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NIST/NIH Vitamin D Metabolites Quality Assurance Program Report of Participant Results: Summer 2014 Comparability Study (Exercise 9)

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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 ninth exercise of this program, the Summer 2014 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) 2973 Vitamin D Metabolites in Frozen Human Serum (High Level) and VitDQAP-I (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 ninth study are reported along with a summary of the analytical methods used.

OVERVIEW OF THE SUMMER 2014 COMPARABILITY STUDY

For the Summer 2014 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. Standard Reference Material (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 VitDQAP-I, and Vial B was SRM 2973 Vitamin D Metabolites in Frozen Human Serum (High Level), 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 53 participants and 63 datasets (nine participants provided data from two or more methods) in the Summer 2014 comparability study. Twenty-three of the datasets originated from immunoassay (IA) techniques, including 15 from chemiluminescence immunoassay (CLIA), two from enzyme immunoassay (EIA), five from radioimmunoassay (RIA), and one from chemiluminescent enzyme immunoassay (CLEIA). **Appendix A-1** summarizes the IA methods used by the participants. Forty of the datasets originated from liquid chromatographic (LC) methods; of those, 35 were from LC with tandem mass spectrometric detection ($LC\text{-}MS^n$), 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 are summarized in **Appendix B**. The IA methods do not distinguish between $25(OH)D_3$ and $25(OH)D_2$, and 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 $25(OH)D_3$ in addition to $25(OH)D_{Total}$. The $25(OH)D_2$ concentration was below the detection limit for most reported methods in the control and study materials. Ten LC participants reported results for 3-epi-$25(OH)D_3$.

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 THE NIST METHOD USED TO EVALUATE THE CONTROL AND STUDY MATERIALS

NIST used isotope dilution LC-MS/MS (ID-LC-MS/MS) [1] to determine the vitamin D metabolites ($25(OH)D_3$, $25(OH)D_2$, and 3-epi-$25(OH)D_3$) 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).

The NIST values for $25(OH)D_3$, $25(OH)D_2$, and 3-epi-$25(OH)D_3$ in VitDQAP-I (Vial A) are reported with expanded uncertainties (*U*) that approximate a 95 % confidence interval. *U* incorporates components for measurement variability and measurement uncertainty associated with the density of the materials and the purity of the reference standards as well as a 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-I (Vial A), 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 values for $25(OH)D_{Total}$ in VitDQAP-I (Vial A) 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 uncertainty (*U*) incorporates measurement uncertainties for the two analytes.

For SRM 2973 (Vial B), the reported NIST value for $25(OH)D_3$ is a certified value. 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 [2]. The NIST values for $25(OH)D_2$, 3-epi-$25(OH)D_3$, and $25(OH)D_{Total}$ in SRM 2973 (Vial B) are reference values. A NIST reference value is a best estimate of the true value provided by NIST where all known or suspected sources of bias have not been fully investigated by NIST [2]. More detailed information about the characterization of SRM 2973 and the components of the expanded uncertainty may be found in the Certificate of Analysis, located on the NIST website [3].

[1] Tai, S. S.-C.; Bedner, M.; Phinney, K.W.; *Anal. Chem.* **2010** *82*, 1942-1948.
[2] 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
[3] http://www.nist.gov/srm/index.cfm

SUMMER 2014 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 VitDQAP-I (Vial A), SRM 2973 (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 VitDQAP-I (Vial A), SRM 2973 (Vial B), and SRM 968d L1 (Control).

Lab	Method	VitDQAP-I Vial A	SRM 2973 Vial B	SRM 968d L1 Control
017	CLIA	33.8	41.8	14.1
026	LC-MS/MS	30.7	38.3	12.0
030a	RIA	35.1	44.5	14.2
056a	LC-MS/MS	33.4	42.7	12.8
056b	LC-MS/MS	30.3	38.1	12.8
060	LC-MS/MS	28.0	37.0	12.0
110	LC-UV	32.5	43.3	12.6
116	LC-MS/MS	35.1	43.3	15.1
119	LC-MS/MS	30.5	37.6	n/r
150	LC-MS/MS	28.2	37.1	12.4
161b	LC-MS/MS	33.0	41.0	13.0
180	RIA	30.4	36.5	15.1
187	LC-MS/MS	33.8	44.8	12.1
188	CLIA	42.9	50.2	12.3
189	LC-UV	39.4	48.7	8.6
194	LC-MS/MS	33.9	38.8	12.1
196	CLIA	29.8	37.1	13.2
197	LC-MS/MS	30.3	40.8	12.4
199	LC-MS/MS	31.4	39.6	13.3
200	RIA	23.0	27.8	12.7
204a	CLIA	31.0	43.2	14.9
204b	LC-MS/MS	30.8	41.5	12.7
209	LC-MS/MS	34.0	48.8	12.4
210a	RIA	32.1	40.2	17.4
210b	CLIA	30.9	44.8	n/r
211	LC-MS/MS	37.8	49.1	12.6
212	LC-MS/MS	31.9	42.9	12.8
213a	CLIA	35.2	48.4	7.4
213b	EIA	25.2	32.3	13.9
214a	RIA	17.1	16.6	6.3
214b	CLIA	29.4	38.1	13.0
214c	LC-MS/MS	31.3	38.6	12.2
215	LC-MS/MS	34.8	40.8	14.0
216	LC-MS/MS	37.2	41.3	12.3
217	LC-MS/MS	39.2	48.8	16.4
218a	CLIA	31.1	40.7	14.0
218b	LC-MS/MS	38.1	42.1	14.7
220	LC-MS/MS	31.0	38.4	12.5
221a	LC-MS/MS	30.1	35.3	14.9
221b	LC-UV	34.3	42.7	38.5
222	CLIA	35.3	40.1	15.0
225	LC-MS/MS	32.9	44.6	13.4
228a	LC-MS/MS	37.0	45.7	12.0
242	LC-MS/MS	30.0	36.9	12.8
243a	LC-UV	29.8	37.0	12.9
243b	LC-MS/MS	30.0	37.0	12.6
244	LC-MS/MS	34.0	40.0	16.0
249	LC-MS/MS	30.4	41.2	12.8
251	LC-MS/MS	37.0	54.0	n/r
253	LC-MS/MS	30.8	39.6	12.2
255	LC-MS/MS	33.4	41.0	12.9
256	CLIA	33.0	35.8	11.4
257	CLIA	30.5	42.0	5.9
258	CLIA	33.9	64.4	13.6
259	LC-MS/MS	34.4	45.2	10.3
261	CLIA	24.2	37.4	6.6
262	CLIA	27.5	39.1	20.2
263	CLIA	33.7	37.6	12.8
264	LC-MS/MS	41.6	39.8	14.0
265	LC-MS/MS	35.0	44.0	13.0
266a	LC-UV	41.3	50.8	16.4
266b	EIA	29.2	38.3	18.5
267	CLEIA	30.4	39.3	12.4

