

## Method evaluation study of a new generation of vitamin D assays

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### Abstract

**Introduction:** Recently several diagnostic manufacturers have launched new 25-hydroxy-vitamin D (25(OH)D) assays, which are aligned to the National Institute of Standards and Technology (NIST) Standard Reference Materials (SRM) (NIST, Gaithersburg, Maryland). The aim of this study was to compare the performance of one liquid chromatography-tandem mass spectrometry (LC-MS/MS) method, one enzyme linked immunosorbent assay (ELISA), and one recalibrated and previous version of a chemiluminescence immunoassay (CLIA).

**Material and methods:** Serum-aliquots of 198 patient samples from routine 25(OH)D analysis were measured by the ClinMass® LC-MS/MS Complete Kit (RECIPE Chemicals + Instruments GmbH, Munich, Germany), the ORGENTEC 25(OH)D<sub>3</sub>/D<sub>2</sub> ELISA (ORGENTEC Diagnostika GmbH, Mainz, Germany), the recalibrated Immunodiagnostic Systems (IDS)-iSYS 25(OH)D<sup>S</sup> and the previous used IDS-iSYS 25(OH)D CLIA (Immunodiagnostic Systems Ltd, Boldon, United Kingdom). Bland-Altman and Deming regression analyses were calculated for methods comparison of all tested 25(OH)D assays. The LC-MS/MS method was defined as the reference method. Within-run and between-run precision measurements were performed for all methods with three different concentration levels.

**Results:** Compared to the LC-MS/MS method, the new IDS-iSYS 25(OH)D<sup>S</sup> and ORGENTEC 25(OH)D<sub>3</sub>/D<sub>2</sub> assay demonstrated mean relative biases of 16.3% and 17.8%. The IDS-iSYS 25(OH)D assay showed the lowest mean bias of 1.5%. Deming regression analyses of the recalibrated IDS-iSYS 25(OH)D<sup>S</sup> and the ORGENTEC 25(OH)D<sub>3</sub>/D<sub>2</sub> assay showed proportional differences, when compared to the reference method. All assays showed a within-run and between-run imprecision of ≤ 20% at each of the evaluated concentration levels.

**Conclusions:** The evaluated standardized immunoassays and LC-MS/MS are useful methods for measuring 25(OH)D serum-levels in clinical laboratories.

**Key words:** vitamin D; immunoassays; liquid chromatography-tandem mass spectrometry; reference standards; quality improvement

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### Introduction

The clinical interest in the physiological importance of the steroid hormone vitamin D and its possible roles in the pathophysiological processes of many diseases have increased the demand for the measurement of vitamin D and its metabolites (1). On the one hand, vitamin D deficiency results in abnormalities in bone metabolism known as rickets, osteomalacia, and osteoporosis (2,3). On the other hand, it is associated with non-skeletal diseases such as type one diabetes mellitus (2,4,5), multiple sclerosis (2), cancer (2,5), hypertension (5) or cardiovascular disease (5,6).

In the liver vitamin D is converted to the 25-hydroxy-vitamin D (25(OH)D) and transported in the circulation by the vitamin-D binding protein (DBP). In the kidneys the biologically active form 1,25-dihydroxy-vitamin D (1,25(OH)<sub>2</sub>D) is created from 25(OH)D (7). This active form has a circulating half-life of only 4-6 hours and serum-levels of about 1000 fold less than 25(OH)D (1,8,9). The major circulating form of vitamin D is 25(OH)D, which has a half-life of approximately 2-3 weeks (8). Therefore, the total 25(OH)D is principally used as the biomarker indicating the vitamin D status (9,10).

In the past, 25(OH)D measurements have proven to be a major challenge with a wide spread variation in the results (1). The sometimes huge between-method discrepancies have been known for many years from data obtained from the International Vitamin D External Quality Assessment Scheme (DEQAS) (11-13). The DEQAS was already founded in 1989 and meanwhile has become the largest vitamin D quality assessment program worldwide (14).

To overcome the problem of inter-laboratory as well as inter-assay discrepancies, the Vitamin D Standardization Program (VDSP) was established in 2011. This program is conducted as a collaboration between the US Office of Dietary Supplements (ODS) of the National Institute of Health (NIH), the Center for Disease Control and Prevention (CDC), the National Centre for Environmental Health (NCEH), the National Institute of Standards and Technology (NIST), and the Belgian Laboratory for Analytical Chemistry, Faculty of Pharmaceutical Sciences, Ghent University (10,13). The NIST, in collaboration with the ODS, has developed and certificated Standard Reference Materials (SRM 2972 and 972a) for vitamin D metabolites in human serum (10,15).

