NISTIR 7893

NIST/NIH Vitamin D Metabolites Quality Assurance Program Report of Participant Results: Summer 2011 Comparability Study (Exercise 4)

Katrice A. Lippa
Mary Bedner
Susan S.-C. Tai

http://dx.doi.org/10.6028/NIST.IR.7893

National Institute of Standards and Technology
U.S. Department of Commerce

NISTIR 7893

NIST/NIH Vitamin D Metabolites Quality Assurance Program Report of Participant Results: Summer 2011 Comparability Study (Exercise 4)

Katrice A. Lippa
Mary Bedner
Susan S.-C. Tai
Chemical Sciences Division
Material Measurement Laboratory

http://dx.doi.org/10.6028/NIST.IR.7893

December 2012

U.S. Department of Commerce
Rebecca Blank, Acting Secretary

National Institute of Standards and Technology
Patrick D. Gallagher, Under Secretary of Commerce for Standards and Technology and Director

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 fourth exercise of this program, the Summer 2011 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 968e Fat-Soluble Vitamins, Carotenoids, and Cholesterol in Human Serum (Level 1, Level 2, and Level 3) and SRM 968d Fat-Soluble Vitamins, Carotenoids and Cholesterol in Human Serum (Level 1). 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 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 fourth exercise are reported along with a summary of the analytical methods used.

OVERVIEW OF THE SUMMER 2011 COMPARABILITY STUDY

For the Summer 2011 Comparability Study (Exercise 4) of 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 concentration values for $25(OH)D_2$, $25(OH)D_3$, and a total concentration of 25-hydroxyvitamin D ($25(OH)D_{Total} = 25(OH)D_2 + 25(OH)D_3$) for each of four samples (vials A, B, C, and D) of human serum (study materials). In this exercise, vials A, B, and C were SRM 968e Fat-Soluble Vitamins, Carotenoids, and Cholesterol in Human Serum Level 1 (SRM 968e L1), Level 2 (SRM 968e L2), and Level 3 (SRM 968e L3), respectively. Vial D was SRM 968d Fat-Soluble Vitamins, Carotenoids and Cholesterol in Human Serum Level 1 (SRM 968d L1). All materials consisted of blended human serum pools with endogenous 25(OH)D levels.

There were a total of 41 participants and 45 datasets (four participants provided data for two different methods) in the Summer 2011 exercise. Seventeen of the datasets originated from immunoassay (IA) techniques, including three from enzyme immunoassay (EIA), nine from chemiluminescence immunoassay (CLIA), and five from radioimmunoassay (RIA). **Appendix A-1** summarizes the immunoassay methods used by the participants. Twenty-eight of the datasets originated from liquid chromatographic (LC) methods; of those, 22 were from LC with tandem mass spectrometric detection (LC-MS/MS), one was from LC-MS (orbitrap), and five were from LC with ultraviolet absorbance detection (LC-UV). A summary of the LC methods used by the participants may be found in **Appendices A-2** and **A-3**. From here, LC-MS/MS and LC-MS are collectively referred to as $LC\text{-}MS^n$.

The raw data received from all participants is summarized in **Appendix B.** For all study materials, the immunoassay methods reported values for $25(OH)D_{Total}$ only, whereas LC participants provided values for $25(OH)D_2$, $25(OH)D_3$, and $25(OH)D_{Total}$. However, all four serum materials contain very low levels of $25(OH)D_2$ (participant reported values ranging from 0.1 ng/mL to 0.4 ng/mL), and most of the LC labs indicated this analyte was below their quantitation limit of <1 ng/mL to <5 ng/mL. Therefore, the $25(OH)D_{Total}$ values reported in **Appendix B** are the same as the $25(OH)D_3$ values in the serum materials for the majority of LC participants. 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 $25(OH)D_2$ was below the quantitation limit ($\approx$ 0.5 ng/mL) in all materials for the NIST method.

SUMMER 2011 EXERCISE RESULTS AND DISCUSSION

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

Participants were asked to analyze the control materials to qualify their assays prior to measuring the study materials. 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.** Of the 45 datasets received for the Summer 2011 exercise, only 26 reported values for the both 25(OH)D_2 and 25(OH)D_3 ethanolic controls; of those, four were from immunoassay methods and 22 were from LC methods. Three additional participants provided values for only the 25(OH)D_3 ethanolic controls. Overall, the control solutions appeared more compatible with the LC methods, and several of the immunoassay participants reported that the calibration solutions were not compatible with their method and did not provide values.

The community results are summarized at the bottom of **Table 1** for all reported methods, the LC methods only, and the LC-MS^n methods only. The community results include the total number of quantitative values reported (N), the median value for each analyte, the MADe (the 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 IA methods because of the limited number of data reported (N = 4). **Table 1** also presents the NIST certified values with expanded uncertainties corresponding to 95% confidence.

Table 1. Summary of participant data and community results 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
032	LC-UV	261.5
056	LC-MS/MS	227.3
110	LC-UV	233.4
116	LC-MS/MS	241.8
119	LC-MS	243.2
128	LC-MS/MS	n/r
139	LC-UV	241.5
184	LC-MS/MS	236.5
185	LC-MS/MS	238.6
186	LC-MS/MS	n/r
187	LC-MS/MS	235.0
188	CLIA	401.5
189	LC-UV	n/r
195	LC-MS/MS	242.0
196	CLIA	210.4
197	LC-MS/MS	234.3
198a	LC-MS/MS	245.5
199	LC-MS/MS	233.0
200	RIA	216.0
202	LC-MS/MS	241.0
209	LC-MS/MS	240.6
210a	RIA	209.4
211	LC-MS/MS	238.0
212	LC-MS/MS	248.8
215	LC-MS/MS	226.7
217	LC-MS/MS	220.8
220	LC-MS/MS	232.4
221a	LC-MS/MS	253.0
221b	LC-UV	177.0
All methods	N	26
	Median	237.3
	MADe	8.0
	CV%	3.4
LC methods	N	22
	Median	238.3
	MADe	7.3
	CV%	3.0
LC-MS^n	N	18
	Median	238.3
	MADe	6.6
	CV%	2.8
	NIST Value	238.6
	U_{95}	3.9

$25(OH)D_3$ (ng/mL)

		SRM 2972
Lab	Method	Value
032	LC-UV	344.0
056	LC-MS/MS	321.3
110	LC-UV	331.7
116	LC-MS/MS	331.0
119	LC-MS	352.6
128	LC-MS/MS	333.5
139	LC-UV	330.5
184	LC-MS/MS	338.5
185	LC-MS/MS	334.8
186	LC-MS/MS	320.0
187	LC-MS/MS	337.0
188	CLIA	381.5
189	LC-UV	280.9
195	LC-MS/MS	330.0
196	CLIA	452.0
197	LC-MS/MS	342.5
198a	LC-MS/MS	308.6
199	LC-MS/MS	329.0
200	RIA	319.1
202	LC-MS/MS	334.5
209	LC-MS/MS	332.8
210a	RIA	299.1
211	LC-MS/MS	303.8
212	LC-MS/MS	341.7
215	LC-MS/MS	386.2
217	LC-MS/MS	394.3
220	LC-MS/MS	324.4
221a	LC-MS/MS	365.0
221b	LC-UV	214.0
	N	29
	Median	332.8
	MADe	16.6
	CV%	5.0
	N	25
	Median	332.8
	MADe	13.2
	CV%	4.0
	N	20
	Median	334.0
	MADe	12.0
	CV%	3.6
	NIST Value	334.0
	U_{95}	5.2

