

**Report prepared by**

Eric A. Whitsel

Robert Angel

Rick O'Hara

Lixin Qu

Kathryn Carrier

Kathleen Mullan Harris

# Neurodegeneration



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 neurodegeneration at the Wave V home exam. This document is one in a set of Wave V user guides. User guides are also available to describe protocols for the following biological measures at Wave V:

- Anthropometrics
- Cardiovascular Measures
- Glucose Homeostasis
- Medication Use – Home Exam
- Inflammation and Immune Function
- Lipids
- Renal Function
- Baroreflex Sensitivity & Hemodynamic Recovery

## Acknowledgement

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## 1. Introduction

Wave V measures of neurodegeneration 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 Neurodegeneration Biomarker Concentrations

- Neurofilament Light (NfL, pg/ml)
- Tau (pg/ml)

Moreover, the restricted use Add Health Wave V data include two constructed measures designed to facilitate analysis and interpretation of neurodegeneration biomarker concentrations:

- Flag Indicating the NfL Concentration Type
- Flag Indicating the Tau Concentration Type

## 2. General Overview of Data Collection

All Wave V venous blood samples were collected during home exams performed by FEs from two Add Health data collection partners: Examination Management Services, Inc. (2016–2017) and Hooper Holmes, Inc. (2018–2019). All FEs were trained and certified using a custom program specific to the Add Health protocol. FEs used a 7" Samsung Galaxy Tab 4 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 respondents. 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 respondent. All respondents 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, 91.8% of the respondents agreed to and completed the blood draw. Of the remainder, 6.5% refused, 1.3% agreed but the blood draw was unsuccessful, and < 1% had exams terminated before the blood draw (see the blood draw status variable **H5BLOOD** in the *bdemo5* 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° C (on ice or frozen cold packs) for up to two hours. Subsequent processing involved centrifuging specific tubes then aliquoting

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serum and plasma into color-coded transport tubes pre-labelled with unique barcode identifiers linking the blood to a particular respondent. 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 a pre-specified panel of metabolic, hematologic, inflammatory, immune, and renal biomarkers. 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, e.g., of the neurodegeneration biomarkers described herein.

#### 3.2 Equipment

Before exams, FEs were shipped a Visit Supply Kit (Exhibit 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) 6 ml sodium fluoride/potassium oxalate (NaF/KOx)-containing vacutainer tube, if needed for the glucose sub-study (see Section 4.1.2.1)
- (1) 3- or 4- ml potassium ethylenediaminetetraacetic acid (EDTA)-containing vacutainer tube
- (1) 10 ml EDTA-containing vacutainer tube
- (1) 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 the vacutainer and transport tubes. As of February 2018, their 3 ml EDTA vacutainer tube (Cat #367835) was no longer available, so Add Health switched to the 4 ml EDTA vacutainer tube (Cat #367844).



**Exhibit 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 respondents were comfortable giving blood, respondents 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. Scanning it automatically captured a unique, eight-digit code, thereby linking the respondent to the transport tubes / labels within it, the corresponding ODK questionnaire data, and ultimately to LCBR results.

Following standard phlebotomy protocols, FEs asked respondents 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 respondents had objections, venipuncture was performed on the best potential vein and whole blood was collected, as summarized below:

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- Put on nitrile gloves.
- Have the respondent 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 respondent 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.
- Quickly push the first vacutainer tube (using the sequence in the table above) onto the needle in the holder, puncturing the center of the stopper.
- Release the tourniquet after the flow is established or if the respondent becomes uncomfortable. The respondent 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 respondent 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 respondent to sit quietly for a few minutes. Due to a fasting blood draw encourage the respondent 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.

Either 5 or 6 tubes of blood were collected per respondent, depending on eligibility for a separate glucose sub-study (see Measures of Glucose Homeostasis User Guide, Section 4.1.2.1). Collection order, tube type, and processing information are listed below (Exhibit 2).

