplacing a printout of data with identifiers) that meets the definition of a reportable event will be reported to the NIH IRB according to Human Research Protections Program (HRPP) Policy 801.
- Additionally, subjects may decide at any point not to have their samples stored. In this case, the PI will destroy all known remaining samples and report what was done to both the subject and to the IRB. This decision will not affect the individual's participation in other protocols at NIH.

10 Data Sharing Plan

Human data generated in this study will be shared for future research as follows:

- De-identified data in an NIH-funded or approved public repository, including
    - genetic data in the database of Genotypes and Phenotypes (dbGaP).
    - gene expression and chromatin profiling data in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO).
    - sequencing data in the NCBI Sequence Read Archive (SRA).
    - flow cytometry data in FlowRepository.
- De-identified data in another public repository.
- Identified data in the Biomedical Translational Research Information System (BTRIS, automatic for activities in the CC).

- De-identified or identified data with approved outside collaborators under appropriate agreements.

This study is not expected to generate the amount of genetic data that triggers the NIH Genomic Data Sharing Policy, which applies to all NIH-funded research that generates large-scale human or non-human genomic data, as well as the use of these data for subsequent research.

Data will be shared before publication through presentations at meetings and seminars; raw and complete data sets will not be shared until the time of publication or shortly thereafter.

11 Compensation Plan for Subjects

Compensation will be dependent on the study visits that a subject chooses to have (Table 1). For each visit, subjects will be compensated at a rate of $20/hour for the first hour, $10/hour for each subsequent hour, and $20 stool collection. Additionally, a completion bonus of $50 will be given to subjects who attend all required study visits through day 100, and another $50 will be given to subjects who attend at least 4 optional monthly visits beyond day 100. Subjects will receive additional $20 bonuses for attending visits on exactly day 14 and day 28.

Total compensation is up to $360 with all bonuses and all optional monthly visits through 1 year after vaccination. Subjects will receive the same compensation for repeating the study schedule in subsequent influenza seasons.

Table 1. Compensation plan for each year of study participation.

Visit	Screen	Day -7	Day 0	Day 1	Day 7	Day 14	Day 28	Day 70	Day 100	Bonus #1	Optional Monthly Visits	Bonus #2 [a]
Time (hours)	2	1	1	1	1	1	1	1	1	-	1	-
Amount per Visit	$30	$20	$20	$20	$20	$40 [b]	$40 [b]	$20	$20	$50	$20	$50
Optional Stool	-	$20 [c]	($20 [c])	-	-	-	$20	-	$20	-	-	-
Total Amount	$30	$70	$90	$130	$150	$190	$250	$270	$310	$360	-	-

Additional visits related to participation will be compensated at a rate of $20/hour for the first hour and $10/hour for each subsequent hour.

[a] Completion Bonus #2 for subjects who attend at least 4 optional monthly visits beyond day 100.

[b] Includes $20 bonus for subjects who attend study visits on exactly day 14 and day 28 (no window).

[c] Stool may be collected once at either day -7 or day 0.

12 Assessment of Safety

AEs and other reportable events are defined in NIH HRPP Policy 801.

12.1 Toxicity Scale

The PI will grade the severity of each AE according to the FDA Guidance for Industry "Toxicity Grading Scale for Healthy Adult and Adolescent Volunteers Enrolled in Preventive Vaccine Clinical Trials," September 2007, which can be found at:
https://www.fda.gov/media/73679/download

12.2 Recording/Documentation

AEs related to the study vaccine will be recorded for 7 days after vaccination. AEs from other research procedures (i.e., blood draw) that occur within 24 hours after the procedure will also be recorded. AEs will be recorded and reviewed in a timely manner by the research team and reported as outlined below. The start date, the stop date, the severity of each reportable event, and the PI's judgment of the AE's relationship and expectedness to the study agent/intervention will also be recorded in the Clinical Research Information Management System of the NIAID (CRIMSON).

