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NIST/NIH Vitamin D Metabolites Quality Assurance Program Report of Participant Results: Summer 2013 Comparability Study (Exercise 7)

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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 seventh exercise of this program, the Summer 2013 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 SRM 972a Vitamin D Metabolites in Human Serum Level 4 and VitDQAP-II (a material designed for the VitDQAP). SRM 968e 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 seventh study are reported along with a summary of the analytical methods used.

OVERVIEW OF THE SUMMER 2013 COMPARABILITY STUDY

For the Summer 2013 comparability study of the VitDQAP, pooled human serum control and study samples 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 calibration or verification. The blinded study samples consisted of two vials, Vial A and Vial B. Vial A was VitDQAP-II, which contains only endogenous 25-hydroxyvitamin D (25(OH)D) levels. Vial B was SRM 972a Vitamin D Metabolites in Human Serum Level 4 (SRM 972a L4), which contains endogenous levels of 25-hydroxyvitamin D_2 ($25(OH)D_2$) and 25-hydroxyvitamin D_3 ($25(OH)D_3$) but was fortified with 3-epi-25-hydroxyvitamin D_3 (3-epi-$25(OH)D_3$). Participants were asked to provide individual concentration values for $25(OH)D_2$, $25(OH)D_3$, and 3-epi-$25(OH)D_3$ along with a total concentration of 25(OH)D ($25(OH)D_{Total} = 25(OH)D_2 + 25(OH)D_3$) for the control and each study sample.

There were a total of 44 participants and 49 datasets (five participants provided data from two different methods) in the Summer 2013 comparability study. Sixteen of the datasets originated from immunoassay (IA) techniques, including 13 from chemiluminescence immunoassay (CLIA) and three from radioimmunoassay (RIA). Note that none of the participants used enzyme immunoassay (EIA) in this study. **Appendix A-1** summarizes the IA methods used by the participants. Thirty-three of the datasets originated from liquid chromatographic (LC) methods; of those, 30 were from LC with tandem mass spectrometric detection ($LC\text{-}MS^n$), and three were from LC with ultraviolet absorbance detection (LC-UV). A summary of the LC methods used by the participants may be found in **Appendices A-2** and **A-3**.

The raw data received from all participants are summarized in **Appendix B**. All datasets from the immunoassay methods reported a single value for $25(OH)D_{Total}$ whereas LC participants provided values for $25(OH)D_2$, $25(OH)D_3$, and 3-epi-25(OH)D3 as well as $25(OH)D_{Total}$ in VitDQAP-II (Vial A), SRM 972a L4 (Vial B), and SRM 968d L1 (Control).

Appendix B also provides the summarized NIST results for each of the serum materials.

The control material (SRM 968d L1) and the two study samples (SRM 972a L4 and VitDQAP-II) all contain low levels of $25(OH)D_2$, thus $25(OH)D_3$ represents the predominant metabolite contributing to $25(OH)D_{Total}$. However, the two study materials, VitDQAP-II (Vial A) and SRM 972a L4 (Vial B), both contain measurable amounts of 3-epi-$25(OH)D_3$.

SUMMER 2013 COMPARABILITY STUDY RESULTS AND DISCUSSION

25(OH)D_{Total} in VitDQAP-II (Vial A), SRM 972a L4 (Vial B), and SRM 968d L1 (Control)

A summary of the individual participant data for 25(OH)D_{Total} in VitDQAP-II (Vial A), SRM 972a L4 (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 the three study materials. For SRM 972a L4 (Vial B), the NIST result is the sum of the certified values for 25(OH)D_3 and 25(OH)D_2 with the corresponding 95% confidence limits (U_{95}).

The NIST values for 25(OH)D_3 in VitDQAP-II (N = 8) and SRM 968d L1 (N = 5) were obtained using an LC-MS/MS reference measurement procedure (RMP)[1] recognized by the Joint Committee for Traceability in Laboratory Medicine (JCTLM). The NIST value for 25(OH)D_2 was also obtained using the RMP for VitDQAP-II (N = 5), but for SRM 968d L1 the value was well below the limit of quantitation and was estimated to be 0.1 ng/mL (N = 1). The NIST values for 25(OH)D_{Total} in VitDQAP-II (Vial A) and SRM 968d L1 (Control) reported in **Table 1** are the sum of the individual 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 and includes components for measurement variability and measurement uncertainty associated with the density of the materials.

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.

[1] Tai, S. S.-C., Bedner, M. and Phinney, K.W. *Anal. Chem.* **2010** *82*, 1942-1948.

Table 1. Summary of participant data for 25(OH)D_{Total} (ng/mL) in VitDQAP-II (Vial A), SRM 972a L4 (Vial B), and SRM 968d L1 (Control).

		VitDQAP-II	SRM 972a L4	SRM 968d L1
Lab	Method	Vial A	Vial B	Control
017	CLIA	36.8	27.0	13.0
030	RIA	33.6	26.3	12.8
056	LC-MS/MS	36.4	27.5	12.3
060	LC-MS/MS	39.4	30.9	12.8
086a	CLIA	40.7	30.7	14.0
110	LC-UV	30.1	44.7	13.2
116	LC-MS/MS	36.7	24.4	16.5
119	LC-MS/MS	41.7	65.3	15.1
128	LC-MS/MS	24.8	33.5	12.3
139	LC-UV	44.2	64.4	14.7
183b	CLIA	37.0	29.2	13.5
187	LC-MS/MS	39.6	59.7	12.5
188	CLIA	47.0	35.2	13.6
194	LC-MS/MS	43.4	64.5	12.5
196	CLIA	40.9	29.6	14.6
197	LC-MS/MS	33.9	46.7	12.8
198a	LC-MS/MS	49.7	56.6	11.4
198c	CLIA	40.8	26.6	15.4
199	LC-MS/MS	41.5	71.0	12.7
200	RIA	30.8	22.9	12.8
209	LC-MS/MS	42.4	49.7	13.0
210a	RIA	38.5	34.5	8.5
210b	CLIA	40.8	39.8	< 3.0
211	LC-MS/MS	42.0	58.0	15.3
213a	CLIA	49.2	49.8	9.0
214b	CLIA	39.6	28.8	13.1
214c	LC-MS/MS	36.1	53.7	12.1
215	LC-MS/MS	40.4	57.2	13.9
216	LC-MS/MS	38.2	29.3	12.6
217	LC-MS/MS	37.2	54.0	12.8
218a	CLIA	37.5	28.6	12.8
218b	LC-MS/MS	42.3	42.7	13.1
220	LC-MS/MS	39.0	59.0	13.0
221a	LC-MS/MS	35.5	25.1	16.9
222	CLIA	51.6	34.8	12.4
225	LC-MS/MS	44.6	65.9	11.2
228a	LC-MS/MS	34.6	51.6	12.4
231	LC-UV	41.3	56.4	n/r
241	LC-MS/MS	43.3	68.0	14.9
242	LC-MS/MS	35.1	30.5	11.9
244	LC-MS/MS	36.5	43.1	12.5
247a	CLIA	50.0	34.1	13.1
248	LC-MS/MS	43.0	55.0	14.0
249	LC-MS/MS	36.4	29.1	12.4
250	LC-MS/MS	44.3	67.7	13.9
253	LC-MS/MS	41.7	33.0	14.1
254a	LC-MS/MS	40.5	59.5	12.9
254b	CLIA	37.5	29.6	12.5
255	LC-MS/MS	50.1	60.4	16.4
All methods	N	49	49	47
	Median	40.4	42.7	12.9
	MADe	4.7	20.2	0.9
	CV%	11.7	47	6.9
IA methods	N	16	16	15
	Median	40.2	29.6	13.0
	MADe	4.3	4.7	0.7
	CV%	10.7	16	5.7
LC methods	N	33	33	32
	Median	40.4	54.0	12.9
	MADe	4.7	15.6	0.8
	CV%	11.7	28.8	6.3
LC-MS^n	N	30	30	30
	Median	40.0	53.9	12.8
	MADe	4.9	16.2	0.7
	CV%	12.2	30.1	5.8
	NIST Value	37.5	30.0	12.5
	U_{95}	1.0	1.0	0.3

For all participant datasets, the single reported values for 25(OH)D_{Total} in VitDQAP-II (Vial A), SRM 972a L4 (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 (○). For the LC results in all three figures, the majority of the data points are from LC-MS^n methods. However, the LC-UV results were sorted separately and are plotted at the right end of the LC results as labeled.

