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ABSTRACT

The National Institute of Standards and Technology (NIST) has 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 fifth exercise of this program, the Winter 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 1950 Metabolites in Human Plasma, SRM 972a Vitamin D Metabolites in Human Serum (Level 2), 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 study, and laboratories were identified by code numbers known only to them. The results from this fifth study are reported along with a summary of the analytical methods used.

OVERVIEW OF THE WINTER 2012 COMPARABILITY STUDY

For the Winter 2012 Comparability Study of VitDQAP (Exercise 5), 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 plasma or serum (study materials). In this study, vial A was SRM 1950 Metabolites in Human Plasma, vials B and D were duplicate samples of SRM 972a Vitamin D Metabolites in Human Serum Level 2 (SRM 972a L2), and vial C was SRM 968d Fat-Soluble Vitamins, Carotenoids and Cholesterol in Human Serum Level 1 (SRM 968d L1). All materials consisted of blended human plasma or serum pools with endogenous 25(OH)D levels.

There were a total of 51 participants and 57 datasets (six participants provided data for two different methods) in the Winter 2012 study. Seventeen of the datasets originated from immunoassay (IA) techniques, including three from enzyme immunoassay (EIA), eight from chemiluminescence immunoassay (CLIA), and six from radioimmunoassay (RIA). **Appendix A-1** summarizes the immunoassay methods used by the participants. Forty of the datasets originated from liquid chromatographic (LC) methods; of those, 32 were from LC with tandem mass spectrometric detection (LC-MS/MS), one was from LC-MS (orbitrap), and seven 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 are summarized in **Appendix B**. All datasets from the immunoassay methods reported single values for $25(OH)D_{Total}$ in SRM 1950, SRM 972a L2, and SRM 968d L1. LC participants provided values for $25(OH)D_2$, $25(OH)D_3$, as well as $25(OH)D_{Total}$ in SRM 1950, SRM 972a L2, 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.

SRM 1950, SRM 972a L2, and SRM 968d L1 contain low levels of $25(OH)D_2$ (reported participant values ranging from 0.2 ng/mL to 1.7 ng/mL), and most of the LC labs indicated this analyte was below their quantitation limit of <1 ng/mL to <7 ng/mL. Therefore, the $25(OH)D_{Total}$ values reported in **Appendix B** are the same as the $25(OH)D_3$ values in the serum and plasma materials for the majority of LC participants.

Appendix B also provides 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.

WINTER 2012 COMPARABILITY STUDY 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 57 datasets received for the Winter 2012 study, only 36 reported values for the ethanolic controls; of those, three were from immunoassay methods and 33 were from LC methods. 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%). The consensus results using robust statistics (i.e., median and MADe) were not calculated for the data from the IA methods because of the limited number of data reported.

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.

		SRM 2972 25(OH)D_2
Lab	Method	Value
056	LC-MS/MS	230.6
060	LC-MS/MS	n/r
062	RIA	n/r
110	LC-UV	237.1
116	LC-MS/MS	245.1
119	LC-MS	182.4
124	LC-MS/MS	47.0
139	LC-UV	239.7
160a	LC-MS/MS	170.0
184	LC-MS/MS	240.5
185a	LC-MS/MS	238.6
186	LC-MS/MS	238.0
194	LC-MS/MS	240.5
195	LC-MS/MS	241.0
196	CLIA	198.6
197	LC-MS/MS	234.0
198a	LC-MS/MS	207.4
199	LC-MS/MS	245.0
200	RIA	278.1
202	LC-MS/MS	241.5
209	LC-MS/MS	248.7
210a	RIA	230.0
211	LC-MS/MS	240.4
212	LC-MS/MS	231.5
216	LC-MS/MS	258.0
217	LC-MS/MS	233.8
218b	LC-MS/MS	240.9
221a	LC-MS/MS	249.7
223	LC-MS/MS	241.2
225	LC-MS/MS	235.9
228a	LC-MS/MS	240.0
231	LC-UV	268.7
234	LC-MS/MS	246.0
241	LC-MS/MS	212.0
242	LC-MS/MS	245.0
243	LC-UV	245.9
244	LC-MS/MS	222.0
245	LC-UV	240.8
All methods	N	36
	Median	240.2
	MADe	8.5
	CV%	3.5
LC methods	N	33
	Median	240.4
	MADe	7.0
	CV%	2.9
LC-MS^n	N	28
	Median	240.2
	MADe	8.0
	CV%	3.3
	NIST Value	238.6
	U_{95}	3.9

