

DEVELOPMENT OF AN ELISA FOR THE DIRECT MEASUREMENT OF FREE 25OH VITAMIN D

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1. Introduction

Almost all circulating 25OH Vitamin D in serum is bound to Vitamin D Binding Protein DBP (88%) and Albumin (12%). A very small fraction, approximately 0.04% of the 25OH Vitamin D, circulates in the free, non-protein bound form.¹

The concentration of DBP is not constant and can be influenced by a number of factors including obesity,² pregnancy,³ the use of oral contraceptives,⁴ hormone replacement therapy,⁵ liver disease,⁶ renal disease,⁷ proteinuria⁸ and intensive care.⁹

Furthermore, common genetic polymorphisms in the DBP gene produce variant proteins that differ in their affinity for 25OH Vitamin D.^{10,11}

For thyroid and steroid hormones it has been shown that their *in vivo* physiological activity correlates better with the free, non-protein bound fraction, than with the total concentration of the hormone in plasma. In such conditions the measurement of the concentration of the free circulating hormone provides more physiologically relevant information. This notion is known as the "free hormone hypothesis".^{12,13} Particularly in situations in which the level of binding proteins is elevated or decreased, the measurement of total circulating hormone may lead to a wrong diagnosis.

The bioavailability of 25OH Vitamin D may be more complex, since it is a precursor that is metabolized in the kidney to the active 1,25(OH)₂ Vitamin D. However, literature data suggest a role for the measurement of free 25 OH Vitamin D.¹⁴⁻¹⁷

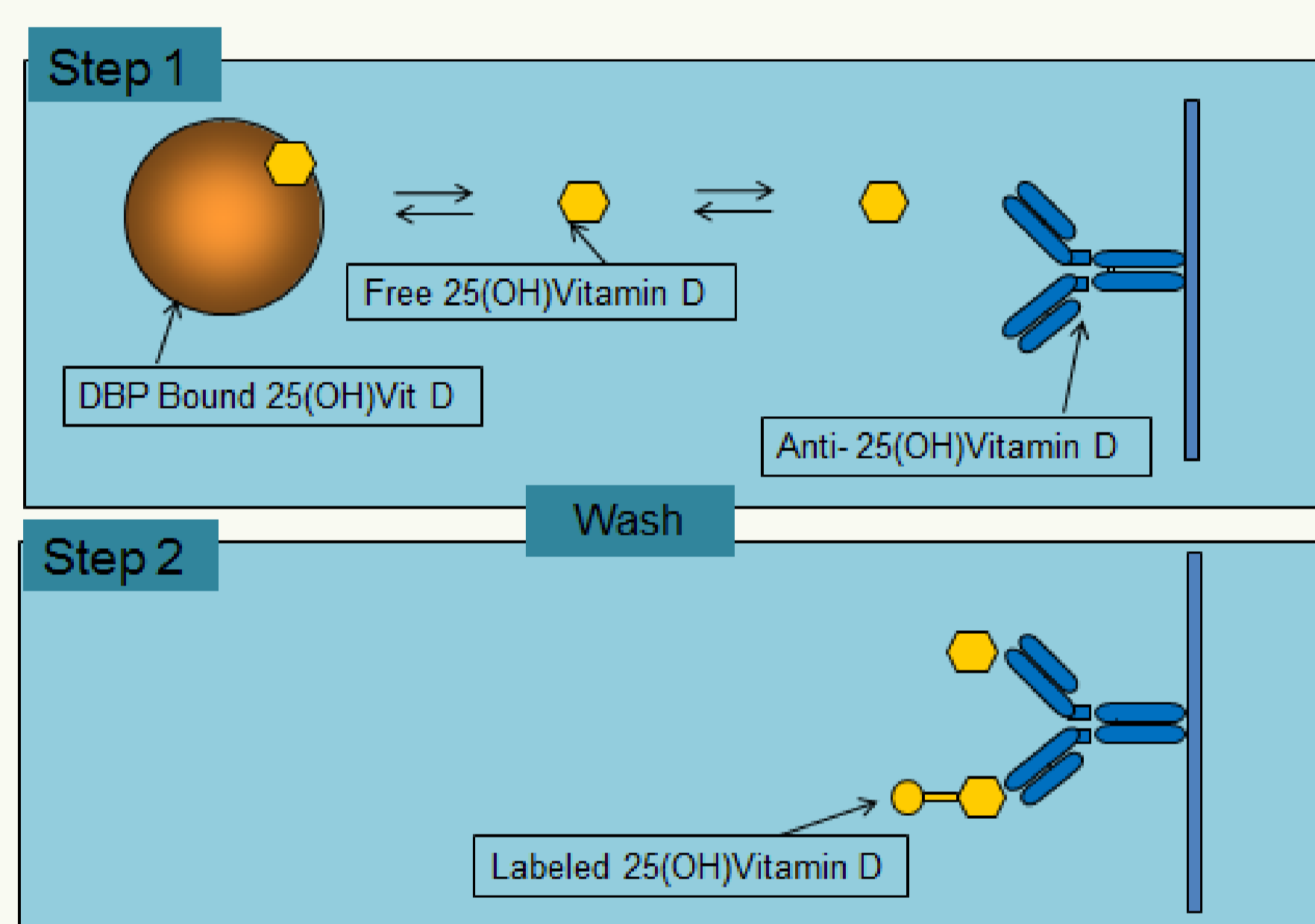
Here we describe an ELISA that offers the possibility to measure the concentration of free 25OH Vitamin D in serum.

