

# Noncompetitive Immunoassay Detection System for Haptens on the Basis of Antimetatype Antibodies

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**BACKGROUND:** Small molecules classified as haptens are generally measured by competitive immunoassay, which is theoretically inferior to noncompetitive sandwich immunoassay in terms of sensitivity and specificity. We created a method for developing sandwich immunoassays to measure haptens on the basis of antimetatype antibodies.

**METHODS:** We generated antimetatype monoclonal antibodies against a hapten-antibody immunocomplex using an ex vivo antibody development system, the Autonomously Diversifying Library (ADLib) system. We selected 2 haptens, estradiol ( $E_2$ ) and 25-hydroxyvitamin D [25(OH)D], as analytes. Sandwich immunoassays for these 2 haptens were developed by use of a 96-well microtiter plate and a fully automated chemiluminescence analyzer, and the performances of these immunoassays were investigated.

**RESULTS:** The developed assays exhibited sensitivity high enough to detect target haptens in serum samples. The limit of detection of the ELISA for  $E_2$  was 3.13 pg/mL, and that of the fully automated chemiluminescent enzyme immunoassay (CLEIA) system was 2.1 ng/mL for 25(OH)D. The cross-reactivity with immunoreactive derivatives was effectively improved compared with the competitive assay. The CVs for the sandwich ELISA for  $E_2$  were 4.2%–12.6% (intra-assay) and 6.2%–21.8% (total imprecision). The CVs for the sandwich CLEIA for 25(OH)D were 1.0%–2.3% (intra-assay) and 1.9%–3.5% (total imprecision). In particular, the sandwich CLEIA for 25(OH)D showed correlations of  $r = 0.99$  with both LC-MS/MS and a commercially available  $^{125}\text{I}$  RIA.

**CONCLUSIONS:** Our method represents a potentially simple and practical approach for routine assays of haptens, including vitamins, hormones, drugs, and toxins.

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A hapten is a small molecule that reacts with a specific antibody but cannot elicit an immune response unless bound to a carrier protein or other large antigenic molecule. Haptens such as hormones, vitamins, drugs, and toxins play a wide variety of roles in biology, and accurate measurement of them is essential. Estradiol ( $E_2$ )<sup>1</sup> and 25-hydroxyvitamin D [25(OH)D] are haptens that are routinely measured for clinical purposes.  $E_2$  is a female sex hormone produced by the ovaries (1), and serum concentrations of  $E_2$  are used to assess ovarian function in women with menstrual disorders, precocious or delayed puberty, and assisted reproduction, as well as to monitor the effect of aromatase inhibitor treatment in breast cancer patients and determine postmenopausal status (2–4). Vitamin D is a fat-soluble, secosteroid hormone that regulates bone metabolism (5). Vitamin D deficiency leads to rickets and osteomalacia and is also associated with increased risk of many types of cancer (6), cardiovascular diseases (7), autoimmune diseases (8), diabetes (9), infectious diseases (10), and dementia (11). Circulating 25(OH)D is widely recognized as the best indicator of vitamin D status.

Until now, haptens have been measurable only by use of competitive immunoassays because their limited molecular sizes impede simultaneous binding of 2 antibodies to 1 hapten molecule. The analytical sensitivity and specificity of a competitive immunoassay depend on the affinity and specificity of only 1 antihapten antibody, which rarely exceeds the range of  $10^{10} \text{ M}^{-1}$  as the affinity constant (12) and often exhibits cross-reactivity to immunological derivatives. On the other hand, the noncompetitive sandwich immunoassay format permits the reaction of analytical target to excess amount of antibodies and the double recognition of the target with the primary and labeled antibodies. Because of these advantages, the sandwich immunoassay is in general superior to the competitive immunoassay in analytical sensitivity and specificity (13).

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Received September 4, 2014; accepted February 2, 2015.

Previously published online at DOI: 10.1373/clinchem.2014.232728

<sup>1</sup> Nonstandard abbreviations:  $E_2$ , estradiol; 25(OH)D, 25-hydroxyvitamin D; mAb, monoclonal antibody; CMO, carboxymethylxime; AP, alkaline phosphatase; ADLib, Autonomously Diversifying Library; 1,25(OH)<sub>2</sub>-D<sub>3</sub>, 1,25-dihydroxyvitamin D<sub>3</sub>; AMPPD, 3-(2'-spiroadamantane)-4-methoxy-4-(3'-phosphoryloxy)phenyl-1,2-dioxetane disodium salt; DEQAS, International Vitamin D Quality Assessment Scheme; ID, isotope dilution; CLEIA, chemiluminescent enzyme immunoassay; LOD, limit of detection; bio- $E_2$ , biotinylated  $E_2$ .

There have been attempts to develop technologies for the detection of haptens with sandwich immunoassays. Wei et al. demonstrated that tacrolimus, with a molecular weight of 804 Da, could be measured by a true sandwich immunoassay in which 2 antibodies were able to bind to tacrolimus simultaneously without overlap of the binding sites (14). Other methodologies for smaller haptens include idiometric assay with anti-idiotypic antibodies (15–17), antimetatype antibody-based immunoassay (18, 19), and open sandwich immunoassay (20, 21). Although these approaches have achieved good performance in measuring several haptens, the difficulties of developing and identifying these unconventional antibodies by classic immunization procedures have been major limitations for their wide application.

