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NIST/NIH Vitamin D Metabolites Quality Assurance Program Report of Participant Results: Summer 2012 Comparability Study (Exercise 6)

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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 sixth exercise of this program, the Summer 2012 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 and SRM 972a Vitamin D Metabolites in Human Serum Level 1 and Level 3. 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 study, and laboratories were identified by code numbers known only to them. The results from this sixth study are reported along with a summary of the analytical methods used.

OVERVIEW OF THE SUMMER 2012 COMPARABILITY STUDY

For the Summer 2012 Comparability Study (Exercise 6) 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 results for single measurements of each of these solutions. In addition, participants were asked to determine 25-hydroxyvitamin D in four samples of human serum (study materials). Individual concentration values for $25(OH)D_2$, $25(OH)D_3$, and 3-epi-25-hydroxyvitamin D_3 (3-epi-$25(OH)D_3$) were requested along with a total concentration of 25-hydroxyvitamin D ($25(OH)D_{Total} = 25(OH)D_2 + 25(OH)D_3$) for each of four samples A, B, C, and D. In this study, A, B, C, and D were all blended human serum pools with endogenous 25(OH)D levels. Vial A was SRM 972a Vitamin D Metabolites in Human Serum Level 1 (SRM 972a L1), and vial B was SRM 972a Level 3 (SRM 972a L3). Vials C and D were duplicate samples of SRM 968d Fat-Soluble Vitamins, Carotenoids and Cholesterol in Human Serum Level 1 (SRM 968d L1).

There were a total of 48 participants and 56 datasets (eight participants provided data for two different methods) in the Summer 2012 study. Eighteen of the datasets originated from immunoassay (IA) techniques, including three from enzyme immunoassay (EIA), 11 from chemiluminescence immunoassay (CLIA), and four from radioimmunoassay (RIA). **Appendix A-1** summarizes the IA methods used by the participants. Thirty-eight of the datasets originated from liquid chromatographic (LC) methods; of those, 32 were from LC with tandem mass spectrometric detection (LC-MS^n), and six 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**.

The raw data received from all participants are summarized in **Appendices B-1** and **B-2**. All datasets from the immunoassay methods reported single values for $25(OH)D_{Total}$ in SRM 972a L1, SRM 972a L3, and SRM 968d L1. LC participants provided values for $25(OH)D_2$, $25(OH)D_3$, and 3-epi-25(OH)D3 as well as $25(OH)D_{Total}$ in SRM 972a L1, SRM 972a L3, and SRM 968d L1. 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.

Both SRM 972a L1 and SRM 968d L1 contain low levels of $25(OH)D_2$ (reported values ranging from 0.2 ng/mL to 0.7 ng/mL), and most of the LC labs indicated this analyte was below their quantitation limit of <1 ng/mL to <7 ng/mL. Conversely, SRM 972a L3 has a high level of $25(OH)D_2$, and all but two of the LC participants reported values; in addition, one LC participant reported that the $25(OH)D_2$ was below their detection limit of 4 ng/mL. In addition, two LC participants provided values for 3-epi-$25(OH)D_3$ in all three materials, one participant provided a value for SRM 972a L3 only, and two labs indicated that this analyte was below their quantitation limit of 2 ng/mL and 7 ng/mL in all three materials (**Appendix B-2**).

Appendices B-1 and **B-2** also provide the summarized results from the National Institute of Standards and Technology (NIST) for each of the serum materials. The $25(OH)D_2$ in SRM 968d L1 was below the quantitation limit (≈ 0.5 ng/mL) for the NIST method; 3-epi-$25(OH)D_3$ was detected in SRM 968d L1 but not quantitated.

SUMMER 2012 COMPARABILITY STUDY RESULTS AND DISCUSSION

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

For the Summer 2012 study, the control solutions were only provided to participants who requested them on their enrollment forms. A summary of the participant data for $25(OH)D_2$ and $25(OH)D_3$ in the SRM 2972 control solutions is provided in **Table 1.** The majority of the datasets received for the Summer 2012 study were from LC methods, and many participants using IA methods did not request the calibration solutions because of compatibility issues with their assays.

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%). The community results were not calculated for the data from the IA methods because of the limited number of data reported (N = 2).

The control materials were characterized at NIST using both gravimetry and LC-MS. **Table 1** presents the NIST certified values with expanded uncertainties corresponding to 95% confidence for SRM 2972. Participants were provided these values both on the shipping package and within the data reporting sheet so that they could qualify their methods prior to analyzing the study samples.

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)

Lab	Method	SRM 2972 Value
030	RIA	257.0
056	LC-MS/MS	246.7
060	LC-MS/MS	210.1
110	LC-UV	239.2
128	LC-MS/MS	n/r
139	LC-UV	256.0
183b	CLIA	262.4
185a	LC-MS/MS	238.6
194	LC-MS/MS	240.5
197	LC-MS/MS	238.0
198a	LC-MS/MS	228.2
199	LC-MS/MS	249.4
202	LC-MS/MS	246.0
211	LC-MS/MS	229.0
216	LC-MS/MS	229.9
218b	LC-MS/MS	239.1
228a	LC-MS/MS	217.3
231	LC-UV	245.3
242	LC-MS/MS	239.4
243	LC-UV	240.6
244	LC-MS/MS	215.0
248	LC-MS/MS	238.6
249	LC-MS/MS	238.4
250	LC-MS/MS	281.6
253	LC-MS/MS	235.5
All methods	N	24
	Median	239.2
	MADe	10.7
	CV%	4.5
LC methods	N	22
	Median	238.9
	MADe	10.1
	CV%	4.2
LC-MS^n	N	18
	Median	238.5
	MADe	11.6
	CV%	4.9
	NIST Value	238.6
	U_{95}	3.9

25(OH)D_3 (ng/mL)

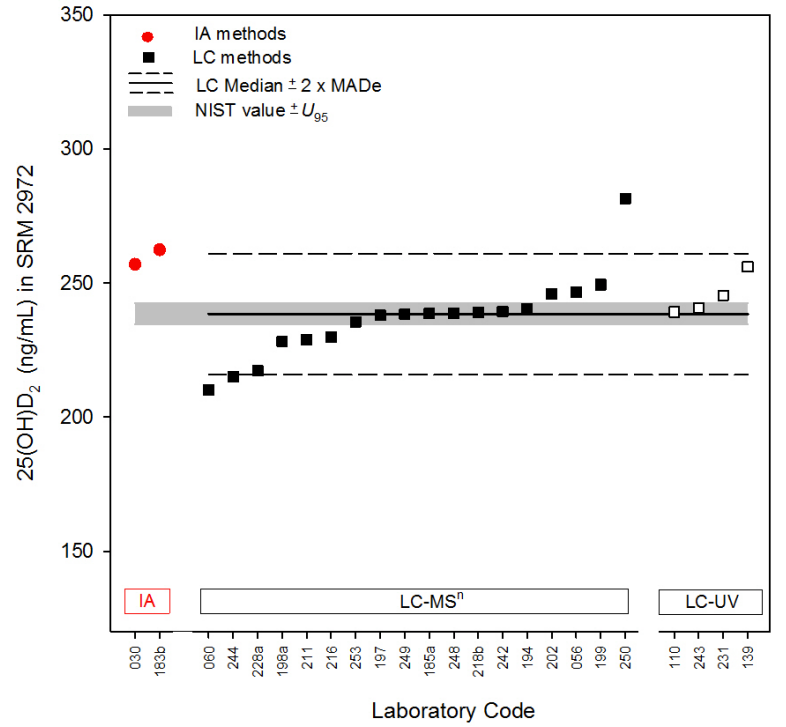
Lab	Method	SRM 2972 Value
030	RIA	408.5
056	LC-MS/MS	346.3
060	LC-MS/MS	348.1
110	LC-UV	335.2
128	LC-MS/MS	332.0
139	LC-UV	335.0
183b	CLIA	336.8
185a	LC-MS/MS	334.8
194	LC-MS/MS	335.0
197	LC-MS/MS	343.0
198a	LC-MS/MS	324.1
199	LC-MS/MS	329.9
202	LC-MS/MS	345.0
211	LC-MS/MS	280.0
216	LC-MS/MS	343.3
218b	LC-MS/MS	336.0
228a	LC-MS/MS	331.5
231	LC-UV	294.5
242	LC-MS/MS	333.4
243	LC-UV	331.7
244	LC-MS/MS	331.0
248	LC-MS/MS	334.8
249	LC-MS/MS	324.2
250	LC-MS/MS	382.0
253	LC-MS/MS	307.0
	N	25
	Median	334.8
	MADe	7.3
	CV%	2.2
	N	23
	Median	334.8
	MADe	7.3
	CV%	2.2
	N	19
	Median	334.8
	MADe	12.2
	CV%	3.6
	NIST Value	334.0
	U_{95}	5.2

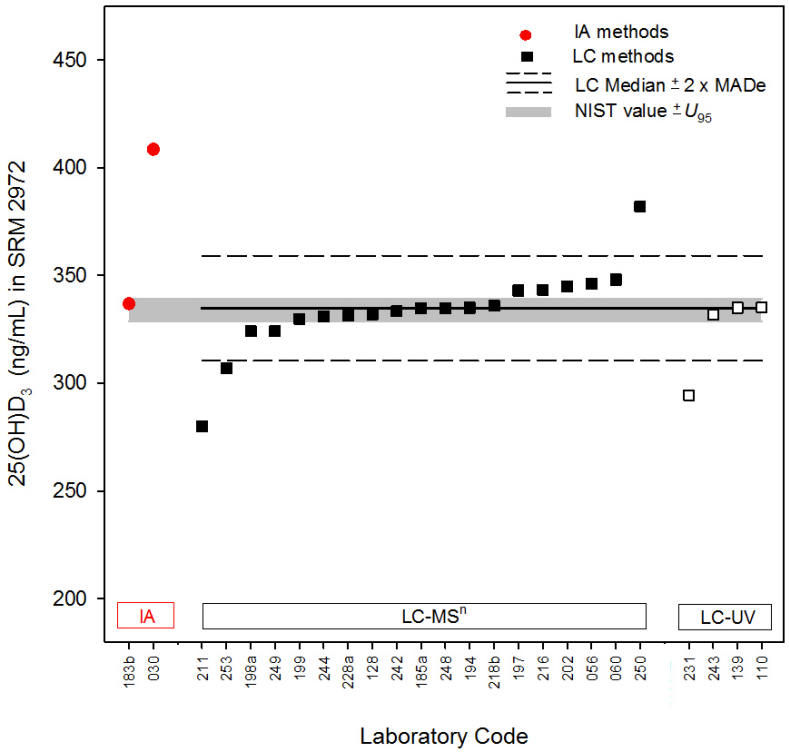
For all participant datasets, the single data values reported for 25(OH)D_2 and 25(OH)D_3 in the control solutions, SRM 2972, are plotted in **Figure 1**. The results from immunoassay methods are displayed with closed red circles (●), and the results from the LC-based methods are displayed with closed black squares (■).

