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NIST/NIH Vitamin D Metabolites Quality Assurance Program Report of Participant Results: Summer 2010 Comparability Study (Exercise 2)

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ABSTRACT

The National Institute of Standards and Technology (NIST) recently established a Vitamin D Metabolites Quality Assurance Program (VitDQAP) in collaboration with the National Institutes of Health (NIH) Office of Dietary Supplements. Participants in the second exercise of this program, the Summer 2010 Comparability Study, were asked to use the methodology of their choice to measure concentrations of 25-hydroxyvitamin D in control and study materials distributed by NIST. The study materials consisted of SRM 968d Fat-Soluble Vitamins, Carotenoids and Cholesterol in Human Serum (Level 1 and Level 2). SRM 2972, which is comprised of separate ethanolic calibration solutions with known concentrations of $25(OH)D_2$ and $25(OH)D_3$, was provided as a control material. Participants provided their data to NIST, where it was compiled and evaluated for trueness relative to the NIST value, within-laboratory precision, and concordance within the participant community. A report of results was provided to all participants of the exercise, and laboratories were identified by code numbers known only to them. The results from this second exercise are reported along with a summary of the analytical methods used.

OVERVIEW OF SUMMER 2010 COMPARABILITY STUDY

For the Summer 2010 Comparability Study (Exercise 2) of the NIST/NIH Vitamin D Metabolites Quality Assurance Program (VitDQAP), control and human serum study samples were distributed to participants for evaluation. SRM 2972, which is comprised of separate ethanolic solutions with known concentrations of 25-hydroxyvitamin D_2 ($25(OH)D_2$) and 25-hydroxyvitamin D_3 ($25(OH)D_3$), was provided as a control material for assay calibration or verification. Participants were asked to provide single results for each of these solutions. In addition, participants were asked to determine $25(OH)D_2$, $25(OH)D_3$, and a total concentration of 25-hydroxyvitamin D ($25(OH)D_{Total} = 25(OH)D_2 + 25(OH)D_3$) in each of four samples (vials A, B, C, and D) of human serum (study materials). In this exercise, vials A and B were duplicate samples of SRM 968d Fat-Soluble Vitamins, Carotenoids and Cholesterol in Human Serum Level 1 (SRM 968d L1), which is a blended human serum pool with endogenous vitamin D levels. Vials C and D were duplicate samples of SRM 968d Level 2 (SRM 968d L2), which is a blended human serum pool that contains endogenous vitamin D levels but has augmented α-tocopherol (vitamin E) and β-carotene (vitamin A) levels.

In the Summer 2010 exercise, there were a total of 37 participants and 39 datasets (two participants provided data for two different methods). Sixteen of the datasets originated from immunoassay (IA) techniques, including six from enzyme immunoassay (EIA), five from chemiluminescence immunoassay (CLIA), and five from radioimmunoassay (RIA). **Appendix A-1** summarizes the immunoassay methods used by the participants. Twenty-three of the datasets originated from liquid chromatographic (LC) methods; of those, 17 were from LC with tandem mass spectrometric detection (LC-MS/MS), five were from LC with ultraviolet absorbance detection (LC-UV), and one was from LC with electrochemical detection (LC-EC). A summary of the LC methods used by the participants may be found in **Appendices A-2** and **A-3**.

The raw data received from all participants is summarized in **Appendix B.** For SRM 968d L1 and SRM 968d L2, the immunoassay methods reported values for $25(OH)D_{Total}$ only, whereas LC-based methods reported values for $25(OH)D_{Total}$, $25(OH)D_2$, and $25(OH)D_3$. Both materials contained very low levels of $25(OH)D_2$ with only four and seven of the LC participants reporting values in SRM 968d L1 and SRM 968d L2, respectively. Two of these participants reported $25(OH)D_2$ values at low levels ranging from 0.04 ng/mL to 3.60 ng/mL, but most labs indicated this analyte was below their quantitation limit of <1 ng/mL to <7 ng/mL. For the majority of the participants using LC, the $25(OH)D_{Total}$ data reported in **Appendix B** is the same as their reported data for $25(OH)D_3$ for SRM 968d L1 and SRM 968d L2. Both LC and immunoassay datasets provided individual values for $25(OH)D_2$ and $25(OH)D_3$ in the ethanolic controls because the analytes were in separate solutions.

Appendix B also provides the summarized NIST results for each of the serum materials. The $25(OH)D_2$ in both SRM 968d L1 and SRM 968d L2 was below the quantitation limit (≈ 0.5 ng/mL) for the NIST method.

SUMMER 2010 EXERCISE RESULTS AND DISCUSSION

25(OH)D_2 and 25(OH)D_3 in the control solutions (SRM 2972)

A summary of the individual participant data for 25(OH)D_2 and 25(OH)D_3 in the SRM 2972 control solutions is provided in **Table 1.** The community results are summarized at the bottom of the table for all reported methods, the immunoassay methods only, LC methods only, and the LC-MS/MS methods only. The community results include the total number of quantitative values reported (N), the median value for each analyte, the MADe (median absolute deviation estimate, a robust estimate of the standard deviation), and the percent coefficient of variation (CV%). Consensus statistics were not calculated for the data from the UV and EC methods because of the limited number of data reported (4 and 1 values, respectively). **Table 1** also presents the NIST certified values with expanded uncertainties corresponding to 95% confidence.

For all participant datasets, the single data values reported for 25(OH)D_2 and 25(OH)D_3 in the control solutions, SRM 2972, are plotted in **Figure 1**. The results from immunoassay methods are displayed with closed red circles (●). The results from the LC-based methods are displayed with black squares and are segregated by MS/MS detection (■) and UV/EC detection (□). From the single reported values for all datasets for a given technique (IA or LC), the consensus median and the consensus variability (2 × MADe) were determined (reported in **Table 1**). For each of the techniques within both graphs, the solid lines (——) represent the consensus median and the dotted lines (- - - - -) represent the consensus variability (2 × MADe). The laboratories with results that fall between the two dotted lines are within the consensus variability area for their technique (IA or LC). The graphs reveal that the consensus variability range for the participants who reported results using IA methods is quite large for both analytes. Several other IA participants reported that the calibration solutions were not compatible with their method and did not provide values. Overall, the control solutions appeared more compatible with the LC methods, which exhibited less consensus variability. The NIST certified value is provided by a grey-shaded bar that represents the value and its associated uncertainty (± U_{95}); these "target" values were provided to participants in the reporting sheet.

