MEDROXYPROGESTERONE ACETATE ELISA

5131MPA[7]01.21

A competitive enzyme immunoassay for
screening and quantitative analysis of
Medroxyprogesterone acetate (MPA)
in kidney fat

EUROPROXIMA MEDROXYPROGESTERONE ACETATE ELISA

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BRIEF INFORMATION

The Medroxyprogesterone (MPA) ELISA is a competitive enzyme immunoassay for the screening of kidney fat for the presence of this synthetic progestagen. The test is based on antibodies against MPA. The ELISA kit contains a 96 well microtiter plate as well as all essential reagents to perform the test. A method for an efficient extraction of MPA from kidney fat is included in the kit manual.

1. INTRODUCTION

Medroxyprogesterone acetate (MPA), megestrol acetate, melengestrol acetate and chlormadinon acetate are synthetic derivatives of progesterone and are also called acetylgestagens. The acetylgestagens can be used as growth promoters in meat production, both in cattle and in pigs. The use of acetylgestagens as growth promoters leads to a faster growth of the animals and to an increase of feed conversion efficiency. Acetylgestagens are permitted as growth promoters in certain countries, e.g. the USA. However, in the EU the use of growth promoters, inclusive acetylgestagens, is prohibited.
Acetylgestagens are apolar steroids. These steroids are particularly concentrated in fat tissue within the body of the animals. Therefore, the fat and especially kidney fat is used as matrix for screening for the presence of acetylgestagens
The antiserum used in this ELISA is directed against MPA, however the antiserum also shows cross-reactivity against 17α-Acetoxyprogesterone (82%), Megestrol acetate (50 %), Chlormadinone acetate (50%) and Melengestrol acetate (25 %).

2. PRINCIPLE OF THE MEDROXYPROGESTERONE ACETATE (MPA) ELISA

The kit is based on a microtiter plate (12 strips, each 8 wells), precoated with sheep antibodies to rabbit IgG. In one incubation step, specific antibodies (Rabbit anti-MPA), enzyme labelled MPA (enzyme conjugate) and MPA standards or sample are added to the precoated wells. The specific antibodies are bound by the immobilised sheep anti-rabbit antibodies and at the same time free MPA (in the standard solution or in the sample) and enzyme labelled MPA 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 visualized 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 sulfuric acid. The colour intensity is measured photometrically at 450 nm and is inversely proportional to the MPA concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The MPA-ELISA utilizes antibodies raised in rabbits against protein conjugated MPA. The reactivity pattern of the antibody is as follows:

Medroxyprogesterone acetate	100%
17α-Acetoxyprogesterone	82%
Megestrol acetate	50%
Melengestrol acetate	25%
Chlormadinone acetate	50%
Medroxyprogesterone	< 0.1%
Progesterone	< 0.1%

The cross-reactivities are determined in a buffer system. The reported values may be different in samples due to matrix effects.
The test cannot discriminate between analytes and cross-reactive substances.

The limit of detection (LOD) and the detection capability (CCβ) are determined under optimal conditions. Cut-off criteria need critical consideration.

Matrix	Procedure	LOD ng/g	CCβ ng/g
Kidney fat	8.1	0.1	0.3

If the sample is found to be non-compliant, the results shall be verified by re-analysis of the sample using a confirmatory method.

4. HANDLING AND STORAGE

- Kit and kit components should be stored at 2°C to 8°C in a dark place. For repeated use store kit components as specified under chapter 9.
- After the expiry date of the kit and/or components has passed, no further quality guarantee is valid.
- Bring all kit components including the microtiter plate to ambient (room) temperature before use.
- Dilute the kit components immediately before use, but after the components are brought to ambient temperature.
- Avoid condensation in the wells of the plate. Bring the sealed plate to ambient temperature before opening the plate sealing.
- The substrate chromogen solution can be stored in a refrigerator (2ºC to 8ºC) until the expiry date stated on the label.
- Exposure of the chromogen solution to light should be avoided.

Degeneration of the reagents may have occurred when the following phenomena are observed:

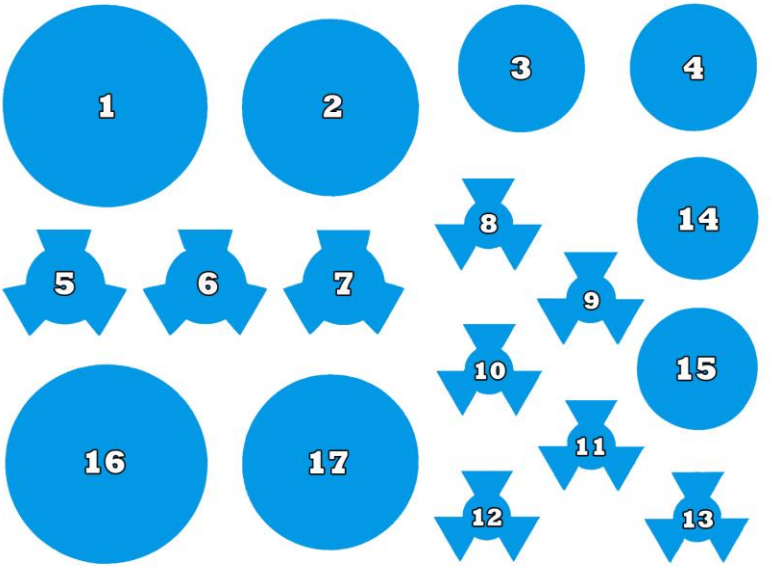
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

5. KIT CONTENTS

Manual

One 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 9.