Order	Tube Type	Centrifuged	Resultant supernatant	Resultant precipitate	Use
1	8.5 ml SST	Yes	Serum	Discarded	Assays*: glucose, total cholesterol, high- & low-density lipoprotein-cholesterol, triglycerides, high sensitivity C reactive protein, creatinine & cystatin C
2	10 ml EDTA	Yes	Plasma	RBC/buffy coat	Assays†: DNA Archival: for future use
3	3 or 4 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	6 ml NaFl/KOx	Yes	Plasma	Discarded	Assay: glucose sub-study
6	10 ml PAXgene	No	N/A	N/A	Archival: for future use

\*Also included neurofilament light as described below. †Also included tau as described below.

**Exhibit 2. Tubes of Blood Collected**

### 3.3.2 Blood Processing

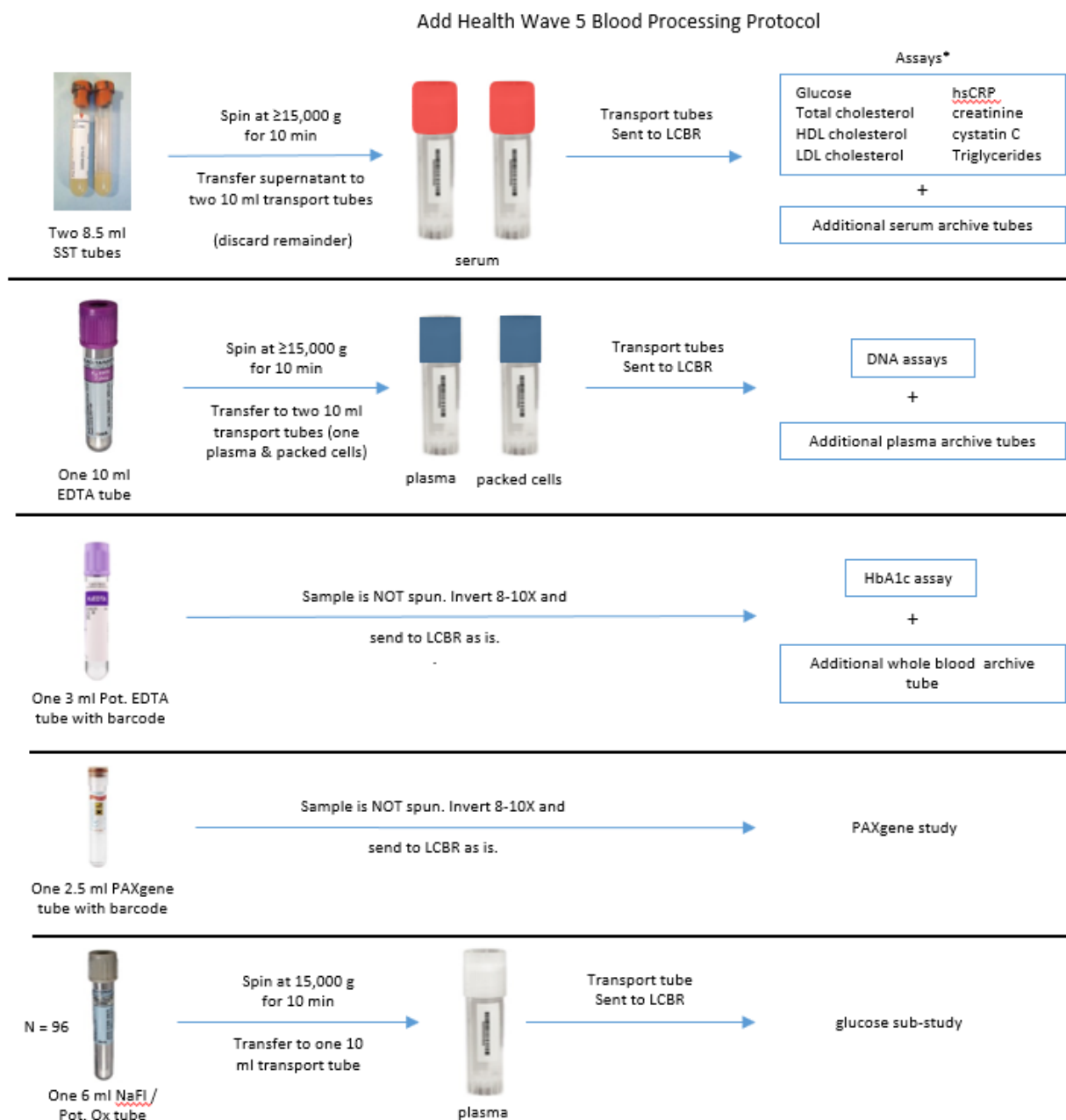
The venous blood draw concluded the home exam. After cleaning up all supplies and equipment, FEs left the exam sites and were allowed a maximum of two hours before processing the blood which was chilled at 4° C (on ice or frozen cold packs) in the interim.