A direct comparison of results for 25(OH)D_2 and 25(OH)D_3 in the SRM 2972 control solutions is provided in the Youden plot in **Figure 2**. Laboratories that are within the consensus range for both the 25(OH)D_2 and 25(OH)D_3 ethanolic controls are within the blue consensus box in **Figure 2**. Conversely, laboratories that fall outside of (or on the edge of) the blue consensus box are highlighted with their laboratory code numbers. The NIST values are denoted with a red diamond symbol (◆). The Youden line centered on the NIST values is illustrated by a red line (——) that represents the relative ratio of the NIST values (334.8/238.6) for 25(OH)D_3 and 25(OH)D_2 across the magnitude of the y- and x-axis, respectively. Participant data (numbers 193, 175, 139, 198a, 191, 196) that are near the Youden line but are above or below the consensus box may suggest that these measurements are biased high or low due to a calibration error. However, correlation with the Youden line may be complicated for the control solutions because separate calibration solutions are likely prepared for measurement of 25(OH)D_2 and 25(OH)D_3, particularly for LC-based methods.

Table 1. Summary of participant data for 25(OH)D_2 (ng/mL) and 25(OH)D_3 (ng/mL) in the SRM 2972 control solutions.

25(OH)D_2 (ng/mL)

		SRM 2972
Lab	Method	Value
030	RIA	239.9
032	LC-UV	215.0
056	LC-MS/MS	255.3
062	RIA	245.0
110	LC-UV	247.0
116	LC-MS/MS	237.3
139	LC-UV	328.9
150	LC-MS/MS	218.0
169	LC-ECD	241.9
175	CLIA	169.5
182	LC-MS/MS	235.2
183a	LC-MS/MS	199.0
184	LC-MS/MS	230.2
187	LC-MS/MS	210.0
191	RIA	303.3
193	EIA	123.6
194	LC-MS/MS	242.5
195	LC-MS/MS	237.0
196	CLIA	364.2
197	LC-MS/MS	247.5
198a	LC-MS/MS	286.7
199	LC-MS/MS	244.0
200	RIA	245.0
202	LC-MS/MS	234.9
203	LC-UV	206.8
204	CLIA	189.0
All methods	N	26
	Median	238.6
	MADe	19.0
	CV%	8.0
IA methods	N	8
	Median	242.4
	MADe	84.7
	CV%	34.9
LC methods	N	18
	Median	237.2
	MADe	15.0
	CV%	6.3
LC-MS/MS	N	13
	Median	237.0
	MADe	10.4
	CV%	4.4
	NIST Value	238.6
	U_{95}	3.9

25(OH)D_3 (ng/mL)

		SRM 2972
Lab	Method	Value
030	RIA	341.9
032	LC-UV	333.0
056	LC-MS/MS	334.8
062	RIA	575.9
110	LC-UV	315.0
116	LC-MS/MS	364.6
139	LC-UV	368.5
150	LC-MS/MS	291.0
169	LC-ECD	339.5
175	CLIA	254.0
182	LC-MS/MS	326.6
183a	LC-MS/MS	326.0
184	LC-MS/MS	325.0
187	LC-MS/MS	331.0
191	RIA	465.8
193	EIA	191.2
194	LC-MS/MS	346.0
195	LC-MS/MS	330.0
196	CLIA	562.0
197	LC-MS/MS	337.5
198a	LC-MS/MS	406.4
199	LC-MS/MS	336.0
200	RIA	373.0
202	LC-MS/MS	352.8
203	LC-UV	377.6
204	CLIA	337.0
	N	26
	Median	337.3
	MADe	20.6
	CV%	6.1
	N	8
	Median	357.5
	MADe	157.0
	CV%	43.9
	N	18
	Median	335.4
	MADe	14.7
	CV%	4.4
	N	13
	Median	334.8
	MADe	13.0
	CV%	3.9
	NIST Value	334.0
	U_{95}	5.2

Figure 1. 25(OH)D_2 and 25(OH)D_3 values in SRM 2972 for immunoassay, LC-MS/MS and LC-UV or LC-EC methods. The grey-shaded bars represent the ranges bound by the NIST certified values with ± U_{95} expanded uncertainty.