The 3-epi-25(OH)D<sub>3</sub> (C<sub>3</sub>-epimer) is a vitamin D metabolite, which is considered as a confounder in 25(OH)D measurements. The biological role and its clinical significance are still unknown. The presence of the C<sub>3</sub>-epimer is considered to affect the quantification of 25(OH)D<sub>3</sub> measurements in routine LC-MS/MS methods, especially in infant populations (16). For that reason, the measurement of this metabolite is one important objective of the VDSP (10).

The recent release of a new generation of 25(OH)D assays, which are aligned to the NIST SRM, is anticipated to show an improved analytical performance. Considering the difficulties of 25(OH)D measurements in the past, the hypothesis of this study was, that the standardization of this new generation of 25(OH)D assays represents an improvement of inter-assay accordance in daily clinical 25(OH)D determination.

The aim of our study was to compare the previous version of one chemiluminescence immunoassay (CLIA), which is not aligned to the NIST SRM, the new follow-up version of this CLIA and one new enzyme linked immunosorbent assay (ELISA), both aligned to the NIST SRM 2972, and at least one new liquid chromatography-mass tandem spectrometry (LC-MS/MS) kit, which is aligned to the new NIST SRM 972a and able to separate and detect the C<sub>3</sub>-epimer. The LC-MS/MS method was defined as the reference method. The 25(OH)D measurements of the new vitamin D assays were based on 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> detection.

## Materials and methods

### Subjects

For the method evaluation of the above mentioned 25(OH)D assays, leftover blood samples from routine 25(OH)D analysis of 198 patients, who were admitted to the General Hospital Steyr (Steyr, Austria), were analyzed. Blood sampling was performed from August 14 to October 09, 2013 at the Hospital's wards after an overnight fasting state (12 h). VACUETTE® Z Serum Sep Clot Activator tubes (4 mL) with inert separator gel (Greiner Bio-one International GmbH, Kremsmünster, Austria) were used for blood draw from a peripheral vein. At the Institute of Laboratory Medicine the serum samples were centrifuged with 2200 x g for 10 minutes, divided in aliquots and stored at -30 °C until the 25(OH)D measurements and precision studies were performed in December 2013.

The study is in accordance with the ethical standards of the Declaration of Helsinki. The ethical approval was provided by the Ethical Committee of Upper Austria, Linz, Austria (trial registration number: K-39-13).

### Methods

*Immunodiagnostic Systems (IDS)-iSYS 25(OH)D assay (Immunodiagnostic Systems Ltd, Boldon, United Kingdom)*

This automated immunoassay is the previous version of the new follow-up IDS-iSYS 25(OH)D<sup>S</sup> CLIA

as listed below. It is based on chemiluminescence technology. The assay was performed on the IDS-iSYS Multi-Discipline Automated Analyzer. 10  $\mu$ L of serum aliquots were automatically pipetted and subjected to a pre-treatment step with NaOH (part of the reagent used for CLIA and ELISA methods) to denature the DBP inside the IDS-iSYS Multi-Discipline Automated Analyzer. The extraction procedure of 25(OH)D from the DBP was followed by analysis. This assay is not aligned to the NIST SRM (NIST, Gaithersburg, Maryland). The measurement range of this assay is 5-140 ng/mL (information of the manufacturer). The IDS-iSYS 25(OH)D control set (IS-2730) (Immunodiagnostic Systems Ltd, Boldon, United Kingdom) was used for quality control (QC).

*IDS-iSYS 25(OH)D<sup>S</sup> assay (Immunodiagnostic Systems Ltd, Boldon, United Kingdom)*

In December 2013, this new recalibrated version replaced the IDS-iSYS 25(OH)D assay on the market. This immunoassay also was performed on the IDS-iSYS Multi-Discipline Automated Analyzer. According to the previous version of this assay, the denaturation of the DBP was also done with NaOH (part of the reagent used for CLIA and ELISA methods) inside the analyzer. In contrast, this assay is aligned to the NIST SRM 2972 (NIST, Gaithersburg, Maryland). The measurement range of this assay is 7-125 ng/mL (information of the manufacturer). The IDS-iSYS 25(OH)D<sup>S</sup> Control Set (IS-2730S) (Immunodiagnostic Systems Ltd, Boldon, United Kingdom) was used for QC.

