

## **PROGESTERONE ELISA**

**A microtiter plate based competitive enzyme immunoassay for screening and quantitative analysis of Progesterone in milk and serum samples**

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**11. LITERATURE:**

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**12. ORDERING INFORMATION**

For ordering the Progesterone ELISA kit please use cat. code 5081PROG.

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EuroProxima B.V.	TEL: + 31 26 3630364
Beijerinckweg 18	FAX: + 31 26 3645111
NL 6827 BN Arnhem	Web-site: <a href="http://www.europroxima.com">http://www.europroxima.com</a>
The Netherlands	E-mail: <a href="mailto:info@europroxima.com">info@europroxima.com</a>

**BRIEF INFORMATION**

The Progesterone ELISA is a competitive enzyme immunoassay for the determination of the concentration of Progesterone in milk and serum samples. With this ELISA-kit 96 analyses can be performed. Samples and standards are measured in duplicate which means that in total 40 milk samples can be analysed. The ELISA kit contains all the reagents, including a standard, required to perform the test.

**1. INTRODUCTION**

Progesterone is a good marker for determining the functional status of the corpus luteum in cattle. It's measurement in milk serves as a valuable diagnostic tool in identifying ovarian conditions such as silent heat, lack of cyclicity, cystic follicles, retained corpora lutea and irregular cycles (1-4).

The apparent concentration of Progesterone in milk is dependent upon the fat content. The concentration of Progesterone in fat is 50-100 times higher than in the aqueous phase of whole milk (5). To obtain more precise physiological Progesterone values, it is preferable to estimate it's concentration in fat-free skim milk. The range of Progesterone in skim milk is 0.2-12.5 ng/ml. For routine classification purposes, values <0.4 ng/ml correspond to the early follicular phase, 0.4-1.0 ng/ml to the early or ending luteal phase, and >1.0 ng/ml skim milk indicates full luteal activity (6).

In the Progesterone-ELISA, defatted milk samples can be analysed directly.

**2. PRINCIPLE OF THE PROGESTERONE ELISA**

The microtiter plate based ELISA kit consists of one plate (12 strips, 8 wells each) precoated with sheep antibodies to rabbit IgG. In one incubation step, specific antibody (rabbit anti-Progesterone), enzyme labelled Progesterone (enzyme conjugate) and Progesterone standards or samples are added to the precoated wells. The specific antibodies are bound by the immobilized rabbit antibodies and at the same time progesterone (in the standard solution or in the milk sample) and enzyme labelled progesterone compete for the specific antibody binding sites (competitive enzyme immunoassay).

After an incubation time of one hour, the non-bound (enzyme labelled) reagents are removed in a washing step.

The amount of bound enzyme conjugate is visualised by the addition of chromogen substrate (tetramethylbenzidine, TMB). Bound enzyme transforms the chromogen into a coloured product. The substrate reaction is stopped by the addition of sulphuric acid. The colour intensity is measured photometrically at 450 nm and is inversely proportional to the hormone concentration in the standard solution or the sample.

### 3. SPECIFICITY AND SENSITIVITY

The progesterone ELISA utilises antibodies raised in rabbits against protein conjugated progesterone.

Cross-reactions:	Progesterone	100%
	Hydroxyprogesterone	< 0.1%
	17 $\beta$ -Testosterone	< 0.1%
	Esterone	< 0.1%
	17 $\beta$ -Nortestosterone	< 0.1%

### 4. HANDLING AND STORAGE

- Store the kit at +2°C to +8°C in a dark place.
- After the expiry date (see kit label) has passed, it is no longer possible to accept any further quality guarantee.
- It is advised to unpack the sealed microtiter plate, reconstitute or dilute the kit components, immediately before use.
- After the lyophilised kit components have been reconstituted, these components are only guaranteed for 1 week (stored at +2°C to +8°C in the dark). For prolonged storage, aliquot reconstituted components and store at -20°C.
- Any direct action of light on the chromogen solution should be avoided.

If the following phenomena are observed, this may indicate a degeneration of the reagents:

- A blue colouring of the chromogen solution before putting it into the wells,
- A weak or absent colour reaction of the first standard (zero standard) (E450nm <0.8).

### 10. INTERPRETATION OF RESULTS

Subtract the mean optical density (O.D.) value of the blank wells A1 and A2 from the individual O.D. of the wells containing the standards and the samples. The O.D. values of the six standards and the samples (mean values of the duplicates) are divided by the mean O.D. value of the zero standard (wells B1 and B2) and multiplied by 100. The zero standard is thus equal to 100% (maximal absorbance) and the other O.D. values are quoted in percentages of the maximal absorbance.

