MEDROXYPROGESTERONE ACETATE ELISA KIT

Cat. No.:DEIA050
Pkg.Size:96T

Intended use

A competitive enzyme immunoassay for quantitative analysis of Medroxyprogesterone acetate (MPA) in kidney fat, food and feed samples.

General Description

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 EC the use of growth promoters, inclusive acetylgestagens, is prohibited. Furthermore, the use of acetylgestagens as feed additives can affect fertility and development in particular animals.
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. Alternatively, the MPA EIA is also suitable to screen food and feed samples or feed additives for the presence of acetylgestagens.
The antiserum used in this EIA is directed against MPA, however the antiserum also shows cross-reactivity against Megestrol acetate (50 %), Melengestrol acetate (25 %) and Chlormadinone acetate (50 %).

Principle Of The Test

The microtitre plate based EIA kit consists of 12 strips, each containing 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 sulphuric acid. The colour intensity is measured photometrically at 450 nm and is inversely proportional to the MPA concentration in the sample.

Reagents And Materials Provided

1 sealed microtitre 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 Reagent Preparation.

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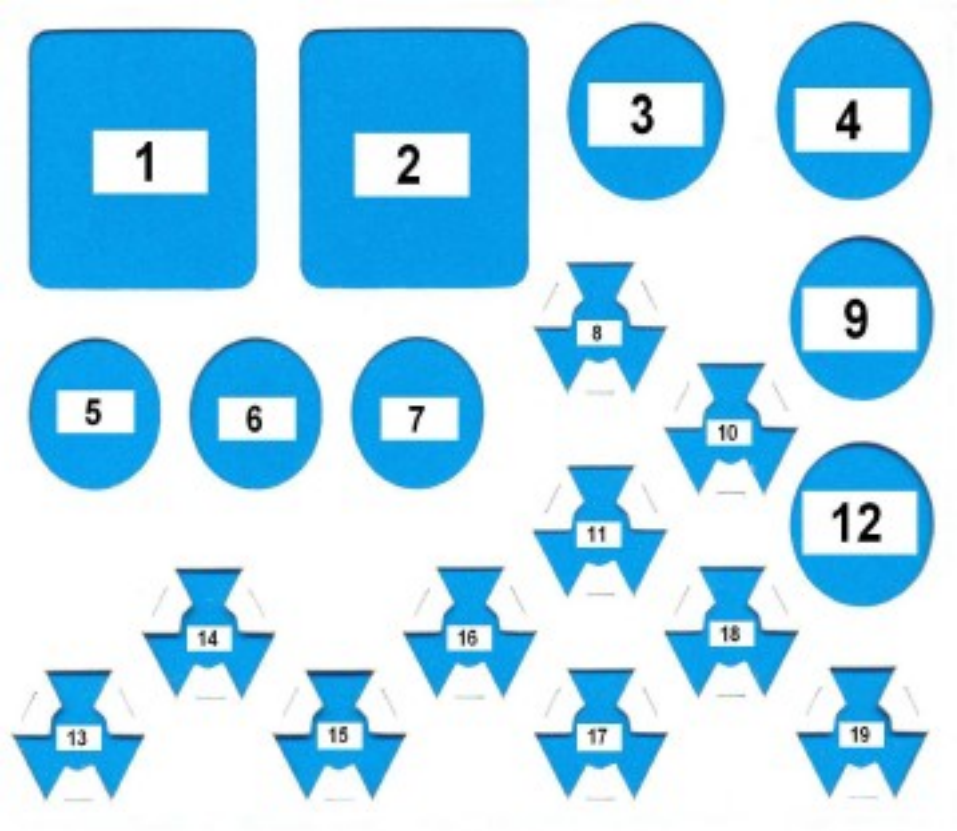

