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NIST/NIH Vitamin D Metabolites Quality Assurance Program Report of Participant Results: Winter 2011 Comparability Study (Exercise 3)

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National Institute of Standards and Technology
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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 third exercise of this program, the Winter 2011 Comparability Study, were asked to use the methodology of their choice to measure concentrations of 25-hydroxyvitamin D in control and study materials distributed by NIST. The study materials consisted of SRM 972 Vitamin D in Human Serum (Level 3) and SRM 968d Fat-Soluble Vitamins, Carotenoids and Cholesterol in Human Serum (Level 1). SRM 2972, which is comprised of separate ethanolic calibration solutions with known concentrations of $25(OH)D_2$ and $25(OH)D_3$, was provided as a control material. Participants provided their data to NIST, where it was compiled and evaluated for trueness relative to the NIST value, within-laboratory precision, and concordance within the participant community. A report of results was provided to all participants of the exercise, and laboratories were identified by code numbers known only to them. The results from this third exercise are reported along with a summary of the analytical methods used.

OVERVIEW OF THE WINTER 2011 COMPARABILITY STUDY

For the Winter 2011 Comparability Study (Exercise 3) of the NIST/NIH Vitamin D Metabolites Quality Assurance Program (VitDQAP), control and human serum study samples were distributed to participants for evaluation. SRM 2972, which is comprised of separate ethanolic solutions with known concentrations of 25-hydroxyvitamin D_2 ($25(OH)D_2$) and 25-hydroxyvitamin D_3 ($25(OH)D_3$), was provided as a control material for assay calibration or verification. Participants were asked to provide single results for each of these solutions. In addition, participants were asked to determine $25(OH)D_2$, $25(OH)D_3$, and a total concentration of 25-hydroxyvitamin D ($25(OH)D_{Total} = 25(OH)D_2 + 25(OH)D_3$) in each of three samples (vials A, B, and C) of human serum (study materials). In this exercise, vial A was SRM 972 Vitamin D in Human Serum Level 3 (SRM 972 L3), which is a blended human serum pool with an endogenous $25(OH)D_3$ level but an augmented $25(OH)D_2$ level. Vials B and C were duplicate samples of SRM 968d Fat-Soluble Vitamins, Carotenoids and Cholesterol in Human Serum Level 1 (SRM 968d L1), which is a blended human serum pool that contains endogenous vitamin D metabolite levels.

In the Winter 2011 exercise, there were a total of 33 participants and 35 datasets (two participants provided data for two different methods). Fourteen of the datasets originated from immunoassay (IA) techniques, including four from enzyme immunoassay (EIA), five from chemiluminescence immunoassay (CLIA), and five from radioimmunoassay (RIA). **Appendix A-1** summarizes the immunoassay methods used by the participants. Twenty-one of the datasets originated from liquid chromatographic (LC) methods; of those, 16 were from LC with tandem mass spectrometric detection (LC-MS/MS) and five were from LC with ultraviolet absorbance detection (LC-UV). A summary of the LC methods used by the participants may be found in **Appendices A-2** and **A-3**.

The raw data received from all participants is summarized in **Appendix B.** For SRM 972 L3 and SRM 968d L1, the immunoassay methods reported values for $25(OH)D_{Total}$ only, whereas LC-based methods reported values for $25(OH)D_2$, $25(OH)D_3$ and $25(OH)D_{Total}$. The LC participant results reveal that SRM 972 L3 contains significant levels of both $25(OH)D_3$ and $25(OH)D_2$ that contribute to $25(OH)D_{Total}$. Conversely, SRM 968d L1 contains very low levels of $25(OH)D_2$ with only two the LC participants reporting values (ranging from 0.18 ng/mL to 1.80 ng/mL), but most labs indicated this analyte was below their quantitation limit of <1 ng/mL to <7 ng/mL. For the majority of the participants using LC, the $25(OH)D_{Total}$ data reported in **Appendix B** is the same as their reported data for $25(OH)D_3$ for SRM 968d L1. Both LC and immunoassay datasets provided individual values for $25(OH)D_2$ and $25(OH)D_3$ in the ethanolic controls because the analytes were in separate solutions.

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

WINTER 2011 EXERCISE RESULTS AND DISCUSSION

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

Participants were asked to analyze the control materials to qualify their assays prior to measuring the study materials. A summary of the individual participant data for 25(OH)D_2 and 25(OH)D_3 in the SRM 2972 control solutions is provided in **Table 1.**

The community results are summarized at the bottom of the table for all reported methods, the immunoassay methods only, the LC methods only, and the LC-MS/MS methods only. 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%). **Table 1** also presents the NIST certified values with expanded uncertainties corresponding to 95% confidence.

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

$25(OH)D_2$ (ng/mL)

		SRM 2972
Lab	Method	Value
030	RIA	252.5
032	LC-UV	261.0
056	LC-MS/MS	239.6
062	RIA	n/r
110	LC-UV	218.0
116	LC-MS/MS	236.8
139	LC-UV	262.2
150	LC-MS/MS	253.0
160b	CLIA	125.0
183a	LC-MS/MS	185.0
183b	CLIA	191.0
185	LC-MS/MS	238.6
194	LC-MS/MS	230.0
195	LC-MS/MS	235.0
196	CLIA	199.5
197	LC-MS/MS	205.0
198a	LC-MS/MS	250.7
199	LC-MS/MS	233.0
200	RIA	187.5
201	EIA	171.0
202	LC-MS/MS	228.2
205	LC-MS/MS	265.4
209	LC-MS/MS	237.6
210	RIA	142.9
211	LC-MS/MS	311.5
212	LC-MS/MS	242.3
All methods	N	25
	Median	235.0
	MADe	26.7
	CV%	11.4
IA methods	N	7
	Median	187.5
	MADe	24.5
	CV%	13.0
LC methods	N	18
	Median	238.1
	MADe	16.7
	CV%	7.0
LC-MS/MS	N	15
	Median	237.6
	MADe	11.3
	CV%	4.7
	NIST Value	238.6
	U_{95}	3.9

