Add Health
The National Longitudinal Study of Adolescent to Adult Health

Report prepared by

Robert A. Angel

Lixin Qu

Jason Grago

Kathryn S. Carrier

Robert A. Hummer

Allison E. Aiello

Eric A. Whitsel

Infection

UNC CAROLINA POPULATION CENTER

CAROLINA POPULATION CENTER | CAROLINA SQUARE - SUITE 210 | 123 WEST FRANKLIN STREET | CHAPEL HILL, NC 27516

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This document summarizes the rationale, equipment, protocol, assay, internal quality control, data cleaning, external quality control, and procedures for the measurement and classification of biomarkers of infection exposure at the Wave VI home exam. Whenever possible, data collection and methods in Wave VI mirrored those of Wave V to ensure comparability of data between waves, although important inter-Wave differences between Waves IV-VI exist and are grey-highlighted herein. This document is one in a set of Wave VI user guides. User guides are also available to describe protocols for the following biological measures at Wave VI:

- Anthropometrics
- Baroreflex Sensitivity & Hemodynamic Recovery
- Biomarker Weights
- Cardiovascular Measures
- Glucose Homeostasis
- Hepatic Injury
- Home Exam – Medication Use
- Home Exam Questionnaire and QC Metrics
- Inflammation and Immune Function
- Lipids
- Neurodegeneration
- Renal Function

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1. Introduction

Wave VI measures of infection were based on venous blood collected via phlebotomy. The blood was collected by field examiners (FEs) certified in phlebotomy, chilled at 4°C during the remainder of the home exam, centrifuged immediately afterward, aliquoted into transport tubes, sent overnight to a laboratory, archived at -80°C, subsequently thawed at 36°C, and then assayed.

Assayed Infection Biomarker Concentrations

- Herpes Simplex Virus IgG (HSV, EU/ml)
- Cytomegalovirus IgG (CMV, EU/ml)
- SARS-CoV-2 Nucleocapsid IgG (AU/ml)
- SARS-CoV-2 Receptor Binding Domain IgG (RBD, AU/ml)
- SARS-CoV-2 Spike IgG (AU/ml)

Moreover, the restricted use Add Health Wave VI data included 8 constructed measures designed to facilitate analysis and interpretation of infection biomarker concentrations:

- Vaccination Status for COVID-19
- Vaccination / Previous Infection Interpretation for COVID-19
- COVID-19 Testing Status
- HSV IgG Concentration Type
- CMV IgG Concentration Type
- SARS-CoV-2 Nucleocapsid IgG Concentration Type
- SARS-CoV-2 RBD IgG Concentration Type
- SARS-CoV-2 Spike IgG Concentration Type

2. General Overview of Data Collection

All Wave VI venous blood samples were collected during home exams performed by ExamOne, a subsidiary of Quest Diagnostics®. All FEs were trained and certified using a custom program specific to the Add Health protocol. FEs used a 7" Samsung Galaxy Tab A7 Lite tablet to record and transmit data. An Add Health data collection application (Open Data Kit or ODK) installed on the tablet guided the FEs through the home exam protocol. In addition, FEs received a series of job aids, both on paper and on the tablet, to serve as quick reference guides when completing the protocol. Each tablet also contained an in-depth Add Health training manual that could be accessed at any time.

FEs conducted home exams among previously consented participants. All FEs were phlebotomy-certified and had at least two years of experience collecting venous blood. Before home exams, FEs were sent a

Visit Supply Kit that included a box for shipping blood to the lab and a Blood Collection Kit containing most required materials for the blood collection. FEs supplied additional materials, as needed (see section 3.2). Protocols for blood collection were dictated to FEs by the handheld 7" Samsung tablet used during all home exams. The tablet gave step-by-step directions for the blood collection and required FEs to enter information about the blood draw for each participant. All participants had the option to decline part or all the blood draw, although declining did not affect their ability to participate in the rest of the home exam. Overall, 90.8% of the participants agreed to and completed the blood draw. Of the remainder, 6.4% refused, 2.1% agreed but the blood draw was unsuccessful, and < 1% had exams terminated before the blood draw (see the blood draw status variable H6BLOOD in the *bdemo6* data set and codebook).

Blood collection was the last step in the home exam. Afterwards, all collection tubes were inverted 8-10 times to distribute the blood and contents of the tubes and then chilled at 4^{o} C (on ice or frozen cold packs) for up to two hours. Subsequent processing involved centrifuging specific tubes then aliquoting serum and plasma into color-coded transport tubes pre-labelled with unique barcode identifiers linking the blood to a particular participant. Then the transport tubes were packaged in a Styrofoam Box with frozen cold packs and shipped overnight via FedEx to the Laboratory for Clinical Biochemistry Research (LCBR) at the University of Vermont. Overnight shipment enabled receipt by LCBR before 10:30 am the next morning. Upon receipt, LCBR documented the arrival of the transport tubes, evaluated their condition, processed them, and either assayed the specimens or aliquoted and archived them in -80°C freezers.

3. Blood Collection

3.1 Rationale

Venous blood was collected to provide Add Health with the biological specimens necessary to assay and interpret pre-specified biomarkers of metabolic, hepatic, renal, amyloid-tau-neurodegenerative (ATN), inflammatory, immune, and infectious exposures, including the measures of exposure to infection or vaccination described herein. It also was collected to establish an archive of serum, plasma, whole blood, RNA, and packed cells capable of supporting future assays and ancillary studies.

3.2 Equipment

Before exams, FEs were shipped a Visit Supply Kit (**Figure 1**) including (1) a cardboard Shipping Box with an inner Styrofoam Box and two cold packs for shipping collected samples to LCBR, (2) a large Tyvek envelope in which to ship the Shipping Box, and (3) a Blood Collection Kit for collecting blood. The Blood Collection Kit contained:

- Biohazard-labelled Ziploc bag
- Latex-free gloves
- 2”x2” gauze
- Latex-free, Band-Aid type adhesive dressings
- Latex-free, strap tourniquet
- Alcohol prep pads, disposable pipets
- Single-use vacutainer (blood collection) tube holder
- 21-gauge Eclipse straight needle
- 21-gauge butterfly needle
- (3) disposable 3 ml graduated transfer pipets
- (2) 8.5 ml serum separation transport (SST) vacutainer tubes
- (1) 3 ml potassium ethylenediaminetetraacetic acid (EDTA)-containing vacutainer tube
- 10 ml EDTA-containing vacutainer tube
- 10 ml PAXgene vacutainer tube (containing 7.5 ml of preservative)
- (4) 10 ml transport tubes with color coded caps
- Extra barcode labels

BD Biosciences (San Jose, CA) supplied all vacutainer tubes, and transport tubes were supplied by Simport Scientific (Quebec, Canada).

Figure 1. Visit Supply and Blood Collection Kits

FEs were responsible for providing ancillary materials for each home exam, including but not limited to a chux-type absorbent under pad, a sharps container, and a cooler with cold packs for keeping samples cold before packaging and shipping them to LCBR.