All FEs centrifuged specific blood collection tubes, including the 8.5 ml SST, 10 ml EDTA, and when collected, the 6 ml NaFl/KOx vacutainer tubes. The 3-4 ml EDTA vacutainer tube used for the HbA1c assay was *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 and (separately, when collected) plasma from the NaFl/KOx vacutainer tubes 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, and a white cap identified the transport tube containing plasma from the Na/Fl/KOx tube. Transport tubes were chilled at 4° C (on ice

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or frozen cold packs) until packaged for shipment to LCBR. Exhibit 3 demonstrates the complete blood processing protocol.



\*Also included serum neurofilament light and plasma tau as described below.

### Exhibit 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

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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 respondent's transport tubes.

### 3.3.3 Shipment of Samples

Immediately before shipment, FEs removed two cold packs from the freezer, sandwiched the transport tubes between them, 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° 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 Neurodegeneration Biomarker Assays

The assays of NfL and tau used archived serum and plasma, respectively. On the day of assay, 1 ml serum and plasma aliquots initially archived at -80°C were thawed in a 36°C water bath for 10 minutes and vortexed. From the aliquots, 75 µl of serum and plasma were sub-aliquoted and the remaining specimens re-archived at -80°C. On average, NfL assays were performed 2.0 years after the initial archival of serum (mean = 738 days; standard deviation = 301 days; range = 308-1345 days) and tau assays 3.7 years after the initial archival of plasma (mean = 1340 days; standard deviation = 289 days; range = 917-1921 days).

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## 4. Assay and Internal Quality Control

### 4.1 NfL [H5NFL]

#### 4.1.1 Rationale

Neurofilament light (NfL) is a cytoskeletal polypeptide that is expressed in neurons. It helps provide structural support for, regulate the diameter of, and control transmission of electrical impulses along the neuronal axon, thereby contributing to regular synaptic function.<sup>1</sup> Significant releases of NfL into both cerebrospinal fluid (CSF) and blood can follow axonal damage secondary to acute traumatic brain injury, stroke, Alzheimer's disease, frontotemporal dementia, amyotrophic lateral sclerosis, multiple sclerosis, and other neurological diseases.<sup>2,3,4</sup> Increases in it are therefore thought to reflect axonal damage and or degeneration. Until recently, NfL concentrations could only be measured in CSF, but new technologies such as the Simoa kit used here also allow it to be measured in serum.