Expected events are described in section 8.1 and the package inserts .[12,13] Events will not be reported unless they are Grade 3 or greater or occur at a severity or frequency greater than expected, at which time they will be reported as UPs.

12.3 Pregnancy

Although pregnancy itself is not an AE, events occurring during pregnancy, delivery, or in the neonate (e.g., congenital anomaly/birth defect) may be AEs or SAEs.

In the event of pregnancy, the following steps will be taken:

- The subject will be withdrawn from the study.
- Inform the subject about available pregnancy registry.
- Report to the IRB.
- Advise research subject to notify the obstetrician of study participation and study vaccine exposure.

12.4 Type and Duration of the Follow-up of Subjects after Adverse Events

AEs related to research procedures will be followed through resolution or until the PI judges that the event has stabilized and no additional follow-up is required. AEs that have not resolved by the end of the follow-up period will be followed until final outcome is known. If it is not possible to obtain a final outcome for an AE (e.g., the subject is lost to follow-up), the reason a final outcome could not be obtained will be recorded by the investigator in CRIMSON.

12.5 Pausing Rules

Pausing is discontinuation of all administrations of a single study vaccine (e.g., Fluvirin or Fluzone High-Dose) until a decision is made to either resume or permanently discontinue administration of that vaccine. Subjects continue to be followed by regularly scheduled protocol assessments during a pause.

The pausing criteria for a study vaccine are:

- 3 or more of the same or similar AEs in different subjects receiving the same vaccine that are grade 3 or above and are unexpected and possibly, probably, or definitely related to the study vaccine.
- Any safety issue that the PI determines should pause administration of the study vaccine.

12.5.1 Reporting a pause

If a pausing criterion is met, a description of the AE(s) or safety issue must be reported by the PI within 1 business day to the IRB.

12.5.2 Resumption of study vaccine after a pause

The PI will determine whether or not it is safe to resume administration of the study vaccine. The PI will notify the IRB of the decision on resumption of the study vaccine.

12.6 Halting Rules for the Protocol

Halting the study requires immediate discontinuation of all study vaccines for all subjects and suspension of enrollment until a decision is made whether or not to continue enrollment and study vaccine administration. Subjects continue to be followed by regularly scheduled protocol assessments during a halt.

The halting rule is:

- Any safety issue that the PI determines should halt the study.

The PI will determine if the study should be halted.

12.6.1 Reporting a halt

If a halting rule is met, a description of the AE(s) or safety issue must be reported by the PI within 1 business day to the IRB.

12.6.2 Resumption of a halted study

The PI will determine if it is safe to resume the study. The PI will notify the IRB of the decision on resumption of the study.

12.7 Study Discontinuation

The IRB, the NIAID, and other oversight bodies as applicable, as part of their duties to ensure that research subjects are protected, may discontinue the study at any time. Subsequent review of serious, unexpected, and related AEs by the IRB may also result in suspension of enrollment and further trial interventions/administration of study agent.

12.8 Premature Withdrawal of a Subject

An individual subject will be withdrawn for any of the following:

- An individual subject's decision. (The investigator should attempt to determine the reason for the subject's decision.)
- Non-compliance with study procedures to the extent that it is potentially harmful to the subject or to the integrity of the study data.
- One of the following changes in the subject's baseline condition after enrollment so that the subject no longer meets one or more eligibility criteria.
    - Unwilling to have samples and data stored for future research.
    - Develops a condition in the exclusion criteria that merits withdrawal per the opinion of the PI.
- The investigator determines that continued participation in the study would not be in the best interest of the subject.

12.9 Replacement of Withdrawn Subjects or Subjects Who Discontinue Study Treatment

Subjects who withdraw or are withdrawn from the study prior to day 14 of their first year of participation will be replaced. If a subject is replaced, all the data collected from that subject will still be included for the safety assessment.

13 Reporting Procedures

13.1 Reporting to the NIH IRB

UPs, non-compliance, and other reportable events will be reported to the NIH IRB according to HRPP Policy 801.