1. **Dilution buffer** (40 ml, ready-to-use)
2. **Rinsing buffer** (30 ml, 20x concentrated)
3. **Substrate solution** (12 ml, ready-to-use)
4. **Stop solution** (15 ml, ready-to-use)
5. **Conjugate solution** (lyophilized, blue cap)
6. **Antibody solution** (lyophilized, yellow cap)
7. **Standard solution** (lyophilized, 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. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Scales and weighing vessels
- Gloves
- Fume hood
- Homogeniser (vortex, mixer)
- Automated microtiter plate washer or 8-channel micropipette 100 – 300 µl
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Microwave
- Micropipettes, 100 – 1000 µl
- Multipipette with 2.5 ml combitips
- Methanol 100%
- 4 ml glass tubes
- Cyclo-hexane
- 15 ml tubes with screw cap (polypropylene)
- Solid phase cartridge (RIDA® C18 Column, Art. Nr. R2002)

7. PRECAUTIONS

- This kit may contain hazardous substances. For hazard notes please refer to the appropriate safety data sheets (SDS).
- 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.
- Do not use components past expiration date and do not use components from different lots.
- Each well is ultimately used as an optical cuvette. Therefore, do not touch the under surface of the wells, prevent damage and dirt.
- All components should be completely dissolved before use. Take special attention to the substrate and rinsing buffer, which crystallize at +4ºC.
- Optimal results will be obtained by strict adherence to this protocol. Careful pipetting and washing throughout this procedure are necessary to maintain good precision and accuracy.

8. SAMPLE PREPARATION

8.1 Kidney fat samples
Transfer approximately 25 g of kidney fat into a vial and heat at 50°C for approximately 1 hour until the fat is melted. Alternatively, the fat can be melted in a microwave for 3 minutes at 360 Watt. Transfer 0.5 g of the melted fat into a glass tube. Add 5.0 ml of cyclo-hexane. Mix for 30 seconds using a vortex. Conduct the solid phase extraction procedure as described below.

Primary solid phase extraction
Activate solid phase cartridges by washing the cartridges subsequently with 5ml cyclo-hexane. Transfer the cyclo-hexane extracts from the kidney fat samples onto the C18 cartridge. Be aware the extracts flow through the cartridges with a speed of 0.5 ml per minute. Wash the cartridges using 5 ml cyclo-hexane. Dry the cartridges for 10 minutes under vacuum. Elute the acetylgestagens using 3.5 ml 80% methanol in distilled water. Add 2 ml of distilled water to the eluate and continue with the second solid phase clean-up procedure as described below.

Solid phase clean-up
Activate solid phase cartridges by washing the cartridges subsequently with 5 ml 100% methanol and with 5 ml 50% methanol in distilled water. Transfer 5.5 ml of the elute obtained after the primary solid phase extraction onto an activated solid phase cartridge. Wash the cartridges using 7.0 ml 50% methanol in distilled water. Dry the cartridges under vacuum. Elute the acetylgestagens using 5.5 ml 80% methanol in distilled water with a speed of 1 ml per minute. Evaporate the elute to dryness at a temperature of 50°C under a mild stream of nitrogen. Dissolve the residue in 500 µl dilution buffer at a temperature of 37°C during regular mixing using a vortex or using an ultrasonic bath. An aliquot of 2 times 50 µl is used in the ELISA test.

9. PREPARATION OF REAGENTS

Before starting the test, allow the reagents to come to ambient temperature. Any reagents not used should be put back into storage immediately at 2°C to 8°C.

Microtiter plate
Return unused strips into the resealable 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 20 ml of diluted rinsing buffer is used (1 ml concentrated rinsing buffer + 19 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
Prepare a dilution range of the Medroxyprogesterone acetate standard.

Reconstitute the lyophilized standard with 2 ml of dilution buffer. The concentration of the reconstituted standard is 40 ng MPA per ml.
Dilute the 40 ng/ml standard 1:2 to obtain the 20 ng/ml standard (take 200ul of the 40 ng/ml standard and add 200 ul buffer).
Dilute the 20 ng/ml standard 1:5 to obtain the 4 ng/ml standard (take 80 ul of the 20 ng/ml standard and add 320 ul buffer).
Continue according to the schedule.

