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NIST/NIH Vitamin D Metabolites Quality Assurance Program Report of Participant Results: Winter 2015 Comparability Study (Exercise 10)

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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 tenth exercise of this program, the Winter 2015 Comparability Study, were asked to use the methodology of their choice to measure concentrations of 25-hydroxyvitamin D in pooled human serum control and study materials distributed by NIST. The study materials consisted of Standard Reference Material (SRM) 972a Vitamin D Metabolites in Frozen Human Serum Level 2 and VitDQAP-III (a material designed for the VitDQAP). SRM 968d Fat-Soluble Vitamins, Carotenoids, and Cholesterol in Human Serum Level 1 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 tenth study are reported along with a summary of the analytical methods used.

OVERVIEW OF THE WINTER 2015 COMPARABILITY STUDY

For the Winter 2015 comparability study of the collaborative National Institute of Standards and Technology and National Institutes of Health (NIST/NIH) Vitamin D Metabolites Quality Assurance Program (VitDQAP), human serum control and study materials were distributed to participants for evaluation. SRM 968d Fat-Soluble Vitamins, Carotenoids, and Cholesterol in Human Serum Level 1 (SRM 968d L1) was provided as a control material for assay validation. For SRM 968d L1 (Control), the participants were provided the NIST target values within the data reporting sheet so that they could qualify their methods prior to analyzing the study samples. The study materials consisted of two vials, each containing a sample of pooled human serum. In this study, Vial A was SRM 972a Vitamin D Metabolites in Frozen Human Serum Level 2 (SRM 972a L2), and Vial B was VitDQAP-III, both of which contain endogenous levels of the vitamin D metabolites. Participants were asked to determine 25-hydroxyvitamin D in each of the human serum control and study samples. Individual concentration values for 25-hydroxyvitamin D_3 ($25(OH)D_3$), 25-hydroxyvitamin D_2 ($25(OH)D_2$), and 3-epi-25-hydroxyvitamin D_3 (3-epi-$25(OH)D_3$) were requested along with a total concentration of 25-hydroxyvitamin D ($25(OH)D_{Total}$ = $25(OH)D_2$ + $25(OH)D_3$).

There were a total of 52 participants and 58 datasets (6 participants provided data from two methods) in the Winter 2015 comparability study. Eighteen (18) of the datasets originated from immunoassay (IA) techniques, including 12 from chemiluminescence immunoassay (CLIA), two from enzyme immunoassay (EIA), three from radioimmunoassay (RIA), and one from chemiluminescence enzyme immunoassay (CLEIA). **Appendix A-1** summarizes the IA methods used by the participants. Forty (40) of the datasets originated from liquid chromatographic (LC) methods; of those, 35 were from LC with tandem mass spectrometric detection (LC-MS/MS), one was from LC-MS, and four were from LC with ultraviolet absorbance detection (LC-UV). The LC-MS/MS and LC-MS methods are collectively referred to as LC-MS^n. A summary of the LC methods used by the participants may be found in **Appendices A-2** and **A-3**. (Note: The methodological information provided on the data reporting sheet was used to update the list from previous comparability studies. For prior participants that did not provide method details for the Winter 2015 study, the information in the appendices were not edited and may not be current.)

The raw data received from all participants are summarized in **Appendix B**. The IA methods do not distinguish between $25(OH)D_3$ and $25(OH)D_2$, and hence IA participants reported single values for $25(OH)D_{Total}$ in the control and study materials. The LC methods measure the vitamin D metabolites separately, and the majority of the LC participants reported values for $25(OH)D_3$ and $25(OH)D_2$ in addition to $25(OH)D_{Total}$; eight LC participants also reported results for 3-epi-$25(OH)D_3$. One participant also reported values for 24(R), 25-dihydroxyvitamin D_3 ($24(R),25(OH)_2D_3$) and 1α, 25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$), which is not represented in **Appendix B**.

Appendix B also provides the summarized NIST results for each of the serum materials. A detailed description of the NIST methods is provided in the next section of this report.

SUMMARY OF NIST METHODS USED TO EVALUATE THE CONTROL AND STUDY MATERIALS

NIST used isotope dilution LC-MS/MS (ID-LC-MS/MS) [1] or a combination of ID-LC-MS/MS and ID-LC-MS [2] procedures to determine the vitamin D metabolites in the control and study materials evaluated in this comparability study. The ID-LC-MS/MS approach is a reference measurement procedure (RMP) for 25(OH)D_3 and 25(OH)D_2 that is recognized by the Joint Committee for Traceability in Laboratory Medicine (JCTLM).

For SRM 972a L2 (Vial A), NIST determined 25(OH)D_3 and 25(OH)D_2 using both ID-LC-MS and the ID-LC-MS/MS RMP. The results for 25(OH)D_3, 25(OH)D_2, 25(OH)D_{Total}, and 3-epi-25(OH)D_3 are a combination of results from the two NIST methods as well as a third method from the Centers for Disease Control and Prevention (CDC) and are certified values. A NIST certified value is a value for which NIST has the highest confidence in its accuracy in that all known or suspected sources of bias have been investigated or taken into account [3]. Detailed information about the characterization of SRM 972a and the components of the expanded uncertainty (*U*) may be found in the Certificate of Analysis, located on the NIST website [4].

The NIST values for 25(OH)D_3, 25(OH)D_2, and 3-epi-25(OH)D_3 in VitDQAP-III (Vial B) and SRM 968d L1 (Control) were determined solely with the ID-LC-MS/MS method. For VitDQAP-III (Vial B), the NIST values for 25(OH)D_3, 25(OH)D_2, and 3-epi-25(OH)D_3 are reported with expanded uncertainties that incorporate components for measurement variability and measurement uncertainty associated with the density of the materials and the purity of the reference standards. In addition, the measurements include an additional 1 % type B uncertainty for unknown systematic errors, which is consistent with the practice used at NIST for clinical measurements [1]. For SRM 968d L1 (Control), the NIST values for 25(OH)D_3 and 3-epi-25(OH)D_3 are reported as described for VitDQAP-III (Vial B), but the value for 25(OH)D_2 was well below the limit of quantitation and was estimated to be 0.1 ng/mL based on one measurement.

The NIST values for 25(OH)D_{Total} in VitDQAP-III (Vial B) and SRM 968d L1 (Control) are the sum of the individual values for 25(OH)D_3 and 25(OH)D_2, and the expanded uncertainties incorporate the measurement uncertainties for the two analytes.

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[1] Tai, S. S.-C.; Bedner, M.; Phinney, K.W.; *Anal. Chem.* **2010** *82*, 1942-1948.
[2] Bedner, M.; Phinney, K.W.; *J. Chromatogr. A* **2012** *1240*, 132–139.
[3] May, W.; Parris, R.; Beck II, C.; Fassett, J.; Greenberg, R.; Guenther, F.; Kramer, G.; Wise, S.; Gills, T.; Colbert, J.; Gettings, R.; MacDonald, B.; NIST Special Publication 260-136 **2000**; http://www.nist.gov/srm/publications.cfm
[4] http://www.nist.gov/srm/index.cfm

WINTER 2015 COMPARABILITY STUDY RESULTS AND DISCUSSION

Results for 25(OH)D_{Total}

A summary of the individual participant data for total 25-hydroxyvitamin D (25(OH)D_{Total}) in SRM 972a L2 (Vial A), VitDQAP-III (Vial B), and SRM 968d L1 (Control) is provided in **Table 1**.

The community results are summarized at the bottom of **Table 1** for all reported methods, the IA methods only, 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 %).

Table 1 also presents the NIST results for 25(OH)D_{Total} in the control and the two study materials.

Table 1. Summary of participant and NIST results for 25(OH)D_{Total} (ng/mL) in SRM 972a L2 (Vial A), VitDQ AP-III (Vial B), and SRM 968d L1 (Control).

		SRM 972a L2	VitDQAP-III	SRM 968d L1
Lab	Method	Vial A	Vial B	Control
026	LC-MS/MS	18.9	33.6	12.7
030a	RIA	19.8	38.4	13.1
056a	LC-MS/MS	18.8	32.3	12.7
056b	LC-MS/MS	18.9	32.5	12.8
060	LC-MS/MS	17.5	27.7	13.2
110	LC-UV	16.2	21.9	12.4
116	LC-MS/MS	21.6	35.0	13.5
119	LC-MS/MS	19.4	40.5	11.5
150	LC-MS/MS	17.0	33.0	11.0
161b	LC-MS/MS	18.5	36.6	13.1
180	RIA	17.8	29.4	13.3
187	LC-MS/MS	21.5	35.2	13.3
188	CLIA	26.6	40.6	15.0
189	LC-UV	20.6	38.0	10.6
194	LC-MS/MS	21.0	28.6	13.0
196	CLIA	18.5	29.9	15.2
197	LC-MS/MS	17.7	30.0	12.2
198a	LC-MS/MS	22.5	37.7	12.8
198c	CLIA	17.1	28.6	5.7
199	LC-MS/MS	20.6	35.3	13.6
204b	LC-MS/MS	18.2	32.1	12.6
209	LC-MS/MS	20.6	36.6	14.1
211	LC-MS/MS	18.8	32.9	12.5
212	LC-MS/MS	18.1	32.9	12.3
214b	CLIA	16.6	28.4	21.2
214c	LC-MS/MS	19.4	32.9	12.2
215	LC-MS/MS	20.4	37.2	13.2
216	LC-MS/MS	19.5	33.7	12.6
217	LC-MS/MS	19.8	37.0	12.6
218a	CLIA	17.6	31.8	13.5
221b	LC-UV	19.3	30.6	14.8
221c	LC-MS	19.3	25.2	13.6
225	LC-MS/MS	21.3	38.1	15.5
228a	LC-MS/MS	17.9	31.2	12.4
231b	CLIA	20.0	30.4	11.9
241	LC-MS/MS	17.7	33.4	11.3
243a	LC-UV	25.3	34.5	12.5
243b	LC-MS/MS	24.3	37.8	12.2
244	LC-MS/MS	17.0	35.0	12.1
249	LC-MS/MS	19.7	31.4	12.1
251	LC-MS/MS	22.0	40.0	n/r
253	LC-MS/MS	20.3	35.4	12.8
255	LC-MS/MS	18.8	32.8	13.2
256	CLIA	16.0	24.6	13.7
258	CLIA	20.9	25.5	17.9
259	LC-MS/MS	18.4	34.3	12.7
261	CLIA	17.3	23.0	14.4
262	CLIA	18.4	31.3	20.9
263	CLIA	18.6	35.0	12.6
267	CLEIA	17.8	32.1	12.6
268a	RIA	16.5	24.8	13.3
268b	EIA	21.1	41.4	21.8
269	LC-MS/MS	18.1	33.7	12.9
270	LC-MS/MS	18.5	26.6	9.3
271	LC-MS/MS	15.0	32.1	11.9
272	LC-MS/MS	19.4	35.4	12.7
273	EIA	17.7	31.8	14.6
274	CLIA	24.7	29.9	21.2