13.2 NIAID-specific Reporting

13.2.1 Reporting to the NIAID clinical director

The PI will report UPs, major protocol deviations, and deaths to the NIAID clinical director according to institutional timelines.

14 Clinical Monitoring Structure

To help ensure that NIH Office of Research Support and Compliance procedures and Good Clinical Practice (GCP) principles are being carried out, a Clinical Trials Management designee within the Office of Clinical Research Policy and Regulatory Operations, Regulatory Compliance and Human Subjects Protection Program will conduct a study initiation visit before study enrollment begins. The purpose of this meeting is to review with the PI and study team designees the roles and responsibilities concerning their commitment to adhere to the requirements of the protocol, especially in terms of the NIH Office of Human Subjects Research Protections reporting requirements for AEs, SAEs, and UPs. In addition, the quality management and data management plan for the study will be reviewed.

14.1 Quality Management Plan

During the study, the PI and study team will be responsible for implementing a quality management plan. Additionally, the study team will be responsible for completing and submitting a summary report on the quality plan to the NIAID clinical director or designee at least annually as detailed in the quality management plan. A courtesy copy will also be sent to Clinical Trials Management.

14.2 Safety Monitoring Plan

The data gathered during this study will be monitored by the PI for safety and compliance with protocol-specified requirements.

15 Statistical Considerations

This open-label, prospective, exploratory study will assess the baseline and post-vaccination immune responses of healthy volunteers to an approved seasonal influenza vaccine. Subjects will undergo blood collections on days -7 and 0 before vaccination as well as regularly after vaccination. Blood samples will be used to assess short- and long-term immunological effects of immunization, including assessment of antibody titer response to vaccination. Subjects may optionally continue study participation annually through multiple influenza seasons.

15.1 Study Objectives

The primary objective of this study is to identify baseline correlates of immune response to the seasonal influenza vaccine in healthy individuals. The secondary objective is to validate the findings of prior study 09-H-0239, specifically the relationship between cell populations and/or gene transcripts at baseline and after vaccination that correlate with vaccine immune response. Exploratory objectives include examining pre- to post-vaccination changes in multiple biomarkers, correlating changes in biomarkers, identifying networks of biomarkers that act in concert, and assessing changes in biomarkers and biomarker relationships over time and over seasons.

15.2 Sample Size Justification

This is a hypothesis-generating study designed to examine relationships between different biomarkers measured at baseline and post-vaccination immune responses. To set the sample size, we used data from a previous study designed to examine biomarkers (09-H-0239).[3] That study identified a significant relationship between Y=adjMFC (a measure of vaccine-induced immune response adjusted for baseline response) and X=ID96_0 (a baseline readout of the frequency a $CD38^+$ $CD20^+$ memory B cell population). The estimated slope was 0.29 with an estimated standard error of 0.12=sqrt[var(R)/((n-1)*var(X))], where var(R)=2.09 is the sample variance of the residuals from the regression line, var(X)=3.03 is the sample variance of X, and n=49 is the sample size of that study.

We used these estimates as a reference to set the sample size for the current study with the objective to identify a *new* biomarker that correlates with adjMFC with a similar or stronger relationship than was observed in 09-H-0239. We assumed the same error variance of var(R)=2.09 and the same sample variance of var(X)=3.03, and we assumed that the true slope is 0.29. We determined the power by using the Wald test where the slope is zero (i.e., the Wald test is B/se(B) where B is the estimated slope and se(B) is the estimated standard error). We assumed that this test is approximately standard normal under the null and approximately normal with mean 0.29/sqrt[var(R)/((n-1)*var(X))] and variance 1 under the alternative.