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

		SRM 2972
Lab	Method	Value
030	RIA	348.0
032	LC-UV	300.9
056	LC-MS/MS	333.4
062	RIA	329.8
110	LC-UV	326.0
116	LC-MS/MS	390.7
139	LC-UV	325.8
150	LC-MS/MS	398.0
160b	CLIA	225.0
183a	LC-MS/MS	329.0
183b	CLIA	262.0
185	LC-MS/MS	334.8
194	LC-MS/MS	336.5
195	LC-MS/MS	332.0
196	CLIA	477.5
197	LC-MS/MS	290.0
198a	LC-MS/MS	337.0
199	LC-MS/MS	331.0
200	RIA	280.6
201	EIA	341.0
202	LC-MS/MS	345.2
205	LC-MS/MS	337.4
209	LC-MS/MS	318.6
210	RIA	269.2
211	LC-MS/MS	423.0
212	LC-MS/MS	333.7
	N	26
	Median	332.7
	MADe	15.4
	CV%	4.6
	N	8
	Median	305.2
	MADe	58.5
	CV%	19.2
	N	18
	Median	333.6
	MADe	9.0
	CV%	2.7
	N	15
	Median	334.8
	MADe	5.6
	CV%	1.7
	NIST Value	334.0
	U_{95}	5.2

n/r = not reported

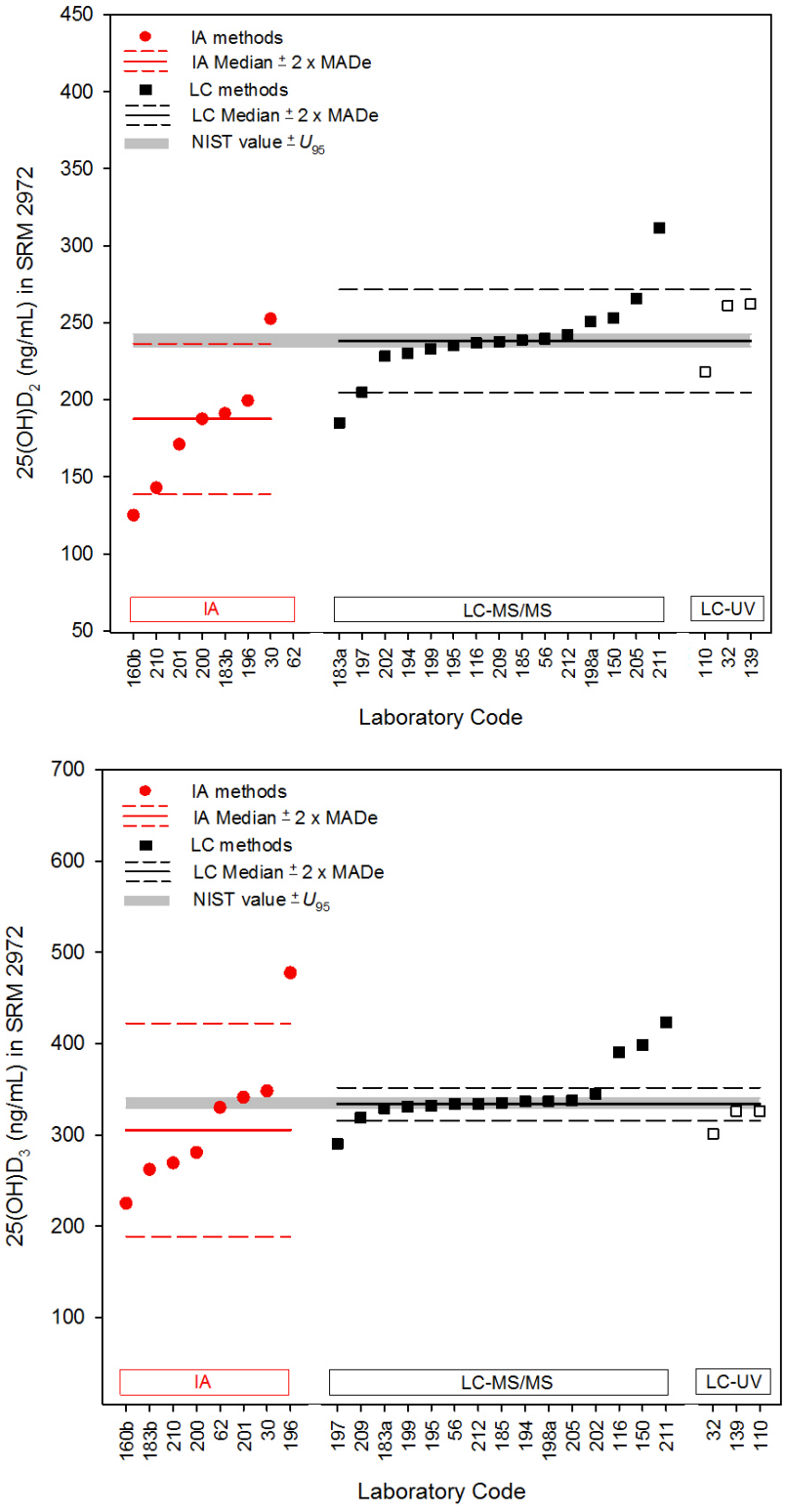
For all participant datasets, the single data values reported for 25(OH)D_2 and 25(OH)D_3 in the control solutions, SRM 2972, are plotted in **Figure 1**. The results from immunoassay methods are displayed with closed red circles (●). The results from the LC-based methods are displayed with black squares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

From the single reported values for all datasets for a given technique (IA or LC), the consensus median and the consensus variability (2 × MADe) were determined (reported in **Table 1**). Consensus statistics were not calculated for the UV results because of the limited number of datasets (N = 3). For each of the techniques within both graphs, the solid lines (——) represent the consensus median, and the dotted lines (- - - - -) represent the consensus variability (2 × MADe).

The laboratories with results that fall between the two dotted lines are within the consensus variability area for their technique (IA or LC). The graphs reveal that the consensus variability range for the participants who reported results using IA methods is quite large for both analytes. Several other IA participants reported that the calibration solutions were not compatible with their method and did not provide values. Overall, the control solutions appeared more compatible with the LC methods, which exhibited less consensus variability.

The NIST certified value is provided by a grey-shaded bar that represents the value and its associated uncertainty (± U_{95}); these "target" values were provided to participants in the reporting sheet.