n/r = not reported

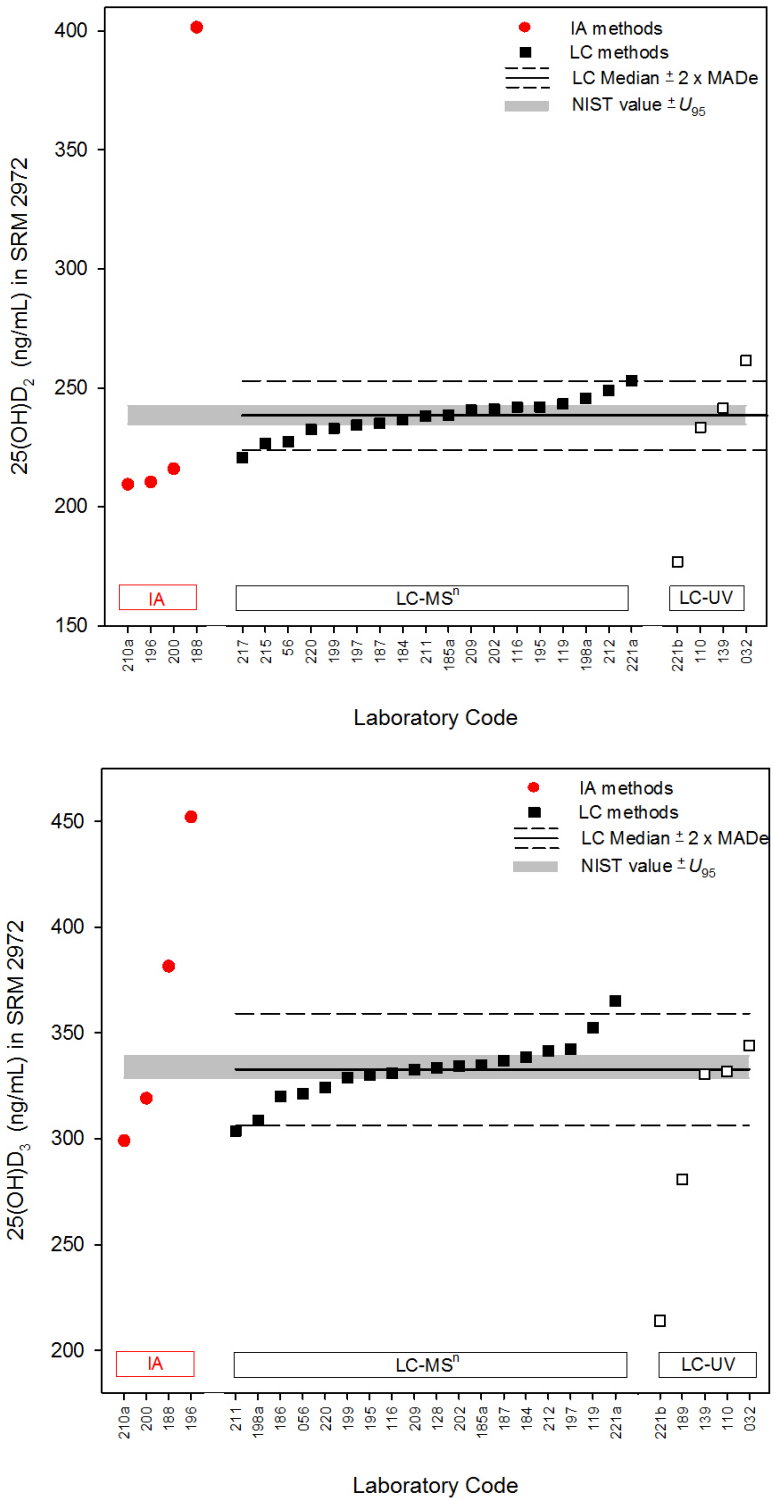
For all participant datasets, the single data values reported for 25(OH)D_2 and 25(OH)D_3 in the control solutions, SRM 2972, are plotted in **Figure 1**. The results from immunoassay methods are displayed with closed red circles (●). The results from the LC-based methods are displayed with black squares and are segregated by MS^n detection (■) and UV 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). For the results from both techniques (IA or LC), the consensus variability is larger for 25(OH)D_3 than for 25(OH)D_2, but the highest variability was obtained for the IA results for 25(OH)D_3.

The grey-shaded bar in **Figure 1** represents the interval in which NIST believes the "true value" exists for these solutions (i.e., NIST value ± approximately 95% confidence intervals (U_{95})). Participants were provided these values both on the SRM 2972 shipping package and within the data reporting sheet. The consensus median value for the LC methods lies within the NIST expanded uncertainty range for both 25(OH)D_2 and 25(OH)D_3. The consensus median value for the IA methods lies outside the NIST expanded uncertainty range for both 25(OH)D_2 and 25(OH)D_3, indicating a potential method bias.

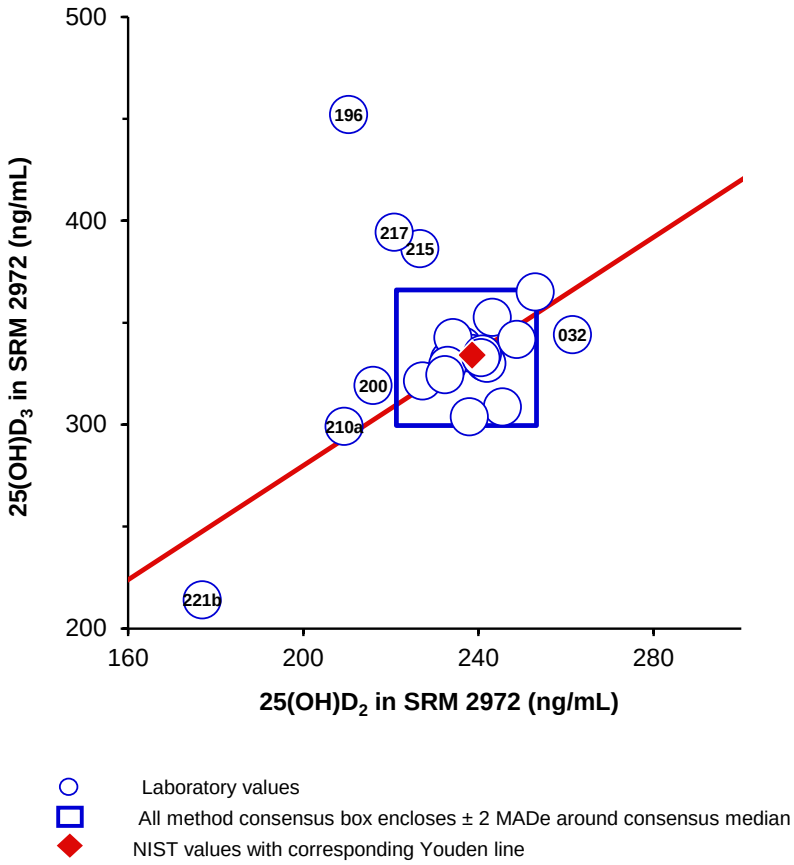
Figure 1. 25(OH)D_2 and 25(OH)D_3 values in SRM 2972 for immunoassay and LC methods. The grey-shaded bars represent the ranges bound by the NIST certified values with ± U_{95} expanded uncertainty.



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**. Laboratory results 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, laboratory results that fall outside of (or on the edge of) the blue consensus boxes are highlighted with their laboratory code numbers (221b, 210a, 200, 196, 217, 215, 32). 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 that are near the Youden line but are clearly above or below the consensus box (number 221b) 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.

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



25(OH)D in SRM 968e L1, SRM 968e L2, SRM 968e L3, and SRM 968d L1

A summary of the individual participant data for $25(OH)D_{Total}$ in samples SRM 968e L1, SRM 968e L2, SRM 968e L3, and SRM 968d L1 (vials A, B, C, and D, respectively) is provided in **Table 2.**

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^n methods only. These summarized results include the total number of quantitative values reported, the median value, the MADe, and the CV%.

