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NIST/NIH Vitamin D Metabolites Quality Assurance Program Report of Participant Results: Winter 2010 Comparability Study (Exercise 1)

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ABSTRACT

The National Institute of Standards and Technology (NIST) has established a Vitamin D Metabolites Quality Assurance Program in collaboration with the National Institutes of Health (NIH) Office of Dietary Supplements (ODS). For this first pilot exercise (Winter 2010 Comparability Study), participants were asked to use the methodology of their choice to measure concentrations of 25-hydroxyvitamin D in control and study materials distributed by NIST. The study material consisted of triplicate samples of SRM 1950 Metabolites in Human Plasma. SRM 2972, which is comprised of separate ethanolic calibration solutions with known concentrations of 25(OH)D_2 and 25(OH)D_3, was provided as a control material. Participants provided their data to NIST, where it was compiled and evaluated for trueness relative to the NIST value, within-laboratory precision, and concordance within the participant community. A report of results was provided to all participants of the exercise, and laboratories were identified by code numbers known only to them. The results from this first pilot exercise are reported along with a summary of the analytical methods used.

OVERVIEW OF WINTER 2010 COMPARABILITY STUDY

For the Winter 2010 Comparability Study (Exercise 1) of the NIST/NIH Vitamin D Metabolites Quality Assurance Program (VitDQAP), control and human serum study samples were distributed to participants for evaluation. SRM 2972, which is comprised of separate ethanolic solutions with known concentrations of 25-hydroxyvitamin D_2 ($25(OH)D_2$) and 25-hydroxyvitamin D_3 ($25(OH)D_3$), was provided as a control material for assay calibration or verification. Participants were asked to provide single results for each of these solutions. In addition, participants were asked to determine $25(OH)D_2$, $25(OH)D_3$, and a total concentration of 25-hydroxyvitamin D ($25(OH)D_{Total} = 25(OH)D_2 + 25(OH)D_3$) in each of three vials A, B, and C, which were triplicate samples of SRM 1950 Metabolites in Human Plasma.

In the Winter 2010 exercise, there were a total of 16 participants and 17 datasets (one participant provided data for two different methods). Ten of these datasets originated from liquid chromatographic (LC) methods; of those, 9 were from LC with tandem mass spectrometric detection (LC-MS/MS) and 1 was from LC with ultraviolet absorbance detection (LC-UV). Seven datasets originated from immunoassay (IA) techniques, including 3 from enzyme immunoassay (EIA), 1 from chemiluminescence immunoassay (CLIA), and 3 from radioimmunoassay (RIA). **Appendices A-1** and **A-2** summarize the immunoassay and LC methods, respectively, used by the participants.

The raw data received from all participants is summarized in **Appendix B.** The data include the results for $25(OH)D_2$, $25(OH)D_3$ and $25(OH)D_{Total}$ in the three vials (A, B and C) of SRM 1950 (study sample) and for $25(OH)D_2$ and $25(OH)D_3$ in the ethanolic calibration solutions. Only the LC-based methods could distinguish between $25(OH)D_2$ and $25(OH)D_3$ in the SRM 1950 samples. All 10 of the LC-based datasets reported values for $25(OH)D_3$, but only two reported values for $25(OH)D_2$. The amount of $25(OH)D_2$ in SRM 1950 was very low and below the detection limit for most of the LC-based methods. The seven datasets from the immunoassay methods could not distinguish between $25(OH)D_2$ and $25(OH)D_3$ and reported the $25(OH)D_{Total}$ in SRM 1950 (study sample). However, both LC and immunoassay datasets provided individual values for $25(OH)D_2$ and $25(OH)D_3$ in the ethanolic controls because the analytes were in separate solutions.

Appendix B also provides the summarized NIST results for both SRM 1950 (study material) and SRM 2972 (controls).