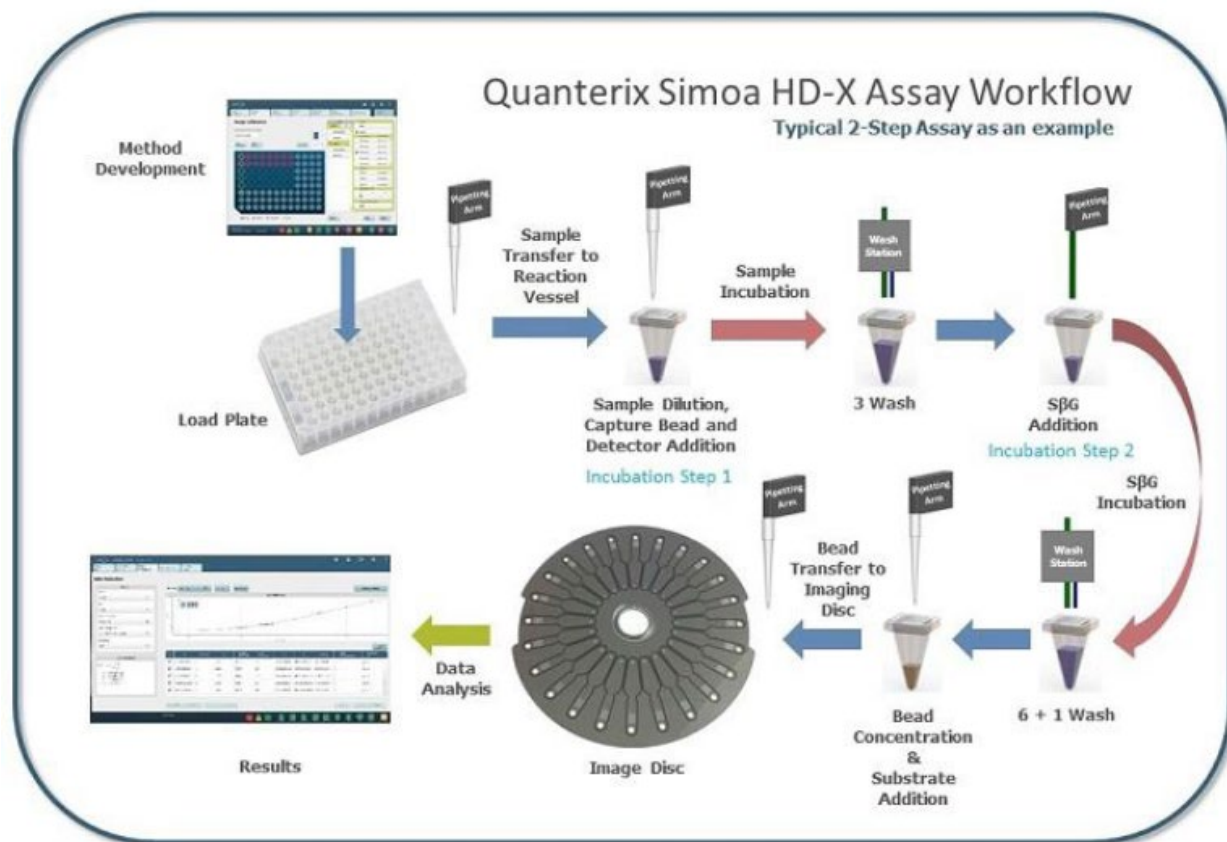
#### 4.1.2 Assay Protocol

All assays were performed at room temperature using a Single molecule array (Simoa™) HD-1 analyzer (Quanterix Corporation, Lexington, MA) and a Quanterix single-plex, 2-step NfL assay. In this assay, NfL antibody capture agents were attached to the surface of 2.7 µm paramagnetic beads included in the assay kit. 75 µl of serum from the thawed 1 ml aliquots was diluted 1:4 using an included sample diluent. All other reagents including calibrator standards and controls were prepared according to manufacturer recommendations. Samples, calibrators, and controls were added to the paramagnetic beads along with the presence of a biotinylated detection antibody. NfL molecules present in the sample were captured by the antibody coated beads and bound with the detection antibody simultaneously. The beads were then washed to remove non-specifically bound proteins and incubated with β-D-galactopyranoside (RGP)-labelled streptavidin.