2. Principle of the Assay

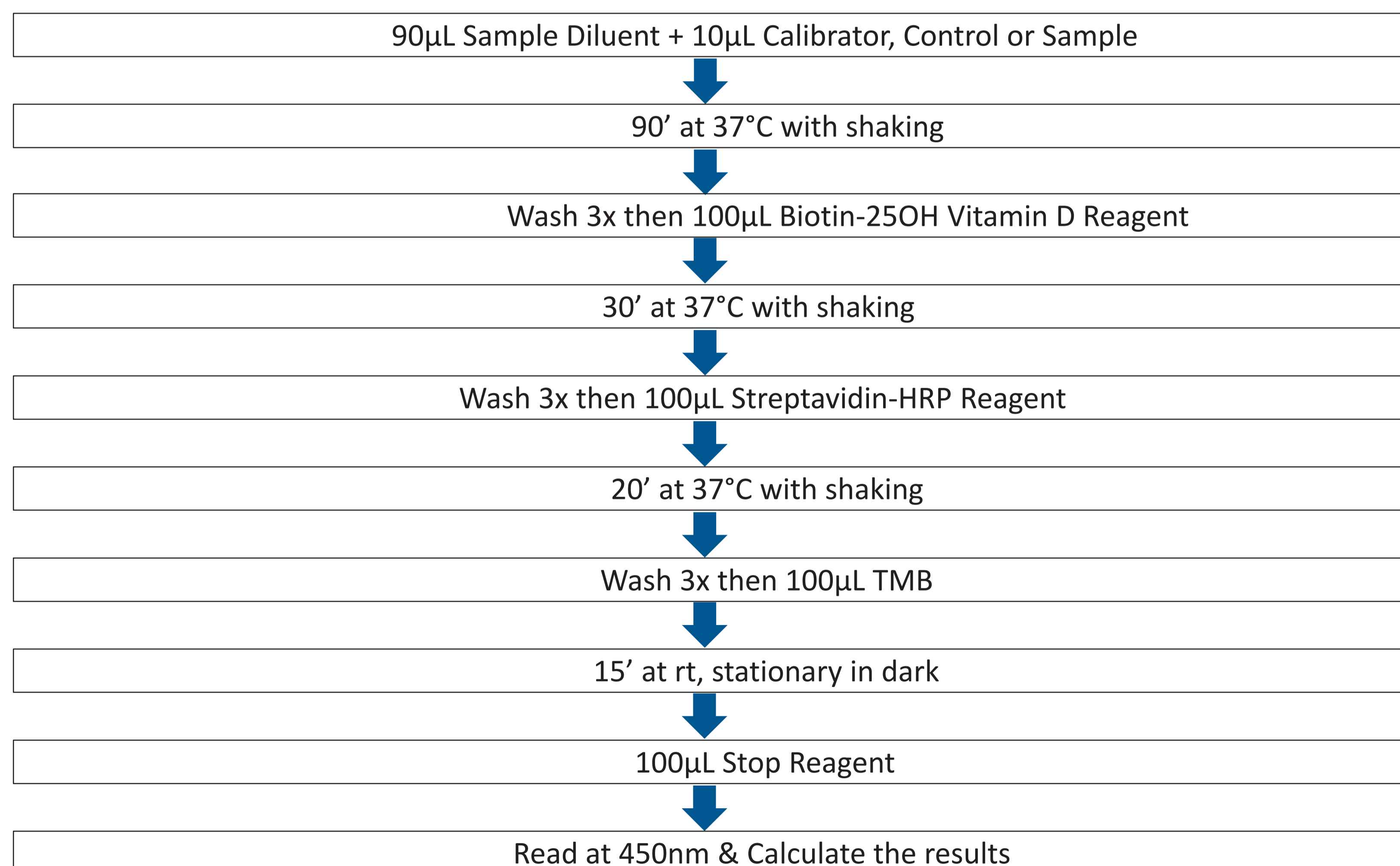
The Free 25OH Vitamin D ELISA is based on a two-step immunoassay procedure. During the first incubation step free 25OH Vitamin D binds to the monoclonal anti-Vitamin D in the microtiter plate. The *in vivo* equilibrium between free and bound 25OH Vitamin D is minimally disturbed by the use of a specific displacement reagent. After washing, a fixed amount of biotinylated 25OH Vitamin D is added to each well. The non-bound biotinylated 25OH Vitamin D is removed by washing and a streptavidin peroxidase conjugate