n/r = not reported or not determined

		VitDQAP-I Vial A	SRM 2973 Vial B	SRM 968d L1 Control
All methods	N	63	63	60
	Median	32.1	40.8	12.8
	MADe	2.9	4.1	1.1
	CV%	9.1	10	8.7
IA methods	N	23	23	22
	Median	30.9	39.3	13.4
	MADe	4.2	4.0	2.0
	CV%	14	10	15
LC methods	N	40	40	38
	Median	33.2	41.1	12.8
	MADe	3.6	4.0	0.8
	CV%	11	9.8	6.4
LC-MS^n	N	35	35	33
	Median	33.0	41.0	12.8
	MADe	3.3	3.9	0.7
	CV%	9.9	9.4	5.8
	NIST Value	32.0	40.1	12.5
	U	0.8	0.8	0.4

n/r = not reported or not determined

For all participant datasets, the single reported values for 25(OH)D_{Total} in VitDQAP-I (Vial A), SRM 2973 (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 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 expanded 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.

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

- For the IA results, two reported values are outside of the consensus variability range (one CLIA, one RIA).
- For the LC results, two reported values are outside of the consensus variability range (one 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 slightly higher than 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.

SRM 2973 (Vial B): **Figure 2**

- For the IA results, five reported values are outside the consensus variability range (three CLIA, two RIA).
- For the LC results, two reported values are outside the consensus variability range (one LC-MS^n, one LC-UV).
- The consensus median values for both the IA and LC results are comparable to the NIST expanded uncertainty range (red lines).

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

- The consensus variability range is larger for the IA results than for the LC results.
- For the IA results, six reported values are outside of the consensus variability range (four CLIA, one EIA, one RIA).
- For the LC results, nine reported values are outside of the consensus variability range (six LC-MS^n, three LC-UV).
- The consensus median value for the IA results is slightly 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 variability range for both IA and LC.