Here we report a method to develop sandwich assays for haptens on the basis of antimetatype antibodies. We focused on the antimetatype antibody-based immunoassay because it is the only nonconventional sandwich immunoassay proposed so far that enables simultaneous recognition of a hapten by 2 antibodies, which could be a key advantage to develop analytically sensitive and specific immunoassays.

## Materials and Methods

### ESTABLISHMENT AND SOURCE OF PRIMARY MONOCLONAL ANTIBODIES

We used anti- $E_2$  mouse monoclonal antibody (mAb) and anti-25(OH)D sheep mAb as primary antibodies for hapten sandwich assays. Anti- $E_2$  mouse mAbs were obtained by immunizing BALB/c mice with  $E_2$ -6-carboxymethyloxime-BSA ( $E_2$ -6CMO-BSA) (Fitzgerald Industries). We used a standard fusion technique with P3U1 myeloma cells to generate hybridoma cell lines. Antibodies specific to  $E_2$  were screened by a competitive ELISA assay with  $E_2$  and alkaline phosphatase (AP)-conjugated  $E_2$  at the C3 portion. Anti-25(OH)D sheep mAb (clone vitD3.5H10), which binds to both 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> equally, was obtained from Bioventix.

### ESTABLISHMENT OF mAbs FOR HAPTEN-ANTIBODY IMMUNOCOMPLEX RECOGNITION WITH THE AUTONOMOUSLY DIVERSIFYING LIBRARY SYSTEM

We established antimetatype chicken mAbs that reacted with hapten–mAb complexes using the Autonomously Diversifying Library (ADLib) system (22, 23). With the ADLib system, in general, the antibodies were isolated *ex vivo* from antibody libraries established by activating immunoglobulin gene diversification of chicken-derived DT40 cells, and the reactivity of candidate antibodies was screened by ELISA with antigen and horseradish peroxidase–conjugated antichickens antibodies. Specifically, 1.5  $\mu$ g each of anti- $E_2$  mouse mAb or anti-25(OH)D

sheep mAb were immobilized on 75  $\mu$ g Dynabeads Protein G (Life Technologies) at 4 °C for 1 h, then 1.5  $\mu$ g each of  $E_2$  or 25(OH)D were mixed at 4 °C for 1 h with the beads to form hapten–antibody complexes. Cells of a DT40 antibody library ( $1.5 \times 10^7$ ) were incubated in 50 mL Iscove modified Dulbecco medium (Life Technologies) containing 9% FBS and 1% chicken serum for 24 h, and the cells were harvested by centrifugation at 240g. The cells were resuspended in 1 mL PBS containing 1% BSA and mixed with the beads immobilized with the hapten–antibody complexes at 4 °C for 30 min. After 3 washes with 1 mL PBS containing 1% BSA by mechanical stirring, the cells adsorbed on the beads were seeded in 96-well plates. Chicken mAbs reactive to hapten–mAb immunocomplex without cross-reactivity to mAb alone were screened by ELISA. Established chicken mAbs in our study were purified by gel filtration chromatography on Superose 6 10/300 GL (GE Healthcare Bio-Sciences).