From the single reported values for all LC datasets for a given technique (IA or LC), the consensus median and the consensus variability (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 approximate 95% confidence interval (2 × MADe). The laboratories with results that fall between the two dashed lines are within the consensus variability area 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_{95}). NIST believes that the "true" value for each material lies within this interval. When these lines are not within the consensus range, then there may be method bias.

Specific results for each of the three study materials are summarized below:

VitDQAP-II (Vial A): **Figure 1**

- For the IA results, four reported values are outside of the consensus variability range (three CLIA, one RIA).
- For the LC results, three reported values are outside of the consensus variability range (two LC-MS^n, one LC-UV).
- The consensus median values for both the LC and IA results agree well with each other but are slightly higher than the NIST expanded uncertainty range (red lines).
- The NIST expanded uncertainty range (red lines) falls within the consensus variability ranges both for LC and IA results.

SRM 972a L4 (Vial B): **Figure 2**

- For the IA results, two reported values are outside the consensus variability range (both CLIA).
- For the LC results, the consensus variability range is very large, and there are no outliers.
- The consensus median value for the IA results agrees well with the NIST expanded uncertainty range (red lines).
- The consensus median value for the LC results is considerably higher (80%) than both the IA median value and the NIST expanded uncertainty range (red lines).
- The NIST expanded uncertainty range (red lines) falls within the consensus variability ranges for both IA and LC results.

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

- For the IA results, two reported values are outside of the consensus variability range (both CLIA).

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

The consensus variability of ≈ 7% to 12% (all methods) for SRM 968d L1 (Control) and VitDQAP-II (Vial A) is consistent with participant performance for other materials containing predominantly 25(OH)D_3 that were evaluated in previous comparability studies of the VitDQAP.

For SRM 972a L4 (Vial B), the LC results are bimodal, where nine reported results agree well with both the NIST value and the reported IA results, but the majority of the LC results (73%) are biased high (**Figure 2**). The bimodal results contribute to the large consensus variability (47%) for this material when the results for all methods are considered (**Table 1**). SRM 972a L4 (Vial B) was fortified with 3-epi-25(OH)D_3, and the NIST-certified value for this vitamin D metabolite is 26.4 ng/mL ± 2.1 ng/mL. The biological significance of 3-epi-25(OH)D_3 is uncertain, and this metabolite is not included in the 25(OH)D_{Total} concentration. Therefore, LC methods that do not chromatographically separate the 3-epi-25(OH)D_3 yield biased results for 25(OH)D_3 and hence 25(OH)D_{Total} because the 3-epi-25(OH)D_3 and the 25(OH)D_3 are diastereomers that are detected by the same multiple reaction monitoring (MRM) ions in MS/MS and absorbance wavelength in UV. Since the majority of the reported LC methods do not separate the 25(OH)D_3 and 3-epi-25(OH)D_3 (**Appendix A-2, A-3**), the median LC value of 54.0 ng/mL for 25(OH)D_{Total} in SRM 972a L4 is biased 80% higher than the NIST value of 30.0 ng/mL ± 1.0 ng/mL. The majority of the IA methods, on the other hand, do not have cross-reactivity with the 3-epi-25(OH)D_3 metabolite and yield an unbiased median result of 29.6 ng/mL for 25(OH)D_{Total} in SRM 972a L4.

VitDQAP-II (Vial A) also has a significant 3-epi-25(OH)D_3 concentration of 3.4 ng/mL ± 0.1 ng/mL, or ≈ 9% of the 25(OH)D_3 concentration of 37.0 ng/mL ± 0.4 ng/mL (NIST values). In theory, bimodal results should have also been obtained for this material, but the 9% bias is indistinguishable in the overall method variability of 12% for the LC results.

Of the nine LC participants that used methods that separate the 3-epi-25(OH)D_3, seven reported values for this metabolite in the control and study materials. The LC method results for 25(OH)D_3 and 3-epi-25(OH)D_3 are presented and discussed in detail later in this report.

Figure 1. 25(OH)D_{Total} levels in VitDQAP-II (Vial A) as determined by immunoassay (CLIA and RIA) and LC (LC-MS^n and LC-UV) methods. The red lines represent the ranges bound by the NIST values with ± estimated U_{95} uncertainty.

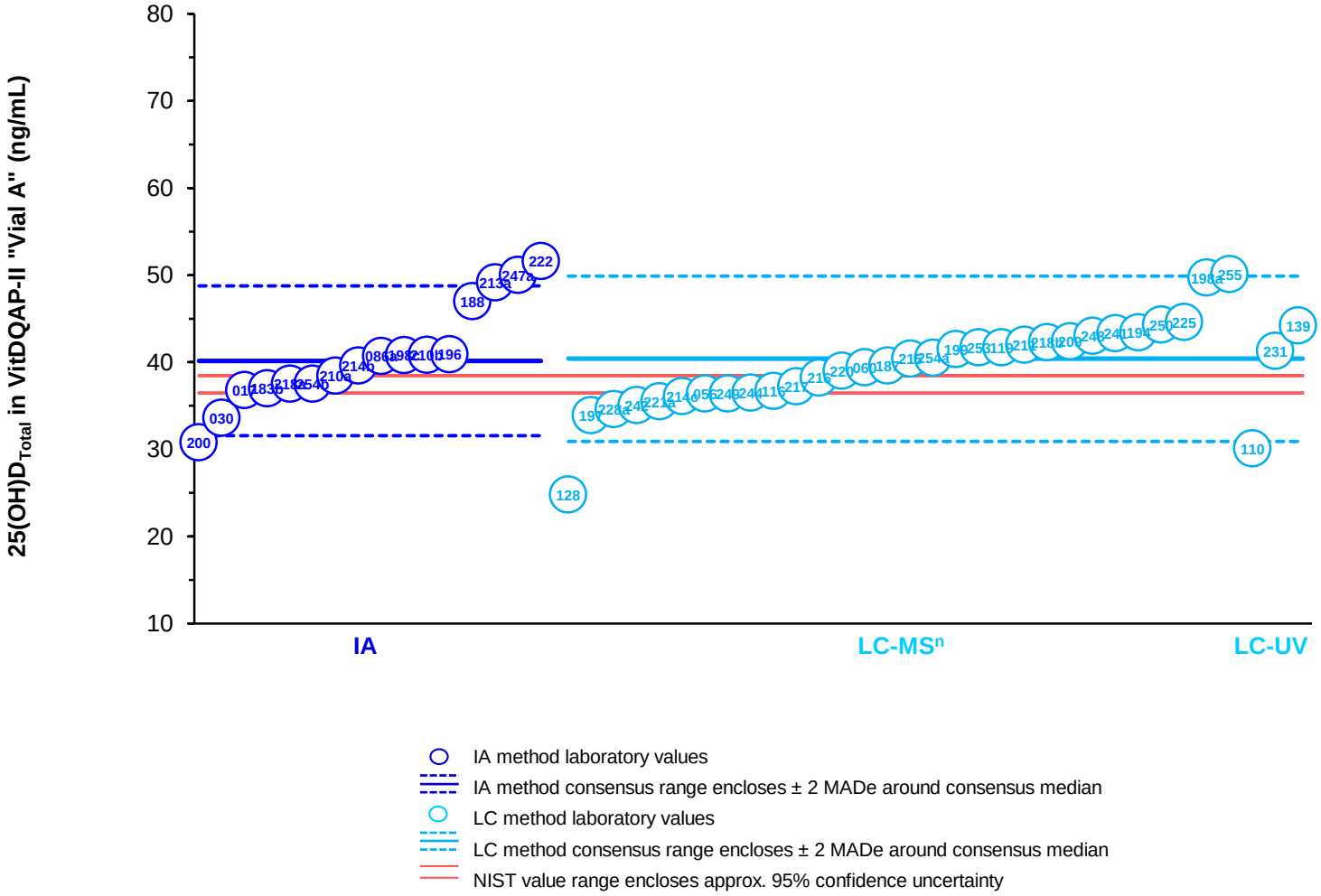


Figure 2. 25(OH)D_{Total} levels in SRM 972a L4 (Vial B) as determined by immunoassay (CLIA and RIA) and LC (LC-MS^n and LC-UV) methods. The red lines represent the ranges bound by the NIST values with ± estimated U_{95} uncertainty.