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

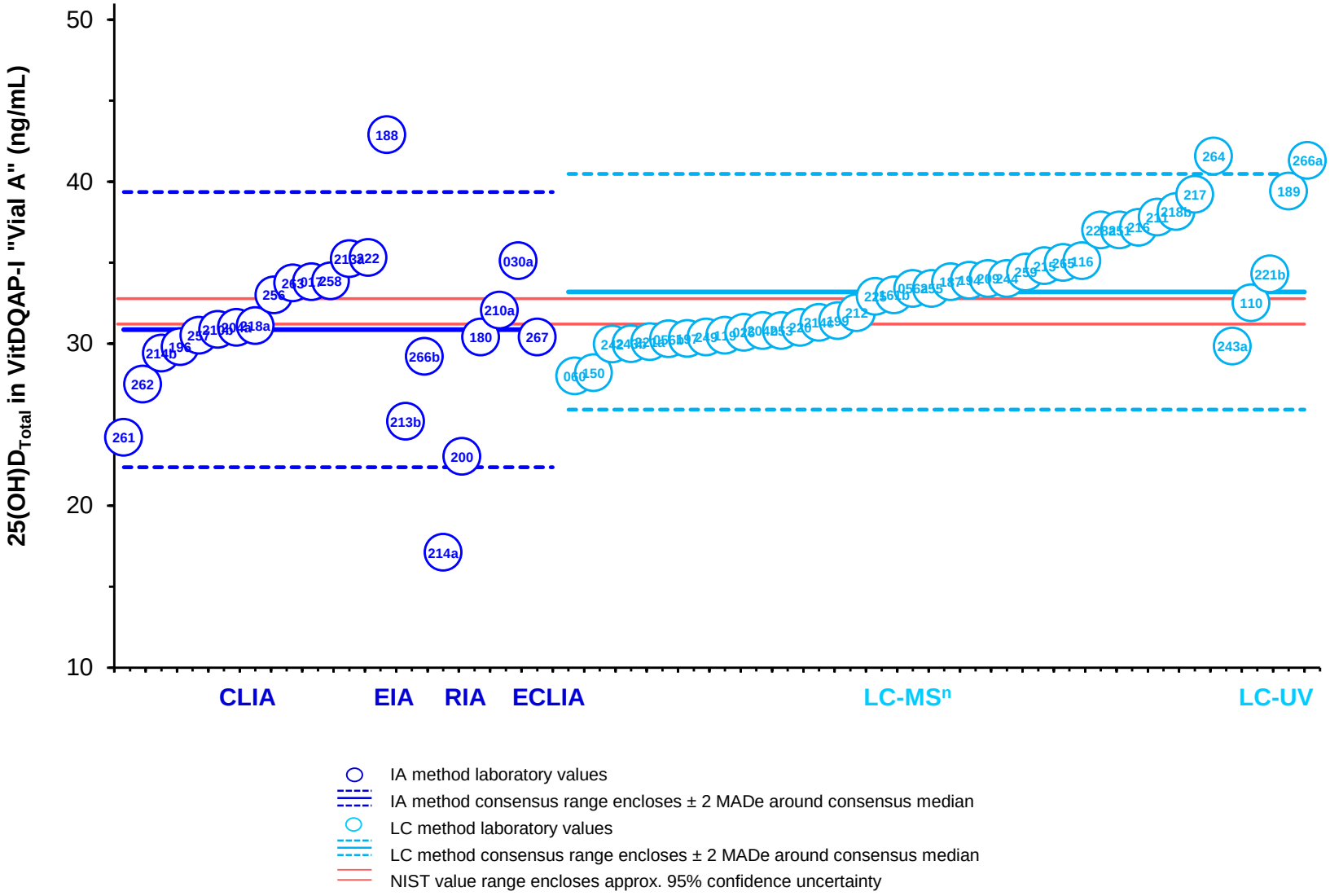


Figure 2. Participant and NIST results for 25(OH)D_{Total} in SRM 2973 (Vial B) as determined by immunoassay (CLIA, EIA, RIA, and CLEIA) and LC (LC-MS^n and LC-UV) methods.

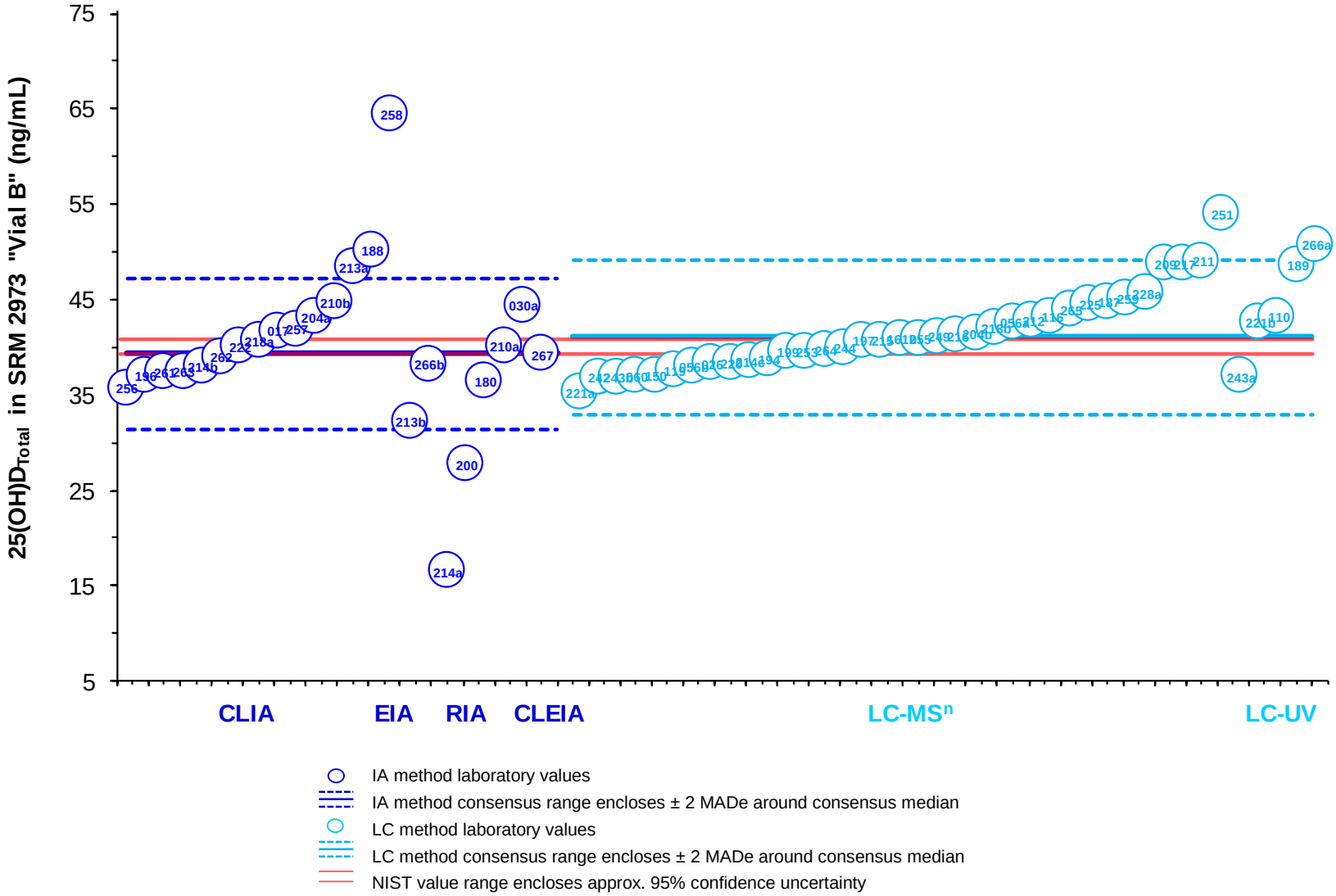


Figure 3. Participant and NIST results for 25(OH)D_{Total} in SRM 968d Level 1 (Control) as determined by immunoassay (CLIA, EIA, RIA, and CLEIA) and LC (LC-MS^n and LC-UV) methods.