From the single reported values for all LC datasets, the consensus median and the consensus variability (2 × MADe) were determined (reported in **Table 1**). In **Figure 1**, the solid lines (——) represent the consensus median and the dashed lines (- - - - -) represent the approximate 95% confidence interval (2 × MADe) for the LC datasets; the laboratories with results that fall between the two dashed lines are within the consensus variability.

The grey-shaded bar in **Figure 1** represents the interval in which NIST believes the "true value" exists for these solutions (i.e., NIST certified values with ± U_{95} expanded uncertainty). 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.

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.





25(OH)D in SRM 972a L1, SRM 972a L3, and SRM 968d L1

A summary of the individual participant data for $25(OH)D_{Total}$ in SRM 972a L1 (vial A), SRM 972a L3 (vial B), and SRM 968d L1 (vials C and D) is provided in **Table 2.** The summarized data also include the average (mean), standard deviation (SD), and percent relative standard deviation (%rSD) of the two reported values for SRM 968d L1.

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 $LC\text{-}MS^n$ methods only. These summarized results include N, the median value, the MADe, and the CV%.

Table 2 also presents the NIST results for the three study materials. For SRM 972a L1 and SRM 972a L3, the NIST result is the sum of the certified values for $25(OH)D_3$ and $25(OH)D_2$ with the corresponding 95% confidence limits (U_{95}). For SRM 968d L1, the NIST value for $25(OH)D_3$ was obtained using an LC-MS/MS reference measurement procedure[a] recognized by the JCTLM (N = 8), and the U_{95} confidence interval includes components for both measurement variability and measurement uncertainty associated with the density. The $25(OH)D_2$ was below the quantitation limit (≈ 0.5 ng/mL) in SRM 968d L1 and was not included in the results for $25(OH)D_{Total}$.

[a] 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 972a L1, SRM 972a L3, and SRM 968d L1.

		SRM 972a L1	SRM 972a L3	SRM 968d L1	SRM 968d L1	SRM 968d L1 Combined		
Lab	Method	Vial A	Vial B	Vial C	Vial D	Mean	SD	%RSD
017	CLIA	28.1	27.7	14.0	14.7	14.4	0.5	3.4
026	LC-MS/MS	31.0	33.5	12.7	13.4	13.1	0.5	3.8
030	RIA	32.4	26.7	15.8	14.4	15.1	1.0	6.6
056	LC-MS/MS	30.9	34.8	12.6	12.3	12.5	0.2	1.7
060	LC-MS/MS	37.1	42.2	15.3	14.6	15.0	0.5	3.3
086a	CLIA	27.4	26.8	14.4	15.7	15.1	0.9	6.1
086b	RIA	32.0	40.0	17.0	17.0	17.0	0.0	0.0
110	LC-UV	21.5	23.3	14.3	17.9	16.1	2.5	16
116	LC-MS/MS	35.1	37.9	15.4	15.5	15.5	0.1	0.5
128	LC-MS/MS	37.0	24.1	15.6	14.1	14.9	1.1	7.1
139	LC-UV	32.0	34.7	13.0	13.6	13.3	0.4	3.2
180	RIA	32.8	34.8	13.7	16.0	14.8	1.6	11
183b	CLIA	28.0	25.0	13.8	13.9	13.9	0.1	0.5
185a	LC-MS/MS	32.1	38.0	14.4	12.3	13.4	1.5	11.1
187	LC-MS/MS	32.7	33.1	13.1	12.9	13.0	0.1	1.1
188	CLIA	31.4	29.4	12.9	14.1	13.5	0.9	6.5
189	LC-UV	37.7	14.0	12.0	10.4	11.2	1.1	10
194	LC-MS/MS	32.3	34.1	12.9	13.1	13.0	0.1	1.1
196	CLIA	31.6	29.2	14.5	15.6	15.1	0.8	5.2
197	LC-MS/MS	32.0	36.3	13.8	14.3	14.1	0.4	2.5
198a	LC-MS/MS	29.8	31.2	12.9	15.2	14.1	1.6	12
198b	EIA	28.2	27.6	12.2	14.1	13.2	1.3	10
199	LC-MS/MS	30.7	33.7	12.7	12.6	12.7	0.1	0.6
202	LC-MS/MS	33.4	35.3	12.9	12.7	12.8	0.1	1.1
209	LC-MS/MS	31.7	39.6	11.9	12.8	12.4	0.6	5.2
210a	RIA	31.4	31.3	12.6	12.8	12.7	0.1	1.2
210b	CLIA	26.8	26.4	13.2	13.7	13.5	0.4	2.6
211	LC-MS/MS	31.5	34.1	12.3	11.7	12.0	0.4	3.5
212	LC-MS/MS	35.4	44.2	14.7	15.1	14.9	0.3	1.9
213a	CLIA	27.5	25.0	3.7	3.7	3.7	0.0	0.8
213b	EIA	28.2	23.8	12.9	12.2	12.5	0.5	4.3
215	LC-MS/MS	32.4	32.4	12.0	13.6	12.8	1.1	8.8
216	LC-MS/MS	30.5	33.4	13.2	12.8	13.0	0.2	1.9
217	LC-MS/MS	30.5	34.1	12.4	12.6	12.5	0.1	1.1
218a	CLIA	29.8	29.2	13.7	14.7	14.2	0.7	5.0
218b	LC-MS/MS	30.4	41.0	14.2	14.5	14.4	0.2	1.5
219	LC-MS/MS	30.0	33.6	12.4	12.7	12.6	0.2	1.7
220	LC-MS/MS	35.0	37.0	14.0	14.0	14.0	0.0	0.0
221a	LC-MS/MS	27.9	31.0	11.6	12.9	12.3	0.9	7.5
221b	LC-UV	24.4	28.0	25.0	24.7	24.9	0.2	0.9
222	CLIA	33.1	35.3	13.5	12.3	12.9	0.8	6.6
228a	LC-MS/MS	45.2	51.4	17.3	17.9	17.6	0.4	2.4
228b	CLIA	30.5	26.7	3.2	3.1	3.2	0.0	1.1
231	LC-UV	26.6	36.9	14.8	14.0	14.4	0.6	3.9
234	LC-MS/MS	34.5	39.2	13.4	14.7	14.1	0.9	6.5
241	LC-MS/MS	30.5	33.7	13.2	12.6	12.9	0.4	3.3
242	LC-MS/MS	28.9	37.8	13.4	12.4	12.9	0.7	5.3
243	LC-UV	30.1	38.8	13.6	12.3	13.0	0.9	7.0
244	LC-MS/MS	29.0	34.0	12.0	11.0	11.5	0.7	6.1
247a	CLIA	38.1	37.1	17.7	14.2	16.0	2.5	16
247b	EIA	31.3	30.5	16.3	16.0	16.1	0.2	1.4
248	LC-MS/MS	31.7	41.1	14.0	14.2	14.1	0.1	1.0
249	LC-MS/MS	30.0	35.0	13.7	11.4	12.6	1.6	13
250	LC-MS/MS	37.0	39.2	13.1	15.2	14.2	1.5	10
251	LC-MS/MS	31.5	33.6	15.2	14.7	15.0	0.4	2.4
253	LC-MS/MS	31.0	35.7	12.9	13.3	13.1	0.3	2.2
All methods	N	56	56	56	56	56		
	Median	31.3	33.9	13.4	13.8	13.4		
	MADe	2.1	5.9	1.2	1.6	1.3		
	CV%	6.6	17	8.9	12	9.5		
IA methods	N	18	18	18	18	18		
	Median	30.9	28.5	13.7	14.2	14.0		
	MADe	3.1	3.1	1.2	2.1	1.6		
	CV%	9.9	11	8.7	15	11		
LC methods	N	38	38	38	38	38		
	Median	31.5	34.8	13.2	13.4	13.1		
	MADe	2.2	3.4	1.2	1.4	1.3		
	CV%	6.8	9.8	9.0	11	9.9		
LC-MS^n	N	32	32	32	32	32		
	Median	31.6	34.9	13.1	13.2	13.0		
	MADe	1.7	2.9	1.1	1.3	1.1		
	CV%	5.4	8.3	8.3	10	8.3		
	NIST Value	29.3	33.2	12.4	12.4	12.4		
	U_{95}	1.1	0.6	0.3	0.3	0.3		

For all participant datasets, the single reported values for 25(OH)D_{Total} in SRM 972a L1 and SRM 972a L3 and the average reported values (± 2 SD) for SRM 968d L1 are plotted in **Figure 2**. The results from immunoassay methods are displayed with closed red circles (●), and the results from the LC-based methods are displayed with closed black squares (■). Each figure also has a legend that indicates which individual methods were used to obtain the reported values: IA, LC-MS^n, or LC-UV.

From the average 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 dashed lines (- - - - -) represent approximate 95% confidence intervals (2 × MADe).

For the IA data for SRM 972a L1, the consensus variability based on MADe is an overestimation of the 95% confidence limits about the median (**Figure 2**). This stems from the non-Gaussian data distribution that contributes to a relatively wide distribution of central 50% of this data, resulting in a large MADe.

For the LC datasets for SRM 972a L1 and for both the LC and IA datasets for SRM 972a L3and SRM 968d L1, the laboratories with results that fall between the two dashed 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 2** are summarized below.