Table 2 also presents the NIST results for the four study materials. For SRM 968e L1, L2, and L3, the NIST results are the certified values for $25(OH)D_3$ with an expanded uncertainty corresponding to the 95% confidence interval (U_{95})[1]. For SRM 968d L1, the NIST value for $25(OH)D_3$ was obtained using an LC-MS/MS reference measurement procedure[2] 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 all materials and was not included in the results for $25(OH)D_{Total}$.

[1] https://www-s.nist.gov/srmors/view_cert.cfm?srm=968E
[2] 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 968e L1, SRM 968e L2, SRM 968e L3, and SRM 968d L1.

		SRM 968e L1	SRM 968e L2	SRM 968e L3	SRM 968d L1
Lab	Method	Vial A	Vial B	Vial C	Vial D
017	CLIA	8.1	15.1	26.5	15.7
026	LC-MS/MS	7.9	14.4	20.1	16.6
032	LC-UV	7.0	12.5	20.4	11.5
056	LC-MS/MS	7.2	12.3	21.8	13.2
086a	CLIA	8.0	16.0	28.0	17.0
086b	RIA	11.0	19.0	29.0	19.0
110	LC-UV	6.5	9.1	13.0	13.6
116	LC-MS/MS	9.5	15.6	24.0	14.3
119	LC-MS	9.4	14.8	21.8	14.6
124	LC-MS/MS	10.1	14.8	24.4	13.9
128	LC-MS/MS	11.2	17.8	27.2	18.1
139	LC-UV	10.1	17.1	25.3	17.9
161	CLIA	6.9	15.0	28.5	17.4
184	LC-MS/MS	6.5	12.3	19.5	13.6
185	LC-MS/MS	8.8	16.1	24.6	14.8
186	LC-MS/MS	9.0	12.0	26.0	16.0
187	LC-MS/MS	7.5	14.4	21.9	13.4
188	CLIA	11.8	20.3	27.8	15.2
189	LC-UV	12.3	16.6	30.4	n/r
191	RIA	7.7	11.3	18.1	14.0
192	EIA	7.5	17.9	27.1	20.1
195	LC-MS/MS	7.3	13.3	21.1	12.7
196	CLIA	7.6	14.9	25.2	15.9
197	LC-MS/MS	7.4	13.7	21.4	13.8
198a	LC-MS/MS	9.6	17.2	22.9	15.2
198b	EIA	9.0	17.0	34.0	16.0
199	LC-MS/MS	9.1	13.0	20.5	13.2
200	RIA	7.0	12.2	18.0	13.9
202	LC-MS/MS	7.8	14.1	23.1	13.6
209	LC-MS/MS	7.6	14.3	20.8	12.8
210a	RIA	12.9	19.7	23.5	18.9
210b	CLIA	9.4	16.1	30.4	17.7
211	LC-MS/MS	5.9	11.7	16.5	10.7
212	LC-MS/MS	8.1	13.4	22.9	13.4
213	EIA	9.3	14.0	18.6	17.7
214a	RIA	7.9	14.4	24.7	13.5
214b	CLIA	7.9	16.4	27.2	15.6
215	LC-MS/MS	8.2	15.0	21.2	14.1
217	LC-MS/MS	8.4	14.7	21.9	14.1
218	CLIA	5.5	15.1	25.2	14.1
219	LC-MS/MS	6.9	14.7	18.6	12.3
220	LC-MS/MS	7.5	13.8	20.0	13.1
221a	LC-MS/MS	7.3	13.3	21.7	12.3
221b	LC-UV	6.3	12.2	22.4	n/r
222	CLIA	11.1	20.3	31.1	15.6
All methods	N	45	45	45	43
	Median	7.9	14.7	22.9	14.1
	MADe	1.5	2.1	3.7	1.9
	CV%	18.8	14	16	13.7
IA methods	N	17	17	17	17
	Median	8.0	16.0	27.1	15.9
	MADe	1.5	2.4	2.8	2.7
	CV%	18.5	15	10	16.8
LC methods	N	28	28	28	26
	Median	7.9	14.2	21.8	13.6
	MADe	1.4	1.6	2.0	1.1
	CV%	17.9	11.0	9.2	8.2
LC-MS^n	N	23	23	23	23
	Median	7.9	14.3	21.8	13.6
	MADe	1.1	1.3	1.9	1.0
	CV%	14.1	9.3	8.8	7.6
	NIST Value	7.09	12.90	19.90	12.38
	U_{95}	0.14	0.30	0.40	0.28

n/r = not reported

For all participant datasets, the single reported values for 25(OH)D_{Total} in SRM 968e L1, SRM 968e L2, SRM 968e L3, and SRM 968d L1 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^n detection (■) and UV detection (□). Each figure also has a legend that indicates which individual methods were used to obtain the reported values: CLIA, EIA, RIA, LC-MS^n, or LC-UV.

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 grey-shaded bar for each figure represents the NIST value and its associated uncertainty (i.e., value ± U_{95}). NIST believes that the "true" value for each material lies within this interval. When this bar is not within the consensus range, then there may be method bias.

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

SRM 968e L1

- For the IA results, all but three datasets are within the consensus variability range.
- For the LC results, all but two datasets are within the consensus variability range.
- The consensus median value is nearly identical for the IA and LC results.
- The consensus median value for both IA and LC results are higher than the NIST expanded uncertainty range (grey-shaded bar).
- The NIST expanded uncertainty range (grey-shaded bar) falls within the consensus variability ranges for both IA and LC.

SRM 968e L2

- For the IA results, all datasets are within the consensus variability range.
- For the LC results, all but two datasets are within the consensus variability range.
- The consensus variability range is larger for the IA results than the LC results.
- The consensus median value for the IA results is higher than the consensus median value for the LC results; both LC and IA median values are higher than the NIST expanded uncertainty range (grey-shaded bar).
- The NIST expanded uncertainty range (grey-shaded bar) falls within the consensus variability ranges for both IA and LC.

SRM 968e L3

- For the IA results, all but four datasets are within the consensus variability range.
- For the LC results, all but four datasets are within the consensus variability range.

- The consensus median value for the IA results is higher than the consensus median value for the LC results; both LC and IA median values are higher than the NIST expanded uncertainty range (grey-shaded bar).
- The NIST expanded uncertainty range (grey-shaded bar) falls within the consensus variability range for LC, but not for IA.

SRM 968d L1

For the IA data for SRM 968d L1, the consensus variability based on MADe is an overestimation of the 95% confidence limits about the median. The non-Gaussian data distribution contributes to a relatively wide range for the central 50% of this data, resulting in a large MADe. Since the consensus variability is not well-described with a MADe estimation, a meaningful assessment of the consensus range, the outlying results, and the agreement with the NIST value is hindered for the IA results.

- For the LC results, all but five datasets are within the consensus variability range.
- The consensus median value for the IA results is higher than the consensus median value for the LC results; both LC and IA median values are higher than the NIST expanded uncertainty range (grey-shaded bar).
- The NIST expanded uncertainty range (grey-shaded bar) falls within the consensus variability range for LC.

Overall, the results for the four study materials are very consistent, with the participant values always higher than the NIST value. In addition, the consensus variability is similar but relatively high for the four materials, ranging from 14% to 19% for all methods (**Table 2**). Similar trends have also been observed for many of the study materials evaluated in previous exercises of VitDQAP. A 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. In addition, a major goal of VitDQAP 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.

It is notable that the NIST method separates 25(OH)D_3 and its 3-epimer, 3-epi-25(OH)D_3, which was not quantitated in the study materials. The 3-epi-25(OH)D_3 coelutes with 25(OH)D_3 using typical chromatographic columns (C8, C18) and is detected by the same multiple reaction monitoring (MRM) ions in MS/MS and absorbance wavelength in UV, leading to a potential bias for LC-based methods. One of the LC-MS/MS participants (number 56) noted using a method that separates 3-epi-25(OH)D_3 and provided values for this analyte in the study materials. However, the 25(OH)D_3 values reported by LC participants that use C8 and C18 columns represent the sum of 25(OH)D_3 and 3-epi-25(OH)D_3, and 25(OH)D_{Total} also includes a contribution from 3-epi-25(OH)D_3. It is unclear how the presence of 3-epi-25(OH)D_3 affects the 25(OH)D_{Total} for immunoassay results.

