A candidate reference method and multiple commutable control materials for serum 25-hydroxyvitamin D measurement

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Abstract

Objectives: The aim of the current study was to establish a reliable candidate reference method for serum 25-hydroxyvitamin D [25(OH)D] measurement and to assess the commutability of multiple control materials among liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods.

Methods: Serum 25-hydroxyvitamin D2 [25(OH)D2] and 25-hydroxyvitamin D3 [25(OH)D3] together with spiked internal standards were extracted with a one-step approach and then analyzed by LC–MS/MS. The commutability assessment for 25(OH)D was conducted according to the Clinical and Laboratory Standards Institute (CLSI) EP14-A3 protocol. 25(OH)D concentrations in 5 levels of unprocessed serum pools, 7 levels of serum pools spiked with 25(OH)D3 or 25(OH)D2, 3 levels of commercial control materials, 2 levels of spiked bovine serum, and 4 levels of external quality assessment (EQA) materials were measured along with 30 single-donor samples using the candidate reference method and two routine LC–MS/MS methods.

Results: The candidate reference method could separate 25(OH)D2 and 25(OH)D3 from 14 potential interfering compounds completely within a 9-min analysis time. Good method precision was obtained, and measurement results on certified reference material NIST SRM 972a were within the uncertainty of the certified values. All candidate materials were assessed commutable for LC–MS/MS methods.

Conclusions: The candidate reference method for serum 25(OH)D measurement is precise, accurate, and robust against interferences and can provide an accuracy base for routine methods. The multiple alternative control materials with commutability among LC–MS/MS methods will facilitate the further standardization for serum 25(OH)D measurement.

Keywords
25-hydroxyvitamin D, accuracy-based external quality assessment, commutability, control materials, liquid chromatography–tandem mass spectrometry, reference method
1 | INTRODUCTION

Obtaining accurate results for serum 25(OH)D determination is critical for the classification of vitamin D nutrient status and the decision on vitamin D supplementation. To improve the performance of 25(OH)D assays, great efforts have been made by the Vitamin D Standardization Program (VDSP) and the Vitamin D External Quality Assessment Scheme (DEQAS) in terms of assay standardization and interference identification. On the whole, the great framework for standardization developed by the VDSP has led to general increase in the accuracy of 25(OH)D methods over the past decade. However, recent studies have revealed that considerable method-related variation remains a problem. The second VDSP intercomparison study demonstrated that there were still 50% of the ligand-binding assays and 47% LC-MS/MS methods failed to meet the VDSP criterion of mean bias ±5%.

To promote assay standardization, reference methods and reference materials are indispensable. So far, three reference methods have been recognized by the Joint Committee for Traceability in Laboratory Medicine (JCTLM). They are critically characterized and able to offer the accuracy base for 25(OH)D measurement. However, more reference measurement services and better EQA scheme design are required as the number of 25(OH)D assays grows. In China, 1200 laboratories participated in the 2021 EQA Program by the National Center for Clinical Laboratory (NCCL), and the overall coefficient of variation (CV) for inter-lab variation exceeded 20% at all concentration levels of 25(OH)D. Therefore, it is necessary for us to develop a 25(OH)D reference method to carry out accuracy-based EQA programs and to identify the source of method-related variation effectively.

 Commutable control materials are essential to avoid the impact of commutability-related biases on the interpretation of EQA results. The VDSP commutability study 2 reported the commutability of unprocessed serum pools among ligand binding assays and LC-MS/MS methods for serum 25(OH)D. However, using unprocessed human serum pools in EQA programs usually means the huge workload for obtaining an adequate amount of serum, the limitation in the coverage of clinically relevant intervals and stricter criteria for storage and shipping. By contrast, using lyophilized materials, materials of animal origin and spiked pools could overcome existing problems for unprocessed human serum pools, and these materials can usually be produced in large quantities at low costs. While it is less likely for manipulated materials to be commutable among ligand binding assays because of significant matrix effects, the testing principle of LC-MS/MS methods and its high specificity may enable manipulated materials to serve as commutable control materials and facilitate better EQA scheme design for LC-MS/MS methods.