3.3 General Protocol

3.3.1 Blood Collection

The blood draw was performed as the final stage of the home exam following collection of anthropometric, cardiovascular, and medication information. After confirming participants were comfortable giving blood, participants were asked to either sit or recline at their discretion. They also were asked if they had problems in the past with blood collection such as fainting, bleeding, or hard-to-find veins. FEs were instructed to ensure the blood collection area was private, uncluttered, and fully prepared before beginning the blood draw. Preparation involved placing the chux pad, organizing the vacutainer tubes/supplies, preparing the cooler to accept the blood samples, and scanning the barcode located on the outside of the Blood Collection Kit and on all vacutainer tubes. Scanning it automatically captured a unique, eight-digit code, thereby linking the participant to the transport tubes / labels within it, the corresponding ODK questionnaire data, and ultimately to LCBR results.

Following standard phlebotomy protocols, FEs asked participants to identify an arm for collecting blood, applied the tourniquet to that arm, and identified a vein in the antecubital fossa for venipuncture. If no vein appeared suitable, FEs asked to try the opposite arm. Unless participants had objections, venipuncture was performed on the best potential vein and whole blood was collected, as summarized below:

- Put on nitrile gloves.
- Have the participant extend his/her arm on the protective pad, palm up and straight at the elbow.
- Inspect the arm. Do not draw blood from an arm that has a rash, open sore, is swollen or shows signs of a recent venipuncture or hematoma. Do not draw blood from an arm that contains an arterial access such as a fistula or shunt.
- Apply the tourniquet several inches above the elbow and palpate for a suitable vein.
- Select a vein that is palpable and well-fixed to surrounding tissue.
- Open the needle assembly unit and attach it to the vacutainer holder.
- Ask the participant to make a tight fist. Cleanse the area with an alcohol wipe using a circular motion and allow the area to air dry.
- Remove the cover from the needle.
- The vein should be fixed or held taut during the puncture. Push the needle firmly and deliberately into the vein. When firmly in the vein, blood appears in the tubing of the needle assembly past the end of the needle.
- Attach the needle holder and quickly push the first vacutainer tube (ordered in Figure 2, below) onto the needle in the holder, puncturing the center of the stopper.

- Release the tourniquet after the flow is established or if the participant becomes uncomfortable. The participant may open his/her fist once blood flow is established.
- When the first vacutainer tube is filled to capacity, remove it from the holder and place the next vacutainer tube in the holder.
- Gently invert each vacutainer tube 8-10 times immediately upon removing each one and while filling the next one. Repeat until all the desired vacutainer tubes are filled.
- Place all filled vacutainer tubes directly into a cooler with ice or ice packs.
- When the last vacutainer tube is filled, remove the tourniquet, carefully withdraw the needle, and cover the venipuncture site with a sterile gauze pad.
- Never apply pressure to the gauze until the needle is clear of the puncture site and away from the arm.
- Have the participant hold the gauze pad with mild pressure and sit quietly for a few minutes.
- Slide the needle safety guard forward to prevent an accidental needle stick. Discard the entire used needle assembly in a sharps container.
- Check the venipuncture site. If it is adequately clotted, remove the gauze and apply a bandage. If after a few minutes, bleeding continues keep direct pressure on the site for 5 minutes.
- Encourage the participant to sit quietly for a few minutes. Due to a fasting blood draw encourage the participant to eat a snack if needed.

When the first attempt at blood collection was unsuccessful, FEs were allowed to ask to draw blood from the opposite arm. However, no more than two blood collection attempts were permitted. Moreover, only the antecubital fossa was acceptable for blood draw. FEs were not allowed to collect blood from any other sites, such as the back of the hand.

5 tubes of blood were collected per participant. Collection order, tube type, and processing information are listed below (**Figure 2**).

Order	Tube Type	Centrifuged	Resultant supernatant	Resultant precipitate	Use
1	8.5 ml SST	Yes	Serum	Discarded	Assay: glucose, total cholesterol, high- & low-density lipoprotein-cholesterol, triglycerides, AST, ALT, creatinine, hsCRP, IL-1β, IL-6, IL-8, IL-10, TNFα, CMV, HSV, SARS-CoV-2 (RBD; spike; nucleocapsid) IgG
2	10 ml EDTA	Yes	Plasma	Packed cells	Assay: Neurofilament light, Tau, GFAP. Archival: packed cells for future use
3	3 ml EDTA	No	N/A	N/A	Assay: hemoglobin A1c Archival: for future use
4	8.5 ml SST	Yes	Serum	Discarded	Archival: for future use
5	10 ml PAXgene	No	N/A	N/A	Archival: for future use

Figure 2. Tubes of Blood Collected

3.3.2 Blood Processing

The venous blood draw concluded the home exam. After cleaning up all supplies and equipment, FEs left the exam sites and were allowed a maximum of two hours before processing the blood which was chilled at 4^{o} C (on ice or frozen cold packs) in the interim.

All FEs centrifuged the 8.5 ml SST and 10 ml EDTA vacutainer tubes. The 3 ml EDTA vacutainer tube used for the HbA1c assay and the PAXgene tube were *not* centrifuged. FEs centrifuged tubes for ≥ 10 min at ≥ 1300 g, depending on the capabilities of their centrifuge. After centrifugation, FEs used the graduated transfer pipettes included in the Blood Collection Kit to aliquot serum from the SST tubes and plasma/packed cells from the 10 ml EDTA tube into 10 ml, round bottom, skirted transport tubes (BD Biosciences, NJ). FEs aliquoted as much supernatant as possible into the transport tubes but avoided disturbing the precipitate layer. A red cap identified transport tubes containing serum from the SST vacutainer tubes, a blue cap identified transport tubes containing plasma from the 10 ml EDTA vacutainer tube. Transport tubes were chilled at 4^{o} C (on ice or frozen cold packs) until packaged for shipment to LCBR. **Figure 3** demonstrates the complete blood processing protocol.

