

Pig and other Species

Progesterone

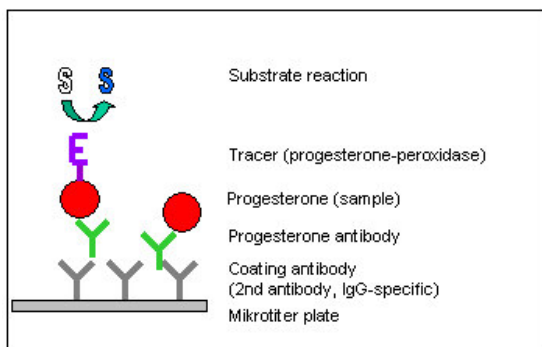
Test Kit

Enzyme Immunoassay for Progesterone in Porcine Serum/Plasma

Quick Guide to Assay Steps

1. Allow reagents to come to room temperature (exception: Tracer Stock Solution and Antibody Stock Solution)
2. Dilute Assay Buffer 5x concentrate
3. Dilute samples with Assay Buffer 1x (e.g. 1:50)
4. Place a sufficient number of antibody coated Strips in a holder
5. Prepare an appropriate volume of Progesterone Tracer Solution (1:250 in Assay Buffer 1x containing 5% Porcine Serum Matrix), and Progesterone Antiserum Solution (1:60 in Assay Buffer 1x)
6. Pipet 50 μ l Tracer Solution into the wells
7. Add 50 μ l Standards, Controls and samples (pre-diluted)
8. Add 50 μ l Antiserum Solution
9. Shake assay plate for about 5 minutes and incubate over night (16-24h) at 2-8°C
10. Dilute Wash Buffer 50x concentrate
11. Wash 4 times with Wash Buffer 1x
12. Pipet 100 μ l Substrate
13. Incubate 45 \pm 10 min at room temperature
14. Add 100 μ l Stop Solution and read optical density at 450 nm (595-650 nm as reference wavelength)

Principle of the Procedure



The progesterone enzyme immunoassay is a competitive ELISA in 96-well microtiter plates (stripwell or massive plate format). Stripwell plates (12 x 8-well strips) are the best choice for few samples whereas 96-well plates are much easier to handle for the measurement of hundreds of samples.

In the first incubation step free progesterone (sample, control or standard) competes with Tracer (horseradish peroxidase conjugated progesterone) for a limited number of antigen binding sites of the progesterone specific antibodies. At the same time the antibodies were fixed to the microtiter plates by a secondary antibody bound to the plate surface.

After removing the excess of test compounds by washing, enzyme activity of the tracer is detected with tetramethylbenzidine (TMB) as substrate.

Reagents and Material Provided

1. **Microtiter Plate** (12 eight-well strips plate or 96 well plate)
2. **Progesterone Standards**
 - A 0 ng/ml
 - B 0.25 ng/ml
 - C 0.5 ng/ml
 - D 1.0 ng/ml
 - E 2.5 ng/ml
 - F 5.0 ng/ml
 - G 25 ng/ml
3. **Low Control:** 1.5 ng/ml (1.2 to 1.8 ng/ml)
High Control: 4 ng/ml (3.2 to 4.8 ng/ml)
4. **50x Wash Buffer**
Containing 0.01% Thimerosal as a preservative
5. **5x Assay Buffer**
BSA in a buffered solution containing dye and 0.01% Thimerosal as a preservative
6. **Tracer Stock Solution**
Containing 50% glycerol to prevent freezing. Store at -18°C to -25°C
7. **Porcine Serum Matrix**
For Tracer Solution
8. **Antiserum Stock Solution**
Containing 50% glycerol to prevent freezing. Store at -18°C to -25°C
9. **Substrate Solution**
One-step TMB substrate
10. **Stop Solution**
1 M HCl

Warnings and Precautions

- For Research Use only. Not for Use in diagnostic procedures.
- Treat samples as potentially biohazardous material. Follow Universal Precautions when handling contents of this kit and any sample.
- Dispose of containers and unused contents in accordance with Federal State and Local regulatory requirements.
- Store assay reagents as indicated.
- Test each sample in duplicate.
- Use of multichannel pipettes or repeat pipettors is recommended to ensure timely delivery of reagents.
- Pipet carefully using only calibrated equipment.
- Perform assay with any validated washing method, preferably, automated plate washer.
- Perform standard curve with each assay.
- Adequate mixing of Standards, Controls, samples and the use of a microtiter plate shaker is required for acceptable assay performance.