After washing, the beads were then transferred to a Simoa image disc and individual beads were sealed within microwells in the array. The addition of β-galactosidase hydrolyzed the RGP substrate in the microwell into a fluorescent product that was then measured by the HD-1 analyzer. The protein concentration in the test sample was determined by counting the number of wells containing both a bead and fluorescent product relative to the total number of wells containing beads. Because Simoa enables concentrations to be determined digitally rather than by measurement of the total analog signal, this approach to detecting single immunocomplexes has been termed a digital enzyme-linked immunosorbent assay (ELISA).<sup>5</sup> A typical assay workflow is illustrated in Exhibit 4.

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**Exhibit 4. 2-Step Assay Protocol**

#### 4.1.3 Internal Quality Control

According to manufacturer recommendations, the HD-1 system was maintained daily by checking reagent levels, waste levels, and cleaning machine components. It also underwent a monthly maintenance routine that included disposing/replacing all fluids in the machine, cleaning fluid paths, running a preloaded maintenance program, and using the Simoa™ Qualification Test assay to verify the sensitivity, precision, and accuracy of the HD-1 system.<sup>6</sup>

Each NfL kit contained reagents for assaying 96 serum samples plus two known (low and high) concentration controls. The controls were kit-specific, but low and high concentrations were typically 10 pg/ml and 925 pg/ml, respectively,<sup>7</sup> and used to process all plates relying on the same kit. The assay was calibrated by running calibrators on each plate, in duplicate, then fitting a four-parameter logistic curve to the assayed concentrations. The lower and upper limits of detection defining the dynamic range for NfL were 0.416 pg/ml and 1504 pg/ml when run at a 1:4 dilution. The range of reported NfL concentrations (H5NfL) was 0.74 - 235.23 pg/ml. The low and high concentration controls had average coefficients of variation (CVs) of 11.98% and 13.74%, respectively.

In addition to the above quality controls, 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 #E050115). The other pool was a serum normal donor pool (Lot #S042715). LCBR

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periodically assayed both pools over the course of Wave V. The plasma and serum NfL concentration mean (CV) based on those assays was 8.74 pg/ml (10.55%) and 12.24 pg/ml (10.91%), respectively. When NfL concentrations exceeded acceptable parameters, the Simoa system was investigated and repaired.

## 4.2 Tau [H5TAU]

### 4.2.1 Rationale

Tau is a microtubule-stabilizing protein primarily localized in central nervous system neurons, but also expressed at low levels in astrocytes and oligodendrocytes. CSF elevation of tau is observed in neurodegenerative disease and brain injury, suggesting its extracellular release during neuronal damage and role as a specific biomarker. Potential movement of elevated CSF tau across the blood-brain barrier also suggests that measurement of tau in blood may provide a convenient peripheral window into the status of the brain.<sup>8</sup> The Simoa™ tau assay employed here uses a combination of monoclonal antibodies that react with both normal and phosphorylated tau. With an epitope in the mid-region of the molecule, the assay recognizes all six tau isoforms in the human brain (molecular weight range = 48,000-67,000 Da), and thus measures total tau.

### 4.2.2 Assay Protocol

All assays were performed at room temperature using a Single molecule array (Simoa™) HD-1 analyzer (Quanterix Corporation, Lexington, MA) and a Quanterix single-plex, 2-step tau assay. In contrast to the NfL assay, tau was measured using plasma, not serum. Otherwise, the assay of tau was identical to that of NfL, described above (Section 4.1.2).<sup>9</sup>

### 4.2.3 Internal Quality Control

The internal quality control protocol for tau was analogous to that of NfL, described above (Section 4.1.3). Each tau kit also contained two known (low and high) concentration controls, typically 1.5 pg/ml and 70 pg/ml.<sup>8</sup> The lower and upper limits of detection defining the dynamic range for tau were 0.380 pg/ml and 332.80 pg/ml when run at 1:4 dilution. However, the range of reported tau concentrations (H5TAU) was 0.05 - 769.95 pg/ml because concentrations outside the dynamic range were in some cases extrapolated by the HD-1 system's Quanterix software. The low and high concentration controls had CVs of 11.13% and 13.67%, respectively.