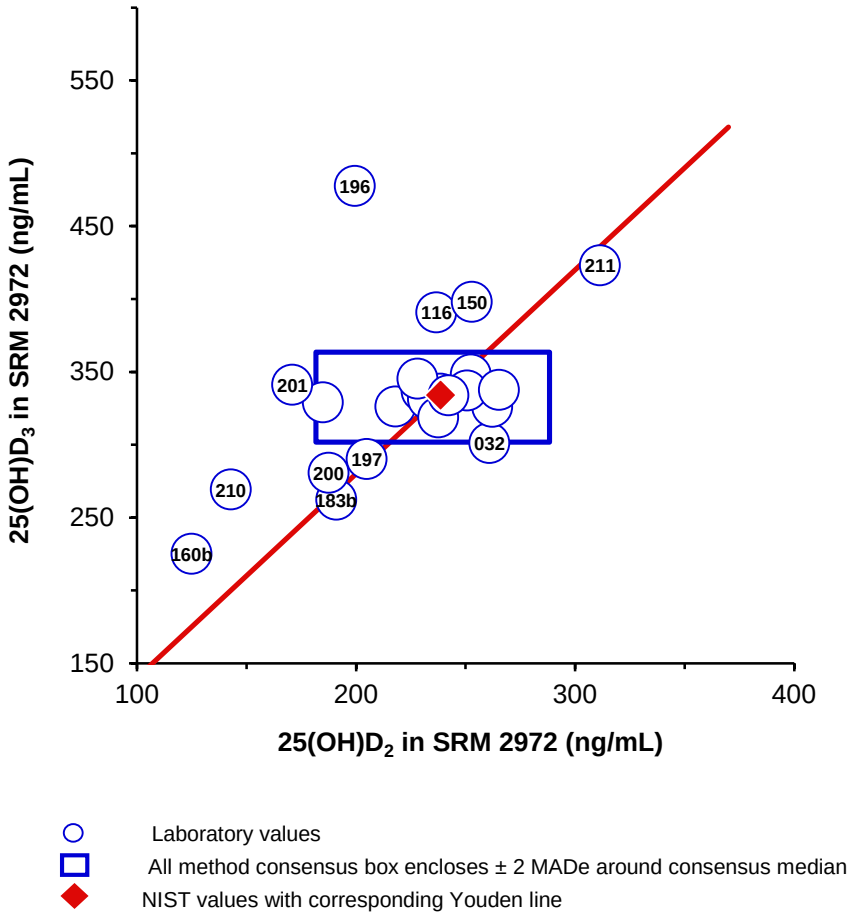
Figure 1. 25(OH)D_2 and 25(OH)D_3 values in SRM 2972 for immunoassay, LC-MS/MS and LC-UV methods. The grey-shaded bars represent the ranges bound by the NIST certified values with ± U_{95} expanded uncertainty.



A direct comparison of results for 25(OH)D_2 and 25(OH)D_3 in the SRM 2972 control solutions is provided in the Youden plot in **Figure 2**. Laboratory results that are within the consensus range for both the 25(OH)D_2 and 25(OH)D_3 ethanolic controls are within the blue consensus box in **Figure 2**. Conversely, laboratory results that fall outside of (or on the edge of) the blue consensus box are highlighted with their laboratory code numbers. The NIST values are denoted with a red diamond symbol (◆). The Youden line centered on the NIST values is illustrated by a red line (——) that represents the relative ratio of the NIST values (334.8/238.6) for 25(OH)D_3 and 25(OH)D_2 across the magnitude of the y- and x-axis, respectively.

Participant data (numbers 160b, 210, 211) that are near the Youden line but are clearly above or below the consensus box may suggest that these measurements are biased high or low due to a calibration error. However, correlation with the Youden line may be complicated for the control solutions because separate calibration solutions are likely prepared for measurement of 25(OH)D_2 and 25(OH)D_3, particularly for LC-based methods.

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



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

A summary of the individual participant data for $25(OH)D_{Total}$ in samples SRM 972 L3 and SRM 968d L1 (vial A and vials B & C, respectively) is provided in **Table 2.** The summarized data include the 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-MS/MS methods only. These summarized results include the total number of quantitative values reported, the median value, the MADe, and the CV%.

Table 2 also presents the NIST results for the two study materials. For SRM 972 L3, the NIST result is the sum of the certified values for $25(OH)D_3$ and $25(OH)D_2$, and the 95% confidence limit (U_{95}) was approximated using the individual uncertainties reported for the two analytes in the Certificate of Analysis.[1] For SRM 968d L1, the NIST value for $25(OH)D_3$ was obtained using an LC-MS/MS reference measurement procedure[2] recognized by the Joint Committee for Traceability in Laboratory Medicine (JCTLM), and the U_{95} confidence interval includes components for both measurement variability (N = 8) and measurement uncertainty associated with the density. The $25(OH)D_2$ was below the quantitation limit (≈ 0.5 ng/mL) in SRM 968d L1 for the NIST method.

[1] https://www-s.nist.gov/srmors/view_cert.cfm?srm=972
[2] Tai, S. S.-C., Bedner, M. and Phinney, K.W. *Anal. Chem.* **2010** *82*, 1942-1948.

Table 2. Summary of participant data for 25(OH)D_{Total} (ng/mL) in SRM 972 L3 and SRM 968d L1.

		SRM 972 L3	SRM 968d L1		SRM 968d L1 Combined		
Lab	Method	Vial A	Vial B	Vial C	Mean	SD	%rSD
017	CLIA	38.1	17.0	18.3	17.7	0.9	5.2
026	LC-MS/MS	43.7	12.4	12.5	12.5	0.1	0.6
030	RIA	41.8	19.5	18.5	19.0	0.7	3.7
032	LC-UV	49.6	11.1	10.8	11.0	0.2	1.9
056	LC-MS/MS	46.2	11.7	12.9	12.3	0.9	7.1
062	RIA	25.5	12.3	12.5	12.4	0.1	1.1
110	LC-UV	16.4	14.3	10.6	12.5	2.6	21
116	LC-MS/MS	47.0	12.2	13.3	12.8	0.8	6.1
139	LC-UV	44.7	13.7	12.6	13.1	0.8	6.0
150	LC-MS/MS	49.0	16.0	15.0	15.5	0.7	4.6
160b	CLIA	31.0	13.0	12.0	12.5	0.7	5.7
180	RIA	29.2	13.1	13.0	13.0	0.1	0.8
183a	LC-MS/MS	55.0	15.0	16.0	15.5	0.7	4.6
183b	CLIA	25.7	13.1	14.5	13.8	1.0	7.2
185	LC-MS/MS	49.3	12.6	12.7	12.6	0.0	0.2
188	CLIA	23.8	14.6	15.2	14.9	0.4	2.8
189	LC-UV	44.2	9.1	9.6	9.4	0.4	3.8
194	LC-MS/MS	46.4	14.7	12.0	13.4	1.9	14
195	LC-MS/MS	52.9	16.0	15.5	15.8	0.4	2.2
196	CLIA	34.8	16.2	16.9	16.6	0.5	3.0
197	LC-MS/MS	41.0	11.0	10.0	10.5	0.7	6.7
198a	LC-MS/MS	47.1	14.7	14.6	14.7	0.1	0.5
198b	EIA	33.0	15.0	16.0	15.5	0.7	4.6
199	LC-MS/MS	41.6	11.5	11.6	11.6	0.1	0.6
200	RIA	32.8	14.0	14.0	14.0	0.0	0.0
201	EIA	22.4	14.6	15.2	14.9	0.4	2.8
202	LC-MS/MS	53.0	14.6	14.3	14.5	0.2	1.5
205	LC-MS/MS	50.4	13.8	13.4	13.6	0.3	2.2
207	LC-UV	37.6	26.3	13.0	19.7	9.4	48
209	LC-MS/MS	39.2	14.4	13.8	14.1	0.4	3.0
210	RIA	34.1	12.3	15.3	13.8	2.1	15
211	LC-MS/MS	61.3	15.2	16.4	15.8	0.8	5.4
212	LC-MS/MS	46.7	13.9	13.7	13.8	0.1	1.0
213	EIA	26.0	17.6	14.6	16.1	2.1	13
214	CLIA	28.5	13.5	13.6	13.6	0.1	0.5
All methods	N	35	35	35	35		
	Median	41.6	14.0	13.7	13.8		
	MADe	11.5	1.8	1.9	2.0		
	CV%	27.5	13	14.1	14.5		
IA methods	N	14	14	14	14		
	Median	30.1	14.3	14.9	14.5		
	MADe	6.3	1.9	1.8	1.8		
	CV%	20.8	13	12.0	13		
LC methods	N	21	21	21	21		
	Median	46.7	13.9	13.0	13.4		
	MADe	4.4	1.9	1.9	1.6		
	CV%	9.5	13.9	14.8	12.2		
LC-MS/MS	N	16	16	16	16		
	Median	47.1	14.2	13.5	13.7		
	MADe	4.9	1.9	1.6	1.7		
	CV%	10.5	13.5	11.5	12.6		
	NIST Value	44.90	12.38	12.38	12.38		
	U_{95}	2.28	0.28	0.28	0.28		