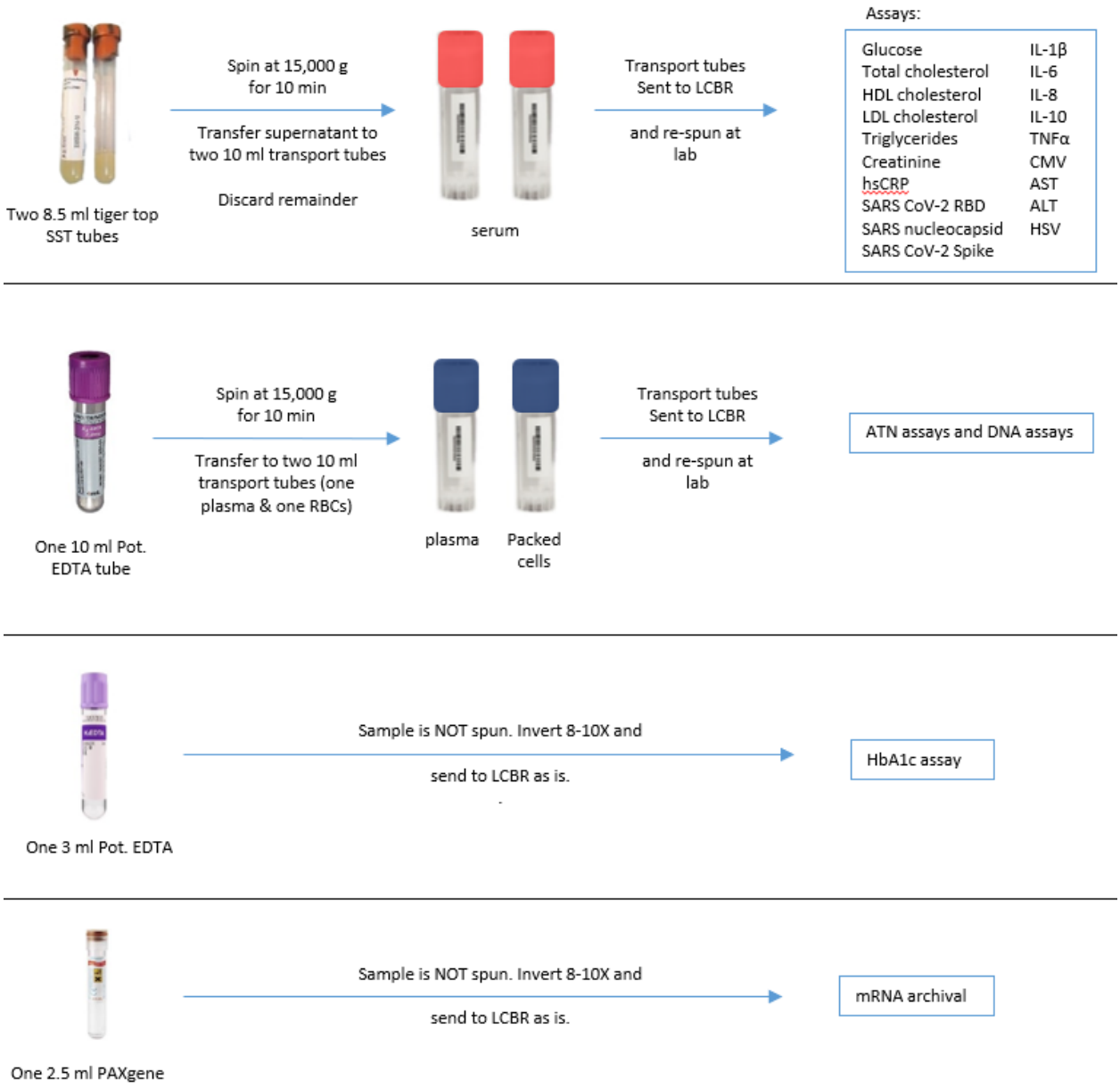

Figure 3. Blood Processing Protocol

After processing the blood, FEs took a loose barcode label provided in the Blood Collection Kit and affixed it to a paper manifest designed to accompany the transport tubes to LCBR. The loose barcode label matched the barcode labels on the transport tubes and the Shipping Box. FEs recorded all vacutainer tubes that were collected and identified all difficulties during blood draw or processing on the manifest as well as in the tablet. The barcode-labelled manifest was designed to be scanned on arrival at LCBR to associate it with an individual participant's transport tubes.

3.3.3 Shipment of Samples

Immediately before shipment, FEs removed two cold packs from the freezer, placed the transport tubes in a sleeve, sandwiched the transport tubes between the ice packs, enclosed the sandwich within the Styrofoam box, placed the manifest on top of the Styrofoam box, sealed the cardboard shipping box around it, put the cardboard shipping box inside the Tyvek envelope, applied a pre-printed FedEx shipping label to the envelope, carried it to a FedEx office, and handed it to a FedEx representative (*in person*) for Priority Overnight shipment to LCBR with arrival the following morning. FEs were not permitted to leave shipments at unattended FedEx drop boxes.

When overnight shipment was impossible, FEs noted this on the manifest and held unboxed transport tubes in a refrigerator approved for biological specimens or cooler with enough cold packs to keep them chilled at 4^{o} C overnight without risk of freezing (or thawing), as is possible on wet or dry ice. The transport tubes were packaged and shipped the next day using freshly frozen cold packs.

3.3.4 Receipt of Samples at LCBR

LCBR technicians specifically trained for Add Health Wave V received and immediately processed samples each morning. They unpacked the shipping boxes one at a time, evaluated the volume and quality of each transport tube, and entered them into a custom-made laboratory information management system (LIMS) program.

After re-centrifuging the serum samples at 4° C for 10 min at 30,000 g, the technicians aspirated the supernatant, discarded all remaining precipitate, transferred the aspirate to pre-labelled tubes, and placed them in a biospecimen refrigerator for archival (in 1 ml aliquots at -80° C) or assay (500 ul aliquot). The LCBR technicians entered all aliquot information into the LIMS system.

3.3.5 Preparation of Samples for Infection Biomarker Assays

The biomarker assays for infectious exposures were run using archived serum samples. On the day of assay, serum aliquots initially archived at -80°C were thawed in a 36°C water bath for 10 minutes, vortexed, and centrifuged. From the aliquots, enough sample was removed to run the specific assay, and the remaining specimens were re-archived at -80°C. The mean (standard deviation) and median archival-assay intervals (days) are listed in **Figure 4**.

	Archive-Assay Interval (days)	
Analyte	**Mean (SD)**	**Median**
HSV	366.7 (169.8)	261.0
CMV	396.4 (133.1)	376.0
SARS-CoV-2	96.0 (62.5)	90.0

Figure 4. Interval between initial archive and assay run date

4. Assay and Internal Quality Control

4.1 Herpes Simplex Virus IgG [H6HSV]

4.1.1 Rationale

Herpes simplex virus types 1 and 2 (HSV-1 and 2) belong to a family of DNA viruses, Herpesviridae, which includes cytomegalovirus (CMV), varicella zoster virus (VZV), and Epstein-Barr virus (EBV), among others. Historically, HSV-1 was linked to oral herpes and HSV-2 to genital herpes, although that pattern has been evolving[1] and both neurotropic viruses have been implicated in a range of other conditions in humans, including Alzheimer's Disease.[2,3,4] HSV-1 and 2 infections can be acquired before birth (congenital), at birth (perinatal) or later in life (postnatal). HSV-1 is approximately 5 times higher in prevalence than HSV-2 in the US. At the infection site, HSV can replicate in epithelial cells; HSV-1 can spread to the spinal cord and brain via peripheral sensory neurons; establish latent infection; and be reactivated / disseminated under stress or immunosuppression. HSV infections are frequent and occasionally severe in immunosuppressed individuals such as patients with AIDS, HIV, cancer, or organ transplants.