The first aim of this study was to develop a candidate reference method for highly accurate quantification of serum 25(OH)D2 and 25(OH)D3 simultaneously. The second aim was to evaluate the commutability of multiple candidate reference materials for 25(OH)D among LC-MS/MS methods.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Certified spiking solution of 25(OH)D3 and 25(OH)D2 was both obtained from Cerilliant Corporation (Texas, USA); [23,24,25,26,27-25(OH)D3 (purity ≥95%) was from IsoSciences (PA, US); [6,19,19-2H3]-25(OH)D2 (purity: ≥95%) was from Sigma-Aldrich (MO, US). Detailed information about chemicals was shown in the File S1.

2.2 | Candidate reference materials

Candidate reference materials tested for commutability: Three levels of commercial lyophilized control materials (BRLyo L1-L3) were from Bio-Rad (CA, US; Lyyochek Immunoassay Plus Control, lot 40,380); four levels of EQA materials were lyophilized human serum-based materials customized from a company. These lyophilized materials were spiked with 25(OH)D3 or 25(OH)D2 and added with preservatives, and 6 vials for each level were redissolved, pooled, and re- aliquoted into 2-ml vials (1 ml/vial). Five levels of frozen serum pools (FSP L1-L5) were prepared from the leftovers of samples in clinical laboratories without anything added and filtered through a 0.22-μm membrane. Seven levels of frozen spiked serum pools (FSSP L1-L7) were prepared from the leftovers of serum samples and spiked with 25(OH)D3 and 25(OH)D2. Two levels of frozen spiked bovine serum (FBS L1-L2) were made by adding 25(OH)D3 and 25(OH)D2 to bovine serum (Sigma-Aldrich). All the serum pools were mixed thoroughly by inverting, aliquoted into 2-mL vials (1 ml/vial), frozen at −80°C, or lyophilized.

2.3 | Serum samples

Single-donor serum samples were collected from 30 healthy people in Beijing hospital. All candidate reference materials and samples were shipped on ice to the laboratories and stored at −80°C until analysis. The study had been reviewed and approved by the Ethics Committee of Beijing Hospital.

2.4 | Calibration and sample preparation

All volumetric steps were gravimetrically controlled. The solution standards for 25(OH)D3 and 25(OH)D2 from Cerilliant Corporation were weighed, diluted with absolute ethanol, and combined to prepare working standard mixtures of 25(OH)D3 and 25(OH)D2.
\textsuperscript{13}C\textsubscript{3}-25(OH)D\textsubscript{3} solids and \textsuperscript{3}H\textsubscript{3}-25(OH)D\textsubscript{2} solids were used to prepare internal standard (IS) mixtures. Detailed description of calibration preparation was in the Supplementary File S1.

For sample preparation, the concentrations of 25(OH)D\textsubscript{3} and 25(OH)D\textsubscript{2} in each serum sample were first determined with an LC-MS/MS method to estimate the needed amount of serum. An appropriate volume of each serum sample (ranging from 100 to 800\,µl) was accurately weighed into a clean 10-ml ampoule, and 30–50\,µl of IS was added to obtain the expected ratio of natural/labeled (ranging from 0.25 to 2.5) for both 25(OH)D\textsubscript{3} and 25(OH)D\textsubscript{2}. Then we added 200\,µl of 0.1 M aqueous Na\textsubscript{2}CO\textsubscript{3} solutions to facilitate the release of 25(OH)D from vitamin D binding protein (VDBP), and water to the mixture to achieve a final volume of 1 ml. This mixture was equilibrated for 1 h at room temperature before the extraction of the two compounds of interest from serum in one-step approach consisting of protein precipitation and liquid–liquid extraction (LLE) with 1 ml of 0.1 M ZnSO\textsubscript{4} methanol/water (v/v, 1/1) solutions and 8 ml of n-hexane. After being shaken for 30 min, the mixture was centrifuged at 1620 g for 5 min. Thereafter, the upper hexane layer was transferred and evaporated under nitrogen, and the residues were reconstituted in 150\,µl mobile phase of 0.1% formic acid in water / methanol (v/v, 30/70) for LC–MS/MS analysis.