*ORGENTEC 25(OH)D<sub>3</sub>/D<sub>2</sub> assay (ORGENTEC Diagnostika GmbH, Mainz, Germany)*

This recently launched assay is based on a competitive ELISA. A single (not duplicate) serum sample was pipetted manually into well number one of an eight-well-micro strip. The extraction of 25(OH)D<sub>2</sub>/D<sub>3</sub> was done automatically inside the Alegria<sup>®</sup> Random Access Analyzer. Serum samples were mixed with tracer reagent (ORGENTEC Diagnostika GmbH, Mainz, Germany) and the 25(OH)D<sub>2</sub>/D<sub>3</sub> was delivered from the DBP. The extraction procedure was followed by analysis. This assay is also aligned to the NIST SRM 2972 (NIST, Gaithers-

burg, Maryland). The measurement range of this assay is 5-170 ng/mL (information of the manufacturer). The 25-OH Vitamin D<sub>3</sub>/D<sub>2</sub> Control Set (ORGENTEC Diagnostika GmbH, Mainz, Germany) was used for QC.

*ClinMass<sup>®</sup> LC-MS/MS Complete Kit (RECIPE Chemicals + Instruments GmbH, Munich, Germany)*

This is a new commercially available LC-MS/MS assay, which is aligned to the NIST SRM 972a (NIST, Gaithersburg, Maryland). The sample pre-treatment for precipitation was followed by LC-MS/MS analysis: for precipitation a 150  $\mu$ L mixture of deuterated internal standard (IS) (25[OH]D<sub>3</sub>-d6) (RECIPE Chemicals + Instruments GmbH, Munich, Germany) and precipitant P (aqueous salt solution) (RECIPE Chemicals + Instruments GmbH, Munich, Germany) was added to 50  $\mu$ L serum samples. After the precipitation step the clear supernatant (40  $\mu$ L) was subjected for further analysis by a Xevo Triple Quadrupole Mass Spectrometer (TQ-S) (Waters Corporation, Milford, Massachusetts) linked to an ACQUITY Ultra Performance Liquid Chromatography (UPLC) (Waters Corporation, Milford, Massachusetts). For the assessment of the vitamin D status the kit is able to distinguish 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> and enables the separation and qualitative detection of C<sub>3</sub>-epimers in a single run. The measurement range of this assay is 5-250 ng/mL (information of the manufacturer). QC was performed by the use of ClinCheck<sup>®</sup> serum controls (RECIPE Chemicals + Instruments GmbH, Munich, Germany). The performance is controlled by an external quality assurance program via participation at DEQAS three times a year.

*Precision studies of the immunoassays*

The precision studies of the immunoassays were performed according to the EP10-A2 Guideline of the Clinical and Laboratory Standards Institute (CLSI): Preliminary Evaluation of Quantitative Clinical Laboratory Methods (17). The 25-OH vitamin D<sub>3</sub>/D<sub>2</sub> control set (IS-2730) (IDS-iSYS 25(OH)D assay), the IDS-iSYS 25(OH)D<sup>S</sup> control set (IS-2730S) (IDS-iSYS 25(OH)D<sup>S</sup> assay), and the 25-OH Vitamin D<sub>3</sub>/D<sub>2</sub> control set (ORGENTEC 25(OH)D<sub>3</sub>/D<sub>2</sub> assay) were used for precision measurements of the im-

munoassays. According to the protocol (17), equal parts of the high- and low-level QC material were mixed to create the mid-level. At least one run per day at five consecutive workdays (from Monday to Friday) with a specific sequence (mid-, high-, low-, mid-, mid-, low-, low-, high-, high-, and mid-level) without change, interruption or intervening samples was analyzed for each immunoassay to determine the between-run precision (N = 5) for each level. In addition to the required measurements in the EP10-A2 protocol, one tenfold measurement (N = 10) of the low-, mid- and high-level of each immunoassay was performed at one day to determine the within-run precision.

#### *Precision studies of the LC-MS/MS method*

The precision studies of the LC-MS/MS method were performed according to the Guidance for Industry – Bioanalytical Method Validation (18). Three serum pools with mixed 25(OH)D<sub>2</sub> (25[OH]D<sub>2</sub> spiked serum pools: pool 1 = 10.9 ng/mL; pool 2 = 21.0 ng/mL; pool 3 = 29.3 ng/mL) and 25(OH)D<sub>3</sub> (pool 1 = 9.57 ng/mL; pool 2 = 19.6 ng/mL; pool 3 = 31.2 ng/mL) of different concentration levels were used to evaluate the distinction and reproducibility of the 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> detection of this method. At least three runs per day at eight analytical days were measured to determine the between-run precision (N = 24) for each serum pool. An eightfold measurement of each serum pool at three analytical days was performed to determine the within-run precision (N = 24).

