Application Note:
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Quantitative Determination of Progesterone in Serum Samples with a Thermo Scientific Varioskan Flash Multimode Reader Using an Enzyme-Linked Immunosorbent Assay

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Introduction

Enzyme-linked immunosorbent assays (ELISA) are widely used in the clinical, agrofood and environmental fields. By taking advantage of the high binding avidity and selectivity of antigen-antibody binding, these assays allow accurate and sensitive analyte quantification with little or no pre analytical sample treatment.

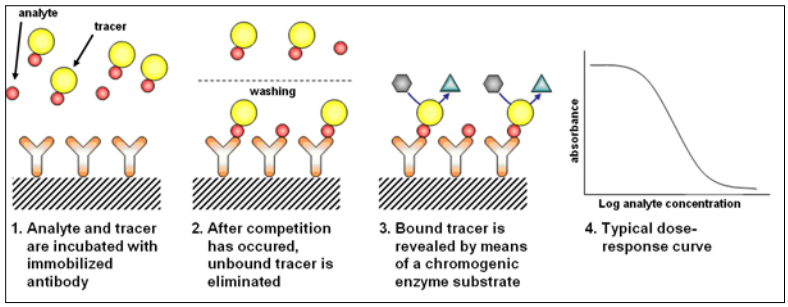

Figure 1. Schematic representation of the assay (1-3) and typical dose-response curve for competitive assay (4)

When small organic molecules need to be detected, the competitive assay format is commonly used. As shown in Figure 1, the method is based on the competition of enzyme-labeled analyte (tracer) and analyte present in the sample binding to a limited number of antibodies immobilized on a solid surface (typically the wells of a microtiter plate). After competition has reached equilibrium, unbound tracer is eliminated by washing, and the enzyme activity of bound tracer is detected by adding a chromogenic enzyme substrate. A typical sigmoidal dose-response curve is obtained by reporting B/B0 (signal obtained for a given standard solution above signal obtained in the absence of standard) against the analyte concentration in logarithm scale.

Progesterone is a C-21 steroid hormone involved in the female menstrual cycle, pregnancy, and embryogenesis of humans and other species. Progesterone levels are relatively low in children and postmenopausal women; adult males have levels similar to those in women during the follicular phase of the menstrual cycle. In women, progesterone levels change depending on the phase of the menstrual cycle or the weeks of pregnancy. Measurements of serum progesterone concentrations can be used in evaluating ovarian function. The serum or plasma progesterone reference values are listed in Table 1.

		Ref. value (ng/ml)
Men		1.0
Women	follicular phase	0.1 – 1.4
	midluteinic phase	4.0 – 25.0
	menopause	< 1.0
	pregnancy	
	weeks 18-21	53 - 76
	weeks 22-25	60 - 86
	weeks 26-29	71 - 133
	weeks 30-33	86 - 142
	weeks 34-37	104 - 175
	weeks 38-41	117 - 187

Table I. Typical reference values of progesterone concentrations in human serum samples.

Materials and Methods

Progesterone was determined in serum samples by using an ELISA kit (Progesterone ELISA kit, prod. no. DKO006, DiaMetra, Milan, Italy) according to the manufacturer's instructions. The kit comprises a 96-well clear microtiter plate coated with anti-progesterone IgG, standard solutions containing 0

(S_0), 0.2 (S_1), 1.0 (S_2), 8.0 (S_3), and 40.0 (S_4) ng/ml of progesterone, progesterone-horseradish peroxidase (HRP) conjugate (tracer), a TMB-based chromogenic substrate for HRP, and a stop solution containing sulphuric acid to stop enzymatic activity. Standard solutions were used to prepare a dose-response curve; two quality controls were included in the assay, QC1 (0.25 ± 0.05 ng/ml) and QC2 (16 ± 1.5 ng/ml), and 8 serum samples were analyzed.

The assay was performed in duplicate. A volume of 50 µl of standard solution, sample, or water (as blank) was dispensed in the wells of the microtiter plate, and then 50 µl of tracer solution (or water for the blank) was added. After incubating one hour at 37°C, wells were washed three times with deionized water, and the plate was then inserted into the Thermo Scientific Varioskan Flash instrument. A volume of 100 µl of TMB substrate was added in all of the wells using the automatic dispenser, then, after incubating 15 minutes at room temperature, 100 µl of stop solution was added via another dispenser. Absorption spectra were recorded between 400 nm and 500 nm (Figure 3), then absorbance measurements were performed at 450 nm.