SRM 972a L1

- For the IA results, the data appear to be non-normally distributed, and the consensus variability is not well-described with a MADe estimation.
- For the LC results, eight datasets are outside of the consensus variability range (four LC-MS^n, four LC-UV).
- The consensus median value for the LC results is slightly higher than the consensus median value for the IA results; both LC and IA median values are ≈ 5% higher than the NIST expanded uncertainty range (grey-shaded bar).
- The NIST expanded uncertainty range (grey-shaded bar) falls within the consensus variability ranges both for LC and IA results.

SRM 972a L3

- For the IA results, four datasets are outside the consensus variability range.
- For the LC results, six datasets are outside of the consensus variability range (four LC-MS^n, two LC-UV).
- The consensus median value for the IA results is considerably lower (≈ 25%) than the consensus median value for the LC results; the IA median value is ≈ 15% lower than the NIST expanded uncertainty range (grey-shaded bar) whereas the LC median value is ≈ 5% higher than the NIST expanded uncertainty range.

- The NIST expanded uncertainty range (grey-shaded bar) falls within the consensus variability ranges for both IA and LC results.

SRM 968d L1

- For the IA results, two mean values are outside of the consensus variability range.
- For the LC results, three mean values are outside of the consensus variability range (one LC-MS^n, two LC-UV).
- The consensus median value for the IA results is marginally higher (**≈** 5%) 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 both LC and IA.

For SRM 972a L1 and SRM 968d L1, the majority of the participant values for $25(OH)D_{Total}$ are higher than the NIST value, regardless of technique used (IA or LC) (**Figure 2**). In addition, the consensus variability is similar for those two materials (**≈** 7% to 10%) when the results from all methods are considered (**Table 2**). However, for SRM 972a L3, the majority of the LC results are higher than the NIST value, whereas the majority of the IA results are lower than the NIST value (**Figure 2**); the consensus variability is 17% for SRM 972a L3 when the results for all methods are considered (**Table 2**). The difference in results for SRM 972a L1 and SRM 968d L1 versus SRM 972a L3 is most likely attributable to the large contribution of $25(OH)D_2$ to $25(OH)D_{Total}$ in the latter material, with the IA methods underrepresenting $25(OH)D_2$.

For SRM 972a L1, the 3-epi-$25(OH)D_3$ is also a significant vitamin D metabolite with a concentration of 1.84 ng/mL ± 0.08 ng/mL, corresponding to **≈** 6% of the $25(OH)D_3$ concentration of 28.8 ng/mL ± 1.1 ng/mL (NIST values). Three of the LC-MS participants also reported values for 3-epi-$25(OH)D_3$ (1.5 ng/mL, 1.9 ng/mL and 2.1 ng/mL) in SRM 972a L1 (**Appendix B-2**). Likewise, the 3-epi-$25(OH)D_3$ also has a significant concentration of 1.18 ng/mL ± 0.13 ng/mL in SRM 972a L3, or **≈** 6% of the $25(OH)D_3$ concentration of 19.8 ± 0.5 ng/mL (NIST values), and two LC labs also provided values for this metabolite (0.7 ng/mL and 1.4 ng/mL). For the methods reported by many LC participants (**Appendix A-2, A-3**), the 3-epi-$25(OH)D_3$ coelutes with $25(OH)D_3$ and is detected by the same multiple reaction monitoring (MRM) ions in MS/MS and absorbance wavelength in UV, leading to a positive bias in the $25(OH)D_{Total}$ results. It is unclear how the presence of 3-epi-$25(OH)D_3$ affects the $25(OH)D_{Total}$ for immunoassay results. Given that the consensus median values for the LC and IA methods are generally **≈**7% to **≈**10% higher than the NIST value, it is likely that some of this bias is attributable to contribution from 3-epi-$25(OH)D_3$.

Figure 2. 25(OH)D_{Total} levels in SRM 972a L1, SRM 972a L3, and SRM 968d L1 as determined by immunoassay (IA) 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.

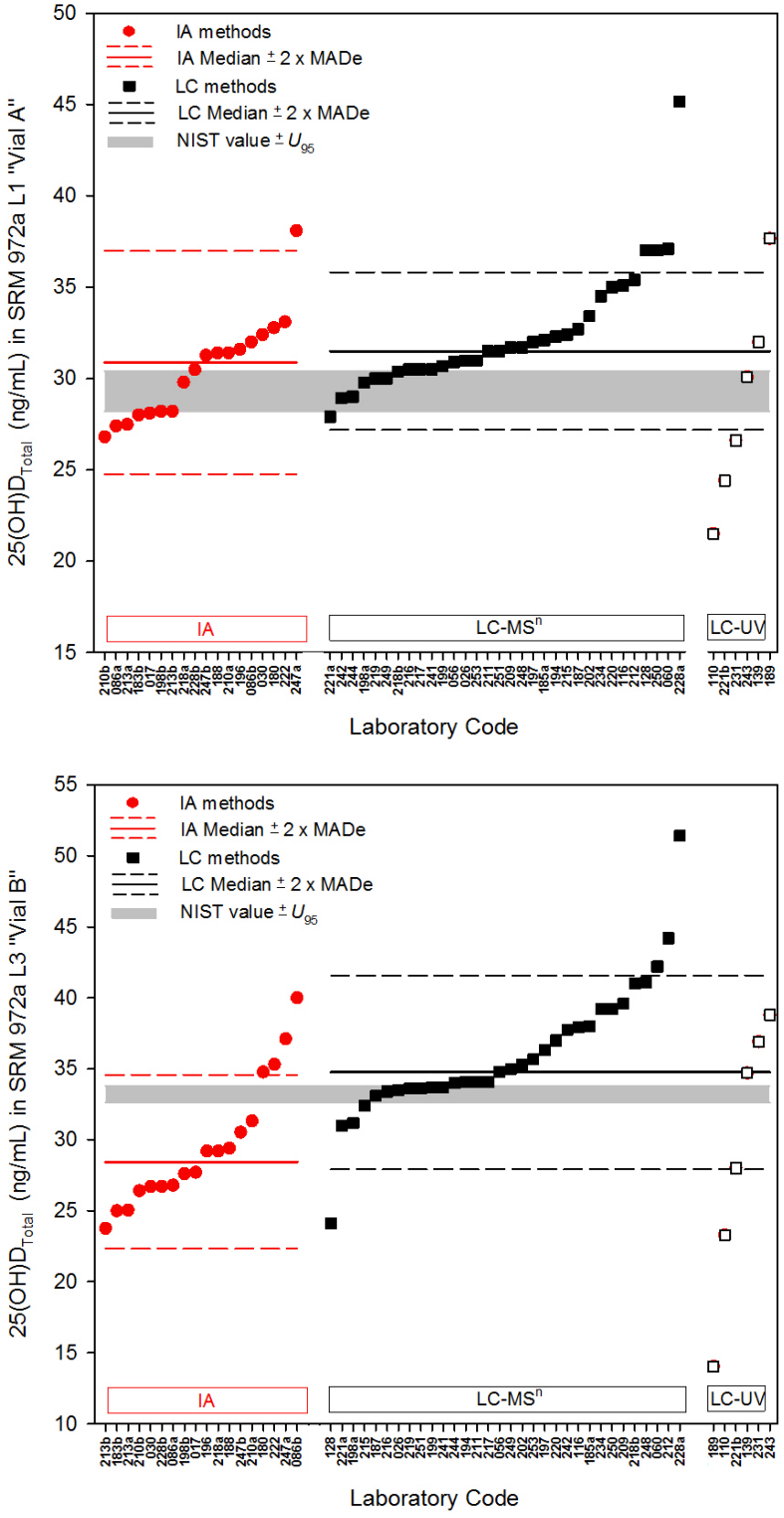


Figure 2 (cont'd). 25(OH)D_{Total} levels in SRM 972a L1, SRM 972a L3, and SRM 968d L1 as determined by immunoassay (IA) and LC (LC-MS^n and LC-UV) methods. The grey-shaded bars represent the ranges bound by the NIST values with ± estimated U_{95} uncertainty. The error bars represent 2 × SD of the duplicate results.

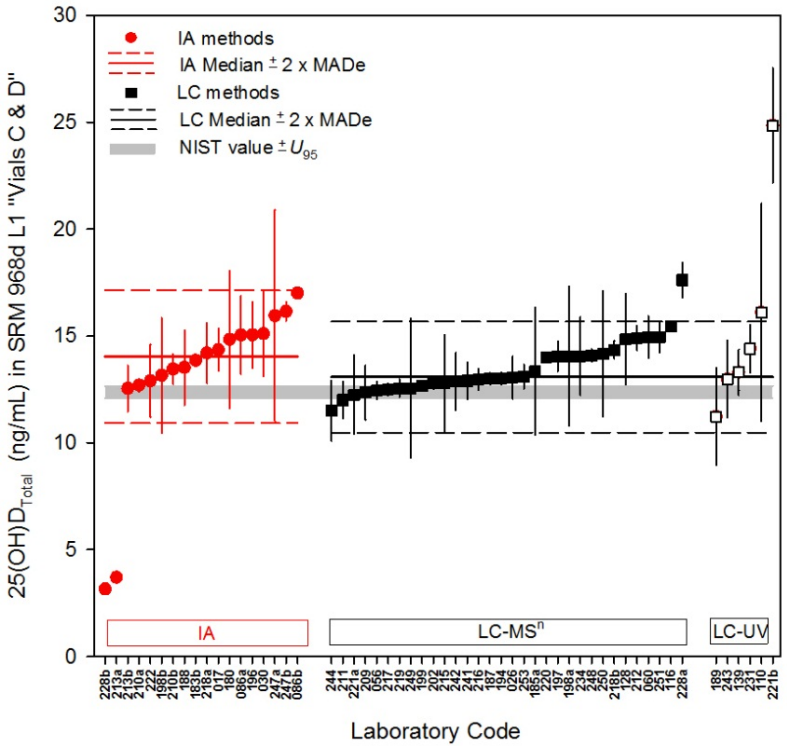


Figure 3 presents direct graphical comparisons of the 25(OH)D_{Total} results for the studied serum materials: A) SRM 972a L3 and SRM 972a L1; B) SRM 968d L1 and SRM 972a L1 and C) SRM 968d L1 and SRM 972a L3. In each plot, there are two blue consensus boxes, one for IA methods and one for LC methods (as indicated). Laboratory results that are within the consensus range for both study materials are within the blue consensus boxes. Conversely, laboratory results that fall outside of (or on the edge of) either of the consensus boxes are not included in the consensus ranges and are highlighted with their laboratory code numbers. In each plot, The NIST values for the materials are denoted with a red diamond symbol (◆), and the Youden line (y=x) centered on the NIST value is illustrated by a red line (——) across the magnitude of the y-axis and x-axis, respectively.