4.1.2 HSV IgG Assay Principle

HSV Immunoglobulin G (IgG) assays were run using a Gold Standard Diagnostics Enzyme Immunoassay Test Kit (720-340, Davis, CA) intended for the qualitative and semi-quantitative detection of IgG antibodies to HSV-1 and/or 2 in human serum by an indirect enzyme immunoassay. The results were objective and were reported in equivalent units per milliliter (EU/ml), which were traceable to in-house reference materials[5].

Diluted samples were incubated with HSV-1 and 2 antigens bound to the solid surface of a microtiter well. If IgG antibodies against HSV were present in the samples, they bound to the antigen forming antigen-antibody complexes. Residual sample was eliminated by aspirating and washing. Conjugate (horseradish peroxidase-labeled anti-human IgG) was added and would bind to these complexes. Unbound conjugate was removed by aspiration and washing. Substrate was then added and incubated. In the presence of bound enzyme, the substrate was converted to an end product. The absorbance of

this end product was read spectrophotometrically at 450 nm (reference 600-630 nm) and directly proportional to the concentration of IgG antibodies to HSV-1 and 2 antigens present in the sample.

4.1.3 HSV IgG Assay Protocol

HSV IgG assays were run in two batches. Batch 1 (n = 2500) was run in 2024, and Batch 2 (n = 3147) was run in 2025. Thawed serum samples were initially diluted 1:101 by adding 2.5 µl of sample to 250 µl of diluent. A known standard of 100 EU/ml was provided in the kit for creation of calibration curves, along with positive and negative controls. The calibrators and standards were provided ready-to-use so no processing or dilution was required. All other reagents were prepared according to the manufacturer's specifications.

All components were allowed to come to room temperature and gently mixed prior to using. 100 µl of samples, standards, and controls were added to respective wells in each 96-well plate. The plates were incubated uncovered at 37°C for one hour. Each well was washed three times with wash solution. 100 µl of conjugate was then added and plates were incubated as before and then washed. 100 µl of substrate was then added and incubated uncovered for 20 minutes. At the end of the incubation, 100 µl of stop solution was added to each well. The plate was then inserted into a Biotek ELx808IU plate reader and the absorbance was read at 450 nm, using a reference wavelength of 600 nm. Any samples that were too high to read on the standard curve were diluted 1:10 and then run again.

Calculation of sample and control results were derived from the following equation, provided by the manufacturer:

$$\frac{\frac{EU}{ml} of\ Calibrator}{Absorbance\ of\ Calibrator}\ X\ \ Aborbance\ of\ Sample\ =\ \frac{EU}{ml} of\ sample$$

Detectable ranges and kit control means and coefficients of variation (CVs) for both batches follow (**Figure 5**).

Batch	Detectable Range	Kit Control 1 (negative)		Kit Control 2 (positive)	
	HSV IgG (EU/ml)	Mean (EU/ml)	CV (%)	Mean (EU/ml)	CV (%)
1	0 - 100	2.57	15.58	56.57	3.97
2	0 - 100	2.31	23.52	57.7	5.31

Figure 5. Limits of detection and Kit Control Means for HSV IgG

Data reduction software at LCBR extrapolated values for some data points beyond the detectable range of the standard curve. Final HSV IgG concentrations (H6HSV) ranged from 0.00 to 120.87 EU/ml. The kit-recommended HSV IgG classification (H6CHSV) follows (**Figure 6**).

HSV IgG (EU/ml)	Classification (H6CHSV)
<16.0	Negative for anti-HSV IgG
16.0 – 19.9	Equivocal for anti-HSV IgG
≥20.0	Positive for anti-HSV IgG

Figure 6. HSV IgG Classification

4.1.4 HSV IgG Internal Quality Control

The BioTek reader was maintained following manufacturer specifications, which included cleaning exposed surfaces, cleaning wavelength filters, lubricating robotic components, and cleaning lamp contacts.

Each assay run included both negative and positive controls, as well as a zero blank well (sample diluent only). All blanks were required to have readings of <0.2 EU/ml, all negative controls had to read <16.0 EU/ml, and positive controls needed to be within the assigned range printed on the vial, which changed per kit lot.

In addition to the daily quality control, LCBR used two pools of samples from twenty normal donors (US Biologicals, Salem, MA) in longitudinal quality control analyses. One pool was an EDTA plasma normal donor pool (Lot #E011221). The other pool was a serum normal donor pool (Lot #S120419). LCBR periodically assayed both pools over the course of Wave VI. The plasma and serum HSV concentration mean (coefficient of variation) based on those assays was 91.09 EU/ml (2.56%) and 87.47 EU/ml (3.04%), respectively. When any concentrations exceeded acceptable parameters, the BioTek reader was investigated and repaired.

4.2 Cytomegalovirus IgG [H6CMV, H6CMVZ]

4.2.1 Rationale

As mentioned above, CMV belongs to a family of DNA viruses, Herpesviridae, which includes HSV-1 and 2, VZV, and EBV. CMV is associated with a range of conditions in humans, including poorer domain-specific cognitive performance, incident mild cognitive impairment, and dementia.[4] CMV infections can be acquired before birth (congenital), at birth (perinatal) or later in life (postnatal). CMV infections are frequent and occasionally severe in immunosuppressed individuals such as patients with AIDS, HIV,

cancer, or organ transplants. Such infections may represent reactivation of latent virus or primary infection introduced by blood transfusion or transplanted organ.[6,7,8,9]

4.2.2 CMV IgG Assay Principle

CMV IgG assays were run using a Gold Standard Diagnostics Enzyme Immunoassay Test Kit (720-320, Davis, CA) intended for the qualitative and semi-quantitative detection of IgG antibodies to CMV in human serum by an indirect enzyme immunoassay. The results were objective and were reported in EU/ml, which were traceable to in-house reference materials.[10]

Diluted samples were incubated with CMV antigen bound to the solid surface of a microtiter well. If IgG antibodies against CMV were present in the samples, they bound to the antigen, forming antigen-antibody complexes. Residual sample was eliminated by aspirating and washing. Conjugate (horseradish peroxidase-labeled anti-human IgG) was added and bound to the complexes. Unbound conjugate was removed by aspiration and washing. Substrate was then added and incubated. In the presence of bound enzyme, the substrate was converted to an end product. The absorbance of this end product was read spectrophotometrically at 450 nm (reference 600-630 nm) and directly proportional to the concentration of IgG antibodies to CMV antigen present in the sample.

4.2.3 CMV IgG Assay Protocol

CMV IgG assays were run in two batches. Batch 1 (n = 2498) was run in 2024, and Batch 2 (n = 3163) was run in 2025. Thawed serum samples were diluted 1:101 by adding 2.5 µl of sample to 250 µl of diluent per manufacturer recommendations. These samples were then diluted one additional time at a 1:10 dilution. Three standards for creating calibration curves were provided in the kit, with known values of 0, 10, and 160 EU/ml. Three controls were also included: a high positive, a low positive, and a negative control. All other reagents were prepared according to manufacturer's specifications.