n/r = not reported or not determined

		SRM 972a L2	VitDQAP-III	SRM 968d L1
		Vial A	Vial B	Control
All methods	N	58	58	57
	Median	18.9	32.9	12.8
	MADe	1.7	3.7	0.9
	CV%	9.1	11	6.9
IA methods	N	18	18	18
	Median	18.1	30.2	14.0
	MADe	1.9	2.7	1.9
	CV%	10	9.1	14
LC methods	N	40	40	39
	Median	19.3	33.7	12.7
	MADe	1.8	2.6	0.7
	CV%	9.3	7.7	5.8
LC-MS^n	N	36	36	35
	Median	19.1	33.7	12.7
	MADe	1.7	2.5	0.7
	CV%	8.7	7.5	5.8
	NIST Value	18.9	32.7	12.5
	U	0.4	0.7	0.4

For all participant datasets, the single reported values for 25(OH)D_{Total} in SRM 972a L2 (Vial A), VitDQAP-III (Vial B), and SRM 968d L1 (Control) are plotted in **Figure 1**, **Figure 2**, and **Figure 3**, respectively. The results from immunoassay methods are displayed with open dark blue circles (○), and the results from the LC-based methods are displayed with open light blue circles (○). The results from the individual methods were sorted separately, as indicated by the x-axis labels.

From the single reported values for all datasets for a given technique (IA or LC), the consensus median and the consensus expanded uncertainty (2 × MADe) were determined. For both of the major techniques (IA or LC) in each figure, the solid lines (——) and (——) represent the consensus median, and the dashed lines (- - - - -) and (- - - - -) represent the consensus expanded uncertainty interval (median ± 2 × MADe). The laboratories with results that fall between the two dashed lines are within the consensus range for their technique (IA or LC).

The red lines (——) in each figure (**Figures 1 – 3**) represent the NIST value and its associated uncertainty (i.e., value ± *U*). NIST has confidence that the "true" value for each material lies within this interval. When these lines are not within the consensus ranges for each technique (IA or LC), then there may be method bias.

Specific results for each of the three study materials are summarized below. Note that the assessment is based on the actual reported values, not the lines and symbols, which have been enlarged to show detail and the laboratory number.

SRM 972a L2 (Vial A): **Figure 1**

- For the IA results, two reported values are outside of the consensus range (both CLIA).
- For the LC results, three reported values are outside of the consensus range (two LC-MS^n, one LC-UV).
- The consensus median value for the IA results is slightly lower than the NIST expanded uncertainty range (red lines).
- The consensus median value for the LC results is comparable to the NIST expanded uncertainty range (red lines).
- The NIST expanded uncertainty range (red lines) falls within the consensus range for both IA and LC.

VitDQAP-III (Vial B): **Figure 2**

- For the IA results, five reported values are outside the consensus range (three CLIA, one EIA, and one RIA).
- For the LC results, six reported values are outside the consensus range (five LC-MS^n, one LC-UV).
- The consensus median value for the IA results is lower than the NIST expanded uncertainty range (red lines).
- The consensus median value for the LC results is slightly higher than the NIST expanded uncertainty range (red lines).

- The NIST expanded uncertainty range (red lines) falls within the consensus ranges for both IA and LC.

SRM 968d L1 (Control): **Figure 3**

- For the IA results, five reported values are outside of the consensus range (four CLIA, one EIA).
- For the LC results, five reported values are outside of the consensus range (three LC-MS^n, two LC-UV).
- The consensus median value for the IA results is higher than the NIST expanded uncertainty range (red lines).
- The consensus median value for the LC results is comparable to the NIST expanded uncertainty range (red lines).
- The NIST expanded uncertainty range (red lines) falls within the consensus range for both IA and LC.

Figure 1. Participant and NIST results for 25(OH)D_{Total} in SRM 972a L2 (Vial A) as determined by immunoassay (CLIA, EIA, RIA, and CLEIA) and LC (LC-MS^n and LC-UV) methods.

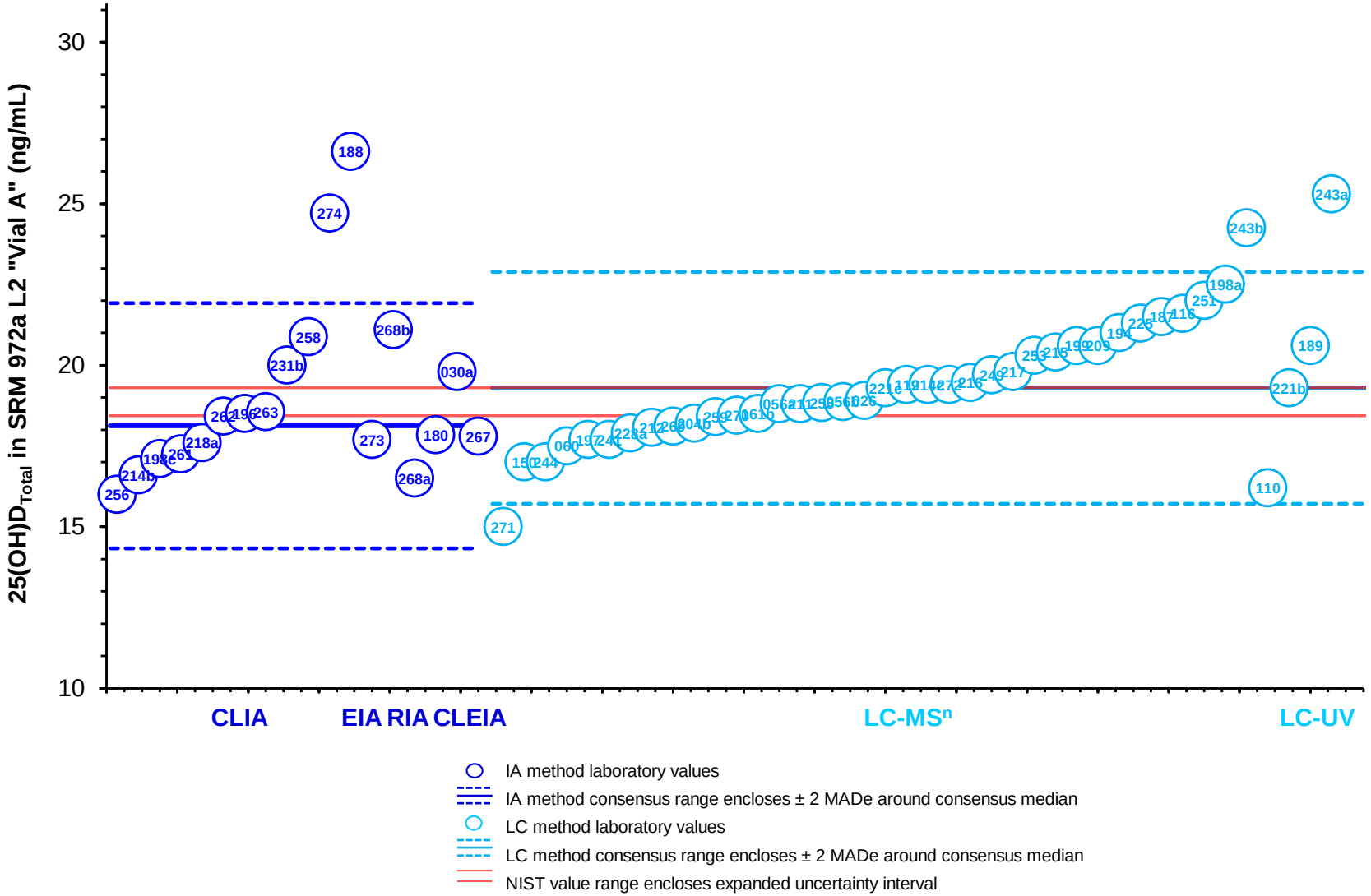


Figure 2. Participant and NIST results for 25(OH)D_{Total} in VitDQAP-III (Vial B) as determined by immunoassay (CLIA, EIA, RIA, and CLEIA) and LC (LC-MS^n and LC-UV) methods.