O.D. standard (or sample)

----- x 100 = % maximal absorbance

O.D. zero standard

#### Calibration curve:

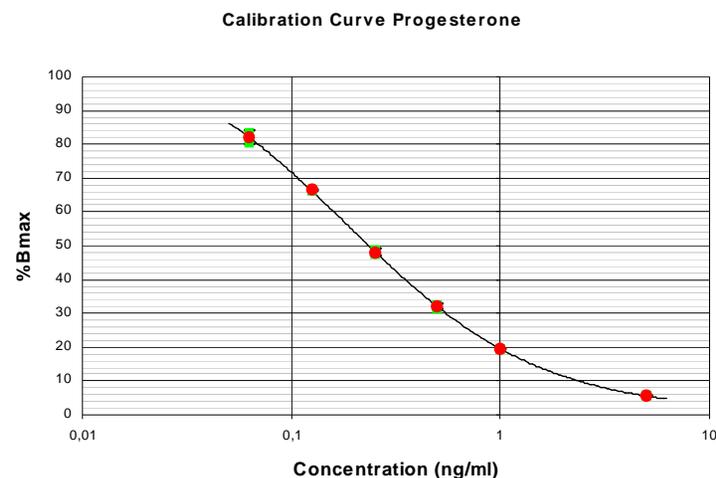
The values (% maximal absorbance) calculated for the standards are plotted (on the Y-axis) versus the Progesterone concentration (ng/ml) on a logarithmic X-axis.

The calibration curve should be linear in the 0.1 - 5 ng/ml range.

#### Milk samples/serum samples:

The amount of Progesterone in the undiluted skim milk sample or serum sample can be read from the calibration curve directly.

Remark: Inconsistency in handling whole milk can have a profound effect in the concentration of Progesterone in skim milk (7).



**Figure 1 : Example of a calibration curve**

## 9. ASSAY PROCEDURE

### Rinsing protocol

In ELISA's, between each immunological incubation step, un-bound components have to be removed efficiently. This is reached by appropriate rinsing. It should be clear that each rinsing procedure must be carried out with care to guarantee inter- and intra-assay results. Basically manual rinsing or rinsing with automatic plate wash equipment can be done as follows:

### *Manual rinsing*

1. Empty the contents of each well by turning the microtiter plate upside down followed by a firm short vertical movement.
2. Fill all the wells to the rims (300 µl) with rinsing solution.
3. This rinsing cycle (1 and 2) should be carried out 3 times.
4. Turn the plate upside down and empty the wells by a firm short vertical movement.
5. Place the inverted plate on absorbent paper towels and tap the plate firmly to remove residual washing solution in the wells.
6. Take care that none of the wells dry out before the next reagent is dispensed.

### *Rinsing with automatic microtiter plate wash equipment*

When using automatic plate wash equipment, check that all wells can be aspirated completely, that the rinsing solution is nicely dispensed reaching the rim of each well during each rinsing cycle. The washer should be programmed to execute three rinsing cycles.

### Assay Protocol

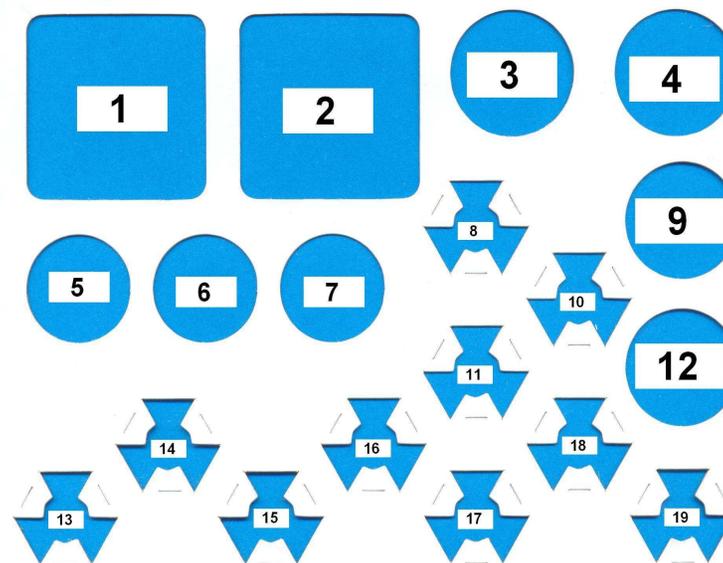
1. Prepare samples according to chapter 7 (Sample treatment) and prepare reagents according to chapter 8. Microtiter plate is ready to use.
2. Pipette 100 µl dilution buffer in duplicate (well A1, A2).  
Pipette 50 µl dilution buffer in duplicate (well B1, B2).  
Pipette 50 µl of each standard dilution in duplicate  
Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate.
3. Add 25 µl conjugate (Progesterone-HRP) to all wells, except wells A1 and A2.
4. Add 25 µl antibody solution to all wells, except wells A1 and A2.
5. Seal the microtiter plate and shake the plate for 1 min.
6. Incubate for 1 hour in the dark at 4°C (2°C - 8°C).
7. Discard the solution from the microtiter plate and wash 3 times with rinsing buffer.
8. Pipet 100 µl substrate solution into each well. Incubate 30 min. at room temperature (20°C - 25°C).
9. Add 100 µl stop solution to each well.
10. Read the absorbance values immediately at 450 nm.