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

SRM 972a L3 and SRM 972a L1 (Figure 3A)

- Laboratory results that are not included in the consensus ranges include numbers 060, 086b, 110, 128, 180, 189, 212, 221b, 222, 231, 247a, and 250.
- The Youden line runs through both the IA and LC consensus boxes for these materials.
- Most of the IA results for both materials are lower than the results from the LC methods, and the IA and LC consensus boxes partially overlap but exhibit significant separation from each other.

SRM 968d L1 and SRM 972a L1 (Figure 3B)

- Laboratory results that are not included in the consensus ranges include numbers 060, 110, 128, 189, 213a, 221b, 228a, 228b, 231, 247a, 250.
- The Youden line runs through the center of both the IA and LC consensus boxes, illustrating that both the IA and LC results are in agreement with each other and with the NIST results for these materials.

SRM 968d L1 and SRM 972a L3 (Figure 3C)

- Laboratory results that are not included in the consensus ranges include numbers 060, 086b, 110, 128, 180, 189, 212, 213a, 221b, 222, 228a, 228b, 247a.
- The Youden line runs through the center of the LC consensus box and through the bottom corner of the IA consensus box for these materials, illustrating that the LC results are in better agreement with the NIST results for these materials.
- In general, the IA results tend to be lower than the LC results for material SRM 972a L3, leading to consensus boxes that partially overlap but exhibit significant separation from each other.

The Youden plots involving SRM 972a L3 reveal separation of the IA and LC consensus boxes, further illustrating the difference in results for the two techniques for the material with high native levels of 25(OH)D_2.

Figure 3. Youden comparison plot of the results for 25(OH)D_{Total} in SRM 972a L3 (Vial B) and SRM 972a L1 (Vial A) for all methods

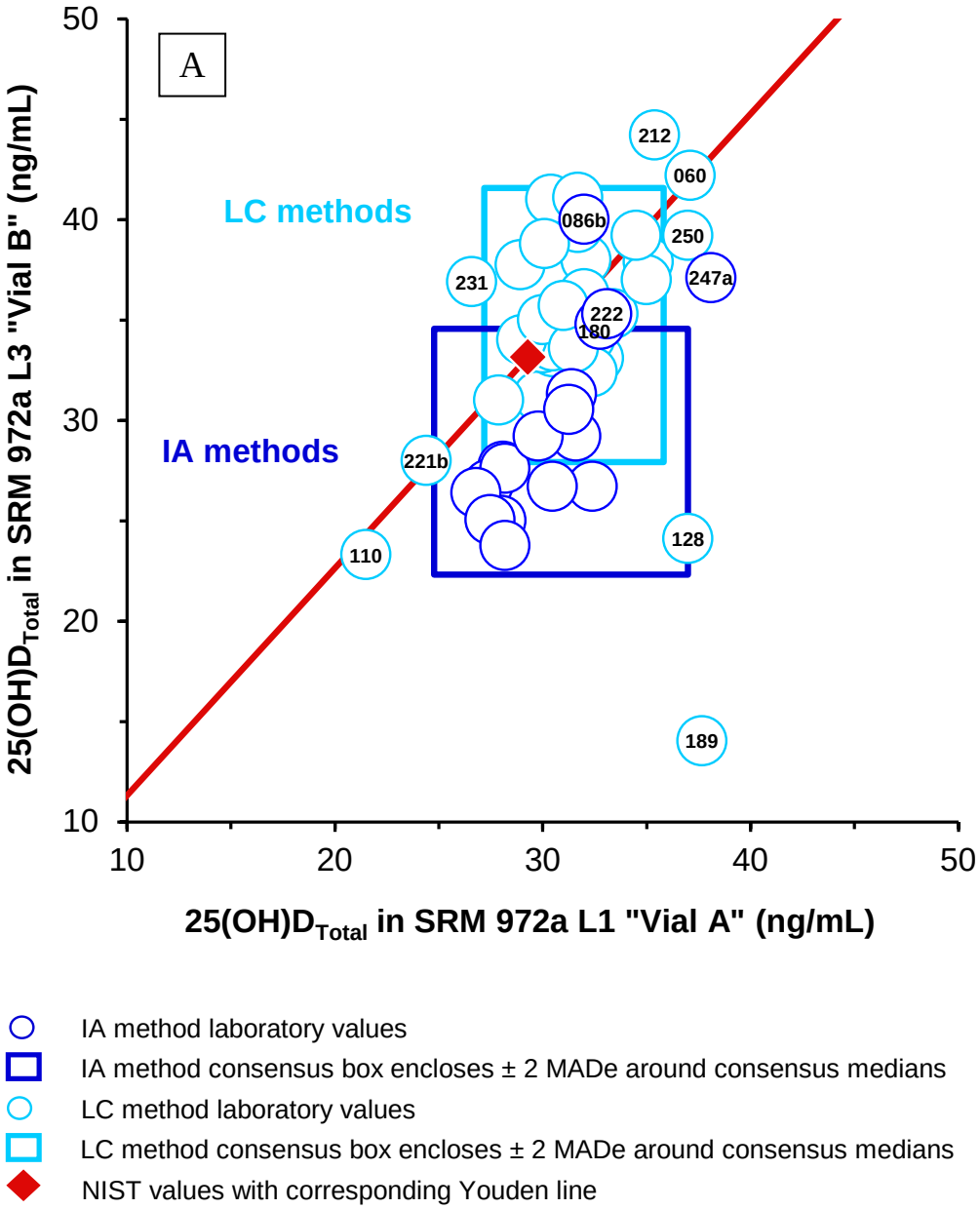


Figure 3 (cont'd). Youden comparison plot of the results for 25(OH)D_{Total} in SRM 968d L1 (Vials C,D) and SRM 972a L1 (Vial A) for all methods

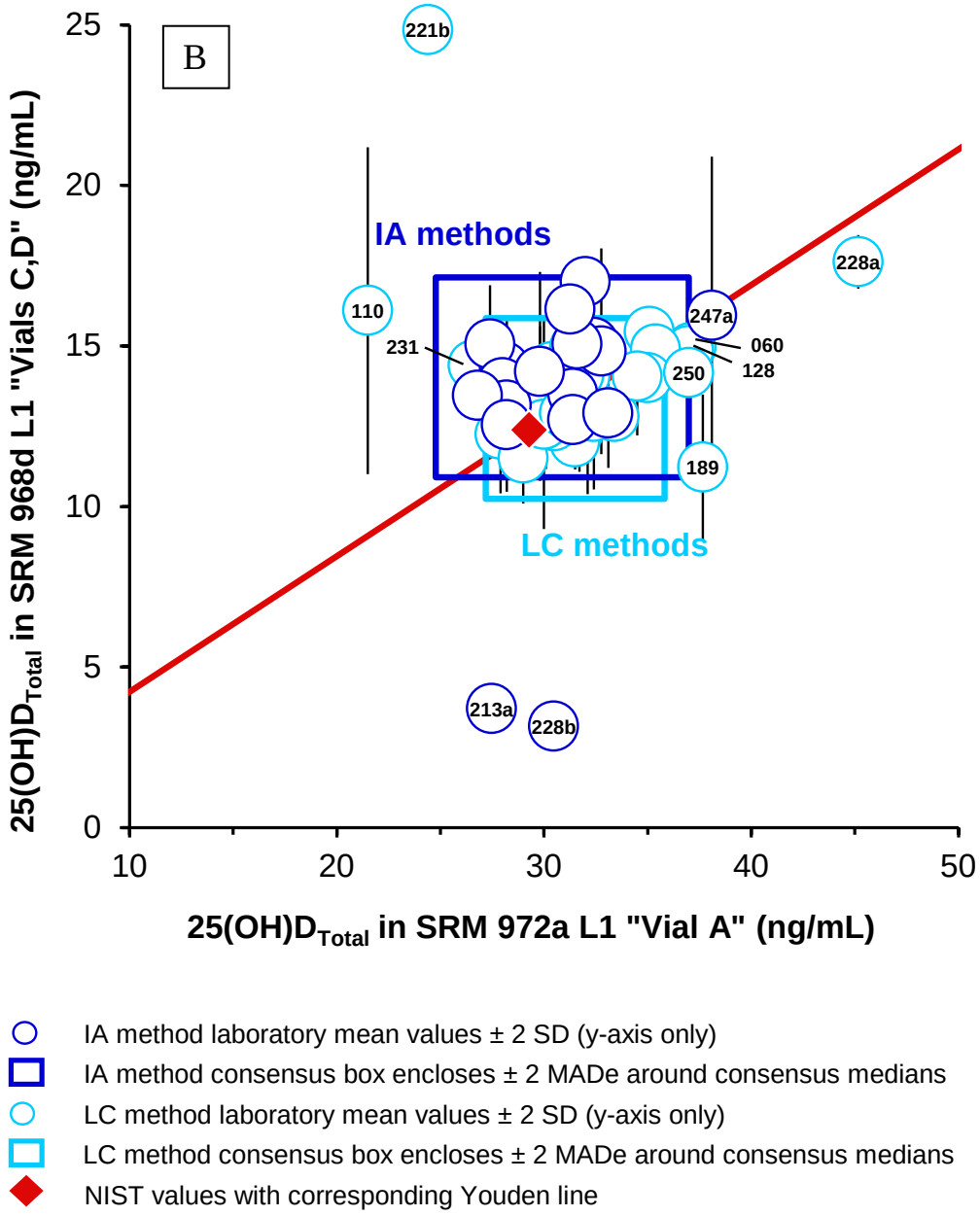
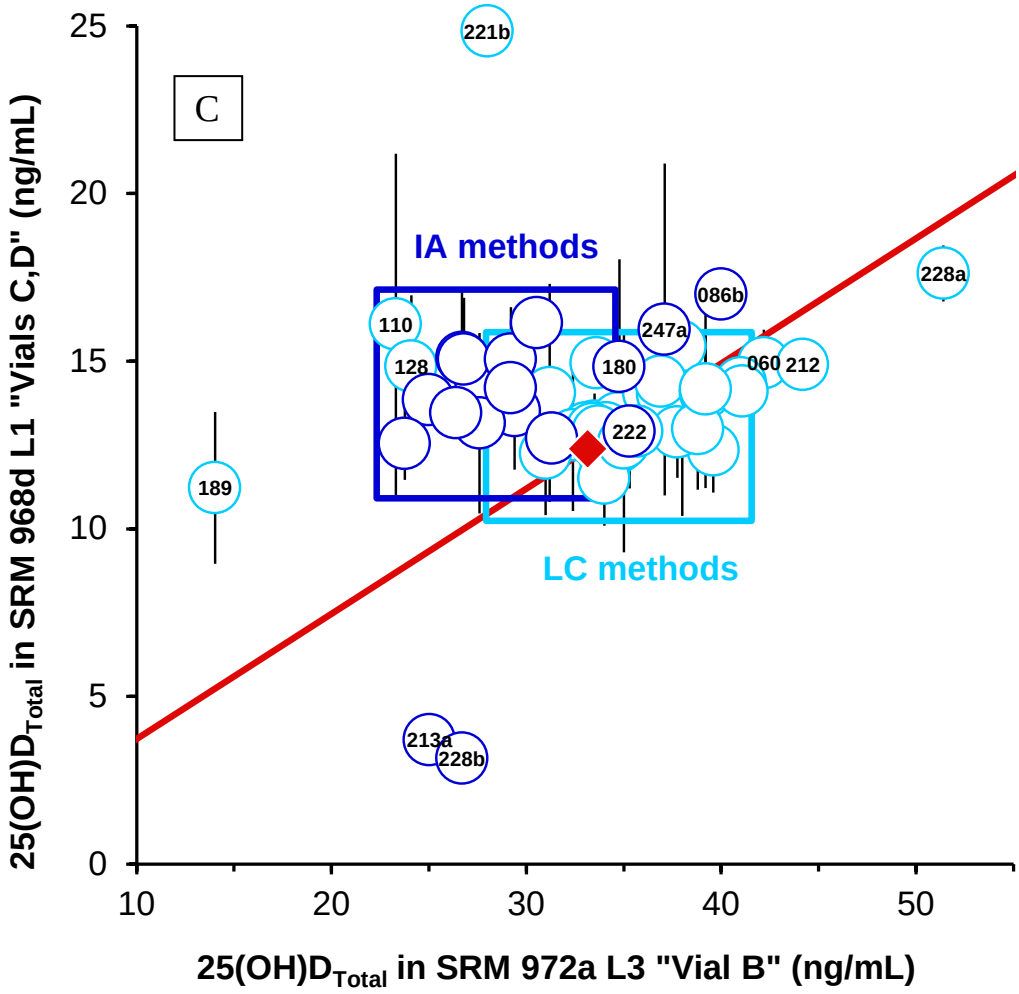