WINTER 2010 EXERCISE RESULTS AND DISCUSSION

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

A summary of the individual participant data for 25(OH)D_2 and 25(OH)D_3 in the SRM 2972 control solutions is provided in **Table 1.** The community results are summarized at the bottom of the table for all reported methods and the LC methods only. The community results include the total number of quantitative values reported (N), the median value, the MADe (the median absolute deviation estimate, a robust estimate of the standard deviation), and the percent coefficient of variation (CV%). Consensus statistics were not calculated for the data from the IA methods because of the limited number of data reported (N = 3). **Table 1** also presents the NIST certified values with expanded uncertainties corresponding to 95% confidence.

For the ethanolic control solutions (SRM 2972), the single data values for 25(OH)D_2 and 25(OH)D_3 reported by each individual laboratory are plotted in **Figures 1a** and **1b**, respectively. The two primary methods of analysis (LC and immunoassay) are displayed separately with closed (●) and open (○) circles, respectively. For each of these graphs, the black solid line (——) represents the consensus median, and the black dotted lines (- - - - -) represent the consensus variability (2 × MADe). The laboratories with results that fall between the two dotted lines are within the consensus variability area. The NIST-assessed values of 238.6 ng/mL ± 3.9 ng/mL and 334.8 ng/mL ± 5.2 ng/mL for 25(OH)D_2 and 25(OH)D_3, respectively, for this control material (SRM 2972) are provided by red squares (■) (with error bars representing ± U_{95}).

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

25(OH)D_2 (ng/mL)

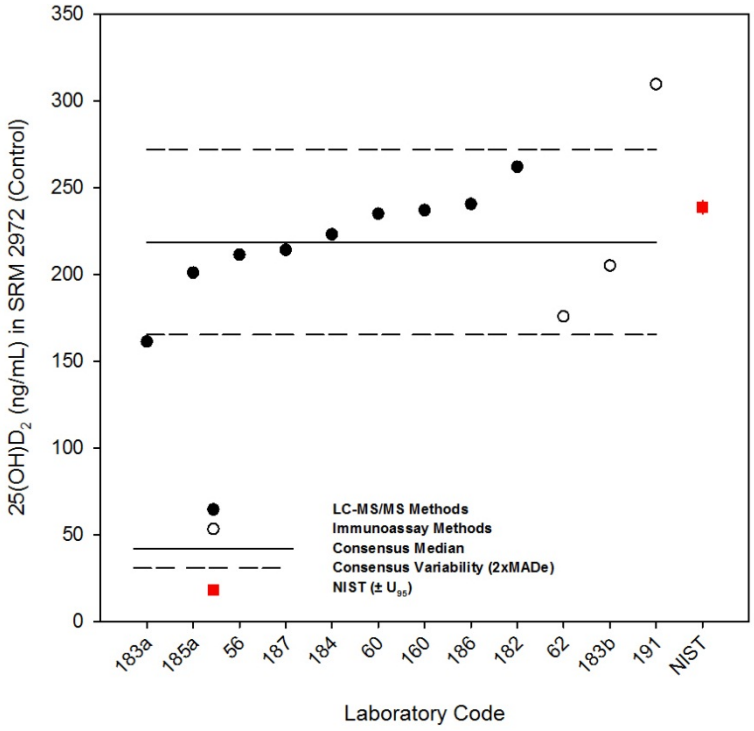
		SRM 2972
Lab	Method	Value
056	LC-MS/MS	211.3
060	LC-MS/MS	235.0
062	RIA	175.7
160	LC-MS/MS	237.0
182	LC-MS/MS	262.0
183a	LC-MS/MS	161.2
183b	CLIA	205.0
184	LC-MS/MS	223.0
185a	LC-MS/MS	201.0
186	LC-MS/MS	240.6
187	LC-MS/MS	214.0
191	RIA	309.6
All methods	N	12
	Median	218.5
	MADe	26.7
	CV%	12.2
LC methods	N	9
	Median	223.0
	MADe	20.8
	CV%	9.3
	NIST Value	238.6
	U_{95}	3.9

25(OH)D_3 (ng/mL)