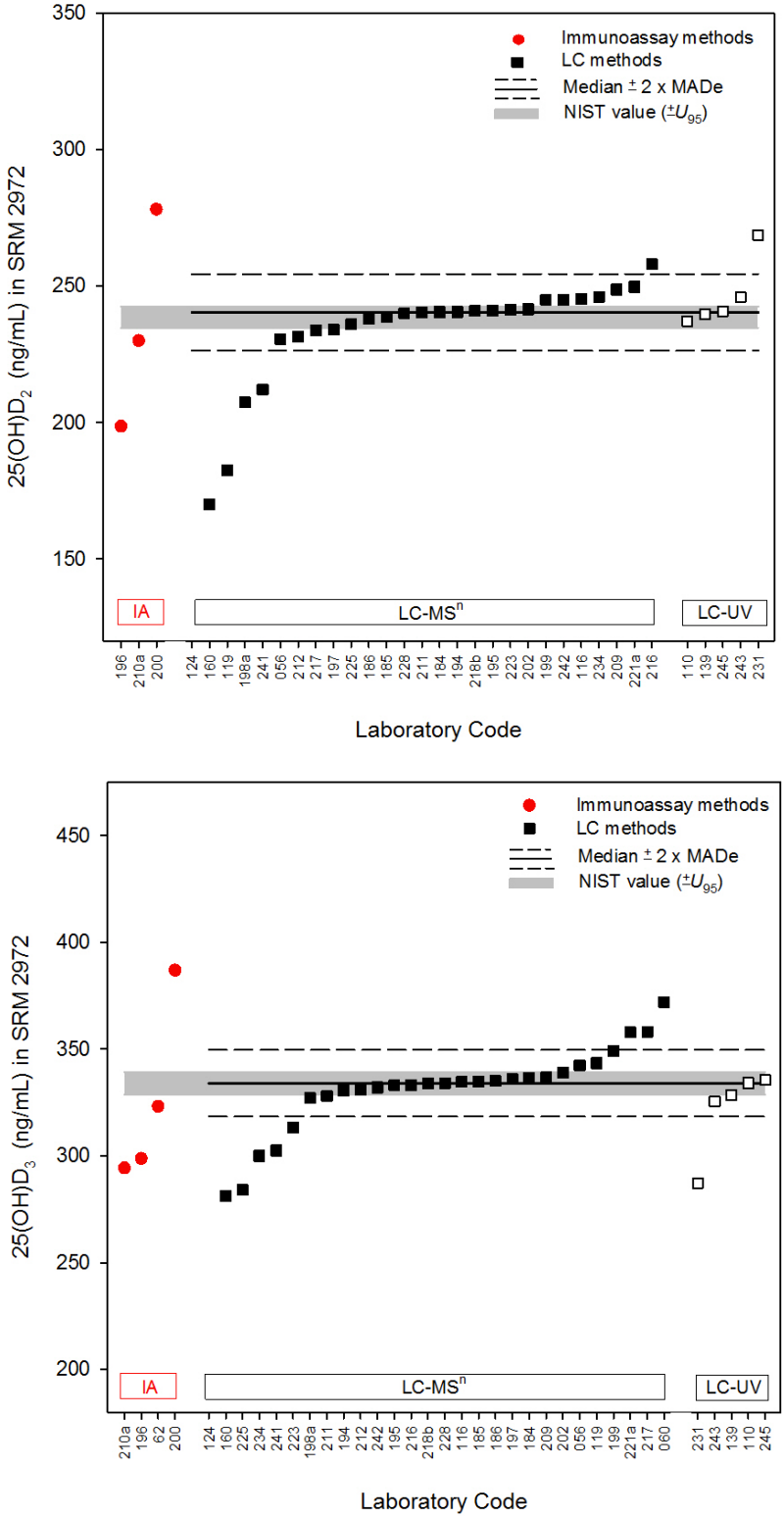
		SRM 2972 25(OH)D3
Lab	Method	Value
056	LC-MS/MS	342.4
060	LC-MS/MS	372.0
062	RIA	323.1
110	LC-UV	334.1
116	LC-MS/MS	334.6
119	LC-MS	343.4
124	LC-MS/MS	123.0
139	LC-UV	328.5
160a	LC-MS/MS	281.0
184	LC-MS/MS	336.5
185a	LC-MS/MS	334.8
186	LC-MS/MS	335.0
194	LC-MS/MS	330.5
195	LC-MS/MS	333.0
196	CLIA	298.8
197	LC-MS/MS	336.0
198a	LC-MS/MS	327.1
199	LC-MS/MS	349.0
200	RIA	386.9
202	LC-MS/MS	339.0
209	LC-MS/MS	336.7
210a	RIA	294.3
211	LC-MS/MS	328.1
212	LC-MS/MS	330.9
216	LC-MS/MS	333.0
217	LC-MS/MS	358.1
218b	LC-MS/MS	333.9
221a	LC-MS/MS	357.8
223	LC-MS/MS	313.2
225	LC-MS/MS	284.1
228a	LC-MS/MS	334.0
231	LC-UV	287.3
234	LC-MS/MS	300.0
241	LC-MS/MS	302.5
242	LC-MS/MS	332.0
243	LC-UV	325.6
244	LC-MS/MS	337.0
245	LC-UV	335.5
	N	38
	Median	333.5
	MADe	8.8
	CV%	2.6
	N	34
	Median	334.0
	MADe	7.8
	CV%	2.3
	N	29
	Median	334.0
	MADe	7.4
	CV%	2.2
	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 value ± approximately 95% confidence intervals (U_{95})). 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 1950, SRM 972a L2, and SRM 968d L1

A summary of the individual participant data for 25(OH)D_{Total} in samples SRM 1950 (vial A), SRM 972a L2 (vials B & D), and SRM 968d L1 (vial C) is provided in **Table 2.** The summarized data also include the mean, standard deviation (SD), and percent relative standard deviation (%rSD) of the two reported values for SRM 972a 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^n methods only. These summarized results include N, the median value, the MADe, and the CV%.

Table 2 also presents the NIST results with approximated 95% confidence limits (U_{95}) obtained for the three study materials.

For SRM 1950, 25(OH)D_{Total} is the sum of the NIST certified and reference values for 25(OH)D_3 and 25(OH)D_2, respectively, and the 95% confidence limit (U_{95}) was approximated using the individual uncertainties reported for the two analytes. Details about the NIST methods and measurements are reported in the Certificate of Analysis for SRM 1950[a].

For SRM 972a L2, the NIST result for 25(OH)D_{Total} is the sum of the certified values for 25(OH)D_3 and 25(OH)D_2, and the 95% confidence limit (U_{95}) incorporates the uncertainties for the two analytes. For SRM 968d L1, the NIST value for 25(OH)D_3 was obtained using an LC-MS/MS reference measurement procedure[b] 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 SRM 968d L1 and was not included in the results for 25(OH)D_{Total}.

[a] https://www-s.nist.gov/srmors/view_cert.cfm?srm=1950
[b] 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 1950, SRM 972a L2, and SRM 968d L1.