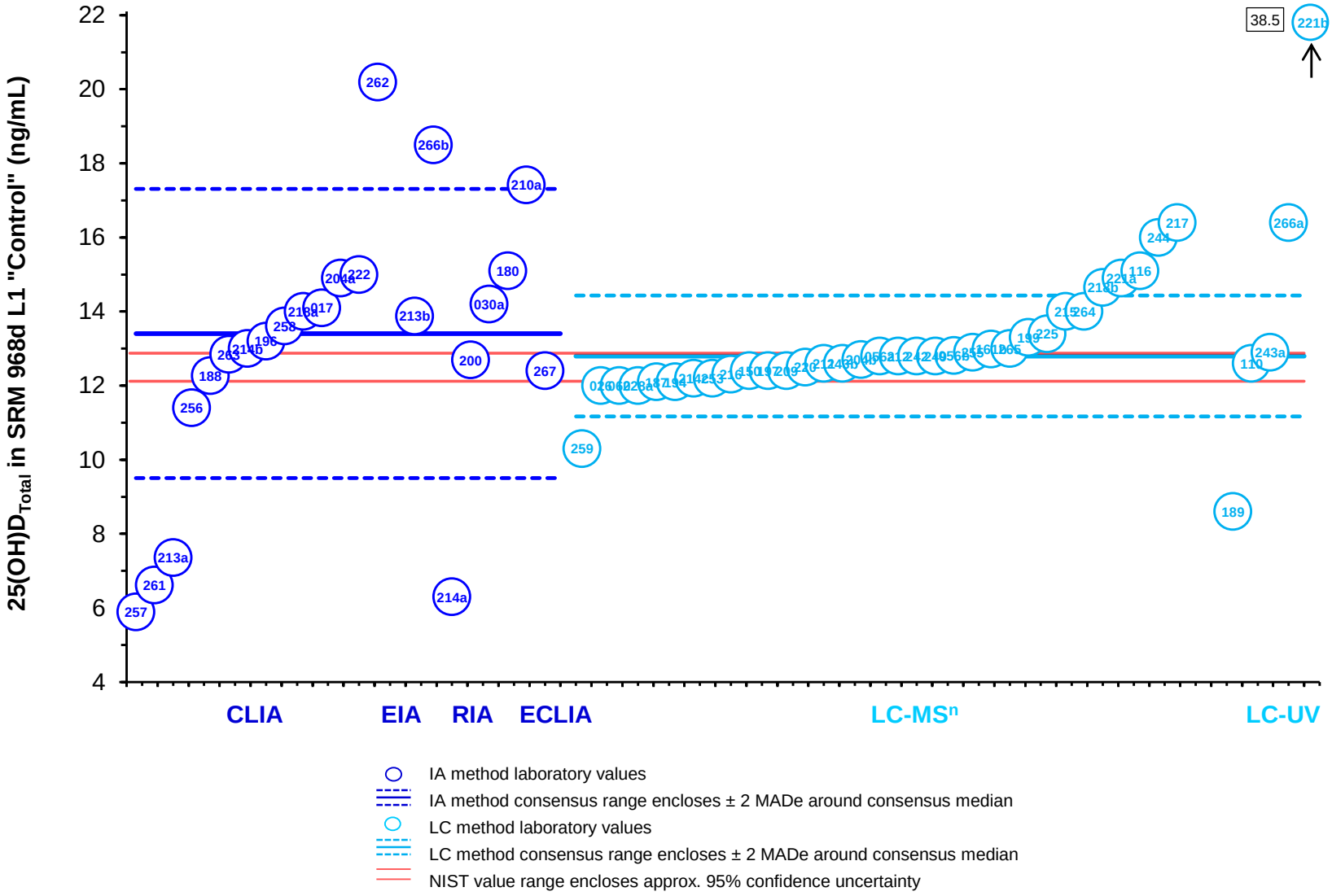


Figure 4 presents direct graphical comparisons of the 25(OH)D_{Total} results for a) VitDQAP-I (Vial A) and SRM 2973 (Vial B), and b) SRM 2973 (Vial B) and SRM 968d L1 (Control). In each plot, there are two blue consensus boxes, one for IA methods and one for LC methods (as indicated). Laboratory results 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.

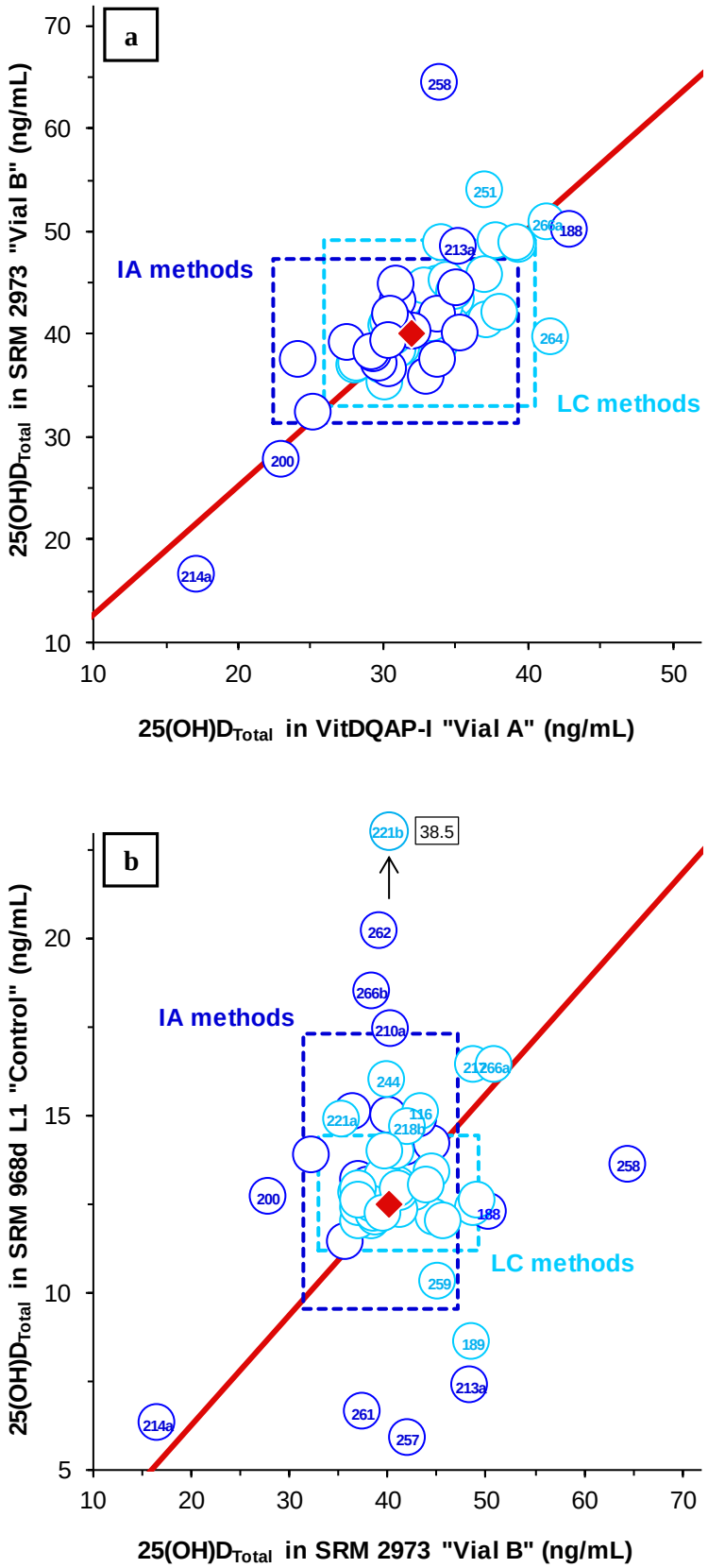
VitDQAP-I (Vial A) and SRM 2973 (Vial B): **Figure 4 a**