For all participant datasets, the single reported values for 25(OH)D_{Total} in SRM 972 L3 and the mean values with error bars (representing the lab mean value ± 2 × SD) for 25(OH)D_{Total} in SRM 968d L1 are plotted in **Figure 3**. The results from immunoassay methods are displayed with closed red circles (●). The results from the LC-based methods are displayed with black squares and are segregated by MS/MS detection (■) and UV detection (□). For the IA and LC techniques within both graphs, the solid lines (——) represent the consensus median and the dotted lines (- - - - -) represent the consensus variability (2 × MADe).

The laboratories with results that fall between the two dotted lines are within the consensus variability area for their technique (IA, or LC). The NIST value for these materials is provided by a grey-shaded bar that represents the value and its associated uncertainty (±U_{95}).

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

SRM 972 L3

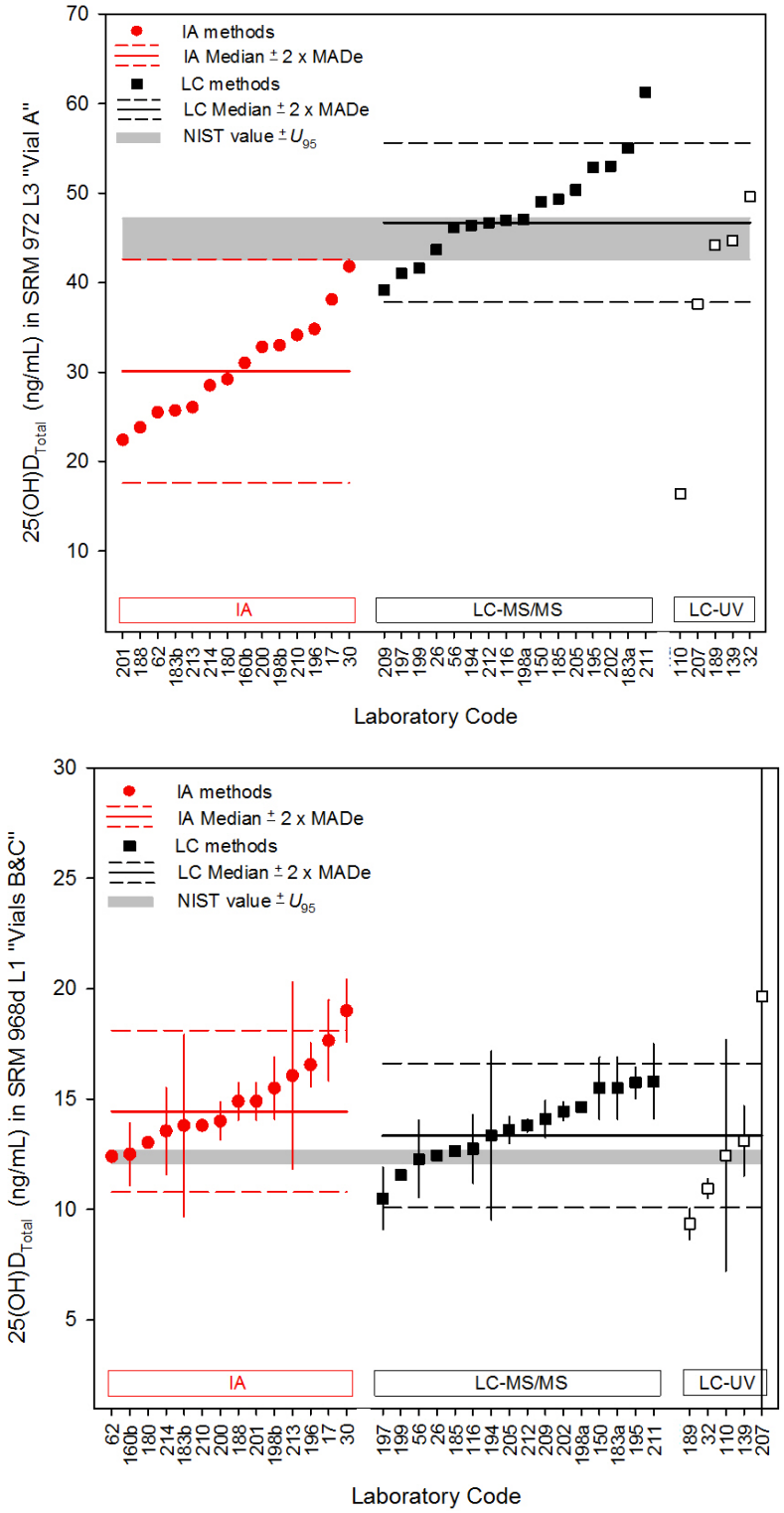
- For the immunoassay results, all laboratory data are within the consensus variability range.
- For the LC results, all but two datasets are within the consensus variability range.
- The consensus median value for the IA results is 36% lower than the median value for all LC methods.
- The consensus median value for the LC results falls within the NIST expanded uncertainty range (grey-shaded bar).
- There is no overlap between the NIST value and the consensus range for IA.

SRM 968d L1

- For both the IA and LC results, all but three of the mean value data points are within the consensus variability range.
- The consensus median for the IA results is 8% higher than the consensus median value for the LC results.
- Both the IA and LC consensus median values are higher than the NIST value.
- The NIST value is included within the consensus ranges for both techniques IA and LC.