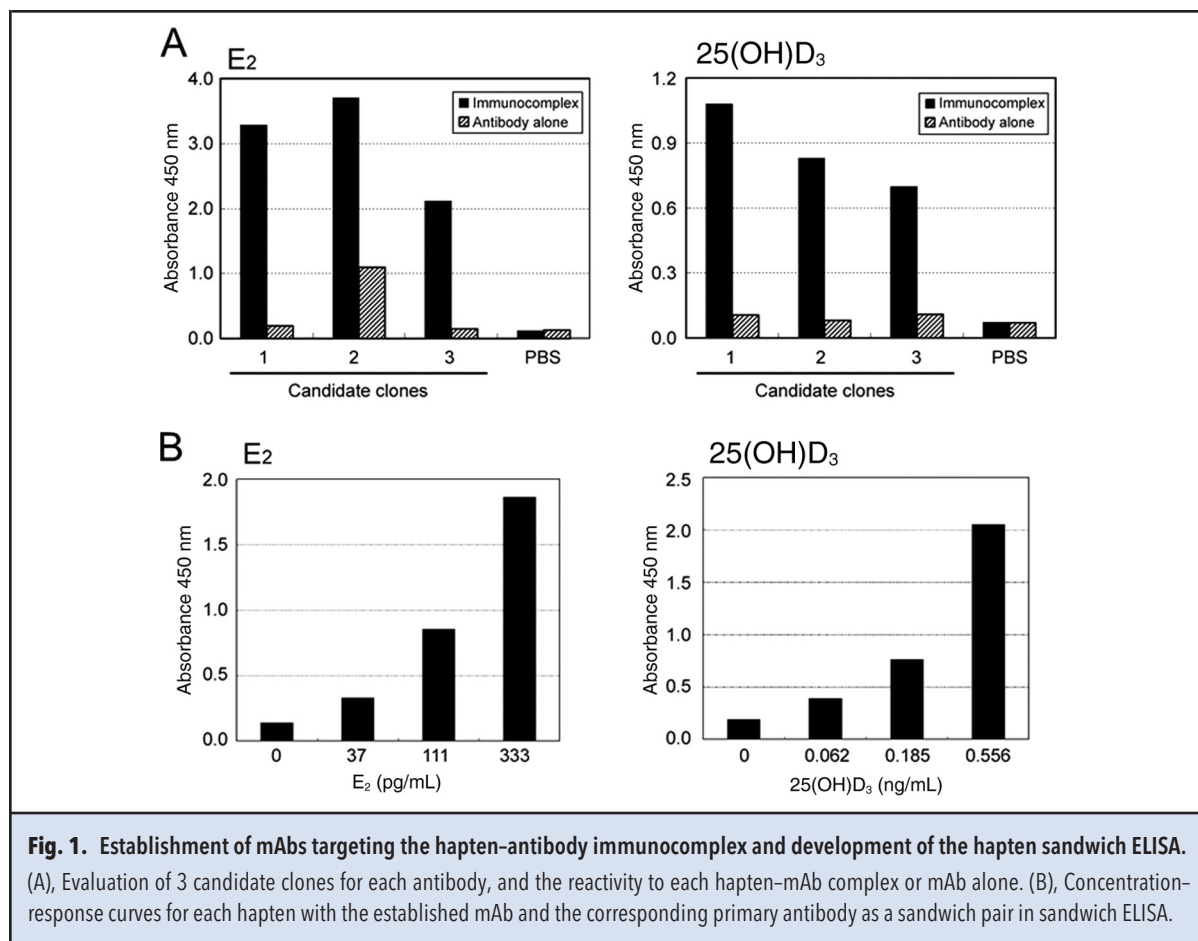
### REAGENTS AND STANDARD PREPARATION

We obtained  $E_2$ ,  $E_2$ -3-sulfate, and biotinylated  $E_2$  ( $E_2$ -6CMO-biotin) from Steraloids. 25(OH)D<sub>3</sub> (Toronto Research Chemicals), 1,25(OH)<sub>2</sub>-D<sub>3</sub> (Cayman Chemical), and 25-hydroxyvitamin D<sub>3</sub> LC-Biotin (Immundiagnostik) were purchased for use as analytes. To prepare the assay calibrator for total 25(OH)D sandwich immunoassay on an automated immunoassay platform, we dissolved crystalline 25(OH)D<sub>3</sub> in 99.9% ethanol to make a stock solution at 10  $\mu$ g/mL, and we prepared calibrators containing 200, 100, 50, 25, 12.5, 6.25, and 3.13 ng/mL 25(OH)D<sub>3</sub> by further diluting the stock solution with horse serum. The concentration of total 25(OH)D in the calibrator with the highest concentration was determined by Quest Diagnostics by use of LC-MS/MS.

We used 3,3',5,5'-tetramethylbenzidine plus substrate-chromogen (Dako) as a substrate for horseradish peroxidase detection in sandwich ELISA, *p*-nitrophenyl phosphate (Wako Pure Chemical Industries) as a substrate for AP detection in competitive ELISA, and 3-(2'-spiroadamantane)-4-methoxy-4-(3''-phosphoryloxy)phenyl-1,2-dioxetane disodium salt (AMPPD) and poly[vinylbenzyl(benzyltrimethylammonium chloride)] (Life Technologies) as a substrate and enhancer, respectively, for AP detection in chemiluminescence assays.

### SAMPLES

To evaluate the imprecision of the prototype sandwich assay for 25(OH)D on the Lumipulse G1200 system, we obtained 2 serum samples from healthy volunteers containing 14.3 or 39.1 ng/mL total 25(OH)D from ProMedDx. In addition, 3 sera from healthy volunteers (purchased from Trina Bioreactives) were pooled, and 700  $\mu$ L 25(OH)D<sub>3</sub> solution at a concentration of 10



$\mu\text{g/mL}$  was spiked into 100 mL pooled serum to prepare a sample containing total 25(OH)D at a concentration of 97.7 ng/mL. Serum aliquots were stored at  $-80^\circ\text{C}$  until used for analysis. We performed quantitative determination of total 25(OH)D in the 3 sera with a 25-hydroxyvitamin D  $^{125}\text{I}$  RIA Kit (DiaSorin). For correlation analysis, we obtained 32 human serum samples from the International Vitamin D Quality Assessment Scheme (DEQAS). DEQAS samples had assigned mean RIA (DiaSorin) and LC-MS/MS concentrations by the supplier. For correlation analysis of the sandwich ELISA for  $\text{E}_2$  with isotope-dilution GC-MS (ID-GC-MS), we obtained sera from healthy women and pregnant women from ProMedDx and Trina Bioreactives and used the sera for the preparation of 23 pooled sera to cover a wide range of  $\text{E}_2$  concentrations. The  $\text{E}_2$  concentrations of the samples were determined in duplicate by ID-GC-MS at Ghent University and the sandwich ELISA for  $\text{E}_2$ .

#### ADDITIONAL PROCEDURES

Descriptions of additional experimental procedures used and an associated reference are provided in Supplemental

Methods, which accompanies the online version of this article at <http://www.clinchem.org/content/vol61/issue4>.

## Results

### ESTABLISHMENT OF mAbs TARGETING THE HAPTEN-ANTIBODY IMMUNOCOMPLEX

Antimetatype chicken mAbs with specific reactivity to hapten-mAb complexes and minimal reactivity to mAbs alone were established for  $\text{E}_2$  and 25(OH)D<sub>3</sub> by use of the ADLib system (Fig. 1A). Clone 1 of the established mAbs against each hapten-mAb immunocomplex was selected as the most specific mAb, and these antibodies were used for the evaluations of hapten sandwich immunoassay.