- IA results that are outside of the consensus range include numbers 188, 200, 213a, 214a, and 258.
- LC results that are outside of the consensus range include numbers 251, 264, and 266.
- The Youden line runs through the center of both the IA and LC consensus boxes, illustrating that both the IA and LC results are in agreement with each other and with the NIST results for these materials.

SRM 2973 (Vial B) and SRM 968d L1 (Control): **Figure 4 b**

- IA results that outside of the consensus range include numbers 188, 200, 210a, 213a, 214a, 257, 258, 261, 262, and 266b
- LC results that are outside of the consensus range include numbers 116, 189, 217, 218b, 221a, 221b, 244, 259, and 266a
- The Youden line runs through the center of the LC consensus box and toward the bottom of the IA consensus box, illustrating that the LC results are in slightly better agreement with the NIST results for these materials.

Figure 4. Youden comparison plot of the results for 25(OH)D_{Total} in a) VitDQAP-I (Vial A) and SRM 2973 (Vial B) and b) SRM 2973 (Vial B) and SRM 968d L1 (Control) for all methods.



LC method results for 25(OH)D_3, 25(OH)D_2, and 3-epi-25(OH)D_3

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}.

Of the 41 LC participants in the Summer 2014 comparability study, all but one lab reported values for 25(OH)D_3, and the study results for 25(OH)D_3 in the study materials and the control are presented in **Table 2**. Neither the study materials nor the control contained a significant concentration of 25(OH)D_2, but the study results for the 12 participants that reported results for this metabolite in at least one of the materials are presented in **Table 3**.

Since VitDQAP-I (Vial A) and SRM 2973 (Vial B) contain relatively high concentrations of 25(OH)D_3 (**Table 2**), the 3-epi-25(OH)D_3 metabolite, which frequently correlates with 25(OH)D_3, is also measureable in these materials. Ten LC participants reported values for the 3-epi-25(OH)D_3 metabolite in at least one of the materials, and the results are presented in **Table 4.** Because the biological significance of 3-epi-25(OH)D_3 remains uncertain, this metabolite is not included in the 25(OH)D_{Total} concentration. Therefore, LC methods that do not chromatographically separate this diastereomer from 25(OH)D_3 yield biased results for 25(OH)D_3 and hence 25(OH)D_{Total}.

The community results are summarized at the bottom of each table (**Table 2 - 4**), 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 %. The NIST values and the expanded uncertainties are also presented at the bottom of each table.

Table 2. Summary of LC participant data and NIST results for 25(OH)D_3 (ng/mL) in VitDQAP-I (Vial A), SRM 2973 (Vial B), and SRM 968d L1 (Control).

25(OH)D_3 (ng/mL)

		VitDQAP-I	SRM 2973	SRM 968d L1
Lab	Method	Vial A	Vial B	Control
026	LC-MS/MS	29.9	37.4	11.6
056a	LC-MS/MS	32.7	41.7	12.6
056b	LC-MS/MS	29.6	37.4	12.6
060	LC-MS/MS	27.4	36.4	11.8
116	LC-MS/MS	35.1	43.3	15.1
119	LC-MS/MS	30.5	37.6	n/r
139	LC-UV	46.2	33.7	12.7
150	LC-MS/MS	28.2	37.1	12.4
161b	LC-MS/MS	33.0	41.0	13.0
187	LC-MS/MS	33.8	44.8	12.1
189	LC-UV	39.4	48.7	8.6
194	LC-MS/MS	33.9	38.8	12.1
197	LC-MS/MS	30.3	40.8	12.4
199	LC-MS/MS	31.4	39.6	13.3
204b	LC-MS/MS	30.8	41.5	12.7
209	LC-MS/MS	34.0	48.8	12.4
211	LC-MS/MS	37.8	49.1	12.6
212	LC-MS/MS	31.9	42.9	12.8
214c	LC-MS/MS	31.3	38.6	12.2
215	LC-MS/MS	34.8	40.8	14.0
216	LC-MS/MS	36.4	40.6	12.2
217	LC-MS/MS	39.2	48.8	16.4
218b	LC-MS/MS	38.1	41.8	14.7
220	LC-MS/MS	31.0	38.4	12.5
221a	LC-MS/MS	30.1	35.3	14.9
221b	LC-UV	34.3	42.7	38.5
225	LC-MS/MS	32.9	44.6	13.4
228a	LC-MS/MS	37.0	45.7	12.0
242	LC-MS/MS	29.5	36.5	12.8
243a	LC-UV	29.5	36.6	12.7
243b	LC-MS/MS	29.3	36.4	12.7
244	LC-MS/MS	34.0	40.0	16.0
249	LC-MS/MS	30.4	41.2	12.8
251	LC-MS/MS	37.0	54.0	n/r
253	LC-MS/MS	30.1	39.0	12.1
255	LC-MS/MS	32.7	40.3	12.7
259	LC-MS/MS	34.4	45.2	10.3
264	LC-MS/MS	41.0	39.2	13.9
265	LC-MS/MS	35.0	44.0	13.0
266a	LC-UV	41.3	50.8	16.4
LC methods	N	40	40	38
	Median	33.0	40.8	12.7
	MADe	3.9	4.7	0.9
	CV%	12	12	6.7
LC-MS^n	N	35	35	33
	Median	32.7	40.8	12.7
	MADe	3.4	3.7	0.8
	CV%	10	9.1	6.4
	NIST Value	31.3	39.4	12.4
	U	0.8	0.8	0.4

n/r = not reported or not determined

Table 3. Summary of LC participant and NIST results for 25(OH)D_2 (ng/mL) in VitDQAP-I (Vial A), SRM 2973 (Vial B), and SRM 968d L1 (Control).