All components were allowed to come to room temperature and gently mixed prior to using. 100 µl of samples, standards, and controls were added to respective wells in each 96-well plate. The plates were incubated uncovered at 37°C for one hour. Each well was washed three times with wash solution included at 37°C for one hour and then washed three times with wash buffer. 100 µl of conjugate was added to each well, and incubated uncovered for one hour, and then wells were washed three times again. 100 µl of substrate was then added and plates were incubated as before but not washed. At the end of the incubation, 100 µl of stop solution was added to each well. The plate was then inserted into a Biotek ELx808IU plate reader and the absorbance was read at 450 nm, using a reference wavelength of 600 nm. Any samples that were too high to read on the standard curve were diluted 1:10 and then run again. Detectable ranges and kit control means and coefficients of variation (CVs) for both batches follow (**Figure 7)**.

Batch	Detectable Range	Kit Control 1		Kit Control 2		Kit Control 3	
	CMV IgG (EU/ml)	Mean (EU/ml)	CV(%)	Mean (EU/ml)	CV(%)	Mean (EU/ml)	CV(%)
1	10 - 1600	1.20	33.46	14.3	5.96	53.1	8.77
2	0.9 - 1600	0.63	39.18	17.43	7.13	71.25	8.62

Figure 7. Limits of Detection and Kit Control Means for CMV IgG

Data reduction software at LCBR extrapolated values for some data points beyond the detectable range of the standard curve. Final CMV concentrations (H6CMV) ranged from 0.18 to 1374.57 EU/ml. The kit-recommended CMV IgG classification (H6CCMV) follows (**Figure 8**).

CMV IgG (EU/ml)	Classification (H6CCMV)
<8.0	Negative for anti-CMV IgG
8.0 – 9.9	Equivocal for anti-CMV IgG
≥10.0	Positive for anti-CMV IgG

Figure 8. Classification of CMV IgG results

4.2.4 CMV IgG Internal Quality Control

The BioTek reader was maintained following manufacturer specifications, which included cleaning exposed surfaces, cleaning wavelength filters, lubricating robotic components, and cleaning lamp contacts.

In addition to the daily quality control, LCBR used two pools of samples from twenty normal donors (US Biologicals, Salem, MA) in longitudinal quality control analyses. One pool was an EDTA plasma normal donor pool (Lot #E011221). The other pool was a serum normal donor pool (Lot #S120419). LCBR periodically assayed both pools over the course of Wave VI. The plasma and serum CMV IgG concentration mean (coefficient of variation) based on those assays was 152.49 EU/ml (9.06%) and 237.12 EU/ml (9.05%), respectively. When concentrations exceeded acceptable parameters, the BioTek reader was investigated and repaired.

4.2.4.1 Inter-Conversion Between Batch 1 and 2 CMV IgG Concentrations

After completing Batch 2 of CMV IgG assays, between-batch differences were noted in the values of the serum and EDTA donor pools. In addition, discrepancies in concentrations were also apparent between

the two batches. As indicated, the mean and percentiles in Batch 1 were lower than in Batch 2 (**Figure 9**). Furthermore, the percent Negative for Anti-CMV IgG was higher in Batch 1 than Batch 2, while the percent Positive for Anti-CMV IgG was lower in Batch 1 than Batch 2 (**Figure 10**).

CMV IgG (EU/ml)							
Batch	**n***	**Mean**	**Minimum**	**25th Percentile**	**50th Percentile**	**75th Percentile**	**Maximum**
1	2060	118.12	0.18	7.57	70.03	164.12	1374.57
2	2975	209.87	0.96	13.15	122.58	306.05	1458.86
* This data includes values that were extrapolated above and below the limits of detection							

Figure 9. CMV IgG Assay Results per Batch

		n* (%)	
CMV IgG (EU/ml)	**Classification**	**Batch 1**	**Batch 2**
<8.0	Negative for Anti-CMV IgG	840 (35.73)	96 (3.78)
8.0 – 9.9	Equivocal for Anti-CMV IgG	132 (5.61)	142 (4.70)
≥ 10	Positive for Anti-CMV IgG	1379 (58.66)	2783 (92.72)
* Counts include values that were extrapolated above and below the limits of detection, as well as undetectable low and high values.			

Figure 10. Batch Differences in CMV IgG Classifications

Upon investigation, LCBR found similar results in a different cohort study that also measured CMV in both 2024 and 2025 using the same assay kit from Gold Standard Diagnostics. A comparison between the EDTA and Serum donor pools for both studies, comparing 2025 to 2024 results yielded nearly identical results. Based on them, it was believed that the standards included in the 2025 Gold Standard Diagnostics assay kits were improperly quantified by the manufacturer. A CMV IgG assay batch identifier (H6CMVB) was therefore constructed (**Figure 11**).

Identification	Description
1	CMV IgG Batch 1
2	CMV IgG Batch 2

Figure 11. CMV IgG Assay Batch Identifier

Thereafter, CMV IgG concentrations from the normal donor pool of 20 serum samples in Batch 2 were plotted against those in Batch 1 (**Figure 12**).

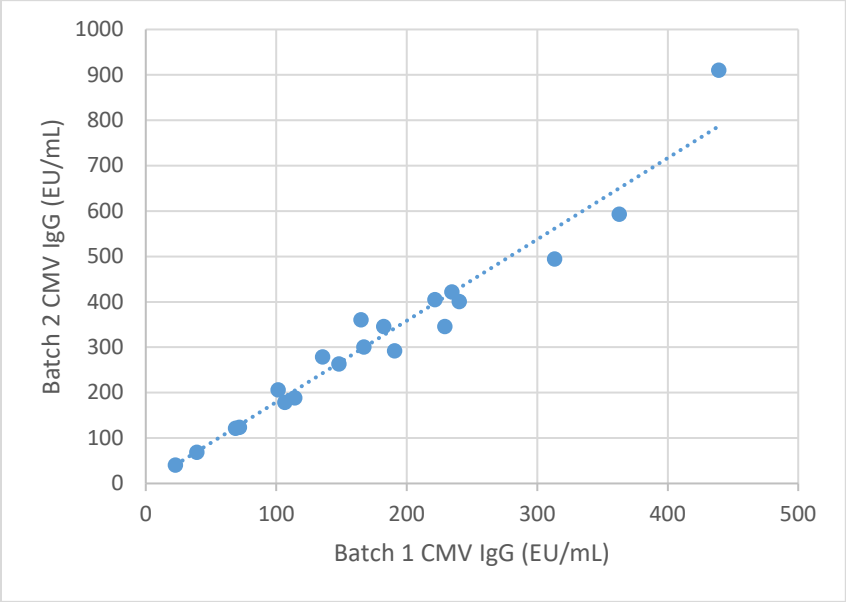

Figure 12. Batch 2 vs. 1 (2025 vs. 2024) CMV IgG Concentrations in 20 Serum Samples

The association between the batched concentrations was modeled by LCBR using linear regression yielding an equation (intercept = 0.0; slope = 1.7921) that was used to convert all Batch 2 to Batch 1 concentrations by dividing them by 1.7921, thereby yielding final CMV IgG concentrations in EU/mL (H6CMV) with similarly negative, equivocal, and positive percentages in both batches. After conversion, the concentrations also were z-transformed by subtracting the overall mean and dividing by the corresponding standard deviation yielding values comparable to those at Wave V. The final z-transformed CMV concentrations (H6CMVZ) ranged from -0.77 to 8.20, in standard deviation units.