#### **Statistical analysis**

Bland-Altman and Deming regression plots were calculated for methods comparison of all tested 25(OH)D assays. The LC-MS/MS method was defined as the reference method. All other methods were compared to the reference method. Within-run and between-run precision at each concentration level were assessed by calculating the mean, the standard deviation (SD) and the coefficient of variation (CV) of the above mentioned replicates and sequences. The CV was calculated based on the formula: CV (%) = 100 × standard deviation (SD)/mean (ng/mL). According to the literature (18),

the precision goal for each concentration level for the within-run and between-run was not to exceed 20% of the CV. Analyse-it<sup>®</sup> software version 2.30 (Analyse-it Software, Ltd, Leeds, United Kingdom) was used for statistical analysis. A P-value < 0.05 was considered statistically significant.

## **Results**

### **Characteristics of the study population**

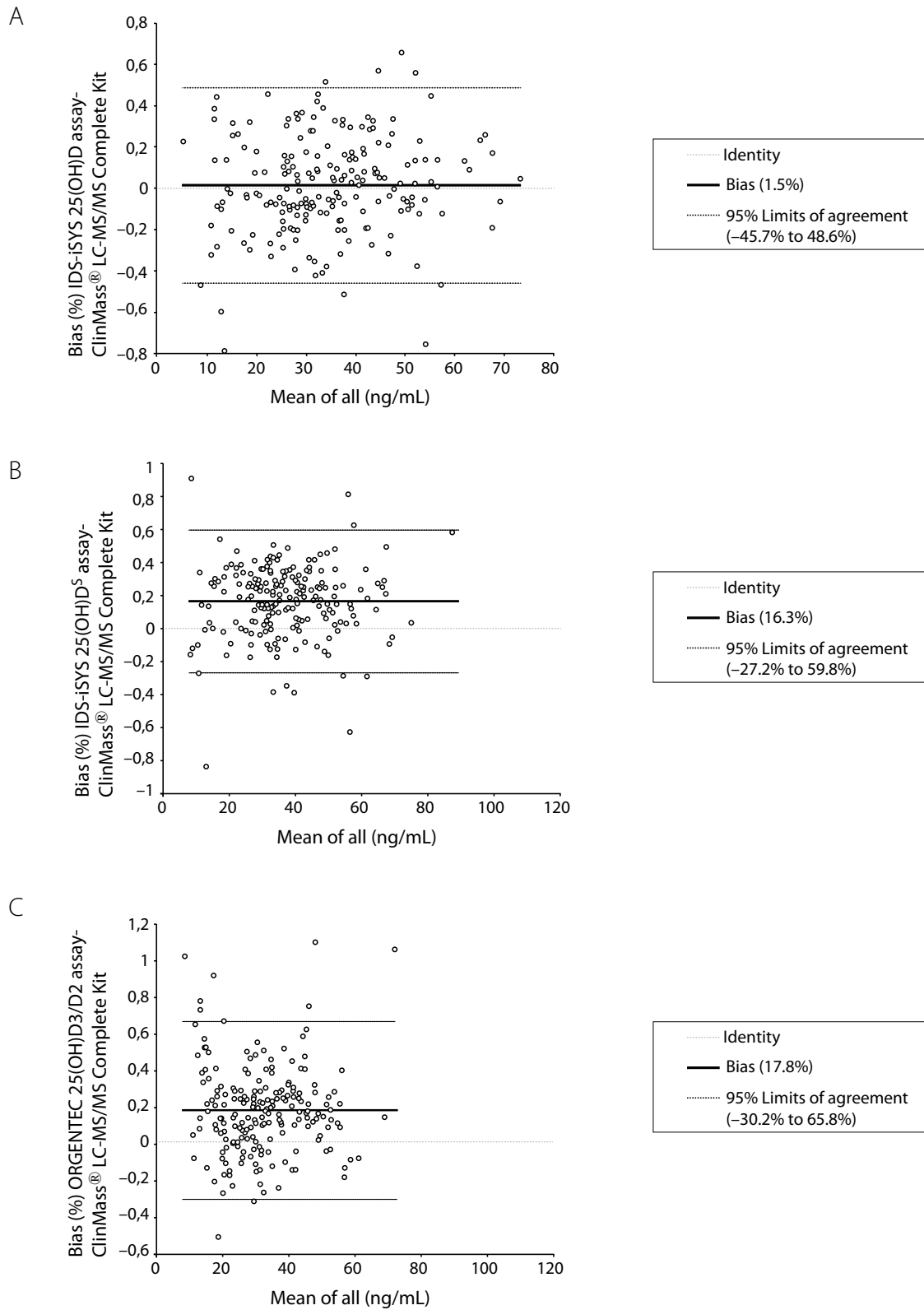
The study population mainly consisted of adults and a total of five adolescents between 15 and 19 years. No newborns or young children were measured. Of all patients (N = 198), 57.6% (N = 114) were female. The median age was 63 (range: 15–91) years.