Reagent Preparation

All reagents should be equilibrated to room temperature (20-26°C) prior to use **except the Tracer Stock Solution and the Antiserum Stock Solution.**

Be sure that possible precipitates in Buffer Solution Concentrates will be re-dissolved before dilution.

Coated Strips

Remove Stripwell frame and the required number of coated strips from the pouch. Ensure that the pouch containing any unused strips is completely resealed and contains desiccant.

Wash Buffer

Prepare required amount of 1x Wash Buffer by diluting 50x Wash Buffer concentrate 1:50 with deionized water. Store at 2-8°C and use 1x Wash Buffer within 2 weeks of preparation.

E.g. add 10 ml 50x Wash Buffer concentrate to 490 ml deionized water.

Assay Buffer

Prepare required amount of 1x Assay Buffer by diluting 5x Wash buffer concentrate 1:5 with deionized water. Store at 2-8°C and use 1x Assay Buffer within 1 months of preparation.

E.g. add 10 ml 5x Assay Buffer concentrate to 40 ml deionized water.

Tracer Solution

Avoid warming of the glycerol containing Stock Solutions!

Prepare Tracer solution before use. Do not store.

Glycerolated Stock Solutions are a bit viscous. For pipetting cut off about 3 mm from top of a pipet tip, aspirate the desired volume and dispense in Assay Buffer 1x. Spill the tip by repeated (5-10 times) aspiration/dispense cycles.

Dilute Progesterone Tracer Stock Solution in Assay Buffer 1:250 and add 5% Porcine Serum Matrix.

Examples:

4 Strips: 1.9 ml Assay Buffer 1x + 8 µl Tracer Stock Solution + 100 µl Serum Matrix

12 Strips: 5.2 ml Assay Buffer 1x + 22 µl Tracer Stock Solution + 275 µl Serum Matrix.

Antiserum Solution

Avoid warming of the glycerol containing Stock Solutions!

Prepare Tracer solution before use. Do not store.

Glycerolated Stock Solutions are a bit viscous. For pipetting cut off about 3 mm from top of a pipet tip, aspirate the desired volume and dispense in Assay Buffer 1x. Spill the tip by repeated (5-10 times) aspiration/dispense cycles.

Dilute Progesterone Antiserum Stock Solution in Assay Buffer 1:60.

Examples:

4 Strips: 2.0 ml Assay Buffer 1x + 33.3 µl Antiserum Stock Solution

12 Strips: 5.5 ml Assay Buffer 1x + 92 µl Antiserum Stock Solution.

Storage

- **Tracer Stock Solution and Antiserum Stock Solution have to be stored at -18°C to -25°C.**
- **Store all other Kit components at 2-8°C.**

Assay Procedure

Read entire product instructions before beginning the assay.

Peroxidase and the TMB Substrate are light sensitive. **Avoid direct sunlight.**
Cover the plate with aluminium foil during incubation steps.

Sample incubation

1. Allow pouch of Coated Strips/Coated Plate to equilibrate to room temperature before opening. Remove Stripwell Frame and the required number of Coated Strips from the pouch. Ensure that the pouch containing unused strips is completely resealed and contains desiccant.
2. Place desired number of Coated Strips in Stripwell Frame just prior to use. Label strips to prevent mix-up in case of accidental removal from Stripwell Frame. Cover the Plate/Strips during all incubation steps.
3. Dilute samples as necessary with Assay Buffer 1x (e.g. 1:50).
4. Pipet 50 µl Tracer Solution to each well by using a multichannel pipette.
5. Add 50 µl Standards, Controls and pre-diluted samples.
6. Add 50 µl Antiserum Solution to each well by using a multichannel pipette.
7. Mix Plate content on a microplate shaker for 5-10 min.
8. Incubate over night (16-24h) at 4°C.

