

MarkVCID Plasma Neurofilament Light Biomarker Kit Protocol

1. Protocol for fluid analysis

The Simoa™ Advantage Assay Kits contain ready-to-use reagents for running 96 tests of target analyte(s) on the Simoa HD-1 Analyzer using Single Molecule Array (Simoa) technology. The following equations and table describe the volume required for each sample. Samples diluted at the bench will be run using the Neat protocol. Plasma or serum samples to be diluted by the instrument will be run using the standard 4x protocol.

The components of the NfL Simoa kit include:

Simoa Advantage Reagent Kit for 96 Tests			
Bead Reagent	1 bottle	Store at 2-8°C	Capture antibody coated beads in buffer with protein stabilizers.
Detector Reagent	1 bottle	Store at 2-8°C	Biotinylated detector antibody in buffer with protein stabilizers.
SBG Reagent	1 bottle	Store at 2-8°C	Streptavidin-β-galactosidase (SBG) in buffer with protein stabilizers.
RGP Reagent	2 bottles	Store at 2-8°C	Resorufin β-D-galactopyranoside (RGP) in buffer with a surfactant.
Calibrators	2 vials per level	Store at -80°C	Antigen in buffer with protein stabilizers.
Controls	2 vials per level	Store at -80°C	Antigen in buffer with protein stabilizers.
Sample Diluent	1 – 2 bottles	Store at 2-8°C	Buffer with protein stabilizers, a heterophilic blocker.

Materials required but not provided

- Simoa HD-1 Analyzer
- Simoa HD-1 System Wash Buffer 1
- Simoa HD-1 System Wash Buffer 2
- Simoa HD-1 Sealing Oil
- Simoa cuvettes*
- Simoa disposable pipettor tips*
- Simoa Discs*

*Cuvettes, Pipettor Tips and Discs are sold as consumable unit “Simoa Disc Kit 2.0”

Step-by-step Simoa Analyzer protocol for SIMOA N4PA Advantage Kit

Prepare Instrument

1. Prepare the instrument by turning on the HD-1 Analyzer. First turn the switch on and then initialize the Simoa software. This should take 3 minutes. The system will tell you when it is ready.
2. If this is the first time the Simoa has been turned on that day, perform pre-run maintenance by going to the maintenance tab, checking the start of day task, and clicking run task. This should take 20 min. If the system has been idle for 4 or more hours, run Idle System Prime, which should take 10 min.
3. To import the assay definition, go to the custom assay tab and click import to select the xml file you wish to use. To import the N4PA assay definition, refer to the lot-specific Certificate of Analysis for the concentration of this lot of calibrators and update in the assay definition. NOTE: If you have not downloaded this assay definition before, you must download this definition from the customer portal and install it on the Simoa Analyzer prior to performing the assay.

Set Up Assay

1. Allow calibrators and controls to come to room temperature. Do NOT heat vial to accelerate thawing.
2. When the calibrators and controls are fully thawed, thoroughly mix by multiple gentle inversions or vortexing. This is critical for accurate calibration.
3. Prepare samples by selecting a Quanterix supplied 96-well plate. Each well should already contain 30uL (i.e. dead volume). Using the below table, calculate the required volume of sample to be added to each well. The table does **not** account for the 30uL already in each well. **Samples will generally be run in singlet on HD-1 and HD-X instruments. Samples run on SR-X instruments must be run in duplicate.**

Calibrator Volume. <i>NOTE: Calibrators should be run in duplicate.</i>	334uL (for 2 replicates in a 96 well plate)
Sample and Control Dilution factor	4X
Undiluted sample and control volume (1 replicate). This is the volume of plasma that will typically be used.	45uL per well
Undiluted sample and control volume (2 replicates)	90uL per well
Total tests per kit	96

To calculate the required volume of sample per well:

Total volume = Dead volume + (number of replicates x protocol aspiration volume)

Volume example calculations:

Onboard sample dilution protocol with 2 replicates:
= 30uL + (2 x 45uL) = 120 uL total/well

4. Mix the thawed samples by gently vortexing OR inverting 10 times. Centrifuge the samples at 10,000 x g for 5 minutes to clear any debris
5. Prepare the plate by pipetting the required volumes (see step 3 above) of calibrators, controls, and samples into the 96 well plates.