### **Immunoassays vs. LC-MS/MS method**

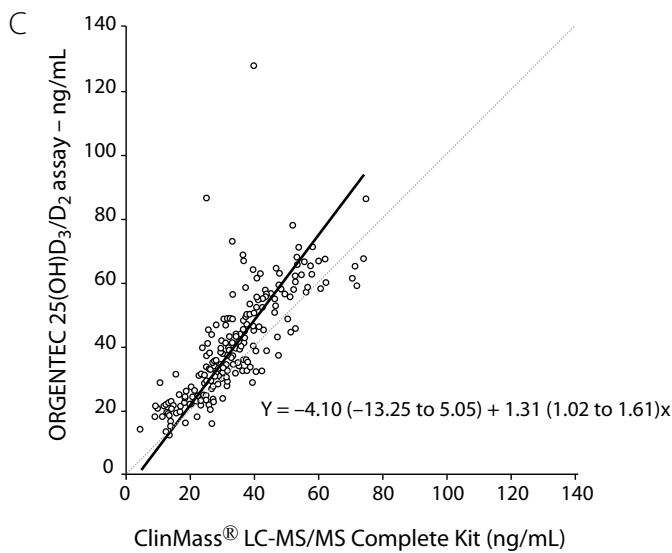
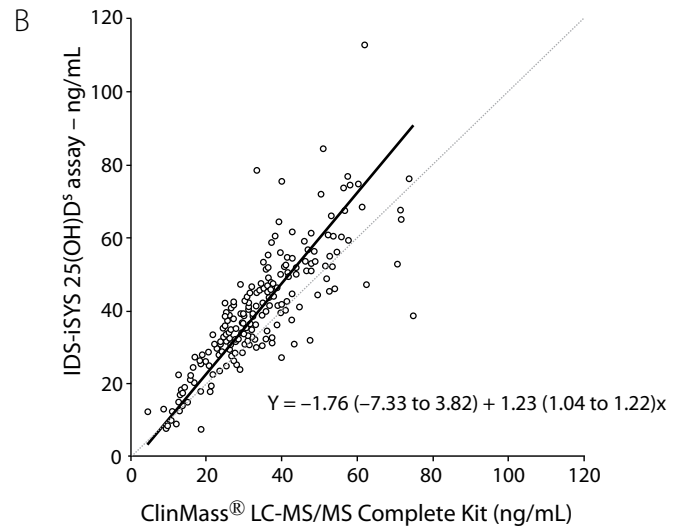
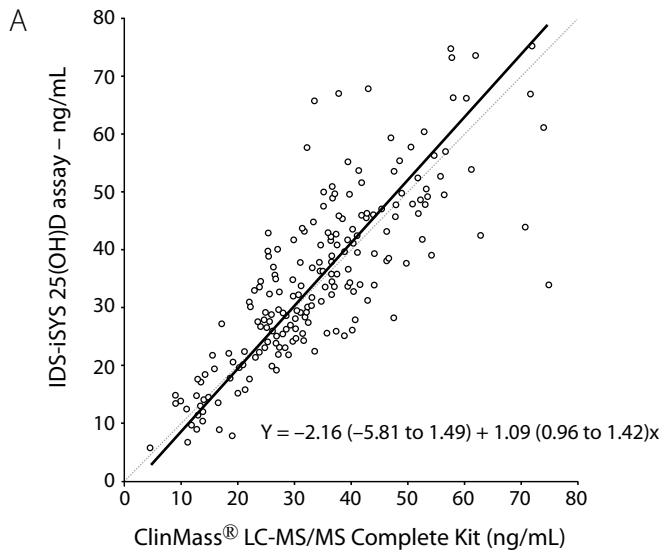
Bland-Altman plots are illustrated in Figure 1 A–C. Compared to the LC-MS/MS method, the new NIST-SRM aligned IDS-iSYS 25(OH)D<sup>S</sup> and ORGENTEC 25(OH)D<sub>3</sub>/D<sub>2</sub> assay demonstrated mean relative biases of 16.3% and 17.8%. The IDS-iSYS 25(OH)D assay showed the lowest mean bias of 1.5%. Deming regression plots are shown in Figure 2 A–C. According to the author's study, the results are as follows: IDS-iSYS 25(OH)D vs. LC-MS/MS:  $y = -2.16 (-5.81 \text{ to } 1.49) + 1.09 (0.96 \text{ to } 1.22)x$ , IDS-iSYS recalibrated vs. LC-MS/MS:  $y = -1.76 (-7.33 \text{ to } 3.82) + 1.23 (1.04 \text{ to } 1.42)x$ , and ORGENTEC vs. LC-MS/MS:  $y = -4.10 (-13.25 \text{ to } 5.05) + 1.31 (1.02 \text{ to } 1.61)x$ . A box-and-whisker plot of all 25(OH)D assay comparisons is illustrated in Figure 3.