We plan to correlate adjMFC with separate distinct collections of biomarkers (e.g., immune phenotyping as measured by flow cytometry or CyTOF, or RNA-seq). For each distinct collection, a multiplicity adjustment would be performed. Power calculations were performed (Table 2) considering various sample sizes, allowing for 5% missing data, and using a two-sided test with an alpha of 0.05 and with an illustrative Bonferroni adjustment for a moderate collection of biomarkers (50; e.g., nontrivial peripheral blood cell subsets measured by flow cytometry or CyTOF). With a sample size of 170 and 10% missing data, we have more than 80% power to identify biomarkers that have true slopes of 0.29 or better even with adjustment for 50 multiple comparisons.

Table 2. Power to detect an association between a baseline biomarker and adjMFC under different sample sizes and multiplicity corrections.

Sample Size	Effective Sample Size (10% missing data)	Power with Adjustment for 50 Comparisons
120	108	.63
170	153	.85
230	207	.96

Sample Size	Effective Sample Size (10% missing data)	Power with Adjustment for 50 Comparisons
280	252	.99

Subjects will have the option to participate for multiple years. Generalized estimating equations (GEE) analyses that use all available measurements from each subject with proper correction for within subject correlation will have greater power than those described in Table 2. Analyses will be stratified by year by including a separate term for each cohort year in the GEE models. Such stratification has a negligible effect on power calculations.

15.3 Description of the Analyses

The primary analysis will be to correlate multiple baseline biomarkers with the vaccine-induced outcome adjMFC using GEE to reflect clustering at the subject level due to multiple measurements over multiple years. Analyses will be stratified by year by including a separate term for each cohort year in the GEE models. Two-sided testing will be performed with an adjustment for multiplicity such as the Bonferroni to control the overall type I error rate at 0.05.

For the first secondary analysis, a similar analysis strategy will be used as in the primary analysis, but the relationship between the vaccine-induced outcome adjMFC and baseline biomarkers (specifically ID_96 [$CD38^+$ $CD20^+$ B cell frequencies and/or transcriptomic signatures]) and post-vaccination biomarkers (specifically ID_87 [plasmablast cell frequencies and/or transcriptomic signatures]) will be examined separately. Specifically, we will evaluate 1) whether the slope between adjMFC and ID96_0 (measured at day 0) is zero using GEE, as described for the primary analysis with no adjustment for multiplicity, and 2) whether the slope between adjMFC and ID_87, measured at day 7/day 0) is zero using GEE, as described for the primary analysis with no adjustment for multiplicity. Both analyses are designed to replicate findings from 09-H-0239.[3]

Analysis of the secondary endpoint on SARS-CoV-2 will involve comparing the results of the above analyses by cohort: 1) those subjects with prior symptomatic SARS-CoV-2 infection, 2) those subjects with prior asymptomatic SARS-CoV-2 infection, and 3) those subjects without a history of SARS-CoV-2 infection.

The exploratory analyses will involve both unsupervised and supervised analyses using the framework developed in 09-H-0239. In addition to that framework, under this protocol we will be able to characterize immune responses and biomarker relationships over multiple seasons in the same individual. The first general aim is to perform unsupervised analysis of the immune states of the baseline along the measured phenotypic dimensions, including PBMC/cell subset

transcriptomes (genes and gene module activities), cell subset frequencies, and relative levels of circulating protein markers. In addition, using data from multiple baseline timepoints (for example, day 28 and beyond might be useful as additional baselines based on comparison to pre-vaccination timepoints to assess the extent of change), we can perform analyses to assess the inter-subject variability and temporal stability of parameters as was done in our earlier study. The parameters that are highly variable among but stable within subjects are good candidates for downstream prediction analysis and correlate discovery.

In addition to unsupervised analyses, specific markers known to be associated with vaccination responses will be assessed including, but not limited to, monocyte frequencies and interferon transcriptomic signatures on day 1, as well as B cell/plasmablast response on day 7.

We will use the same Monte Carlo cross-validation framework from 09-H-0239 to build predictive models and assess immune response correlates by first focusing on antibody titer response as the endpoint. The goal is to link baseline and early response status to antibody response. We will also use variable selection and multiple regression approaches, including Lasso, Ridge regression, and Elastic Net. Variable and model selections will be conducted through cross-validation.