The IA and LC techniques perform differently for SRM 972 L3, which contains native levels of 25(OH)D_3 but an augmented 25(OH)D_2 level. The augmented 25(OH)D_2 is not bound in the serum in the same manner as the native 25(OH)D_3 and 25(OH)D_2, which leads to an underrepresentation on the 25(OH)D_{Total} with the IA methods. The NIST value for SRM 972 L3 was obtained with a combination of LC-MS and LC-MS/MS methods, which explains the better agreement with the participant LC results.

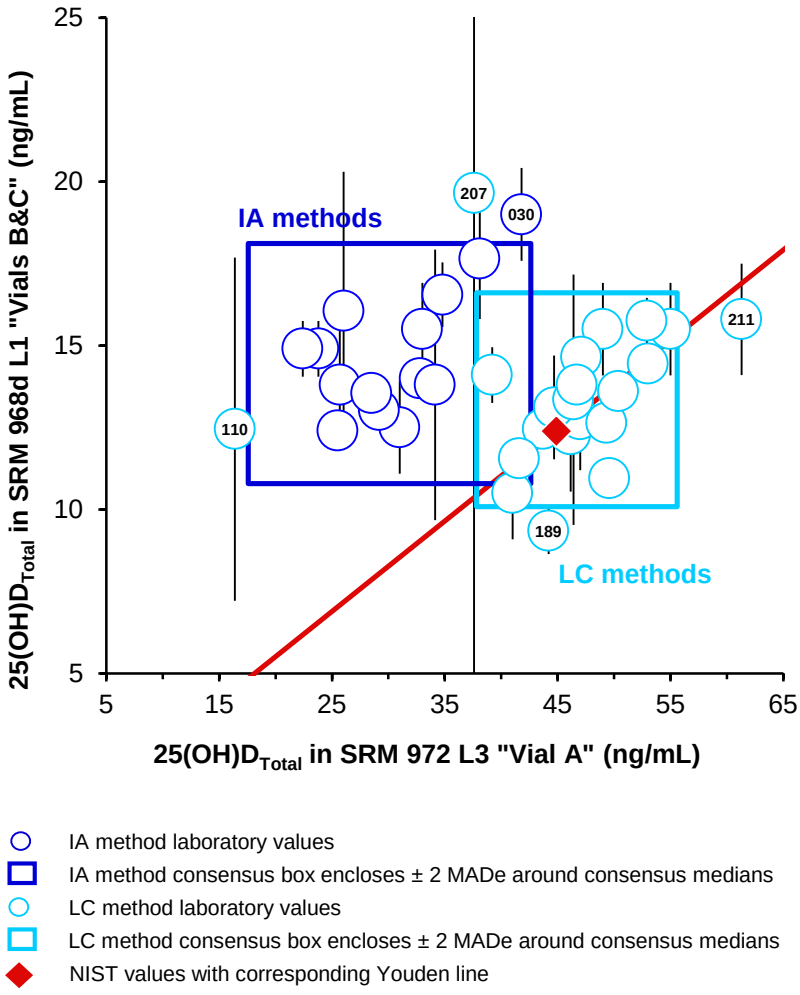
Figure 3. 25(OH)D_{Total} levels in SRM 972 L3 and SRM 968d L1 as determined by immunoassay, LC-MS/MS and LC-UV methods. The grey-shaded bars represent the ranges bound by the NIST values with ± estimated U_{95} uncertainty.



A direct comparison of results for 25(OH)D_{Total} between SRM 972 L3 and SRM 968d L1 is provided in the Youden plot in **Figure 4**. Because of the bimodal results obtained for SRM 972 L3, 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 ranges are not included in the blue consensus box and are highlighted with their laboratory code numbers (numbers 110, 207, 30, 189, 211).

The NIST values for these materials are denoted with a red diamond symbol (◆). The Youden line centered on the NIST values is illustrated by a red line (——), which represents the relative ratio of the NIST values (12.38/44.90) for SRM 968d L1 and SRM 972 L3 across the magnitude of the y- and x-axis, respectively. The Youden line runs directly through the LC consensus box because the NIST values were obtained with LC methods as previously mentioned.

Figure 4. Comparison of results for 25(OH)D_{Total} for all methods. Data that fall outside the consensus boxes are labeled with their laboratory number.

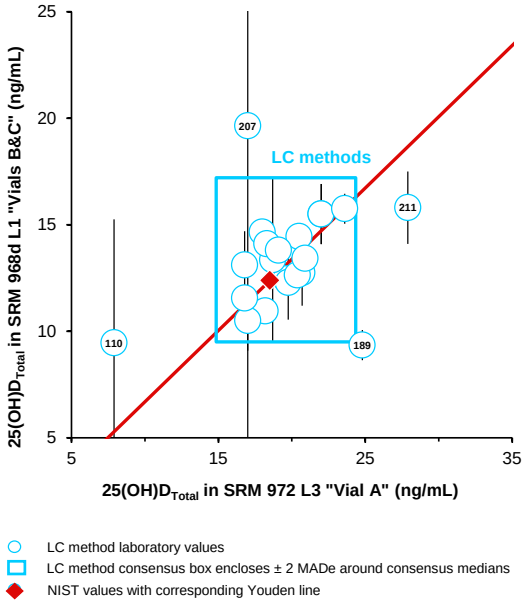


A summary of the individual participant data obtained with LC methods for 25(OH)D_3 in samples SRM 972 L3 and SRM 968d L1 (vial A and vials B & C, respectively) is provided in **Figure 5**, which also includes a Youden plot of the results. Laboratory results that are within the consensus range for both study materials are within the blue consensus box. Laboratory results that fall outside of (or on the edge of) the blue consensus box are highlighted with their laboratory code numbers, which includes three laboratories that use LC-UV (110, 189, 207) and one that uses LC-MS/MS (211). The NIST values are denoted with a red diamond symbol (◆). The Youden line centered on the NIST values is illustrated by a red line (——) that represents the relative ratio of the NIST values (12.38/18.50) for 25(OH)D_3 across the magnitude of the y- and x-axis, respectively.

It is notable that the NIST methods separate 25(OH)D_3 and its 3-epimer, 3-epi-25(OH)D_3, which was detected but not quantitated in either material. The 3-epi-25(OH)D_3 coelutes with 25(OH)D_3 using typical chromatographic columns (C8, C18) and is detected by the same multiple reaction monitoring (MRM) ions in MS/MS and absorbance wavelength in UV, leading to a potential bias for LC-based methods. One of the LC-MS/MS participants (number 56) noted using a method that separates 3-epi-25(OH)D_3 and provided values for this analyte in both study materials. However, the 25(OH)D_3 values reported by the rest of the LC participants 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 5. Summary of participant data obtained with LC methods for 25(OH)D_3 (ng/mL) in SRM 972 L3 and SRM 968d L1 and the resulting Youden comparison plot. Data that fall outside the consensus boxes are labeled with their laboratory number.