### 2.5 | LC–MS/MS analysis and calculation

The LC–MS/MS analysis was performed with atmospheric pressure chemical ionization (APCI) in positive ion mode. Gradient elution was performed on a Shimadzu Shim-pack Velox PFPP column (2.1 x 100 mm, 1.8 \,µm) with a mobile phase consisting of 0.1% formic acid in water (A) and methanol (B) at a flow rate of 0.45 ml/min. Detailed description of solvent gradient and ion pairs were shown in the File S1. MS parameters were summarized in Table S1. Five-point bracketing calibration curves were obtained by plotting peak area ratios of the analyte quantification ion divided by the IS on the y-axis and the corresponding mass ratio on the x-axis. With the calibration equation, y (peak area ratio) = b\times (mass ratio)+a, the mean area ratio of the three injections was used to calculate the mass fraction (ng/g) of 25(OH)D\textsubscript{3} or 25(OH)D\textsubscript{2} in each sample. And the mass fraction (ng/g) was then converted to mass concentration (ng/mL) by multiplying the density of the serum measured with a density meter (DMA 4500 M, Anton Paar, Austria).

### 2.6 | Method validation

To evaluate method imprecision, four pooled serum samples were measured in triplicate in three runs, and the within-day, between-day, and total CV were calculated for each level.\textsuperscript{21} Method true- ness was assessed using NIST 972a Level 1-Level 4 (frozen human serum based) with two preparations per level over 2 days (n = 4).\textsuperscript{22} Trueness was expressed as percent difference from the NIST-certified value. To assess analytical recovery, serum samples at 25(OH)D\textsubscript{3} concentrations of 18.20\,ng/g were spiked with different amounts of 25(OH)D\textsubscript{3} standard solution to final concentrations of 35.45, 62.55, and 118.87\,ng/g, and serum samples at 25(OH)D\textsubscript{2} concentrations of 1.05\,ng/g were spiked with different amounts of 25(OH)D\textsubscript{2} standard solution to final concentrations of 3.08, 6.27, 12.90\,ng/g. The analytical recovery was calculated as the ratio of measured concentrations to expected concentrations. Furthermore, this method was used to measure two samples from the 2020 IFCC external quality assessment scheme for Reference Laboratories in Laboratory Medicine (RELA).\textsuperscript{23} To determine the matrix effect in normal serum, hemolyzed serum, lipemic serum, and EDTA plasma, we compared the peak area of the internal standard added to extracted serum samples with that added to a calibrator.\textsuperscript{24} We tested 14 structural analogues and metabolites of 25(OH)D for potential interference, among which were 3-epi-25(OH)D\textsubscript{3}, alfalcacidol, 7α-hydroxy-4-cholesten-3-one, 3-epi-25(OH)D\textsubscript{2}, and doxercalciferol that have the same molecular weight or fragmentation pattern with 25(OH)D\textsubscript{3} or 25(OH)D\textsubscript{2}. The limit of detection (LOD) and quantification (LOQ) was defined as the amount of 25(OH)D\textsubscript{3} or 25(OH)D\textsubscript{2} with a signal-to-noise (S/N) ratio of 3:1 and 10:1, respectively.