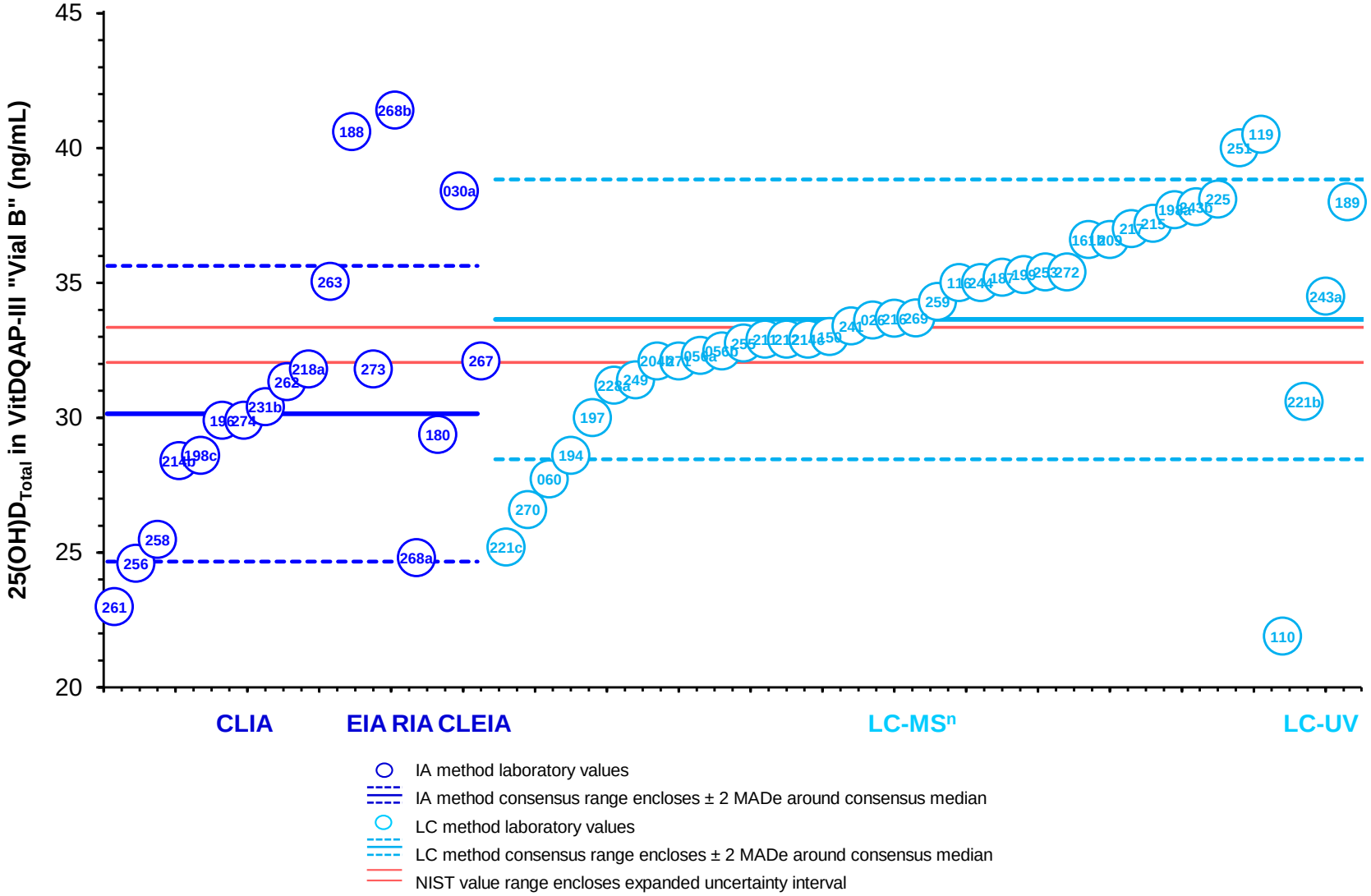


Figure 3. Participant and NIST results for 25(OH)D_{Total} in SRM 968d Level 1 (Control) as determined by immunoassay (CLIA, EIA, RIA, and CLEIA) and LC (LC-MS^n and LC-UV) methods.

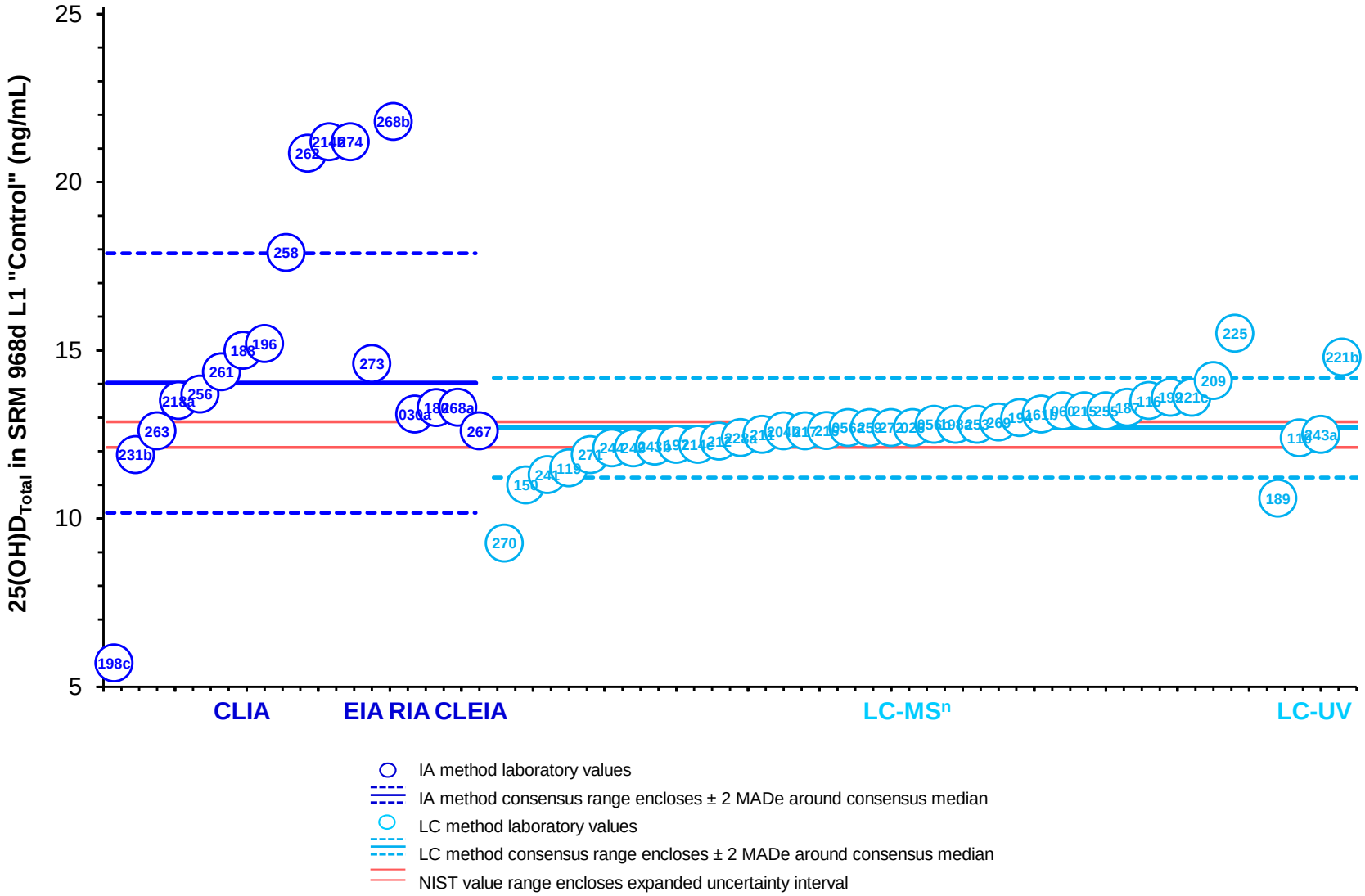


Figure 4 presents direct graphical comparisons of the 25(OH)D_{Total} results for a) SRM 972a L2 (Vial A) and VitDQAP-III (Vial B), and b) VitDQAP-III (Vial B) and SRM 968d L1 (Control). In each plot, there are two blue consensus boxes, one for IA methods and one for LC methods (as indicated). Laboratory 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 the Youden comparison plots are summarized below.

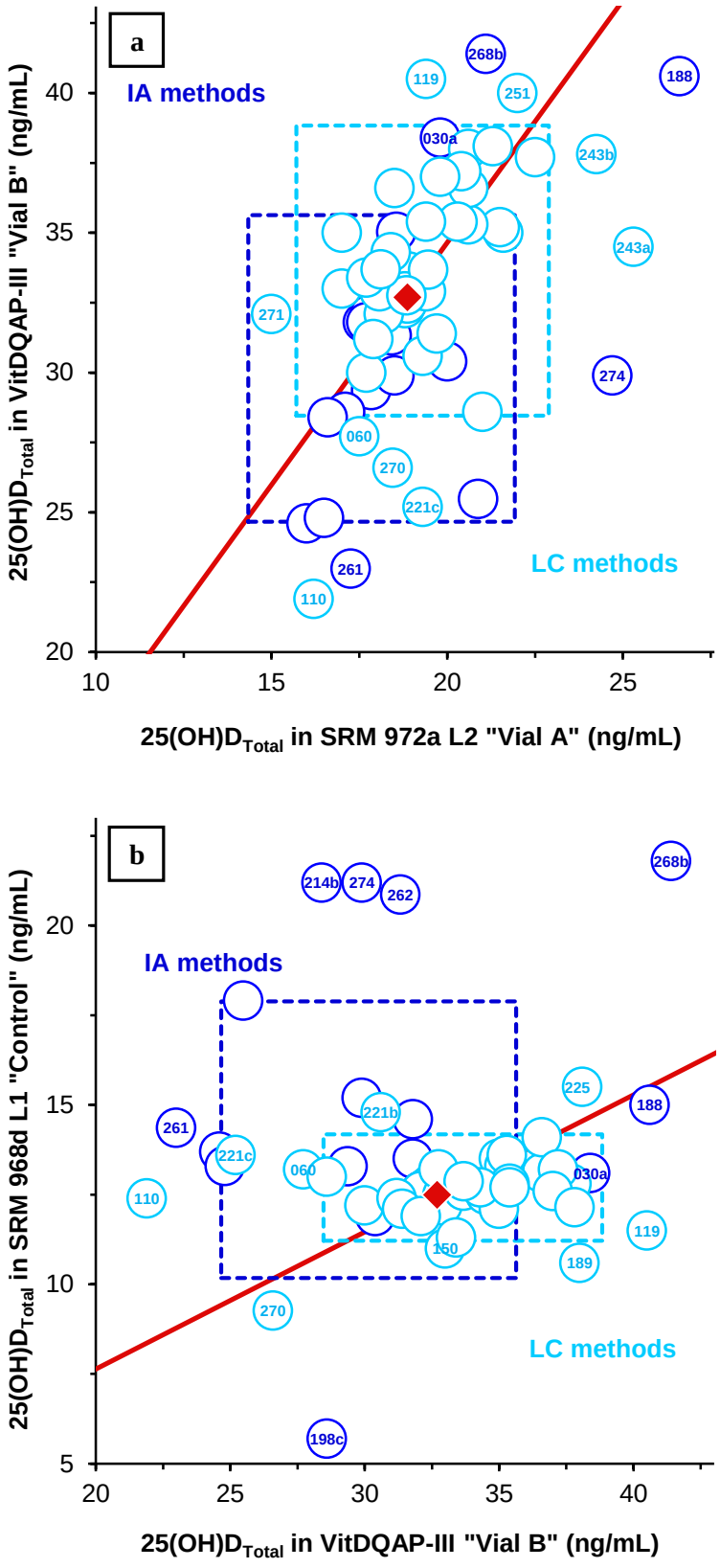
SRM 972a L2 (Vial A) and VitDQAP-III (Vial B): **Figure 4 a**

- IA results that are not included in the consensus ranges include numbers 030a, 188, 261, 268b, and 274
- LC results that are not included in the consensus ranges include numbers 060, 110, 119, 221c, 243a, 243b, 251, 270, and 271
- 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.

