MarkVCID2 Plasma Neurofilament Light (NfL)

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Biomarker Kit Protocol

1. Brief description of the biomarker kit

Neurofilament light (NfL) is part of the neurofilament complex that maintains axonal caliber. Increased concentrations of NfL in CSF and blood products (i.e., serum and plasma) have been related to neuronal damage, and the risk and progression of a variety of neurological disorders, as well as to pathological correlates [1, 2]. This kit includes instructions for the measurement of NfL using the Simoa Quanterix Neuro 4-plex A (N4PA) (Kautz et al., under submission) [3].

Category 1: Susceptibility/Risk

Context of use 1. *Subject selection hypothesis:* NfL is a suitable fluid biomarker to sensitively and specifically identify or risk-stratify participants across the spectrum of cerebral small vessel disease (SVD) at an appropriate stage for inclusion in trials of vascular contributions to cognitive impairment and dementia (VCID).

Category 2: *Disease monitoring*

Context of use 2. *Study outcome hypothesis:* NfL is a suitable fluid biomarker to monitor the progression of cerebral SVD sensitively and efficiently, and therefore the progression of VCID.

During MarkVCID-1, a comprehensive clinical validation was performed for the NfL kit using samples from six MarkVCID-1 sites (UKY, UNM, USC, UTHSA, UCD, and JHU). Replication was sought in three independent samples from the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE, which included data from CARDIA=667 [4], the Framingham Heart Study Offspring and Omni 1 cohorts, n=1487 [5], ARIC=1870 [6], and AGES=1051 [7]), the University of Kentucky ADRC (UKY-ADRC=349), and the University of California Davis ADRC (UCD-ADRC=196) (Kautz et al., under submission) [3].
Circulating NfL levels were related to general cognitive function adjusting for age, sex, education, and any relevant study-specific covariates when required (e.g., study center, race/ethnicity if clustered with study center, or time difference between blood draw and cognitive evaluation). A secondary model additionally adjusted for eGFR (when available), a marker of renal function, as prior studies reporting renal function may influence NfL clearance in addition to previous literature relating poor renal function to cognitive function [8, 9]. Results showed significant associations between higher NfL concentrations and worse general cognitive function in the MarkVCID-1 cohort (**Table 1**). Results were replicated in independent samples. Additional adjustment for eGFR did not materially change the results.

Table 1. Association of circulating log-NfL with general cognitive function in MarkVCID-1 cohort and three independent validation samples

	General Cognitive Function, Beta [95% CI]	
	Model 1[1]	**Model 2[2]**
MarkVCID (n=330)	-0.23 [-0.43; -0.03]	-0.24 [-0.45; -0.03]
CHARGE (n=5,075)	-0.11 [-0.17; -0.06]	-0.11 [-0.17; -0.05]
UKY ADRC (n=349)	-0.22 [-0.41; -0.03]	NA
UCD ADRC (n=199)	-0.32 [-0.56; -0.07]	-0.45 [-0.74; -0.15]

[1]Model 1: Adjusted for age, sex, education, and site-specific covariates.
[2]Model 2: Adjusted for age, sex, education, site-specific covariates, and eGFR (not available in the UKY ADRC sample).

2. Summary of kit instrumental validation results

During MarkVCID-1, plasma NfL was primarily measured using the Quanterix N4PA kit (Kautz et al., under submission) [3], although some measurements were performed using the single molecule kit due to N4PA shortages. No differences were found when measuring NfL using the N4PA kit or single molecule kit (ICC≥0.81, **Figure 1**) or when using the Quanterix Simoa HD-1 or HD-X platforms (R^2≥0.9) to measure plasma NfL (**Figure 2**).