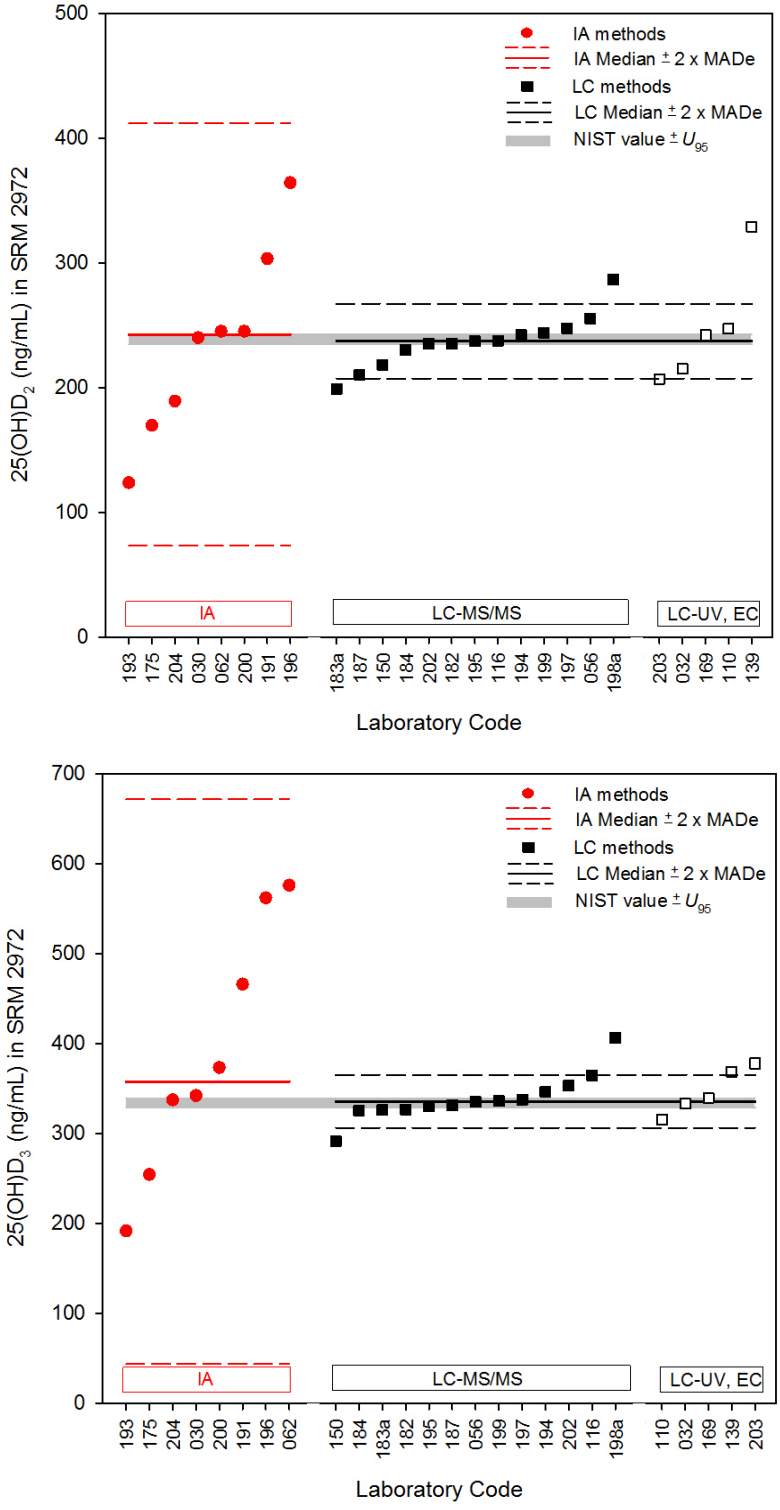
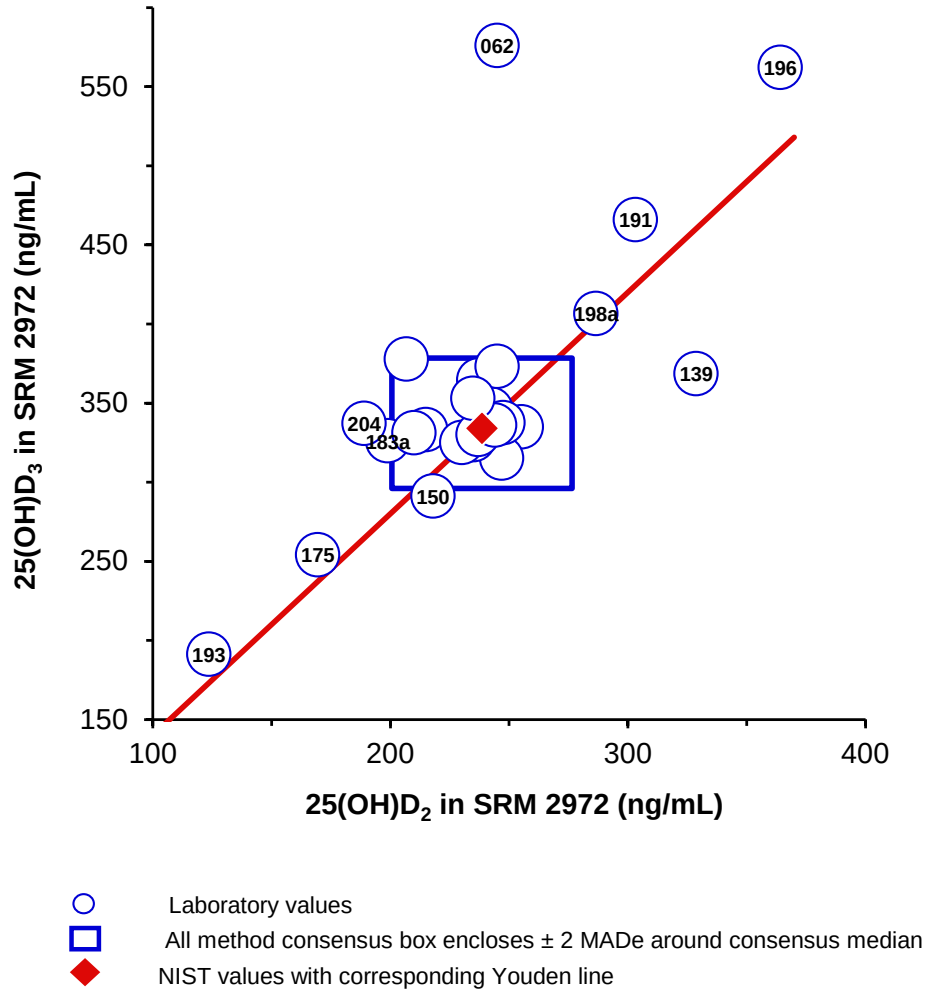


Figure 2. Comparison of results for 25(OH)D_2 and 25(OH)D_3 values in the SRM 2972 control solutions.



25(OH)D in SRM 968d L1 and SRM 968d L2

A summary of the individual participant data for $25(OH)D_{Total}$ in samples SRM 968d L1 and SRM 968d L2 (vials A&B and vials C&D, respectively) is provided in **Table 2.** The summarized data include the average, standard deviation (SD), and percent relative standard deviation (%rSD) of the two reported values for SRM 968d L1 or SRM 968d L2. The community results are summarized at the bottom of the table for all reported methods, the immunoassay methods only, the LC methods only, and the LC-MS/MS methods only. These summarized results include the total number of quantitative values reported, the median value, the MADe, and the percent coefficient of variation. Consensus statistics were not calculated for the data from the UV and EC methods because of the limited number of data reported (5 and 1 values, respectively).

Table 2 also presents the NIST results with approximated 95% confidence limits obtained for SRM 968d L1 and SRM 968d L2. The NIST values for $25(OH)D_3$ were obtained using an LC-MS/MS reference measurement procedure[1] recognized by the Joint Committee for Traceability in Laboratory Medicine (JCTLM), and the U_{95} confidence interval includes components for both measurement variability (N = 8) and measurement uncertainty associated with the density. The $25(OH)D_2$ was below the quantitation limit (≈ 0.5 ng/mL) in both materials and was not included in the results for $25(OH)D_{Total}$.

[1] Tai, S. S.-C., Bedner, M. and Phinney, K.W. *Anal. Chem.* **2010** *82*, 1942-1948.

Table 2: Summary of participant data for 25(OH)D_{Total} (ng/mL) in SRM 968d L1 and SRM 968d L2.