VitDQAP-III (Vial B) and SRM 968d L1 (Control): **Figure 4 b**

- IA results that are not included in the consensus ranges include numbers 030a, 188, 198c, 214b, 261, 262, 268b, and 274
- LC results that are not included in the consensus ranges include numbers 060, 110, 119, 150, 189, 221b, 221c, 225, and 270
  The Youden line runs through the center of the LC consensus box and through the bottom corner of the IA consensus box, illustrating that the LC results are in better agreement with the NIST results than are the IA results for these materials.

Figure 4. Youden comparison plot of the results for $25(OH)D_{Total}$ in a) 972a L2 (Vial A) and VitDQAP-III (Vial B) and b) VitDQAP-III (Vial B) and SRM 968d L1 (Control) for all methods.



Discussion of Results for 25(OH)D_{Total}

In the Winter 2015 comparability study, both SRM 972a L2 (Vial A) and SRM 968d L1 (Control) contain predominantly 25(OH)D_3. The CV %'s of 9.1 % and 6.9 % (all methods) for SRM 972a L2 (Vial) A and SRM 968d L1 (Control), respectively, are consistent with participant performance for other materials containing predominantly 25(OH)D_3 that were evaluated in previous comparability studies of the VitDQAP.

The VitDQAP-III material (Vial B) is different from SRM 972a L2 (Vial A) and SRM 968d L1 (Control) because it contains measurable 25(OH)D_2 in addition to 25(OH)D_3. The metabolite 25(OH)D_2 represents 20 % of the 25(OH)D_{Total} concentration in VitDQAP-III (Vial A), based on the NIST values of 6.5 ng/mL ± 0.2 ng/mL for 25(OH)D_2 and 32.7 ng/mL ± 0.7 ng/mL for 25(OH)D_{Total}. When materials containing appreciable amounts of 25(OH)D_2 (> 13 ng/mL) were evaluated in previous comparability studies of the VitDQAP, the results were bimodal, with the IA methods underrepresenting the 25(OH)D_{Total} concentration. In addition, the CV % (all methods) for those materials was relatively large (approximately 17 % to 28 %). The results for VitDQAP-III (Vial B), do not reveal those same trends: the CV % (all methods) is relatively low (11 %), and the IA method results overlap almost completely with the LC results. The difference in the observed results for the VitDQAP-III material (Vial B) is likely attributable to both the relatively high concentration of 25(OH)D_{Total} and the relatively low concentration of 25(OH)D_2, which causes any effect from the 25(OH)D_2 contribution to be lost in the overall variability of the results. However, the median IA result for VitDQAP-III (Vial B) is biased 7 % and 10 % lower than the NIST and the LC median results, respectively, which may be attributable to the nonequivalent response of many IA methods to 25(OH)D_2.

The Winter 2015 exercise was the first to utilize study materials that were evaluated in previous comparability studies of the VitDQAP. VitDQAP-III (Vial B) was also evaluated in the Winter 2014 comparability study, and SRM 972a L2 (Vial A) was previously evaluated in Winter 2012. **Table 2** provides the program results for each of these two study materials for the labs participating in the current study. Using the results in **Table 2**, labs that participated in the prior studies could assess their performance for these materials over time. In addition, it is informative to compare the summary statistics at the bottom of **Table 2**. For both materials, the median and CV % results are very consistent across both comparability studies in which the materials were evaluated, even though there are fewer labs and hence data points (N) for the prior studies.

Figure 5 presents direct graphical comparisons of the 25(OH)D_{Total} results for 25(OH)D_{Total} in a) VitDQAP-III (Vial B) in the present study (Winter 2015) and in a previous study (Winter 2014 – Vial A) and b) SRM 972a L2 (Vial A) in the present study (Winter 2015) and in a previous study (Winter 2012 – Vial B and D). The features of the plots are the same as described for **Figure 4**. The clustering of results around the NIST value in both **Figure 5 a** and **b** illustrates that there are not consistent within-laboratory biases for VitDQAP-III and SRM 972a L2 over 1 and 3 years, respectively, and that the within-round variability is consistent with the over-time variability. While the vast majority of labs yield results that are within the consensus boxes for their techniques, the labs that fall outside are not in as good statistical control. For VitDQAP-III, these labs include 030a, 060, 110, 119, 188, 251, 259, and 261 (**Figure 5a)**, and for SRM 972a L2, these labs include 188, 189, 216, 228a, and 243a (**Figure 5b)**.

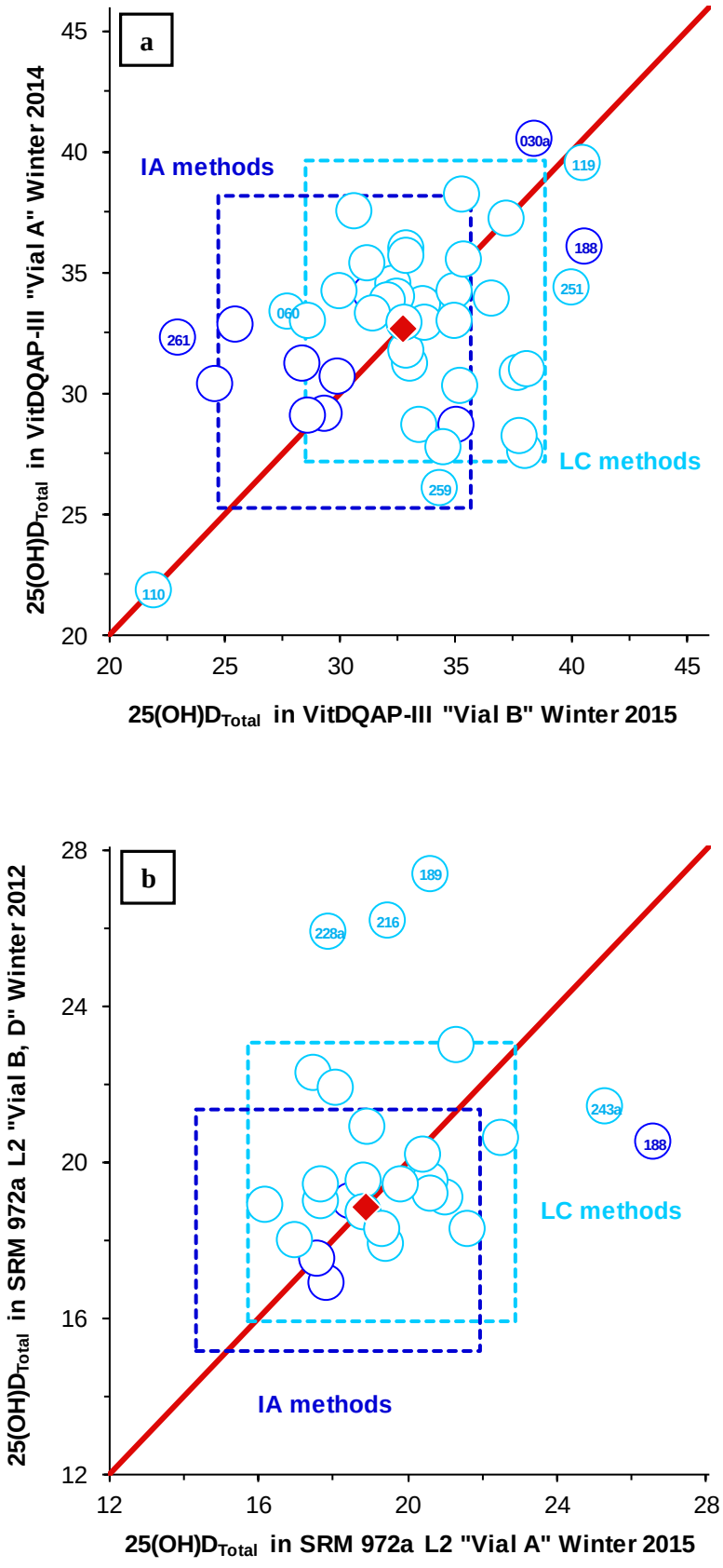
Table 2. Summary of participant data for 25(OH)D_{Total} (ng/mL) in VitDQAP-III in the current study (Vial B) and a prior study (Winter 2014 – Vial A) as well as for SRM 972a L2 in the current study (Vial A) and a prior study (Winter 2012 – Vial B and D).