25(OH)D_2 (ng/mL)

		VitDQAP-I	SRM 2973	SRM 968d L1
Lab	Method	Vial A	Vial B	Control
026	LC-MS/MS	0.8	1.0	0.4
056a	LC-MS/MS	0.8	1.0	0.2
056b	LC-MS/MS	0.7	0.7	0.2
060	LC-MS/MS	0.6	0.6	0.2
216	LC-MS/MS	0.7	0.7	0.2
218b	LC-MS/MS	0.0	0.3	0.0
242	LC-MS/MS	0.5	0.4	n/d
243a	LC-UV	0.5	0.4	n/d
243b	LC-MS/MS	0.5	0.4	n/d
253	LC-MS/MS	0.7	0.6	0.1
255	LC-MS/MS	0.7	0.7	0.2
264	LC-MS/MS	0.6	0.6	0.1
LC methods	N	12	12	9
	Median	0.7	0.6	0.2
	MADe	0.1	0.2	0.1
	CV%	22	31	37
LC-MS^n	N	11	11	9
	Median	0.7	0.6	0.2
	MADe	0.1	0.1	0.1
	CV%	15	20	37
	NIST Value	0.68	0.65	0.1*
	U	0.06	0.02	---

n/d = not detected; *estimated value

Table 4. Summary of LC participant and NIST results for 3-epi-25(OH)D_3 (ng/mL) in VitDQAP-I (Vial A), SRM 2973 (Vial B), and SRM 968d L1 (Control).

3-epi-25(OH)D_3 (ng/mL)

		VitDQAP-I	SRM 2973	SRM 968d L1
Lab	Method	Vial A	Vial B	Control
026	LC-MS/MS	2.7	2.4	0.7
056a	LC-MS/MS	1.8	3.6	1.3
060	LC-MS/MS	1.8	2.4	0.7
150	LC-MS/MS	2.0	3.0	<2
216	LC-MS/MS	2.0	2.3	0.7
242	LC-MS/MS	1.5	1.7	0.6
243a	LC-UV	1.5	1.7	0.7
243b	LC-MS/MS	1.4	1.8	0.6
249	LC-MS/MS	1.5	1.8	0.5
253	LC-MS/MS	1.9	2.1	0.7
LC methods	N	10	10	9
	Median	1.8	2.2	0.70
	MADe	0.4	0.6	0.07
	CV%	20	29	11
LC-MS^n	N	9	9	8
	Median	1.8	2.3	0.70
	MADe	0.2	0.7	0.08
	CV%	13	32	12
	NIST Value	1.7	2.1	0.65
	U	0.1	0.1	0.03

< X = less than a quantitation limit of X

Discussion of the Summer 2014 Comparability Study Results

In the Summer 2014 study of the VitDQAP, $25(OH)D_3$ is the predominant vitamin D metabolite contributing to $25(OH)D_{Total}$ in the two study materials and the control material, which is consistent with most materials evaluated in the VitDQAP. However, the two study materials VitDQAP-I (Vial A) and SRM 2973 (Vial B) both contain relatively high levels of $25(OH)D_3$, with NIST values of 31.3 ng/mL ± 0.8 ng/mL and 39.4 ng/mL ± 0.8 ng, mL, respectively. SRM 2973 (Vial B) contains the highest concentration of $25(OH)D_3$ of any study materials evaluated in the VitDQAP to date.

The consensus variabilities for $25(OH)D_{Total}$ determined by the IA and LC techniques differ for SRM 968d L1 (Control), with values of 15 % and 6.4 %, respectively. When the IA and LC results for SRM 968d L1 (Control) are compared to the results from the previous two studies of the VitDQAP, the consensus variability for the LC methods has remained remarkably consistent around 6 % while the consensus variability for the IA methods has fluctuated between 6 % and 18 %, indicating that IA methods are in less control. However, the IA variability for the VitDQAP-I (Vial A) and SRM 2973 (Vial B) study materials of 14 % and 10 %, respectively, indicate that the IA method performance is relatively consistent for different materials/matrices and $25(OH)D_3$ concentrations. Conversely, the LC method variability of 11 % and 10 %, respectively, for the two study materials VitDQAP-I (Vial A) and SRM 2973 (Vial B) is somewhat higher than for SRM 968d L1 (Control), but still exhibits general consistency with performance on the blinded study samples containing predominantly $25(OH)D_3$ evaluated in previous studies.

The participant performance in the Summer 2014 study is generally consistent with performance in the eight prior studies, indicating that the relatively high levels of $25(OH)D_3$ and hence $25(OH)D_{Total}$ in the two study samples do not pose a particular measurement challenge. To date, the most significant variability in the participant results arose from study samples that contain relatively high levels of the $25(OH)D_2$ and 3-epi-$25(OH)D_3$ metabolites.

