AUTOMATED ELISA FOR DIRECT MEASUREMENT OF FREE 25OH VITAMIN D

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Introduction

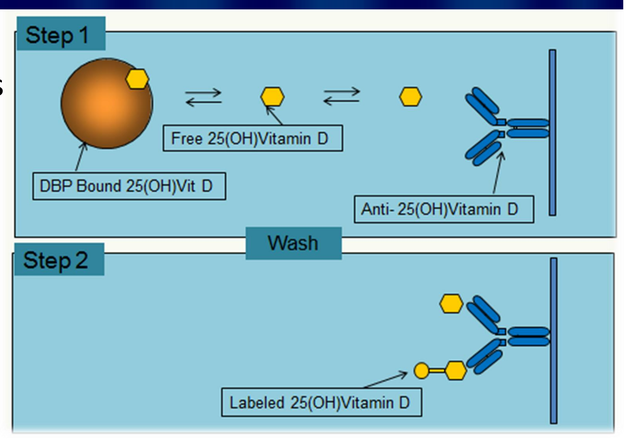
Recent studies suggest that the concentration and genotype of Vitamin D binding protein (DBP) are important factors that determine the bioavailability of 25OH Vit D in blood. It has been suggested that measurement of free, non-protein bound 25OH Vit D in serum, may provide more relevant diagnostic information than total 25OH Vit D, for instance in chronic kidney disease, bladder cancer and pancreatic cancer, or in hemodialysis patients[1-9]. To measure Free 25OH Vit D in blood Future Diagnostics developed a direct ELISA method. Following the first laboratory evaluation phase, a two-step enzyme-linked immunosorbent assay (ELISA) was optimized for the quantification of free 25OH Vit-D assay[10,11]. Modifications were made in the protocol for the coating of the monoclonal anti-25OH Vit-D in the microtiter plates as well as in the formulation of the sample diluent and of the biotinylated Vitamin D conjugate. The optimized assay was validated and showed the following performances: the calibrator range is 0.2-35 pg/ml. Total assay precision is 10.2% at 6.0 pg/ml, 7.6% at 10.9 pg/ml and 5.5% at 24.9 pg/ml. The cross-reactivity of the antibody towards 25OH Vitamin D2 is 77% and the influence of interfering hemoglobin, bilirubin and triglycerides was also verified being lower than 10%. 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 Vit-D.

Sample	Free 25OH Vitamin D (pg/mL)	
	Native	Spiked with DBP 2mg/mL
Sample 1	9.74	3.49
Sample 2	8.09	3.08
Sample 3	11.98	4.18
	Native	Spiked with HSA 60g/L
Sample 4	17.87	9.41
Sample 5	9.99	5.83
Sample 6	10.93	6.14

Here we describe the comparison between automated and manual processing of the ELISA for measuring Free 25OH Vitamin D.

Methods

The principle of the assay. 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 the next step TMB substrate is added. Next the reaction is stopped and the absorbance is measured using a plate reader.



Protocol Manual
- Add 90 µL of sample diluent into the wells.
- Add 10 µl samples into the well.
- Incubate shaking (orbital) 90 min at 37 °C
- Wash
- Add 100 µL biotin-25(OH) VitD into each well.
- Incubate shaking (orbital) 30 min at 37°C.
- Wash
- Add 100 µL of strep-HRP into each well
- Incubate shaking (orbital) for 20 min at 37 °C.
- Wash
- Add 100 µL of TMB substrate into each well.
- Incubate 15 minutes at RT in the dark.
- Add 100 µL Stop Solution into each well.
- Read the absorbance at 450 nm.

Protocol Dynex DS2
- Add 90 µl of sample diluent into the wells.
- Add 10 µl sample in to the well.
- Incubate shaking(linear) 90 min at 37°C
- Wash
- Add 100µl biotin-25(OH) VitD into each well
- Incubate shaking (linear) 30 min at 37°C.
- Wash
- Add 100 µL of strep-HRP into each well.
- Incubate shaking (linear) for 20 min at 37 °C.
- Wash
- Add 100 µL of TMB substrate into each well.
- Incubate 15 minutes at RT in the dark.
- Add 100 µL Stop Solution into each well.
- Shake 5 seconds.
- Read the absorbance at 450 nm