Plasma Sample Clarification Via Centrifugation

In accordance to the Kit Instructions for the Simoa N4PA Advantage Kit, excess fibrin, lipids and particulate matter may interfere with the accuracy of the assay. High speed centrifugation will separate lipids (top layer) and fibrin/particulate matter (pellet formation) such that a clarified plasma sample can be plated for use on the Quanterix HD-1 or HD-X analyzer.

The following procedure efficiently removes these interfering substances (for more details refer to the Plasma & Serum Sample Clarification Via Centrifuge – UVM procedure appendix)

1. Thaw sample at 37°C for 5-7 minutes
2. Vortex sample to mix well
3. Centrifuge sample(s) at 14,000g for 10 minutes
4. Carefully remove the vial from the centrifuge such that the lipid layer and pellet formed is not disturbed.
5. Carefully insert pipette into the clarified serum or plasma. Take care to minimally disturb any lipid layer and/or pellet. Tilting the cryo may be helpful in exposing clarified serum.
6. Aliquot to desired receptacle: cryovial, HD-1/HD-X plate.
7. Freeze aliquots at -80°C until further use. And/or Run on HD-1/HD-X analyzer

Load Instrument and Run Assay

1. Vortex beads for 30 seconds before loading. If beads sit idle for more than 5 minutes, vortex again.
2. Load beads, detector, and SBG reagents into the reagent rack. Make sure that the beads are in one of the shaking positions. Select the load reagent tab and select the reagent lane. Use the barcode reader to assign positions of reagents in the reagent rack. Insert the reagent rack. To load RGP, use the barcode reader to assign RGP and insert the rack labelled with an O (the RGP rack). Touch the Done Loading Reagents tab.
3. Create the plate layout by selecting the Setup Run tab and assigning the Batch Name and assigning the Plate Barcode. Click enter on the computer when done.
4. Assign calibrators by selecting the Assign Calibrators tab. Highlight a single well and select assay. Select a calibrator from the Select Calibrator pop up. Click Ascending/Descending to populate the remaining calibrators. Select the number of replicates per well.
5. To assign samples, select the assign sample tab and highlight all wells that contain any controls or samples. Select assay and assign the number of replicates per well.
6. When steps 3-5 are complete, click on List View to confirm the selections. When confirmed, insert plate rack and select Done With Setup.
7. *If necessary*, load the liquid consumables by filling the secondary containers with System Liquid (DI water), Wash Buffer 1, and Wash Buffer 2.

8. *If necessary*, load cuvettes, tips, and discs. To load cuvettes, place a full stack of 50 in the cuvette chute. Additional stacks must be loaded only when the system says ready. To load tips, go to the System Resources tab and select solid resources. Click Unlock Drawers and load the tips. In the software, tap twice in the positions where you loaded new tip racks. The tip positions in the rack diagram will turn light blue. To load disks, select the System Resources tab and use the barcode scanner to scan the barcode on the wrapper. Remove the blue base plate from the old stack from the disc pole. Load the new stack on an empty disc pole and remove the wrapper and the top disc with the Quanterix logo.
9. *If necessary*, empty solid and liquid waste at the bottom of the cabinet.
10. To start the run, go to the System Resources tab and select all resources. Click Start Run. If the button is not active, check for flags in Resource Details. To monitor the progress of the run, go to the Current Run tab. The run is finished when this tab reads 00:00 and the status line at the bottom left corner says ready.

Post-Run

1. Remove the sample plate, reagents, and RGP.
2. Review the results and export as a CSV file.
3. Perform post-run maintenance by selecting the maintenance tab and checking the End of Day task. Click Run Task. This should take 15 minutes.
4. To shut down the instrument, shutdown the software, followed by turning off the instrument, and finally shutting down the PC.