4.3 Severe Acute Respiratory Syndrome Coronavirus 2 IgG [H6SIGG, H6NCIGG, H6RBDIGG]

4.3.1 Rationale

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is a single-stranded RNA virus that has spike, envelope, membrane, and nucleocapsid proteins. The receptor-binding domain (RBD) of the spike protein attaches to host receptors, thereby initiating SARS-CoV-2 infection and replication. Infection and replication typically cause mild coronavirus disease (COVID-19), the condition at the center of the eponymously named pandemic of 2019-2023 characterized by fatigue, cough, fever, and loss of smell or taste. However, infection can progress, causing pneumonia, respiratory failure, or death, particularly among older, unhealthy, and / or immunosuppressed persons. Moreover, infection can involve persistent symptoms including cognitive difficulties (long COVID-19), underscoring its putative relevance for risk of Alzheimer's disease and related dementias.[11,12,13]

4.3.2 SARS-CoV-2 IgG Assay Principle

Samples were analyzed for SARS-CoV-2 spike, nucleocapsid, and RBD IgG using a highly sensitive electrochemiluminescent immunoassay on the MesoScale Diagnostics (MSD) platform (V-PLEX COVID-19 Coronavirus Panel 2; K15383-U) and read using an MSD QuickPlex 120 (MesoScale Diagnostics, Rockville, MD). This platform allowed for the simultaneous quantification of multiple analytes in a single assay. V-PLEX COVID-19 Serology Kits were available as panels defined by a set of antigens coated on a 10-spot MULTI-SPOT® 96-well plate. Each kit included a reference standard for quantitation, controls, plate(s), anti-human IgG antibodies, and all other reagents necessary to conduct the assay including detection antibody conjugated with an electrochemiluminescent label; calibrators; and diluents and buffers to be used as needed.

The assay principle was as follows: Capture antibodies for each target were printed as arrays in discrete spots at the bottom of each test well in a 96-well plate. Following the addition of sample or controls and capture of the target, detection antibody with an electrochemiluminescent label was added to the wells and an electrical impulse generated light in proportion to bound target at each spot in the array. The MSD QuickPlex 120 then measured the intensity of emitted light, which was proportional to the amount of analyte present in the sample[14] (**Figure 13**). This method had a wide dynamic range, low background, high sensitivity, and it required only a small volume of sample to generate results for multiple analytes in a single assay.

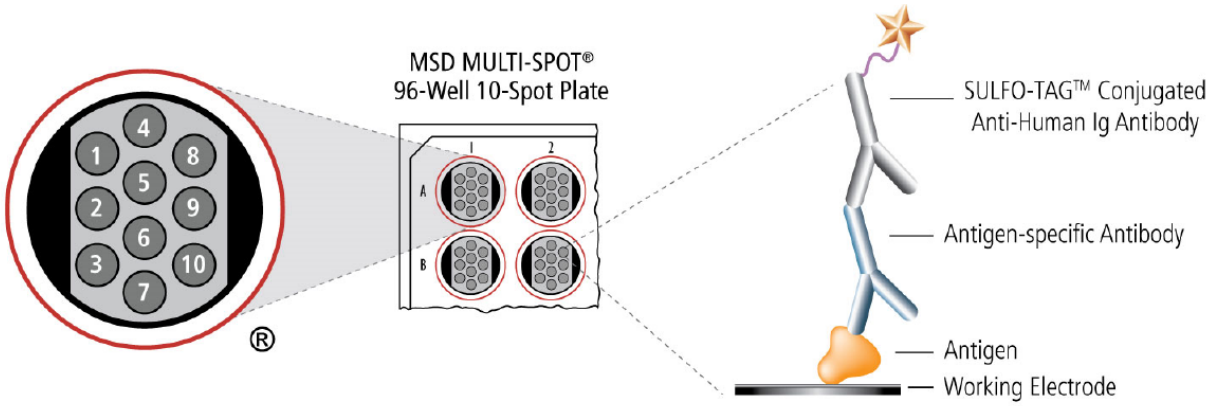

Figure 13. Schematic for V-Plex COVID-19 Serology Kits

4.3.3 SARS-CoV-2 IgG Assay Calibration and Control preparation

A serum-based reference standard supplied in the kit (MSD, COOADK-2) was diluted 1:10 in supplied diluent that yielded the highest recommended calibration concentration in arbitrary units per milliliter (AU/mL). This calibrator was then serially diluted in a 1:4 ratio to make a total of 7 calibrators, plus a

zero blank. These calibrators were used throughout the assay period and were rerun on a continual basis to ensure consistent machine calibration. The calibrators were stable through -80°C freeze-thaw cycles, so only one lot was used throughout the testing period.

MSD also supplied a serology human control pack (MSD, C4381-1) which contained 3 controls of known concentrations of human IgG against antigens in the V-Plex kit. These controls were supplied ready-to-use at working concentrations, so no dilution or processing was required. Again, the controls were stable through multiple freeze-thaw cycles, and thus only one lot was required throughout the testing period. Mean and %CV values for the three controls follow (**Figure 14**).

Analyte	Kit Control 1		Kit Control 2		Kit Control 3	
	Mean (AU/ml)	CV(%)	Mean (AU/ml)	CV(%)	Mean (AU/ml)	CV(%)
Spike IgG	105038.95	3.81	46579.72	4.15	9907.41	3.30
Nucleocapsid IgG	109311.81	3.51	40695.18	3.88	21796.49	5.37
RBD IgG	47086.41	9.80	19055.94	11.26	4563.42	6.54

Figure 14. Mean Values of Human Control Samples (AU/ml)

4.3.4 SARS-CoV-2 Assay Protocol

Serum samples were diluted 1:500 by first adding 3 µl of sample to 297 µl of diluent, vortexing, and then taking 10 µl of the prepared samples and adding it to 490 µl of diluent. Calibration standards and controls were prepared as stated above. All MSD diluents, detection antibodies, wash buffers, phosphate-buffered saline, and read buffers were prepared according to manufacturer recommendations.[14]

All plates were incubated with a blocker solution for one hour at room temperature and then washed three times with wash solution while gently shaking on a plate shaker. The blocker was removed and then 50 µl of calibrator, controls, and samples were added to each well. The plates were sealed with an adhesive plate seal and incubated at room temperature for 2 hours while shaking. Plates were then washed again three times with 250 µl wash buffer, and 50 µl of detection antibody solution was added to each well. The plate was sealed and incubated at room temperature for 1 hour while shaking. The plates were then washed again three times with 150 µl wash buffer, and 150 µl of MSD read buffer was added to each well. The plate was then analyzed on the MSD QuickPlex 120. A 4-parameter logistic curve was generated to fit the signal and calibrator concentrations, and the concentrations of samples were calculated based on the standard curve for each analyte. Data reduction software at LCBR extrapolated values for some data points beyond the detectable range of the standard curve. Analyte ranges (calibrator; detectable; result) and kit-recommended classifications (negative; positive) follow

(**Figures 15-16)**. It is important to note that a natural log or other normalizing transformation may be helpful in analyzing these right-skewed concentrations.