		SRM 1950	SRM 972a L2	SRM 968d L1	SRM 972a L2	SRM 972a L2 Combined		
Lab	Method	Vial A	Vial B	Vial C	Vial D	Mean	SD	%RSD
017	CLIA	24.5	19.2	13.7	17.9	18.5	0.9	5.0
026	LC-MS/MS	29.1	20.6	13.9	21.1	20.9	0.4	1.7
056	LC-MS/MS	25.3	19.2	13.1	19.7	19.5	0.4	1.8
060	LC-MS/MS	33.9	22.4	15.1	22.1	22.3	0.2	1.0
062	RIA	28.7	20.6	13.3	21.2	20.9	0.4	2.0
086a	CLIA	32.9	21.6	14.5	23.3	22.5	1.2	5.4
086b	RIA	33.0	24.0	17.0	28.0	26.0	2.8	11
110	LC-UV	24.4	19.9	38.9	17.9	18.9	1.4	7.5
116	LC-MS/MS	26.1	18.2	13.5	18.4	18.3	0.2	1.0
119	LC-MS	25.5	18.0	12.9	17.8	17.9	0.1	0.8
124	LC-MS/MS	26.3	18.7	15.5	20.3	19.5	1.1	5.8
139	LC-UV	29.6	23.0	22.1	21.8	22.4	0.8	3.8
160a	LC-MS/MS	25.3	18.6	12.0	18.3	18.5	0.2	1.1
161	CLIA	26.7	19.9	17.0	18.5	19.2	1.0	5.2
180	RIA	25.1	16.1	12.1	17.6	16.9	1.1	6.5
184	LC-MS/MS	28.7	20.1	13.6	17.5	18.8	1.8	9.8
185a	LC-MS/MS	27.0	19.3	14.7	32.8	26.1	9.5	37
185b	CLIA	33.3	21.5	13.8	20.2	20.9	0.9	4.4
186	LC-MS/MS	13.0	8.0	7.0	12.0	10.0	2.8	28
188	CLIA	31.5	21.8	16.2	19.1	20.5	1.9	9.3
189	LC-UV	30.9	28.4	10.0	26.4	27.4	1.4	5.2
191	RIA	27.5	18.5	14.4	19.0	18.8	0.3	1.8
194	LC-MS/MS	26.5	20.7	11.4	17.4	19.1	2.3	12
195	LC-MS/MS	26.3	18.7	11.8	18.3	18.5	0.3	1.5
196	CLIA	28.0	18.5	15.3	19.4	19.0	0.6	3.4
197	LC-MS/MS	28.0	19.0	14.0	19.0	19.0	0.0	0.0
198a	LC-MS/MS	29.9	20.7	14.4	20.5	20.6	0.1	0.7
198b	EIA	31.6	20.3	15.3	20.2	20.3	0.1	0.3
199	LC-MS/MS	23.0	20.3	13.1	18.7	19.5	1.1	5.8
200	RIA	25.8	18.9	14.6	18.8	18.9	0.1	0.4
201	EIA	33.4	21.7	16.0	22.8	22.3	0.8	3.5
202	LC-MS/MS	27.2	21.9	14.1	20.6	21.3	0.9	4.3
209	LC-MS/MS	26.7	20.6	13.7	17.8	19.2	2.0	10
210a	RIA	26.8	22.1	15.1	19.6	20.8	1.8	8.7
210b	CLIA	30.4	18.9	15.1	20.2	19.6	0.9	4.7
211	LC-MS/MS	26.9	18.7	13.1	18.7	18.7	0.0	0.0
212	LC-MS/MS	30.8	22.8	14.5	21.0	21.9	1.3	5.8
215	LC-MS/MS	24.0	20.4	12.0	20.0	20.2	0.3	1.4
216	LC-MS/MS	33.5	25.6	17.9	26.7	26.2	0.8	3.0
217	LC-MS/MS	24.8	19.6	13.6	19.2	19.4	0.3	1.5
218a	CLIA	26.4	17.7	14.6	17.2	17.5	0.4	2.0
218b	LC-MS/MS	27.2	24.9	18.3	27.2	26.1	1.6	6.2
219	LC-MS/MS	26.2	19.8	13.4	19.5	19.7	0.2	1.1
220	LC-MS/MS	28.0	22.0	15.0	21.0	21.5	0.7	3.3
221a	LC-MS/MS	26.1	17.5	12.8	19.8	18.7	1.6	8.7
221b	LC-UV	25.3	15.9	51.0	20.6	18.3	3.3	18
223	LC-MS/MS	24.8	18.6	13.3	18.3	18.5	0.2	1.1
225	LC-MS/MS	32.4	24.8	20.1	21.1	23.0	2.6	11.4
228a	LC-MS/MS	28.8	24.6	15.6	27.1	25.9	1.8	6.8
231	LC-UV	30.6	20.8	45.7	21.0	20.9	0.1	0.7
234	LC-MS/MS	25.2	18.9	13.7	18.0	18.5	0.6	3.4
236	CLIA	27.8	17.4	12.5	17.9	17.7	0.4	2.0
241	LC-MS/MS	25.8	19.5	12.5	19.2	19.4	0.2	1.1
242	LC-MS/MS	28.3	20.5	14.5	20.6	20.6	0.1	0.3
243	LC-UV	28.8	21.2	14.8	21.5	21.4	0.2	1.0
244	LC-MS/MS	27.0	18.0	14.0	18.0	18.0	0.0	0.0
245	LC-UV	38.5	33.2	15.9	27.2	30.2	4.2	13.9
All methods	N	57	57	57	57	57		
	Median	27.2	20.1	14.4	19.7	19.5		
	MADe	2.8	2.2	1.6	2.1	1.8		
	CV%	10	11	11	11	9.3		
IA methods	N	17	17	17	17	17		
	Median	28.0	19.9	14.6	19.4	19.6		
	MADe	3.6	2.4	1.2	1.3	1.9		
	CV%	13	12	8	6.9	9.8		
LC methods	N	40	40	40	40	40		
	Median	27.0	20.2	14.0	19.9	19.5		
	MADe	2.5	2.2	1.6	2.3	1.7		
	CV%	9.4	11	11	12	8.8		
LC-MS^n	N	33	33	33	33	33		
	Median	26.7	19.8	13.7	19.5	19.5		
	MADe	2.1	1.6	1.2	1.8	1.5		
	CV%	7.8	8.2	8.7	9.1	7.6		
	NIST Value	25.3	18.9	12.4	18.9	18.9		
	U_{95}	0.8	0.4	0.3	0.4	0.4		

For all participant datasets, the single reported values for 25(OH)D_{Total} in SRM 1950 and SRM 968d L1, and the average reported values (± 2 SD) for SRM 972a L2 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: CLIA, EIA, RIA, 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 the consensus variability (2 × MADe).

For the IA data for material SRM 1950, 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 (**Figure 2**). 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 SRM 1950.

For the LC datasets for SRM 1950 and for both the LC and IA datasets for SRM 972a L2 and 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 1950

- 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, 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 and overlaps the IA data range

SRM 972a L2

- For the IA results, all but one dataset are within the consensus variability range when the average results are considered.
- For the LC results, all but seven datasets are within the consensus variability range when the average results are considered.
- The consensus median values are comparable for both the IA results and LC results and are slightly 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 968d L1

- For the IA results, all datasets are within the consensus variability range.
- For the LC results, nine datasets are outside of the consensus variability range (four LC-MS^n, five LC-UV).
- 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 and overlaps the consensus variability range for IA.