Figure 3 (cont'd). Youden comparison plot of the results for 25(OH)D_{Total} in SRM 968d L1 (Vials C,D) and SRM 972a L3 (Vial B) for all methods



IA method laboratory mean values ± 2 SD (y-axis only)
IA method consensus box encloses ± 2 MADe around consensus medians
LC method laboratory mean values ± 2 SD (y-axis only)
LC method consensus box encloses ± 2 MADe around consensus medians
NIST values with corresponding Youden line

25(OH)D_2 and 25(OH)D_3 in SRM 972a L3 (LC methods only)

Of the two major techniques IA and LC, only the LC techniques can independently measure the 25(OH)D_2 and 25(OH)D_3 components of 25(OH)D_{Total}. SRM 972a L3 contains appreciable concentrations of both metabolites, and a summary of the individual LC participant data for 25(OH)D_2 and 25(OH)D_3 in SRM 972a L3 (vial B) is provided in **Table 3.**

The community results are summarized at the bottom of the table for all LC methods and for the LC-MS^n methods only. These summarized results include N, the median value, the MADe, and the CV%. For the participant results, the consensus variability is much larger for 25(OH)D_2 (17%) than it is for 25(OH)D_3 (6%). The source of the difference in measurement uncertainty for the two metabolites is unclear.

Table 3 also presents the NIST certified values for 25(OH)D_3 and 25(OH)D_2 and the 95% confidence limits (U_{95}) in SRM 972a L3.

Table 3. Summary of LC participant data and community results for 25(OH)D_2 (ng/mL) and 25(OH)D_3 (ng/mL) in SRM 972a L3 (Vial B).

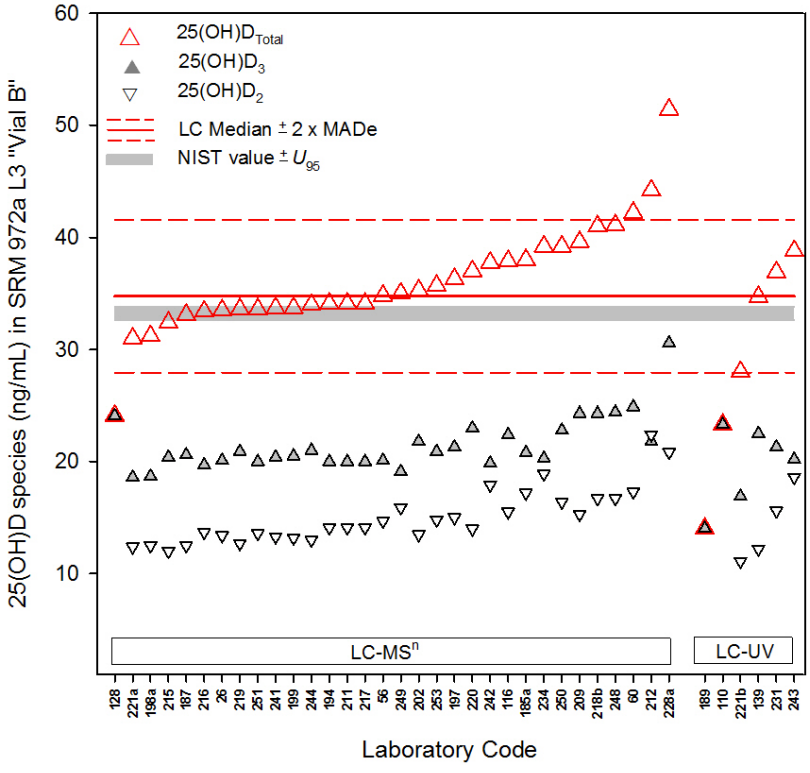
		25(OH)D2	25(OH)D3
		SRM 972a L3	SRM 972a L3
Lab	Method	Vial B	Vial B
026	LC-MS/MS	13.4	20.1
056	LC-MS/MS	14.7	20.1
060	LC-MS/MS	17.3	24.9
110	LC-UV	<4	23.3
116	LC-MS/MS	15.5	22.4
128	LC-MS/MS	n/r	24.1
185a	LC-MS/MS	17.2	20.8
187	LC-MS/MS	12.5	20.6
187	LC-MS/MS	12.5	20.6
189	LC-UV	n/d	14.0
197	LC-MS/MS	15.0	21.3
198a	LC-MS/MS	12.5	18.7
198a	LC-MS/MS	12.5	18.7
199	LC-MS/MS	13.2	20.5
202	LC-MS/MS	13.5	21.8
211	LC-MS/MS	14.1	20.0
212	LC-MS/MS	22.4	21.8
215	LC-MS/MS	12.0	20.4
216	LC-MS/MS	13.7	19.7
217	LC-MS/MS	14.1	20.0
217	LC-MS/MS	14.1	20.0
218b	LC-MS/MS	16.7	24.3
219	LC-MS/MS	12.7	20.9
220	LC-MS/MS	14.0	23.0
221a	LC-MS/MS	12.4	18.6
221b	LC-UV	11.1	16.9
228a	LC-MS/MS	20.8	30.6
231	LC-UV	15.6	21.3
234	LC-MS/MS	18.9	20.3
241	LC-MS/MS	13.3	20.4
242	LC-MS/MS	17.9	19.9
243	LC-UV	18.6	20.2
248	LC-MS/MS	16.7	24.4
249	LC-MS/MS	15.9	19.1
250	LC-MS/MS	16.4	22.8
251	LC-MS/MS	13.6	20.0
253	LC-MS/MS	14.8	20.9
LC methods	N	34	37
	Median	14.1	20.5
	MADe	2.4	1.2
	CV%	17	5.8
LC-MS^n	N	31	32
	Median	14.1	20.6
	MADe	2.4	1.1
	CV%	17	5.2
	NIST Value	13.3	19.8
	U_{95}	0.3	0.5

For the LC participant datasets, the single data values reported for $25(OH)D_2$ and $25(OH)D_3$ in SRM 972a L3 are plotted in **Figure 4** and are displayed with white triangles (▽) and grey triangles (▲), respectively. The values for $25(OH)D_{Total}$ represent the sum of $25(OH)D_2$ and $25(OH)D_3$ and are plotted as red triangles (△). In **Figure 4**, the red solid lines (——) represent the consensus median and the red dashed lines (- - - - -) represent the approximate 95% confidence interval ($2 \times MAD_e$) for all LC method results.

The laboratories with results that fall between the two dashed lines are within the consensus variability range. For SRM 972a L3, there are three outlying LC results (one LC-MS^n, two LC-UV) that underestimated the $25(OH)D_{Total}$ either because they did not measure or did not detect the $25(OH)D_2$. For the LC methods, independent, accurate measurements of both $25(OH)D_2$ and $25(OH)D_3$ are required to obtain accurate values for $25(OH)D_{Total}$.