		SRM 972 L3	SRM 968d L1		SRM 968d L1 Combined		
Lab	Method	Vial A	Vial B	Vial C	Mean	SD	%rSD
026	LC-MS/MS	19.6	12.4	12.5	12.5	0.1	0.6
032	LC-UV	18.2	11.1	10.8	11.0	0.2	1.9
056	LC-MS/MS	19.8	11.7	12.9	12.3	0.9	7.1
110	LC-UV	7.9	11.5	7.4	9.5	2.9	31
116	LC-MS/MS	20.7	12.2	13.3	12.8	0.8	6.1
139	LC-UV	16.8	13.7	12.6	13.1	0.8	6.0
150	LC-MS/MS	22.0	16.0	15.0	15.5	0.7	4.6
183a	LC-MS/MS	22.0	15.0	16.0	15.5	0.7	4.6
185	LC-MS/MS	20.4	12.6	12.7	12.6	0.0	0.2
189	LC-UV	24.8	9.1	9.6	9.4	0.4	3.8
194	LC-MS/MS	18.7	14.7	12.0	13.4	1.9	14
195	LC-MS/MS	23.6	16.0	15.5	15.8	0.4	2.2
197	LC-MS/MS	17.0	11.0	10.0	10.5	0.7	6.7
198a	LC-MS/MS	18.0	14.7	14.6	14.7	0.1	0.5
199	LC-MS/MS	16.8	11.5	11.6	11.6	0.1	0.6
202	LC-MS/MS	20.5	14.6	14.3	14.5	0.2	1.5
205	LC-MS/MS	20.9	13.6	13.2	13.4	0.3	2.2
207	LC-UV	17.0	26.3	13.0	19.6	9.4	48
209	LC-MS/MS	18.3	14.4	13.8	14.1	0.4	3.0
211	LC-MS/MS	27.9	15.2	16.4	15.8	0.8	5.4
212	LC-MS/MS	19.1	13.9	13.7	13.8	0.1	1.0
LC methods	N	21	21	21	21		
	Median	19.6	13.7	13.0	13.4		
	MADe	2.4	2.2	1.9	1.9		
	CV%	12.1	15.9	14.8	14.4		
LC-MS/MS	N	16	16	16	16		
	Median	20.1	14.2	13.5	13.6		
	MADe	2.3	1.9	1.6	1.6		
	CV%	11.6	13.5	11.5	12.0		
	NIST Value	18.50	12.38	12.38	12.38		
	U_{95}	1.10	0.28	0.28	0.28		



Correlation of 25(OH)D in SRM 972 L3 and SRM 968d L1 with Clinical Ranges

As indicated in **Table 2**, the NIST values for $25(OH)D_{Total}$ in SRM 972 L3 and SRM 968d L1 are 44.90 ng/mL ± 2.28 ng/mL and 12.38 ng/mL ± 0.28 ng/mL, respectively. According to the current guidance regarding 25(OH)D levels and human health (obtained from the NIH website and presented in **Table 3**), the NIST data indicate that the concentration in SRM 972 L3 is consistent with "adequate" levels of vitamin D, but SRM 968d L1 is in the "inadequate" range. The median participant results (for all methods) of 41.6 ng/mL and 13.8 ng/mL for SRM 972 L3 and SRM 968d L1, respectively, also indicate that the concentrations in these materials are consistent with "adequate" and "inadequate" 25(OH)D, respectively. However, the range of 25(OH)D values reported by program participants of 16.4 ng/mL to 61.3 ng/mL and 9.4 ng/mL to 19.7 ng/mL for SRM 972 L3 and SRM 968d L1, respectively, resulted in an overall program CV% ranging from 15% to 28% (**Table 2**).

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)

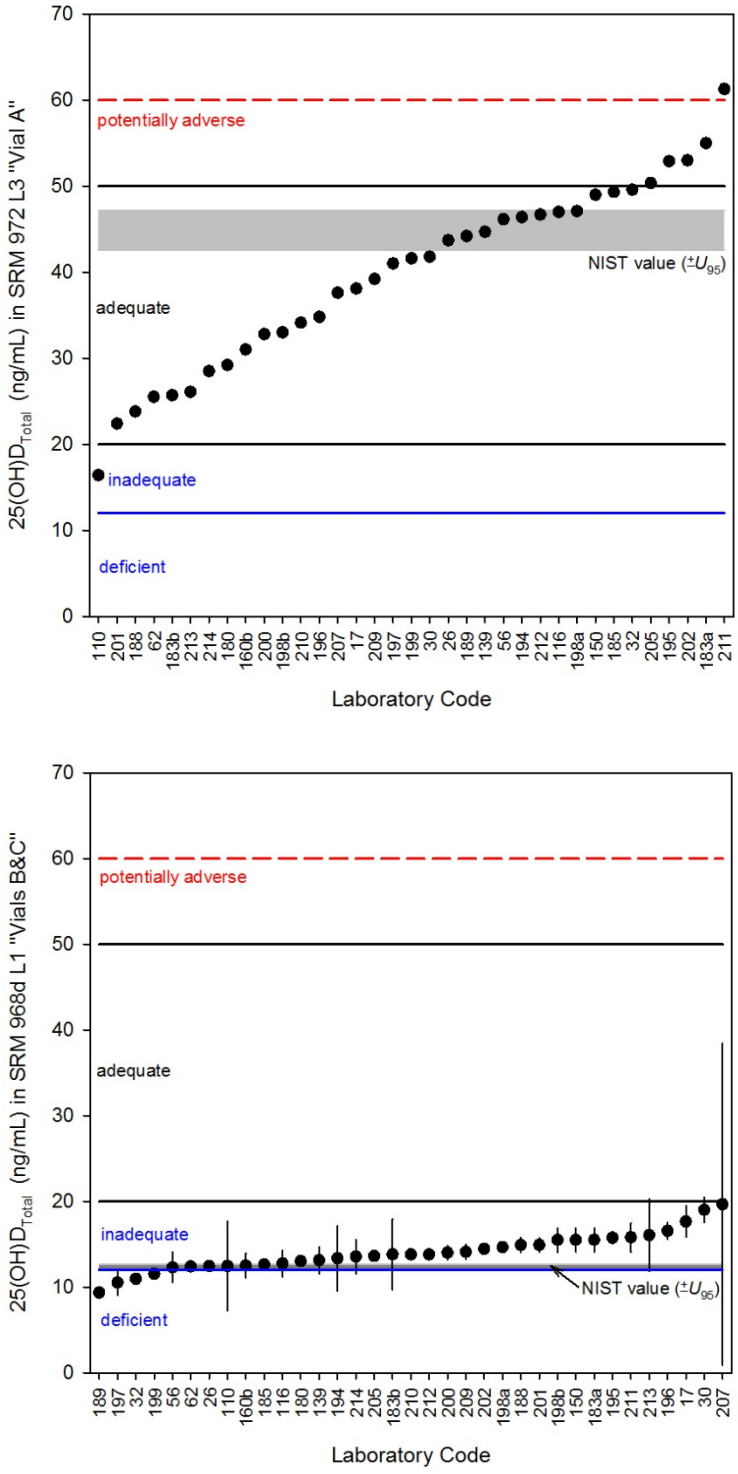
Table from http://ods.od.nih.gov/factsheets/vitamind#h4