Literature references

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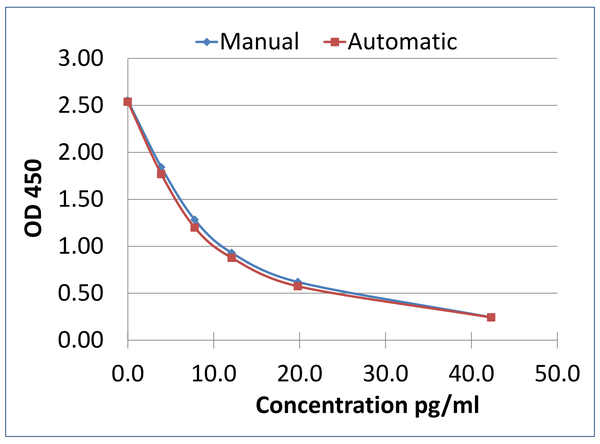
[10] Heureux N., Mathieu F., Swinkels L., Huijs T., Lindhout E., Martens M. OPTIMIZATION OF AN ELISA FOR THE DIRECT MEASUREMENT OF FREE 25OH VITAMIN D. ASBMR Poster 2015
[11] **Patent Future Diagnostics: PCT/NL2011/050219. Immunoassay for FREE Vitamin D**

Results

Calibration Curve



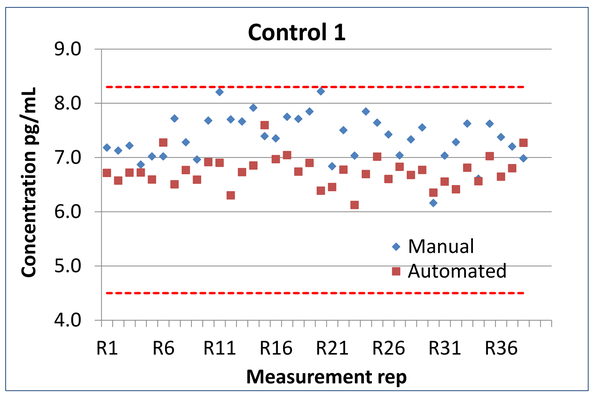
Curve shape parameters		OD 450		B/B0	
Calibrator	pg/ml	Manual	Auto	Manual	Auto
Cal A	0.0	2.551	2.539	100%	100%
Cal B	3.9	1.842	1.769	72%	70%
Cal C	7.8	1.282	1.199	50%	47%
Cal D	12.1	0.930	0.878	36%	35%
Cal E	19.8	0.620	0.575	24%	23%
Cal F	42.3	0.244	0.243	10%	10%

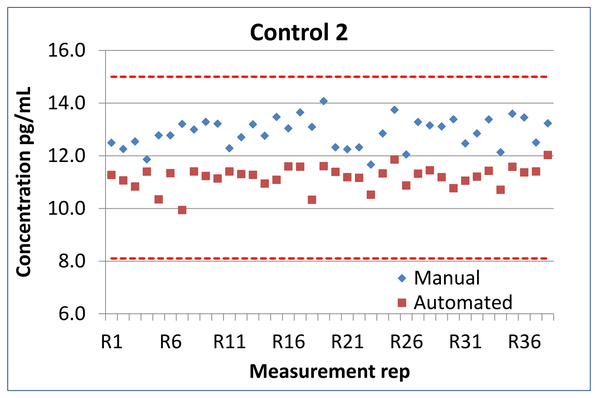
Limit of Blank & Limit of Detection

The LoB and LoD in pg/mL were determined according to CLSI EP17-A2. For LoB 38 reps were measured. For LoD 4*38 reps were measured.