Plate Layout Setting

A calibration curve, obtained by analyzing standard solutions provided by the kit, was produced employing Thermo Scientific SkanIt Software using the "Quantitative Curve Fit" function. Use of this function required that the blanks, calibrators and unknown samples were defined in the plate layout

Protocol definition

Absorbance measurements for all of the standard and sample wells were performed at 450 nm. To ensure that the maximum absorbance occurred at this wavelength, absorbance spectra were recorded between 400 nm and 500 nm (Figure 3).

STEP 1.
Dispensing step. Dispenser 1, dispensing volume 100 µl, dispensing speed "Medium," dispensing position "L1," tip priming "Automatic." Dispenses 100 µl of TMB substrate into each well.

STEP 2.
Shaking step, Duration 5 s, speed 60 rpm, diameter 5 mm, shaking mode "End with ON time" Shakes the plate for five seconds using 60 rpm speed and 5 mm diameter

STEP 3.
Incubation step, Incubation time 15 min, temperature 24°C, select "Leave the instrument at this temperature." Incubates the plate for 15 min. in room temperature

STEP 4.
Dispensing step, dispenser 2, dispensing volume 100 µl, Dispensing speed "Medium," dispensing position "L2," tip priming "Automatic." Dispenses 100 µl of stop solution into each well.

STEP 5.
Area definition step 1.
This step is used to limit the following action to only certain group of the wells defined in layout. Only those wells that are used to measure the absorption spectra are selected.

STEP 6.
Photometric spectral scanning step under the Area 1 step, Scanning wavelengths: Start wavelength 400 nm, end wavelength 500 nm, step size 5 nm, bandwidth 5 nm, measurement time 100 ms.

STEP 7.
Photometric measurement step. Wavelength 450 nm, bandwidth 5 nm, measurement time 100 ms.

Step structure of the protocol is shown in Figure 2.

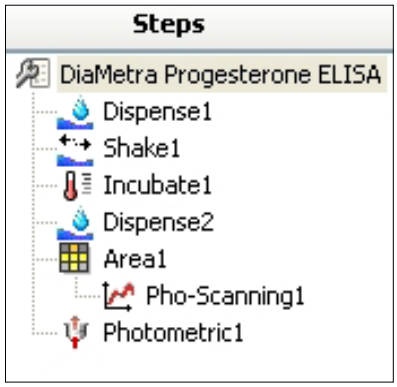

Figure 2. Protocol step structure of the ELISA assay.

Result

Absorbance spectra in the range of 400-500 nm were recorded in all the calibrator wells as shown in Figure 3. The absorption maximum was at 450 nm, which is the wavelength recommended by the manufacturer and which is typically used for this substrate.

Progesterone concentration in serum samples was calculated using "Quantitative Curve Fit" in the "Results" action panel. This step calculates the concentration of the samples based on a standard curve made from a series of calibrators with known concentrations. Any dilutions defined for unknowns in the layout are taken into account in the calculations.

$$y = d + \frac{a - d}{1 + (x/c)^b}$$

The calibration curve was fitted using a four-parameter logistic function with y as the signal, x the concentration, a the minimum signal (asymptote below), d the maximum signal (asymptote above), c then concentration at the inflection point, and b a slope-related term at the inflection point.

The dose-response curve is shown in Figure 4.