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

A graphical representation of the single reported values for $25(OH)D_{Total}$ in SRM 972 L3 and the mean values with error bars (representing the lab mean value ± 2 × SD) for $25(OH)D_{Total}$ in SRM 968d L1 overlaid with the clinical ranges for 25(OH)D from **Table 3** is presented in **Figure 6**. The majority of the participant results for SRM 972 L3 indicate this study material has adequate 25(OH)D, but the reported results range from inadequate to potentially adverse levels of 25(OH)D. For SRM 968d L1, the range of reported values is smaller but includes values that are in the deficient as well as inadequate regions.

As indicated in previous reports, 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. Another goal of the program is to achieve better agreement between the participant consensus median value and the NIST value and to better understand the sources of bias between the results. The significant difference in results between the IA and LC results for SRM 972 L3 indicates that study materials with strictly native levels of 25(OH)D will be critical to help meet these goals in future exercises.

Figure 6. 25(OH)D_{Total} levels in SRM 972 L3 and SRM 968d L1 superimposed over clinically relevant serum 25-hydroxyvitamin D (25(OH)D_{Total}) concentration levels as reported by NIH (**Table 2**). 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.

Participant Number	*IA Method*	*Sample Preparation*	*Detection*
17	CLIA	n/r	n/r
30	RIA	50 µL sample was extracted with 500 µL acetonitrile	n/r
62	RIA	RIA kit; sample was extracted	n/r
160b	CLIA	Samples were thawed, swirled, and analyzed.	n/r
180	RIA	Samples were prepared per vendor sample extraction protocol	I^{125} detection using Gamma counter
183b	CLIA	None; calibrators diluted to get samples in analytical range	n/r
188	EIA	n/r	n/r
196	CLIA	The human serum samples were analyzed neat	n/r
198b	EIA	n/r	n/r
200	RIA	Sample was extracted	n/r
201	EIA	Sample was thawed and gently mixed prior to analysis	OD reading at 450 nm
210	RIA	n/r	n/r
213	EIA	n/r	n/r
214	CLIA	n/r	n/r

n/r = not reported
OD = optical density

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

Participant Number	*Internal Standard (IS)*	*Sample Preparation*	*Chromatographic Conditions*	*Detection: MRM ions*
26	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 and 25(OH)D_3-d_6	Samples were extracted with hexane, evaporated, then reconstituted with 69% methanol	PFP column (100 x 2.1 mm; 1.9 μm); isocratic elution; flow 0.4 mL/min	25(OH)D_3 383/365; 25(OH)D_3-d_6 389/371; 25(OH)D_2 395/377; 25(OH)D_2-d_3 398/380
116	25(OH)D_3 -d_6	Serum proteins were precipitated with methanol	LC column; isocratic separation with 95% methanol, 5% water; flow 0.6 mL/min; online SPE	25(OH)D_3 383/211; 25(OH)D_3-d_6 *389/211*; 25(OH)D_2 395/269
150	Deuterated stable isotope compounds	The samples were liquid-liguid extracted, centrifuged, separated, evaporated, and reconstituted in mobile phase	LC column (100 x 2.1 mm); Isocratic separation with 74% methanol, 26% water; flow 0.5 mL/min	2 MRM transitions per analyte
183a	25(OH)D_3 -d_6	IS (25 μL) was added to sample (150 μL), followed by protein precipiation and extraction with 0.1 mol/L $ZnSO_4$ (150 μL), methanol (300 μL), and hexane (750 μL); extract dried and dissolved with 70% methanol, 30% water with 2 mmol/L $NH_4C_2H_3O_2$	C8 column (50 x 2.1 mm); isocratic elution with 73% methanol/ 27% water; flow 0.4 mL/min	25(OH)D_3 401/159, 401/383; 25(OH)D_2 413/82, 413/395
185	25(OH)D_2-d_6 and 25(OH)D_3-d_6	n/r	C18 (50 x 2 mm); methanol/water gradient	25(OH)D_2 413/355; 25(OH)D_3 401/365
194	25(OH)D_3 -d_6	Proteins precipitated with acetonitrile, top layer aspirated then evaporated under nitrogen and reconstituted in methanol	C8 column (50 x 2 mm); isocratic elution with 98% acetonitrile, 2% water	25(OH)D_3 383/211; 25(OH)D_2 395/119
195	25(OH)D_2-d_3 and 25(OH)D_3-d_6	Samples extracted then derivatized	LC column (30 x 2.1 mm); gradient with methanol/water	n/r
197	25(OH)D_3 -d_6	Precipitating agent added (200 μL with 20 ng IS) to each serum (200 μL), calibrator and control sample followed by mixing, centrifugation, and analysis	C18 column (50 x 4.6 mm; 5 μm); 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 um); isocratic elution with 85% methanol (0.1% formic acid); flow 0.5 mL/min	25(OH)D_3 401/383, 401/365; 25(OH)D_2 413/395, 413/355; 25(OH)D_3-d_6 407/389, 407/371
199	25(OH)D_3-d_6	n/r	n/r	n/r
202	d_6-labeled compound	Sample was extracted	C18 column (50 x 2.1 mm); Gradient with 10% acetonitrile (0.1% formic acid), 90% methanol; flow 0.3 mL/min	n/r
205	25(OH)D_2-d_3 and 25(OH)D_3-d_3	Proprietary method	Proprietary conditions	n/r