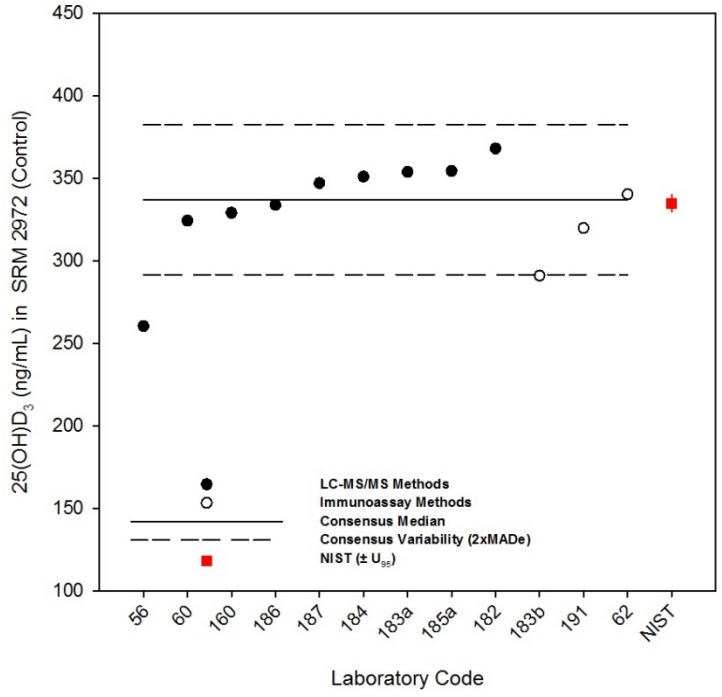
		SRM 2972
Lab	Method	Value
056	LC-MS/MS	260.4
060	LC-MS/MS	324.2
062	RIA	340.3
160	LC-MS/MS	329.0
182	LC-MS/MS	368.0
183a	LC-MS/MS	353.8
183b	CLIA	291.0
184	LC-MS/MS	351.0
185a	LC-MS/MS	354.4
186	LC-MS/MS	333.8
187	LC-MS/MS	347.0
191	RIA	319.8
	N	12
	Median	337.1
	MADe	22.8
	CV%	6.8
	N	9
	Median	347.0
	MADe	19.6
	CV%	5.6
	NIST Value	334.0
	U_{95}	5.2

Figure 1. Winter 2010 exercise results for (a) 25(OH)D_2 and (b) 25(OH)D_3 in the ethanolic control (SRM 2972).

(a) 25(OH)D_2



(b) 25(OH)D_3



25(OH)D in the study material SRM 1950

A summary of the individual participant data for 25(OH)D_{Total} in SRM 1950 is provided in **Table 2.** The summarized data include the mean, standard deviation (SD), and relative percent standard deviation (%rSD) of the three reported values (vials A, B, and C) for SRM 1950.

The community results are summarized at the bottom of the table for all reported methods, the IA methods only, the LC methods only and the LC-MS/MS methods only. These summarized results include the total number of quantitative values reported, the median value, the MADe, and the percent coefficient of variation.

The table also presents the sum of the NIST certified and reference values for 25(OH)D_3 and 25(OH)D_2, respectively, for SRM 1950 (study sample) with approximated 95% confidence limits (U_{95}). NIST results for SRM 1950 were obtained using data from both LC-MS and LC-MS/MS techniques; details about the methods and measurements are reported in the Certificate of Analysis for SRM 1950[1].

For each participant who used an LC method, the calculated average (mean) value and error bars (representing the lab mean value ± 2 × SD) for 25(OH)D_3 in SRM 1950 (study sample) are plotted in **Figure 2.** The data for 25(OH)D_2 are not provided in graphical form because only two laboratories reported values that were above their detection limits. When the error bars for each data point are considered, all laboratory data are within the consensus variability (2 × MADe).