Figure 3. 25(OH)D_{Total} levels in SRM 968e L1, SRM 968e L2, SRM 968e L3 and SRM 968d L1 as determined by immunoassay (CLIA, EIA and RIA)and LC (LC-MS^n and LC-UV) methods. The grey-shaded bars represent the ranges bound by the NIST values with ± estimated U_{95} uncertainty.

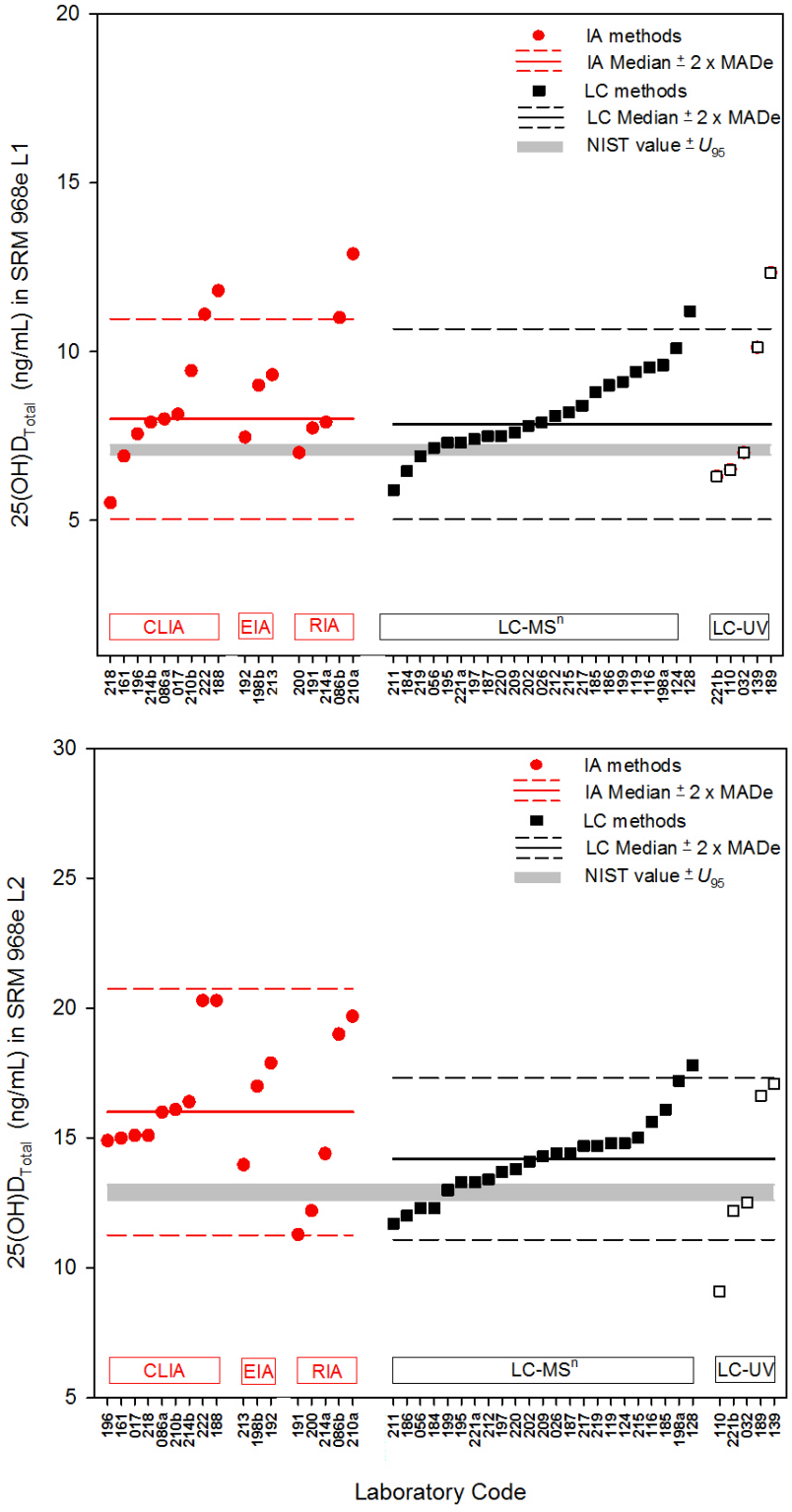
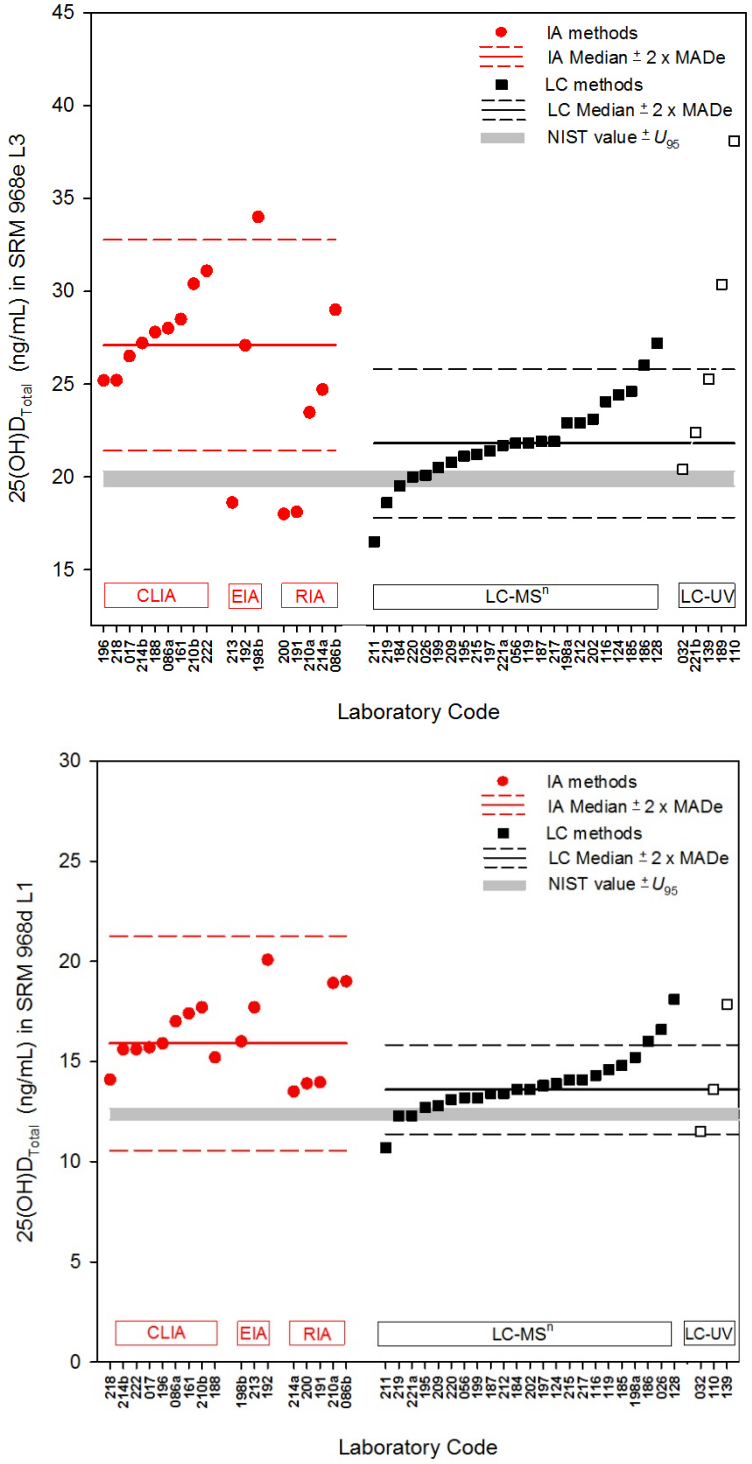


Figure 3 (cont'd). 25(OH)D_{Total} levels in SRM 968e L1, SRM 968e L2, SRM 968e L3 and SRM 968d L1 as determined by immunoassay (CLIA, EIA and RIA) and LC (LC-MS^n and LC-UV) methods. The grey-shaded bars represent the ranges bound by the NIST values with ± estimated U_{95} uncertainty.