Overall, the results for the three study materials are consistent, with the majority of the participant values higher than the NIST value. In addition, the consensus variability is similar but relatively high for the three materials, ranging from 9.3% to 11% when all methods are considered (**Table 2**). Similar trends have also been observed for many of the study materials evaluated in previous studies of the 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 detected in all study materials but quantitated in SRM 972a L2 only (1.29 ng/mL ± 0.06 ng/mL). 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 that ranged from ≈ 3% to ≈ 6% of 25(OH)D_{Total} 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 2. Plots of the single reported values for 25(OH)D_{Total} in SRM 1950 and SRM 968d L1, and the average reported values (± 2 SD) for SRM 972a L2 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.

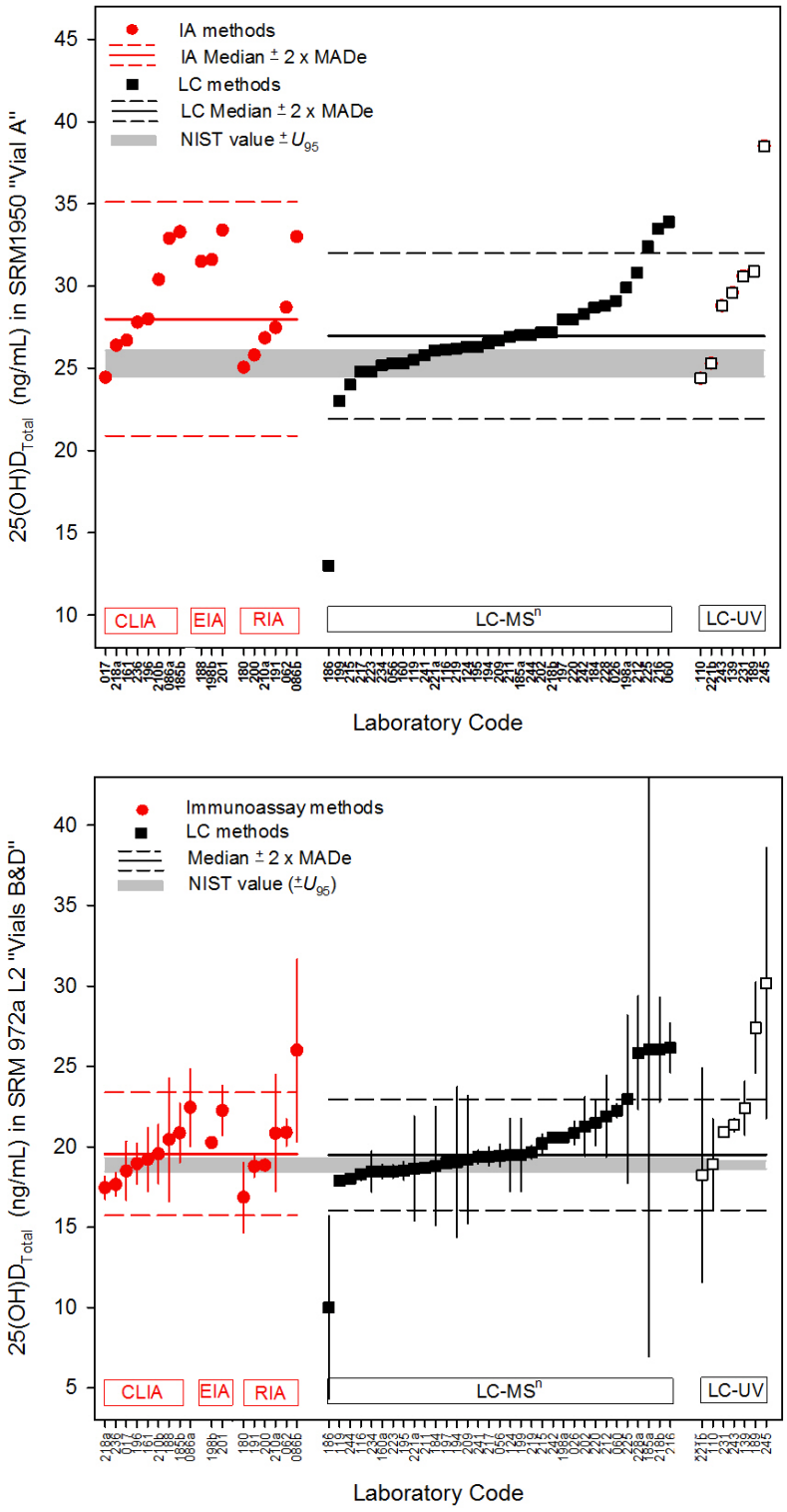


Figure 2 (cont'd). Plots of the single reported values for 25(OH)D_{Total} in SRM 1950 and SRM 968d L1, and the average reported values (± 2 SD) for SRM 972a L2 as determined by immunoassay (CLIA, EIA and RIA) and LC (LC-MS^n and LC-UV) methods. The grey-shaded bars represent the ranges bound by the NIST values with ± estimated U_{95} uncertainty.