### **Precision studies of the immunoassays**

The results of the precision studies of the immunoassays are demonstrated in Table 1. All immunoassays showed a within-run and between-run imprecision of ≤ 20% at each concentration level (low, mid, high). The highest within-run CV (19.0%) was observed at the low-level precision measurements with the IDS-iSYS 25(OH)D<sup>S</sup> assay. The highest between-run CV (19.1%) was shown at the low-level precision measurements with the IDS-iSYS 25(OH)D assay.

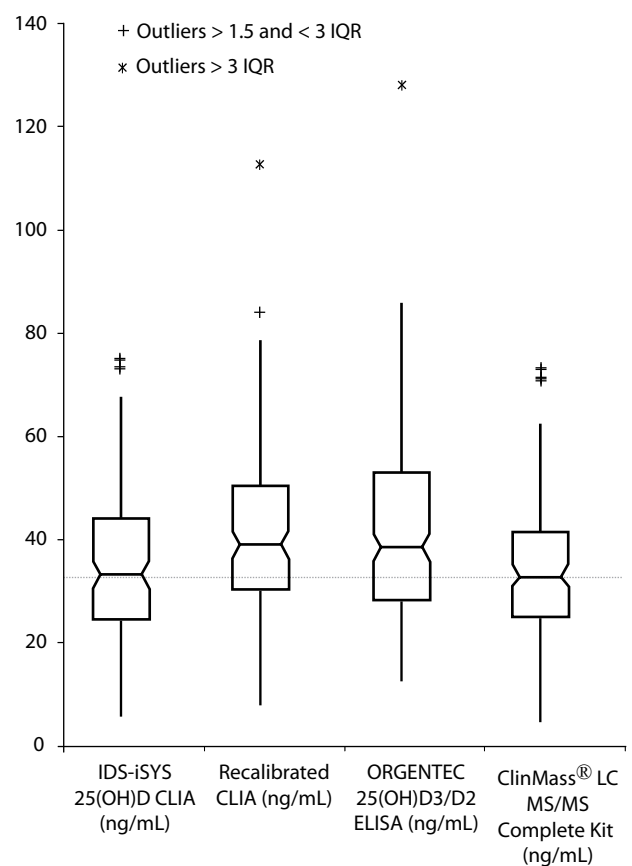


**FIGURE 1 A-C.** Bland-Altman plots of 25(OH)D assay comparisons. The mean relative bias in percent for all immunoassays compared to the LC-MS/MS (reference method) is represented in plain line. The limits of agreement are illustrated by dashed lines. ng/mL x 2.5 = nmol/L.



**FIGURE 2 A-C.** Deming regression plots of 25(OH)D assay comparisons. Bold lines represent Deming regression lines. The regression equation with the included 95% confidence intervals for the slope and intercept is presented next to the regression lines.

CI - confidence interval. ng/mL x 2.5 = nmol/L.



**FIGURE 3.** Box-and-whisker plot of 25(OH)D assay comparisons. The central boxes represent the 25th to 75th percentile range. The lines inside the boxes show the median value for each method. The straight line shows the median value as measured by the liquid chromatography-tandem mass spectrometry (reference method). Recalibrated chemiluminescence immunoassay - IDS-iSYS 25(OH)DS assay, IQR - interquartile range. ng/mL x 2.5 = nmol/L.

### Precision studies of the LC-MS/MS method

The results of the precision studies of the LC-MS/MS method are shown in Table 2. All within-run and between-run precision measurements of each 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> concentration level had a CV of ≤ 10%.