### SPECIFICITY OF THE ESTABLISHED ELISA SYSTEM FOR EACH HAPTEN

The concentration-dependent signals for the sandwich ELISAs for  $\text{E}_2$  and 25(OH)D<sub>3</sub> are shown in Fig. 1B. Because sandwich immunoassays are expected to achieve higher specificity than competitive immunoassays owing to

**Table 1. Comparison of specificities between the sandwich and competitive assays for E<sub>2</sub>.**

| Analyte                   | Sandwich assay                |                     | Competitive assay             |                     |
|---------------------------|-------------------------------|---------------------|-------------------------------|---------------------|
|                           | Additive concentration, ng/mL | Cross-reactivity, % | Additive concentration, ng/mL | Cross-reactivity, % |
| Estrone                   | 27                            | 0.001               | 10                            | 0.118               |
| Estriol                   | 27                            | 0.043               | 10                            | 0.243               |
| E <sub>2</sub> -3-sulfate | 27                            | Not detected        | 10                            | 5.833               |

the use of 2 antibodies targeting 2 distinct recognition sites (13), we next compared the specificities of the established E<sub>2</sub> sandwich ELISA and the E<sub>2</sub> competitive immunoassay with the major cross-reactants as analytes (Table 1; Fig. 2, A and B). Although the E<sub>2</sub> competitive assay exhibited good specificity overall, it showed high reactivity to E<sub>2</sub>-3-sulfate, with approximately 5.8% cross-reactivity at 10 ng/mL. In contrast, the sandwich ELISA showed little or no cross-reactivity to all E<sub>2</sub>-related molecules tested, including E<sub>2</sub>-3-sulfate, indicating that the sandwich principle with antihapten-immunocomplex antibodies substantially improved specificity, as expected.

Similar results were obtained in the immunoassay for 25(OH)D. Although the competitive assay showed almost the same reactivity to 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>-D<sub>3</sub>, the sandwich ELISA detected 25(OH)D<sub>3</sub> specifically, without detection of 1,25(OH)<sub>2</sub>-D<sub>3</sub> (Fig. 2, C and D; online Supplemental Table 1).

#### DETECTION LIMIT OF E<sub>2</sub> ELISA SYSTEM

Next, we evaluated the sensitivity of the sandwich assay for E<sub>2</sub>, for which there is an increasing clinical demand for highly sensitive assays (24). The detection limit of the sandwich assay for E<sub>2</sub> was assessed by measuring aliquots of 4 samples with E<sub>2</sub> concentrations of 0, 1.56, 3.13, and 6.25 pg/mL in 6 replicates (Fig. 2E). The limit of detection (LOD) in the E<sub>2</sub> sandwich assay was estimated to be approximately 3.13 pg/mL because the mean minus 1.96 SD of the sample with 3.13 pg/mL E<sub>2</sub> was larger than the mean plus 1.96 SD of blank measurements.

#### MECHANISM OF HAPTEN RECOGNITION BY THE NOVEL SANDWICH ELISA SYSTEM

Haptens such as E<sub>2</sub> and 25(OH)D are generally considered to be too small for the development of sandwich assays. One possible mechanism of hapten recognition by our novel sandwich ELISA system may involve recognition of the epitope composed of both the hapten and the variable region of the antibody in the complex by the established mAb. Alternatively, the established mAb may recognize a conformation change in the primary antibody induced by binding