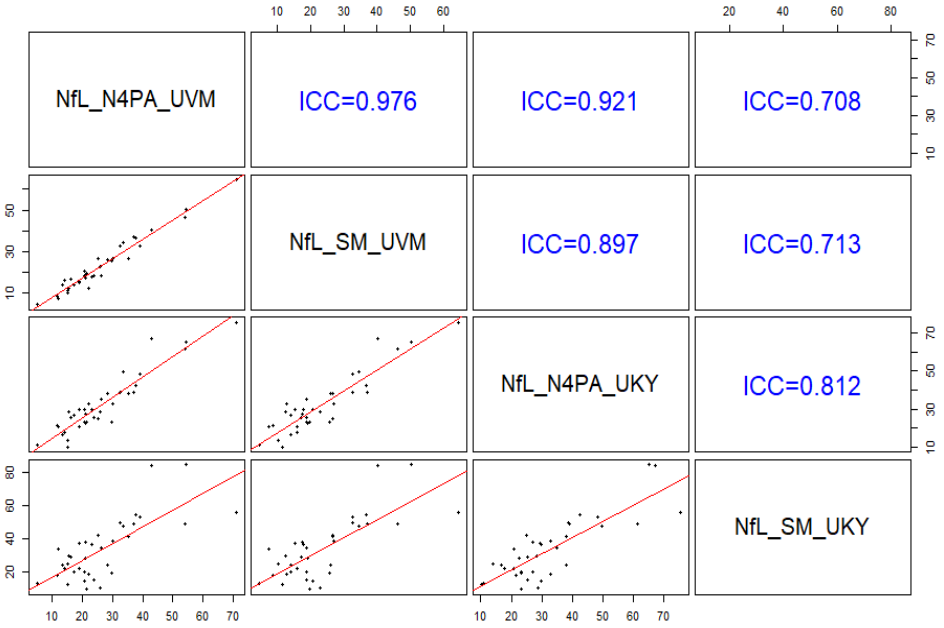

Figure 1. Regression plots (lower panel) and ICC (upper panel) between NfL measured with the N4PA and single-molecule (SM) Quanterix assays

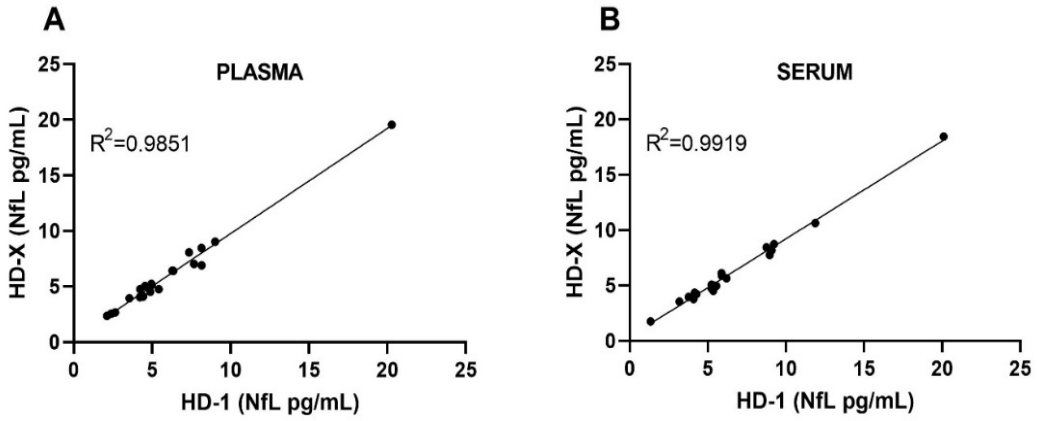

Figure 2. Comparison of NfL assayed with HD-1 and HD-X Quanterix instruments in plasma (A) or serum (B) samples from n=40 CARDIA participants. The Quanterix N4PA kit was used for this comparison.

To determine intra-plate and inter-site variability between different Quanterix HD-1/HD-X instruments during MarkVCID-1, plasma samples from 36 MarkVCID participants were shipped to UKY, UCSF, UTHSA, and UVM and measured for NfL in duplicate. When examining intraplate variation for matched samples measured at these 4 different sites, CV values were consistently low and ranged from 3.59% to 5.07%. There were no significant differences in CV % between sites as measured by 1-way ANOVA ($p>0.5$). Our analysis across sites indicated an excellent ICC for reproducibility, with the overall ICC=0.93 (**Figure 3**).