The grey-shaded bars in **Figure 4** represent the interval in which NIST believes the "true value" exists for these solutions (i.e., NIST value ± approximately 95% confidence intervals (U_{95})). The median results for both $25(OH)D_2$ and $25(OH)D_3$ are higher than the NIST expanded uncertainty range; however, the NIST value falls within the consensus range for the LC methods for both $25(OH)D_2$ and $25(OH)D_3$.

Figure 4. $25(OH)D_2$, $25(OH)D_3$ and $25(OH)D_{Total}$ levels in SRM 972a L3 as determined by 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.



Correlation of 25(OH)D in SRM 972a L1, SRM 972a 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 4**.

Table 4. 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 972a L1, SRM 972a L3, and SRM 968d L1 overlaid with the clinical ranges for 25(OH)D from **Table 4** are presented in **Figure 5**. Specific results as assessed from **Figure 5** are summarized below:

SRM 972a L1

- All of the participant results are in the adequate 25(OH)D concentration range, but the range or reported values is large (from 21.5 ng/mL to 45.2 ng/mL).
- The NIST value (29.3 ng/mL ± 1.1 ng/mL) is in the adequate 25(OH)D concentration range.

SRM 972a L3

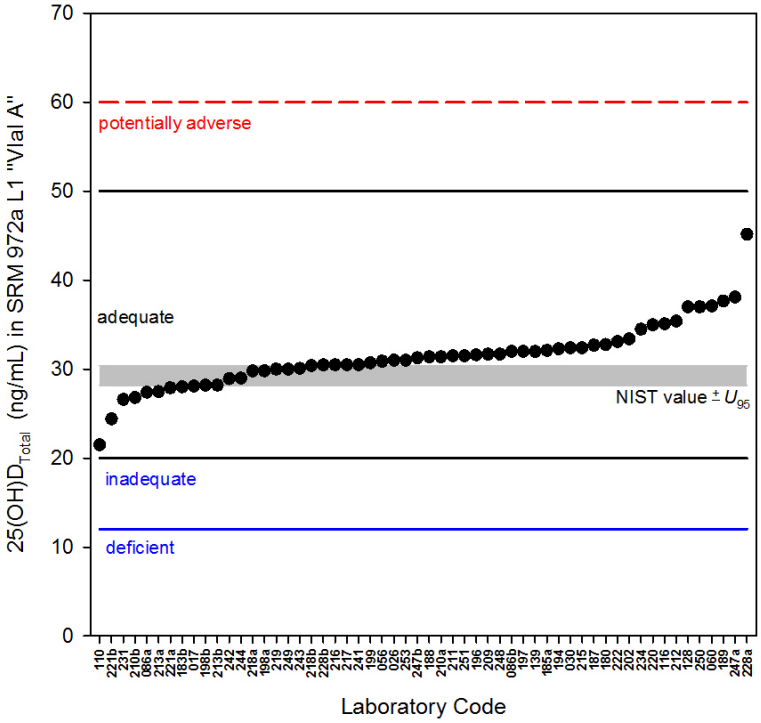
- The majority of the participant results are in the adequate range, but there is one result in each of the inadequate and potentially adverse ranges; the range of participant results is large (from 14.0 ng/mL to 51.4 ng/mL).
- The NIST value (33.2 ng/mL ± 0.6 ng/mL) is in the adequate 25(OH)D concentration range.

SRM 968d L1

- The majority of participant results are in the inadequate 25(OH)D concentration range, but results in the deficient and adequate concentration ranges were also reported.
- The NIST value (12.4 ng/mL ± 0.3 ng/mL) is in the inadequate 25(OH)D concentration range.

The consensus CV% of the participant results from all methods was ≈ 10% for SRM 972a L1 and 968d L1 and 17% for SRM 972a L3 (**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 972a L1, SRM 972a 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.



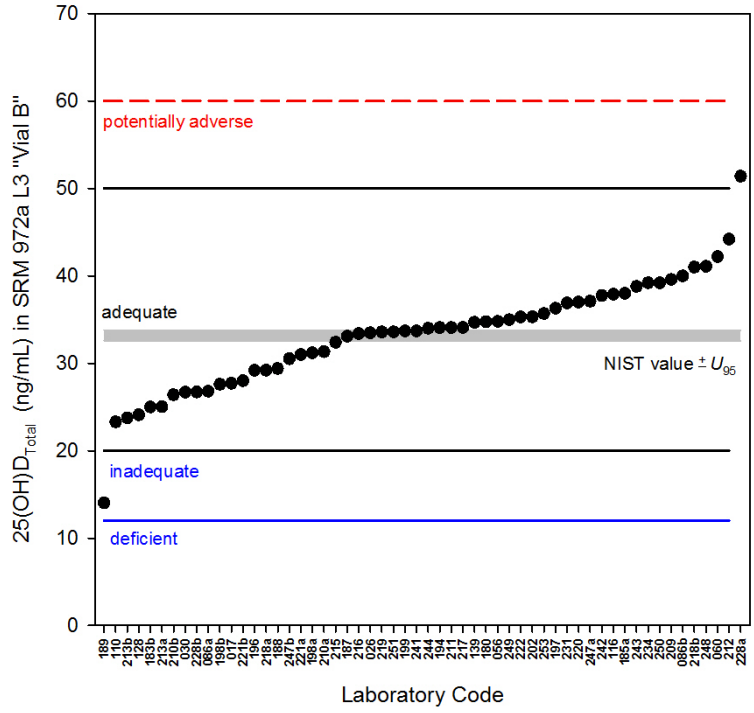
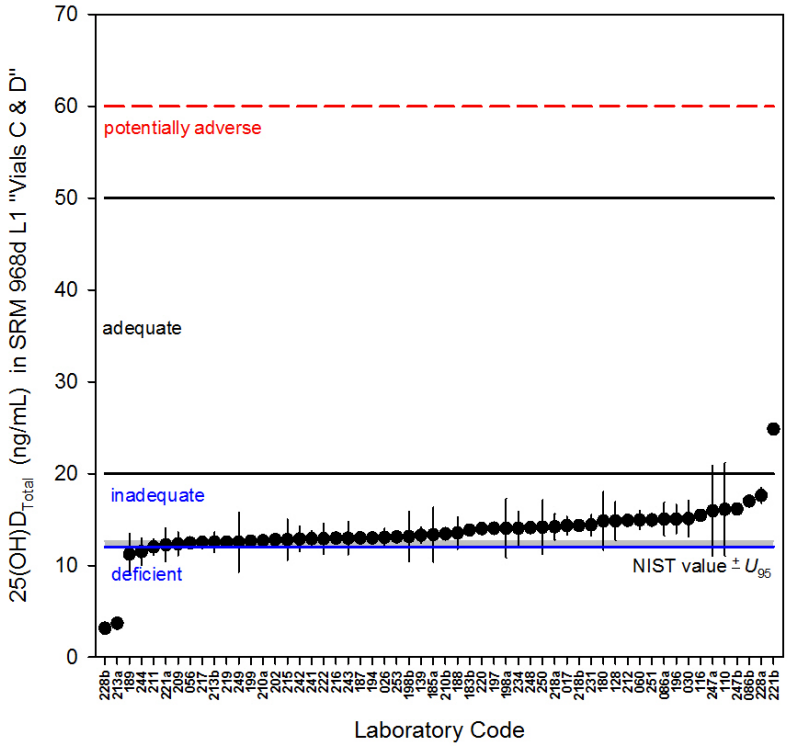


Figure 5 (cont'd) 25(OH)D_{Total} levels in SRM 972a L1, SRM 972a L3, and SRM 968d L1 superimposed over clinically-relevant serum 25-hydroxyvitamin D (25(OH)D_{Total}) concentration levels as reported by NIH (Table 3). The grey-shaded bars represent the ranges bound by the NIST values with ± estimated 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
30	RIA	Sample was extracted with acetonitrile (50 µL sample + 500 µL acetonitrile); controls measured using water as the diluent	Gamma counter with data reduction software
86a	CLIA	n/r	n/r
86b	RIA	n/r	n/r
180	RIA	Samples extracted with acetonitrile	I^{125} detection
183b	CLIA	n/r	n/r
188	CLIA	None	n/r
196	CLIA	The human serum samples were analyzed neat; calibration solutions were diluted 1:4 in a diluent mix and analyzed	n/r
198b	EIA	n/r	n/r
210a	RIA	Sample was extracted with acetonitrile	n/r
210b	CLIA	n/r	n/r
213a	EIA	Sample was thawed and gently mixed prior to analysis	n/r
213b	CLIA	Sample was thawed and gently mixed prior to analysis	n/r
218a	CLIA	Direct analysis	n/r
222	CLIA	n/r	n/r
228b	CLIA	n/r	n/r
247a	CLIA	Sample was thawed, mixed well and used in the assay	n/r
247b	EIA	Sample was thawed, mixed well and used in the assay	UV at 450 nm with a reference filter at 630 nm