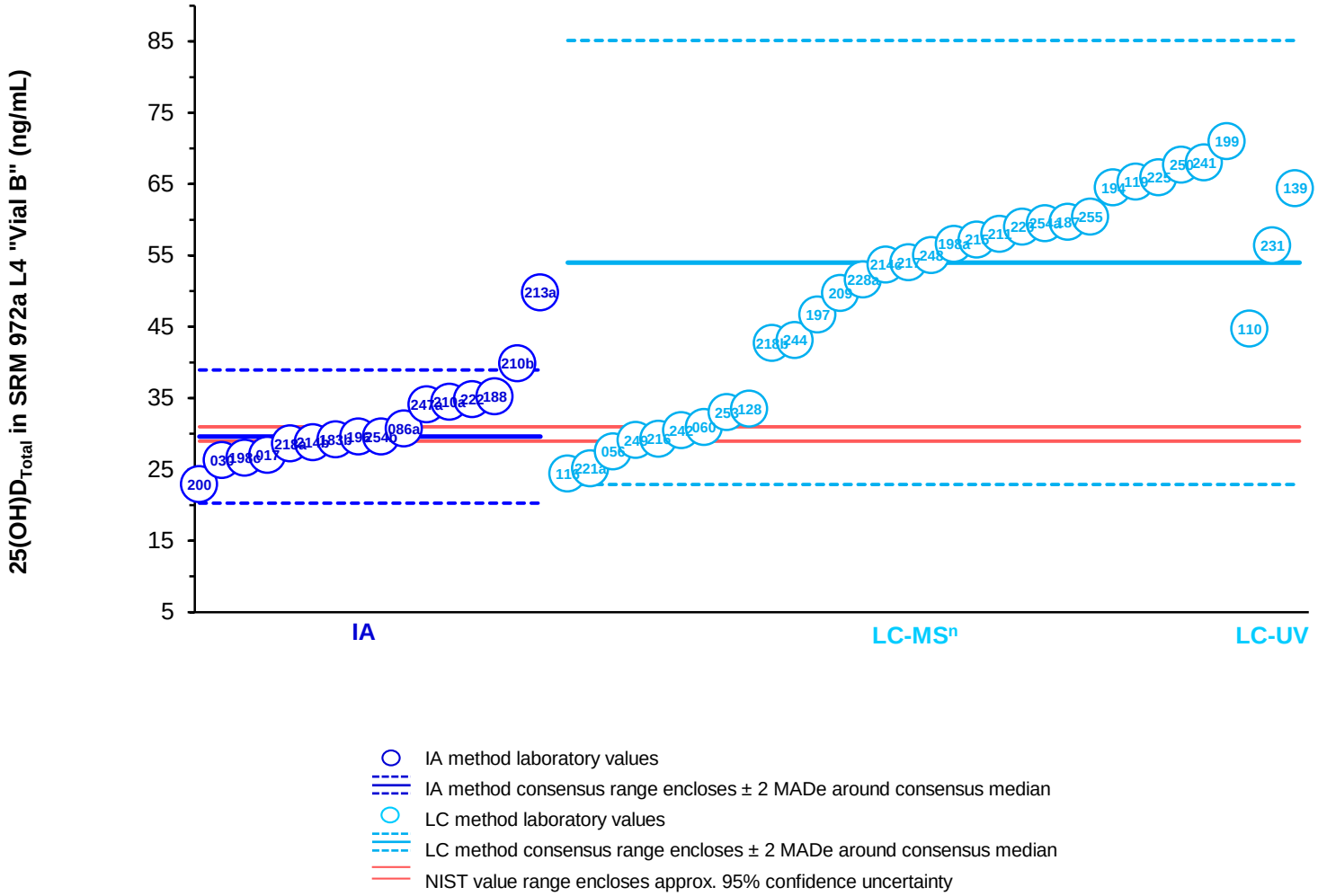


Figure 3. 25(OH)D_{Total} levels in SRM 968d L1 (Control) as determined by immunoassay (CLIA and RIA) and LC (LC-MS^n and LC-UV) methods. The red lines represent the ranges bound by the NIST values with ± estimated U_{95} uncertainty.