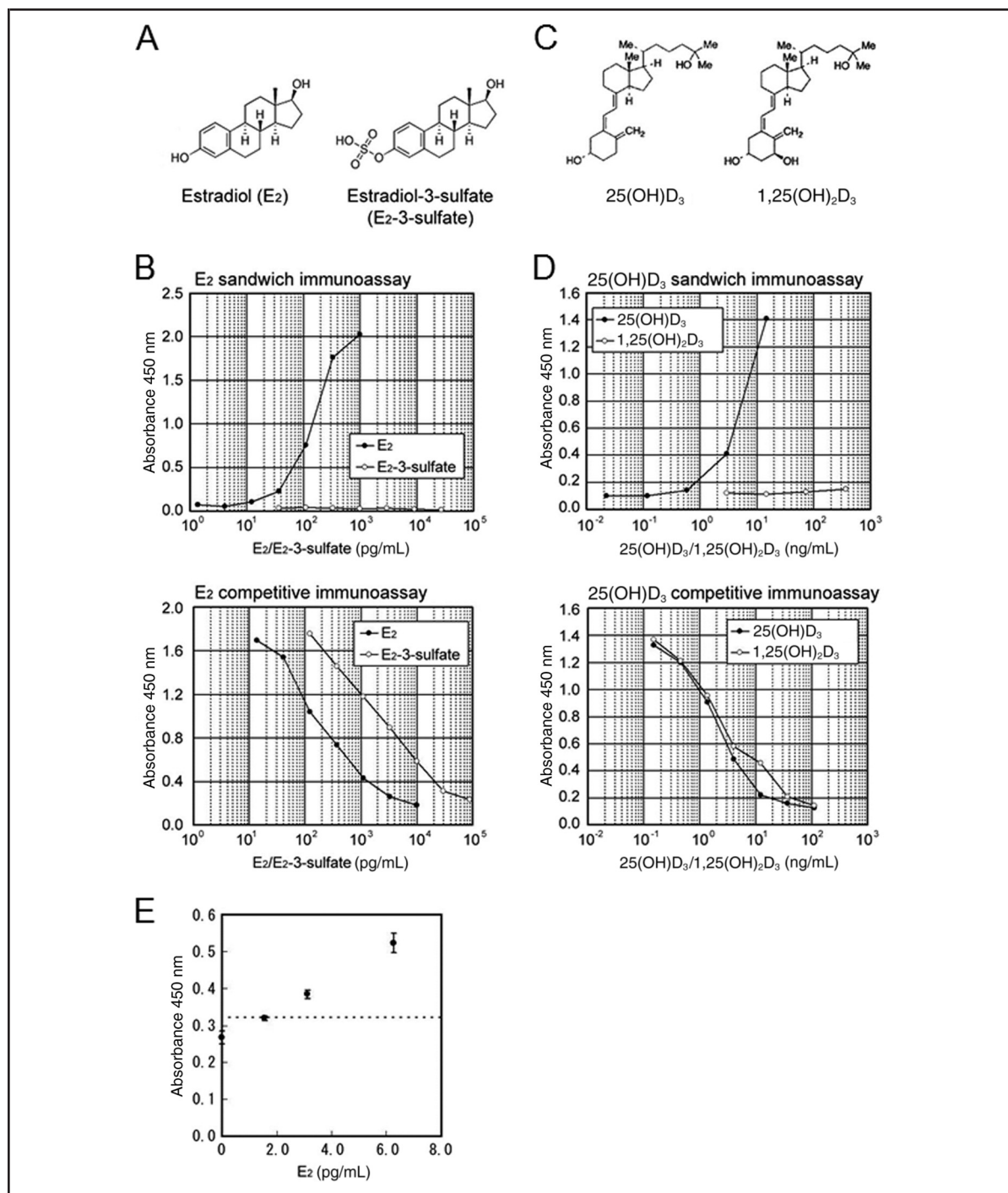
with the hapten. To confirm the mechanism of hapten recognition by the sandwich ELISA system for E<sub>2</sub>, competitive assays were conducted with E<sub>2</sub> biotinylated at the C6 position (bio-E<sub>2</sub>). When a 3-fold excess of bio-E<sub>2</sub> was added into the E<sub>2</sub> sandwich assay solution, the E<sub>2</sub> signal was completely diminished (Fig. 3A). Because streptavidin-conjugated AP reacted in this condition, bio-E<sub>2</sub> was supposed to be captured by the primary antibody in place of E<sub>2</sub>. These results indicated that the anti-immunocomplex antibody recognized the part of E<sub>2</sub> harboring the C6 position in the E<sub>2</sub>-mAb immunocomplex. A similar result was obtained for the 25(OH)D sandwich assay. Although 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>-D<sub>3</sub> biotinylated at the C3 position [bio-25(OH)D<sub>3</sub>] both formed immunocomplexes with the primary antibody (data not shown), the established anti-immunocomplex antibody reacted much more weakly with the bio-25(OH)D<sub>3</sub> immunocomplex than with the 25(OH)D<sub>3</sub> immunocomplex (Fig. 3B). These results indicated that the anti-immunocomplex antibody recognized the part of 25(OH)D<sub>3</sub> harboring the C3 position in the 25(OH)D<sub>3</sub>-mAb immunocomplex.

#### IMPRECISION OF SANDWICH ELISA FOR E<sub>2</sub>

We determined the intra-assay CV of sandwich ELISA for E<sub>2</sub> by analyzing 20 replicates of samples containing 50, 400, and 1200 pg/mL of E<sub>2</sub> in a single run. Four replicates of the same samples were also measured in 5 independent runs to determine total assay CV. The ranges for intra-assay and total CVs were 4.2%–12.6% and 6.2%–21.8%, respectively (see online Supplemental Table 2). Although the sandwich ELISA was performed manually, it showed acceptable imprecision.

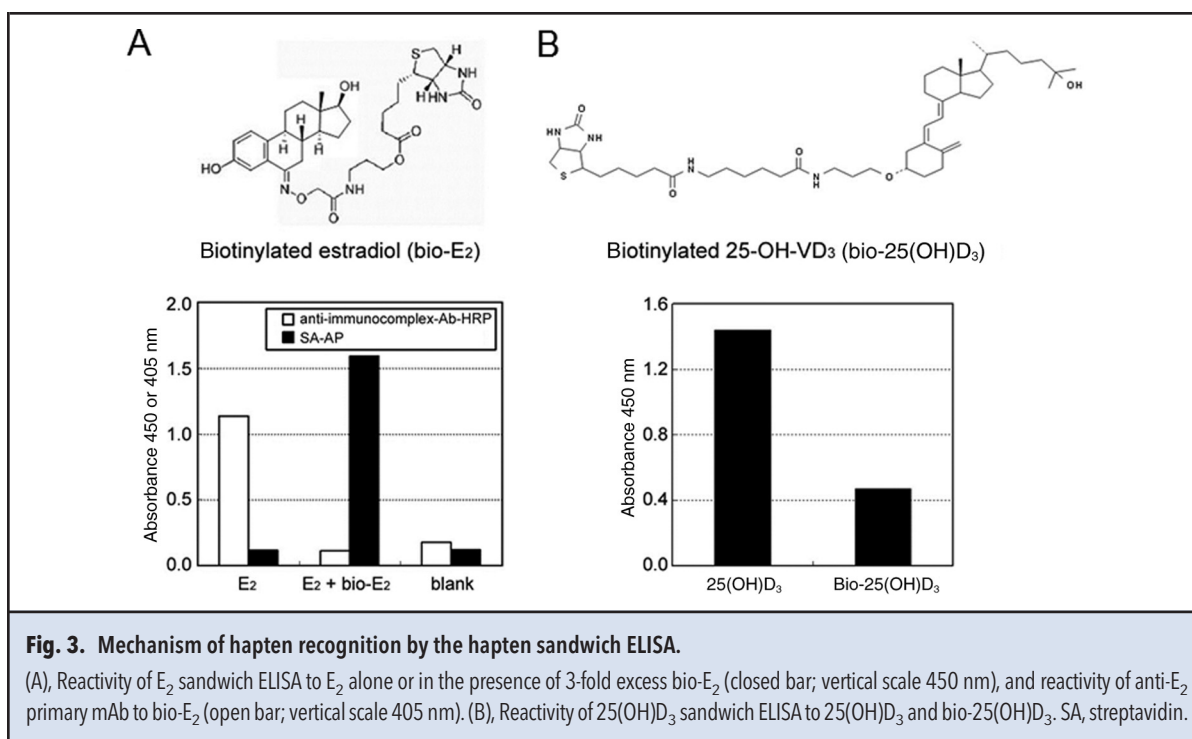
#### CORRELATIONS OF THE SANDWICH ELISA FOR E<sub>2</sub> WITH ID-GC-MS