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

Laboratory Number	*IA Method*	*Sample Preparation*	*Vendor/kit**
17	CLIA	n/r	A
30a	RIA	Samples were extracted with acetonitrile	B
180	RIA	Samples were extracted with acetonitrile	B
188	CLIA	n/r	C
196	CLIA	No sample preparation required	A
200	RIA	Samples were extracted	B
204a	CLIA	n/r	A
210a	RIA	Sample was extracted with acetonitrile	B
210b	CLIA	n/r	D
213a	CLIA	Sample was thawed and gently mixed prior to analysis	D
213b	EIA	Samples, calibrators, and controls processed per manufacturer's protocol	E
214a	RIA	n/r	F
214b	CLIA	n/r	A
218a	CLIA	Direct analysis	n/r
222	CLIA	n/r	C
256	CLIA	n/r	A
257	CLIA	Sample was thawed at room temperature until analysis	D
258	CLIA	n/r	G
261	CLIA	No sample preparation required	G
262	CLIA	n/r	H
263	EIA	On board displacement	I
266b	EIA	n/r	E
267	CLEIA	n/r	J

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)	APPI 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)	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
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 reconstitued 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	APCI
187	n/r	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
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 deuteriated 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)
218b	25(OH)D_2-d_3 and 25(OH)D_3-d_3	Sample was extracted, filtered, centrifuged, etc.	Phenyl column (50 mm × 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; phospholipids removed with SPE	C18 column (20 mm × 2.1 mm, 2.7 µm); 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 the IS	CN column (50 mm × 3.0 mm; 1.8 µm); methanol/water gradient at 50 °C	APCI 25(OH)D_3 383/211; 25(OH)D_3-d_6 389/211; 25(OH)D_2 395/209
225	25(OH)D_3-d_6	Liquid-liquid extraction	C8 column (50 mm × 2.1 mm; 1.7 µm); gradient with methanol/water; flow 0.4 mL/min	25(OH)D_3 401/107; 25(OH)D_2 413/83
228a	^{13}C-labeled compound	Proteins precipitated	n/r	n/r
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 mm × 2 mm; 3 µm); isocratic elution with 18 % water/ 82 % methanol/ 0.1 % formic acid; flow 0.35 mL/min	APCI 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

243b	25(OH)D_3-d_6	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	PFP column (150 mm × 2 mm); isocratic separation with 85 % methanol/15 % water; flow 0.3 mL/m	APCI 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_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); isocratic separation with 77.5 % methanol/22.5 % water; flow 0.2 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 x 2.1 mm); gradient with methanol; flow 0.5 mL/min	25(OH)D_3 607/298; 25(OH)D_2 619/298
259	deuterium labeled 25(OH)D_3	Liquid-liquid extraction using hexane	C8 column; gradient with methanol/water/0.1 % formate	n/r
264	clozapine	Samples were extracted with tert-butyl methyl ether, evaporated, and derivatized with 4-phenyl-1,2,4-triazoline-3,5-dione	Reversed-phase column (150 mm × 4.6 mm); isocratic separation with 85 % methanol/15 % water/0.3 % formic acid; flow 1.4 mL/min	n/r
265	n/r	n/r	n/r	n/r

