Supplementary Online Content


**eAppendix.** Expanded assay methods

This supplementary material has been provided by the authors to give readers additional information about their work.
eAppendix> Expanded Assay Methods

Plasma samples were coded using unique identifiers and stored until being forwarded to Rules-Based Medicine, Inc. (RBM) who measured the levels of 77 molecules using Luminex platform-based multiplex immunoassays (see Table 1 for a list of analytes measured).

Covalent attachment of the capture reagent to the microspheres is achieved with standard carbodiimide chemistry using carboxyl functional groups located on the surface of each 5.6-µm microsphere and primary amines within the capture reagent. Coupling chemistry is performed on large numbers of individual microspheres (10^7-10^9 microspheres/mL) simultaneously within each unique set. All assays were validated in a multiplex environment for the fundamental assay parameters of least detectable dose, precision, cross-reactivity, correlation, linearity, spike-recovery, standard range, matrix interferences, freeze-thaw stability and bench-top stability. Full assay validation documents can be requested from Rules-Based Medicine, Inc.

The data analysis and verification software that runs on the automated Luminex platform requires the detection of a minimum of 50 beads per analyte per sample. The median fluorescence intensity (MFI) value of the measured beads is then derived for each protein in the multiplexed assay. During the automated run, high concentration standards (loaded during the sample plating) are serially diluted to produce two sets of eight-point calibrators that incorporate every analyte in the multiplex. After the plate has been read, this dual set of standard concentration values is fitted using a set of proprietary curve-fitting routines. The algorithms use four and five parameter equations to produce the best description of the standard values and are specifically tailored to include the “difficult-to-fit” points at the low and high ends of the curve. The MFI for each sample is then plotted on the standard curve and the final concentration is derived by multiplying by the sample dilution factor. In addition, all assays have unique sets of three-level controls (high, medium, and low concentration) for each of the analytes within the multiplex. These controls are developed by RBM to mimic the sample matrix or type.

The least detectable dose (LDD) was determined by adding three standard deviations to the average of the signal for 20 replicate determinations of the standard curve blank. This value was converted to concentration as interpolated from the standard curve and multiplied by the dilution factor used for testing plasma or serum samples. Any value below the LDD but above the lower assay limit (LAL) was reported. The LAL is defined as each assay’s working sensitivity in plasma as defined by the lowest concentration calibrator used for quantitation. The lower limit of quantification (LLOQ) was defined as the point at which the coefficient of variation (CV) for samples was 30%. It was determined by making two-fold dilutions of Standard 5 for eight dilutions and assaying these samples in triplicate over three different runs. The CV was calculated and plotted against concentration. The LLOQ was interpolated from this plot and multiplied by the dilution factor.