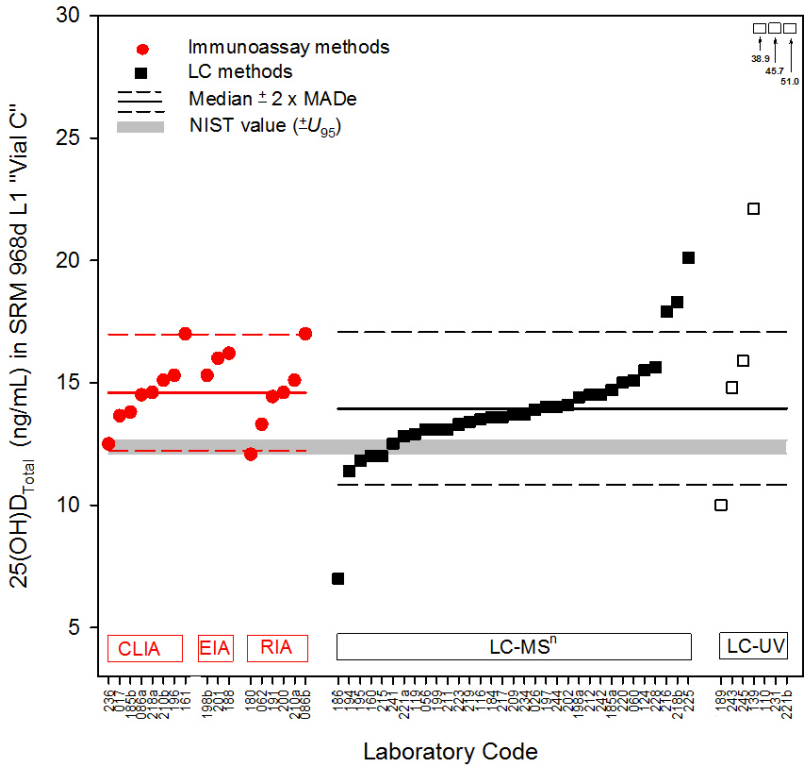


Figure 3 is a direct graphical comparison of the 25(OH)D results for the duplicate samples of SRM 972a L2 (vial B and vial D). For the first Youden plot (Panel A), there are two blue consensus boxes, one for IA methods and one for LC methods (as indicated). Laboratory results that are within the consensus 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 (numbers 186, 180, 225, 245, 189, 216, 218b, 228, 086b and 185a). The NIST value for this material (18.9 ng/mL) is 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- and x-axis, respectively. The Youden line runs through both the IA and LC consensus boxes for these materials.

For the second Youden plot (Panel B), the results for SRM 972a L2 are evaluated with respect to a 10% range relative to the NIST value for this material (18.9 ng/mL). Laboratory results that fall outside of this range are indicative of non-repeatable measurement performance for this material and are highlighted with their laboratory code numbers (numbers 186, 221b, 194, 209, 184, 225, 245, 086b and 185a). In general, the combined results for vial B and vial D from these laboratories had a relative standard deviation ≥ 10% (**Table 2**). The relative distance of the individual laboratory results from the Youden line (y=x) is also indicative of the relative level of imprecision between the two results.

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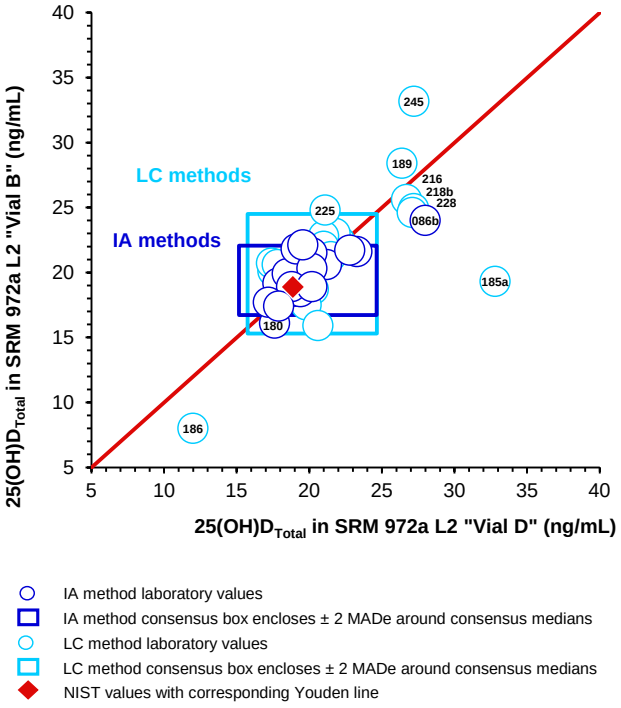
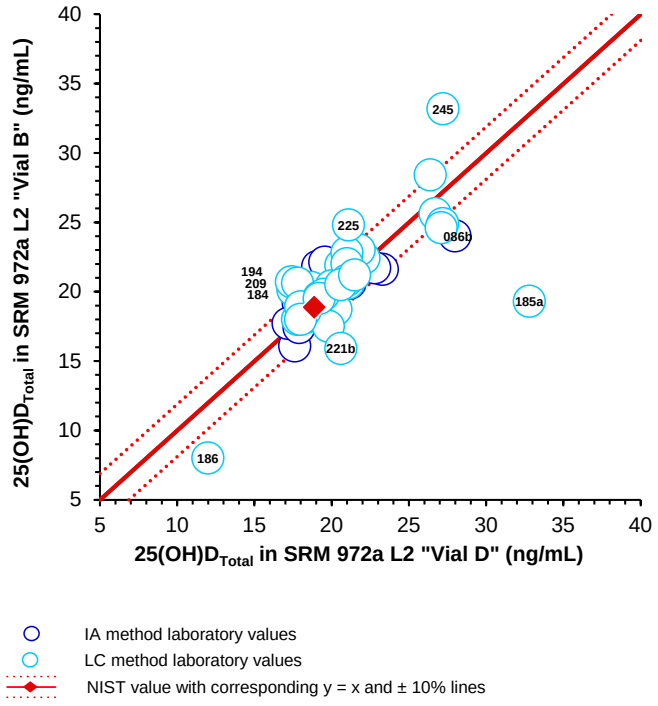


Figure 3. Youden comparison plot of the results for 25(OH)D_{Total} in SRM 972a L2 "Vial B" and "Vial D" for all methods

Panel A: Data that fall outside the consensus boxes are labeled with their laboratory number.



Panel B: Data for the two replicates that are more than 10% discrepant are labeled with their laboratory number.

Correlation of 25(OH)D in SRM 1950, SRM 972a L2, and W012-3 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 single reported values for $25(OH)D_{Total}$ in SRM 1950 and SRM 968d L1 and the mean values with error bars (representing the lab mean value ± 2 × SD) for $25(OH)D_{Total}$ in SRM 972a L2 overlaid with the clinical ranges from **Table 3** are presented in **Figure 4**. Specific results as assessed from **Figure 4** are summarized below:

SRM 1950

- All but one of the participant results are in the adequate 25(OH)D concentration range.
- The NIST value (25.3 ng/mL ± 0.8 ng/mL) is in the adequate 25(OH)D concentration range.