We also plan to include unsupervised analysis without focusing on specific endpoints such as titers (e.g., by assessing the correlation between the most variable baseline states to the most variable response profiles among subjects).

16 Ethics/Protection of Human Subjects

16.1 Informed Consent Process

Informed consent is a process where information is presented to enable persons to voluntarily decide whether or not to participate as a research subject. It is an ongoing conversation between the human research subject and the researchers which begins before consent is given and continues until the end of the subject's involvement in the research. Discussions about the research will provide essential information about the study and include: purpose, duration, experimental procedures, alternatives, risks, and benefits. Coercion and undue influence will be minimized by informing subjects that their decision to join the study will not affect any medical care they are currently receiving, or their eligibility to participate in other research studies at the NIH. Subjects will be given as much time as they need to read the consent form and ask questions of the investigators. Subjects will also be given time to discuss their participation with family members, friends, and other healthcare providers.

Informed consent will be obtained in person in a private setting by a study team member authorized to obtain consent. The subjects will sign the informed consent document prior to undergoing any research procedures. The subjects may withdraw consent at any time throughout

the course of the trial. A copy of the informed consent document will be given to the subjects for their records. The researcher will document the signing of the consent form in the subject's medical record. The rights and welfare of the subjects will be protected by emphasizing to them that the quality of their medical care will not be adversely affected if they decline to participate in this study.

16.2 Considerations for Consent of NIH Staff

Consent for NIH staff will be obtained as detailed above with following additional protections:

Consent from staff will be obtained by an individual independent of the staff member's team whenever possible. Otherwise, the consent procedure will be independently monitored by the CC Department of Bioethics Consultation Service in order to minimize the risk of undue pressure on the staff member.

16.3 Subject Confidentiality

All records will be kept confidential to the extent provided by federal, state, and local law. The study monitors and other authorized individuals may inspect all documents and records required to be maintained by the investigator, including but not limited to, medical records. Records will be kept locked and data will be coded. Any personally identifiable information maintained for this study will be kept on restricted-access computers and networks. Personally identifiable information will only be shared with individuals authorized to receive it under this protocol. Individuals not authorized to receive personally identifiable information will be provided with coded information only, as needed. Clinical information will not be released without written permission of the subject, except as necessary for monitoring by the IRB, NIAID, and the Office for Human Research Protections.

To further protect the privacy of study subjects, a Certificate of Confidentiality has been issued by the NIH. This certificate protects identifiable research information from forced disclosure. It allows the investigator and others who have access to research records to refuse to disclose identifying information on research participation in any civil, criminal, administrative, legislative, or other proceeding, whether at the federal, state, or local level. By protecting researchers and institutions from being compelled to disclose information that would identify research subjects, Certificates of Confidentiality help achieve the research objectives and promote participation in studies by helping assure confidentiality and privacy to subjects.

17 Data Handling and Record Keeping

17.1 Data Capture and Management

Study data will be maintained in CRIMSON and collected directly from subjects during study visits and telephone calls, or will be abstracted from subjects' medical records. Source

documents include all recordings of observations or notations of clinical activities and all reports and records necessary to confirm the data abstracted for this study. Data entry into CRIMSON will be performed by authorized individuals. The investigator is responsible for assuring that the data collected are complete, accurate, and recorded in a timely manner. Study data, including cumulative subject accrual numbers, should be generated via the chosen data capture method and submitted to the IRB as needed.

17.2 Record Retention

The investigator is responsible for retaining all essential documents listed in the International Council on Harmonisation GCP guidelines. Study records will be maintained by the PI for a minimum of 7 years in compliance with institutional, IRB, state, and federal medical records retention requirements, whichever is longest. All stored records will be kept confidential to the extent required by federal, state, and local law.