We used 23 pooled serum samples that covered a wide range of E<sub>2</sub> concentrations to assess correlation of the sandwich E<sub>2</sub> ELISA with ID-GC-MS. The corresponding equation with ID-GC-MS was ELISA = 1.00 × (ID-GC-MS) – 16.78 (95% CI of the intercept –128.35–54.80; 95% CI of the



**Fig. 2. Improved specificity and high sensitivity of the hapten sandwich ELISA.**

(A), Chemical structures and formulas of E<sub>2</sub> and its analog, E<sub>2</sub>-3-sulfate. (B), Reactivity to E<sub>2</sub> and E<sub>2</sub>-3-sulfate were compared in the sandwich immunoassay for E<sub>2</sub> (top) and competitive immunoassay for E<sub>2</sub> (bottom), both of which used the common primary anti-E<sub>2</sub> antibody. (C), Chemical structures and formulas of 25(OH)D<sub>3</sub> and its analog, 1,25(OH)<sub>2</sub>D<sub>3</sub>. (D), Reactivities to 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> were compared in the 25(OH)D<sub>3</sub> sandwich immunoassay (top) and 25(OH)D<sub>3</sub> competitive immunoassay (bottom), both of which used the common primary anti-25(OH)D<sub>3</sub> antibody. (E), Evaluation of sensitivity of the sandwich immunoassay for E<sub>2</sub> with 4 samples containing 0, 1.56, 3.13, or 6.25 pg/mL E<sub>2</sub> (n = 6). The dashed line indicates the mean signal plus 3SD of blank samples.



slope 0.85–1.18), and the correlation coefficient was 0.93 (see online Supplemental Fig. 1).

#### 25(OH)D SANDWICH IMMUNOASSAY ON THE FULLY AUTOMATED ANALYZER

We used the Lumipulse G1200, an automated chemiluminescent enzyme immunoassay (CLEIA) system (25, 26), and developed prototype reagents for the 25(OH)D sandwich immunoassay on this system with anti-25(OH)D antibody-coated magnetic beads, AP-conjugated anti-immunocomplex antibody, and AMPPD as a substrate. When we used serum-based standards of 25(OH)D<sub>3</sub> as assay samples at various concentrations, we observed concentration–response curves (Fig. 4A) comparable to those of the 25(OH)D ELISA (Fig. 1B, right panel). The LOD for the prototype sandwich assay on the fully automated analyzer was estimated according to CLSI Document EP17-A2. The calculated LOD was 2.1 ng/mL. Table 2 shows intra-assay and total assay CVs determined with serum samples containing 14.3, 39.1, and 97.7 ng/mL 25(OH)D. The within-run assay CV was evaluated by measuring 20 replicates in 1 day. Total assay CV was assessed by measuring 4 replicates in 5 days. The ranges for within-run and total CVs were 1.0%–2.3% and 1.9%–3.5%, respectively.