Progesterone concentration in quality controls and samples was

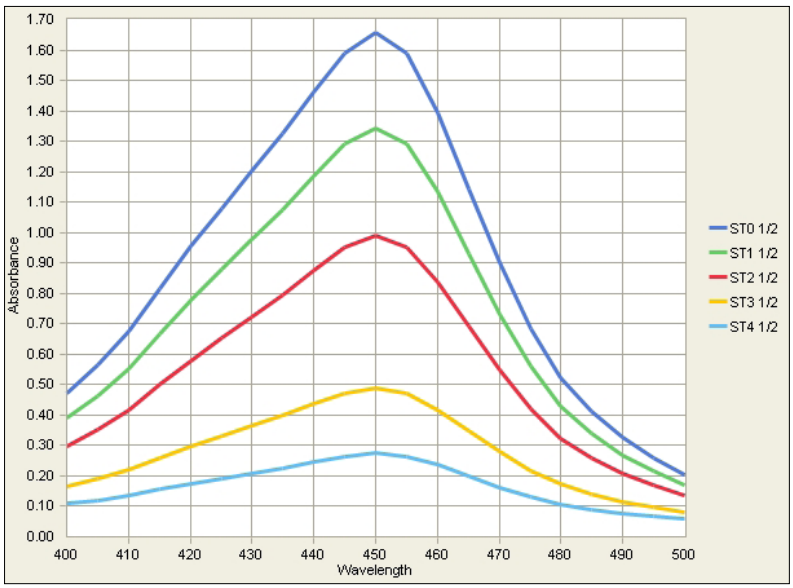

Figure 3. Photometric spectra of the ELISA assay calibrators. Values shown in the figure are average values of the replicate samples.

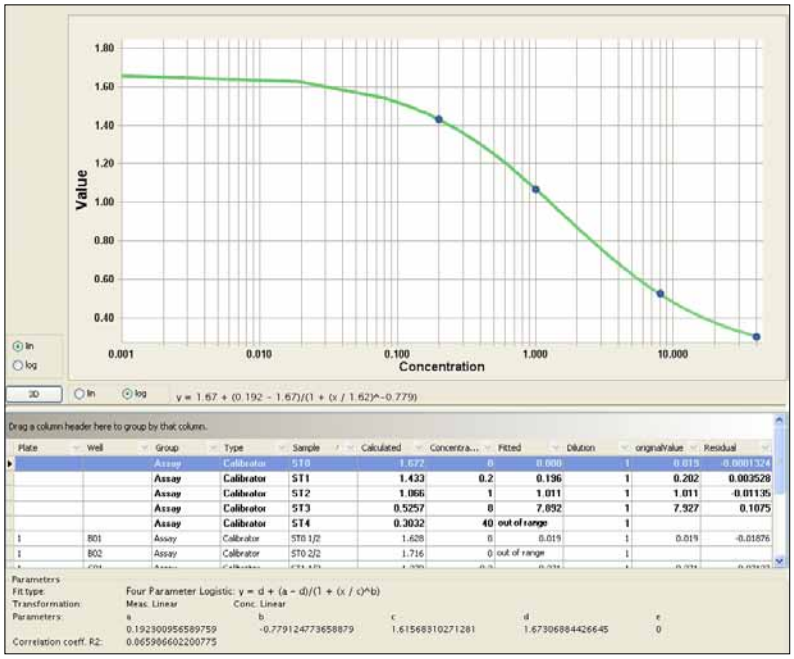

Figure 4. 4PL calibration curve and calculated fitting parameters from the competitive progesterone ELISA assay.

Group	Type	Sample	Calculated
Assay	Blank	blank	0.04751
Assay	Control	CTR1	1.379
Assay	Control	CTR2	0.4066
Assay	Unknown	S1	1.317
Assay	Unknown	S2	1.423
Assay	Unknown	S3	0.8203
Assay	Unknown	S4	0.7843
Assay	Unknown	S5	0.4932
Assay	Unknown	S6	0.6102
Assay	Unknown	S7	0.3871
Assay	Unknown	S8	1.106

Table II. Result summary of the progesterone ELISA assay.

calculated by interpolating their absorbance values on the dose-response curve. Quality controls stayed within the acceptability range, and all the samples were within the calibration range. Summary of the results is shown in Table II.

Conclusion

The detection of bound tracer activity employing the Varioskan® Flash is very convenient, because all the dispensing, incubation, reading steps and calculations can be performed automatically with a suitable SkanIt® Software protocol. These protocols also ensure future measurements are performed and calculated in exactly the same way to create greater standardization of test methods.

Further information

For further information about the Varioskan Flash multimode reader, please refer to the following web pages:

www.thermo.com/readingroom
www.thermo.com/varioskan

References

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