### 2.7 | Measurement of 25(OH)D and commutability assessment

The candidate reference materials and 30 single-donor serum samples were measured using the candidate reference method and two LC–MS/MS assays developed by laboratories of BioSino Biotechnology and Science Inc. (Beijing, China) and Beijing Harmony Health Medical Diagnostics Co., LTD (China). The BioSino method used Waters Acquity TQD LC–MS/MS and Waters UPLC BEN F5 column (1.8 \,µm, 2.1 x 50 mm) for analysis and was unable to separate 3-epi-25(OH)D\textsubscript{3} from 25(OH)D\textsubscript{3}. The Harmony method used AB SCIEX TRIPLE QUAD 6500+ and Phenomenex Kinetex F5 column (2.6 \,µm, 3.0 x 50 mm) and was able to quantify 3-epi-25(OH)D\textsubscript{3} and 24,25(OH)\textsubscript{2}D\textsubscript{3}. All 51 samples were measured in duplicate on the same day, with the testing order of analyzing all samples first in ascending and then descending order. IQC materials were measured in pre-analytical, analytical, and post-analytical testing phases following the validation of the calibration curves by the instrument. The Intra-run CVs were <2.1% and <2.0% for 3 concentrations between 6.8 and 94\,ng/ml using the Harmony method and BioSino method, respectively. The commutability study was carried out according to the CLSI EP14-A3 protocol.\textsuperscript{19,25} Ordinary linear regression (OLR) and 95% prediction intervals (PIs) were performed using the mean of replicates for each of single-donor samples. Measurement results of the candidate reference materials were compared with the limits of the PIs, and those with results within the PIs were classified as commutable. Statistical analyses and calculations were carried out using Microsoft Excel 2010 (Microsoft Corporation).
3 | RESULTS

3.1 | Method validation

The mean within-run and total imprecision assessed from four pooled serum were 0.97% (ranges, 0.64%–1.25%) and 1.13% (ranges, 0.97%–1.25%) for 25(OH)D3, and 1.68% (ranges, 1.24%–2.18%) and 1.98% (ranges, 1.24%–2.77%) for 25(OH)D2, respectively (Table 1). We assessed the trueness of the developed method using certified reference material NIST SRM 972a. As shown in Table 2, the results were in good agreement with the certified values within the uncertainties.22 The mean relative recoveries (±CV) were 100.8±0.8% to 101.0±0.6% and 99.9±1.2% to 101.0±1.5% for 25(OH)D3 and 25(OH)D2, respectively (Table 3). In addition, our method showed relative deviations of −0.8% and −1.2% (n = 2) from the mean results of all 12 laboratories for 25(OH)D3 in sample A and B, according to the data from the 2020 IFCC RELA. Measurement uncertainties of the results were estimated according to the ISO Guide (GUM).26 The estimated relative expanded measurement uncertainty was 1.0%–1.1% for 25(OH)D3 and 1.2%–2.1% for 25(OH)D2 at four concentration levels, respectively. A detailed description of potential sources of uncertainty and their contributions were shown in the Table S2.

From the matrix effect results, there was no apparent matrix influence on analyte ionization for both 25(OH)D3 and 25(OH)D2. The average ion intensity differences were −1.13%, −2.08% to −4.15%, and −4.67% for 25(OH)D3, and 2.20%, 0.23% to −0.55% and −1.77% for 25(OH)D2 in serum, hemolyzed serum, lipemic serum, and EDTA plasma, respectively. As shown in Figure 1, 14 compounds were able to be separated from 25(OH)D3 and 25(OH)D2 completely with relative retention time of greater than 1.1 or less than 0.52. Detailed information about MS conditions and relative retention time of 14 compounds was provided in the Table S3. As 15 μl of 1.7 ng/g 25(OH)D3 standard solution produced a S/N of 49.4 (CV = 7.8%, n = 8), the LOD and LOQ for 25(OH)D3 were estimated to be 0.001 ng (S/N of 3:1) and 0.004 ng (S/N of 10:1) in amount, respectively. And injection of 15 μl of 2.0 ng/g 25(OH)D2 standard solution produced a S/N of 24.1 (CV = 11.8%, n = 8), so the LOD (S/N of 3:1) and LOQ (S/N of 10:1) for 25(OH)D2 were estimated to be 0.003 and 0.010 ng.