	40	20	4	2	0.4	0.2	0
Buffer		200 µl	320 µl	200 µl	320 µl	200 µl	200 µl
Standard	400µl	200 µl	80 µl	200 µl	80 µl	200 µl	

Conjugate solution
Reconstitute the vial of lyophilized conjugate (MPA-HRP) 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 lyophilized 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.

10. ASSAY PROCEDURE

Rinsing protocol

In ELISA's, between each immunological incubation step, unbound 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 good inter- and intra-assay results.
Basically, manual rinsing or rinsing with automatic plate wash equipment can be performed as follows:

Manual rinsing

1. Empty the contents of each well by turning the microtiter plate upside down and remove residual liquid by striking the plate against a paper towel.
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 rinsing solution from 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. Three rinsing cycles should be executed.

Assay Protocol

1. Prepare samples according to chapter 8 and prepare reagents according to chapter 9.
   Microtiter plate is ready-to-use, do not wash.

2. Pipette 100 µl dilution buffer in duplicate (well H1, H2).
   Pipette 50 µl dilution buffer in duplicate (well A1, A2).
   Pipette 50 µl of each standard dilution in duplicate (well B1,2 to G1,2).

3. Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate.

4. Pipette 25 µl conjugate (MPA-HRP) to all wells, except wells H1 and H2.

5. Pipette 25 µl antibody solution to all wells, except wells H1 and H2.

6. Seal the microtiter plate and shake the plate for a few seconds on a microtiter plate shaker.

7. Incubate for 1 hour in the dark at 37°C.

8. Discard the solution from the microtiter plate and wash 3 times with rinsing buffer.

9. Pipette 100 µl substrate solution into each well.

10. Incubate 30 minutes at 20°C - 25°C.

11. Pipette 100 µl stop solution to each well.

12. Read the absorbance values immediately at 450 nm.

11. INTERPRETATION OF RESULTS

Subtract the mean optical density (O.D.) of the wells H1 and H2 (Blank) 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/Bmax (wells A1 and A2) and multiplied by 100. The zero standard/Bmax is thus made 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 = percentage maximal absorbance
O.D. zero standard/Bmax

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

Alternative for calibration curve:
The absorption value of the standards is plotted on the Y-axis versus the concentration on the X-axis. The Y-axis is in logit the Y-axis is logarithmic.

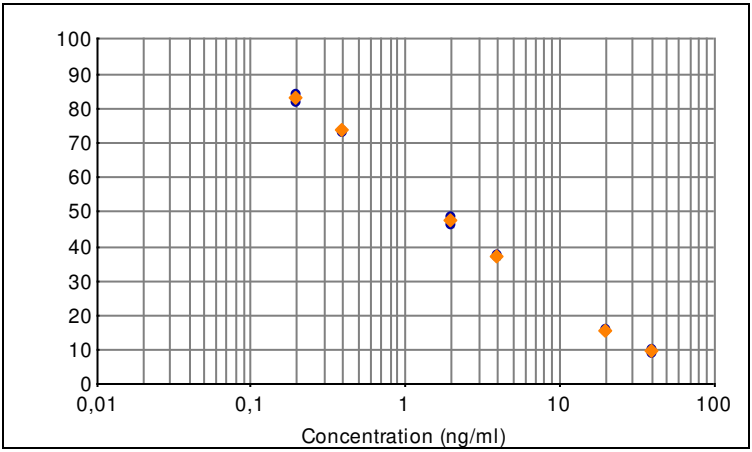


Figure 1: Example of a calibration curve

The amount of Medroxyprogesterone acetate in the samples is expressed as MPA equivalents. The MPA equivalents in the extracts (ng/g) corresponding to the percentage maximal absorbance of each extract can be read from the calibration curve.

Calculation

8.1 Kidney fat
Calculation
The results obtained can directly be expressed as ng MPA equivalents per g or per ml of sample

12. LITERATURE

Lindfors E., Bäckman C. Determination of acetyl gestagens in fat using automated solid phase extraction and normal phase HPLC. Proceedings of the EuroResidue III conference. Edited by N. Haagsma and A. Ruiter. Velhoven, the Netherlands. (1996), 646-649.

Hädrich J., Jarvers J., and Podestàt U. Immunoenzymatischer Nachweis von Acetylgestagenen in Fettgewebe. Validierung des Verfahrens gemaβ DIN EN ISO/IEC 17025 und nach den Vorgaben der Entscheidung (EWG) Nr. 93/256 am Beispiel von 6α-Methyl-17α-hydroxyprogesteronacetat. Deutsche Lebensmittel-Rundschau: 97, 11, 409-414, 2001.

13. ORDERING INFORMATION

For ordering the Medroxyprogesterone acetate ELISA kit, please use cat. code 5131MPA.

14. REVISION HISTORY

The manual is adapted to a new layout of the test kit. Several textual changes are added.

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