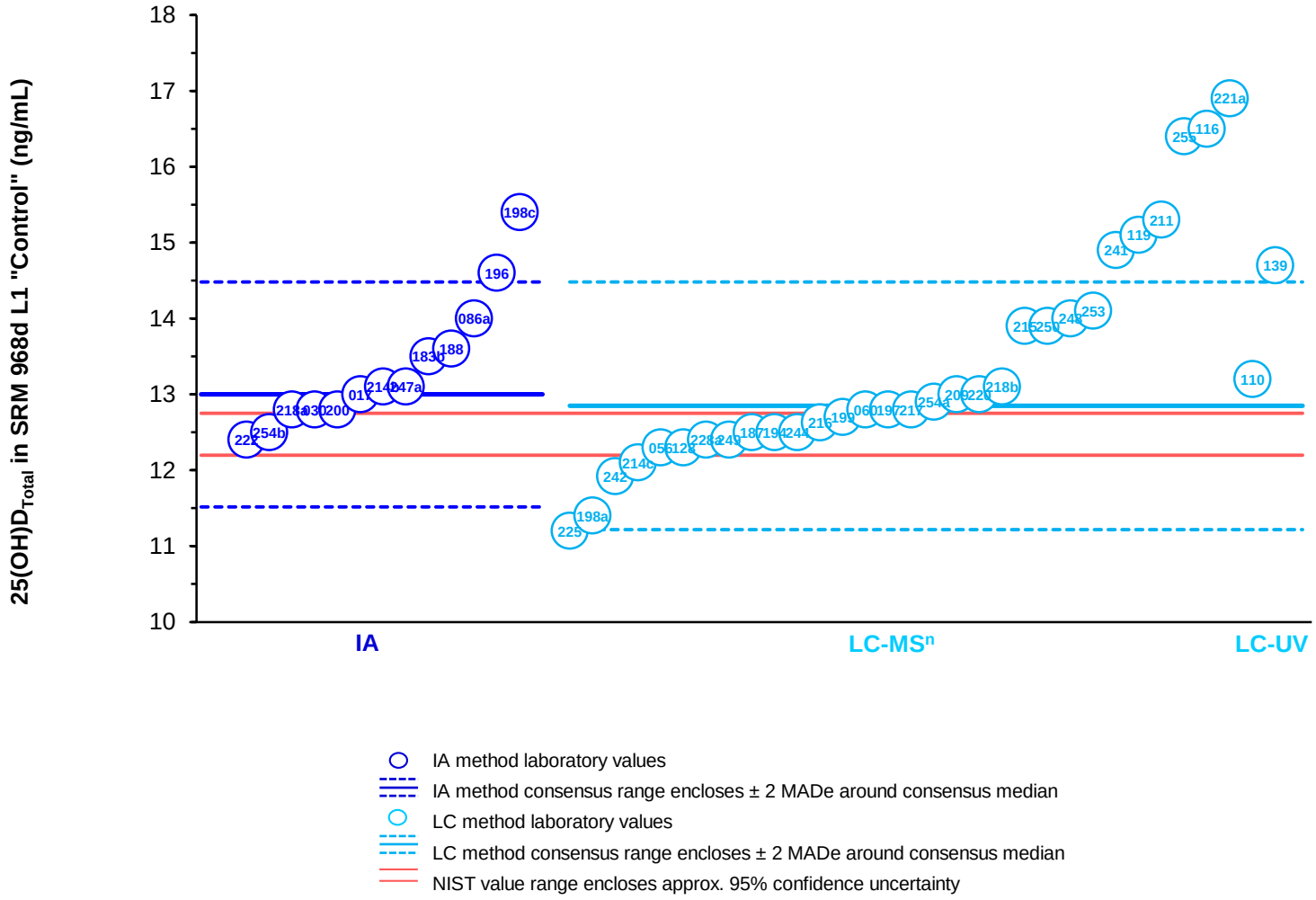


Figure 4 presents direct graphical comparisons of the 25(OH)D_{Total} results for A) VitDQAP-II (Vial A) and SRM 972a L4 (Vial B), and B) SRM 972a L4 (Vial B) and SRM 968d L1 (Control). In each plot, there are two blue consensus boxes, one for IA methods and one for LC methods (as indicated). Laboratory 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.

VitDQAP-II (Vial A) and SRM 972a L4 (Vial B): **Figure 4 A**

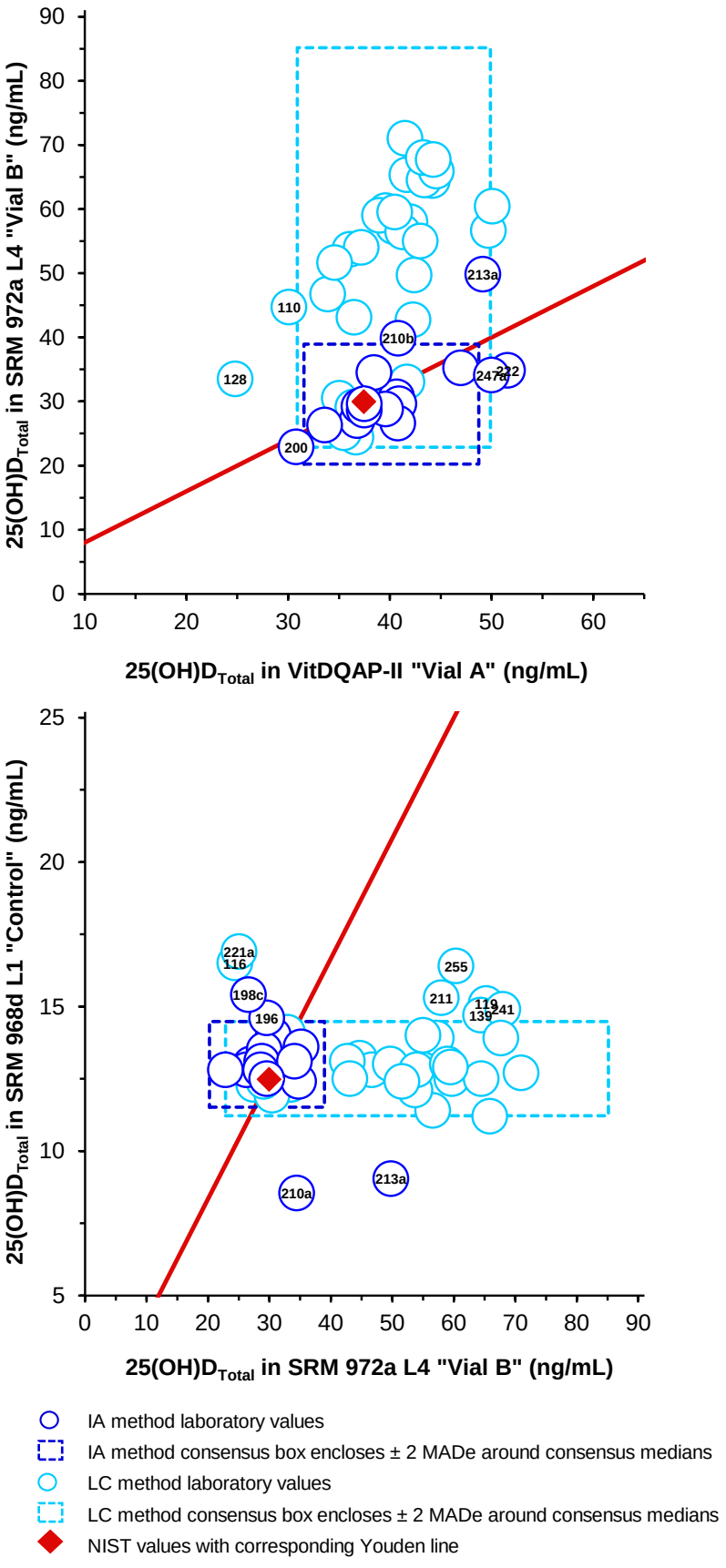
- Laboratory results that are not included in the consensus ranges include numbers 110, 128, 200, 210b, 213a, 222, and 247a
- The Youden line runs through the center of the IA consensus box and through the bottom corner of the LC consensus box for these materials, illustrating that the IA results are in better agreement with the NIST results for these materials.
- Most of the LC results for SRM 972a L4 are higher than both the Youden line and the IA consensus box; however, the LC box overlaps both the Youden line and the IA consensus box because the LC consensus box is very large in the SRM 972a L4 dimension (y-axis).

SRM 972a L4 (Vial B) and SRM 968d L1 (Control): **Figure 4 B**

- Laboratory results that are not included in the consensus ranges include numbers 116, 119, 139, 196, 198c, 210a, 211, 213a, 221a, 241, and 255
- The Youden line runs through the center of the IA consensus box and through the left corner of the LC consensus box for these materials, illustrating that the IA results are in better agreement with the NIST results for these materials.
- Most of the LC results for SRM 972a L4 are higher than both the Youden line and the IA consensus box; however, the LC box overlaps both the Youden line and the IA consensus box because the LC consensus box is very large in the SRM 972a L4 dimension (x-axis).

Both of these Youden plots involving SRM 972a L4 (Vial B) reveal separation of the IA results and the majority of the LC results, further illustrating the difference in results for the two techniques for the material with high levels of 3-epi-25(OH)D_3.

Figure 4. Youden comparison plot of the results for 25(OH)D_{Total} in
A) VitDQAP-II (Vial A) and SRM 972a L4 (Vial B) and
B) 972a L4 (Vial B) and SRM 968d L1 (Control) for all methods



25(OH)D_3 and 3-epi-25(OH)D_3 in VitDQAP-II (Vial A), SRM 972a L4 (Vial B), and SRM 968d L1 (Control): LC methods only

Of the two major techniques IA and LC, only the LC methods can independently measure the 25(OH)D_2 and 25(OH)D_3 components of 25(OH)D_{Total}, and therefore 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}. The 25(OH)D_2 metabolite does not contribute significantly to the 25(OH)D_{Total} in either of the two study materials or the control. However, both VitDQAP-II (Vial A) and SRM 972a L4 (Vial B) contain significant concentrations of the 3-epi-25(OH)D_3 metabolite. Therefore, the 3-epi-25(OH)D_3 needs to be separated from 25(OH)D_3 to avoid a significant measurement bias.

In the Summer 2013 comparability study, all but one of the LC participants reported values for 25(OH)D_3, and the reported values are summarized in **Table 2**.