	Concentration (AU/mL)		
Analyte	**Calibrator Range**	**Detectable Range**	**Result Range**
Spike IgG	0.043 –- 720	10.71 – 3,600,000	6.15 – 1,784,906
Nucleocapsid IgG	0.042 – 700	8.3 – 3,500,000	14.87 – 1,820,111
RBD IgG	0.017 – 290	9.97 – 1,450,000	9.30 – 1,753,332

Figure 15. Standard Curve and Detectable Range of SARS-CoV-2 analytes

	Analyte Concentration (AU/mL)			
Classification	**Spike IgG (H6CSIGG)**	**Nucleocapsid IgG (H6CNCIGG)**	**RBD IgG (H6CRBDIGG)**	**Classification**
0	< 1960	< 5000	< 538	Negative
1	≥ 1960	≥ 5000	≥ 538	Positive

Figure 16. SARS-CoV-2 Analyte Classifications

4.3.5 SARS-CoV-2 IgG Internal Quality Control

All assays were run by the same technicians, using a single lot number of reagents from MSD and the same set of calibrators and controls that were manufactured at the beginning of the assay period. The MSD Quick-Plex 120 is a maintenance free, self-check analyzer with built-in status monitor. The status monitor maintained and recorded the functional status of the onboard camera which must operate at -25°C. Operational equipment was relayed to end user as a constant blue "okay" light. If the unit failed self-maintenance checks, the analyzer displayed a red light and would not function. A calibration curve was generated on each kit plate and was evaluated by running the human control pack listed above that had defined ranges for each level (low, high) of kit quality control per analyte on the panel. Quality control data was run every 36 samples.

In addition to the daily quality control, LCBR used two pools of samples from twenty normal donors (US Biologicals, Salem, MA) in longitudinal quality control analyses. One pool was an EDTA plasma normal donor pool (Lot #E011221). The other pool was a serum normal donor pool (Lot #S120419). LCBR periodically assayed both pools over the course of the assay period. The plasma and serum concentration means and coefficients of variation (CVs) based on those assays follow (**Figure 17**). When concentrations exceeded acceptable parameters, the MSD analyzer was investigated and repaired.

Analyte	Serum		Plasma	
	Mean (AU/ml)	CV (%)	Mean (AU/ml)	CV (%)
Spike IgG	106.10	11.45	2,663.1	4.19
Nucleocapsid IgG	708.21	6.32	2,305.38	7.17
RBD IgG	67.65	33.91	1,186.15	9.29

Figure 17. Plasma and Serum Control Values

5. External Quality Control

5.1 Reliability of HSV, CMV and SARS-CoV-2 IgG

Within race/ethnicity- and sex-stratified random samples of 136, 126, and 24 Add Health participants among whom venous blood was collected twice, on average 13.1 (95% confidence interval: 11.9-14.2), 13.1 (11.9-14.3), and 13.2 (10.8-15.7) days apart, typically by the same FE and at approximately the same time of day, the reliability of log-transformed HSV IgG (EU/ml), CMV IgG (EU/ml), and SARS-CoV-2 IgG (AU/mL) was estimated as an intra-class correlation coefficient (ICC, 95% confidence interval) (**Figure 18**). The estimates suggest that the home exam venous blood collected at Add Health Wave VI yields highly reliable measures of HSV, CMV, and SARS-CoV-2 IgG.

Analyte*	N	ICC	95% CI
HSV IgG	136	0.99	(0.99, 0.99)
CMV IgG	126	0.96	(0.95, 0.98)
SARS-CoV-2 Spike IgG	24	1.00	(0.99,1.00)
Nucleocapsid IgG	24	1.00	(0.99,1.00)
RBD IgG	24	0.99	(0.99,1.00)
***Log-transformed.**			

Figure 18. Reliability of HSV, CMV, and SARS-CoV-2 IgG

6. Coronavirus Disease and Vaccinations [H6EQ**]

Section E of the home exam asked questions about past coronavirus disease (COVID-19)—including acute and long COVID-19 symptomology—and vaccinations for COVID-19 and other diseases. The

COVID-19 vaccine data included numbers, dates, and types of COVID-19 vaccines. Please refer to Section 8.2 for a full variable list.

7. Constructed Measures

7.1 COVID-19 Vaccination Status [H6COVVC]

COVID-19 vaccination status was determined from the self-reported answers to three questions: H6EQ06A, H6EQ07, and H6EQ08, as follows:

COVID-19 Vaccination Status (H6COVVC)	COVID-19 Vaccination Past 2 Weeks? (H6EQ06A)		COVID-19 Vaccination Ever? (H6EQ07)		COVID-19 Vaccination Doses (H6EQ08)
Yes	1	or	1	or	(≥ 1 or -9998)
No	0	and	0	and	< 1
Unknown	(., 0, or -9995)	and	(., 0, or -9996)	and	(. or -9997)

-9995 = question not asked. -9996 = question refused. -9997 = question skipped. -9998 = don't know.

Figure 19. COVID-19 Vaccination Status

7.2 Interpretation of Vaccination Status and IgG Positivity [H6COVINTP]

COVID-19 vaccination status and SARS-CoV-2 spike and nucleocapsid IgG concentrations can be used to help differentiate Wave VI participants vaccinated against and / or infected by COVID-19 within days to weeks of vaccination or acute SARS-CoV-2 infection, as previously described by the Centers for Disease Control and Prevention (CDC) (**Figure 20**).[15] However, these are research assay, not clinical test results, and their interpretation can be affected by many factors. For example, potential false positive or false negative results, failure to develop detectable IgG after vaccination or infection, and waning of IgG with time after infection or vaccination should be considered when interpreting these assay results. Indeed, a negative IgG test does not rule out previous infection and some people infected with SARS-CoV-2 may not develop measurable IgG, thereby limiting the sensitivity of IgG assays to detect previous infection.

COVID-19 Vaccination Status (H6COVVC)	Spike IgG Classification (H6CSIGG)	Nucleocapsid IgG Classification (H6CNCIGG)	CDC Interpretation* (H6COVINTP)
Yes	Positive	Positive	Vaccinated & previous infection
Yes	Positive	Negative	Vaccinated & no previous infection
Yes	Negative	Negative	Vaccinated & no previous infection
No	Positive	Positive	Unvaccinated & previous infection
No	Positive	Negative	Unvaccinated & previous infection
No	Negative	Positive	Unvaccinated & previous infection
No	Negative	Negative	Unvaccinated & no previous infection
Unknown	Positive	Positive	Unknown vaccine status & previous infection
Unknown	Positive	Negative	Vaccinated & no previous infection
Unknown	Negative	Negative	Unvaccinated & no previous infection

*See caveats in Section 7.2. CDC = Centers for Disease Control.