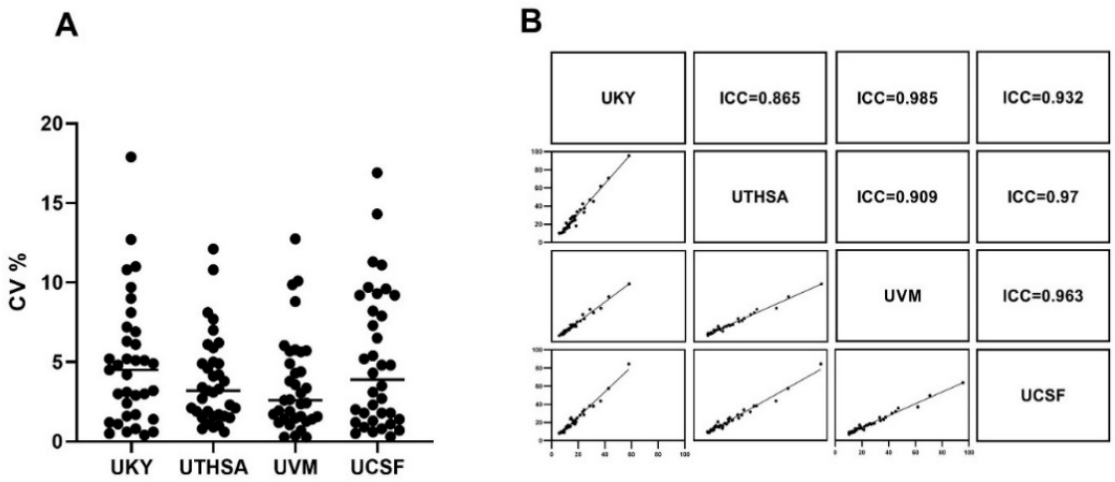

Figure 3. Intra-plate and inter-site reproducibility. Matched plasma aliquots from 36 MarkVCID participants were assayed for NfL in duplicate using the N4PA kit on one plate at 4 MarkVCID sites: UKY, UTHSA, UVM, and UCSF. CV % was measured within duplicate samples at each site (A). ICC between the 4 sites was measured to assess reproducibility between instruments (B).

To determine the inter-plate reliability of plasma NfL measurements generated by the Quanterix Simoa instruments, n≥10 participants' samples were measured in singlicate using n≥3 different plates at 3 different sites (UTHSA, UCD, or UVM). Each site ran a different set of samples, with UTHSA running 10 samples from their MarkVCID site, UCD running 10 plasma samples from their ADRC, and UVM running samples from healthy donors (20 serum and 20 plasma, unmatched) that were purchased in bulk from a commercial vendor (Innovative Research, Inc., Novi, MI). Results showed excellent inter-plate repeatability. The average CV ranged from 5.96 to 11.54%, again demonstrating consistently low CVs.

Table 1. Inter-plate reliability

Sample (fraction)	N	Plates	Average CV	Min CV	Max CV
UTHSA MarkVCID (plasma)	10	3	11.54%	3.15%	26.03%
UCD ADRC (plasma)	11	3	5.96%	1.74%	8.79%
UVM (plasma)	20	4	9.00%	3.34%	16.65%
UVM (serum)	20	4	9.85%	5.70%	19.84%

To test the within-participant variability of NfL over a short period of time, 4-10 participants from each MarkVCID site provided 3 fasting blood samples over a period of 30 days that were collected at least 5 days apart. UVM measured NfL in plasma samples from USC (n=4), UNM (n=4), and JHU (n=4), while UKY (n=8), UCSF (n=10), and UTHSA (n=10) measured their samples on-site. All samples were measured in duplicate. Results show consistent within-participant NfL levels, with a mean ICC=0.968. Analysis of variance with a mixed-effects model showed no significant within participant differences for NfL over time (p=0.258), demonstrating excellent short-term NfL concentration repeatability (**Figure 4**).