For all participant datasets, the calculated average (mean) values and error bars (representing the lab mean value ± 2 × SD) for 25(OH)D_{Total} in SRM 1950 (study sample) are plotted in **Figure 3.** From the mean values for all datasets, the consensus median and the consensus variability (2 × MADe) were determined. When the error bars for each data point are considered, all laboratory data are within the consensus variability. Both primary methods of analysis (LC, immunoassay) provide similar mean values and uncertainties for SRM 1950 (study sample).

The precision (as %rSD) for the three replicate analyses of SRM 1950 (study sample) ranged from 1% to 10% for the individual laboratories, where over 2/3 of the laboratories that participated had method precision under 5%. When all reported data for SRM 1950 (study sample) were considered, the consensus variability was ≈ 14% and the consensus median was biased ≈ 7% higher than the NIST-assessed value for this material. A goal for this program is to determine the cause of any bias and to achieve better agreement between the consensus median value and the NIST-assessed value. While the precision of most individual laboratories was acceptable, another program goal is to reduce the consensus variability to better represent the community's measurement capability while also recognizing that a 'fit-for-purpose' variability-level may exist. One approach to meet this goal is to strive for within-laboratory precision of less than 5% for all participants.

[1] https://www-s.nist.gov/srmors/view_cert.cfm?srm=1950

Table 2. Summary of participant data for 25(OH)D_{Total} (ng/mL) in SRM 1950.

		SRM 1950	SRM 1950	SRM 1950	SRM 1950 Combined		
Lab	Method	Vial A	Vial B	Vial C	Mean	SD	%rSD
056	LC-MS/MS	24.9	24.2	24.3	24.5	0.4	1.5
060	LC-MS/MS	26.5	26.7	27.1	26.8	0.3	1.1
062	RIA	22.6	24.3	23.4	23.4	0.9	3.6
160	LC-MS/MS	26.3	29.2	27.3	27.6	1.5	5.3
180	RIA	25.4	27.0	25.5	26.0	0.9	3.5
182	LC-MS/MS	24.0	26.0	29.0	26.3	2.5	9.6
183a	LC-MS/MS	31.3	29.8	29.3	30.1	1.0	3.5
183b	CLIA	30.0	29.1	29.8	29.6	0.5	1.6
184	LC-MS/MS	23.8	28.3	27.1	26.4	2.3	8.8
185a	LC-MS/MS	26.4	26.7	27.0	26.7	0.3	1.3
186	LC-MS/MS	27.6	25.3	23.5	25.5	2.0	8.0
187	LC-MS/MS	25.5	24.8	26.2	25.5	0.7	2.7
188	CLIA	28.0	28.0	25.0	27.0	1.7	6.4
189	LC-UV	29.8	29.3	28.6	29.2	0.6	2.1
190	EIA	32.5	30.4	30.4	31.1	1.2	3.9
191	RIA	29.9	30.4	30.1	30.1	0.2	0.8
192	EIA	23.5	23.4	24.2	23.7	0.4	1.7
All methods	N	17	17	17	17		
	Median	26.4	27.0	27.1	26.7		
	MADe	3.5	3.2	3.1	1.8		
	CV%	13.2	12	11	7		
IA methods	N	7	7	7	7		
	Median	28.0	28.0	25.5	27.0		
	MADe	3.8	3.5	3.0	4.6		
	CV%	13.7	13	12	17		
LC methods	N	10	10	10	10		
	Median	26.3	26.7	27.1	26.6		
	MADe	2.0	2.6	1.8	1.6		
	CV%	7.6	9.7	6.6	5.9		
LC-MS/MS	N	9	9	9	9		
	Median	26.3	26.7	27.1	26.4		
	MADe	1.9	2.4	1.3	1.3		
	CV%	7.3	8.9	4.9	5.1		
	NIST Value	25.30	25.30	25.30	25.30		
	U_{95}	0.79	0.79	0.79	0.79		

Figure 2. Winter 2010 exercise results for 25(OH)D_3 using only LC methods in SRM 1950 (study sample).