		VitDQAP-III		SRM 972a L2	
		Winter 2015	Winter 2014	Winter 2015	Winter 2012
Lab	Method	Vial B	Vial A	Vial A	Vial B, D
026	LC-MS/MS	33.6	33.7	18.9	20.9
030a	RIA	38.4	40.5	19.8	X
056a	LC-MS/MS	32.3	34.5	18.8	19.5
056b	LC-MS/MS	32.5	34.0	18.9	X
060	LC-MS/MS	27.7	33.4	17.5	22.3
110	LC-UV	21.9	21.8	16.2	18.9
116	LC-MS/MS	35.0	34.2	21.6	18.3
119	LC-MS/MS	40.5	39.5	19.4	17.9
150	LC-MS/MS	33.0	31.2	17.0	X
161b	LC-MS/MS	36.6	X	18.5	X
180	RIA	29.4	29.1	17.8	16.9
187	LC-MS/MS	35.2	30.3	21.5	X
188	CLIA	40.6	36.1	26.6	20.5
189	LC-UV	38.0	27.6	20.6	27.4
194	LC-MS/MS	28.6	33.0	21.0	19.1
196	CLIA	29.9	30.7	18.5	19.0
197	LC-MS/MS	30.0	34.2	17.7	19.0
198a	LC-MS/MS	37.7	30.8	22.5	20.6
198c	CLIA	28.6	29.1	17.1	X
199	LC-MS/MS	35.3	38.2	20.6	19.5
204b	LC-MS/MS	32.1	33.8	18.2	X
209	LC-MS/MS	36.6	33.9	20.6	19.2
211	LC-MS/MS	32.9	36.0	18.8	18.7
212	LC-MS/MS	32.9	35.7	18.1	21.9
214b	CLIA	28.4	31.2	16.6	X
214c	LC-MS/MS	32.9	31.7	19.4	X
215	LC-MS/MS	37.2	37.2	20.4	20.2
216	LC-MS/MS	33.7	32.9	19.5	26.2
217	LC-MS/MS	37.0	X	19.8	19.4
218a	CLIA	31.8	33.7	17.6	17.5
221b	LC-UV	30.6	37.5	19.3	18.3
221c	LC-MS	25.2	X	19.3	X
225	LC-MS/MS	38.1	31.0	21.3	23.0
228a	LC-MS/MS	31.2	35.4	17.9	25.9
231b	CLIA	30.4	X	20.0	X
241	LC-MS/MS	33.4	28.7	17.7	19.4
243a	LC-UV	34.5	27.8	25.3	21.4
243b	LC-MS/MS	37.8	28.2	24.3	X
244	LC-MS/MS	35.0	33.0	17.0	18.0
249	LC-MS/MS	31.4	33.3	19.7	X
251	LC-MS/MS	40.0	34.4	22.0	X
253	LC-MS/MS	35.4	35.5	20.3	X
255	LC-MS/MS	32.8	32.9	18.8	X
256	CLIA	24.6	30.4	16.0	X
258	CLIA	25.5	32.8	20.9	X
259	LC-MS/MS	34.3	26.1	18.4	X
261	CLIA	23.0	32.3	17.3	X
262	CLIA	31.3	34.1	18.4	X
263	CLIA	35.0	28.7	18.6	X
267	CLEIA	32.1	X	17.8	X
268a	RIA	24.8	X	16.5	X
268b	EIA	41.4	X	21.1	X
269	LC-MS/MS	33.7	X	18.1	X
270	LC-MS/MS	26.6	X	18.5	X
271	LC-MS/MS	32.1	X	15.0	X
272	LC-MS/MS	35.4	X	19.4	X
273	EIA	31.8	X	17.7	X
274	CLIA	29.9	X	24.7	X

X = did not participate in that study

		VitDQAP-III		SRM 972a L2	
		Winter 2015	Winter 2014	Winter 2015	Winter 2012
		Vial B	Vial B	Vial A	Vial B, D
All methods	N	58	45	58	27
	Median	32.9	33.0	18.9	19.4
	MADe	3.7	3.3	1.7	1.6
	CV%	11	10	9	8.4
IA methods	N	18	12	18	4
	Median	30.2	31.7	18.1	18.3
	MADe	2.7	3.2	1.9	1.6
	CV%	9.1	10	10	8.5
LC methods	N	40	33	40	23
	Median	33.7	33.4	19.3	19.5
	MADe	2.6	3.1	1.8	1.8
	CV%	7.7	9	9.3	9.1
LC-MS^n	N	36	29	36	19
	Median	33.7	33.7	19.1	19.5
	MADe	2.5	2.5	1.7	1.6
	CV%	7.5	7.5	8.7	8.4
	NIST Value	32.7	32.7	18.9	18.9
	U	0.7	0.7	0.4	0.4

Figure 5. Youden comparison plot of the results for 25(OH)D_{Total} (ng/mL) in a) VitDQAP-III (Vial B) in the present study (Winter 2015) and in a previous study (Winter 2014 – Vial A) and b) SRM 972a L2 (Vial A) in the present study (Winter 2015) and in a previous study (Winter 2012 – Vial B and D).



LC method results for 25(OH)D_3, 25(OH)D_2, and 3-epi-25(OH)D_3 in VitDQAP-III (Vial B)

Of the two major techniques IA and LC, only the LC methods can measure the individual vitamin D metabolites. Given that 25(OH)D_{Total} is the sum of 25(OH)D_2 and 25(OH)D_3, LC methods require accurate, unbiased measurements of both 25(OH)D_2 and 25(OH)D_3 to obtain the correct values for 25(OH)D_{Total}. In the Winter 2015 comparability study, only VitDQAP-III (Vial B) contained a significant concentration of the 25(OH)D_2 metabolite, and the results for the individual metabolites in that study material are detailed below.

Of the 40 LC participants in the Winter 2015 comparability study, all reported values for 25(OH)D_3 and all but two reported values for 25(OH)D_2 in VitDQAP-III (Vial B). Since VitDQAP-III (Vial B) contains relatively high amounts of 25(OH)D_3 (NIST value 26.2 ng/mL ± 0.6 ng/mL), the 3-epi-25(OH)D_3 metabolite is also measureable in this material. Eight LC participants reported values for the 3-epi-25(OH)D_3 metabolite. The study results and the NIST values for 25(OH)D_3, 25(OH)D_2, and 3-epi-25(OH)D_3 in VitDQAP-III (Vial B) are presented in **Table 3**.

The single reported values for 25(OH)D_3 and 25(OH)D_2 in VitDQAP-III (Vial B) are plotted in **Figure 5 a** and **b**, respectively. The results from LC-MS^n and LC-UV were sorted separately, as indicated by the x-axis labels. In each plot, the consensus median is represented by the solid line (——), and the expanded uncertainty interval (2 × MADe), is represented by the dashed lines (- - - -). The laboratories with results that fall between the two dashed lines are within the consensus variability range.

The red lines (——) in **Figures 5 a** and **b** represent the NIST value and its associated uncertainty (i.e., value ± *U*). NIST has confidence that the "true" value for each metabolite lies within this interval. When these lines are not within the consensus range, then there may be method bias.

Specific results for 25(OH)D_3 and 25(OH)D_2 in VitDQAP-III (Vial B) are summarized below:

25(OH)D_3 in VitDQAP-III (Vial B): **Figure 5 a**

- Seven reported values are outside of the consensus variability range (five LC-MS^n, two LC-UV).
- The consensus median value is slightly higher than the NIST expanded uncertainty range (red lines).
- The NIST expanded uncertainty range (red lines) falls within the consensus variability range.

25(OH)D_2 in VitDQAP-III (Vial B): **Figure 5 b**

- Four reported values are outside of the consensus variability range, all from LC-MS^n.
- The consensus median value is in good agreement with the NIST expanded uncertainty range (red lines).

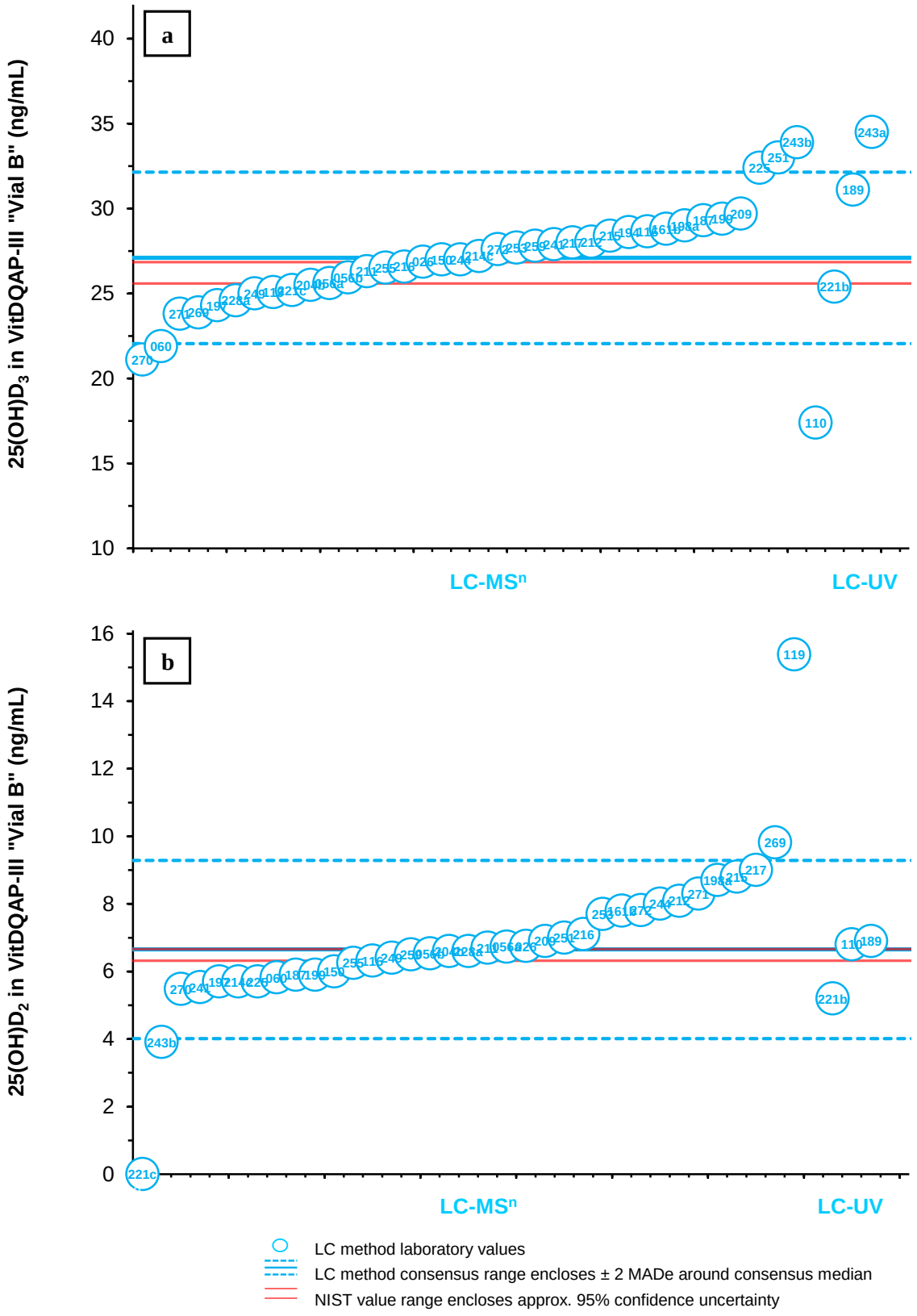
Table 3. Summary of LC participant and NIST results for 25(OH)D_3, 25(OH)D_2, and 3-epi-25(OH)D_3 (ng/mL) in VitDQAP-III (Vial B).