		SRM 968d L1		SRM 968d L2		SRM 968d L1 Combined			SRM 968d L2 Combined		
Lab	Method	Vial A	Vial B	Vial C	Vial D	Mean	SD	%rSD	Mean	SD	%rSD
017	CLIA	11.4	12.5	8.8	9.2	12.0	0.8	6.5	9.0	0.2	2.7
026	LC-MS/MS	13.1	11.1	9.8	11.6	12.1	1.4	11.7	10.7	1.3	11.9
030	RIA	13.6	13.7	9.2	8.9	13.7	0.1	0.5	9.1	0.2	2.3
032	LC-UV	13.5	14.6	18.4	18.5	14.1	0.8	5.5	18.5	0.1	0.4
056	LC-MS/MS	13.7	11.0	11.3	11.0	12.4	1.9	15.5	11.2	0.2	1.9
062	RIA	14.2	14.1	16.9	17.2	14.2	0.1	0.5	17.1	0.2	1.2
110	LC-UV	7.6	9.9	7.0	6.8	8.8	1.6	18.6	6.9	0.1	2.0
116	LC-MS/MS	13.3	12.6	13.1	10.7	13.0	0.5	3.8	11.9	1.7	14.3
128	LC-MS/MS	12.6	12.1	12.7	12.2	12.4	0.4	2.9	12.5	0.4	2.8
139	LC-UV	9.2	9.1	n/d	94.8	9.2	0.1	0.8	94.8	n/a	n/a
141	EIA	9.7	12.1	4.1	5.5	10.9	1.7	15.6	4.8	1.0	20.6
150	LC-MS/MS	15.0	15.1	12.2	11.3	15.1	0.1	0.5	11.8	0.6	5.4
169	LC-ECD	19.9	20.1	19.6	19.0	20.0	0.1	0.7	19.3	0.4	2.2
175	CLIA	15.7	15.8	11.1	11.3	15.8	0.1	0.4	11.2	0.1	1.3
180	RIA	17.3	16.6	14.1	14.3	17.0	0.5	3.0	14.2	0.1	0.9
182	LC-MS/MS	11.7	11.5	12.0	12.3	11.6	0.1	1.2	12.2	0.2	1.7
183a	LC-MS/MS	13.5	14.9	12.0	12.8	14.2	1.0	7.0	12.4	0.6	4.6
183b	CLIA	14.2	14.3	9.8	10.0	14.3	0.1	0.5	9.9	0.1	1.4
184	LC-MS/MS	12.5	12.7	23.9	22.5	12.6	0.1	1.1	23.2	1.0	4.3
185	LC-MS/MS	14.7	14.8	12.6	12.7	14.7	0.1	0.5	12.6	0.0	0.3
186	LC-MS/MS	18.6	19.0	15.8	13.0	18.8	0.3	1.5	14.4	2.0	13.7
187	LC-MS/MS	14.1	12.9	11.9	12.3	13.5	0.8	6.3	12.1	0.3	2.4
188	CLIA	14.6	13.6	10.6	10.5	14.1	0.7	5.0	10.6	0.1	0.7
189	LC-UV	15.9	12.0	11.5	12.6	14.0	2.8	19.8	12.1	0.8	6.5
191	RIA	14.3	14.3	9.8	9.8	14.3	0.0	0.0	9.8	0.0	0.0
193	EIA	16.4	16.4	11.6	12.1	16.4	0.0	0.0	11.9	0.4	3.0
194	LC-MS/MS	12.5	13.7	10.9	11.9	13.1	0.8	6.5	11.4	0.7	6.2
195	LC-MS/MS	12.5	12.6	14.4	12.1	12.6	0.1	0.6	13.3	1.6	12.3
196	CLIA	13.9	13.8	9.7	9.9	13.9	0.1	0.5	9.8	0.1	1.3
197	LC-MS/MS	16.0	15.0	12.0	13.0	15.5	0.7	4.6	12.5	0.7	5.7
198a	LC-MS/MS	12.6	15.8	11.2	8.5	14.2	2.3	15.9	9.9	1.9	19.4
198b	EIA	15.0	16.0	11.0	10.0	15.5	0.7	4.6	10.5	0.7	6.7
199	LC-MS/MS	11.3	12.8	10.8	11.8	12.0	1.0	8.5	11.3	0.7	6.0
200	RIA	13.9	13.9	10.3	10.0	13.9	0.0	0.0	10.2	0.2	2.1
201	EIA	18.5	17.1	12.8	12.6	17.8	1.0	5.6	12.7	0.1	1.1
202	LC-MS/MS	13.6	12.8	11.6	11.2	13.2	0.6	4.5	11.4	0.3	2.4
203	LC-UV	17.2	17.5	17.1	18.0	17.3	0.2	0.9	17.6	0.6	3.6
204	CLIA	13.1	13.2	10.2	9.7	13.2	0.1	0.5	10.0	0.4	3.6
206	EIA	16.4	16.2	11.7	11.5	16.3	0.1	0.5	11.6	0.2	1.5
All methods	N	39	39	38	39	39			39		
	Median	13.9	13.8	11.6	11.8	14.0			11.8		
	MADe	1.9	1.9	1.9	1.9	2.1			1.9		
	CV%	13.9	14	16.0	16	14.9			15.8		
IA methods	N	16	16	16	16	16			16		
	Median	14.3	14.2	10.5	10.0	14.2			10.3		
	MADe	1.4	1.9	1.4	1.4	1.7			1.6		
	CV%	9.9	14	13.6	14	12			15		
LC methods	N	23	23	22	23	23			23		
	Median	13.5	12.8	12.0	12.3	13.2			12.2		
	MADe	1.5	2.6	1.4	1.1	1.5			1.3		
	CV%	11.0	20.6	11.7	9	11.2			10.7		
LC-MS/MS	N	17	17	17	17	17			17		
	Median	13.3	12.8	12.0	12.1	13.1			12.1		
	MADe	1.2	1.9	1.0	1.0	1.5			0.9		
	CV%	8.9	14.8	8.6	9	11.3			7.7		
	NIST Value	12.38	12.38	10.37	10.37	12.38			10.37		
	U_{95}	0.28	0.28	0.23	0.23	0.28			0.23		

n/d = not detected; n/a = not applicable

For all participant datasets, the mean values and error bars (representing ± 2 × SD) for 25(OH)D_{Total} in SRM 968d L1 and SRM 968d L2 are plotted in **Figure 3**. The results from immunoassay methods are displayed with closed red circles (●). The results from the LC-based methods are displayed with black squares and are segregated by MS/MS detection (■) and UV/EC detection (□). From the mean values for all datasets for a given technique (IA or LC), the consensus median and the consensus variability (2 × MADe) were determined (reported in **Table 2**). For each of the techniques within both graphs, the solid lines (——) represent the consensus median and the dotted lines (- - - - -) represent the consensus variability (2 × MADe). Note that consensus statistics were not calculated for the UV and EC results because of the limited number of datasets. The laboratories with results that fall between the two dotted lines are within the consensus variability area for their technique (IA or LC). The NIST value for these materials is provided by a grey-shaded bar that represents the value and its associated uncertainty (± U_{95}).

Specific results as assessed from **Figure 3** are summarized below.