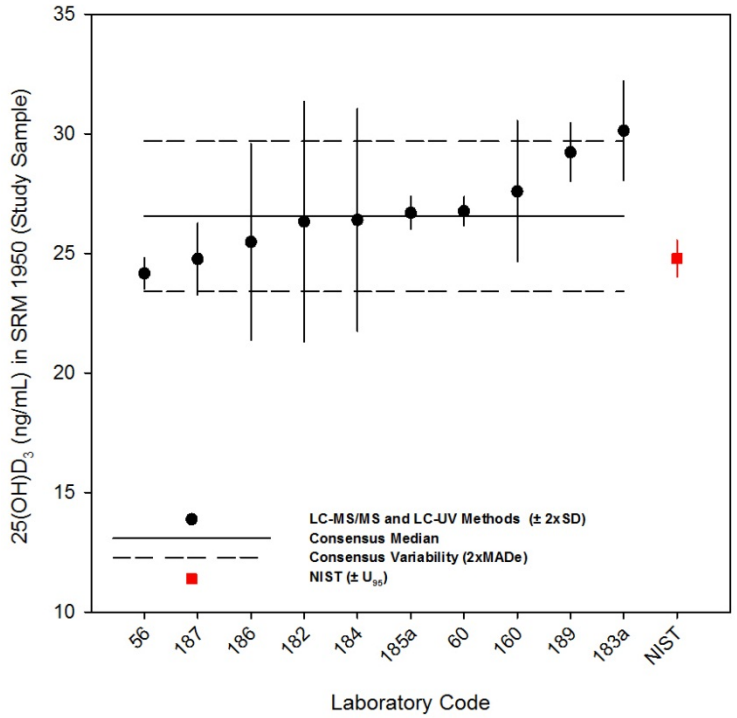
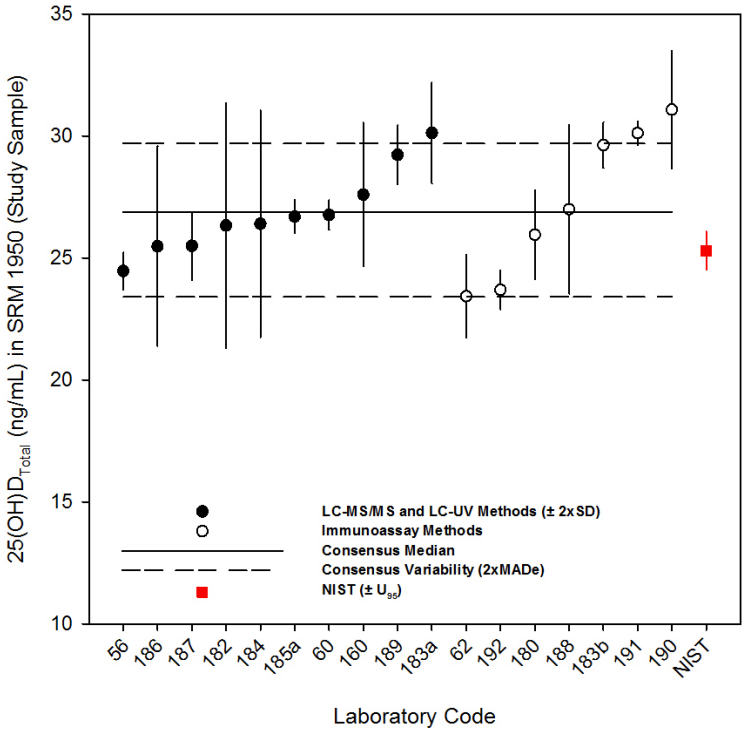


Figure 3. Winter 2010 exercise results for 25(OH)D_{Total} in SRM 1950 (study sample).