Figure 20. Interpretation of Vaccination Status and Assay Results

7.3 Concentration Type Variables

All assay concentrations were categorized based on their corresponding limits of detection (LODs) and extrapolation beyond them, then flagged as tabulated below (**Figures 21-26**). *Warning: Users should recognize extrapolated concentrations as such and exercise caution when working with them. Moreover, theoretical and computational recommendations for properly analyzing left- and right-censored concentrations falling beyond limits of detection under both frequentist and Bayesian frameworks can be found elsewhere.*[16]

7.3.1 Flag for HSV IgG Concentration Type [H6HSVFL]

Code	Description
1	HSV concentration is below the lower LOD (<0 EU/ml) - missing
3	HSV concentration is within the LODs (0 -100 EU/ml)
4	HSV concentration is above the upper LOD (> 100 EU/ml), but extrapolated

LOD = limit of detection.

Figure 21. Flag for HSV Concentration Type

7.3.2 Flag for CMV IgG Concentration Type [H6CMVFL]

Code	Description
1	CMV concentration is below the lower LOD (batch1:<10, batch2:<0.9 EU/ml) -missing
2	CMV concentration is below the lower LOD (batch1:<10, batch2:<0.9 EU/ml), but extrapolated
3	CMV concentration is within the LODs (batch1: 10-1600, batch2: 0.9-1600 EU/ml)
5	CMV concentration is above the upper LOD (> 1600 EU/ml) – missing
LOD = limit of detection.	

Figure 22. Flag for CMV Concentration Type

7.3.3 Flag for Testing Status of SARS-CoV-2 [H6COVFL]

Code	Description
0	Sample present but SARS-CoV-2 not tested
1	Sample present and SARS-CoV-2 tested

Figure 23. Flag for SARS-CoV-2 Testing Status

7.3.4 Flag for SARS-CoV-2 Spike IgG Concentration Type [H6SIGGFL]

Code	Description
2	Spike IgG concentration is below the lower LOD (< 10.71 AU/ml), but extrapolated
3	Spike IgG concentration is within the LODs (10.71 – 3,600,000 AU/ml)
LOD = limit of detection.	

Figure 24. Flag for SARS-CoV-2 Spike IgG Concentration Type

7.3.5 Flag for SARS-CoV-2 Nucleocapsid IgG Concentration Type [H6NCIGGFL]

Code	Description
3	NC IgG concentration is within the LODs (8.3 – 3,500,000 AU/ml)
LOD = limit of detection.	

Figure 25. Flag for SARS-CoV-2 Nucleocapsid IgG Concentration Type

7.3.6 Flag for SARS-CoV-2 Receptor Binding Domain IgG Concentration Type [H6RBDIGGFL]

Code	Description
1	RBD IgG concentration is below the lower LOD (< 9.97 AU/ml) - missing
2	RBD IgG concentration is below the lower LOD (< 9.97 AU/ml), but extrapolated
3	RBD IgG concentration is within the LODs (9.97 – 1,450,000 AU/ml)
4	RBD IgG concentration is above the upper LOD (> 1,450,000 AU/ml), but extrapolated
LOD = limit of detection.	

Figure 26. Flag for SARS-CoV-2 Receptor Binding Domain IgG Concentration Type

8. The Infection Data File (binfect6.sas7bdat)

8.1 Structure

The structure of the disseminated infection data file is flat. This means that it is a participant-level data file, where each participant has one and only one record. The participant's identifying number (the AID variable) will appear in each data file only once.

8.2 Contents

The infection data file (binfect6.sas7bdat) includes the variables below, which are described in the corresponding codebook documentation that also contains frequencies.

Variable Name	Variable Description
AID	Participant Identifier
H6HSV	Herpes simplex virus IgG (HSV, EU/mL)
H6CHSV	HSV IgG classification
H6CMV	Cytomegalovirus IgG (CMV, EU/mL)
H6CMVZ	CMV Z-Score (CMV, STD)
H6CCMV	CMV IgG classification
H6CMVB	CMV IgG assay batch
H6SIGG	Spike IgG (AU/mL)
H6CSIGG	Spike IgG classification (CDC)
H6NCIGG	Nucleocapsid IgG (AU/mL)
H6CNCIGG	Nucleocapsid IgG classification (CDC)
H6RBDIGG	Receptor Binding Domain IgG (AU/mL)
H6CRBDIGG	Receptor Binding Domain IgG classification (CDC)

H6EQ01	EQ01 Ever diagnosed with COVID-19
H6EQ02	EQ02 Ever used COVID-19 medication
H6EQ03	EQ03 Ever diagnosed with long COVID-19
H6EQ04	EQ04 Long COVID-19 symptoms
H6EQ05M	EQ05M Month of symptoms started
H6EQ05YR	EQ05YR Year of symptoms started
H6EQ06A	EQ06a COVID-19 vaccine in past two weeks
H6EQ06B	EQ06b Influenza vaccine in past two weeks
H6EQ06C	EQ06c Shingles vaccine in past two weeks
H6EQ06D	EQ06d Tetanus vaccine in past two weeks
H6EQ06E	EQ06e Other vaccine in past two weeks
H6EQ07	EQ07 Ever received COVID-19 vaccine
H6EQ08	EQ08 Number of doses of COVID-19 vaccination
H6EQ09M	EQ09M Month of first COVID-19 vaccination
H6EQ09YR	EQ09YR Year of first COVID-19 vaccination
H6EQ10M	EQ10M Month of last COVID-19 vaccination
H6EQ10YR	EQ10YR Year of last COVID-19 vaccination
H6EQ11A	EQ11a Pfizer COVID-19 vaccine
H6EQ11B	EQ11b Moderna COVID-19 vaccine
H6EQ11C	EQ11c Janssen COVID-19 vaccine
H6EQ11D	EQ11d Novavax COVID-19 vaccine
H6EQ11E	EQ11e AstraZeneca COVID-19 vaccine
H6EQ11F	EQ11f Unsure/unknown COVID-19 vaccine
H6EQ11G	EQ11g Other COVID-19 vaccine
H6COVVC	Ever been vaccinated for COVID-19
H6COVINTP	CDC interpretation of vaccination status and IgG positivity
H6HSVFL	HSV IgG concentration type
H6CMVFL	CMV IgG concentration type
H6COVFL	SARS-CoV-2 IgG tested/not tested
H6SIGGFL	SARS-CoV-2 Spike IgG concentration type
H6NCIGGFL	SARS-CoV-2 Nucleocapsid IgG concentration type
H6RBDIGGFL	SARS-CoV-2 Receptor Binding Domain IgG concentration type

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