		25(OH)D_3	25(OH)D_2	3-epi-25(OH)D_3
Lab	Method	Vial B	Vial B	Vial B
026	LC-MS/MS	26.9	6.8	2.2
056a	LC-MS/MS	25.6	6.7	1.5
056b	LC-MS/MS	25.9	6.5	n/r
060	LC-MS/MS	21.9	5.8	1.7
110	LC-UV	17.4	6.8	n/r
116	LC-MS/MS	28.7	6.3	<4.0
119	LC-MS/MS	25.1	15.4	n/r
150	LC-MS/MS	27.0	6.0	n/r
161b	LC-MS/MS	28.8	7.8	n/r
187	LC-MS/MS	29.3	5.9	n/r
189	LC-UV	31.1	6.9	n/r
194	LC-MS/MS	28.6	<7.0	n/r
197	LC-MS/MS	24.3	5.7	n/r
198a	LC-MS/MS	29.0	8.7	n/r
199	LC-MS/MS	29.4	5.9	n/r
204b	LC-MS/MS	25.5	6.6	n/d
209	LC-MS/MS	29.7	6.9	n/r
211	LC-MS/MS	26.3	6.7	n/r
212	LC-MS/MS	28.0	8.1	n/r
214c	LC-MS/MS	27.2	5.7	n/r
215	LC-MS/MS	28.4	8.8	n/r
216	LC-MS/MS	26.6	7.1	1.7
217	LC-MS/MS	28.0	9.0	n/r
221b	LC-UV	25.4	5.2	n/r
221c	LC-MS	25.2	0.0	n/r
225	LC-MS/MS	32.4	5.7	n/r
228a	LC-MS/MS	24.6	6.6	2.3
241	LC-MS/MS	27.9	5.5	1.1
243a	LC-UV	34.5	n/d	n/d
243b	LC-MS/MS	33.9	3.9	n/d
244	LC-MS/MS	27.0	8.0	n/r
249	LC-MS/MS	25.0	6.4	1.3
251	LC-MS/MS	33.0	7.0	n/r
253	LC-MS/MS	27.7	7.7	n/r
255	LC-MS/MS	26.5	6.3	n/r
259	LC-MS/MS	27.8	6.5	n/r
269	LC-MS/MS	23.9	9.8	n/r
270	LC-MS/MS	21.1	5.5	n/r
271	LC-MS/MS	23.8	8.3	n/r
272	LC-MS/MS	27.6	7.8	1.3
LC methods	N	40	38	8
	Median	27.1	6.7	1.6
	MADe	2.5	1.3	0.4
	CV%	9.3	20	28
LC-MS^n	N	36	35	8
	Median	27.1	6.6	1.6
	MADe	2.3	1.3	0.4
	CV%	8.6	20	28
	NIST Value	26.2	6.5	1.6
	U	0.6	0.2	0.1

n/r = not reported or not determined; n/d = not detected

< x = less than a reported quantitation limit of x

Figure 5. Participant and NIST results for a) 25(OH)D_3 and b) 25(OH)D_2 in VitDQAP-III (Vial B).



Dihydroxyvitamin D_3 Metabolites

The Winter 2015 comparability study is the first in which a participant, Lab 269, reported results for two dihydroxyvitamin D_3 metabolites, 24, 25-dihydroxyvitamin D_3 ($24,25(OH)_2D_3$) and 1α, 25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$), in each of the study materials. The results provided by participant 269 for these metabolites are provided in the following table:

Results reported by Lab 269	**$24,25(OH)_2D_3$ (ng/mL)**	**$1\alpha,25(OH)_2D_3$ (ng/mL)**
SRM 972a L2 (Vial A)	1.40 ± 0.07	0.0405 ± 0.0095
VitDQAP-III (Vial B)	2.50 ± 0.16	0.0442 ± 0.0084
SRM 968d L1 (Control)	0.687 ± 0.032	0.0628 ± 0.0068

NIST has developed a candidate RMP for the determination of $24R,25(OH)_2D_3$ and has provided reference values for this metabolite in SRM 972a. For SRM 972a L2 (Vial A), the NIST reference value is 1.41 ± 0.05 ng/mL (95 % confidence interval), which agrees well with Lab 269's value. NIST has not developed a method for the $1\alpha,25(OH)_2D_3$ metabolite to date.

Conclusions from the Winter 2015 Comparability Study of the VitDQAP

The Winter 2015 comparability study was the tenth exercise and marked the five-year point for the VitDQAP. Over those five years and ten studies, the participant performance was consistent for study materials that contain predominantly $25(OH)D_3$; the CV was in the range from 7 % to 19 %, and the median values were biased slightly high relative to the NIST values. In the Winter 2015 comparability study, both SRM 972a L2 (Vial A) and SRM 968d L1 (Control) also contained predominantly $25(OH)D_3$. The median participant results (all methods) for these materials agreed well with the NIST values but otherwise follow these longstanding trends. In addition, Winter 2015 represented the second study in which SRM 972a L2 (Vial A) was evaluated in the VitDQAP. **Table 2** and **Figure 5 b** contains the program results for this material in both studies and demonstrates the consistency of the participant results for SRM 972a L2.

When VitDQAP-III (Vial B) was evaluated in the Winter 2014 study, it was the first study material that had an 'intermediate' concentration of $25(OH)D_2$ (NIST value 6.5 ng/mL ± 0.2 ng/mL) in addition to a significant concentration of $25(OH)D_3$ (NIST value 26.3 ng/mL ± 0.7 ng/mL). The material was first selected for study because it was anticipated that the IA methods would underrepresent the $25(OH)D_{Total}$ concentration due to nonequivalent response to the $25(OH)D_2$ metabolite. To the contrary, in both the Winter 2014 and the current Winter 2015 studies the IA results overlapped almost completely with the LC results, and any effect from the $25(OH)D_2$ metabolite was lost in the overall variability of the results for the VitDQAP-III study material (Vial B). As when previously evaluated, the median IA result for VitDQAP-III (Vial B) was biased lower than the median LC and NIST results, which is the only indication of potential non-equivalent response to the $25(OH)D_2$ metabolite. The consistency of the participant results for VitDQAP-III (Vial B) are also evident from the results provided in **Table 2** and **Figure 5 a**.

Appendix A-1. Summary of immunoassay methods used as reported by the study participants.

Laboratory Number	*IA Method*	*Sample Preparation*	*Vendor/kit**
30a	RIA	Samples were extracted with acetonitrile	A
180	RIA	Samples were extracted with acetonitrile	A
188	CLIA	n/r	B
196	CLIA	No sample preparation required	C
198c	CLIA	n/r	n/r
214b	CLIA	n/r	C
218a	CLIA	Direct analysis	C
231b	CLIA	n/r	B
256	CLIA	n/r	C
258	CLIA	n/r	D
261	CLIA	No sample preparation required	D
262	CLIA	n/r	E
263	EIA	On board displacement	F
267	CLEIA	n/r	G
268a	RIA	n/r	H
268b	EIA	n/r	I
273	EIA	n/r	n/r
274	CLIA	n/r	D

n/r = not reported

*NIST cannot endorse or recommend commercial products, therefore individual vendors/kits are indicated with a unique letter but not identified