Table 1 The precision evaluation of the candidate reference method

| Sample  | Set | Mean, ng/g | Grand mean, ng/g | Within-run CV, % | Total CV, % |
|---------|-----|------------|-----------------|-----------------|-------------|
| 25(OH)D3 | Conc 1 | 6.26 | 6.29 | 1.25 | 1.25 |
|         | Conc 2 | 19.12 | 18.98 | 0.88 | 0.97 |
|         | Conc 3 | 26.97 | 26.73 | 1.09 | 1.2 |
|         | Conc 4 | 33.55 | 33.12 | 0.64 | 1.23 |
| 25(OH)D2 | Conc 1 | 0.75 | 0.76 | 1.58 | 2.77 |
|         | Conc 2 | 1.05 | 1.05 | 2.18 | 2.18 |
|         | Conc 3 | 2.99 | 2.99 | 1.24 | 1.24 |
|         | Conc 4 | 5.16 | 5.17 | 1.73 | 1.73 |

3.2 | Measurement of 25(OH)D and commutability assessment

Total 25(OH)D concentrations by the candidate reference method ranged from 11.28 to 59.34 ng/ml in 30 single-donor samples and ranged from 13.53 to 56.6 ng/ml in candidate materials. The 3-epi-25(OH)D3 concentration (mean±SD, ng/ml) was 1.80±1.01 for single-donor samples, and 1.05±0.61 for candidate materials, respectively. Correlations between measurement results by routine LC–MS/MS methods and those by the candidate reference method were analyzed with OLR. The slope (95% CI), intercept (95% CI), correlation coefficient (r), and S_{xy} were 0.97 (0.91 to 1.03), 0.43 (−2.03 to 2.53), 0.99, 1.95 for Harmony method and 0.97 (0.90 to 1.05), 0.80 (−1.86 to 3.70), 0.98, 2.37 for BioSino method. Commutability of candidate materials among LC–MS/MS methods is summarized in Figure 2 and Table 4. All materials are commutable for LC–MS/MS assays.

4 | DISCUSSION

We developed a candidate reference method and provided a comprehensive and careful method validation for its performance characteristics. Compared to previous reference methods,11–13,27 our method has shorter analysis time while completely separating 25(OH)D from 14 structural analogs with similar molecular masses or ionization patterns to 25(OH)D3 and 25(OH)D2. Additionally, we used protein precipitation combining LLE to prepare samples instead of using repeated LLE in previous studies.11–13 Compared to the repeated LLE process that requires adding organic solvents twice, centrifuging, and extracting the lower layer twice, our approach only
requires these steps to be performed once. Therefore, the sample preparation procedure is simplified while producing clean extract for analysis, as evidenced by the absence of any apparent ion suppression or enhancement effect. In the process of method development, we also compared protein precipitation combining LLE with other sample preparation techniques including protein precipitation combining solid-phase extraction, solid–liquid extraction, and phospholipid removal plate. The results for comparison were shown in Table S4 and S5, and it is observed that the protein precipitation combining LLE produced the highest S/N ratios for both 25(OH)D3 and 25(OH)D2. Furthermore, the developed method was in agreement with the imprecision and trueness reported by previous

### Table 2 - Trueness of the developed method for quantification of 25(OH)D2 and 25(OH)D3 using NIST SRM 972a with certified values

|         | SRM 972a-Level 1 | SRM 972a-Level 2 | SRM 972a-Level 3 | SRM 972a-Level 4 |
|---------|------------------|------------------|------------------|------------------|
| 25(OH)D3 |                  |                  |                  |                  |
| n\(^a\)  | 4                | 4                | 4                | 4                |
| Target±U,\(^b\) ng/g | 28.1±1.1         | 17.7±0.4         | 19.4±0.4         | 28.8±0.9         |
| Mean (CV), ng/g | 28.2 (0.4%)      | 17.3 (1.3%)      | 19.1 (1.1%)      | 27.9 (0.6%)      |
| Trueness, % | 100.4            | 97.9             | 98.2             | 97               |
| 25(OH)D2 |                  |                  |                  |                  |
| n\(^b\)  | 0                | 4                | 4                | 0                |
| Target±U,\(^b\) ng/g | -                | 0.79±0.08        | 12.9±0.3         | -                |
| Mean (CV), ng/g | -                | 0.75 (5.9%)      | 13.2 (1.2%)      | -                |
| Trueness, % | -                | 94.9             | 102.3            | -                |

\(^a\)Number of independent analysis measurement.