Substrate incubation

9. Wash 4 times:
Empty Strips/Plate. Add at least 250µl of Wash Buffer 1x to each well.
Repeat three more times for a total of four washes.
Preferably, an automated microtiter plate washer should be used.
Vigorously blot the Strips/Plate dry on paper towels after the last wash.
10. Pipet 100 µl of Substrate Solution to each well.
11. Incubate 45 ± 10 min at room temperature.

Stop/Read

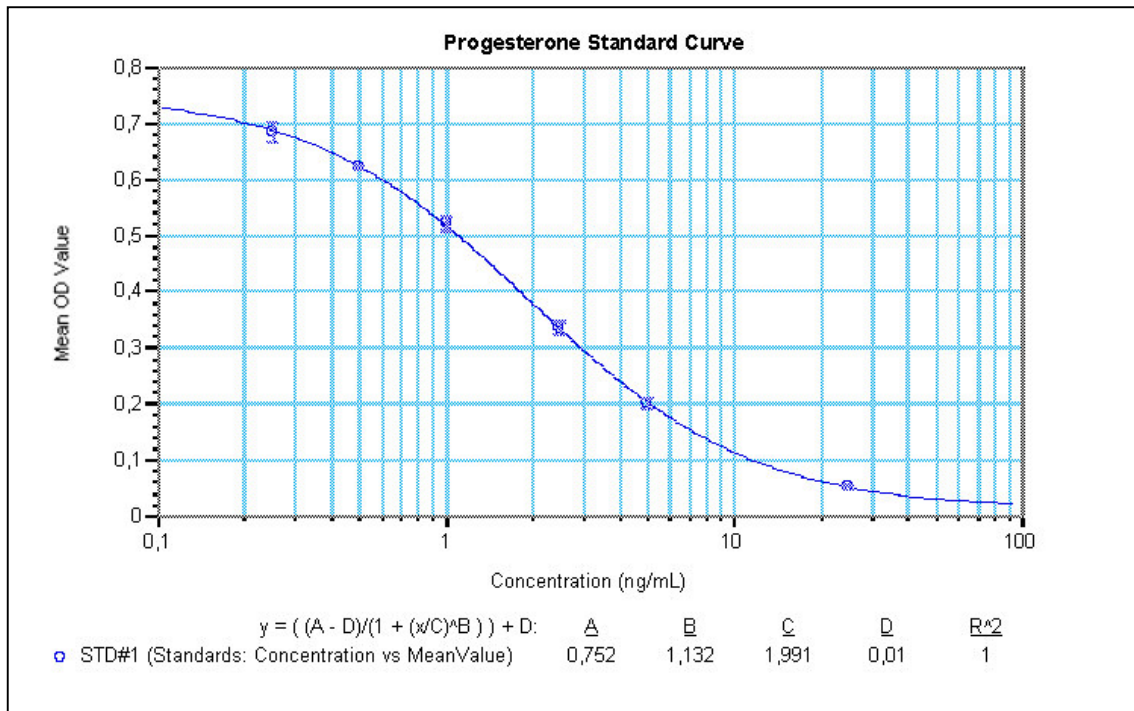
12. Add 100 µl of Stop Solution to each well. Add Stop solution in the same pattern and time intervals as the Substrate Solution addition.
13. Read optical density at 450 nm (use 595-650 nm as reference wavelength, if possible). Assure that no large bubbles are present in wells and that the bottom of the Strips/Plate are clean. Strips/Plate should be read immediately (within 5 min).
14. Use appropriate computer software for calibration curve to analyse assay results (e.g. 4-Parameter Method).
15. Determine concentration of samples and Controls from the standard curve. Multiply the values with the dilution factor if pre-dilution of the samples was different to 1:50.
16. Control values should be within the range specified.