is added. In a next step TMB chromogenic substrate is added. The reaction is then stopped by addition of Stop solution and the absorbance is measured using a plate reader.

The concentration of free 25OH Vitamin D in the calibrators was determined using a rate dialysis technique.



3. Assay Protocol



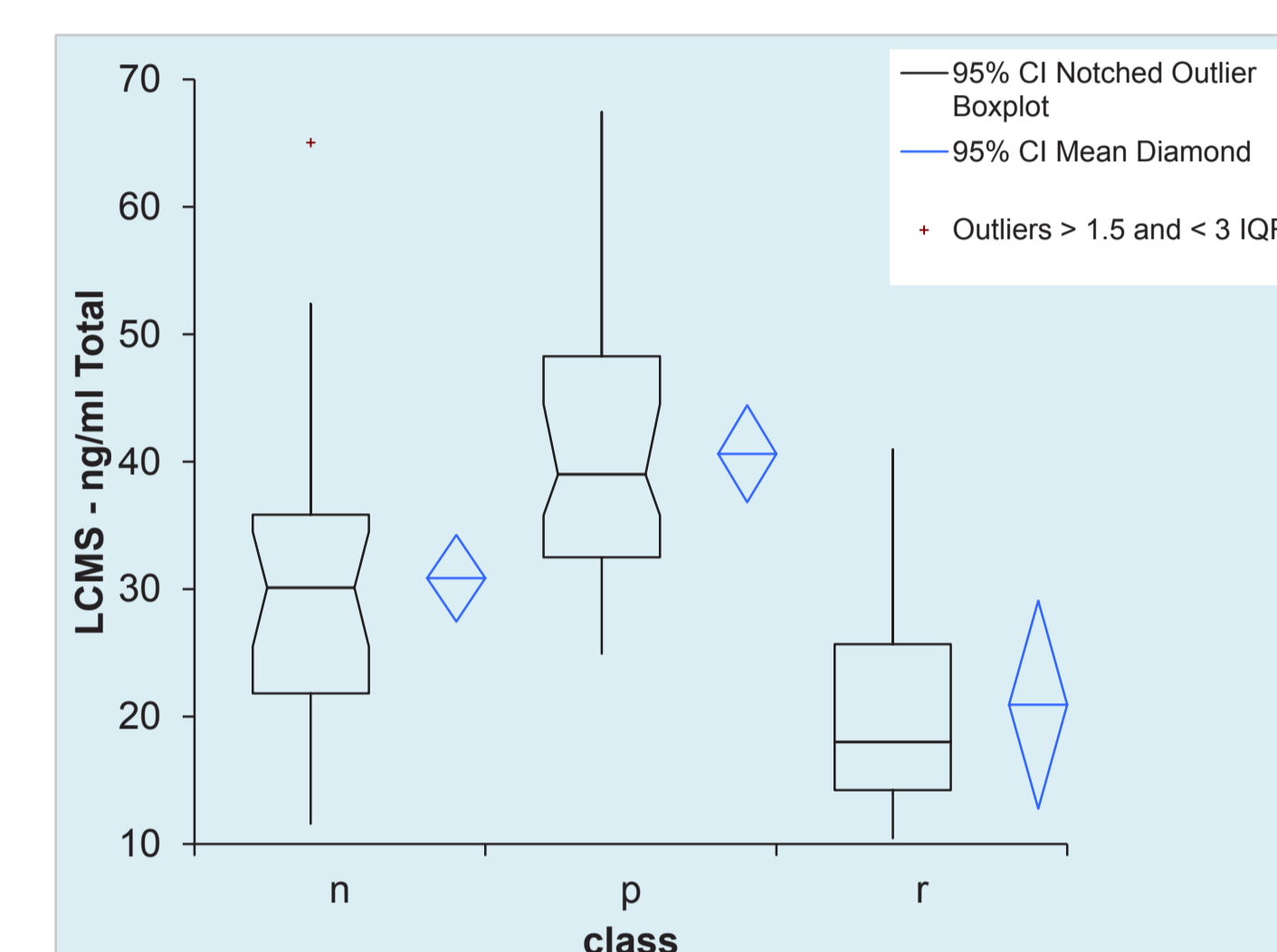
4. Assay Performance

Range	0-40 pg/mL
Precision	<10%