	Manual	Automated
LoB	0.96	0.52
LoD	1.83	1.28

Control samples - Precision

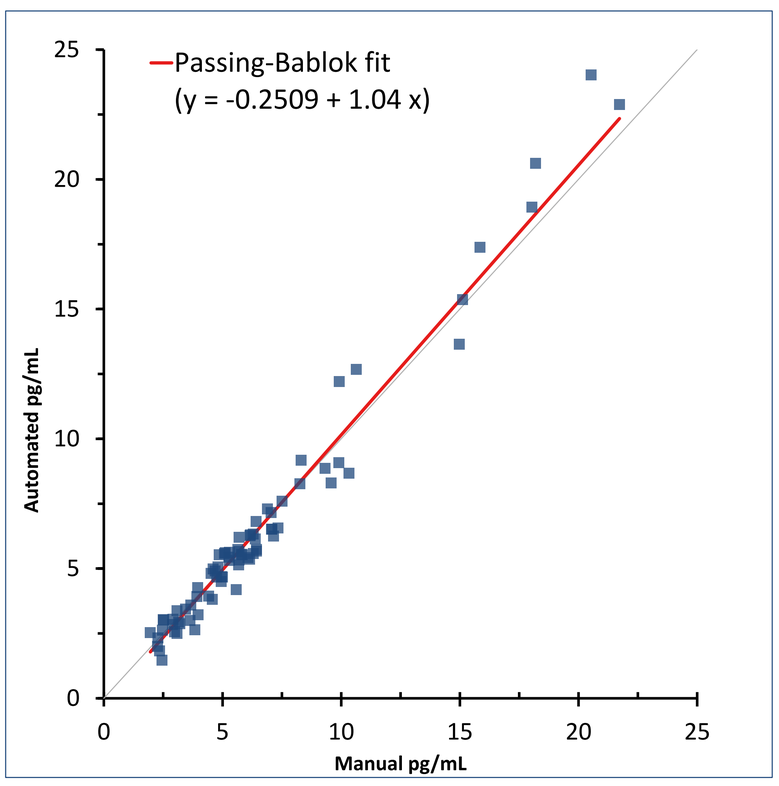




Control 1			
	Mean	SD	CV
Manual	7.37	0.43	5.8%
Automated	6.74	0.28	4.2%

Control 2			
	Mean	SD	CV
Manual	12.87	0.56	4.4%
Automated	11.18	0.42	3.7%

Sample correlation



In total 78 random samples were measured in the manual and in the automated assay. The slope, intercept and r show substantial equivalency between the manual and automated performed assay.

Correlation	
Pearson's r	0.986
Fisher 95% CI	0.979 to 0.991

Conclusion

The Free 25OH Vit-D assay that reproducibly determines the level of Free 25OH Vit D in serum was validated on an open ELISA platform. The manual and automated results were compared in terms of Dose response curve, LoB, LoD, Precision, and Correlation and show substantial equivalency.

The Free 25OH Vitamin-D ELISA eliminates the need to calculate the bioavailable 25OH Vitamin D from the measured DBP, HSA and total Vit-D concentrations.

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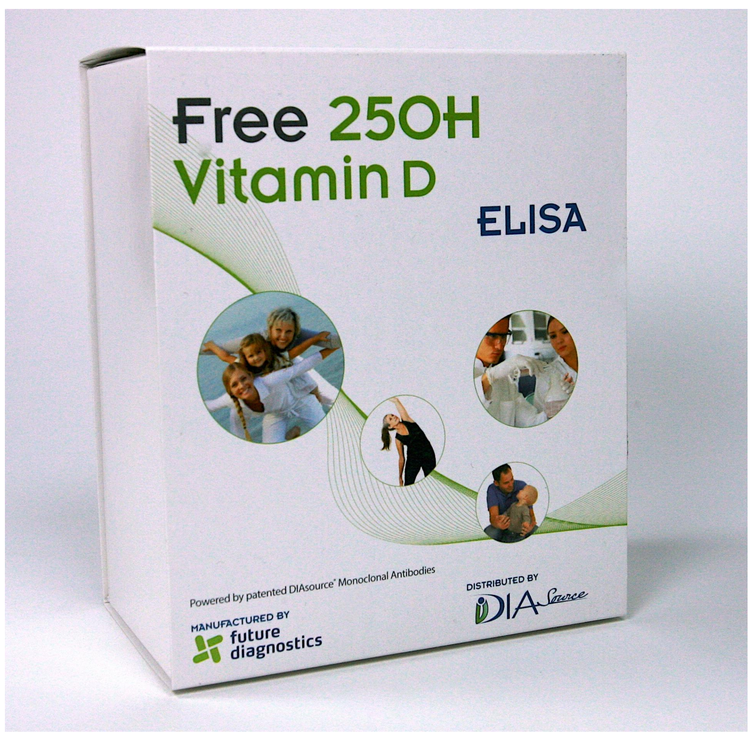
Future Diagnostics Solutions Free 25OH Vitamin D assay.
Why calculate when you can measure?



Future Diagnostics Solutions Intra Operative-intact-PTH assay.
It’s more than saving time