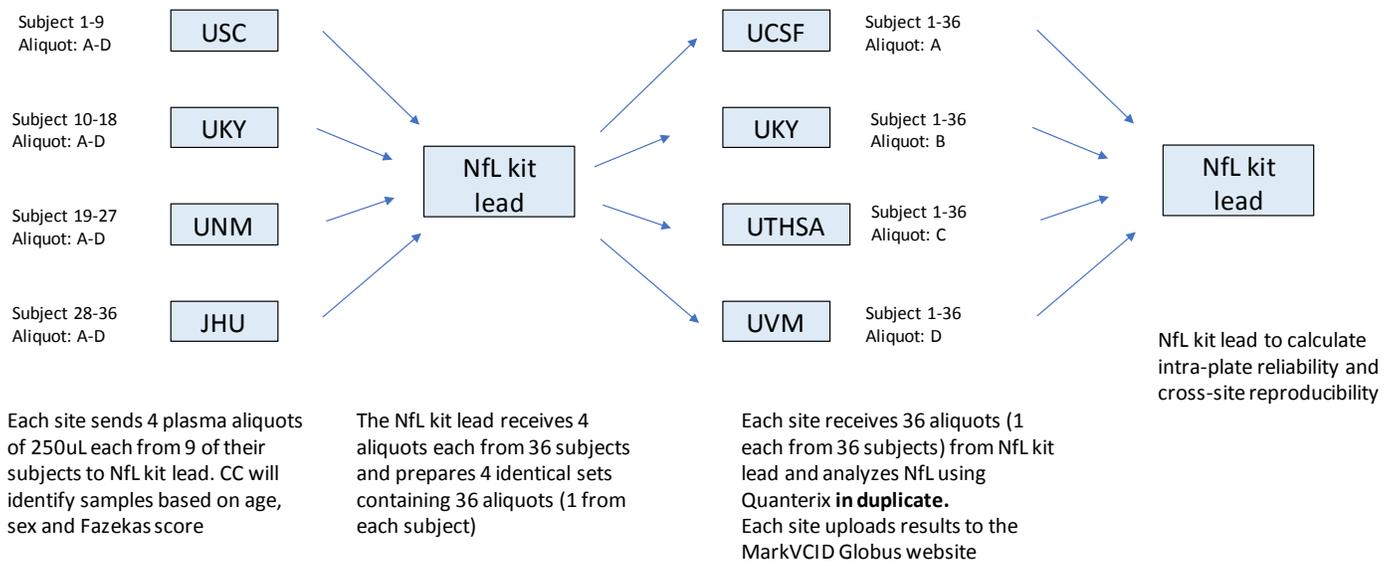
Instrumental validation

1. Intra-plate / Cross-site

USC, UKY, UNM, and JHU to ship 4 frozen 250 uL plasma aliquots from n=9 subjects to the NfL lead site (UTHSA). Lead site to redistribute 1 aliquot per subject to UCSF, UKY, UTHSA and UVM for assessment of NfL **in duplicate**.

Contributing samples: USC, UKY, UNM, JHU

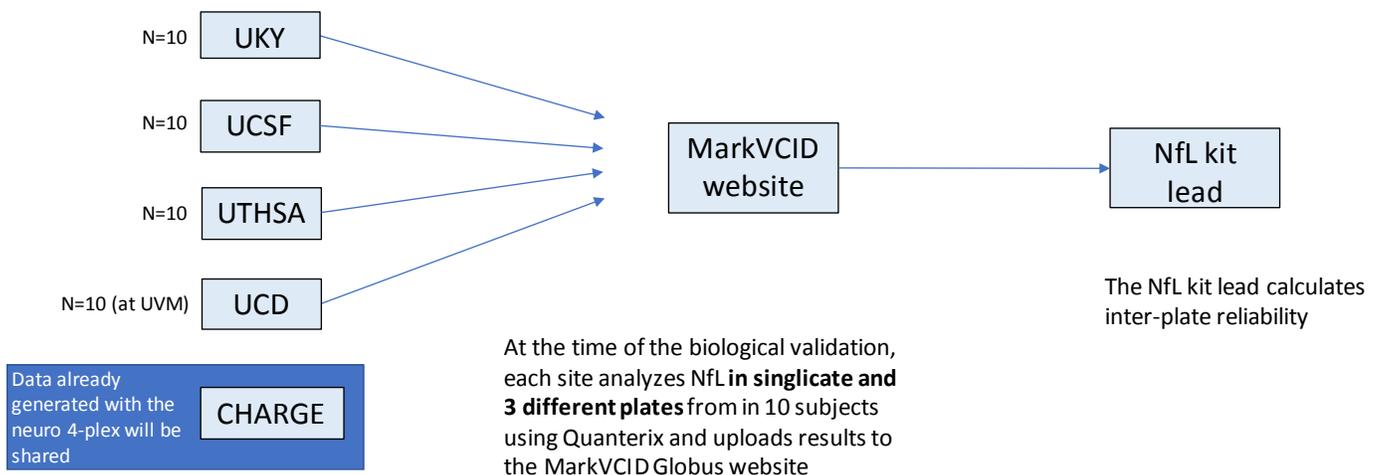
Performing analyses using Quanterix: UCSF, UKY, UTHSA, UVM