SRM 972a L2

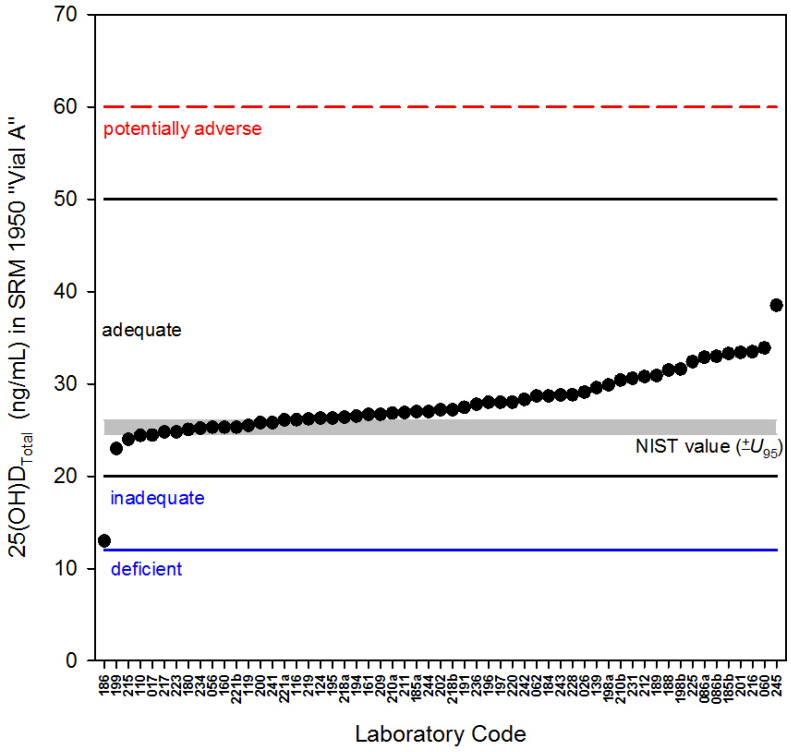
- The participant results are almost equally split between the inadequate and adequate 25(OH)D concentration ranges.
- The NIST value (18.9 ng/mL ± 0.4 ng/mL) is in the inadequate 25(OH)D concentration range.

SRM 968d L1

- The range of participant results for SRM 968d L1 is larger than for the other materials.
- The majority of participant results are in the inadequate 25(OH)D concentration range, but several also reported deficient and adequate concentration values.
- 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 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 4. 25(OH)D_{Total} levels in SRM 1950, SRM 972a L2, 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. The error bars represent 2 × SD of the duplicate results.



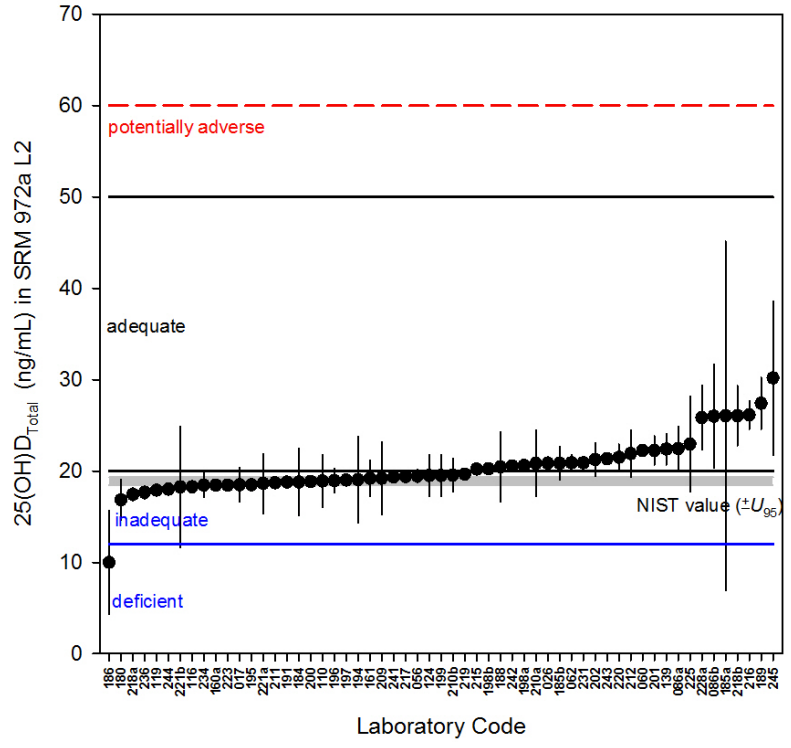
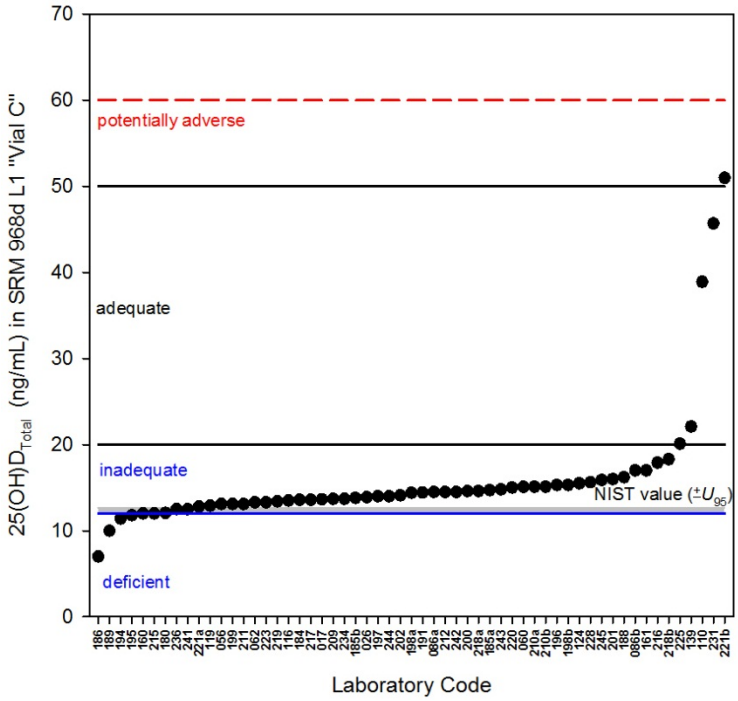


Figure 4 (cont'd) 25(OH)D_{Total} levels in SRM 1950, SRM 972a L2, and SRM 968d L1 superimposed over clinically-relevant serum 25-hydroxyvitamin D (25(OH)D_{Total}) concentration levels as reported by NIH (Table 3). The grey-shaded bars represent the ranges bound by the NIST values with ± estimated U_{95} uncertainty.