18 Scientific References

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13. Fluvirin (influenza virus vaccine) [package insert]. Seqirus Vaccines Limited, Liverpool, UK. (2017).
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Appendix A. Schedule of Procedures/Evaluations

Evaluation	Study Day (window) [a]									
	Screening	Baseline	Vaccination	Follow-up						Optional Follow-up
	(-30 to -7)	-7 (±2)	0	1 (±0)	7 (±0)	14 (±1)	28 (±2)	70 (±2)	100 (±7) [b]	Monthly [c]
Informed consent	X									
Clinical Procedures and Evaluations										
History, physical exam with height and weight (for BMI), and vital signs	X									
Electronic history/medication questionnaire		X [d]	X	X	X	X	X	X	X	X
Electronic AE questionnaire				X	X					
Vaccination			X [e]							
Stool collection (optional) [f]		X	X				X		X	
Clinical Laboratory Evaluations	Blood Volume (mL)									
Urine/serum pregnancy test (women of childbearing potential)	4 [g]	4 [d]								
SARS-CoV-2 antibody	X									
CBC with differential	3 [h]	3 [d]	3	3	3	3	3	3	3	3
TBNK lymphocyte phenotyping	3 [h]	3 [d]	3	3	3	3	3	3	3	3
Acute care, mineral, and hepatic panels	4 [h]									
Anti-CMV IgG, IgM antibody and anti-EBV antibody panel	4 [h]									
Anti-HIV 1/2 antibody, antibody to hepatitis B surface antigen, and anti-hepatitis C virus antibody	8 [h]									
Research Laboratory Evaluations	Blood Volume (mL)									
Serum isolation		4	4	4	4	4	4	4	4	4
PBMC isolation		60	90	40	40	40	40	40	40	40
Whole-blood RNA isolation		6	6	6	6	6	6	6	6	6
Daily Blood Volume	26	80	106	56	56	56	56	56	56	56
Cumulative Blood Volume	26	106	212	268	324	380	436	492	548	

Abbreviations: AE, adverse event; BMI, body mass index; CBC, complete blood count; CMV, cytomegalovirus; EBV, Epstein-Barr virus; HIV, human immunodeficiency virus; Ig, immunoglobulin; PBMC, peripheral blood mononuclear cell; RNA, ribonucleic acid; TBNK = T, B, and natural killer cells.

[a] After the first year of participation, subjects may repeat the study schedule annually through the 2023-24 influenza season. Screening will be repeated each year except the informed consent process and the history/physical exam/vital signs, which will be replaced with the electronic history/medication questionnaire. Subjects who are ineligible for that year will remain on study and may return to participate the following year if they are eligible.

[b] Subjects withdrawn before day 100 will be encouraged to have an early termination visit to complete procedures for the day 100 visit.

[c] Once per calendar month starting the calendar month after the day 100 visit, as requested up until 1 year following vaccination (up to 8 optional visits per year).

[d] To be performed if baseline is not the same day as screening.
[e] Prior to vaccination, subjects will complete the history/medication questionnaire and blood draw, and will come to the clinic to review the questionnaire. After vaccination, subjects will be observed for AEs for 15 minutes. If there are no concerns about AEs after this period, subjects will be discharged.
[f] Optionally, stool will be collected prior to vaccination (once at either day -7 or day 0), at day 28, and at day 100. Samples will be self-collected at the subject's domicile 24 hours before or after a study visit. Sampling kits with verbal and written instructions for collection will be provided to each subject prior to selected visits.
[g] Unless performed at NIH within the previous 2 days.
[h] Unless performed at NIH within the previous 30 days. Screening labs may be repeated at the discretion of the principal investigator within 30 days.

Appendix B. Electronic Study Questionnaires

Evaluation for Adverse Events: Days 1 and 7

Since receiving your flu vaccine, have you had any of the following symptoms?