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	PFP column (100 x 3.2 mm); isocratic separation with 82% methanol, 18% water; flow 0.4 mL/min	25(OH)D_2 413/355; 25(OH)D_3 401/365; 3-epi-25(OH)D_3 401/365
56	25(OH)D_2-d_3; 25(OH)D_3-d_6; 3-epi-25(OH)D_3-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; 3-epi-25(OH)D_3 383/365
60	25(OH)D_3 -d_6	IS was added and serum (150 µL) proteins were precipitated with ternary solvent, followed by centrifugation, evaporation, and reconstitution	C18 column (150 x 3.0 mm); gradient with water, methanol and acetonitrile (0.05% formic acid); flow 0.55 mL/min	25(OH)D_3 383/211; 25(OH)D_3-d_6 389/211; 25(OH)D_2 395/270
116	25(OH)D_3 -d_6	Serum proteins were precipitated, followed by centrifugation and injection of the supernatant	2-dimensional LC-MS/MS	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
185a	25(OH)D_2-d_6 and 25(OH)D_3-d_6	Liquid-liquid extraction; 40 µL sample	C18 column; methanol/water gradient	MRM
187	n/r	n/r	n/r	n/r
194	25(OH)D_3 -d_6	Proteins precipitated with acetonitrile, top layer removed, evaporated, and reconstituted with methanol	C8 column (50 x 2mm)	25(OH)D_2 395.3/119.0; 25(OH)D_3 383.4/211.3
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); column temp 45°C; gradient with water and methanol; flow 1.0 mL/min	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	n/r	n/r	n/r	n/r
202	d_6-labeled compound	Sample was extracted	C18 column (50 x 2.1 mm); gradient with water (10 mmol/L ammonium formate) and methanol (0.1% formic acid); flow 0.4 mL/min	n/r
209	25(OH)D_3 -d_6	Proteins were precipitated with $ZnSO_4$ in methanol	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	Proteins precipitated with acetonitrile containing IS followed by centrifugation	Column (33 x 4.6 mm; 3 µm)	25(OH)D_3 383/365 (quant), 383/257 (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 containing the IS (350 µL)	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 and $ZnSO_4$; supernatant extracted using solid phase extraction	C18 column (50 x 2.1mm; 2.6 µm) column; gradient with water (0.1% formic acid, 5 mmol/L ammonium formate) and methanol (0.05% formic acid)	25(OH)D_3 401/383; 25(OH)D_2 413/395; 25(OH)D_3-d_6 407/389
216	25(OH)D_2-d_3 and 25(OH)D_3-d_6	Samples extracted using liquid-liquid extraction then labeled with a derivatization reagent	C18 column (200 x 2.1 mm); gradient from 25% water (0.05% formic acid) to 50% acetonitrile (0.05% formic acid); flow 0.2 mL/min	n/r
217	25(OH)D_3-d_6	Protein precipitation with $ZnSO_4$ in methanol followed by solid phase extraction	C8 column (50 x 2.1 mm; 1.7 µm); 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_2 413/83 (quant), 413/395 (qual)
218b	25(OH)D_2-d_3 and 25(OH)D_3-d_3	Sample was extracted, filtered, centrifuged, etc.	Phenyl column (50 x 2.1 mm; 1.7 µm); flow 0.45 mL/min	25(OH)D_3 401; 25(OH)D_2 413
219	25(OH)D_3-d_6	Online SPE	n/r	n/r
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); sample filtered then phospholipids removed with solid phase extraction	C18 column (20 x 2.1mm, 2.7µm); gradient with water and acetonitrile; flow 1 mL/min; column 40 °C	n/r
221a	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
228a	n/r	Proteins precipitated followed by centrifugation	C18 column; gradient with water and methanol	25(OH)D_3 401; 25(OH)D_2 413
234	25(OH)D_3-d_6	The samples are protein crashed using acetonitrile	A turbo column is used for cleanup followed by a C18 analytical column; isocratic separation with water and methanol	25(OH)D_3 383/365; 25(OH)D_2 395/209; 25(OH)D_3-d_6 389/211
241	25(OH)D_3-d_6	Acetonitrile containing the IS (100 µL) added to sample (50 µL) to precipate proteins, followed by mixing, sonication, and centrifugation.	C8 column (50 x 2 mm; 3 µm); gradient starting with 50% methanol (0.1% formic acid), 50% water (0.1% formic acid)	25(OH)D_3 383/211 (quant), 383/229 (qual); 25(OH)D_2 395/119 (quant), 395/211 (qual); 25(OH)D_3-d_6 389/211
242	25(OH)D_3-d_6	Ethanol containing the IS (75 µL) and acetonitrile (500 µL) added to sample (400 µL) to precipate proteins, followed by extraction with heptane, evaporation, and reconsitution in methanol.	Reversed-phase column (150 x 2 mm); gradient with acetonitrile/water; flow 0.35 mL/min	25(OH)D_3 401/383; 25(OH)D_2 413/395; 25(OH)D_3-d_6 407/389
244	25(OH)D_3-d_6	Protein precipitation followed by filtration	CN column; mobile phase consisting of distilled water (formic acid) and methanol	25(OH)D_2 395/269; 25(OH)D_3 383/211
248	25(OH)D_2-d_3 and 25(OH)D_3-d_3	IS was added and the sample was precipitated with acetonitrile, centrifuged, and injected	C18 column (100 x 2.1 mm; 2.5 µm); gradient with water and methanol, each containing 2 mmol/L ammonium acetate	25(OH)D_3 383/257 (quant), 383/365 (qual); 25(OH)D_2 395/377 (quant), 395/269 (qual); 25(OH)D_3-d_3 386/257 (quant), 386/368 (qual); 25(OH)D_2-d_3 398/380 (quant), 398/271 (qual)
249	25(OH)D_2-d_3 and 25(OH)D_3-d_6	Proteins precipitated followed by centrifugation and SPE	PFP column (100 x 2.1 mm; 1.8 µm); gradient separation with water (2 mmol/L ammonium acetate, 0.1% formic acid) and methanol (0.3% formic acid); flow 0.3 mL/min	25(OH)D_3 401/159; 25(OH)D_3-d_6 407/159; 25(OH)D_2 413/159; 25(OH)D_2-d_3 416/83

250	25(OH)D_2-d_3 and 25(OH)D_3-d_3	Protein crash followed by SPE	Phenyl column (50 x 2.1 mm); gradient with water and methanol, each containing 2 mmol/L ammonium acetate and 0.1% formic acid; flow 0.45 mL/min	25(OH)D_3 401/159, 401/365; 25(OH)D_2 413/355, 413/83
251	25(OH)D_2-d_3 and 25(OH)D_3-d_3	Protein precipitation followed by SPE	Phenyl column (50 x 2.1 mm; 1.7 µm); gradient with water and methanol, each containing 2 mmol/L ammonium acetate and 0.1% formic acid; flow 0.45 mL/min	25(OH)D_3 401/159 (quant), 401/365 (qual); 25(OH)D_2 413/355 (quant), 413/83 (qual); 25(OH)D_3-d_3 404/162; 25(OH)D_2-d_3 416/358
253	*d*-labeled isotope	The sample was extracted with acetonitrile/trifluroacetic acid and then centrifuged	C18 column (50 x 2.1 mm); isocratic separation with water and methanol; flow 0.4 mL/min	25(OH)D_2 619/298; 25(OH)D_3 607/298

MRM = multiple reaction monitoring

PFP = pentafluorophenyl

n/r = not reported

quant = quantitative ions

qual = qualitative ions

SPE = solid phase extraction

CN = cyano

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

Laboratory Number	*Internal Standard (IS)*	*Sample Preparation*	*Chromatographic Conditions*	*Wavelength*
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%)	268 nm
139	Proprietary	The sample was extracted, centrifuged and injected	Reversed-phase column, isocratic separation with proprietary mobile phase; flow 1 mL/min	264 nm
189	Added before extraction	Proteins were disrupted and precipitated; analytes were extracted using solid-phase extraction	Reversed-phase column (150 x 4.6 mm); isocratic separation with commercial mobile phase; flow 0.7 mL/min	265 nm
221b	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; 47 °C	275 nm
231	1alpha(OH)D_3	Samples were extracted with hexane/dichloromethane, evaporated and reconstituted with mobile phase (phosphate buffer/acetonitrile)	Reversed-phase column (250 x 4.5 mm; 5μm), isocratic separation with 14% phosphate buffer, 86% acetonitrile; flow 1.2 mL/min	265 nm
243	Laurophenone	Reagent 1 containing the ethanolic IS (400 μL) added to sample (400 μL), followed by vortexing the precipitation reagent (500 μL) and sampling of the supernatant	Reversed-phase column (150 x 3 mm); isocratic separation with 65% acetonitrile, 35% water; flow 1 mL/min	264 nm