Appendix A-2. Summary of LC-MS^n methods as reported by the study 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 mm × 3.2 mm); isocratic elution with 82 % methanol/18 % water; flow 0.4 mL/min	25(OH)D_3 401/365; 25(OH)D_2 413/355; 3-epi-25(OH)D_3 401/365
56a	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 mm × 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; 3-epi-25(OH)D_3-d_3 386/368
56b	n/r	n/r	n/r	n/r
60	25(OH)D_3 -d_6	IS was added, and then samples were extracted with acetonitrile, evaporated, and reconstituted with 90 % methanol/10 % water	PFP column (100 mm × 3.0 mm; 2.6 µm); gradient with water, methanol and acetonitrile (0.05 % formic acid)	25(OH)D_3 383/211; 25(OH)D_3-d_6 389/211; 25(OH)D_2 413/355; 3-epi-25(OH)D_3 401/383
116	25(OH)D_3 -d_6	Serum proteins were precipitated with methanol	Online SPE; reversed-phase column; isocratic elution with 95 % methanol/5 % water; flow 0.6 mL/min	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	Samples were mixed with ethanol containing the IS, equilibrated, mixed, extracted with hexane, evaporated, and reconstituted in methanol	C18 column (150 mm × 3.0 mm; 2.7 µm); Gradient with water and methanol (0.1 % formic acid)	Exact mass system 25(OH)D_3 383.32932/365.31897; 25(OH)D_3-d_6 389.36658; 25(OH)D_2 395.32946/377.31894
150	25(OH)D_2-d_6 and 25(OH)D_3-d_3	Sample (200 µL) was mixed with IS solution, liquid-liquid extracted, centrifuged, supernatant evaporated, and reconstituted in mobile phase	PFP column (100 mm × 3.0 mm; 2.6 µm); isocratic separation with 74 % methanol/26 % water (2 mmol/L ammonium acetate, 0.1 % formic acid); flow 0.5 mL/min	25(OH)D_3 401/383, 401/365; 25(OH)D_2 413/395, 413/365
161b	25(OH)D_3 -d_6	Protein precipitation	Reversed-phase column (50 mm × 2.1 mm; 2.6 µm); gradient with methanol and water (0.1 % formic acid); flow 0.5 mL/min	25(OH)D_3 383/211; 25(OH)D_2 395/269
187	deuterated standards for 25(OH)D_2 and 25(OH)D_3	SPE	C18 column (50 mm × 2.1 mm; 3 µm); gradient with methanol and water	25(OH)D_2 413/395; 25(OH)D_3 401/383
194	25(OH)D_3 -d_6	Proteins precipitated with acetonitrile, top layer removed, evaporated, and reconstituted with methanol	C8 column (50 mm × 2 mm); isocratic elution with 70 % acetonitrile/ 30 % water; flow 0.7 mL/min	25(OH)D_2 395/119; 25(OH)D_3 383/211
197	25(OH)D_3 -d_6	Precipitating agent added (200 µL with 20 ng IS) to each serum sample (200 µL), calibrator and control sample followed by mixing, centrifugation, and analysis	C18 column (50 mm × 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 $ZnSO_4$ addition, hexane extraction, centrifugation, evaporation under N_2, and reconstitution in methanol (0.1 % formic acid)	C18 column (50 mm 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	proprietary	proprietary	proprietary	proprietary
204b	25(OH)D_2-d_3; 25(OH)D_3-d_6; 3-epi-25(OH)D_3-d_3	Protein crash with 73 % methanol followed by liquid-liquid extraction with hexane, centrifugation, evaporation, and reconstitution in mobile phase	PFP column (100 mm × 2.1 mm; 1.9 µm); column temperature 30 °C; isocratic elution with 73 % methanol/27 % water; flow 0.4 mL/min	APCI 25(OH)D_3 383/365, 383/257; 25(OH)D_2 395/377, 395/209; 3-epi-25(OH)D_3 383/365, 383/257
209	25(OH)D_3 -d_6	Proteins were precipitated with 5 % $ZnSO_4$ in methanol	C8 column (50 mm × 2 mm; 5 µm); gradient with water/methanol; flow 0.7 mL/min	APCI 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	Turbulent flow column (32 mm x 4.6 mm; 3 µm)	25(OH)D_3 383/365 (quant), 383/257 (qual); 25(OH)D_2 395/209 (quant), 395/377 (qual)
212	25(OH)D_3-d_6	Serum (100 µL) proteins precipitated using 5 % methanol/95 % acetonitrile containing the IS (350 µL)	C8 column (50 mm × 2 mm; 3 µm); gradient of 60 % to 98 % acetonitrile (0.1 % formic acid)	25(OH)D_3 383/229, 383/211; 25(OH)D_2 395/269, 395/119
214c	25(OH)D_3-d_6	Samples were extracted with hexane, centrifuged, evaporated, and filtered	Column (50 mm × 2.1 mm); isocratic elution with 85 % methanol/ 15 % water/ 0.1 % formic acid; flow 0.3 mL/min	25(OH)D_3 401/383; 25(OH)D_3-d_6 407/389; 25(OH)D_2 413/395
215	25(OH)D_3-d_6	Protein precipitation with methanol/isopropanol and $ZnSO_4$; supernatant extracted using SPE	C18 column (50 mm × 2.1 mm; 2.6 µm) column; gradient with water (0.1 % formic acid, 5 mmol/L ammonium formate) and methanol (0.05 % formic acid)	ESI 25(OH)D_3 401/383; 25(OH)D_2 413/395; 25(OH)D_3-d_6 407/389
216	Derivatized deuterated standard	Samples extracted using liquid-liquid extraction then labeled with a derivatization reagent	Reversed-phase column (150 mm × 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 SPE	C8 column (50 mm × 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)
221c	25(OH)D_2-d_6 and 25(OH)D_3-d_3	Protein crash with acetonitrile containing IS; SPE extraction; elution with methanol/acetonitrile solution; evaporation; reconstitution with acetonitrile	PFP column (50 mm × 3.0 mm; 2.7 µm); elution with methanol/water/formic acid; column 40 °C	LC-MS SIM 25(OH)D_3 383; 25(OH)D_2 395; 25(OH)D_3-d_6 389; 25(OH)D_2-d_6 401
225	25(OH)D_3-d_6	Liquid-liquid extraction	PFP column (100 mm × 2.1 mm); gradient with methanol/water	25(OH)D_3 401/107; 25(OH)D_2 413/83
228a	n/r	n/r	n/r	n/r
241	25(OH)D_3-d_6	Acetonitrile containing the IS (100 µL) added to sample (200 µL) to precipitate proteins, followed hexane extraction, centrifugation, evaporation, and reconstitution with 50 % methanol	PFP column (100 mm × 2.1 mm; 2.6 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

243b	25(OH)D_3-d_6	Samples (400 µL) were mixed with solution containing the IS (400 µL) and the mobile phase (500 µL); samples were centrifuged; supernatant was diluted; portion (50 µL) was injected	PFP column (150 mm × 2 mm); isocratic separation with 85 % methanol/15 % water; flow 0.3 mL/min	25(OH)D_3 383/257; 25(OH)D_2 395/269; 25(OH)D_3-d_6 389/263;
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_3 383/211; 25(OH)D_3-d_6 389/211; 25(OH)D_2 395/269
249	25(OH)D_2-d_3; 25(OH)D_3-d_6; 3-epi-25(OH)D_3-d_3	Serum was deproteinated with NaOH and 90 % acetonitrile/ 10 % methanol followed by SPE	PFP column (100 mm × 2.1 mm; 1.8 µm); gradient separation with water (2 mmol/L ammonium acetate) and methanol; flow 0.35 mL/min	25(OH)D_3 401/159; 25(OH)D_2 413/159
251	25(OH)D_2-d_3 and 25(OH)D_3-d_3	Protein precipitation followed by SPE	Phenyl column (50 mm × 2.1 mm; 1.7 µm); gradient with water and methanol (0.1 % formic acid, 2 mmol/L ammonium acetate); flow 0.45 mL/min	25(OH)D_3 401/159 (quant), 401/365 (qual); 25(OH)D_2 413/83 (quant), 413/355 (qual); 25(OH)D_3-d_3 404/162; 25(OH)D_2-d_3 416/358
253	25(OH)D_2-d_3 and 25(OH)D_3-d_3	The sample was extracted, centrifuged, and derivatized	C18 column (150 mm × 2.1 mm); gradient separation with water and methanol; flow 0.4 mL/min	25(OH)D_2 588; 25(OH)D_3 576
255	deuterium labeled compound	Samples were extracted and derivatized with 4-phenyl-1,2,4-triazoline-3,5-dione	Reversed-phase column (50 mm × 2.1 mm); gradient with methanol; flow 0.5 mL/min	25(OH)D_3 607/298; 25(OH)D_2 619/298
259	25(OH)D_3-d_6	Liquid-liquid extraction using hexane	C8 column; gradient with methanol/water/0.1 % formate; column temperature 40 °C	25(OH)D_3 401/355; 25(OH)D_2 413/355; 25(OH)D_3-d_6 407/371
269	25(OH)D_3-d_6	Samples were spiked with IS(s), deprotonated with acetonitrile, filtered, dried, derivatized with 4-phenyl-1,2,4-triazoline-3,5-dione overnight at 4°C, dried, reconstituted with cyclohexyldodecylurea, and filtered	C18 column (100 mm × 2.1 mm; 1.7 µM); gradient separation with 0.1 % formic acid and water (10 %)/acetonitrile (90 %); flow 0.25 mL/min	24,25(OH)$_2$$D_3$-$d_6$ 580/298 24,25(OH)$_2$$D_3$ 574/298 1a,25(OH)$_2$$D_3$-$d_6$ 580/314 1a,25(OH)$_2$$D_3$ 574/314 25(OH)D3-d_6 564/298 25(OH)D_3 558/298 25(OH)D_2 570/ 298
270	25(OH)D_3-d_6	Samples were precipitated, centrifuged, evaporated, reconstituted, centrifuged, and upper layer injected	C18 column (300 mm × 4.6 mm; 3.5 µM); isocratic separation with 50 % water/ 50 % methanol; flow 1.0 mL/min	25(OH)D_3 401/383; 25(OH)D_2 413/395; 25(OH)D_3-d_6 407/389
271	25(OH)D_3-d_6	Protein precipitation	C8 column (3 µm); gradient with water/acetonitrile/0.1 % formic acid; flow 0.7 mL/min	25(OH)D_3 383/229; 25(OH)D_2 395/269
272	Isotopically labeled internal standards	Samples were precipitated and centrifuged before injection	Analytical column and trap column from a kit; separation using a binary gradient system and an additional isocratic pump	25(OH)D_3 383/365, 383/299; IS (1): 386/257, 386/232; 25(OH)D_2 395/269, 395/251; 3-epi-25(OH)D_3 383/257, 383/299; 3-epi-25(OH)D_2 395/269, 395/251; IS (2): 386/257, 386/232

C18 = octadecyl; C8 = octyl; PFP = pentafluorophenyl; SPE = solid phase extraction; CN = cyano;

MRM = multiple reaction monitoring; quant/qual = quantitative/qualitative ions; n/r = not reported;

APPI = atmospheric pressure photoionization; APCI = atmospheric pressure chemical ionization; ESI = electrospray ionization

Appendix A-3. Summary of LC-UV methods as reported by the study participants.