**TABLE 1.** Precision studies for all 25(OH)D immunoassays tested.

Assay	QC material*	Within-run			Between-run		
		Mean (ng/mL)†	SD‡	CV (%)§	Mean (ng/mL)	SD	CV (%)
IDS-iSYS 25(OH)D	Low-level	7.7	1.2	15.5	9.4	1.8	19.1
	Mid-level	38.8	3.9	10.5	3.9	3.9	10.0
	High-level	66.8	4.1	6.1	65.5	4.9	7.4
IDS-iSYS 25(OH)D <sup>5</sup>	Low-level	15.2	2.9	19.0	9.8	1.6	16.3
	Mid-level	48.7	4.5	9.0	49.5	5.5	11.1
	High-level	78.1	5.9	7.5	78.2	6.8	8.6
ORGENTEC 25(OH)D <sub>3</sub> /D <sub>2</sub>	Low-level	19.2	1.2	6.3	16.6	1.1	6.6
	Mid-level	38.8	4.8	12.3	35.6	2.1	5.8
	High-level	60.3	3.6	5.9	62.3	1.9	3.0

\*Quality control material of the assay manufacturers; †mean concentration in nanograms per milliliter (ng/mL x 2.5 = nmol/L); ‡standard deviation; §coefficient of variation in percent.

**TABLE 2.** Precision studies for the LC-MS/MS (reference method).

25(OH)D <sub>2</sub> /D <sub>3</sub>	Serum pools*	Within-run			Between-run		
		Mean (ng/mL)†	SD‡	CV (%)§	Mean (ng/mL)	SD	CV (%)
25(OH)D <sub>2</sub>	Pool 1	10.3	0.8	7.4	9.5	0.8	5.1
	Pool 2	20.1	1.4	6.9	19.2	1.4	5.1
	Pool 3	27.8	1.7	6.0	24.9	1.7	3.9
25(OH)D <sub>3</sub>	Pool 1	11.5	0.8	8.7	10.7	0.9	8.8
	Pool 2	21.6	1.4	7.3	21.2	1.4	6.4
	Pool 3	32.3	1.7	6.9	31.5	1.5	4.7

\*Three serum pools mixed with different 25(OH)D<sub>2</sub> (25[OH]D<sub>2</sub> spiked) and 25(OH)D<sub>3</sub> concentration levels; †mean concentration in nanograms per milliliter (ng/mL x 2.5 = nmol/L); ‡standard deviation; §coefficient of variation in percent.

### 25(OH)D<sub>2</sub> and C<sub>3</sub>-epimer detection by the LC-MS/MS method

In all blood samples (N = 198), no 25(OH)D<sub>2</sub> and no C<sub>3</sub>-epimer were detected with the LC-MS/MS method.

### Discussion

The present study compared one previous version and three new recently released NIST-SRM aligned 25(OH)D assays. Compared to the NIST-SRM aligned LC-MS/MS method, the new IDS-iSYS 25(OH)D<sup>5</sup> CLIA demonstrated a lower mean bias

(16.3%) than the new ORGENTEC 25(OH)D<sub>3</sub>/D<sub>2</sub> ELISA (17.8%). In addition, Deming regression analyses of the recalibrated IDS-iSYS 25(OH)D<sup>5</sup> and the ORGENTEC 25(OH)D<sub>3</sub>/D<sub>2</sub> assay showed proportional differences, when compared to the reference method. The IDS-iSYS 25(OH)D CLIA showed the lowest mean bias (1.5%) compared to the LC-MS/MS.

Interestingly, the previous version of the CLIA (not aligned to the NIST-SRM) showed the better agreement with the reference method compared to the NIST-SRM aligned immunoassays, expecting that the standardization of 25(OH)D assays would rep-

resent an improvement of inter-assay accordance in daily clinical 25(OH)D determination. Considering the results of this work, it cannot be recommended, that only NIST-SRM aligned methods should be used for routine 25(OH)D measurements.

However, recent studies with commercially available 25(OH)D assays before standardization have reported significant bias between 25(OH)D assays (19,20). For example, one immunochemical method performed on the Architect i2000 (Abbott GmbH, Vienna, Austria) showed a mean bias of 27.0% compared to the LC-MS/MS method (19). Furthermore own published data of a previous work presented a significant negative absolute mean bias of -22.8 nmol/L between the Cobas® Vitamin D<sub>3</sub> assay (Roche Diagnostics GmbH, Mannheim, Germany) and the LC-MS/MS method, leading to a misjudgment of the actual 25(OH)D status of a patient (21). Meanwhile the manufacturer has withdrawn this assay from the market. High inter-assay disagreement in the 25(OH)D measurements (20,22) can lead to an underestimation (23) or overestimation (24) of the 25(OH)D serum-levels. The lack of standardization, matrix effects, poor antibody specificity, and cross-reactivity with other 25(OH)D metabolites could be possible reasons for reported high inter-assay disagreement before standardization (21).