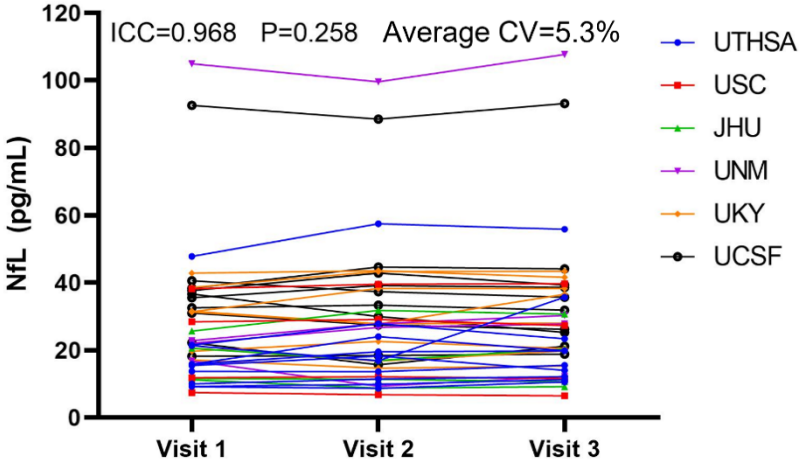

Figure 4. NfL levels in 3 repeated blood draws within 30 days. 40 MarkVCID participants from UTHSA, USC, JHU, UNM, UKY, and UCSF had their blood drawn 3 times (visit 1, 2, and 3) over the course of 30 days. NfL was measured in duplicate using the Neuro-4 Plex A (UTHSA, USC, UNM, JHU) or single molecule NfL kit (UKY, UCSF).

Finally, to assess the effect of fasting status on plasma NfL levels, fasting and non-fasting (2 hours post-breakfast) blood samples were collected from an independent sample of n=10 older adults across the spectrum of vascular disease. Plasma NfL was measured at UTHSA and UKY using the single molecule Quanterix NfL assay. Although NfL concentrations were significantly elevated in fasting conditions (p=0.03), results tracked consistently (ICC≥ 0.87) within participants (**Figure 5**).