Laboratory Number	*Internal Standard (IS)*	*Sample Preparation*	*Chromatographic Conditions*	*Wavelength*
110	n/a	Samples (500 µL) were mixed with ethanol (500 µL), extracted twice with hexane/methylene chloride (5:1), evaporated, and reconstituted	C18 column (2.1 mm × 100 mm; 1.8 µm); gradient with acetonitrile/methanol (85:15) and isopropanol (100 %)	267 nm
189	unidentified	Protein precipitation followed by SPE	Reversed-phase column (150 mm × 4.6 mm); isocratic separation; flow 0.7 mL/min	265 nm
221b	laurophenone	Protein crash with acetonitrile solution containing IS, followed by SPE, elution with methanol/acetonitrile solution, evaporation, and reconstitution with acetonitrile	CN column (150 mm × 5 mm; 3.5 µm); elution with methanol/water/formic acid; column temperature 47 °C	275 nm
243a	dodecanophenone	Samples (400 µL) were mixed with solution containing the IS (400 µL), precipitation reagent was added (500 µL), and portion of upper layer (50 µL) was injected	C18 column (100 mm × 3 mm); isocratic elution with water and isobutanol; flow 1.2 mL/min; column temperature 25 °C	264 nm

C18 = octadecyl; SPE = solid phase extraction; CN = cyano; n/a = not applicable

Appendix B. Raw participant data and NIST results for $25(OH)D_2$, $25(OH)D_3$, $25(OH)D_{Total}$, and 3-epi-$25(OH)D_3$ in SRM 972a L2 (Vial A), VitDQAP-III (Vial B), and SRM 968d L1 (Control).

		$25(OH)D_2$ (ng/mL)			$25(OH)D_3$ (ng/mL)			$25(OH)D_{Total}$ (ng/mL)			3-epi-$25(OH)D_3$ (ng/mL)		
		SRM 972a L2	VitDQAP-III	SRM 968d L1	SRM 972a L2	VitDQAP-III	SRM 968d L1	SRM 972a L2	VitDQAP-III	SRM 968d L1	SRM 972a L2	VitDQAP-III	SRM 968d L1
Lab	Method	Vial A	Vial B	Control	Vial A	Vial B	Control	Vial A	Vial B	Control	Vial A	Vial B	Control
026	LC-MS/MS	0.7	6.8	0.3	18.2	26.9	12.4	18.9	33.6	12.7	1.5	2.2	0.6
030a	RIA	n/a	n/a	n/a	n/a	n/a	n/a	19.8	38.4	13.1	n/a	n/a	n/a
056a	LC-MS/MS	0.6	6.7	0.6	18.2	25.6	12.1	18.8	32.3	12.7	1.2	1.5	0.7
056b	LC-MS/MS	0.8	6.5	<0.6	18.1	25.9	12.8	18.9	32.5	12.8	n/r	n/r	n/r
060	LC-MS/MS	0.9	5.8	0.2	16.6	21.9	13.0	17.5	27.7	13.2	1.3	1.7	0.9
110	LC-UV	3.5	6.8	n/d	12.8	17.4	12.4	16.2	21.9	12.4	n/r	n/r	n/r
116	LC-MS/MS	<3.3	6.3	<3.3	21.6	28.7	13.5	21.6	35.0	13.5	<4.0	<4.0	<4.0
119	LC-MS/MS	n/d	15.4	n/d	19.4	25.1	11.5	19.4	40.5	11.5	n/r	n/r	n/r
150	LC-MS/MS	<2	6.0	<2	17.0	27.0	11.0	17.0	33.0	11.0	n/r	n/r	n/r
161b	LC-MS/MS	<4	7.8	<4	18.5	28.8	13.1	18.5	36.6	13.1	n/r	n/r	n/r
180	RIA	n/a	n/a	n/a	n/a	n/a	n/a	17.8	29.4	13.3	n/a	n/a	n/a
187	LC-MS/MS	0.0	5.9	0.0	21.5	29.3	13.3	21.5	35.2	13.3	n/r	n/r	n/r
188	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	26.6	40.6	15.0	n/a	n/a	n/a
189	LC-UV	0.0	6.9	0.0	20.6	31.1	10.6	20.6	38.0	10.6	n/r	n/r	n/r
194	LC-MS/MS	<7.0	<7.0	<7.0	21.0	28.6	13.0	21.0	28.6	13.0	n/r	n/r	n/r
196	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	18.5	29.9	15.2	n/a	n/a	n/a
197	LC-MS/MS	<5	5.7	<5	17.7	24.3	12.2	17.7	30.0	12.2	n/r	n/r	n/r
198a	LC-MS/MS	<5.0	8.7	<5.0	22.5	29.0	12.8	22.5	37.7	12.8	n/r	n/r	n/r
198c	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	17.1	28.6	5.7	n/a	n/a	n/a
199	LC-MS/MS	<2.0	5.9	<2.0	20.6	29.4	13.6	20.6	35.3	13.6	n/r	n/r	n/r
204b	LC-MS/MS	n/d	6.6	n/d	18.2	25.5	12.6	18.2	32.1	12.6	n/d	n/d	n/d
209	LC-MS/MS	<1.0	6.9	<1.0	20.6	29.7	14.1	20.6	36.6	14.1	n/r	n/r	n/r
211	LC-MS/MS	0.0	6.7	0.0	18.8	26.3	12.5	18.8	32.9	12.5	n/r	n/r	n/r
212	LC-MS/MS	<2	8.1	<2	18.1	28.0	12.3	18.1	32.9	12.3	n/r	n/r	n/r
214b	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	16.6	28.4	21.2	n/a	n/a	n/a
214c	LC-MS/MS	<1.0	5.7	<1.0	19.4	27.2	12.2	19.4	32.9	12.2	n/r	n/r	n/r
215	LC-MS/MS	<2	8.8	<2	20.4	28.4	13.2	20.4	37.2	13.2	n/r	n/r	n/r
216	LC-MS/MS	0.8	7.1	0.2	18.7	26.6	12.5	19.5	33.7	12.6	1.3	1.7	0.8
217	LC-MS/MS	<0.8	9.0	<0.8	19.8	28.0	12.6	19.8	37.0	12.6	n/r	n/r	n/r
218a	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	17.6	31.8	13.5	n/a	n/a	n/a
221b	LC-UV	0.0	5.2	0.0	19.3	25.4	14.8	19.3	30.6	14.8	n/r	n/r	n/r
221c	LC-MS	0.0	0.0	0.0	19.3	25.2	13.6	19.3	25.2	13.6	n/r	n/r	n/r
225	LC-MS/MS	<5	5.7	<5	21.3	32.4	15.5	21.3	38.1	15.5	n/r	n/r	n/r
228a	LC-MS/MS	n/d	6.6	n/d	17.9	24.6	12.4	17.9	31.2	12.4	1.8	2.3	0.75
231b	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	20.0	30.4	11.9	n/a	n/a	n/a
241	LC-MS/MS	0.7	5.5	0.3	17.0	27.9	11.0	17.7	33.4	11.3	0.7	1.1	0.7
243a	LC-UV	n/d	n/d	n/d	25.3	34.5	12.5	25.3	34.5	12.5	n/d	n/d	n/d
243b	LC-MS/MS	n/d	3.9	n/d	24.3	33.9	12.2	24.3	37.8	12.2	1.6	n/d	n/d
244	LC-MS/MS	0.0	8.0	0.0	17.0	27.0	12.1	17.0	35.0	12.1	n/r	n/r	n/r
249	LC-MS/MS	0.0	6.4	0.0	19.7	25.0	12.1	19.7	31.4	12.1	1.6	1.3	0.5
251	LC-MS/MS	<4	7.0	n/r	22.0	33.0	n/r	22.0	40.0	n/r	n/r	n/r	n/r
253	LC-MS/MS	0.9	7.7	0.2	19.4	27.7	12.6	20.3	35.4	12.8	n/r	n/r	n/r
255	LC-MS/MS	0.9	6.3	0.1	18.0	26.5	13.1	18.8	32.8	13.2	n/r	n/r	n/r
256	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	16.0	24.6	13.7	n/a	n/a	n/a
258	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	20.9	25.5	17.9	n/a	n/a	n/a
259	LC-MS/MS	n/d	6.5	n/d	18.4	27.8	12.7	18.4	34.3	12.7	n/r	n/r	n/r
261	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	17.3	23.0	14.4	n/a	n/a	n/a
262	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	18.4	31.3	20.9	n/a	n/a	n/a
263	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	18.6	35.0	12.6	n/a	n/a	n/a
267	CLEIA	n/a	n/a	n/a	n/a	n/a	n/a	17.8	32.1	12.6	n/a	n/a	n/a
268a	RIA	n/a	n/a	n/a	n/a	n/a	n/a	16.5	24.8	13.3	n/a	n/a	n/a
268b	EIA	n/a	n/a	n/a	n/a	n/a	n/a	21.1	41.4	21.8	n/a	n/a	n/a
269	LC-MS/MS	2.8	9.8	2.1	15.3	23.9	10.8	18.1	33.7	12.9	n/r	n/r	n/r
270	LC-MS/MS	1.4	5.5	0.9	17.1	21.1	8.3	18.5	26.6	9.3	n/r	n/r	n/r
271	LC-MS/MS	<4	8.3	<4	15.0	23.8	11.9	15.0	32.1	11.9	n/r	n/r	n/r
272	LC-MS/MS	0.6	7.8	0.0	18.8	27.6	12.7	19.4	35.4	12.7	1.5	1.3	0.9
273	EIA	n/a	n/a	n/a	n/a	n/a	n/a	17.7	31.8	14.6	n/a	n/a	n/a
274	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	24.7	29.9	21.2	n/a	n/a	n/a

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

	NIST Value	0.81	6.5	0.1*	18.1	26.2	12.4	18.9	32.7	12.5	1.3	1.6	0.7
	U	0.06	0.2	---	0.4	0.6	0.4	0.4	0.7	0.4	0.1	0.1	0.03

*estimated value (no uncertainty determined)