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

Laboratory Number	*IA Method*	*Sample Preparation*	*Detection*
17	CLIA	n/r	n/r
62	RIA	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
180	RIA	Samples prepared per manufacturer's instructions	I^{125} detection
185b	CLIA	n/r	n/r
188	EIA	None	n/r
191	RIA	Samples were prepared as per kit protocol	I^{125} detection using Gamma counter
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
200	RIA	Sample was extracted	n/r
201	EIA	n/r	n/r
210a	RIA	Sample was extracted with acetonitrile	n/r
210b	CLIA	n/r	n/r
218a	CLIA	Direct analysis	n/r
236	CLIA	Centrifuged	n/r

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
60	25(OH)D_3 -d_6	Serum proteins were precipitated with acetonitrile containing the IS, followed by centrifugation and injection of the supernatant	n/r	25(OH)D_3 383/211; 25(OH)D_3-d_6 389/211; 25(OH)D_2 395/269
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
119	25(OH)D_3 -d_6	IS was added and serum (150 µL) proteins were precipitiated with methanol (150 µL), followed by hexane extraction (1.5 mL), evaporation, and reconstitution with methanol (150 µL)	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 25(OH)D_3 401.33824; 25(OH)D_3-d_6 407.37907; 25(OH)D_2 413.33929
124	25(OH)D_2-d_6 and 25(OH)D_3-d_6	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
160	25(OH)D_2-d_6 and 25(OH)D_3-d_6	IS added to sample, followed by centrifugation, evaporation, reconstitution, mixing, and centrifugation (all using filter plate)	C18 column (50 x 2.1 mm); gradient with methanol/water; flow 0.7 mL/min	MS/MS at *m/z* 413.3/337.3
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 mM 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
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
186	25(OH)D_3 -d_6	Sample was deproteinized in acetone, extracted with 3 volumes of hexanes, evaporated, and reconstituted in methanol/water	C18 column (50 x 2.1 mm; 1.7µm)	25(OH)D_3 401/383 (quant), 401/159 (qual); 25(OH)D_3-d_6 407/159
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
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	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 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 $ZnSO_4$ 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	Extraction with acetonitrile containing IS followed by centrifugation	Column (33 x 4.6 mm; 3 µm)	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 IS	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_2 413/88 (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	Samples were protein crashed in conjunction with internal standard addition, vortexed, centrifuged	Automated 2-dimensional 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); 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	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
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
223	25(OH)D_2-d_3 and 25(OH)D_3-d_6	Protein precipitation with methanol followed by liquid-liquid extraction with cyclohexane:ethyl acetate (9:1); supernatant evaporated then derivatized with 4-phenyl-1,2,4-triazoline-3,5-dione in acetonitrile	C18 column (100 x 2.1 mm; 1.7 µm); gradient with water, acetonitrile, 0.01% formic acid	25(OH)D_3 558/298; 25(OH)D_3-d_6 564/298; 25(OH)D_2 570/298; 25(OH)D_2-d_3 573/301

225	25(OH)D_3-d_6	Extracted with hexane	C8 column (50 x 2.1 mm; 1.7 µm); gradient with water/methanol; flow 0.4 mL/min	n/r
228	25(OH)D_3-d_6	Proteins precipitated with isopropanol:acetonitrile:methanol (200:720:80), followed by centrifugation and removal of supernatant	C18 (30 x 3.0 mm); gradient with water (ammonium acetate/formic acid) amd methanol; flow 0.8 mL/min	25(OH)D_3 401; 25(OH)D_2 413
234	25(OH)D_3-d_6	The samples are protein crashed using acetonitrile and separated from the protein	A turbo column is used for cleanup followed by a C18 analytical column; water and methanol mobile phase	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

MRM = multiple reaction monitoring
quant = quantitative ions
qual = qualitative ions
PFP = pentafluorophenyl
CN = cyano
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*
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	LC 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	1-alpha(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
245	Proprietary	Precipant added to sample, followed by addition of IS, mixing, and centrifugation.	Flow 1 mL/min	264 nm