Of the 33 LC participants, nine used methods that separated the 3-epi-25(OH)D_3 interference from 25(OH)D_3 (**Appendix A-2, A-3**). Of those nine, seven participants reported values for 3-epi-25(OH)D_3 in the study samples and in the control, and the results are summarized in **Table 3.**

For both **Table 2** and **Table 3**, the community results are summarized at the bottom of the table for all LC methods and for the LC-MS^n methods only. These summarized results include N, the median value, the MADe, and the CV%. **Table 2** and **Table 3** also present the NIST values and the 95% confidence limits (U_{95}) for 25(OH)D_3 and 3-epi-25(OH)D_3, respectively, in the study and control materials.

For the participant results for SRM 972a L4 (Vial B), the consensus variability is large for 25(OH)D_3 (30%). The source of the measurement uncertainty for 25(OH)D_3 is the wide-ranging, bimodal results from labs that separate the 3-epi-25(OH)D_3 and those that do not. However, the results for the seven participants that measure 3-epi-25(OH)D_3 exhibit relatively low variability for VitDQAP-II (Vial A) and SRM 972a L4 (Vial B) with a CV ≈ 8%.

Table 2. Summary of LC participant data and community results for 25(OH)D_3 (ng/mL) in the study samples and control.

		VitDQAP-II	SRM 972a L4	SRM 968d L1
Lab	Method	Vial A	Vial B	Control
056	LC-MS/MS	35.8	26.8	12.1
060	LC-MS/MS	39.4	30.9	12.8
110	LC-UV	30.1	44.7	13.2
116	LC-MS/MS	36.7	24.4	16.5
119	LC-MS/MS	41.7	65.3	15.1
128	LC-MS/MS	24.8	33.5	12.3
187	LC-MS/MS	39.6	59.7	12.5
194	LC-MS/MS	43.4	64.5	12.5
197	LC-MS/MS	33.9	46.7	12.8
198a	LC-MS/MS	49.7	56.6	11.4
199	LC-MS/MS	41.5	71.0	12.7
209	LC-MS/MS	42.4	49.7	13.0
211	LC-MS/MS	42.0	58.0	15.3
214c	LC-MS/MS	36.1	53.7	12.1
215	LC-MS/MS	40.4	56.8	13.9
216	LC-MS/MS	37.8	28.9	12.5
217	LC-MS/MS	37.2	54.0	12.8
218b	LC-MS/MS	42.3	42.7	13.1
220	LC-MS/MS	39.0	59.0	13.0
221a	LC-MS/MS	35.5	25.1	16.9
225	LC-MS/MS	44.6	65.9	11.2
228a	LC-MS/MS	34.6	51.6	12.4
231	LC-UV	41.3	56.4	n/r
241	LC-MS/MS	43.3	68.0	14.9
242	LC-MS/MS	35.1	30.5	11.9
244	LC-MS/MS	36.5	43.1	12.5
248	LC-MS/MS	42.7	55.4	14.1
249	LC-MS/MS	36.4	29.1	12.4
250	LC-MS/MS	44.3	67.7	13.9
253	LC-MS/MS	41.2	32.5	13.9
254a	LC-MS/MS	40.3	59.4	12.9
255	LC-MS/MS	49.5	59.9	16.1
LC methods	N	32	32	31
	Median	40.0	53.9	12.8
	MADe	4.9	15.9	0.7
	CV%	12.2	29.5	5.8
LC-MS^n	N	29	29	29
	Median	40.3	54.0	12.8
	MADe	4.6	16.8	0.7
	CV%	11.4	31.0	5.8
	NIST Value	37.0	29.4	12.4
	U_{95}	0.4	0.9	0.3

Table 3. Summary of LC participant data and community results for 3-epi-25(OH)D_3 (ng/mL) in the study samples and control.

		VitDQAP-II	SRM 972a L4	SRM 968d L1
Lab	Method	Vial A	Vial B	Control
056	LC-MS/MS	3.5	24.3	1.8
060	LC-MS/MS	3.3	28.7	0.7
116	LC-MS/MS	5.8	27.3	<4.0
216	LC-MS/MS	3.1	27.0	0.6
242	LC-MS/MS	2.6	20.7	0.6
249	LC-MS/MS	3.2	28.5	0.4
253	LC-MS/MS	3.4	25.8	0.7
LC methods	N	7	7	6
	Median	3.3	27.0	0.7
	MADe	0.3	2.2	0.1
	CV%	7.6	8.2	13.6
LC-MS^n	N	7	7	6
	Median	3.3	27.0	0.7
	MADe	0.3	2.2	0.1
	CV%	7.6	8.2	13.6
	NIST Value	3.4	26.4	0.66
	U_{95}	0.1	2.1	0.02

Figure 5 and **Figure 6** present direct graphical comparisons of the LC results for 25(OH)D_3 and 3-epi-25(OH)D_3, respectively, for A) VitDQAP-II (Vial A) and SRM 972a L4 (Vial B), and B) SRM 972a L4 (Vial B) and SRM 968d L1 (Control). In each plot, there is one blue consensus box for the LC results. Laboratory results that are within the consensus range for both study materials are within the blue consensus box. Conversely, laboratory results that fall outside of (or on the edge of) the consensus box are not included in the consensus range 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.

25(OH)D_3 in VitDQAP-II (Vial A) and SRM 972a L4 (Vial B): **Figure 5 A**

- Laboratory results that are not included in the consensus range include numbers 110 and 128.
- The Youden line runs through the cluster of LC results that separate the 25(OH)D_3 and 3-epi-25(OH)D_3, illustrating the better agreement of these results with the NIST values for these materials.
- Most of the LC results for SRM 972a L4 are higher than the Youden line; however, the Youden line overlaps the bottom of the LC consensus box, which is very large in the SRM 297a L4 dimension (y-axis).

25(OH)D_3 in SRM 972a L4 (Vial B) and SRM 968d L1 (Control): **Figure 5 B**

- Laboratory results that are not included in the consensus range include numbers 116, 119, 211, 221a, 225, 241, and 255.
- The Youden line runs through the cluster of the LC results that separate the 25(OH)D_3 and 3-epi-25(OH)D_3, illustrating the better agreement of these results with the NIST values for these materials.
- Most of the LC results for SRM 972a L4 are higher than the Youden line; however, the Youden line overlaps the left corner of the LC consensus box, which is very large in the SRM 297a L4 dimension (x-axis).

3-epi-25(OH)D_3 in VitDQAP-II (Vial A) and SRM 972a L4 (Vial B): **Figure 6 A**

- Laboratory results that are not included in the consensus range include numbers 116 and 242.
- The Youden line runs through the center of the consensus box, indicating good agreement of the LC results with the NIST values for these materials.

3-epi-25(OH)D_3 in SRM 972a L4 (Vial B) and SRM 968d L1 (Control): **Figure 6 B**

- Only three of the laboratory results are included in the consensus range for these materials because of the variability of the results for SRM 968d L1 (y-axis).
- The Youden line runs through the center of the consensus box, indicating good agreement of the LC results with the NIST values for these materials even with the higher variability of results for SRM 968d L1 (y-axis).

Figure 5. Youden comparison plot of the results for 25(OH)D_3 in A) VitDQAP-II (Vial A) and SRM 972a L4 (Vial B) and B) SRM 972a L4 (Vial B) and SRM 968d L1 (Control) for LC methods.