The C<sub>3</sub>-epimer is one of the vitamin D metabolites, which is considered to be a potential confounder in 25(OH)D measurements, especially in infants (16). In the present study, no newborns or young children were included. Although the C<sub>3</sub>-epimer is also described in adults (25,26), no C<sub>3</sub>-epimer was detected with the LC-MS/MS method.

Not only the C<sub>3</sub>-epimer but also other vitamin D metabolites are considered as possible reasons for significant inter-assay differences of previous published 25(OH)D comparative studies. Many chemiluminescence assays use antibodies to measure 25(OH)D in unextracted serum. These antibodies also recognize other vitamin D metabolites, such as 24, 25(OH)<sub>2</sub>D. Such assays not only provide the total 25(OH)D but also include the metabolites (27,28). Furthermore, some of these assays do not

always recognize 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> (27). An increased antibody specificity could be one potential reason for the improvement of the reproducibility and comparability of the new NIST SRM aligned 25(OH)D assay generation.

According to the literature (18), the within-run and between-run precision goal for each concentration level of this method evaluation study was a CV of ≤ 20%. Based on these criteria, all evaluated 25(OH)D methods demonstrated acceptable within- and between-run precision measurements. The poorest within-run precision with a CV of 19.0% was observed at the low-level precision measurements with the IDS-iSYS 25(OH)D<sup>S</sup> and the highest between-run CV with 19.1% was shown at the low-level precision measurements with the IDS-iSYS 25(OH)D assay. The low level precision measurements were performed close to the detection limits (IDS-iSYS 25[OH]D<sup>S</sup> assay: 7 ng/mL; IDS-iSYS 25[OH]D assay: 5 ng/mL; ORGENTEC 25(OH)D<sub>3</sub>/D<sub>2</sub> assay: 5 ng/mL) of these immunoassays. Consequently, the precision measurements at this concentration levels demonstrated the highest CV values. Using the imprecision data in the light of total error, additional error sources, which can affect the 25(OH)D measurements in daily practice, must be considered. Matrix effects could be one additional component of error, which affect individual patient samples in 25(OH)D assays. It is caused by interfering substances present in the sample assay tubes, which are not present in the standard assay tubes (21). In the present study, it was shown that biases still exist between different measurement principles and procedures after standardization.

Since the LC-MS/MS is considered to distinguish 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub>, we performed a different protocol for precision studies of this method (18). We used three serum pools (mixed with 25[OH]D<sub>2</sub> and 25[OH]D<sub>3</sub>) of three different concentration levels to evaluate the distinction and reproducibility of the 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> detection of this commercially available 25(OH)D assay. All within-run and between-run precision measurements of each 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> concentration level had a CV of ≤ 10%.



In comparative studies, the LC-MS/MS is widely used as the reference method (12). It is a reliable diagnostic tool and able to distinguish 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> (9,29). A low batch-to-batch variation and a low limit of detection are further advantages of this method (30). In the present study, we used an LC-MS/MS method, which has met the performance target set by the international DEQAS Advisory Panel in 2013 and 2014 (data not shown). The method was aligned to the NIST SRM 972a and enabled the separation and qualitative detection of the C<sub>3</sub>-epimers in a single analytical run. Nevertheless the biggest problem in 25(OH)D measurements in the last few years was the lack of a common standard. Furthermore not all LC-MS/MS methods used in previous comparative studies (20) could separate C<sub>3</sub>-epimers. The LC-MS/MS method in particular was recommended to be aligned to the NIST SRM and to be able to discriminate the C<sub>3</sub>-epimer (10,20). The strength of this study is that these recommendations of the VSDP have been completely fulfilled. The reported new generation of 25(OH)D assays (except the previous used IDS-iSYS 25[OH]D assay) tested are aligned to the NIST SRM 2972 or 972a.

The limitation of this study is that the precision studies of the immunoassays and the precision studies of the LC-MS/MS method were not performed with the same protocol. Therefore, the within-run and between-run precision measurements of the immunoassays are not comparable with the LC-MS/MS method.

In conclusion, the new generation of the NIST SRM aligned immunoassays and LC-MS/MS evaluated in this study are useful methods for measuring 25(OH)D serum-levels in clinical laboratories. The performance characteristics are suitable for routine diagnostic purposes.

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### Potential conflict of interest

Research funding cost for reagents were reimbursed by Immunodiagnostic Systems Ltd (Boldon, United Kingdom), ORGENTEC Diagnostika GmbH (Mainz, Germany), and RECIPE Chemicals + Instruments GmbH (Munich, Germany).

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