\(^b\)The target value and the uncertainty listed in the 2021 NIST certificates of analysis.

### Table 3 - Relative recovery of added 25(OH)D3 and 25(OH)D2 for the developed method

| Conc Added, ng/g | Expected result, ng/g | Mean detected, ng/g | Mean relative recovery, % | CV (%), n = 2 |
|------------------|-----------------------|---------------------|---------------------------|--------------|
| 25(OH)D3         |                       |                     |                           |              |
| 0                | -                     | 18.2                | 18.2                      | -            |
| 1                | 17.25                 | 35.45               | 35.73                     | 100.8        |
| 2                | 44.35                 | 62.55               | 63.02                     | 100.8        |
| 3                | 100.67                | 118.87              | 120.1                     | 101.0        |
| 25(OH)D2         |                       |                     |                           |              |
| 0                | -                     | 1.05                | 1.05                      | -            |
| 1                | 2.03                  | 3.08                | 3.11                      | 101.0        |
| 2                | 5.22                  | 6.27                | 6.26                      | 99.9         |
| 3                | 11.84                 | 12.9                | 13.02                     | 101.0        |

### Figure 1 - The chromatogram for 25(OH)D3, 25(OH)D2, and 14 compounds tested for potential interference
reference methods.\textsuperscript{11–13} In the light of all these characteristics, the candidate reference method can serve as an accuracy base to facilitate the improvement of routine 25(OH)D measurement.

In the section of commutability study, all manipulated control materials showed commutability among three LC-MS/MS methods, although these methods used different sample preparation techniques. This may be attributed to the high specificity of LC-MS/MS and strong extraction ability for 25(OH)D in the sample preparation steps.\textsuperscript{4,28} The results are of concern in promoting the standardization of LC-MS/MS assays for 25(OH)D measurement and helping them achieve the highest level of accuracy, because these manipulated materials have obvious advantages in the cost, storage stability, and availability compared to unprocessed serum pools, which will facilitate a better EQA scheme design, including improvement in the number of specimens per distribution, number of distributions per year and concentration intervals covered.\textsuperscript{30} As more and more commercial LC-MS/MS assays and laboratory-developed LC-MS/MS methods are applied to measure 25(OH)D and many of them use home-made calibrators,\textsuperscript{29} it is critical for accuracy-based EQA programs to be suitably designed to assess and monitor their performance. The multiple commutable control materials promise to accelerate the standardization of LC-MS/MS methods for 25(OH)D measurement.

In conclusion, the candidate reference method for serum 25(OH)D measurement is precise, accurate, and robust against interferences, and can provide an accuracy base for routine methods. Frozen human serum pools spiked with 25(OH)D3 or 25(OH)D2, spiked bovine serum, and commercial control materials are assessed as commutable for LC-MS/MS methods. Obvious advantages of these alternative control materials will facilitate a better EQA scheme design and promote the further standardization for serum 25(OH)D measurement.

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CONFLICT OF INTEREST
The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT
Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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REFERENCES
1. Deluca HF. History of the discovery of vitamin D and its active metabolites. Bonekey Rep. 2014;3:479.
2. Holick MF, Binkley NC, Bischoff-Ferrari HA, et al. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab. 2011;96(7):1911-1930.