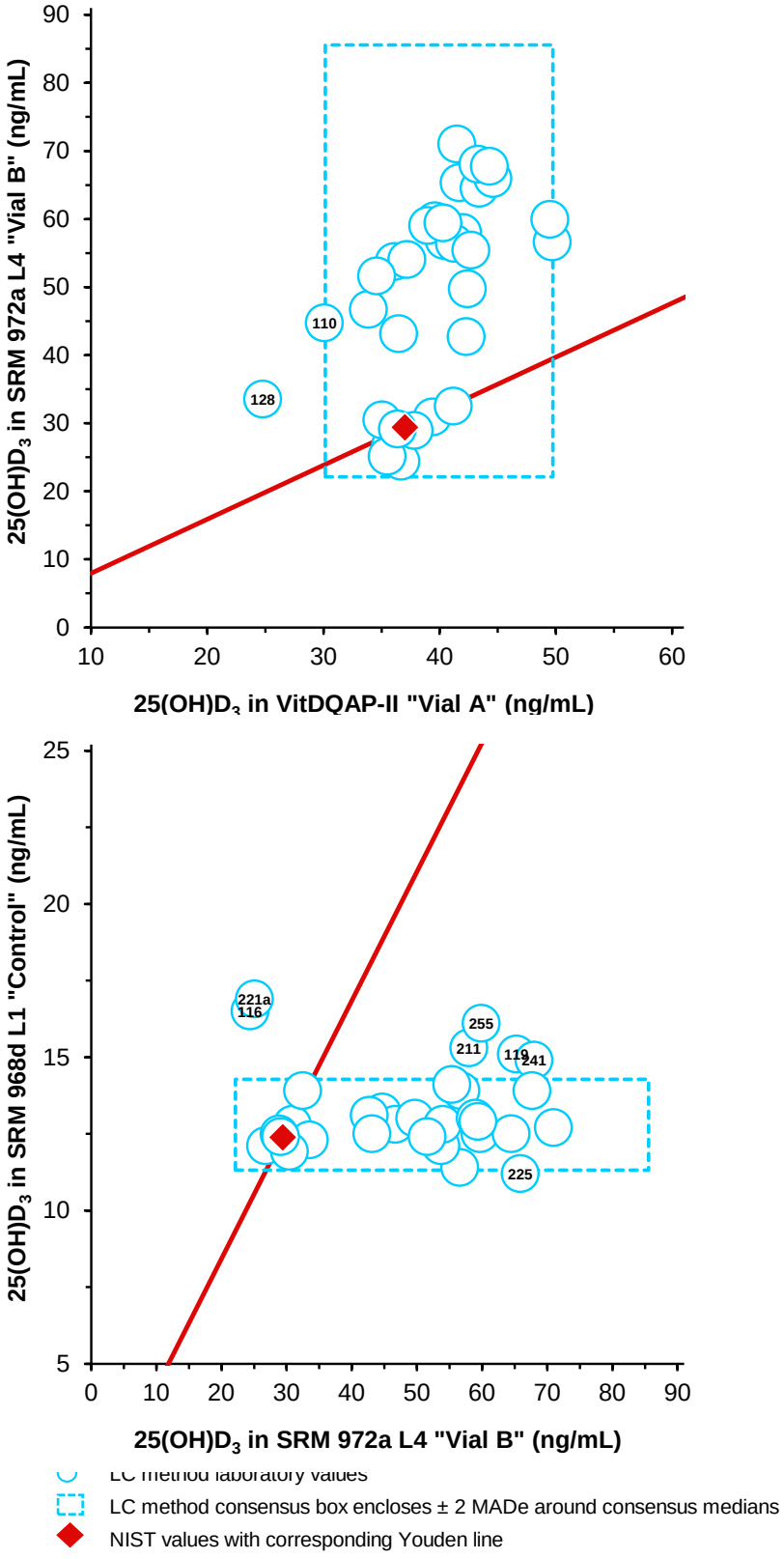
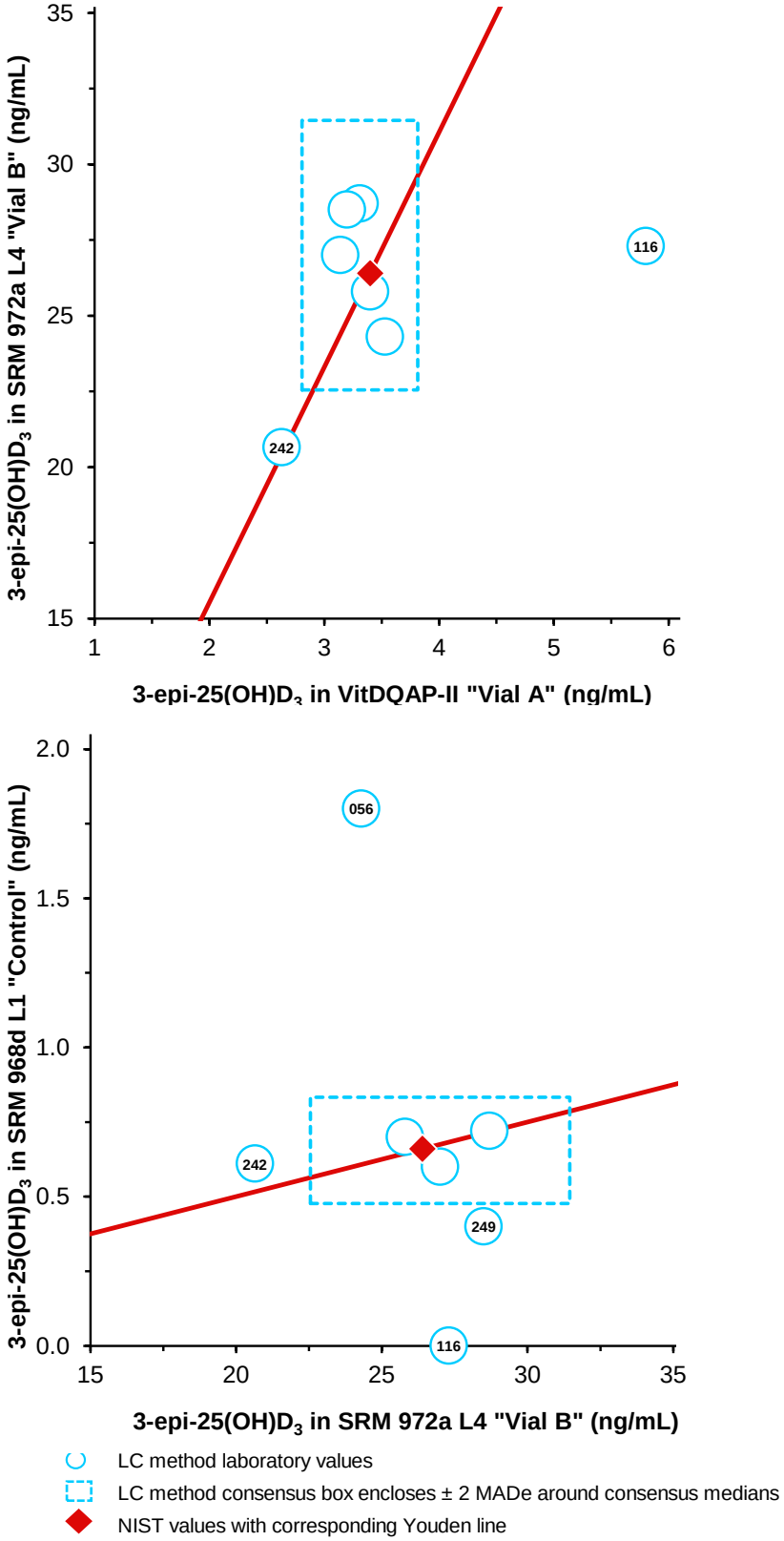


Figure 6. Youden comparison plot of the results for 3-epi-25(OH)D_3 in A) VitDQAP-II (Vial A) and SRM 972a L4 (Vial B) and B) SRM 972a L4 (Vial B) and SRM 968d L1 (Control) for LC methods.



Conclusions from the Summer 2013 Comparability Study of the VitDQAP

In the six previous comparability studies of the VitDQAP, 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 Summer 2013 comparability study, both VitDQAP-II (Vial A) and SRM 968d L1 (Control) also contain predominantly $25(OH)D_3$. The participant results for VitDQAP-II (Vial A) and SRM 968d L1 (Control) had a CV of 12% and 7%, respectively, and the all-method median values were biased slightly high relative to the NIST values, indicating the performance for these two materials is comparable to similar materials previously evaluated in the VitDQAP.