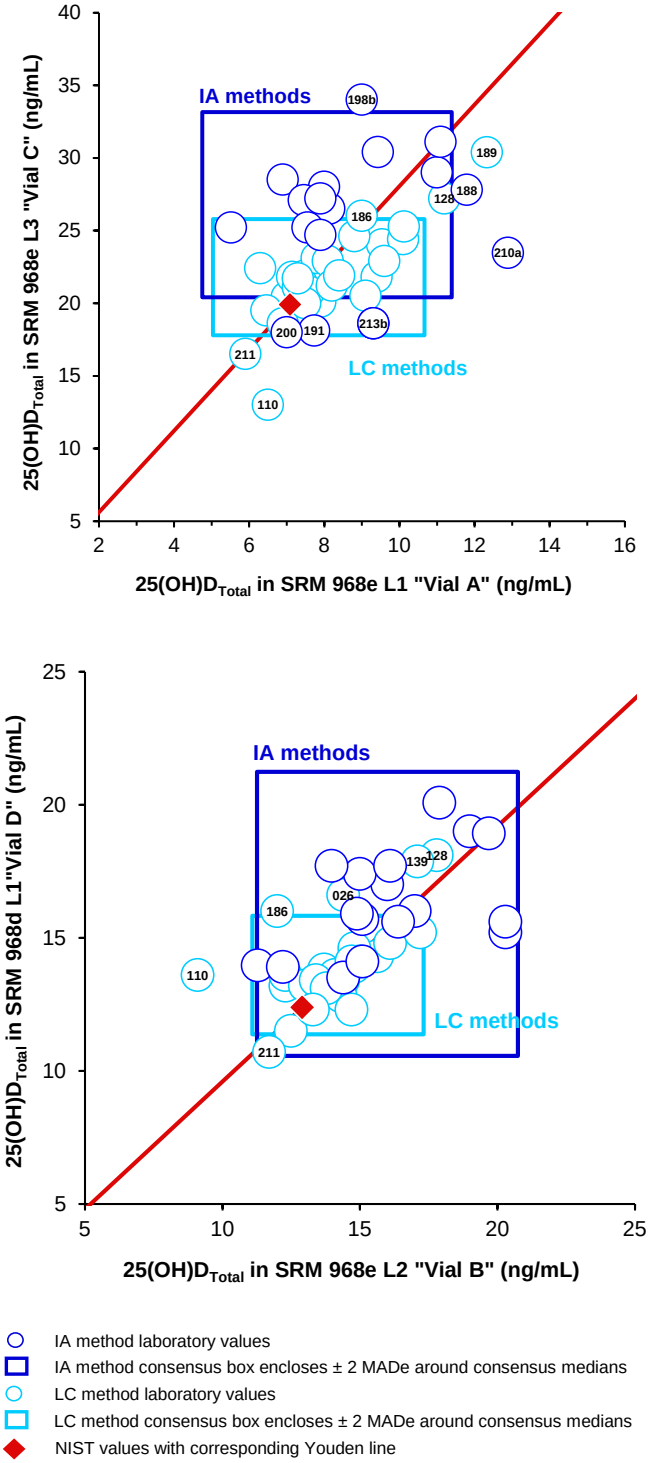


A direct comparison of the results for SRM 968e L1 and SRM 968e L3, which represent the lowest and highest 25(OH)D_{Total} levels in the Summer 2011 study samples, respectively, is provided in the Youden plot in **Figure 4.** Also in this figure is a Youden plot comparing the results for SRM 968e L2 and SRM 968d L1, which both have comparable 25(OH)D_{Total} levels. There are two blue consensus boxes in each plot, 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 each plot). Conversely, laboratory results 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 lines centered on the NIST values are illustrated by a red line (——), which represents the relative ratio of the NIST values (19.90/7.09 for SRM 968e L3/SRM 968e L1 and 12.38/12.90 for SRM 968d L1/SRM 968e L2) across the magnitude of the y- and x-axis, respectively. The Youden lines run through both the IA and LC consensus boxes for these materials.

Since the four study materials for the Summer 2011 exercise had very low levels of 25(OH)D_2, the results for 25(OH)D_{Total} and 25(OH)D_3 are the same for the majority of the LC participants. Therefore, the Youden plots comparing the results for 25(OH)D_3 obtained by the LC participants are nearly identical to the plots in **Figure 4** and are not presented separately in this report.

Figure 4. Youden comparison plots of the results for 25(OH)D_{Total} in SRM 968e L1 and SRM 968e L3 and in SRM 968e L2 and SRM 968d L1 for all methods. Data that fall outside the consensus boxes are labeled with their laboratory number.



Correlation of 25(OH)D in SRM 968e L1, SRM 968e L2, SRM 968e L3, and SRM 968d L1 with Clinical Ranges

The current guidance regarding 25(OH)D concentrations and human health (obtained from the NIH website) is presented in **Table 3**.

Table 3. Serum 25-Hydroxyvitamin D [25(OH)D] Concentrations and Health [1]

ng/mL	nmol/L	Health Status
<12	<30	Associated with vitamin D deficiency, leading to rickets in infants and children and osteomalacia in adults
12–20	30-50	Generally considered inadequate for bone and overall health in healthy individuals
≥ 20	≥ 50	Generally considered **adequate** for bone and overall health in healthy individuals
>50	>125	Emerging evidence links potentially adverse effects to such high levels, particularly >150 nmol/L (>60 ng/mL)

Table from http://ods.od.nih.gov/factsheets/vitamind#h4

[1] Institute of Medicine, Food and Nutrition Board. Dietary Reference Intakes for Calcium and Vitamin D. Washington, DC: National Academy Press, 2010.

Graphical representations of the participant and NIST results for SRM 968e L1, SRM 968e L2, SRM 968e L3, and SRM 968d L1 overlaid with the clinical ranges for 25(OH)D from **Table 3** are presented in **Figure 5**.

Specific results as assessed from **Figure 5** are summarized below:

SRM 968e L1

- The majority of the participant results are in the deficient 25(OH)D concentration range, but two are in the inadequate range.
- The NIST value (7.09 ng/mL ± 0.14 ng/mL) is in the deficient 25(OH)D concentration range.

SRM 968e L2

- The majority of the participant results are in the inadequate 25(OH)D concentration range, but some reported deficient as well as adequate concentration values.
- The NIST value (12.90 ng/mL ± 0.30 ng/mL) is in the inadequate 25(OH)D concentration range.

SRM 968e L3

- The range of participant results for SRM 968e L3 is larger than for the other materials.
- The majority of participant results are in the adequate 25(OH)D concentration range, but several also reported inadequate concentration values.
- The NIST value (19.90 ng/mL ± 0.40 ng/mL) is in the inadequate 25(OH)D concentration range.

SRM 968d L1

- The majority of the participant results are in the inadequate 25(OH)D concentration range, but some reported deficient as well as adequate concentration values.
- The NIST value (12.38 ng/mL ± 0.28 ng/mL) is in the inadequate 25(OH)D concentration range.

The consensus CV% of the participant results from all methods ranged from 14% to 19% for the study materials (**Table 2**). Large consensus variability has implications regarding the accuracy of 25(OH)D measurements for the diagnosis of vitamin D status, particularly given the narrow ranges associated with vitamin D deficiency and inadequacy.