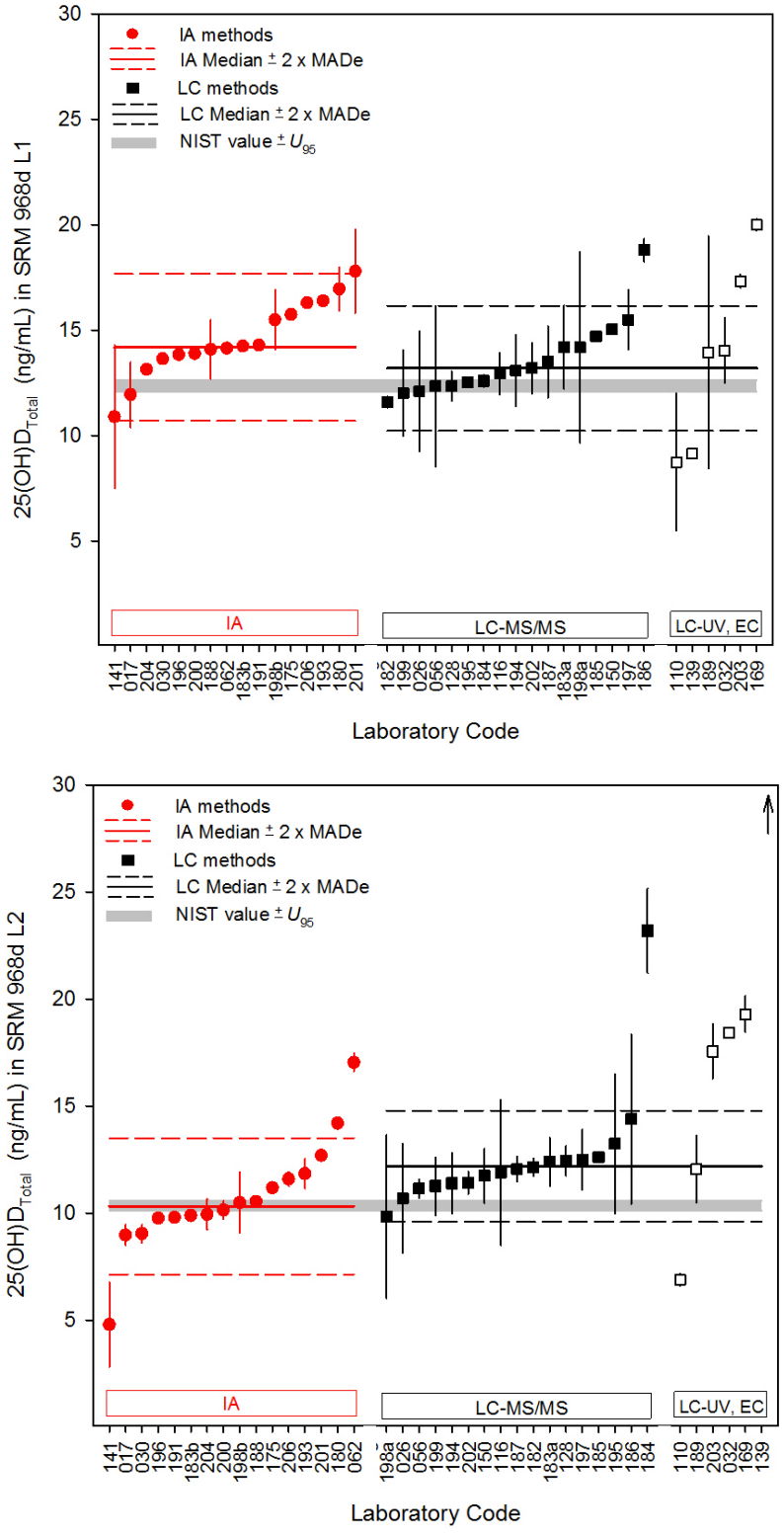
SRM 968d L1

- For the immunoassay results, all laboratory data are within the consensus variability when the error bars for each data point are considered.
- For the LC-MS/MS results, all but one of the datasets are within the consensus variability.
- The LC-UV and LC-EC techniques provide more variable results for 25(OH)D, with only three of the six data points falling in the consensus range when the error bars are considered.
- The median value for the IA results is slightly higher than the median value for the LC-methods.
- The consensus median results for both IA and LC methods are higher than the NIST value.
- The NIST value is included within the consensus ranges for both IA and LC methods.

SRM 968d L2

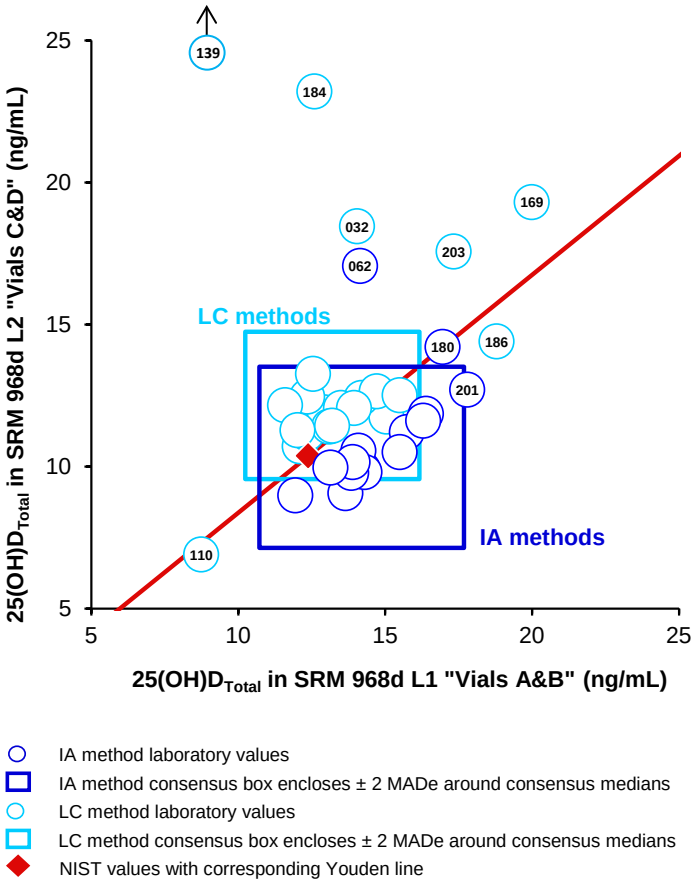
- The IA results exhibit an asymmetrical distribution towards higher levels of 25(OH)D.
- For the LC-MS/MS results, all but one of the datasets are within the consensus variability.
- The LC-UV and LC-EC techniques provide more variable results for 25(OH)D, with only one dataset in the consensus range.
- The consensus median for the LC method results is higher than the IA consensus median (opposite of what was observed for SRM 968d L1).
- The LC consensus median is higher than the NIST value, whereas the consensus median for the IA results is directly comparable with the NIST value.
- The NIST value is included within the consensus ranges for both IA and LC methods.

Figure 3. 25(OH)D_{Total} levels in SRM 968d L1 and SRM 968d L2 as determined by immunoassay, LC-MS/MS, and LC-UV or LC-EC methods. Data that extend beyond the y-axis scale are denoted by an arrow. The grey-shaded bars represent the ranges bound by the NIST values with ± estimated U_{95} uncertainty.



A direct comparison of results for 25(OH)D_{Total} between SRM 968d L1 and SRM 968d L2 is provided in the Youden plot in **Figure 4**. There are two blue consensus boxes, one for IA methods and one for LC methods (as indicated). Laboratory results that are within the consensus range for both study materials are within the blue consensus boxes in **Figure 4**. Conversely, laboratories that fall outside of (or on the edge of) either of the consensus ranges are not included in the blue consensus boxes and are highlighted with their laboratory code numbers. The NIST values for these materials are denoted with a red diamond symbol (◆). The Youden line centered on the NIST values is illustrated by a red line (——), which represents the relative ratio of the NIST values (10.37/12.38) for SRM 968d L2 and SRM 968d L1 across the magnitude of the y- and x-axis, respectively. Participant data (numbers 110, 180, 201, 186, 203, 169) that are near the Youden line but are above or below the consensus box suggest that these measurements may be biased high or low due to a calibration error. Laboratories that fall into this category may want to investigate how their calibrants are prepared, the purity of the calibrant material(s), and/or the method used to evaluate calibrant concentration (e.g., gravimetry vs. UV-spectrophotometry). Several laboratories (numbers 62, 32, 184 and 139 (data not observable on y-axis scale)) provided results that were relatively consistent with consensus for the SRM 968d L1 material but not for SRM 968d L2 material.