In longitudinal quality control analyses of tau, LCBR also used two pools of samples from twenty normal donors (US Biologicals, Salem, MA). One pool was an EDTA plasma normal donor pool (Lot #E050115). The other pool was a serum normal donor pool (Lot #S042715). LCBR periodically assayed both pools over the course of Wave V. The plasma and serum tau concentration mean (CV) based on those assays was 2.84 pg/ml (10.68%) and 0.12 pg/ml (28.16%), respectively. It should be noted that although the serum donor pool was run as a matter of course, the tau assay is designed to process plasma only; thus the low mean and high CV, in serum. When tau concentrations exceeded acceptable parameters, the Simoa system was investigated and repaired.

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## 5. External Quality Control

### 5.1 Reliability

Within a race/ethnicity- and sex-stratified random sample of 105 Add Health respondents among whom venous blood was collected twice, on average 14.3 (95% confidence interval: 13.1-15.5) days apart, typically by the same FE and at approximately the same time of day, the reliability of NfL (pg/ml) and tau (pg/ml) was estimated as an intra-class correlation coefficient (ICC, 95% confidence interval) [Exhibit 5]. The estimates suggest that the home exam venous blood collected at Add Health Wave V yields a highly reliable measure of NfL and slightly less reliable measure of tau.

Measure	N	ICC	95% CI
NfL (pg/ml)	105	0.92	(0.90, 0.95)
Tau (pg/ml)	105	0.82	(0.76, 0.88)

Exhibit 5. Reliability of NfL and tau

## 6. Constructed Variables

All NfL and tau concentrations were categorized based on their corresponding limits of detection (LODs) and extrapolation beyond them, then flagged as tabulated below (Exhibit 6-7). *Warning: Users should recognize extrapolated concentrations as such and exercise caution if choosing to work 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.*<sup>10</sup>

### 6.1. Flag Indicating the NfL Concentration Type [H5NFLFL]

Code	Description
1	NfL concentration is <u>below</u> the lower LOD (< 0.416 pg/ml) – missing
2	NfL concentration is <u>below</u> the lower LOD (< 0.416 pg/ml), but extrapolated
3	NfL concentration is within the LODs (0.416 - 1504 pg/ml)
4	NfL concentration is <u>above</u> the upper LOD (> 1504 pg/ml), but extrapolated
5	NfL concentration is <u>above</u> the upper LOD (> 1504 pg/ml) – missing

Exhibit 6. Flag Indicating the NfL Concentration Type

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## 6.2. Flag Indicating the Tau Concentration Type [H5TAUFL]

Code	Description
1	Tau concentration is <u>below</u> the lower LOD (< 0.380 pg/ml) – missing
2	Tau concentration is <u>below</u> the lower LOD (< 0.380 pg/ml), but extrapolated
3	Tau concentration is within the LODs (0.380 – 332.80 pg/ml)
4	Tau concentration is <u>above</u> the upper LOD (> 332.80 pg/ml), but extrapolated
5	Tau concentration is <u>above</u> the upper LOD (> 332.80 pg/ml) – missing

**Exhibit 7. Flag Indicating the Tau Concentration Type**

## 7. The Neurodegeneration Biomarker Data File (bneuro5.xpt)

### 7.1. Structure

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

### 7.2. Contents

The neurodegeneration biomarker data file includes the variables below, which are described in the corresponding codebook documentation that also contains frequencies.

<b><u>Variable Name</u></b>	<b><u>Variable Description</u></b>
AID	Respondent identifier
H5NFL	Neurofilament Light (NfL, pg/ml)
H5NFLFL	FLAG: NfL Concentration Type
H5TAU	Tau (pg/ml)
H5TAUFL	FLAG: Tau Concentration Type

## 8. References

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