Figure 5. 25(OH)D_{Total} levels in SRM 968e L1, SRM 968e L2, SRM 968e L3, and SRM 968d L1 superimposed over clinically-relevant serum 25-hydroxyvitamin D (25(OH)D_{Total}) concentration levels as reported by NIH (Table 3). The grey-shaded bars represent the ranges bound by the NIST values with ± estimated U_{95} uncertainty.

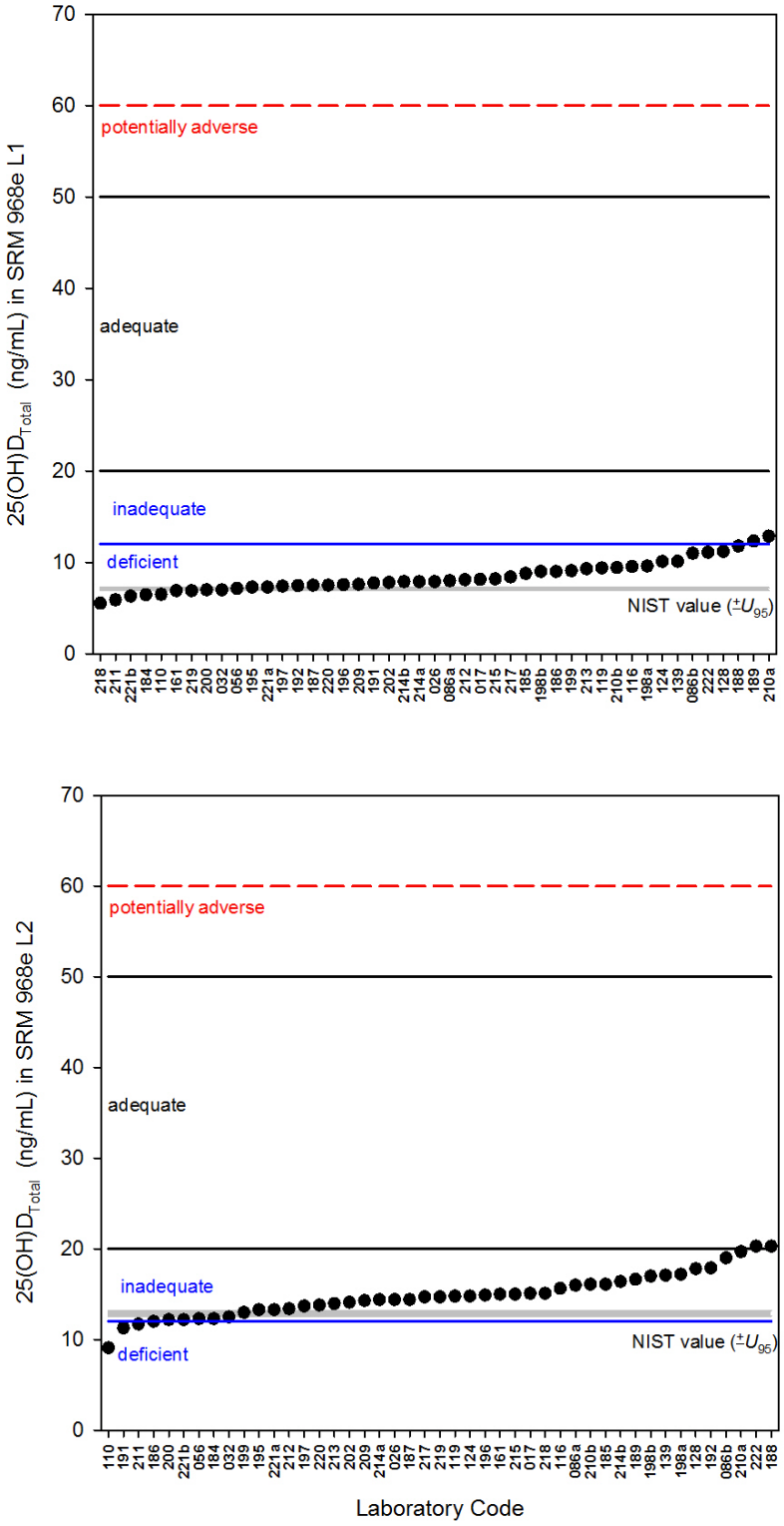
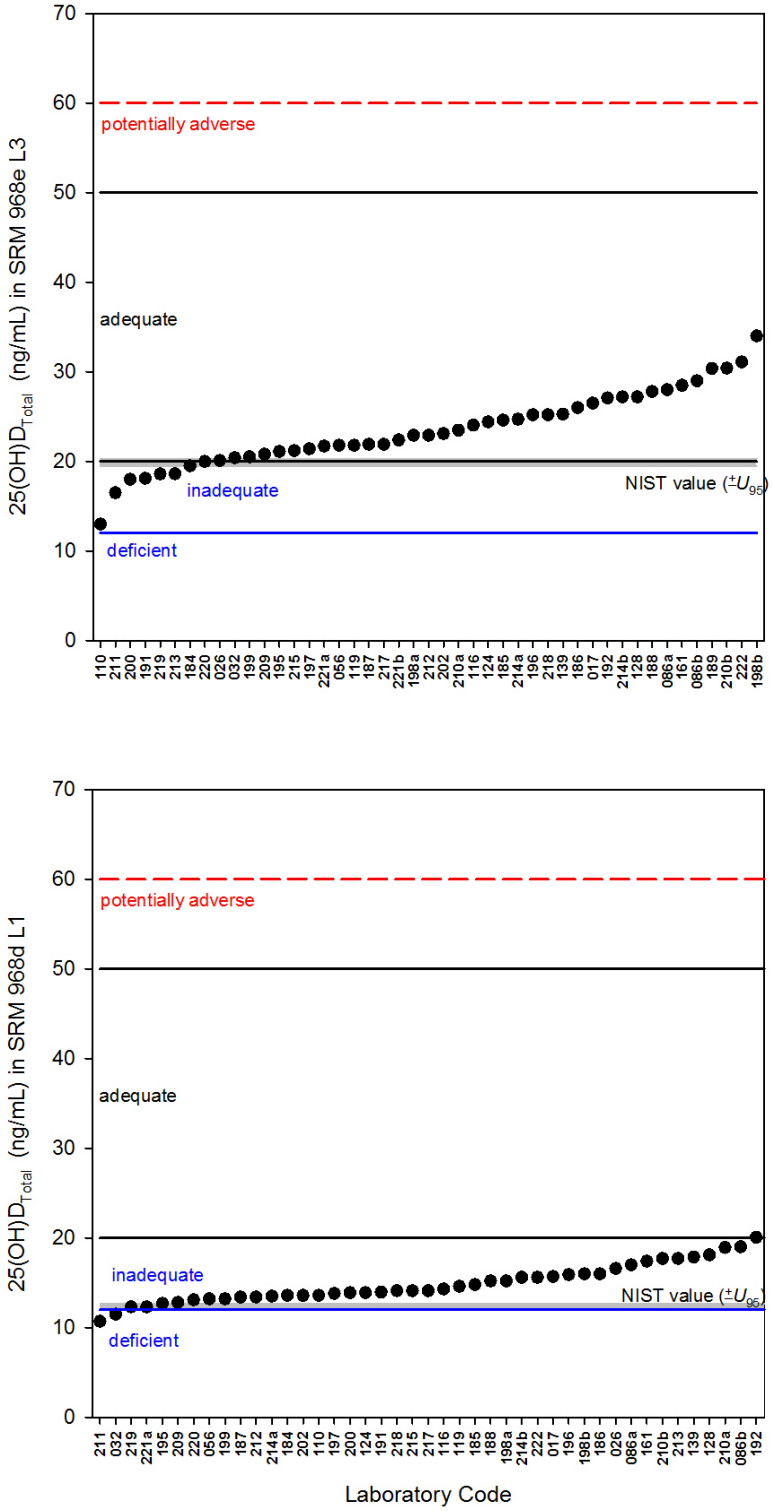


Figure 5 (cont'd) 25(OH)D_{Total} levels in SRM 968e L1, SRM 968e L2, SRM 968e L3 and SRM 968d L1 superimposed over clinically-relevant serum 25-hydroxyvitamin D (25(OH)D_{Total}) concentration levels as reported by NIH (Table 3). The grey-shaded bars represent the ranges bound by the NIST values with ± estimated U_{95} uncertainty.