For SRM 972a L4 (Vial B), however, which contained similar concentrations of both $25(OH)D_3$ and 3-epi-$25(OH)D_3$, the results were not comparable. The majority of the LC methods did not separate the 3-epi-$25(OH)D_3$ interference from the $25(OH)D_3$ measurement and obtained results for $25(OH)D_{Total}$ that were biased significantly high, which led to the largest all-method CV (47%) to date for any material evaluated in the VitDQAP. While VitDQAP-II (Vial A) also contained a measureable amount of 3-epi-$25(OH)D_3$ and should have produced similar results, the 3-epi-$25(OH)D_3$ was low enough ($\approx$ 3.4 ng/mL) that the bias was not observable in the overall method CV of 12% for that study material. Participants that use LC methods are encouraged to utilize chromatographic conditions and columns that separate the 3-epi-$25(OH)D_3$ interference to eliminate this potential measurement bias.

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

Laboratory Number	*IA Method*	*Sample Preparation*	*Vendor/kit**
17	CLIA	n/r	A
30	RIA	Samples were extracted with acetonitrile	D
86a	CLIA	n/r	A
183b	CLIA	n/r	A
188	CLIA	None	B
196	CLIA	The human serum samples were analyzed neat	A
198c	CLIA	n/r	n/r
200	RIA	n/r	D
210a	RIA	Sample was extracted with acetonitrile	D
210b	CLIA	n/r	C
213a	CLIA	Sample was thawed and gently mixed prior to analysis	C
214b	CLIA	n/r	n/r
218a	CLIA	Direct analysis	n/r
222	CLIA	n/r	B
247a	CLIA	Sample was thawed, mixed well and used in the assay	B
254b	CLIA	n/r	A

n/r = not reported

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

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

Laboratory Number	*Internal Standard (IS)*	*Sample Preparation*	*Chromatographic Conditions*	*Detection: MRM ions*
56	25(OH)D_2-d_3; 25(OH)D_3-d_6; 3-epi-25(OH)D_3-d_3	Samples were extracted with hexane, evaporated, then reconstituted with 69% methanol	PFP column (100 x 2.1 mm; 1.9 µm); isocratic elution; flow 0.4 mL/min	25(OH)D_3 383/365; 25(OH)D_3-d_6 389/371; 25(OH)D_2 395/377; 25(OH)D_2-d_3 398/380; 3-epi-25(OH)D_3 383/365
60	25(OH)D_3 -d_6	IS was added, and then samples were extracted with acetonitrile, evaporated, and reconstituted with 80% methanol/20% water	PFP column (100 x 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_2 413/355; 3-epi-25(OH)D3 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 mixed with ethanol containing the IS, equilibrated, mixed, extracted with hexane, evaporated, and reconstituted in mobile phase	C18 column (150 x 3.0 mm; 2.7 µm); Gradient with water and methanol (0.1% formic acid)	25(OH)D_3 401/383; 25(OH)D_3-d_6 407/371 and 407/389; 25(OH)D_2 395/209 and 395/251
128	n/r	n/r	n/r	n/r
187	n/r	SPE	C18 column (50 x 2.1 mm; 3 µm)	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 x 2mm); 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 (200 µL), calibrator and control sample followed by mixing, centrifugation, and analysis	C18 column (50 x 4.6 mm; 5 µm); column temp 45°C; gradient with water and methanol; flow 1.0 mL/min	n/r
198a	25(OH)D_3 -d_6	Proteins precipitated with methanol, followed by hexane extraction, centrifugation, evaporation under N_2, and reconstitution in methanol (0.1% formic acid)	C18 column (50 x 2.1 mm; 3.5 µm); isocratic elution with 85% methanol (0.1% formic acid); flow 0.5 mL/min	25(OH)D_3 401/383, 401/365; 25(OH)D_2 413/395, 413/355; 25(OH)D_3-d_6 407/389, 407/371
199	n/r	n/r	n/r	n/r
209	25(OH)D_3 -d_6	Proteins were precipitated with $ZnSO_4$ in methanol	C8 column (50 x 2 mm; 5 µm); gradient with water/methanol; flow 0.7 mL/min	25(OH)D_3 383/229,383/211; 25(OH)D_3-d_6 389/211; 25(OH)D_2 395/269, 395/119
211	25(OH)D_3-d_6	Proteins precipitated with acetonitrile containing IS followed by centrifugation	Turbulent flow column (32 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)
214c	25(OH)D_3-d_6	Samples were extracted with hexane, centrifuged, evaporated, and filtered	Column (50 x 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 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	Derivatized deuteriated standard	Samples extracted using liquid-liquid extraction then labeled with a derivatization reagent	Revered-phase column (150 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 SPE	C8 column (50 x 2.1 mm; 1.7 µm); gradient of 70% to 98% methanol (with 0.1% formic acid); flow 0.4 mL/min	25(OH)D_3 401/159 (quant), 401/383 (qual); 25(OH)D_2 413/83 (quant), 413/395 (qual)
218b	25(OH)D_2-d_3 and 25(OH)D_3-d_3	Sample was extracted, filtered, centrifuged, etc.	Phenyl column (50 x 2.1 mm; 1.7 µm); flow 0.45 mL/min	25(OH)D_3 401; 25(OH)D_2 413
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 SPE	C18 column (20 x 2.1mm, 2.7µm); gradient with water and acetonitrile; flow 1 mL/min; column 40 °C	MRM with dehydrated precursor and product ions
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
225	n/r	n/r	n/r	n/r
228a	D8-labeled compound	Proteins precipitated	n/r	n/r
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	Water with 0.1% formic acid (500 µL) and the IS (400 µL) were added to the sample (400 µL), followed by centrifugation and dilution of supernatant with water	PFP column (150 x 2 mm; 3 µm); isocratic elution with 18% water/ 82% methanol/ 0.1% formic acid; flow 0.35 mL/min	25(OH)D_3 383/257; 25(OH)D_2 395/269; 25(OH)D_3-d_6 389/263; 3-epi-25(OH)D_3 383/257; 3-epi-25(OH)D_2 395/269
244	25(OH)D_3-d_6	Protein precipitation followed by filtration	CN column; mobile phase consisting of distilled water (formic acid) and methanol	25(OH)D_2 395/269; 25(OH)D_3 383/211
248	25(OH)D_2-d_3 and 25(OH)D_3-d_3	Serum is precipitated with methanol/$ZnSO_4$ containing the IS and then with acetonitrile, centrifuged, and injected	Reversed-phase column (75 x 2.1 mm; 2.5 µm); gradient with water and methanol; flow 0.55 mL/min	25(OH)D_3 383/257, 383/365; 25(OH)D_2 395/269, 395/377; 25(OH)D_3-d_3 386/257, 386/368; 25(OH)D_2-d_3 398/380, 398/272
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 x 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
250	n/r	Protein crash followed by SPE	Phenyl column (50 x 2.1 mm); gradient with 15% water and 85% methanol; flow 0.45 mL/min	MRM
253	25(OH)D_2-d_3 and 25(OH)D_3-d_3	The sample was extracted, centrifuged, and derivatized	C18 column (150 x 2.1 mm); isocratic separation with 22.5% water/ 77.5% methanol; flow 0.2 mL/min	25(OH)D_2 588; 25(OH)D_3 576
254a	25(OH)D_3-d_6	IS was added to each sample (200 µL) and mixed; acetontrile was added, followed by mixing, centrifugation, and injection	C8 column (50 x 2.0 mm; 3 µm); elution with water and acetonitrile, each containing 0.1% formic acid	25(OH)D_3 383/229, 383/211; 25(OH)D_2 395/269, 395/119; 25(OH)D_3-d_6 389/211
255	deuterium labeled compound	Samples were extracted and derivatized with 4-phenyl-l ,2,4-triazoline-3,5-dione	Reversed-phase column (50 x 2.1 mm); gradient with methanol; flow 0.5 mL/min	25(OH)D_3 607/298; 25(OH)D_2 619/298