Appendix A-1. Summary of immunoassay methods used by VitDQAP Winter 2010 participants

Participant Number	*Immunoassay*	*Sample Preparation*	*Detection*
62	RIA	Samples were extracted, centrifuged	Gamma counter
180	RIA	Samples were prepared per vendor's sample extraction protocol	I^{125} detection using gamma counter
183b	CLIA	n/r	n/r
188	EIA	n/r	n/r
190	EIA	Calibrators, controls and test specimens were all diluted with biotin-labeled 25(OH)D and analyzed in duplicate	Microplate reader
191	RIA	n/r	n/r
192	EIA	n/r	Software was used to convert the absorbance values obtained at 450 nm to get the concentration

n/r = not reported

Appendix A-2. Summary of LC methods used by VitDQAP Winter 2010 participants

Participant Number	*Internal Standard (IS)*	*Sample Preparation*	*Chromatographic Conditions*	*Detection*
56	$25(OH)D_2$-d_3 and $25(OH)D_3$-d_6	Samples were extracted with acetonitrile, centrifuged, then filtered	Online SPE with C8 column (2.1 x 30 mm); separation on C18 column (2.1 x 50 mm); step gradient with methanol/water; flow 0.5 mL/min	MS/MS MRM: $25(OH)D_3$ *m/z* 401/383; $25(OH)D_3$-d_6 *m/z* 407/389; $25(OH)D_2$ m/z 413/395; $25(OH)D_2$-d_3 m/z 416/398
60	$25(OH)D_3$ -d_6	IS solution was added to plasma (150 μL), and proteins were precipiated by addition of a ternary extraction solvent. Sample cetrifuged, supernatant transfered to new vial, dried, and dissolved in mobile phase	C18 column (3.0 x 150 mm); A: 0.05% formic acid in water, B: 0.05% formic acid in methanol/acetonitrile (80/20, volume fraction); isocratic elution with 92% B from 0-2 min, step gradient to 100% B (2-8 min), equilibration (8-13) min; flow 0.55 mL/min	MS/MS (positive) MRM: $25(OH)D_3$ *m/z* 383/211; $25(OH)D_3$-d_6 *m/z* 389/211; $25(OH)D_2$ *m/z* 395/270
160	$25(OH)D_2$-d_6 and $25(OH)D_3$-d_6	Samples were extracted in acetonitrile, centrifuged and filtered	2-Dimensional separation CN column (2.1x 50mm) (1st Dimension), C18 column (2.1x 50mm) (2nd Dimension); gradient with 10 mmol/L formic acid in water and 10 mmol/L formic acid in methanol	MS/MS MRM: $25(OH)D_3$ m/z 401/365; $25(OH)D_3$-d_6 m/z 407/371; $25(OH)D_2$ m/z 413/337; $25(OH)D_2$-d_6 m/z 419/337
182	$25(OH)D_3$ -d_6	Proteins were precipitated with acetonitrile/methanol (3:1) and IS directly in 96 well plate. Plate was covered, mixed manually, and centrifuged	C18 column (2.1 x 50 mm); gradient elution from 60%-100% methanol with formic acid and ammonium acetate modifiers	MS/MS MRM: $25(OH)D_3$ *m/z* 401/365 (quant), *m/z* 401/383 (qual); $25(OH)D_2$ *m/z* 413/355 (quant), *m/z* 413/271 (qual)
183a	$25(OH)D_3$ -d_6	IS (25 μL) was added to plasma (150 μL), followed by protein precipiation and extraction with 0.1 mol/L $ZnSO_4$ (150 μL), methanol (300 μL), and hexane (750 μL); extract dried and dissolved with 70% methanol, 30% water with 2 mmol/L ammonium acetate	C8 column (2.1 X 50 mm); isocratic elution with 73% methanol/ 27% water; flow 0.4 mL/min	MS/MS MRM: $25(OH)D_3$ *m/z* 401/159, 401/383; $25(OH)D_2$ *m/z* 413/82, 413/395
184	$25(OH)D_3$ -d_6	Plasma (200 μL) extracted with acetonitrile and IS (700 μL); mixed, centrifuged, and filtered	C18 column (100 x 2.1 mm; 5μm); linear gradient from 60% B to 98% B over 2 min (A: 0.1% formic acid in water, B: methanol with 0.1% formic acid and 5 mmol/L ammonium acetate)	MS/MS (APCI) MRM: $25(OH)D_3$ *m/z* 383/257; $25(OH)D_3$-d_6 *m/z* 389/263; $25(OH)D_2$ *m/z* 395/209
185	n/r	n/r	n/r	MS/MS
186	$25(OH)D_3$ -d_6	Proteins were removed by addition of $ZnSO_4$, followed by methanol extraction and centrifugation; analytes were liquid/liquid extracted with three volumes of hexane, dried, and dissolved in methanol/water (65:35)	Phenyl column (2.1 x 100; 1.7um); 40°C; gradient: 65-85 % B over 3 min (A: 0.1% formic acid in water; B: 0.1% formic acid in methanol); flow 0.45 mL/min	MS/MS MRM: $25(OH)D_3$ *m/z* 401/159; $25(OH)D_2$ *m/z* 413/395
187	n/r	n/r	n/r	MS/MS
189	Obtained from kit supplier	Proteins were precipitated, samples centrifuged, analytes in the supernatant were extracted using SPE cartridges	Commercially obtained reagent set and column; isocratic elution; flow 0.7mL/min	UV at 265 nm

