Novel sandwich immunoassay for quantification of 25-hydroxy vitamin D in human blood

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Introduction

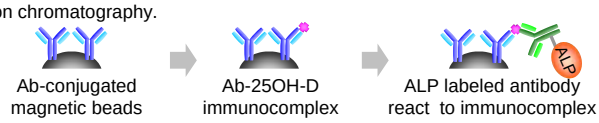
Vitamin D is a key regulator of calcium homeostasis and bone remodeling, and also known to be involved in several non-bone diseases. The most reliable vitamin D marker called 25-hydroxy vitamin D (25OH-D) can be measured by competitive immunoassay, HPLC, and liquid chromatography tandem mass spectrometry (LC-MS/MS). Demand for automated 25OH-D assay continues to grow as the test number increases, but currently available automated immunoassays reportedly fail to meet accuracy and specificity requirements.

Here we show a novel sandwich immunoassay for 25OH-D using an antibody that specifically recognizes immunocomplex consisting of 25OH-D and anti-25OH-D antibody. Anti-25OH-D antibody-conjugated magnetic beads were mixed with specimens in treatment solution to form immunocomplex, and the complex was quantified using an alkaline phosphatase-labeled secondary antibody recognizing the immunocomplex. All reactions were executed on fully automated chemiluminescence analyzer (LUMIPULSE, Fujirebio Inc.).

Materials and Procedure

Antibodies

Anti-25OH-D antibody was used to coat magnetic beads. We have developed an monoclonal antibody that specifically recognizes the immunocomplex formed by 25OH-D and anti-25OH-D antibody. For the antibody establishment, ADLib (Autonomously Diversifying Library) system, an in vitro antibody generation system developed by Chiome Bioscience Inc. was used. ADLib system utilizes gene diversified DT40 cell libraries. Antibody was conjugated with alkaline phosphatase (ALP) and purified by gel filtration chromatography.



Standards Preparation

Crystalline 25OH-D3 was dissolved into 99.9 % EtOH to make stock solution, and standards were prepared by further diluting the solution with horse serum. The concentration of the ethanolic stock solution is determined using UV spectrophotometer.

Serum samples

Human serum samples were obtained from Trina Bioreactives. Serum aliquots were stored at -80℃. Quantitative determination of serum 25OH-D was performed by DiaSorin 25 hydroxyvitamin D ^{125} I RIA, and the concentration of 25OH-D were between 8 and 174 ng/mL.

Chemiluminescent Sandwich Immunoassay

The principles of the assay are two-step sandwich assay. All reactions were executed on fully automated chemiluminescence analyzer **LUMIPULSE G1200** (Fujirebio Inc., Tokyo, Japan). Human specimens were mixed with anti-25OH-D antibody-conjugated magnetic beads in treatment solution. After washing, the immunocomplex was quantified using an ALP-labeled second antibody against the complex.

- **Time to first result: 30 min**
- **Tests per hour: 120**
- **Sample volume: 10 – 30 uL**

Reaction cartridge

Results

Figure 1. Dose- response curve of the chemiluminescent sandwich Immunoassay for 25OH-D3 standard solutions.

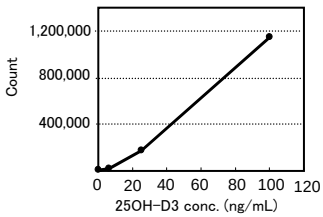


Table 1. Precision and Sensitivity of the LUMIPULSE assay

The precision of the assay was determined using 3 human serum specimens with 25OH-D level ranging from 14 to 97.7 ng/mL. Each specimen was assayed in 20 replicates per run and 4 replicates per run on 5 days to determine the within-run CV and total CV, respectively. Precision ranges of our sandwich assay were 1.0-2.3% for within-run, and 1.9-3.5% for total precision. The limit of detection was 1.3 ng/mL, and the limit of quantification (LOQ) was 3.1ng/mL. LOQ was defined as the sample concentration at which CV < 10%, and determined from the precision profile.

Precision

Sample	n	Conc. (RIA, ng/mL)	Within-run CV (%)	Total CV (%)
L	20	14.3	1.4	2.2
M	20	39.1	1.0	1.9
H	20	97.7	2.3	3.5

Sensitivity

Limit of detection	1.3 ng/mL
Limit of quantification	< 3.1 ng/mL

Figure 2. Comparison of LUMIPULSE assay and DiaSorin RIA in 25OH-D measurements using correlation graph (a) and Bland and Altman plot (b).

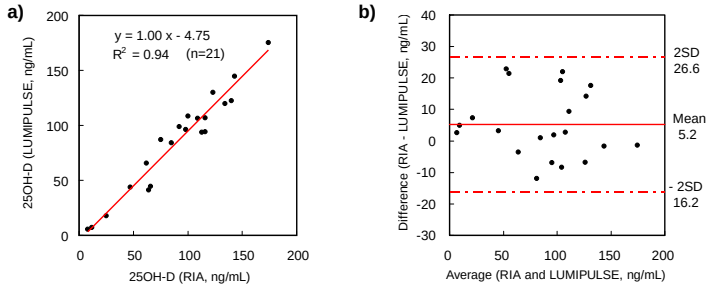
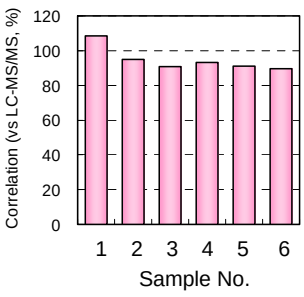
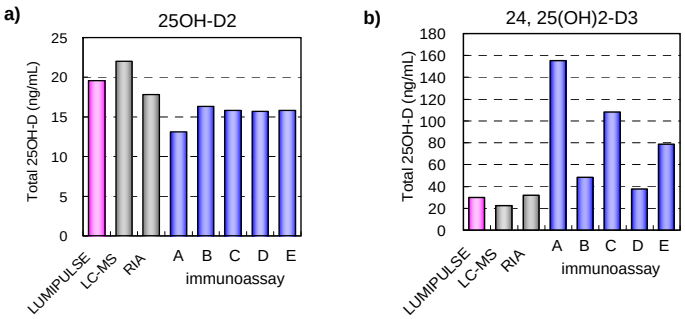


Figure 3. Accuracy of the LUMIPULSE assay



Quantitative determination of serum 25OH-D was performed by LUMIPULSE and LC-MS/MS, and the concentration of 25OH-D were between 11.0 and 30.6 ng/mL.

Figure 4. Reactivity evaluation for 25OH-D2 and 24, 25(OH)2-D3



a) Serum endogenously contained 14.5ng/mL 25OH-D2 (approx. 65% of total 25OH-D).
b) Serum were spiked with 24S,25(OH)$_2$-D3. Using two antibodies, our assay showed improved specificity against immunoreactive derivatives such as 24S,25(OH)$_2$-D3.

Conclusion

Our sandwich assay for 25OH-D specifically detected 25OH-D immunocomplex in a dose-dependent manner, and demonstrated significant correlation with the conventional methods. Assay performance was significantly improved by converting the immunoassay principle from competitive to sandwich. Our novel assay would provide high-throughput, accurate and specific immunoassay for 25OH-D.