2. Inter-plate reliability

This analysis will be performed during the biological validation. UKY, UCSF, UTHSA, UCD, and CHARGE will assess NfL in plasma samples from n=10 subjects on different plates **in singlicate**.

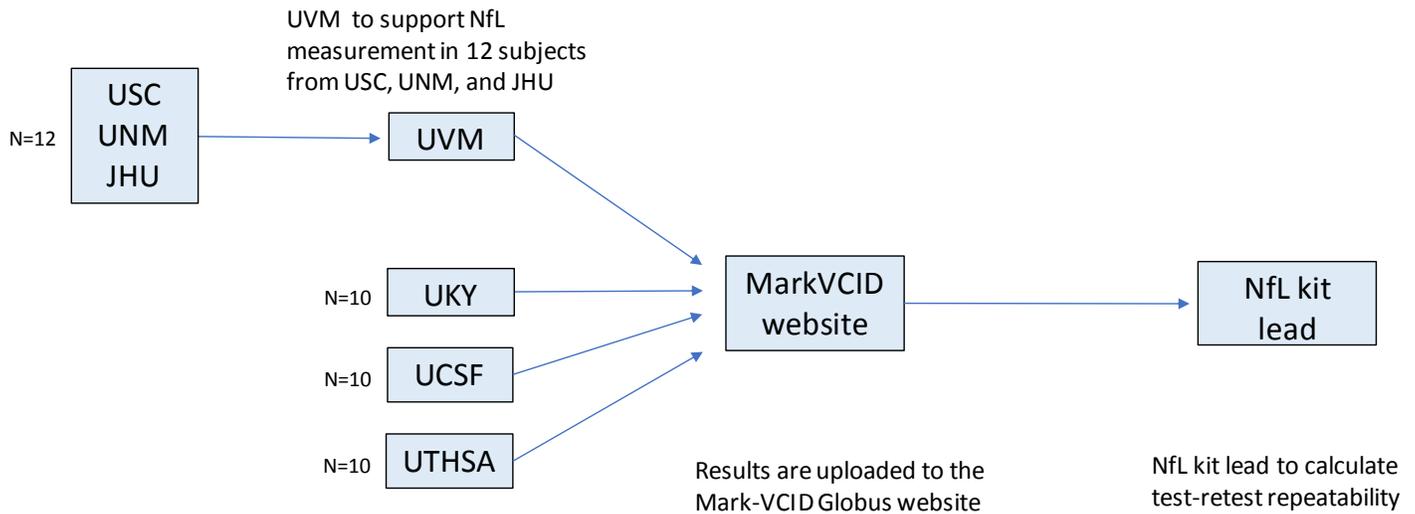
Contributing samples: UKY, UCSF, UTHSA, UCD, CHARGE
Performing analyses using Quanterix: UKY, UCSF, UTHSA, UVM



3. Test-retest repeatability (same subject and site)

- USC, UNM and JHU to ship 3 frozen 250 uL plasma aliquots from n=12 subjects combined to UVM, each derived at 3 different time points (at least 5 days apart from one another and completed within 30 days), to assess NfL **in duplicate**.
- UKY, UCSF and UTHSA to assess NfL **in duplicate** from n=10 subjects in samples collected at 3 different time points (at least 5 days apart from one another and completed within 30 days) at their own site using Quanterix.

Contributing samples: USC, UNM, JHU, UKY, UCSF
Performing analyses using Quanterix: UVM, UKY, UCSF



Each site analyzes NfL **in duplicate** at **3 different time points** from 10 subjects, at their own site using Quanterix, or send it to UVM if Quanterix is not available