## 5. KIT CONTENTS

### Manual

1 sealed microtiter plate (12 strips, 8 wells each), coated with antibodies to rabbit IgG. Ready to use.

Position of the reagents in the kit. For preparation of the reagents see Chapter 8.



1. **Dilution buffer** (40 ml, Ready to use)
2. **Rinsing buffer** (30 ml, 20 times concentrated)
3. **Substrate solution** (12 ml, Ready to use)
4. **Stop solution** (15 ml, Ready to use)
5. **Conjugate** (lyophilised, blue cap)
6. **Antibody** (lyophilised, yellow cap)
7. **Standard** (lyophilised, black cap)
8. not in use
9. not in use
10. not in use
11. not in use
12. not in use
13. not in use
14. not in use
15. not in use
16. not in use
17. not in use
18. not in use
19. not in use

## 6. PRECAUTIONS

- The stop solution contains 0.5 M sulphuric acid. Do not allow the reagent to get into contact with the skin and/or eyes.
- Avoid contact of all biological materials with skin and mucous membranes.
- Do not pipette by mouth.
- Do not eat, drink, smoke, store or prepare foods, or apply cosmetics within the designated work area.
- TMB is toxic by inhalation, in contact with skin and if swallowed; observe care when handling the substrate.
- Do not use components past expiration date and do not intermix components from different serial lots.
- Each well is ultimately used as an optical cuvette. Therefore, do not touch the outside or inside of the wells, prevent damage and dirt.
- Optimal results will be obtained by strict adherence to this protocol.
- Careful pipetting and washing throughout this procedure are necessary to maintain precision and accuracy.

## 7. SAMPLE TREATMENT

### 7.1 Milk

- Frozen milk samples are thawed at 45°C and incubated at this temperature for 1 h.
- Fresh milk samples are incubated at 45°C for 1 h. also.
- The samples are centrifuged at 2000 x g for 20 min under refrigerated conditions in an inverted position to facilitate easy decantation of the skim milk after centrifugation.
- Use 50 µl in the ELISA test.

### 7.2 Serum

- Incubate serum during 30 min. at 50°C.
- Dilute 1 ml serum with 1 ml sample dilution buffer\* and extract with 2 ml Diethylether.
- Centrifuge 5 min. at 4°C 2000 x g .
- 1 ml of the Diethylether phase is evaporated to dryness under a slow flow of nitrogen.
- Dissolve in 0.5 ml dilution buffer and use 50 µl in the ELISA test.

#### \* Sample dilution buffer

Na <sub>2</sub> HPO <sub>4</sub>	0.77 g
KH <sub>2</sub> PO <sub>4</sub>	0.18 g
NaCl	8.94 g
PH	7.4 (7.3 – 7.5)

## 8. PREPARATION OF REAGENTS

The reagents included in the kit are sufficient to carry out 96 analyses (including standard analyses). Each standard and sample is analysed in duplicate.

Before beginning the test, the reagents should be brought up to ambient temperature. Any reagent not used should be put back into storage immediately at prescribed temperature.

- Microtiter plate:  
Return unused strips into the resealable ziplock bag with desiccant and store at 2°C to 8°C for use in subsequent assays. Retain also the strip holder.
- Rinsing buffer: The rinsing buffer is delivered 20 times concentrated. Prepare dilutions freshly before use. Per strip 40 ml of diluted rinsing buffer is used (2 ml concentrated rinsing buffer + 38 ml distilled water).
- Substrate solution: The substrate solution (ready to use) precipitates at 4°C. Take care that this vial is at room temperature (keep in the dark) and mix the content before pipetting into the wells.
- Standard solutions: Prepare a dilution range of the progesterone standard. Add 2.0 ml of dilution buffer to the vial of progesterone standard and mix. Pipette 400 µl of this progesterone solution into a glass tube. This standard solution contains 5 ng progesterone per ml. Pipette 100 µl of this solution and add 400 µl of dilution buffer (concentration of progesterone 1.0 ng/ml). Continue to make a dilution range of 1.0, 0.5, 0.25, 0.125 and 0.0625 ng/ml in dilution buffer. Keep the solutions of the dilution range in the dark at room temperature until use.
- Conjugate solution: Reconstitute the vial of lyophilised conjugate (PROG-HRPO) with 4 ml dilution buffer, mix thoroughly and keep in the dark until use. Store the vial immediately after use in the dark at 2°C to 8°C.
- Antibody solution: Reconstitute the vial of lyophilised antibodies with 4 ml dilution buffer, mix thoroughly and keep in the dark until use. Store the vial immediately after use in the dark at 2°C to 8°C.