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 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
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
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	264 nm
266a	alpha calcidol	Proteins precipitated with acetonitrile and methanol, extracted with SPE, eluted with ethyl acetate, evaporated, and reconstituted in acetonitrile	C18 column (4.6 mm × 150 mm; 2.5 µm); gradient with acetonitrile and methanol; flow 0.5 mL/min	265 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-$25(OH)D_3$, and in VitDQAP-I (Vial A), SRM 2973 (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)		
		VitDQAP-I	SRM 2973	SRM 968d L1	VitDQAP-I	SRM 2973	SRM 968d L1	VitDQAP-I	SRM 2973	SRM 968d L1	VitDQAP-I	SRM 2973	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	33.8	41.8	14.1	n/a	n/a	n/a
026	LC-MS/MS	0.8	1.0	0.4	29.9	37.4	11.6	30.7	38.3	12.0	2.7	2.4	0.7
030a	RIA	n/a	n/a	n/a	n/a	n/a	n/a	35.1	44.5	14.2	n/a	n/a	n/a
056a	LC-MS/MS	0.8	1.0	0.2	32.7	41.7	12.6	33.4	42.7	12.8	1.8	3.6	1.3
056b	LC-MS/MS	0.7	0.7	0.2	29.6	37.4	12.6	30.3	38.1	12.8	n/r	n/r	n/r
060	LC-MS/MS	0.6	0.6	0.2	27.4	36.4	11.8	28.0	37.0	12.0	1.8	2.4	0.7
110	LC-UV	n/r	n/r	n/r	n/r	n/r	n/r	32.5	43.3	12.6	n/r	n/r	n/r
116	LC-MS/MS	<3.3	<3.3	<3.3	35.1	43.3	15.1	35.1	43.3	15.1	<4.0	<4.0	<4.0
119	LC-MS/MS	n/d	n/d	n/d	30.5	37.6	n/r	30.5	37.6	n/r	n/r	n/r	n/r
139	LC-UV	n/r	n/r	n/r	46.2	33.7	12.7	n/r	n/r	n/r	n/r	n/r	n/r
150	LC-MS/MS	<2	<2	<2	28.2	37.1	12.4	28.2	37.1	12.4	2.0	3.0	<2
180	RIA	n/a	n/a	n/a	n/a	n/a	n/a	30.4	36.5	15.1	n/a	n/a	n/a
187	LC-MS/MS	<1.5	<1.5	<1.5	33.8	44.8	12.1	33.8	44.8	12.1	n/r	n/r	n/r
188	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	42.9	50.2	12.3	n/a	n/a	n/a
189	LC-UV	0.0	0.0	0.0	39.4	48.7	8.6	39.4	48.7	8.6	n/r	n/r	n/r
194	LC-MS/MS	<7.0	<7.0	<7.0	33.9	38.8	12.1	33.9	38.8	12.1	n/r	n/r	n/r
196	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	29.8	37.1	13.2	n/a	n/a	n/a
197	LC-MS/MS	<5	<5	<5	30.3	40.8	12.4	30.3	40.8	12.4	n/r	n/r	n/r
199	LC-MS/MS	<2.0	<2.0	<2.0	31.4	39.6	13.3	31.4	39.6	13.3	n/r	n/r	n/r
200	RIA	n/a	n/a	n/a	n/a	n/a	n/a	23.0	27.8	12.7	n/a	n/a	n/a
204a	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	31.0	43.2	14.9	n/a	n/a	n/a
204b	LC-MS/MS	n/d	n/d	n/d	30.8	41.5	12.7	30.8	41.5	12.7	n/d	< 2.4	n/d
209	LC-MS/MS	<1.0	<1.0	<1.0	34.0	48.8	12.4	34.0	48.8	12.4	n/r	n/r	n/r
210a	RIA	n/a	n/a	n/a	n/a	n/a	n/a	32.1	40.2	17.4	n/a	n/a	n/a
210b	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	30.9	44.8	n/r	n/a	n/a	n/a
211	LC-MS/MS	0.0	0.0	0.0	37.8	49.1	12.6	37.8	49.1	12.6	n/r	n/r	n/r
212	LC-MS/MS	<2	<2	<2	31.9	42.9	12.8	31.9	42.9	12.8	n/r	n/r	n/r
213a	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	35.2	48.4	7.4	n/a	n/a	n/a
213b	EIA	n/a	n/a	n/a	n/a	n/a	n/a	25.2	32.3	13.9	n/a	n/a	n/a
214a	RIA	n/a	n/a	n/a	n/a	n/a	n/a	17.1	16.6	6.3	n/a	n/a	n/a
214b	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	29.4	38.1	13.0	n/a	n/a	n/a
214c	LC-MS/MS	<1.0	<1.0	<1.0	31.3	38.6	12.2	31.3	38.6	12.2	n/r	n/r	n/r
215	LC-MS/MS	<2	<2	<2	34.8	40.8	14.0	34.8	40.8	14.0	n/r	n/r	n/r
216	LC-MS/MS	0.7	0.7	0.2	36.4	40.6	12.2	37.2	41.3	12.3	2.0	2.3	0.7
217	LC-MS/MS	<2	<2	<2	39.2	48.8	16.4	39.2	48.8	16.4	n/r	n/r	n/r
218a	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	31.1	40.7	14.0	n/a	n/a	n/a
218b	LC-MS/MS	0.0	0.3	0.0	38.1	41.8	14.7	38.1	42.1	14.7	n/r	n/r	n/r
220	LC-MS/MS	n/d	n/d	n/d	31.0	38.4	12.5	31.0	38.4	12.5	n/r	n/r	n/r
221a	LC-MS/MS	<2.0	<2.0	<2.0	30.1	35.3	14.9	30.1	35.3	14.9	n/r	n/r	n/r
221b	LC-UV	0.0	0.0	0.0	34.3	42.7	38.5	34.3	42.7	38.5	n/r	n/r	n/r
222	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	35.3	40.1	15.0	n/a	n/a	n/a
225	LC-MS/MS	<5	<5	<5.0	32.9	44.6	13.4	32.9	44.6	13.4	n/r	n/r	n/r
228a	LC-MS/MS	n/d	n/d	n/d	37.0	45.7	12.0	37.0	45.7	12.0	n/r	n/r	n/r
242	LC-MS/MS	0.5	0.4	n/d	29.5	36.5	12.8	30.0	36.9	12.8	1.5	1.7	0.6
243a	LC-UV	0.5	0.4	n/d	29.5	36.6	12.7	29.8	37.0	12.9	1.5	1.7	0.7
243b	LC-MS/MS	0.5	0.4	n/d	29.3	36.4	12.7	30.0	37.0	12.6	1.4	1.8	0.6
244	LC-MS/MS	<5	<5	<5	34.0	40.0	16.0	34.0	40.0	16.0	n/r	n/r	n/r
249	LC-MS/MS	<0.8	<0.8	<0.8	30.4	41.2	12.8	30.4	41.2	12.8	1.5	1.8	0.5
251	LC-MS/MS	<4	<4	n/r	37.0	54.0	n/r	37.0	54.0	n/r	n/r	n/r	n/r
253	LC-MS/MS	0.7	0.6	0.1	30.1	39.0	12.1	30.8	39.6	12.2	1.9	2.1	0.7
255	LC-MS/MS	0.7	0.7	0.2	32.7	40.3	12.7	33.4	41.0	12.9	n/r	n/r	n/r
256	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	33.0	35.8	11.4	n/a	n/a	n/a
257	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	30.5	42.0	5.9	n/a	n/a	n/a
258	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	33.9	64.4	13.6	n/a	n/a	n/a
259	LC-MS/MS	<2	<2	<2	34.4	45.2	10.3	34.4	45.2	10.3	n/r	n/r	n/r
261	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	24.2	37.4	6.6	n/a	n/a	n/a
262	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	27.5	39.1	20.2	n/a	n/a	n/a
263	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	33.7	37.6	12.8	n/a	n/a	n/a
264	LC-MS/MS	0.6	0.6	0.1	41.0	39.2	13.9	41.6	39.8	14.0	n/r	n/r	n/r
265	LC-MS/MS	0.0	0.0	0.0	35.0	44.0	13.0	35.0	44.0	13.0	n/r	n/r	n/r
266a	LC-UV	0.0	0.0	0.0	41.3	50.8	16.4	41.3	50.8	16.4	n/r	n/r	n/r
266b	EIA	n/a	n/a	n/a	n/a	n/a	n/a	29.2	38.3	18.5	n/a	n/a	n/a
267	CLEIA	n/a	n/a	n/a	n/a	n/a	n/a	30.4	39.3	12.4	n/a	n/a	n/a
	NIST Value	0.68	0.65	0.1*	31.3	39.4	12.4	32.0	40.1	12.5	1.7	2.1	0.65
	U	0.06	0.02	---	0.8	0.8	0.4	0.8	0.8	0.4	0.1	0.1	0.03

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

*estimated value (no uncertainty determined)