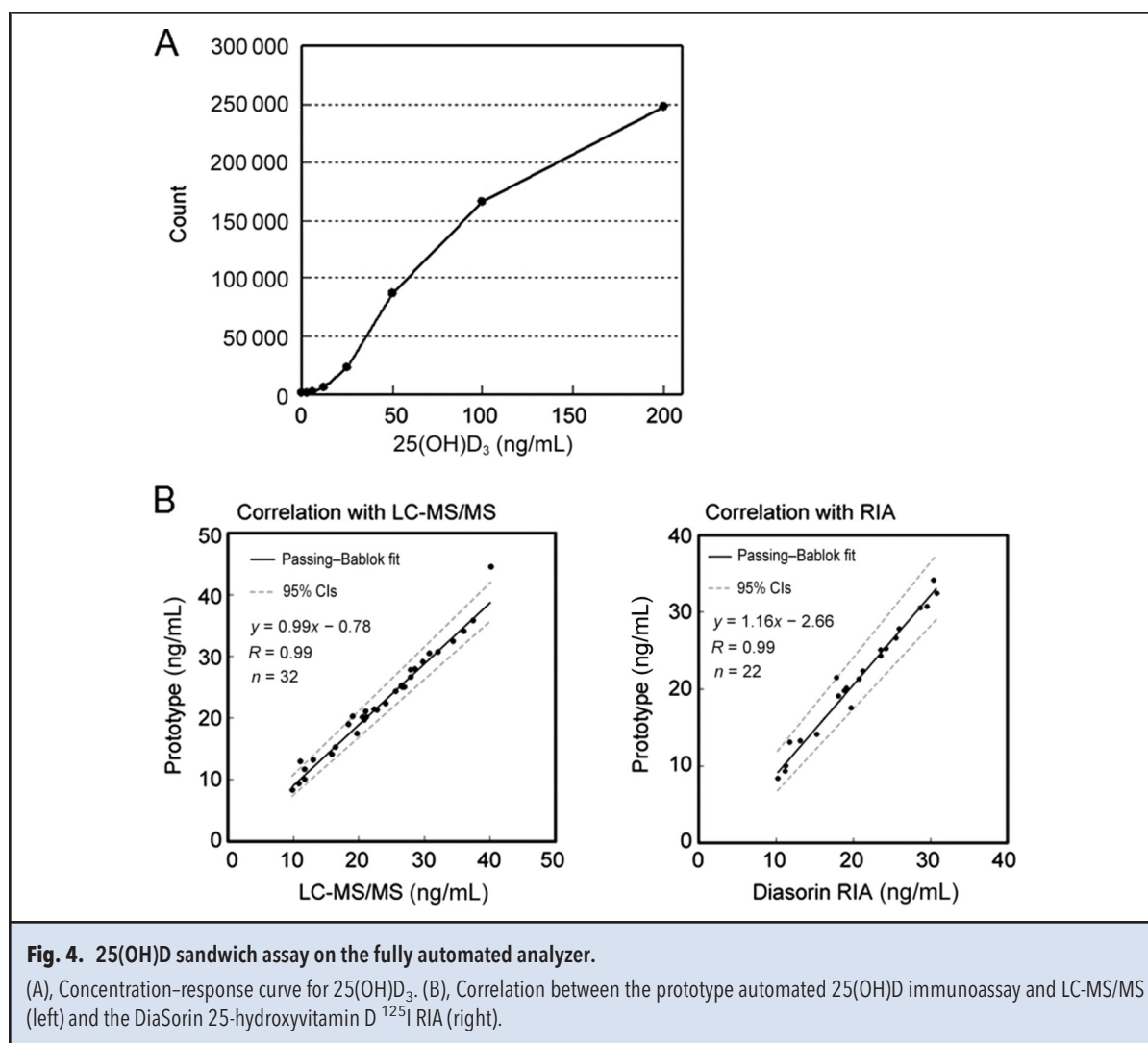
#### CORRELATIONS OF LC-MS/MS AND <sup>125</sup>I RIA WITH THE 25(OH)D ASSAY

We tested 32 human serum samples from DEQAS with prototype Lumipulse 25(OH)D reagents to assess corre-

lations with LC-MS/MS and <sup>125</sup>I RIA. To perform Passing–Bablok regression analysis, the measured values were compared with LC-MS/MS values and <sup>125</sup>I RIA values provided by the supplier (Fig. 4B). The corresponding equation with LC-MS/MS was prototype = 0.99 × (LC-MS/MS) – 0.78 (95% CI of the intercept 0.94–1.04; 95% CI of the slope –1.81 to 0.41). The correlation coefficient was 0.99. A comparison with RIA yielded the following regression equation: prototype = 1.16 × (RIA) – 2.66 (95% CI of the intercept 1.08–1.24; 95% CI of the slope –4.20 to –0.72). The results obtained with our prototype Lumipulse 25(OH)D reagents correlated with those of LC-MS/MS and RIA for quantification of 25(OH)D.

#### Discussion

In this study, we created a method to establish sandwich immunoassays for haptens on the basis of antimetatype antibodies. Previous studies showed that the antimetatype mAbs specific to hapten–mAb immunocomplexes could be established even following classic immunization procedures (18, 19). However, there have been few successful examples of antimetatype mAbs so far, indicating that this conventional strategy has essential limitations. It is not easy to immunize animals with hapten–mAb immunocomplexes, because the immunocomplexes can undergo time-dependent natural dissociation, plus some immunization procedures such as preparation of water-in-oil emulsion with antigens may facilitate dissociation.



To overcome such limitations, we used the ADLib *ex vivo* antibody development system to establish antimetatype antibodies. Antibody diversification in this system is automatically promoted by DNA recombination in the antibody locus, which provides this system with the potential to generate antibodies with various specificities independently of antigen stimulation (22, 23). This sys-

**Table 2.** Imprecision of the prototype sandwich assay for 25(OH)D on the Lumipulse G1200 system.

| Sample | n  | Concentration, ng/mL by Lumipulse | Intra-assay 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         |

tem also enables rapid antibody selection by affinity isolation like the magnetic beads–based immunoprecipitation method, which is supposed to avoid undesirable dissociation of the hapten–antibody immunocomplexes when used as bait. With this strategy, we successfully obtained antimetatype mAbs for 2 haptens, E<sub>2</sub> and 25(OH)D<sub>3</sub>, that reacted to the hapten–mAb immunocomplex but not to the primary mAb alone (Fig. 1A). The ELISAs developed with these antimetatype mAbs detected each hapten in a concentration-dependent manner as expected (Fig. 1B).