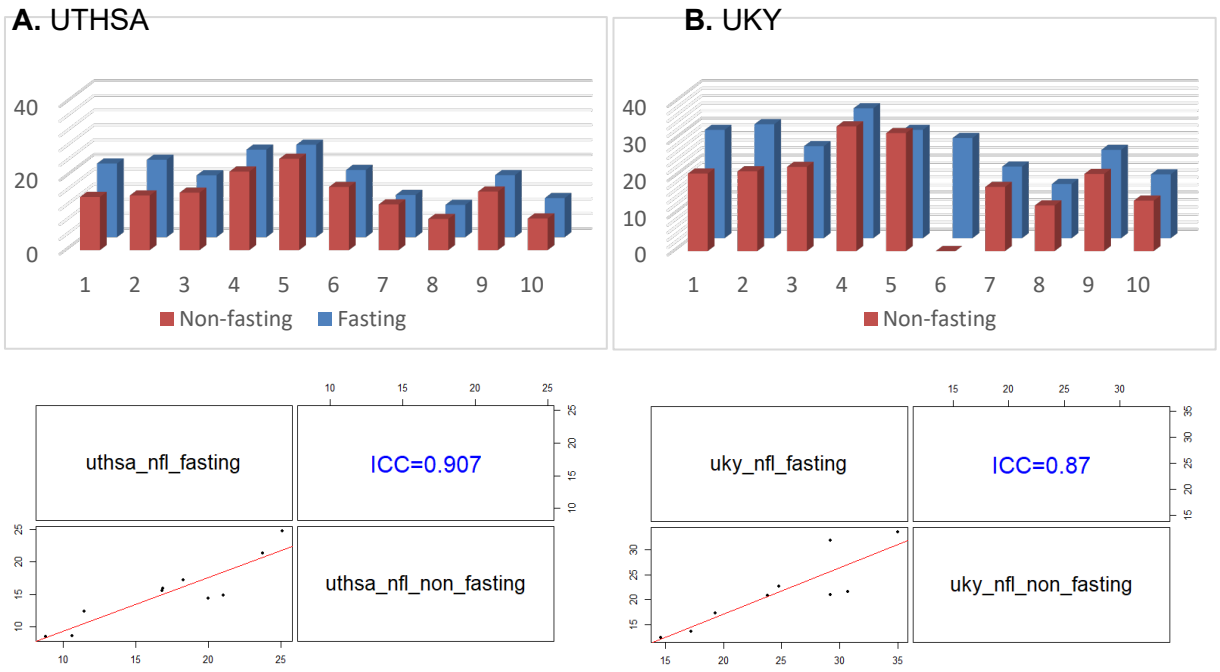

Figure 5. Comparison of NfL measured in fasting (blue) and non-fasting (red) conditions at UTHSA (A) and UKY (B). The upper panel shows NfL concentrations per site, the lower panel shows consistency

3. Protocol for fluid sample acquisition

All prospective plasma samples should be collected according to the MarkVCID-2 fluid best practices SOP. Plasma samples must not be hemolyzed, have obvious microbial contamination, or have been freeze-thawed prior to shipping. Samples must be shipped using dry ice and following the MarkVCID-2 “sharing and dissemination of fluid samples” shipping protocol.

4. Additional data collection required for analysis

Data collection should include age, sex, either cystatin-c (preferred) or creatinine to derive eGFR, and fasting status at the time of the blood draw that will be used for NfL measurement.

5. Protocol for fluid assay

The Simoa™ Advantage Assay Kits contain ready-to-use reagents for running 96 tests of target analyte(s) on the Simoa HD-X Analyzer using Single Molecule Array (Simoa) technology. The NfL MarkVCID kit can be performed using the Simoa Quanterix N4PA multiplex or NfL singleplex kits to measure NfL. This protocol describes the use of the Simoa Quanterix N4PA kit. The tables below describe the supplies and volume required for each sample. Plasma samples for this kit will be diluted by the instrument using the standard 4x protocol.

The N4PA assay is temperature sensitive. Assay sensitivity and precision are compromised when laboratory temperatures exceed 22°C. Prior to running the assay, the laboratory temperature should be confirmed to be ≤22°C and the instrument should be configured for increased heat dissipation if using the HD-1 instrument. Plasma samples that are hemolyzed and/or show evidence of microbial contamination should not be used.

The components of the N4PA Simoa kit are presented in **Table 2**.

Table 2. 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-ß-galactosidase (SBG) in buffer with protein stabilizers.
RGP Reagent	2-3 bottles	Store at 2-8°C	Resorufin ß-D-galactopyranoside (RGP) in buffer with a surfactant.
Calibrators	2 vials per level	Store at -80°C	Antigen in buffer with protein stabilizers.
Controls	1-2 vials per level	Store at -80°C	Antigen in buffer with protein stabilizers.
Sample Diluent	2 bottles	Store at 2-8°C	Buffer with protein stabilizers, a heterophilic blocker.
X-Pierce XP-100 plate seal	1 sheet	Store at 2-8°C or room temperature	Reduces sample evaporation during Quanterix HD-X run

Materials required but not provided:

- Simoa HD-X Analyzer
- Simoa HD-X System Wash Buffer 1 (cat #100486)
- Simoa HD-X System Wash Buffer 2 (cat #100487)
- Simoa HD-X Sealing Oil (cat #100206)
- Simoa cuvettes*
- Simoa disposable pipettor tips*
- Simoa Discs*

*Cuvettes, Pipettor Tips and Discs are sold as consumable unit “Simoa Disc Kit 2.0” (cat #103347)

Protocol Summary

NfL kit HD-X Quanterix Simoa Quick Reference Protocol:

1. Turn on Quanterix HD-X and perform start of day maintenance
2. Ensure that the most recent N4PA assay definition is available on the instrument
3. Allow calibrators, controls, and plasma to come to room temperature
4. Secure RGP vials in a heated shaker at 30°C for ≥30 minutes at 800 rpm
5. Centrifuge plasma samples at 14,000xg for 10 minutes at RT
6. Transfer 185uL of calibrator into 2 adjacent wells of the 96-well Quanterix plate
7. Add 75uL of plasma into each well, following the plate diagram
8. Add 110uL of controls into the indicated wells on the plate diagram
9. Seal plate using X-Pierce XP-100 plate seal (included in N4PA kit)
10. Scan N4PA buffers into the HD-X instrument
11. Load plate into the HD-X instrument and set-up the run
12. Start run
13. Collect data on USB drive

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

Prepare Instrument

1. Prepare the instrument by turning on the HD-X Analyzer. To do this, first turn the computer on, followed by the HD-X power switch, and then initialize the Simoa software found on the desktop. Log onto the instrument. Initialization should take 3 minutes. The system will tell you when it is ready.
2. If this is the first time the Simoa HD-X 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. Follow any prompts the instrument provides. The start of day task should take 20 min to complete. If the system has been idle for 4 or more hours, run Idle System Prime, which should take 10 min to complete.
3. If this is your first time running the N4PA assay, you must download the N4PA definition from the customer portal and install it on the Simoa HD-X. To import the assay definition, go to the custom assay tab and click import to select the xml file you wish to use. Before each run, refer to the lot-specific Certificate of Analysis (COA; found on the Quanterix website) for the concentration of this lot of calibrators and update the concentrations in the assay definition.

Set Up Assay

1. Secure RGP vials in a heated shaker at 30°C for ≥30 minutes at 800 RPM. One bottle of RGP is sufficient for up to 48 tests on the HD-X. If running 1 plate (i.e., 96 samples), prepare 2 RGP vials.
2. Allow calibrators and controls to come to room temperature. Do NOT heat vial to accelerate thawing.
3. When the calibrators and controls are fully thawed, thoroughly mix by vortexing. This is critical for accurate calibration.
4. Prepare samples by selecting a Quanterix supplied 96-well plate. Using **Table 3**, determine the required volume of sample to be added to each well. Calibrators and controls will be run in duplicate. Samples will generally be run in singlicate. If running 1 plate, the total must be ≤96 total tests when including calibrators and controls.
5. Mix the thawed samples by gently **vortexing**. Centrifuge the samples at **14,000 x g for 10 minutes at room temperature** to clear any debris.
    i. Carefully remove the vial from the centrifuge such that the lipid layer and pellet formed is not disturbed.
    ii. 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. Prepare the plate by pipetting the required volumes (see step 3 above) of calibrators, controls, and samples into the 96 well plates.
    i. Note: Each Quanterix plate well can contain a maximum of 300uL. Due to this, calibrators must be plated in separate wells for each replicate (i.e., 185uL in 2 adjacent wells).
    ii. Note: Avoid generating bubbles while pipetting. Pop any bubbles that appear using a pipet tip. Reverse pipetting is recommended while loading the plate.
7. Seal the plate using the X-Pierce XP-100 plate seal provided in the kit. Carefully position the circles so they are centered over the wells.

Table 3. Assay volume guide	
Calibrator Volume. *NOTE*: Calibrators should be run in duplicate.	185uL per well in 2 adjacent wells (370uL total)
Sample and Control Dilution factor	4X
Undiluted sample and control volume (1 replicate). This is the volume of plasma that will typically be used.	75uL per well
Undiluted sample and control volume (2 replicates)	110uL per well
Total tests per kit	96

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. Do this again for the replicate calibrators. Make sure that each well is marked as 1 replicate.
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 (typically 1-2). Controls are also added under the sample tab.
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. Make sure that the plate rack position matches the position selected under the calibrator tab.
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, click Unlock Drawers, 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.

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