LoB	0.7 pg/mL
LoD	1.9 pg/mL

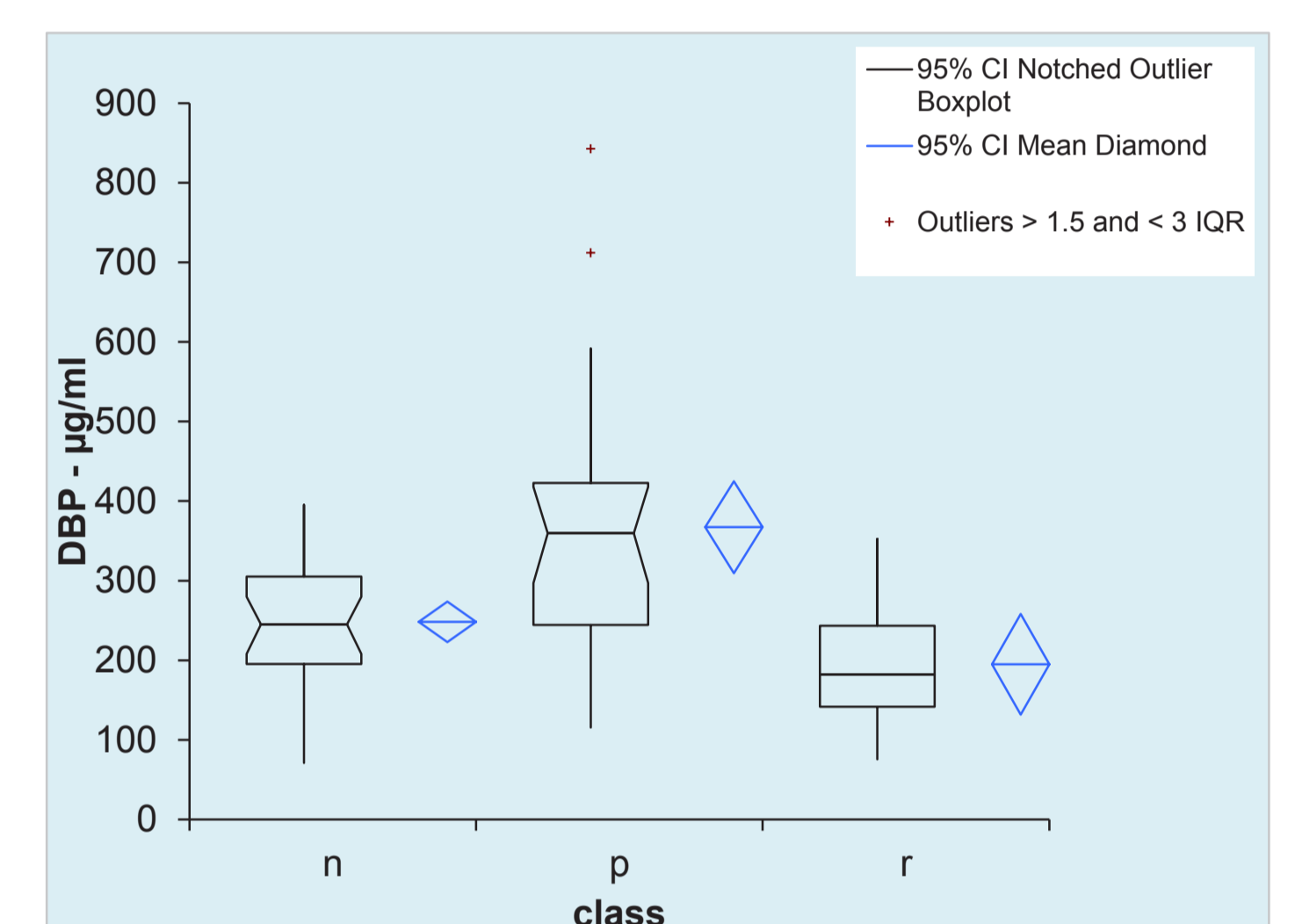
Additional experiments have shown that the addition of albumin or Vitamin D Binding Protein to serum leads to a decrease in the observed level of free 25OH Vitamin D.

Sample	Free 25OH Vitamin D (pg/mL)
Native sample 1	3.25
Native sample 1 + 60 g/L Albumin	1.95
Native sample 2	13.0 pg/mL
Native sample 2 + 1 g/L DBP	5.8 pg/mL