TABLE 4 Commutability of the candidate materials based on the CLSI EP14-A3 protocol

| Assays       | EQA L1-L4 | BRlyo L1-L3 | FSP L1-L5 | FSSP L1-L7 | FBS L1-L2 |
|--------------|-----------|------------|-----------|------------|-----------|
| Harmony assay| C         | C          | C         | C          | C         |
| BioSino assay| C         | C          | C         | C          | C         |

FIGURE 2 Commutability of the candidate materials based on the CLSI EP14-A3 protocol. The X-axis and Y-axis represent the total 25(OH)D concentrations. FSP L1-L5, five levels of frozen serum pools; BRlyo L1-L3, three levels of commercial lyophilized control materials from Bio-Rad; FBS L1-L2, two levels of frozen spiked bovine serum; FSSP L1-L7, seven levels of frozen spiked serum pools; EQA L1-L4, four levels of EQA materials customized from a company.
3. Shen Y, Pu L, Si S, et al. Vitamin D nutrient status during pregnancy and its influencing factors. Clin Nutr (Edinburgh, Scotland). 2020;39(5):1432-1439.

4. Bjerg LN, Halsegren JR, Hansen SH, Morris HA, Jorgensen NR. An evaluation of total 25-hydroxyvitamin D assay standardization: where are we today? J Steroid Biochem Mol Biol. 2019;190:224-233.

5. Burdette CQ, Camara JE, Nalin F, et al. Establishing an accuracy basis for the vitamin D external quality assessment scheme (DEQAS). J AOAC Int. 2017;100(5):1277-1287.

6. Wise SA, Camara JE, Sempos CT, et al. Vitamin D standardization program (VDSP) intralaboratory study for the assessment of 25-hydroxyvitamin D assay variability and bias. J Steroid Biochem Mol Biol. 2021;212:105917.

7. Cavalier E, Souberbielle JC. Vitamin D and its metabolites: from now and beyond. EJIFCC. 2018;29(2):105-110.

8. Wise SA, Camara JE, Burdette CQ, et al. Interlaboratory comparison of 25-hydroxyvitamin D assays: vitamin D standardization program (VDSP) Intercomparison study 2 - part 1 liquid chromatography - tandem mass spectrometry (LC-MS/MS) assays - impact of 3-epi-25-hydroxyvitamin D3 on assay performance. Anal Bioanal Chem. 2021;414:333-349.

9. Wise SA, Camara JE, Burdette CQ, et al. Interlaboratory comparison of 25-hydroxyvitamin D assays: vitamin D standardization program (VDSP) Intercorrelation study 2 - part 2 ligand binding assays - impact of 25-hydroxyvitamin D2 and 24R,25-dihydroxyvitamin D3 on assay performance. Anal Bioanal Chem. 2021;414:351-366.

10. Erdman P, Palmer-Toy DE, Horowitz G, Hoofnagle A. Accuracy-based vitamin D survey: six years of quality improvement guided by proficiency testing. Arch Pathol Lab Med. 2019;143(12):1531-1538.

11. Tai SS, Bedner M, Phinney KW. Development of a candidate reference measurement procedure for the determination of 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 in human serum using isotope-dilution liquid chromatography-tandem mass spectrometry. Anal Chem. 2010;82(5):1942-1948.

12. Stepman HC, Vanderroost A, Van Uyttendaele K, Thiennout LC. Candidate reference measurement procedures for serum 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 by using isotope-dilution liquid chromatography-tandem mass spectrometry. Clin Chem. 2011;57(3):441-448.

13. Mineva EM, Schleicher RL, Chaudhary-Webb M, et al. A candidate reference measurement procedure for quantifying serum concentrations of 25-hydroxyvitamin D(3) and 25-hydroxyvitamin D(2) using isotope-dilution liquid chromatography-tandem mass spectrometry. Anal Bioanal Chem. 2015;407(19):5615-5624.

14. The national external quality assessment (EQA) in China. Available from: https://www.clnet.com.cn/.

15. Lo SF, Alter D. Reemphasizing the importance of commutability. Arch Pathol Lab Med. 2021;145(2):129a.

16. Greg Miller W, Greenberg N, Budd J, Delatour V. The evolving role of commutability in metrological traceability. Clin Chem Acta. 2021;514:84-89.