	No	Yes (If yes, please specify one of the following.)			
Redness at the site of injection (2.5 cm or more)		2.5-5 cm	5.1-10 cm	More than 10 cm	Emergency room visit or hospitalization
Swelling or hardening at the site of injection (2.5 cm or more)		2.5-5 cm	5.1-10 cm	More than 10 cm	Emergency room visit or hospitalization
Pain at the site of injection		Does not interfere with daily activity	Repeated use of over-the-counter pain reliever OR **interferes with some** daily activity	Any use of prescription pain reliever OR **prevents** daily activity	Emergency room visit or hospitalization
Headache		Does not interfere with daily activity	Repeated use of over-the-counter pain reliever for more than 24 hours OR **interferes with some** daily activity	Any use of prescription pain reliever OR **prevents** daily activity	Emergency room visit or hospitalization
Fever (temperature above 100.4°F)		100.4-101.1°F	101.2-102.0°F	102.1-104°F	Over 104°F
Myalgia (body aches)		Does not interfere with daily activity	**Interferes with some** daily activity	**Prevents** daily activity	Emergency room visit or hospitalization
Any other symptoms? (If so, please describe):		Free Text			

Evaluation for Changes in Health History and Medications: Days -7, 0, 1, 7, 14, 28, 70, 100, and optional monthly visits

Since your last clinic visit, have you had any of the following changes?

	Yes	No
Started any new medications, including prescription or over-the-counter medications, vitamins, or herbal supplements?	If Yes, what? When? Dose?	
Stopped taking any medications, including prescription or over-the-counter medications, vitamins, or herbal supplements?	If Yes, what? When?	
Received any vaccinations?	If Yes, what? When?	
Had a blood transfusion?		
Had any surgical procedures?	If Yes, what?	
Had any symptoms of infections?	If Yes, what?	
Had any new medical diagnoses given to you by another health care provider?	If Yes, what?	
If female: Are you currently pregnant?		
Have you enrolled in or participated in any other research protocols since your last visit?	If Yes, what protocol?	

Appendix C. Instructions for Self-Collection of Stool Samples

Purpose: The collection of stool for study of the gut microbiome

You will be given a kit for the collection of stool. You may use the kit to collect stool during your visit, or you may take it for collection of stool at home. The clinic staff will review the instructions with you and address any questions or concerns you may have.

- Please take the freezer packs out of your bag and put them into your **freezer** as soon as you arrive home from today's visit. This way, the freezer pack will help the samples stay cold on the day you return to the clinic with your sample. These packs are re-usable, so please do not throw them away.

- Please place the bag and its contents in a secured area, away from children and pets until you are ready to use it.

- In general, you should try to collect the stool samples as close as possible to the time of your clinic visit.

Your sample collection visit is scheduled on: _____

- After you have collected your stool samples, place them in the transport bag with the frozen freezer packs and place entire bag in the **refrigerator**.

- Bring the bag with stool samples and freezer packs to the clinical center as soon as possible.

****

Before you start, locate the sample collection kit.
Please WASH YOUR HANDS before you begin.

****

STEP 1 – DEPOSITING STOOL INTO THE COLLECTION CONTAINER

- Open collection paper carefully in the direction of the arrows.
- Attach tape to top of toilet seat
- Do not let collection paper touch water.
- Without urinating into the container, sit down and pass bowel movement into collection paper.

STEP 2 – COLLECTION OF STOOL INTO TUBE

- Put on gloves.
- Using the collection spoon built into the cap of the vial, collect small scoops of stool from each end and the middle. Collect until tube is at least halfway full. Be sure the scoop will fit in the container. The collection scoop will stay attached to the cap.
- Replace cap and make sure the vial is tightly closed.
- Place the tube into provided plastic blue bag.
- Discard the collection container. One can flush the paper once it is soft/wet or throw it in the trash.
- Remove gloves and wash hands

STEP 3 – STORAGE AND TRANSPORT

- Remove ice pack from the freezer and place in provided Styrofoam container.
- Place the bag with collection tube in the provided Styrofoam container with the icepack as soon as possible.
- Place the entire container in your **refrigerator.**
- Wash hands.