The control values are intended to verify the validity of the curve and sample results. Each laboratory should establish its own parameters for acceptable assay limits. If the control values are NOT within this limits, the assay results should be considered as questionable and the samples should be repeated.

Standard concentrations are calculated for serum/plasma samples pre-diluted 1:50 in Assay Buffer.

Example Data and Standard Curve

Sample	Concentration	Wells	Values	MeanValue	Std.Dev.	CV%
St01	0,000	A5	0,804	0,753	0,044	5,9
		A6	0,773			
		H5	0,733			
		H6	0,703			
St02	0,250	B5	0,697	0,685	0,017	2,4
		B6	0,673			
St03	0,500	C5	0,624	0,621	0,003	0,5
		C6	0,619			
St04	1,000	D5	0,530	0,521	0,012	2,3
		D6	0,512			
St05	2,500	E5	0,343	0,335	0,011	3,3
		E6	0,327			
St06	5,000	F5	0,207	0,200	0,009	4,5
		F6	0,194			
St07	25,000	G5	0,049	0,051	0,003	5,6
		G6	0,053			



Sample	Wells	OD Values	Concentration	Mean Conc.	Std.Dev.	CV%
Low Ctrl	A7	0,428	1,591	1,616	0,035	2,2
	B7	0,421	1,641			
High Ctrl	C7	0,238	4,078	4,081	0,005	0,1
	D7	0,238	4,085			

Characteristics of the Progesterone Assay

The assay based on the antiserum described by D.F.M. Van de Wiel and W. Koops [1] and Prakash *et al.* [2]. The assay was validated for porcine samples in a PhD thesis at the University of Göttingen [3] and used in several studies [4, 5].

Samples

Porcine plasma or serum

Pre-dilution needed for the assay: 1:20 or higher. Standard concentrations are calculated for serum/plasma samples pre-diluted 1:50.

Measuring range

0.25 to 15 ng/ml (50 µl sample: serum pre-diluted 1:50)

Normal progesterone ranges in sows

< 1,5 ng/ml no or minor activity of corpus luteum, non-pregnant

> 4 ng/ml high corpus luteum activity, pregnancy possible

Antiserum was raised in rabbits against progesterone-7 α -carboxyethylthioether-BSA.

Finale dilution 1:180,000

Tracer

Progesterone-6 β -hydroxyhemisuccinate-(horseradish peroxidase)

Cross-reactivity:

Progesterone 100%, 5 α -pregnan-3,20-dione 25%, 11 α -progesterone 5.6%.

Testosterone, 5 α -DHT, 17 β estradiol, estrone, 17 α -progesterone and cortisol < 0.1%.

Intraassay CV (n=20) 8.8%

Interassay CV (n=10) 8.9%

Recovery (%) of different amount of progesterone added to porcine serum samples

1 ng/ml	88%
5 ng/ml	128%
25 ng/ml	89%

References

- [1] Van de Wiel DFM, Koops W (1986) Development and validation of an enzyme immunoassay for progesterone in bovine milk or blood plasma. *Anim Reprod Sci* 10, 201-213.
- [2] Prakash BS, Meyer HHD, Van de Wiel DFM (1988) Sensitive enzyme immunoassay of progesterone in skim milk using second antibody technique. *Anim Reprod Sci* 16, 225-235.
- [3] Möller R (1991) Enzymimmunosassay mit zweitem Antikörper für Progesteron und porcinem LH. PhD thesis, University of Göttingen.
- [4] Wallenhorst S, Holtz W (2002) Embryo collection in prepubertal gilts and attempts to develop an improved embryo transfer technique. *Vet Rec* 150, 749-751.
- [5] Dziuk PJ, Parvizi N, Ellendorff F (1999) Concentrations of free steroids in the jugular and hepatic portal veins of pigs after ingestion of testosterone, estrogen, or progesterone or transplantation of ovaries to the intestine. *Domest Anim Endocrinol* 17, 29-38.