17. Camara JE, Wise SA, Hoofnagle AN, et al. Assessment of serum total 25-hydroxyvitamin D assay commutability of standard reference materials and College of American Pathologists Accuracy-Based Vitamin D (ABVD) scheme and vitamin D external quality assessment scheme (DEQAS) materials: vitamin D standardization program (VDSP) commutability study 2. Anal Bioanal Chem. 2021;413(20):5067-5084.

18. Badrick T, Miller WG, Panteghini M, et al. Interpreting EQA-understanding why commutability of materials matters. Clin Chem. 2022;68(4):494-500.

19. Zeng J, Qi T, Wang S, et al. Commutability of control materials for external quality assessment of serum apolipoprotein A-I measurement. Clin Chem Lab Med. 2018;56(5):789-795.

20. Meng Q, Zhou W, Zhang C, et al. Serum triglyceride measurements: the commutability of reference materials and the accuracy of results. Clin Chem Lab Med. 2017;55(9):1284-1290.

21. Wang Y, Zhang T, Zhao H, et al. Measurement of serum progesterone by isotope dilution liquid chromatography tandem mass spectrometry: a candidate reference method and its application to evaluating immunoassays. Anal Bioanal Chem. 2019;411(11):2363-2371.

22. National Institute of Standards and Technology. Certificate of Analysis, Standard Reference Material 972a: Vitamin D Metabolites in Frozen Human Serum. NIST; 2021.

23. External quality control for Reference Laboratories. Available from: http://dkgl-rfb.de:81/

24. Long Q, Zhang T, Yan Y, et al. Measurement of serum 17-hydroxyprogesterone using isotope dilution liquid chromatography-tandem mass spectrometry candidate reference method and evaluation of the performance for three routine methods. Clin Chem Lab Med. 2021;59(3):523-532.

25. Pittas AG, Dawson-Hughes B, Sheehan P, et al. Vitamin D supplementation and prevention of type 2 diabetes. N Engl J Med. 2019;381(6):520-530.

26. <iso_iec_guide_98-3_2008e-.uncertainty-of-measurement--part-3-guide-to-the-expression-of-uncertainty-in-measurement-gum1995.pdf>.

27. Tai SS, Nelson MA. Candidate reference measurement procedure for the determination of 25-dihydroxyvitamin D3 in human serum using isotope-dilution liquid chromatography-tandem mass spectrometry. Anal Chem. 2015;87(15):7964-7970.

28. Makris K, Bhattoa HP, Cavalier E, et al. Recommendations on the measurement and the clinical use of vitamin D metabolites and vitamin D binding protein - a position paper from the IFCC committee on bone metabolism. Clin Chim Acta. 2021;517:171-197.

29. Lingxiao S, Jinfei M, Qingying Y, Pengyu L, Huafen L. The application of certified reference materials for clinical mass spectrometry. J Clin Lab Anal. 2022;36(4):e24301.

30. van den Ouweland JM, Beijers AM, van Daal H. Fast separation of 25-hydroxyvitamin D3 from 3-epi-25-hydroxyvitamin D3 in human serum by liquid chromatography-tandem mass spectrometry: variable prevalence of 3-epi-25-hydroxyvitamin D3 in infants, children, and adults. Clin Chem. 2011;57(11):1618-1619.

31. Aghajafari F, Field CJ, Rabi D, et al. Plasma 3-Epi-25-hydroxycholecalciferol can Alter the assessment of vitamin D status using the current reference ranges for pregnant women and their newborns. J Nutr. 2016;146(1):70-75.

32. Berger SE, Van Rompay MI, Gordon CM, et al. Investigation of the C-3-epi-25(OH)D3(3) of 25-hydroxyvitamin D(3) in urban schoolchildren. Applied physiology, nutrition, and metabolism =. Physi Appl Nutr Metab. 2018;43(3):259-265.

SUPPORTING INFORMATION
Additional supporting information can be found online in the Supporting Information section at the end of this article.