Figure 4. Comparison of results for 25(OH)D_{Total} for all methods.



As indicated in **Figure 3** and **Table 2**, the NIST values for SRM 968d L1 and SRM 968d L2 are 12.38 ng/mL ± 0.28 ng/mL, and 10.37 ng/mL ± 0.23 ng/mL, respectively. According to the current guidance regarding 25(OH)D levels and human health (obtained from the NIH website and presented in **Table 3**), the NIST data indicate that the concentrations in both materials are consistent with "inadequate" levels of vitamin D, but SRM 968d L2 may also be consistent with "deficient" 25(OH)D. SRM 968d L1 and SRM 968d L2 were selected because they are in the clinically-important, narrow range in which accurate and reliable measurements of 25(OH)D are important. The median participant results (for all methods) of 14.0 ng/mL and 11.8 ng/mL for SRM 968d L1 and SRM 968d L2, respectively, also indicate that the concentrations in these materials are consistent with "inadequate" 25(OH)D. However, the range of 25(OH)D values reported by program participants of 8.8 ng/mL to 20.0 ng/mL and 4.8 ng/mL to 94.8 ng/mL for SRM 968d L1 and SRM 968d L2, respectively, resulted in an overall program CV% of ≈ 16% (**Table 2**). Because the concentration ranges that distinguish inadequate and adequate levels of 25(OH)D are narrow (**Table 3**), relatively precise within-laboratory measurements are necessary.

One of the goals of the program is to reduce the consensus variability to better represent the community's measurement capability while also recognizing that a "fit-for-purpose" variability-level may exist. Another goal of the program is to achieve better agreement between the participant consensus median value and the NIST value and to better understand the sources of bias between the results.

One source of bias in the LC-MS/MS measurements is contribution from 3-epi-25(OH)D_3, which coelutes with 25(OH)D_3 using typical chromatographic columns (C8, C18) and responds to the same multiple reaction monitoring (MRM) conditions. The NIST method separates 25(OH)D_3 and 3-epi-25(OH)D_3, which was detected but not quantitated in either material. This bias was estimated to be approximately 6% and 7% at NIST for SRM 968d L1 and SRM 968d L2, respectively, which might explain the difference observed for SRM 968d L1 but does not fully account for the difference observed for SRM 968d L2 (LC-MS/MS median 7% and 17% higher than the NIST value for SRM 968d L1 and SRM 968d L2, respectively). One of the LC-MS/MS participants (Lab 56) also noted the presence of 3-epi-25(OH)D_3 in both study materials. It is unclear how the presence of 3-epi-25(OH)D_3 affects the immunoassay results.

Table 3. Serum 25-Hydroxyvitamin D [25(OH)D] Concentrations and Health

ng/mL	nmol/L	Health Status
<10-11	<25-27.5	Associated with vitamin D deficiency, leading to rickets in infants and children and osteomalacia in adults [1,2]
<10-15	<25-37.5	Generally considered inadequate for bone and overall health in healthy individuals [1,2]
≥15	≥37.5	Generally considered adequate for bone and overall health in healthy individuals [1]
Consistently >200	Consistently >500	Considered potentially toxic, leading to hypercalcemia and hyperphosphatemia, although human data are limited. In an animal model, concentrations ≤400 ng/mL (≤1,000 nmol/L) demonstrated no toxicity [3,4].

Table from http://ods.od.nih.gov/factsheets/vitamind/

[1] Institute of Medicine, Food and Nutrition Board. Dietary Reference Intakes: Calcium, Phosphorus, Magnesium, Vitamin D, and Fluoride. Washington, DC: National Academy Press, 1997

[2] Scientific Advisory Committee on Nutrition. Update on Vitamin D. Position Statement by the Scientific Advisory Committee on Nutrition. London: The Stationery Office, Limited, 2007

[3] Jones G. Pharmacokinetics of vitamin D toxicity. Am J Clin Nutr 2008;88:582S-6S. [PubMed abstract]

[4] Shepard RM, DeLuca HF. Plasma concentrations of vitamin D3 and its metabolites in the rat as influenced by vitamin D3 or 245-hydroxyvitamin D3 intakes. Arch Biochem Biophys 1980;202:43-53. [PubMed abstract]

Appendix A-1. Summary of immunoassay methods used by participants.

Participant Number	*IA Method*	*Sample Preparation*	*Detection*
17	CLIA	n/r	n/r
30	RIA	50 µL sample was extracted with 500 µL acetonitrile	n/r
62	RIA	RIA kit	Gamma counter
141	EIA	Sample diluted 1:20 followed by 25(OH)D releasing reagent; ELISA plate precoated with 25(OH)D - sample added; free 25(OH)D on plate competes to bind with antibody; a peroxidase conjugate added	OD reading at 450nm; color is inversely proportional to 25(OH)D in sample
175	CLIA	1st incubation (10 min): 25-OH-D is dissociated from its binding protein and binds to the specific antibody on the solid phase; tracer (vitamin D linked to an isoluminol derivative) is added 10 min later. 2nd incubation: unbound material removed with a wash cycle; starter reagents added; a flash chemiluminescent reaction initated	Photomultiplier measures relative light units, which is inversely proportional to the concentration of 25(OH)D present in calibrators, controls or samples.
180	RIA	Samples were prepared per vendor's sample extraction protocol	I^{125} detection using gamma counter
183b	CLIA	None; calibrators diluted to get samples in analytical range	n/r
188	EIA	n/r	n/r
191	RIA	n/r	n/r
193	EIA	Samples centrifuged	OD reading
196	CLIA	The human serum samples were thawed and analyzed	n/r
198b	EIA	n/r	n/r
200	RIA	Sample was extracted	n/r
201	EIA	Sample was thawed and swirled prior to analysis	OD reading at 450 nm
204	CLIA	Sample was well-mixed prior to analysis	n/r
206	EIA	n/r	n/r

OD = optical density

n/r = not reported

Appendix A-2. Summary of LC-MS/MS methods used by participants.