209	25(OH)D_3 -d_6	Proteins in the samples (100 µL) were precipitated with 5% $ZnSO_4$ in methanol containing IS (200 µL), followed by an incubation period, centrifugation, and analysis of the supernatant (10 µL).	C8 column (50 x 2 mm; 5 µm); gradient with water/methanol; flow 0.7 mL/min	25(OH)D_3 383/229,383/211; 25(OH)D_3-d_6 389/211; 25(OH)D_2 395/269, 395/119
211	25(OH)D_3 -d_6	Extraction with acetonitrile containing IS followed by centrifugation	Column (33 x 4.6 mm; 3 µm); turboflow with methanol/water gradient	25(OH)D_3 383/365 (quant), 383/257 (qual); 25(OH)D_2 395/377 (quant), 395/209 (qual)
212	25(OH)D_3 -d_6	IS (100 µL) added to sample (200 µL) then extracted with hexane (1 mL). Hexane (700 µL) evaporated and reconstituted with 1:1 methanol:water (150 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

PFP = pentafluorophenyl
MRM = multiple reaction monitoring
SPE = solid phase extraction
quant = quantitative ions
qual = qualitative ions
n/r = not reported

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

Participant Number	***Internal Standard (IS)***	***Sample Preparation***	***Chromatographic Conditions***	***Wavelength***
32	Proprietary	Samples were extracted with filtration	C18 column (300 x 3.9 mm; 4 µm); proprietary mobile phase; flow 0.7mL/min	265 nm
110	n/r	n/r	n/r	n/r
139	Proprietary	The sample was extracted, centrifuged and injected directly onto LC column	Required reagents, column, controls and calibrators supplied in "kit" form	264 nm
189	Obtained from kit supplier	Proteins were precipitated, analytes were extracted using SPE cartridges	LC (150 x 4.6 mm) column; isocratic separation with commercial mobile phase; flow 0.7mL/min	265 nm
207	n/r	n/r	n/r	n/r

n/r = not reported
SPE = solid phase extraction

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

		$25(OH)D_2$ (ng/mL)			$25(OH)D_3$ (ng/mL)			$25(OH)D_{Total}$ (ng/mL)			$25(OH)D_2/D_3$ (ng/mL)	
		SRM 972 L3	SRM 968d L1	SRM 968d L1	SRM 972 L3	SRM 968d L1	SRM 968d L1	SRM 972 L3	SRM 968d L1	SRM 968d L1	SRM 2972	
Lab	Method	Vial A	Vial B	Vial C	Vial A	Vial B	Vial C	Vial A	Vial B	Vial C	$25(OH)D_2$	$25(OH)D_3$
017	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	38.1	17.0	18.3	n/r	n/r
026	LC-MS/MS	24.1	<1.0	<1.0	19.6	12.4	12.5	43.7	12.4	12.5	n/r	n/r
030	RIA	n/a	n/a	n/a	n/a	n/a	n/a	41.8	19.5	18.5	252.5	348.0
032	LC-UV	31.4	n/d	n/d	18.2	11.1	10.8	49.6	11.1	10.8	261.0	300.9
056	LC-MS/MS	26.4	n/d	n/d	19.8	11.7	12.9	46.2	11.7	12.9	239.6	333.4
062	RIA	n/a	n/a	n/a	n/a	n/a	n/a	25.5	12.3	12.5	n/r	329.8
110	LC-UV	7.3	1.4	1.8	7.9	11.5	7.4	16.4	14.3	10.6	218.0	326.0
116	LC-MS/MS	26.3	< 3.3	< 3.3	20.7	12.2	13.3	47.0	12.2	13.3	236.8	390.7
139	LC-UV	27.9	n/d	n/d	16.8	13.7	12.6	44.7	13.7	12.6	262.2	325.8
150	LC-MS/MS	27.0	<2	<2	22.0	16.0	15.0	49.0	16.0	15.0	253.0	398.0
160b	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	31.0	13.0	12.0	125.0	225.0
180	RIA	n/a	n/a	n/a	n/a	n/a	n/a	29.2	13.1	13.0	n/r	n/r
183a	LC-MS/MS	33.0	<4	<4	22.0	15.0	16.0	55.0	15.0	16.0	185.0	329.0
183b	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	25.7	13.1	14.5	191.0	262.0
185	LC-MS/MS	28.9	n/d	n/d	20.4	12.6	12.7	49.3	12.6	12.7	238.6	334.8
188	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	23.8	14.6	15.2	n/r	n/r
189	LC-UV	19.4	n/d	n/d	24.8	9.1	9.6	44.2	9.1	9.6	n/r	n/r
194	LC-MS/MS	27.7	<7.0	<7	18.7	14.7	12.0	46.4	14.7	12.0	230.0	336.5
195	LC-MS/MS	29.3	<4.0	<4.0	23.6	16.0	15.5	52.9	16.0	15.5	235.0	332.0
196	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	34.8	16.2	16.9	199.5	477.5
197	LC-MS/MS	24.0	<5	<5	17.0	11.0	10.0	41.0	11.0	10.0	205.0	290.0
198a	LC-MS/MS	29.1	<5.0	<5.0	18.0	14.7	14.6	47.1	14.7	14.6	250.7	337.0
198b	EIA	n/a	n/a	n/a	n/a	n/a	n/a	33.0	15.0	16.0	n/r	n/r
199	LC-MS/MS	24.8	<4	<4	16.8	11.5	11.6	41.6	11.5	11.6	233.0	331.0
200	RIA	n/a	n/a	n/a	n/a	n/a	n/a	32.8	14.0	14.0	187.5	280.6
201	EIA	n/a	n/a	n/a	n/a	n/a	n/a	22.4	14.6	15.2	171.0	341.0
202	LC-MS/MS	32.5	n/d	n/d	20.5	14.6	14.3	53.0	14.6	14.3	228.2	345.2
205	LC-MS/MS	29.5	0.2	0.2	20.9	13.6	13.2	50.4	13.8	13.4	265.4	337.4
207	LC-UV	20.6	n/d	n/d	17.0	26.3	13.0	37.6	26.3	13.0	n/r	n/r
209	LC-MS/MS	21.0	<1.0	<1.0	18.3	14.4	13.8	39.2	14.4	13.8	237.6	318.6
210	RIA	n/a	n/a	n/a	n/a	n/a	n/a	34.1	12.3	15.3	142.9	269.2
211	LC-MS/MS	33.4	n/d	n/d	27.9	15.2	16.4	61.3	15.2	16.4	311.5	423.0
212	LC-MS/MS	27.6	n/d	n/d	19.1	13.9	13.7	46.7	13.9	13.7	242.3	333.7
213	EIA	n/a	n/a	n/a	n/a	n/a	n/a	26.0	17.6	14.6	n/r	n/r
214	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	28.5	13.5	13.6	n/r	n/r

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

	NIST Value	26.40	<0.5	<0.5	18.50	12.38	12.38	44.90	12.38	12.38	238.6	334.8
	U_{95}	2.00	0.0	0.0	1.10	0.28	0.28	2.28	0.28	0.28	3.9	5.2