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

Laboratory Number	*IA Method*	*Sample Preparation*	*Detection*
17	CLIA	n/r	n/r
86a	CLIA	n/r	n/r
86b	RIA	n/r	n/r
161	CLIA	Sample incubated for 30 min with anti-25(OH)D antibodies attached to paramagnetic particles in a buffer that dissociates 25(OH)D from binding proteins. Magnetic separation and washing removes unbound reagents. Trigger reagent used to initiate the chemiluminescent reaction.	Relative light units (from luminometer) are compared to a stored master curve to determine the concentration of 25(OH)D
188	CLIA	Samples were thawed, swirled, and analyzed. Controls were diluted with phosphate-buffered saline (PBS) (100µL control + 400 µL PBS).	n/r
191	RIA	Samples were prepared as per kit protocol	I^{125} detection using Gamma counter
192	EIA	n/r	OD reading at 450 nm
196	CLIA	The human serum samples were analyzed neat; calibration solutions were diluted 1:4 in water and analyzed.	n/r
198b	EIA	n/r	n/r
200	RIA	Sample was extracted	n/r
210a	RIA	Sample was extracted	n/r
210b	CLIA	n/r	n/r
213b	EIA	n/r	n/r
214a	RIA	Sample was extracted	n/r
214b	CLIA	n/r	n/r
218a	CLIA	n/r	n/r
222	CLIA	n/r	n/r

OD = optical density
n/r = not reported

Appendix A-2. Summary of LC-MS^n methods reported by participants.

Laboratory Number	*Internal Standard (IS)*	*Sample Preparation*	*Chromatographic Conditions*	*Detection: MRM ions*
26	25(OH)D_2-d_6 and 25(OH)D_3-d_6	Liquid-liquid extraction method	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; 25(OH)D_3-d_6; 3-epi-25(OH)D_2-d_3	Samples were extracted with hexane, evaporated, then reconstituted with 69% methanol	PFP column (100 x 2.1 mm; 1.9 µm); 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
119	25(OH)D_3 -d_6	Serum (150 µL) proteins were precipitiated with methanol containing IS (200 µL), followed by hexane extraction (1 mL), evaporation, and reconstitution with methanol	C18 column (150 x 3.0 mm, 2.7 µm); gradient with methanol and water (0.1% formic acid); flow 0.65 mL/min	Orbitrap MS Detection at $[M+H]^+$: 25(OH)D_3 401.34141; 25(OH)D_3-d_6 407.37907; 25(OH)D_2 413.34141
124	deuterated 25(OH)D_2 and deuterated 25(OH)D_3	Solid-phase extraction	Phenyl column (50 x 2.1 mm; 1.7µm), gradient with methanol/water (both with ammonium acetate and formic acid)	n/r
128	n/r	n/r	n/r	n/r
184	25(OH)D_3 -d_6	Serum (200 µL) treated with acetonitrile containing IS (700 µL); mixed, centrifuged, and filtered	C18 column (100 x 2.1mm; 5µm); Linear gradient from 40% A (0.1% formic acid in water) and 60% B (0.1% formic acid/5 mmol/L ammonium acetate in methanol) 98% B in 2 min	25(OH)D_3 383/257; 25(OH)D_3-d_6 389/263; 25(OH)D_2 395/209
185	25(OH)D_2-d_6 and 25(OH)D_3-d_6	Liquid-liquid extraction; 100 µL sample	C18 column; methanol/water gradient	MRM
186	25(OH)D_3 -d_6	Deproteinized with cold methanol; liquid/liquid extraction with hexane	C18 column (50 x 2.1 mm; 1.7µm), 45° C, methanol/water gradient	25(OH)D_3 401/159 (quant), 401/383 (qual); 25(OH)D_3-d_6 407/159
187	n/r	n/r	n/r	n/r
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 µm); 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_3-d_6	n/r	n/r	n/r
202	d_6-labeled compound	Sample was extracted	C18 column (50 x 2.1 mm); gradient with 10 % acetonitrile (0.1% formic acid), 90% methanol; flow 0.3 mL/min	n/r

209	25(OH)D_3 -d_6	Proteins were precipitated with $ZnSO_4$, followed by centrifugation and analysis	C8 column (50 x 2 mm; 5 µm); gradient with water/methanol; flow 0.7 mL/min	25(OH)D_3 383/229,383/211; 25(OH)D_3-d_6 389/211; 25(OH)D_2 395/269, 395/119
211	25(OH)D_3-d_6	Extraction with acetonitrile containing IS followed by centrifugation	Column (33 x 4.6 mm; 3 µm); turboflow with methanol/water gradient	25(OH)D_3 383/365 (quant), 383/357 (qual); 25(OH)D_2 395/377 (quant), 395/209 (qual)
212	25(OH)D_3-d_6	Serum (100 µL) precipitated with 5:95 methanol:acetonitrile (350 µL) containing the deuterated internal standard.	C8 column (50 x 2mm; 3 µm); gradient starting with 60% acetonitrile (0.1% formic acid), 40% water (0.1% formic acid)	25(OH)D_3 383/229,383/211; 25(OH)D_3-d_6 389/211; 25(OH)D_2 395/269, 395/119
215	25(OH)D_3-d_6	Protein precipitation with methanol/isopropanol followed by liquid/liquid extraction with hexane	C18 column (50 x 2.1mm; 2.6 µm); gradient from 85% to 100% methanol; flow 0.25 mL/min	n/r
217	25(OH)D_3-d_6	Protein precipitation with $ZnSO_4$ in methanol followed by SPE extraction	C8 column (50 x 2.1 mm; 1.7µm); gradient of 70% to 98% methanol (with 0.1% formic acid); flow 0.4 mL/min	25(OH)D_3 401/159 (quant), 401/383 (qual); 25(OH)D_2 413/88 (quant), 413/395 (qual)
219	25(OH)D_3-d_6	Samples were protein crashed in conjunction with internal standard addition, vortexed, centrifuged	Automated 2-D system	25(OH)D_3 401/365; 25(OH)D_2 413/355; 25(OH)D_3-d_6 407/371
220	25(OH)D_2-d_3 and 25(OH)D_3-d_6	Protein crash with 90% methanol, 10% $ZnSO_4$ and then acetonitrile (1% formic acid). Precipitated sample is passed through an SPE plate.	C18 column (20 x 2.1mm, 2.7µm); flow 1 mL/min	25(OH)D_3 383/211 (quant), 383/229 (qual); 25(OH)D_2 395/119 (quant), 395/269 (qual); 25(OH)D_3-d_6 389/211; 25(OH)D_2-d_3 398/272
221	25(OH)D_3-d_6	Protein crash with 1% methanol in acetonitrile containing IS	CN column (50 x 3.0 mm; 1.8 µm); methanol/water gradient at 50 °C	25(OH)D_3 383/211; 25(OH)D_3-d_6 389/211; 25(OH)D_2 395/209