Participant Number	*Internal Standard (IS)*	*Sample Preparation*	*Chromatographic Conditions*	*Detection: MRM ions*
26	Deuterated $25(OH)D_2$ and $25(OH)D_3$	Sample was extracted, evaporated, and reconstituted with methanol/water	C18 column (50 x 2.1 mm); isocratic separation with 95% methanol, 5% water; flow 0.2 mL/min	$25(OH)D_2$ 413/355; $25(OH)D_3$ 401/365
56	$25(OH)D_2$-d_3 and $25(OH)D_3$-d_6	Liquid-liquid extraction	PFP column; isocratic elution; flow 0.4 mL/min	$25(OH)D_3$ 383/365; $25(OH)D_3$-d_6 389/371; $25(OH)D_2$ 395/377; $25(OH)D_2$-d_3 398/380
116	$25(OH)D_3$ -d_6	Serum proteins were precipitated with methanol	LC column; Isocratic separation with 95% methanol, 5% water; flow 0.6 mL/min; Online SPE	$25(OH)D_3$ 383/211; $25(OH)D_3$-d_6 *389/211* ; $25(OH)D_2$ 395/269
128	n/r	n/r	n/r	n/r
150	$25(OH)D_2$-d_6 and $25(OH)D_3$-d_3	The samples were liquid-liguid extracted, centrifuged, separated, evaporated under nitrogen, reconstituted in mobile phase, and analyzed	LC column (100 x 2.1 mm); Isocratic separation with 74% methanol, 26% water; flow 0.5 mL/min	$25(OH)D_3$ 401/365, 401/383; $25(OH)D_3$-d_3 404/386; $25(OH)D_2$ 413/355, 413/361; $25(OH)D_2$-d_6 419/401
182	$25(OH)D_3$ -d_6	Proteins were precipitated with acetonitrile and IS directly in 96 well plate	C18 column (50 x 2 mm); Gradient from 60% to 100% methanol	$25(OH)D_3$ 401/365 (quant), 401/383 (qual); $25(OH)D_2$ 413/355 (quant), 413/271 (qual); $25(OH)D_3$-d_6 407/371 (quant), 407/389 (qual)
183a	$25(OH)D_3$ -d_6	IS (25 μL) was added to sample (150 μL), followed by protein precipiation and extraction with 0.1 mol/L $ZnSO_4$ (150 μL), methanol (300 μL), and hexane (750 μL); extract dried and dissolved with 70% methanol, 30% water with 2 mmol/L ammonium acetate	C8 column (50 x 2.1 mm); isocratic elution with 73% methanol, 27% water; flow 0.4 mL/min	$25(OH)D_3$ 401/159, 401/383; $25(OH)D_2$ 413/82, 413/395
184	$25(OH)D_3$ -d_6	Serum (200 μL) extracted with acetonitrile and IS (700 μL); mixed, centrifuged, and filtered	C18 column (100 x 2.1mm; 5μm); linear gradient from 60% B to 98% B over 2 min (A: 0.1% formic acid in water, B: methanol with 0.1% formic acid and 5 mmol/L ammonium acetate)	$25(OH)D_3$ 383/257; $25(OH)D_3$-d_6 389/263; $25(OH)D_2$ 395/209 (APCI)
185	$25(OH)D_2$-d_6 and $25(OH)D_3$-d_6	Liquid-Liquid extraction	C18 (50 x 2 mm); flow 0.4 mL/min; methanol gradient (3.6 min)	n/r
186	$25(OH)D_3$ -d_6	Protein precipitation with methanol, liquid-liquid extraction with hexane followed by centrifugation, evaporation and reconstitution in mobile phase	Phenyl LC column (50 x 2.1 mm; 1.7μm); temp. 35° C	$25(OH)D_3$ 383/159 (ESI)
187	n/r	n/r	n/r	n/r
194	$25(OH)D_3$ -d_6	Proteins precipitated, followed by centrifugation, evaporation of top layer, and reconstitution	C8 column (50 x 2 mm); isocratic elution with 70% acetonitrile/ 30% water; flow 0.7 mL/min	$25(OH)D_3$ 383/211; $25(OH)D_2$ 395/119
195	$25(OH)D_2$-d_3 and $25(OH)D_3$-d_6	Samples extracted then derivatized	LC column (30 x 2.1 mm); gradient with methanol/water	n/r

197	25(OH)D_3 -d_6	Precipitating agent added (200 µL with 20 ng IS) to each serum (200 µL), calibrator and control sample followed by mixing, centrifugation, and analysis	C18 column (50 x 4.6 mm; 5 µm); flow 1.0 mL/min; column temp 45°C; gradient with water and methanol	n/r
198a	25(OH)D_3 -d_6	Proteins precipitated with methanol, followed by hexane extraction, centrifugation, evaporation under N_2, and reconstitution in methanol (0.1% formic acid)	C18 column (50 x 2.1 mm; 3.5 um); isocratic elution with 85% methanol (0.1% formic acid); flow 0.5 mL/min	25(OH)D_3 401/383, 401/365; 25(OH)D_2 413/395, 413/355; 25(OH)D_3-d_6 407/389, 407/371
199	25(OH)D_2-d_3 and 25(OH)D_3-d_6	Sample (100 µL) extracted with acetonitrile (200 µL) containing IS, followed by mixing (15 s), equilibration (30 min @ 4°C), mixing (15 s), centrifugation (10 min), analysis of supernatant	C18 column (50 x 2.1mm, 5 µm); gradient with 0.1% formic acid in water and 0.1% formic acid in methanol; flow 0.7mL/min	25(OH)D_3 383/211, 383/257, 383/365; 25(OH)D_3-d_6 389/211, 389/263, 389/371; 25(OH)D2 395/209, 395/211, 395/269, 395/377; 25(OH)D_2-d_3 398/211, 398/230, 398/272, 398/380
202	D6-labeled compound	Sample was extracted	C18 column (50 x 2.1 mm); Gradient with 10% acetonitrile (containing 0.1% formic acid), 90% methanol; flow 0.3 mL/min	n/r

MRM= multiple reaction monitoring
PFP = pentafluorophenyl phase
SPE = solid phase extraction
quant = quantitative ions
qual = qualitative ions
ESI = electrospray ionization
APCI = atmospheric pressure chemical ionization
n/r = not reported

Appendix A-3. Summary of LC-UV and LC-ED methods used by participants.

Participant Number	*Internal Standard (IS)*	*Sample Preparation*	*Chromatographic Conditions*	*Detection*
32	Proprietary	Samples were extracted with filtration	C18 column (300 x 3.9 mm; 4 µm); proprietary mobile phase; flow 0.7mL/min	UV at 265 nm
110	None	Ethanol (0.3-0.5 mL) used to precipiate proteins in serum (0.3–0.5 mL); sample extracted with two volumes of hexane-dichloromethane, evaporated, then dissolved	LC C18 column (100 x 2.1 mm; 1.8 µm); C18 column (220 x 4.6 mm; 5 µm); Column temp 35°C and 21°C; gradient: from 5% B to 50% B from 0.8 min to 2 min, hold until 3.5 min (A: 85% acetonitrile, 15% methanol, B: isopropanol); flow 0.5 ml/min	UV at 267 nm
139	Proprietary	Samples extracted by precipitation, centrifuged, and an aliquot injected directly	Column and mobile phase are proprietary	UV at 264 nm
169	Proprietary	SPE	Column and mobile phase are proprietary	EC at 450 mv (screening electrode) and 750 mv (measuring electrode)
189	Obtained from kit supplier	Proteins were precipitated, samples centrifuged, analytes in the supernatant were extracted using SPE cartridges	LC column (4.6 x150 mm); isocratic separation with commercial mobile phase; flow 0.7mL/min	UV at 265 nm
203	n/r	Serum (0.5 mL) precipitated with methanol/isopropanol solution, and then extracted with hexane	LC column (150 x 4.6 mm); isocratic separation with methanol/TFA ; flow 0.6 mL/min	UV at 265 nm

SPE = solid phase extraction

TFA = trifluoroacetic acid

n/r = not reported

Appendix B. Raw participant data for $25(OH)D_2$, $25(OH)D_3$ and $25(OH)D_{Total}$.