n/r = not reported

CN = cyano

Appendix B. Raw participant data and NIST results for $25(OH)D_2$, $25(OH)D_3$ and $25(OH)D_{Total}$ in SRM 1950, SRM 972a L2, 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 1950	SRM 972a L2	SRM 968d L1	SRM 972a L2	SRM 1950	SRM 972a L2	SRM 968d L1	SRM 972a L2	SRM 1950	SRM 972a L2	SRM 968d L1	SRM 972a 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	24.5	19.2	13.7	17.9	n/r	n/r
026	LC-MS/MS	<1.0	1.0	<1.0	<1.0	29.1	19.6	13.9	21.1	29.1	20.6	13.9	21.1	n/r	n/r
056	LC-MS/MS	0.5	0.7	n/d	0.9	24.9	18.5	13.1	18.8	25.3	19.2	13.1	19.7	230.6	342.4
060	LC-MS/MS	< 2	< 2	< 2	< 2	33.9	22.4	15.1	22.1	33.9	22.4	15.1	22.1	n/r	372.0
062	RIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	28.7	20.6	13.3	21.2	n/r	323.1
086a	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	32.9	21.6	14.5	23.3	n/r	n/r
086b	RIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	33.0	24.0	17.0	28.0	n/r	n/r
110	LC-UV	<4	<4	<4	<4	22.2	18.1	34.7	16.1	24.4	19.9	38.9	17.9	237.1	334.1
116	LC-MS/MS	< 3.3	< 3.3	< 3.3	< 3.3	26.1	18.2	13.5	18.4	26.1	18.2	13.5	18.4	245.1	334.6
119	LC-MS	n/d	n/d	n/d	n/d	25.5	18.0	12.9	17.8	25.5	18.0	12.9	17.8	182.4	343.4
124	LC-MS/MS	<4.0	<4.0	<4.0	<4.0	26.3	18.7	15.5	20.3	26.3	18.7	15.5	20.3	47.0	123.0
139	LC-UV	n/a	n/a	n/a	n/a	29.6	23.0	22.1	21.8	29.6	23.0	22.1	21.8	239.7	328.5
160a	LC-MS/MS	<1.0	<1.0	<1.0	<1.0	25.3	18.6	12.0	18.3	25.3	18.6	12.0	18.3	170.0	281.0
161	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	26.7	19.9	17.0	18.5	n/r	n/r
180	RIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	25.1	16.1	12.1	17.6	n/r	n/r
184	LC-MS/MS	<1.0	<1.0	<1.0	<1.0	28.7	20.1	13.6	17.5	28.7	20.1	13.6	17.5	240.5	336.5
185a	LC-MS/MS	n/d	n/d	n/d	n/d	27.0	19.3	14.7	32.8	27.0	19.3	14.7	32.8	238.6	334.8
185b	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	33.3	21.5	13.8	20.2	n/r	n/r
186	LC-MS/MS	n/d	n/d	n/d	n/d	13.0	8.0	7.0	12.0	13.0	8.0	7.0	12.0	238.0	335.0
188	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	31.5	21.8	16.2	19.1	n/r	n/r
189	LC-UV	n/d	n/d	n/d	n/d	30.9	28.4	10.0	26.4	30.9	28.4	10.0	26.4	n/r	n/r
191	RIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	27.5	18.5	14.4	19.0	n/r	n/r
194	LC-MS/MS	<7.0	<7.0	<7.0	<7.0	26.5	20.7	11.4	17.4	26.5	20.7	11.4	17.4	240.5	330.5
195	LC-MS/MS	n/d	n/d	n/d	n/d	26.3	18.7	11.8	18.3	26.3	18.7	11.8	18.3	241.0	333.0
196	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	28.0	18.5	15.3	19.4	198.6	298.8
197	LC-MS/MS	<5	<5	<5	<5	28.0	19.0	14.0	19.0	28.0	19.0	14.0	19.0	234.0	336.0
198a	LC-MS/MS	<5.0	<5.0	<5.0	<5.0	29.9	20.7	14.4	20.5	29.9	20.7	14.4	20.5	207.4	327.1
198b	EIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	31.6	20.3	15.3	20.2	n/r	n/r
199	LC-MS/MS	<2	<2	<2	<2	23.0	20.2	13.1	18.7	23.0	20.3	13.1	18.7	245.0	349.0
200	RIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	25.8	18.9	14.6	18.8	278.1	386.9
201	EIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	33.4	21.7	16.0	22.8	n/r	n/r
202	LC-MS/MS	n/d	n/d	n/d	n/d	27.2	21.9	14.1	20.6	27.2	21.9	14.1	20.6	241.5	339.0
209	LC-MS/MS	<1.0	<1.0	<1.0	<1.0	26.7	20.6	13.7	17.8	26.7	20.6	13.7	17.8	248.7	336.7
210a	RIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	26.8	22.1	15.1	19.6	230.0	294.3
210b	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	30.4	18.9	15.1	20.2	n/r	n/r
211	LC-MS/MS	n/d	n/d	n/d	n/d	26.9	18.7	13.1	18.7	26.9	18.7	13.1	18.7	240.4	328.1
212	LC-MS/MS	n/d	<4	n/d	<4	30.8	22.8	14.5	21.0	30.8	22.8	14.5	21.0	231.5	330.9
215	LC-MS/MS	n/d	0.4	n/d	0.4	24.0	20.0	12.0	19.6	24.0	20.4	12.0	20.0	n/r	n/r
216	LC-MS/MS	0.4	0.7	0.2	1.0	33.1	24.9	17.7	25.7	33.5	25.6	17.9	26.7	258.0	333.0
217	LC-MS/MS	< 2	< 2	< 2	< 2	24.8	19.6	13.6	19.2	24.8	19.6	13.6	19.2	233.8	358.1
218a	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	26.4	17.7	14.6	17.2	n/r	n/r
218b	LC-MS/MS	n/d	n/d	n/d	n/d	27.2	24.9	18.3	27.2	27.2	24.9	18.3	27.2	240.9	333.9
219	LC-MS/MS	<4.0	<4.0	<4.0	<4.0	26.2	19.8	13.4	19.5	26.2	19.8	13.4	19.5	n/r	n/r
220	LC-MS/MS	<5.0	<5.0	<5.0	<5.0	28.0	22.0	15.0	21.0	28.0	22.0	15.0	21.0	n/r	n/r
221a	LC-MS/MS	n/d	n/d	n/d	n/d	26.1	17.5	12.8	19.8	26.1	17.5	12.8	19.8	249.7	357.8
221b	LC-UV	n/d	n/d	n/d	n/d	25.3	15.9	51.0	20.6	25.3	15.9	51.0	20.6	n/r	n/r
223	LC-MS/MS	<5	<5	<5	<5	24.8	18.6	13.3	18.3	24.8	18.6	13.3	18.3	241.2	313.2
225	LC-MS/MS	<5.0	<5.0	<5.0	<5.0	32.4	24.8	20.1	21.1	32.4	24.8	20.1	21.1	235.9	284.1
228a	LC-MS/MS	0.8	1.5	0.4	1.7	28.1	23.1	15.2	25.4	28.8	24.6	15.6	27.1	240.0	334.0
231	LC-UV	n/d	n/d	33.0	n/d	30.6	20.8	12.7	21.0	30.6	20.8	45.7	21.0	268.7	287.3
234	LC-MS/MS	<3	<3	<3	<3	25.2	18.9	13.7	18.0	25.2	18.9	13.7	18.0	246.0	300.0
236	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	27.8	17.4	12.5	17.9	n/r	n/r
241	LC-MS/MS	0.4	0.9	n/d	0.9	25.4	18.6	12.5	18.3	25.8	19.5	12.5	19.2	212.0	302.5
242	LC-MS/MS	n/d	n/d	n/d	n/d	28.3	20.5	14.5	20.6	28.3	20.5	14.5	20.6	245.0	332.0
243	LC-UV	n/d	n/d	n/d	n/d	28.8	21.2	14.8	21.5	28.8	21.2	14.8	21.5	245.9	325.6
244	LC-MS/MS	<5	<5	<5	<5	27.0	18.0	14.0	18.0	27.0	18.0	14.0	18.0	222.0	337.0
245	LC-UV	n/d	n/d	n/d	n/d	38.5	33.2	15.9	27.2	38.5	33.2	15.9	27.2	240.8	335.5
	NIST Value	0.52	0.81	<0.5	0.81	24.8	18.1	12.4	18.1	25.3	18.9	12.4	18.9	238.6	334.8
	U_{95}	0.17	0.06	0.00	0.06	0.8	0.4	0.3	0.4	0.8	0.4	0.3	0.4	3.9	5.2

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