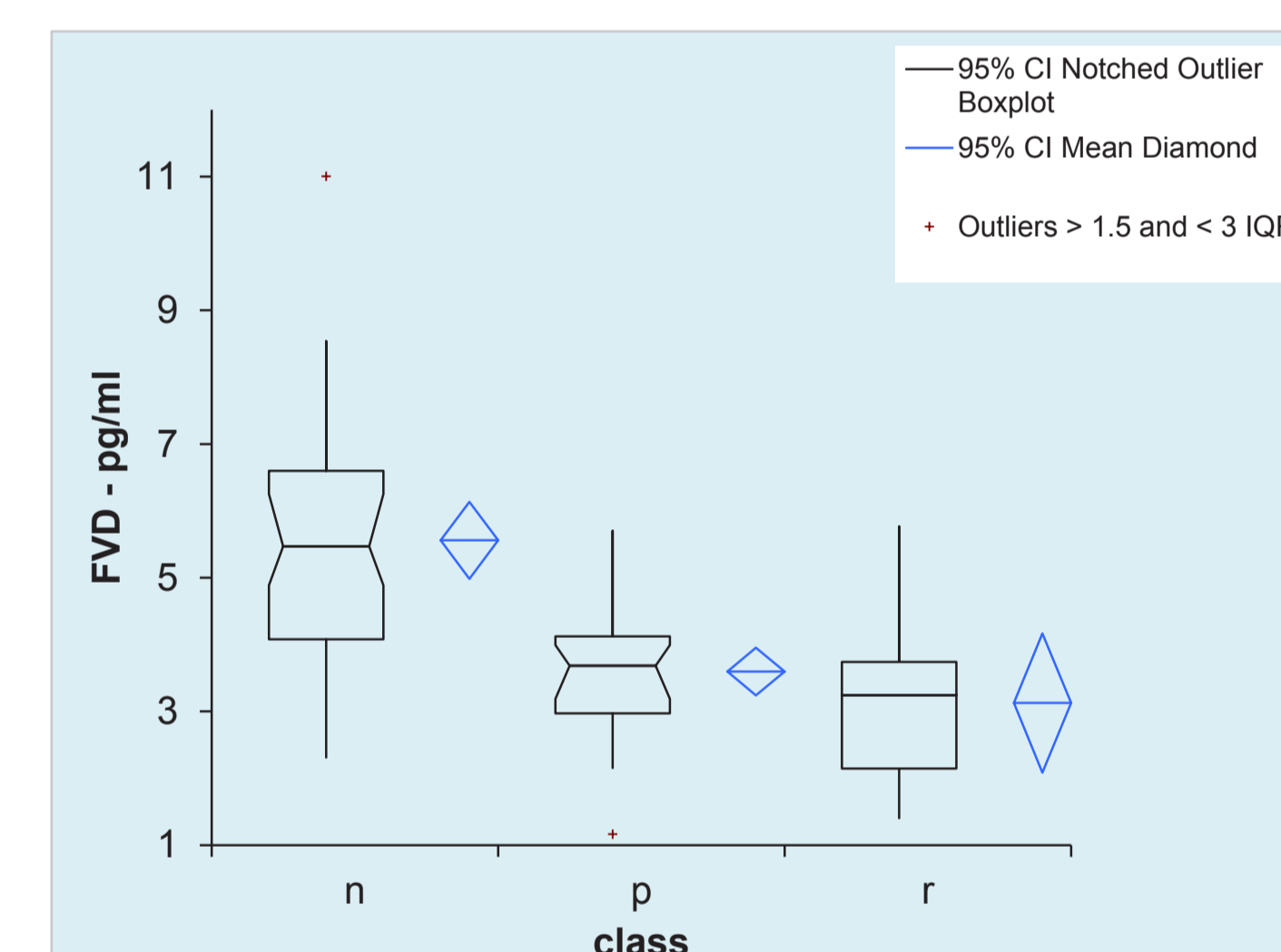
5. Pregnancy and Renal Failure



Box and Whisker Plot of Total 25OH Vitamin D concentration by LC-MS



Box and Whisker Plot of DBP concentration by ELISA



Box and Whisker Plot of free 25OH Vitamin D concentration by ELISA

Total 25OH Vitamin D, DBP and free 25OH Vitamin D concentrations were measured in normal controls "n" (n=45), Pregnant "p" (n=31) and Renal Failure "r" patients (n=9).

Total 25OH Vitamin D levels are higher in pregnancy than in controls. However, levels of free 25OH Vitamin D suggest that the Vitamin D status in pregnancy is lower than in controls. The higher concentration of DBP in pregnancy samples corroborates these results.

6. Discussion

We present a practical, automatable immunoassay for the measurement of free 25OH Vitamin D in serum.

The addition of albumin and DBP to serum leads to a decrease of the observed level of free 25OH Vitamin D. This demonstrates that the assay results reflect the level of free 25OH Vitamin D.

Measurement of free 25OH Vitamin D provides a different and possibly more relevant perspective on the Vitamin D status in pregnancy and other conditions of altered Vitamin D binding.

7. References

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