		$25(OH)D_2$ (ng/mL)				$25(OH)D_3$ (ng/mL)				$25(OH)D_{Total}$ (ng/mL)				$25(OH)D_2/D_3$ (ng/mL)	
		SRM 968d L1	SRM 968d L1	SRM 968d L2	SRM 968d L2	SRM 968d L1	SRM 968d L1	SRM 968d L2	SRM 968d L2	SRM 968d L1	SRM 968d L1	SRM 968d L2	SRM 968d L2	SRM 2972	
Lab	Method	Vial A	Vial B	Vial C	Vial D	Vial A	Vial B	Vial C	Vial D	Vial A	Vial B	Vial C	Vial D	$25(OH)D_2$	$25(OH)D_3$
017	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	11.4	12.5	8.8	9.2	n/r	n/r
026	LC-MS/MS	< 1.0	< 1.0	< 1.0	< 1.0	13.1	11.1	9.8	11.6	13.1	11.1	9.8	11.6	n/r	n/r
030	RIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	13.6	13.7	9.2	8.9	239.9	341.9
032	LC-UV	n/d	n/d	3.5	3.6	13.5	14.6	14.9	14.9	13.5	14.6	18.4	18.5	215.0	333.0
056	LC-MS/MS	0.3	n/d	0.6	0.6	13.4	11.0	10.7	10.4	13.7	11.0	11.3	11.0	255.3	334.8
062	RIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	14.2	14.1	16.9	17.2	245.0	575.9
110	LC-UV	n/d	n/d	n/d	n/d	7.6	9.9	7.0	6.8	7.6	9.9	7.0	6.8	247.0	315.0
116	LC-MS/MS	< 3.3	< 3.3	< 3.3	< 3.3	13.3	12.6	13.1	10.7	13.3	12.6	13.1	10.7	237.3	364.6
128	LC-MS/MS	n/d	n/d	n/d	n/d	12.6	12.1	12.7	12.2	12.6	12.1	12.7	12.2	n/r	n/r
139	LC-UV	n/r	n/r	n/r	n/r	9.2	9.1	n/d	94.8	9.2	9.1	n/d	94.8	328.9	368.5
141	EIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	9.7	12.1	4.1	5.5	n/r	n/r
150	LC-MS/MS	n/d	n/d	n/d	n/d	15.0	15.1	12.2	11.3	15.0	15.1	12.2	11.3	218.0	291.0
169	LC-ECD	7.0	7.6	9.6	9.1	12.9	12.5	10.0	9.9	19.9	20.1	19.6	19.0	241.9	339.5
175	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	15.7	15.8	11.1	11.3	169.5	254.0
180	RIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	17.3	16.6	14.1	14.3	n/r	n/r
182	LC-MS/MS	0.6	0.7	1.3	1.4	11.1	10.8	10.7	10.9	11.7	11.5	12.0	12.3	235.2	326.6
183a	LC-MS/MS	< 4	< 4	< 4	< 4	13.5	14.9	12.0	12.8	13.5	14.9	12.0	12.8	199.0	326.0
183b	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	14.2	14.3	9.8	10.0	n/r	n/r
184	LC-MS/MS	< 1.0	< 1.0	1.1	< 1.0	12.5	12.7	22.8	22.5	12.5	12.7	23.9	22.5	230.2	325.0
185	LC-MS/MS	n/d	n/d	n/d	n/d	14.7	14.8	12.6	12.7	14.7	14.8	12.6	12.7	n/r	n/r
186	LC-MS/MS	n/r	n/r	n/r	n/r	18.6	19.0	15.8	13.0	18.6	19.0	15.8	13.0	n/r	n/r
187	LC-MS/MS	n/d	n/d	1.0	0.9	14.1	12.9	10.9	11.4	14.1	12.9	11.9	12.3	210.0	331.0
188	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	14.6	13.6	10.6	10.5	n/r	n/r
189	LC-UV	n/d	n/d	n/d	n/d	15.9	12.0	11.5	12.6	15.9	12.0	11.5	12.6	n/r	n/r
191	RIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	14.3	14.3	9.8	9.8	303.3	465.8
193	EIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	16.4	16.4	11.6	12.1	123.6	191.2
194	LC-MS/MS	< 7	< 7	< 7	< 7	12.5	13.7	10.9	11.9	12.5	13.7	10.9	11.9	242.5	346.0
195	LC-MS/MS	< 4.0	< 4.0	< 4.0	< 4.0	12.5	12.6	14.4	12.1	12.5	12.6	14.4	12.1	237.0	330.0
196	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	13.9	13.8	9.7	9.9	364.2	562.0
197	LC-MS/MS	< 5	< 5	< 5	< 5	16.0	15.0	12.0	13.0	16.0	15.0	12.0	13.0	247.5	337.5
198a	LC-MS/MS	< 5	< 5	< 5	< 5	12.6	15.8	11.2	8.5	12.6	15.8	11.2	8.5	286.7	406.4
198b	EIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	15.0	16.0	11.0	10.0	n/r	n/r
199	LC-MS/MS	0.04	0.05	0.4	0.4	11.3	12.7	10.4	11.4	11.3	12.8	10.8	11.8	244.0	336.0
200	RIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	13.9	13.9	10.3	10.0	245.0	373.0
201	EIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	18.5	17.1	12.8	12.6	n/r	n/r
202	LC-MS/MS	n/d	n/d	n/d	n/d	13.6	12.8	11.6	11.2	13.6	12.8	11.6	11.2	234.9	352.8
203	LC-UV	n/d	n/d	n/d	n/d	17.2	17.5	17.1	18.0	17.2	17.5	17.1	18.0	206.8	377.6
204	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	13.1	13.2	10.2	9.7	189.0	337.0
206	EIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	16.4	16.2	11.7	11.5	n/r	n/r

*n/a = not applicable (for immunoassay methods); n/r = not reported; n/d = not detected; < X = less than a reported quantitation limit of X

	NIST Value	<0.5	<0.5	<0.5	<0.5	12.38	12.38	10.37	10.37	12.38	12.38	10.37	10.37	238.6	334.8
	U_{95}	0.0	0.0	0.0	0.0	0.28	0.28	0.23	0.23	0.28	0.28	0.23	0.23	3.9	5.2