Our results demonstrated that the antimetatype antibodies actually recognized a part of each hapten molecule. The antimetatype antibody for the E<sub>2</sub> sandwich assay reacted with the E<sub>2</sub>–mAb immunocomplex and exhibited minimum reaction with the immunocomplex when E<sub>2</sub> was replaced with E<sub>2</sub> biotinylated at the C6

position (Fig. 3A). These results support that the antimetatype antibody recognizes the part of E<sub>2</sub> harboring the C6 position in the E<sub>2</sub>-mAb immunocomplex. These results also support that the antimetatype antibody compensates for the insufficient specificity of the anti-E<sub>2</sub> primary mAb. Because E<sub>2</sub> conjugated to BSA at the C6 position was used for the generation of the primary mAb, the mAb inevitably lacks the ability to recognize the structure near the C6 position of E<sub>2</sub>. The cross-reactivity of the primary mAb to E<sub>2</sub>-3-sulfate can be attributed to such a defect in generating antihapten primary mAbs, and the established antimetatype mAb effectively overcomes this problem (Fig. 2B; Table 1). Similarly, the primary mAb for 25(OH)D showed cross-reactivity to 1,25(OH)<sub>2</sub>-D<sub>3</sub> and 25(OH)D<sub>3</sub> biotinylated at C3, which was eliminated by the established antimetatype mAb in the 25(OH)D<sub>3</sub> sandwich immunoassay (Figs. 2D and 3B; online Supplemental Table 1). These results show that the established sandwich assays for E<sub>2</sub> and 25(OH)D<sub>3</sub> take advantage of the double recognition of 2 antibodies, a key feature of the sandwich immunoassay.

The sandwich assays also showed good analytical sensitivity, especially for E<sub>2</sub>, for which there is an increasing clinical and research demand for high-sensitivity measurement. Highly sensitive E<sub>2</sub> assays are increasingly required for the assessment of inborn errors of sex-steroid metabolism, disorders of puberty, estrogen deficiency in men, and therapeutic drug monitoring, in the context of either low-dose female hormone replacement therapy or anti-estrogen treatment (24). High sensitivity is especially required when measuring E<sub>2</sub> concentrations in postmenopausal women or elderly men whose E<sub>2</sub> concentrations are low (<50 pg/mL). The sandwich assay for E<sub>2</sub> achieved an LOD of approximately 3.13 pg/mL in the ELISA format, sufficient to meet the clinical demands. Establishment of highly sensitive immunoassays may also create new clinical value, as has been shown for high-sensitivity cardiac troponin assays, which have demonstrated the prognostic relevance of quantitatively minor increases in this biomarker, making the clinical decision limit for cardiac troponin progressively lower (27). In the case of E<sub>2</sub> measurement, as the assay sensitivity has been improved, it has been recognized that serum E<sub>2</sub> measurement is associated not only with ovarian function in women with menstrual disorders, precocious or delayed puberty, and assisted reproduction but also with other diseases including coronary artery disease, stroke, and breast cancer (24). Our E<sub>2</sub> assay could contribute to future clinical studies on such E<sub>2</sub>-related diseases.

We chose the vitamin D assay as the target for the fully automated CLEIA system in this study because there is an increasing demand for vitamin D routine testing owing to rising vitamin D deficiency rates worldwide and increasing evidence of serum vitamin

D concentrations as a general health indicator (5, 28). Although automated 25(OH)D competitive immunoassays have been commercially available from various manufacturers, they are unsatisfactory in specificity, accuracy, and imprecision (29). Therefore, we sought to overcome these challenges with anti-25(OH)D-mAb immunocomplexes. Our data showed that the anti-immunocomplex antibody was applicable as the secondary antibody on the automated immunoassay analyzer (Lumipulse G1200). Concentration-response curves showed that intraassay CVs were <2.5%; total CVs were ≤3.5% across the 3 different concentration samples (Table 2). These results indicate that the prototype 25(OH)D has good imprecision for routine assay compared with the reported CVs with 6 different commercial automated competitive immunoassays (30). Additionally, our sandwich assay for 25(OH)D had satisfactory high-precision performance compared with available 25(OH)D immunoassays (29, 31, 32). Our 25(OH)D immunoassays showed good correlation with LC-MS/MS measurements (Fig. 4B, left). Additionally, the correlation between 25(OH)D assays on the Lumipulse G1200 and a 25-hydroxyvitamin D <sup>125</sup>I RIA, the most commonly used 25(OH)D immunoassay kit, was also high (Fig. 4B, right). Although further analytical and clinical performance validation studies with appropriate samples, such as samples compliant with the CDC Vitamin D standardization program, are necessary and currently planned, our study showed that the prototype 25(OH)D assay has an acceptable performance for clinical diagnostic application.

In conclusion, we report a novel method to establish hapten sandwich assays that use antimetatype mAbs recognizing hapten-mAb immunocomplex, and the first automated sandwich immunoassay for measuring 25(OH)D. Our method enables the systematic establishment of high-throughput sandwich immunoassays for small molecules with high sensitivity and specificity.

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**Author Contributions:** All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

**Authors' Disclosures or Potential Conflicts of Interest:** Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:

**Employment or Leadership:** None declared.

**Consultant or Advisory Role:** None declared.

**Stock Ownership:** K. Omi, Chiome Bioscience.

**Honoraria:** None declared.

**Research Funding:** None declared.



**Expert Testimony:** None declared.

**Patents:** K. Omi, WO2013042426 A1; T. Ando, WO2013042426 A1; T. Sakyu, WO2013042426 A1, WO2014122972 A1, T. Shirakawa, WO2013042426 A1; Y. Uchida, WO2013042426 A1, WO2014122972 A1, A. Oka, WO2013042426 A1.

**Role of Sponsor:** No sponsor was declared.

**Acknowledgments:** We deeply thank Chiome Bioscience Inc. for its cooperation with the ADLib system in this study. We thank M. Wakabayashi, N. Sudo, and Dr. S. Kojima in Development Department, Fujirebio Inc. for helping with experiments.

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