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

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

		$25(OH)D_2$ (ng/mL)			$25(OH)D_3$ (ng/mL)			$25(OH)D_{Total}$ (ng/mL)			$25(OH)D_2/D_3$ (ng/mL)	
		SRM 1950	SRM 1950	SRM 1950	SRM 1950	SRM 1950	SRM 1950	SRM 1950	SRM 1950	SRM 1950	SRM 2972	
Lab	Method	Vial A	Vial B	Vial C	Vial A	Vial B	Vial C	Vial A	Vial B	Vial C	$25(OH)D_2$	$25(OH)D_3$
056	LC-MS/MS	0.5	0.4	<0.4	24.4	23.8	24.3	24.9	24.2	24.3	211.3	260.4
060	LC-MS/MS	<2	< 2	< 2	26.5	26.7	27.1	26.5	26.7	27.1	235.0	324.2
062	RIA	n/a	n/a	n/a	n/a	n/a	n/a	22.6	24.3	23.4	175.7	340.3
160	LC-MS/MS	<1.0	<1.0	<1.0	26.3	29.2	27.3	26.3	29.2	27.3	237.0	329.0
180	RIA	n/a	n/a	n/a	n/a	n/a	n/a	25.4	27.0	25.5	n/r	n/r
182	LC-MS/MS	<2	<2	<2	24.0	26.0	29.0	24.0	26.0	29.0	262.0	368.0
183a	LC-MS/MS	<4	<4	<4	31.3	29.8	29.3	31.3	29.8	29.3	161.2	353.8
183b	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	30.0	29.1	29.8	205.0	291.0
184	LC-MS/MS	<1.0	<1.0	<1.0	23.8	28.3	27.1	23.8	28.3	27.1	223.0	351.0
185a	LC-MS/MS	n/d	n/d	n/d	26.4	26.7	27.0	26.4	26.7	27.0	201.0	354.4
186	LC-MS/MS	n/r	n/r	n/r	27.6	25.3	23.5	27.6	25.3	23.5	240.6	333.8
187	LC-MS/MS	0.7	0.8	0.7	24.8	24.0	25.5	25.5	24.8	26.2	214.0	347.0
188	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	28.0	28.0	25.0	n/r	n/r
189	LC-UV	n/d	n/d	n/d	29.8	29.3	28.6	29.8	29.3	28.6	n/r	n/r
190	EIA	n/a	n/a	n/a	n/a	n/a	n/a	32.5	30.4	30.4	n/r	n/r
191	RIA	n/a	n/a	n/a	n/a	n/a	n/a	29.9	30.4	30.1	309.6	319.8
192	EIA	n/a	n/a	n/a	n/a	n/a	n/a	23.5	23.4	24.2	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	0.52	0.52	0.52	24.78	24.78	24.78	25.30	25.30	25.30	238.6	334.8
	U_{95}	0.17	0.17	0.17	0.77	0.77	0.77	0.79	0.79	0.79	3.9	5.2