n/r = not reported

CN = cyano

Appendix B-1. Raw participant data and NIST results for $25(OH)D_2$, $25(OH)D_3$ and $25(OH)D_{Total}$ in SRM 972a L1, SRM 972a 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 972a L1	SRM 972a L3	SRM 968d L1	SRM 968d L1	SRM 972a L1	SRM 972a L3	SRM 968d L1	SRM 968d L1	SRM 972a L1	SRM 972a L3	SRM 968d L1	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	28.1	27.7	14.0	14.7	n/r	n/r
026	LC-MS/MS	<1.0	13.4	<1.0	<1.0	31.0	20.1	12.7	13.4	31.0	33.5	12.7	13.4	n/r	n/r
030	RIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	32.4	26.7	15.8	14.4	257.0	408.5
056	LC-MS/MS	0.7	14.7	n/d	n/d	30.2	20.1	12.6	12.3	30.9	34.8	12.6	12.3	246.7	346.3
060	LC-MS/MS	< 2.00	17.3	n/d	n/d	37.1	24.9	15.3	14.6	37.1	42.2	15.3	14.6	210.1	348.1
086a	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	27.4	26.8	14.4	15.7	n/r	n/r
086b	RIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	32.0	40.0	17.0	17.0	n/r	n/r
110	LC-UV	<4	<4	<4	<4	19.9	23.3	14.3	15.3	21.5	23.3	14.3	17.9	239.2	335.2
116	LC-MS/MS	< 3.3	15.5	< 3.3	< 3.3	35.1	22.4	15.4	15.5	35.1	37.9	15.4	15.5	n/r	n/r
128	LC-MS/MS	n/r	n/r	n/r	n/r	37.0	24.1	15.6	14.1	37.0	24.1	15.6	14.1	n/r	332.0
139	LC-UV	n/d	12.2	n/d	n/d	32.0	22.5	13.0	13.6	32.0	34.7	13.0	13.6	256.0	335.0
180	RIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	32.8	34.8	13.7	16.0	n/r	n/r
183b	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	28.0	25.0	13.8	13.9	262.4	336.8
185a	LC-MS/MS	n/d	17.2	2.1	n/d	32.1	20.8	12.3	12.3	32.1	38.0	14.4	12.3	238.6	334.8
187	LC-MS/MS	n/d	12.5	n/d	n/d	32.7	20.6	13.1	12.9	32.7	33.1	13.1	12.9	n/r	n/r
188	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	31.4	29.4	12.9	14.1	n/r	n/r
189	LC-UV	n/d	n/d	n/d	n/d	37.7	14.0	12.0	10.4	37.7	14.0	12.0	10.4	n/r	n/r
194	LC-MS/MS	<7	14.1	<7	<7	32.3	20.0	12.9	13.1	32.3	34.1	12.9	13.1	240.5	335.0
196	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	31.6	29.2	14.5	15.6	n/r	n/r
197	LC-MS/MS	<5	15.0	<5	<5	32.0	21.3	13.8	14.3	32.0	36.3	13.8	14.3	238.0	343.0
198a	LC-MS/MS	<5	12.5	<5	<5	29.8	18.7	12.9	15.2	29.8	31.2	12.9	15.2	228.2	324.1
198b	EIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	28.2	27.6	12.2	14.1	n/r	n/r
199	LC-MS/MS	< 2	13.2	< 2	< 2	30.7	20.5	12.7	12.6	30.7	33.7	12.7	12.6	249.4	329.9
202	LC-MS/MS	n/d	13.5	n/d	n/d	33.4	21.8	12.9	12.7	33.4	35.3	12.9	12.7	246.0	345.0
209	LC-MS/MS	<1.0	15.3	<1.0	<1.0	31.7	24.3	11.9	12.8	31.7	39.6	11.9	12.8	n/r	n/r
210a	RIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	31.4	31.3	12.6	12.8	n/r	n/r
210b	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	26.8	26.4	13.2	13.7	n/r	n/r
211	LC-MS/MS	n/d	14.1	n/d	n/d	31.5	20.0	12.3	11.7	31.5	34.1	12.3	11.7	229.0	280.0
212	LC-MS/MS	n/d	22.4	n/d	n/d	35.4	21.8	14.7	15.1	35.4	44.2	14.7	15.1	n/r	n/r
213a	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	27.5	25.0	3.7	3.7	n/r	n/r
213b	EIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	28.2	23.8	12.9	12.2	n/r	n/r
215	LC-MS/MS	0.4	12.0	n/d	n/d	32.0	20.4	12.0	13.6	32.4	32.4	12.0	13.6	n/r	n/r
216	LC-MS/MS	0.5	13.7	0.2	0.2	30.0	19.7	13.0	12.6	30.5	33.4	13.2	12.8	229.9	343.3
217	LC-MS/MS	< 2	14.1	< 2	< 2	30.5	20.0	12.4	12.6	30.5	34.1	12.4	12.6	n/r	n/r
218a	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	29.8	29.2	13.7	14.7	n/r	n/r
218b	LC-MS/MS	n/d	16.7	n/d	n/d	30.4	24.3	14.2	14.5	30.4	41.0	14.2	14.5	239.1	336.0
219	LC-MS/MS	<4.0	12.7	<4.0	<4.0	30.0	20.9	12.4	12.7	30.0	33.6	12.4	12.7	n/r	n/r
220	LC-MS/MS	<5	14.0	<5	<5	35.0	23.0	14.0	14.0	35.0	37.0	14.0	14.0	n/r	n/r
221a	LC-MS/MS	n/d	12.4	n/d	n/d	27.9	18.6	11.6	12.9	27.9	31.0	11.6	12.9	n/r	n/r
221b	LC-UV	n/d	11.1	n/d	n/d	24.4	16.9	25.0	24.7	24.4	28.0	25.0	24.7	n/r	n/r
222	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	33.1	35.3	13.5	12.3	n/r	n/r
228a	LC-MS/MS	n/d	20.8	n/d	n/d	45.2	30.6	17.3	17.9	45.2	51.4	17.3	17.9	217.3	331.5
228b	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	30.5	26.7	3.2	3.1	n/r	n/r
231	LC-UV	n/d	15.6	n/d	n/d	26.6	21.3	14.8	14.0	26.6	36.9	14.8	14.0	245.3	294.5
234	LC-MS/MS	< 3.0	18.9	< 3.0	< 3.0	34.5	20.3	13.4	14.7	34.5	39.2	13.4	14.7	n/r	n/r
241	LC-MS/MS	n/d	13.3	n/d	n/d	30.5	20.4	13.2	12.6	30.5	33.7	13.2	12.6	n/r	n/r
242	LC-MS/MS	n/d	17.9	n/d	n/d	28.9	19.9	13.4	12.4	28.9	37.8	13.4	12.4	239.4	333.4
243	LC-UV	n/d	18.6	n/d	n/d	30.1	20.2	13.6	12.3	30.1	38.8	13.6	12.3	240.6	331.7
244	LC-MS/MS	<5	13.0	<5	<5	29.0	21.0	12.0	11.0	29.0	34.0	12.0	11.0	215.0	331.0
247a	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	38.1	37.1	17.7	14.2	n/r	n/r
247b	EIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	31.3	30.5	16.3	16.0	n/r	n/r
248	LC-MS/MS	< 3	16.7	< 3	<3	31.7	24.4	14.0	14.2	31.7	41.1	14.0	14.2	238.6	334.8
249	LC-MS/MS	n/d	15.9	n/d	n/d	30.0	19.1	13.7	11.4	30.0	35.0	13.7	11.4	238.4	324.2
250	LC-MS/MS	<2.4	16.4	<2.4	<2.4	37.0	22.8	13.1	15.2	37.0	39.2	13.1	15.2	281.6	382.0
251	LC-MS/MS	<4	13.6	<4	<4	31.5	20.0	15.2	14.7	31.5	33.6	15.2	14.7	n/r	n/r
253	LC-MS/MS	n/d	14.8	n/d	n/d	31.0	20.9	12.9	13.3	31.0	35.7	12.9	13.3	235.5	307.0

*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	0.54	13.30	<0.5	<0.5	18.80	19.80	12.38	12.38	29.30	33.20	12.38	12.38	238.6	334.0
	U_{95}	0.06	0.30	0.00	0.00	1.10	0.50	0.28	0.28	1.10	0.60	0.28	0.28	3.9	5.2

Appendix B-2. Raw participant data and NIST results for 3-epi-25(OH)D_3 in SRM 972a L1, SRM 972a L3, and SRM 968d L1

		epi-25(OH)D_3 (ng/mL)			
		SRM 972a L1	SRM 972a L3	SRM 968d L1	SRM 968d L1
Lab	Method	Vial A	Vial B	Vial C	Vial D
017	CLIA	n/r	n/r	n/r	n/r
026	LC-MS/MS	1.5	0.7	0.3	0.4
030	RIA	n/r	n/r	n/r	n/r
056	LC-MS/MS	1.9	1.2	0.6	0.7
060	LC-MS/MS	n/r	n/r	n/r	n/r
086a	CLIA	n/r	n/r	n/r	n/r
086b	RIA	n/r	n/r	n/r	n/r
110	LC-UV	n/r	n/r	n/r	n/r
116	LC-MS/MS	n/r	n/r	n/r	n/r
128	LC-MS/MS	n/r	n/r	n/r	n/r
139	LC-UV	n/r	n/r	n/r	n/r
180	RIA	n/r	n/r	n/r	n/r
183b	CLIA	n/r	n/r	n/r	n/r
185a	LC-MS/MS	n/r	n/r	n/r	n/r
187	LC-MS/MS	n/r	n/r	n/r	n/r
188	CLIA	n/r	n/r	n/r	n/r
189	LC-UV	n/r	n/r	n/r	n/r
194	LC-MS/MS	<7	<7	<7	<7
196	CLIA	n/r	n/r	n/r	n/r
197	LC-MS/MS	n/r	n/r	n/r	n/r
198a	LC-MS/MS	n/r	n/r	n/r	n/r
198b	EIA	n/r	n/r	n/r	n/r
199	LC-MS/MS	n/r	n/r	n/r	n/r
202	LC-MS/MS	n/r	n/r	n/r	n/r
209	LC-MS/MS	n/r	n/r	n/r	n/r
210a	RIA	n/r	n/r	n/r	n/r
210b	CLIA	n/r	n/r	n/r	n/r
211	LC-MS/MS	n/r	n/r	n/r	n/r
212	LC-MS/MS	n/r	n/r	n/r	n/r
213a	CLIA	n/r	n/r	n/r	n/r
213b	EIA	n/r	n/r	n/r	n/r
215	LC-MS/MS	n/r	n/r	n/r	n/r
216	LC-MS/MS	n/r	n/r	n/r	n/r
217	LC-MS/MS	<2	<2	<2	<2
218a	CLIA	n/r	n/r	n/r	n/r
218b	LC-MS/MS	n/r	n/r	n/r	n/r
219	LC-MS/MS	n/r	n/r	n/r	n/r
220	LC-MS/MS	n/r	n/r	n/r	n/r
221a	LC-MS/MS	n/r	n/r	n/r	n/r
221b	LC-UV	n/r	n/r	n/r	n/r
222	CLIA	n/r	n/r	n/r	n/r
228a	LC-MS/MS	n/r	n/r	n/r	n/r
228b	CLIA	n/r	n/r	n/r	n/r
231	LC-UV	n/r	n/r	n/r	n/r
234	LC-MS/MS	n/r	n/r	n/r	n/r
241	LC-MS/MS	n/r	n/r	n/r	n/r
242	LC-MS/MS	n/r	n/r	n/r	n/r
243	LC-UV	n/r	n/r	n/r	n/r
244	LC-MS/MS	n/r	n/r	n/r	n/r
247a	CLIA	n/r	n/r	n/r	n/r
247b	EIA	n/r	n/r	n/r	n/r
248	LC-MS/MS	n/r	n/r	n/r	n/r
249	LC-MS/MS	2.1	n/r	n/r	n/r
250	LC-MS/MS	n/r	n/r	n/r	n/r
251	LC-MS/MS	n/r	n/r	n/r	n/r
253	LC-MS/MS	n/r	n/r	n/r	n/r

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

	NIST Value	1.84	1.18	<0.5	<0.5
	U_{95}	0.08	0.13	0.00	0.00