MRM = multiple reaction monitoring

PFP = pentafluorophenyl

quant = quantitative ions

qual = qualitative ions

SPE = solid phase extraction

n/r = not reported

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

Laboratory Number	*Internal Standard (IS)*	*Sample Preparation*	*Chromatographic Conditions*	*Wavelength*
32	Proprietary	Samples were extracted with filtration; The controls were evaporated with a known ratio of internal standard, reconstituted, and injected	C18 column (300 x 3.9 mm; 4 µm); proprietary mobile phase; flow 0.7mL/min	265 nm
110	n/r	Samples were extracted twice with hexane/methylene chloride (5:1), evaporated and reconstituted	Ultra-fast LC; gradient with acetonitrile/methanol (85:15) and isopropanol (100%)	n/r
139	Proprietary	The sample was extracted, centrifuged and injected directly onto LC column	Required reagents, column, controls and calibrators supplied in "kit" form.	264 nm
189	Added before extraction	Proteins were disrupted and precipitated; analytes were extracted using solid-phase extraction	LC column (150 x 4.6 mm); isocratic separation with commercial mobile phase; flow 0.7mL/min	265 nm
221	Laurophenone	Protein crash with acetonitrile (contaning IS), followed by extraction on C-18 sorbent, elution with methanol/acetonitrile, evaporation, and reconstitution with acetonitrile	CN column (150 x 4.6 mm; 3.5 µm); methanol/water/formic acid mobile phase; 50 °C	275 nm

n/r = not reported

Appendix B. Raw participant data for 25(OH)D_2, 25(OH)D_3 and 25(OH)D_{Total} in SRM 968e L1, SRM 968e L2, SRM 968e L3, and SRM 968d L1 and the control solutions, SRM 2972.

		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 968e L1	SRM 968e L2	SRM 968e L3	SRM 968d L1	SRM 968e L1	SRM 968e L2	SRM 968e L3	SRM 968d L1	SRM 968e L1	SRM 968e L2	SRM 968e L3	SRM 968d L1	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	8.1	15.1	26.5	15.7	n/r	n/r
026	LC-MS/MS	<1.0	<1.0	<1.0	1.0	7.9	14.4	20.1	15.6	7.9	14.4	20.1	16.6	n/r	n/r
032	LC-UV	<5	<5	<5	<5	7.0	12.5	20.4	11.5	7.0	12.5	20.4	11.5	261.5	344.0
056	LC-MS/MS	<1.2	<1.2	<1.2	<1.2	7.2	12.3	21.8	13.2	7.2	12.3	21.8	13.2	227.3	321.3
086a	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	8.0	16.0	28.0	17.0	n/r	n/r
086b	RIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	11.0	19.0	29.0	19.0	n/r	n/r
110	LC-UV	<4.0	<4.0	<4.0	<4.0	6.5	9.1	13.0	13.6	6.5	9.1	13.0	13.6	233.4	331.7
116	LC-MS/MS	< 3.3	< 3.3	< 3.3	< 3.3	9.5	15.6	24.0	14.3	9.5	15.6	24.0	14.3	241.8	331.0
119	LC-MS	< 1.0	< 1.0	< 1.0	< 1.0	9.4	14.8	21.8	14.6	9.4	14.8	21.8	14.6	243.2	352.6
124	LC-MS/MS	<4.0	<4.0	<4.0	<4.0	10.1	14.8	24.4	13.9	10.1	14.8	24.4	13.9	n/r	n/r
128	LC-MS/MS	n/d	n/d	n/d	n/d	11.2	17.8	27.2	18.1	11.2	17.8	27.2	18.1	n/r	333.5
139	LC-UV	n/d	n/d	n/d	n/d	10.1	17.1	25.3	17.9	10.1	17.1	25.3	17.9	241.5	330.5
161	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	6.9	15.0	28.5	17.4	n/r	n/r
184	LC-MS/MS	<1.0	<1.0	<1.0	<1.0	6.5	12.3	19.5	13.6	6.5	12.3	19.5	13.6	236.5	338.5
185	LC-MS/MS	n/d	n/d	n/d	n/d	8.8	16.1	24.6	14.8	8.8	16.1	24.6	14.8	238.6	334.8
186	LC-MS/MS	n/d	n/d	n/d	n/d	9.0	12.0	26.0	16.0	9.0	12.0	26.0	16.0	n/r	320.0
187	LC-MS/MS	n/d	n/d	n/d	n/d	7.5	14.4	21.9	13.4	7.5	14.4	21.9	13.4	235.0	337.0
188	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	11.8	20.3	27.8	15.2	401.5	381.5
189	LC-UV	n/d	n/d	n/d	n/d	12.3	16.6	30.4	n/r	12.3	16.6	30.4	n/r	n/r	280.9
191	RIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	7.7	11.3	18.1	14.0	n/r	n/r
192	EIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	7.5	17.9	27.1	20.1	n/r	n/r
195	LC-MS/MS	n/d	n/d	n/d	n/d	7.3	13.3	21.1	12.7	7.3	13.3	21.1	12.7	242.0	330.0
196	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	7.6	14.9	25.2	15.9	210.4	452.0
197	LC-MS/MS	<5	<5	<5	<5	7.4	13.7	21.4	13.8	7.4	13.7	21.4	13.8	234.3	342.5
198a	LC-MS/MS	<5.0	<5.0	<5.0	<5.0	9.6	17.2	22.9	15.2	9.6	17.2	22.9	15.2	245.5	308.6
198b	EIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	9.0	17.0	34.0	16.0	n/r	n/r
199	LC-MS/MS	<4	<4	<4	<4	9.1	13.0	20.5	13.2	9.1	13.0	20.5	13.2	233.0	329.0
200	RIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	7.0	12.2	18.0	13.9	216.0	319.1
202	LC-MS/MS	n/d	n/d	n/d	n/d	7.8	14.1	23.1	13.6	7.8	14.1	23.1	13.6	241.0	334.5
209	LC-MS/MS	<1.0	<1.0	<1.0	<1.0	7.6	14.3	20.8	12.8	7.6	14.3	20.8	12.8	240.6	332.8
210a	RIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	12.9	19.7	23.5	18.9	209.4	299.1
210b	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	9.4	16.1	30.4	17.7	n/r	n/r
211	LC-MS/MS	n/d	n/d	n/d	n/d	5.9	11.7	16.5	10.7	5.9	11.7	16.5	10.7	238.0	303.8
212	LC-MS/MS	n/d	n/d	n/d	n/d	8.1	13.4	22.9	13.4	8.1	13.4	22.9	13.4	248.8	341.7
213	EIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	9.3	14.0	18.6	17.7	n/r	n/r
214a	RIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	7.9	14.4	24.7	13.5	n/r	n/r
214b	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	7.9	16.4	27.2	15.6	n/r	n/r
215	LC-MS/MS	0.3	0.4	0.2	0.1	7.9	14.6	21.0	14.0	8.2	15.0	21.2	14.1	226.7	386.2
217	LC-MS/MS	0.4	0.3	0.3	0.1	8.0	14.4	21.6	14.0	8.4	14.7	21.9	14.1	220.8	394.3
218	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	5.5	15.1	25.2	14.1	n/r	n/r
219	LC-MS/MS	<4.0	<4.0	<4.0	<4.0	6.9	14.7	18.6	12.3	6.9	14.7	18.6	12.3	n/r	n/r
220	LC-MS/MS	<5.0	<5.0	<5.0	<5.0	7.5	13.8	20.0	13.1	7.5	13.8	20.0	13.1	232.4	324.4
221a	LC-MS/MS	n/d	n/d	n/d	n/d	7.3	13.3	21.7	12.3	7.3	13.3	21.7	12.3	253.0	365.0
221b	LC-UV	n/d	n/d	n/d	n/d	6.3	12.2	22.4	n/r	6.3	12.2	22.4	n/r	177.0	214.0
222	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	11.1	20.3	31.1	15.6	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	7.09	12.90	19.90	12.38	7.09	12.90	19.90	12.38	238.6	334.8
	U_{95}	0.0	0.0	0.0	0.0	0.14	0.30	0.40	0.28	0.14	0.30	0.40	0.28	3.9	5.2