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

Appendix A-3. Summary of LC-UV methods used by 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 x 100 mm; 1.8 µm); gradient with acetonitrile/methanol (85:15) and isopropanol (100%)	267 nm
139	Proprietary	The sample was extracted, centrifuged and injected	Reversed-phase column heated to 40 °C, isocratic separation with proprietary mobile phase; flow 1 mL/min	264 nm
231	1alpha(OH)D_3	Samples were extracted with hexane/dichloromethane, evaporated and reconstituted with mobile phase (phosphate buffer/acetonitrile)	Reversed-phase column (250 x 4.5 mm; 5µm), isocratic separation with 14% phosphate buffer, 86% acetonitrile; flow 1.2 mL/min	265 nm

n/a = not applicable

Appendix B-1. Raw participant data and NIST results for 25(OH)D_2, 25(OH)D_3, 3-epi-25(OH)D_3, and 25(OH)D_{Total} in VitDQAP-II (Vial A), SRM 972a L4 (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)			epi-25(OH)D_3 (ng/mL)		
		VitDQAP-II	SRM 972a L4	SRM 968d L1	VitDQAP-II	SRM 972a L4	SRM 968d L1	VitDQAP-II	SRM 972a L4	SRM 968d L1	VitDQAP-II	SRM 972a L4	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
017	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	36.8	27.0	13.0	n/r	n/r	n/r
030	RIA	n/a	n/a	n/a	n/a	n/a	n/a	33.6	26.3	12.8	n/r	n/r	n/r
056	LC-MS/MS	0.6	0.7	0.2	35.8	26.8	12.1	36.4	27.5	12.3	3.5	24.3	1.8
060	LC-MS/MS	<0.5	<0.5	<0.5	39.4	30.9	12.8	39.4	30.9	12.8	3.3	28.7	0.7
086a	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	40.7	30.7	14.0	n/r	n/r	n/r
110	LC-UV	<2	<2	<2	30.1	44.7	13.2	30.1	44.7	13.2	n/r	n/r	n/r
116	LC-MS/MS	<3.3	<3.3	<3.3	36.7	24.4	16.5	36.7	24.4	16.5	5.8	27.3	<4.0
119	LC-MS/MS	n/r	n/r	n/r	56.0	87.0	20.0	56.0	87.0	20.0	n/r	n/r	n/r
128	LC-MS/MS	n/a	n/a	n/a	24.8	33.5	12.3	24.8	33.5	12.3	n/r	n/r	n/r
139	LC-UV	n/r	n/r	n/r	n/r	n/r	n/r	44.2	64.4	14.7	n/r	n/r	n/r
183b	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	37.0	29.2	13.5	n/r	n/r	n/r
187	LC-MS/MS	< 1.5	< 1.5	< 1.5	39.6	59.7	12.5	39.6	59.7	12.5	n/r	n/r	n/r
188	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	47.0	35.2	13.6	n/r	n/r	n/r
194	LC-MS/MS	<7	<7	<7	43.4	64.5	12.5	43.4	64.5	12.5	n/r	n/r	n/r
196	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	40.9	29.6	14.6	n/r	n/r	n/r
197	LC-MS/MS	<0.5	<0.5	<0.5	33.9	46.7	12.8	33.9	46.7	12.8	n/r	n/r	n/r
198a	LC-MS/MS	<5.0	<5.0	<5.0	49.7	56.6	11.4	49.7	56.6	11.4	n/r	n/r	n/r
198c	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	40.8	26.6	15.4	n/r	n/r	n/r
199	LC-MS/MS	< 2	< 2	< 2	41.5	71.0	12.7	41.5	71.0	12.7	n/r	n/r	n/r
200	RIA	n/a	n/a	n/a	n/a	n/a	n/a	30.8	22.9	12.8	n/r	n/r	n/r
209	LC-MS/MS	<1.0	<1.0	<1.0	42.4	49.7	13.0	42.4	49.7	13.0	n/r	n/r	n/r
210a	RIA	n/a	n/a	n/a	n/a	n/a	n/a	38.5	34.5	8.5	n/r	n/r	n/r
210b	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	40.8	39.8	< 3.0	n/r	n/r	n/r
211	LC-MS/MS	0.0	0.0	0.0	42.0	58.0	15.3	42.0	58.0	15.3	n/r	n/r	n/r
213a	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	49.2	49.8	9.0	n/r	n/r	n/r
214b	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	39.6	28.8	13.1	n/r	n/r	n/r
214c	LC-MS/MS	<1.0	<1.0	<1.0	36.1	53.7	12.1	36.1	53.7	12.1	n/r	n/r	n/r
215	LC-MS/MS	0.0	0.4	0.0	40.4	56.8	13.9	40.4	57.2	13.9	n/r	n/r	n/r
216	LC-MS/MS	0.4	0.4	0.1	37.8	28.9	12.5	38.2	29.3	12.6	3.1	27.0	0.6
217	LC-MS/MS	<2	<2	<2	37.2	54.0	12.8	37.2	54.0	12.8	n/r	n/r	n/r
218a	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	37.5	28.6	12.8	n/r	n/r	n/r
218b	LC-MS/MS	0.0	0.0	0.0	42.3	42.7	13.1	42.3	42.7	13.1	n/r	n/r	n/r
220	LC-MS/MS	<5.0	<5.0	<5.0	39.0	59.0	13.0	39.0	59.0	13.0	n/r	n/r	n/r
221a	LC-MS/MS	0.0	0.0	0.0	35.5	25.1	16.9	35.5	25.1	16.9	n/r	n/r	n/r
222	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	51.6	34.8	12.4	n/r	n/r	n/r
225	LC-MS/MS	<5.0	<5.0	<5.0	44.6	65.9	11.2	44.6	65.9	11.2	n/r	n/r	n/r
228a	LC-MS/MS	n/d	n/d	n/d	34.6	51.6	12.4	34.6	51.6	12.4	n/r	n/r	n/r
231	LC-UV	n/d	n/d	n/d	41.3	56.4	n/r	41.3	56.4	n/r	n/r	n/r	n/r
241	LC-MS/MS	< 0.5	< 0.5	n/d	43.3	68.0	14.9	43.3	68.0	14.9	n/r	n/r	n/r
242	LC-MS/MS	n/d	n/d	n/d	35.1	30.5	11.9	35.1	30.5	11.9	2.6	20.7	0.6
244	LC-MS/MS	<5	<5	<5	36.5	43.1	12.5	36.5	43.1	12.5	n/r	n/r	n/r
247a	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	50.0	34.1	13.1	n/r	n/r	n/r
248	LC-MS/MS	<3.0	<3.0	<3.0	42.7	55.4	14.1	43.0	55.0	14.0	n/r	n/r	n/r
249	LC-MS/MS	<0.8	<0.8	<0.8	36.4	29.1	12.4	36.4	29.1	12.4	3.2	28.5	0.4
250	LC-MS/MS	< 2.5	< 2.5	< 2.5	44.3	67.7	13.9	44.3	67.7	13.9	n/r	n/r	n/r
253	LC-MS/MS	0.5	0.5	0.2	41.2	32.5	13.9	41.7	33.0	14.1	3.4	25.8	0.7
254a	LC-MS/MS	0.1	0.1	0.0	40.3	59.4	12.9	40.5	59.5	12.9	n/r	n/r	n/r
254b	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	37.5	29.6	12.5	n/r	n/r	n/r
255	LC-MS/MS	0.6	0.5	0.3	49.5	59.9	16.1	50.1	60.4	16.4	n/r	n/r	n/r

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

	NIST Value	0.44	0.55	0.1*	37.0	29.4	12.4	37.5	30.0	12.5	3.4	26.4	0.66
	U_{95}	0.04	0.10	---	0.4	0.9	0.3	1.0	1.0	0.3	0.1	2.1	0.02

*estimated value (no uncertainty determined)