1. Dilution buffer (40 ml, Ready to use)
2. Rinsing buffer (30 ml, 20 times concentrated)
3. Substrate solution (12 ml, Ready to use)
4. Stop solution (15 ml, Ready to use)
5. Conjugate solution (lyophilised, blue cap)
6. Antibody solution (lyophilised, yellow cap)
7. Standard solution (lyophilised, 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

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Storage

1. Store the kit at + 2°C to + 8°C in a dark place.
2. After the expiry date (see kit label) has passed, it is no longer possible to accept any further quality guarantee.
3. Before opening the sealed plate, the plate should be at ambient temperature. This is to avoid condensation in the ELISA plate after the plate is transported from the refrigerator to room temperature.
4. Reconstitute or dilute the kit components immediately before use, but after the components are at ambient temperature.
5. After the lyophilised kit components have been reconstituted, these components have to be used directly or can be stored in a refrigerator for maximally one week (stored at + 2°C to + 8°C in the dark). When no refrigerator is available or when the conjugate has to be stored for longer than one week, the conjugate should be stored in aliquots at –20°C immediately after the first use. Alternatively, after reconstitution of the antibody and conjugate components, aliquots of these solutions can be prepared. The aliquots can be stored in a freezer (–20°C) for at least one year. The substrate and standard solutions can be stored in a refrigerator (+ 2°C to + 8°C) until the expiry date stated on the label.
6. Any direct action of light on the chromogen solution should be avoide. 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 maximum binding (zero standard)
(E450nm < 0.8).

Specimen Collection And Handling

Sample treatment will be conducted according to J. Hädrich et al . In this manuscript an ISO validated method is described for screening for acetylgestagens in kidney fat using the EuroProxima MPA ELISA test. A similar method is given for screening of food (e.g. beverages) and feed samples for acetylgestagens.

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 min 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 sec using a vortex. Conduct the solid phase extraction procedure as described in paragraph 5.

2 Muscle, meat and sausages

Homogenise approximately 25 g of muscle, meat or sausage sample. Transfer 1.0 g of the homogenised sample into a glass tube. Add 10.0 ml of cyclo- hexane. Mix for 30 sec using a vortex. Use 5 ml of the clean cyclo-hexane extract for the solid phase extraction procedure as described in paragraph 5.

3 Feed samples

Homogenise approximately 25 g of feed sample. Transfer 1.0 g of the homogenised sample into a glass tube. Add 7.0 ml of 80 % methanol in distilled water. Mix for 30 sec using a vortex. Mix for another 10 min using a shaker. Add 4.0 ml of distilled water and mix again for 30 sec using a vortex. Transfer 5.5 ml of the clean extract onto an activated C18 cartridge. Conduct the solid phase clean-up procedure as described in paragraph 6.

4 Beverages

Transfer 0.5 ml of a beverage sample into a glass tube. Extract the acetylgestagens from the sample with 2 times 2.5 ml of cyclo -hexane. Collect the cyclo-hexane phases into a new glass tube, evaporate to dryness at a temperature of approximately 40ºC under a mild stream of nitrogen. Dissolve the dry residue in 5 ml 50 % methanol in distilled water. Mix for 30 sec using a vortex. Mix for another 3 min using an ultrasonic bath. Conduct the solid phase clean-up procedure as described in paragraph 6.

5 Primary solid phase extraction

Transfer the cyclo-hexane extracts from the kidney fat samples (paragraph 1) or from the muscle, meat or sausage samples (paragraph 2) onto a non- activated C18 cartridge (E.g. Bond-Elut C18, LRC 10 ml, 500 mg; Varian, art. Nr.: 1211-3027). Be

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aware the extracts flow through the cartridges with a speed of 0.5 ml per min. Wash the cartridges using 3 ml cyclo-hexane. Dry the cartridges for 10 min 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 in paragraph 6.

6 Solid phase clean-up

Activate solid phase cartridges (Bond-Elut C18, LRC 10 ml, 500 mg; Varian, art. Nr.: 1211-3027) by washing the cartridges subsequently with 5 ml methanol and with 5 ml 50 % methanol in distilled water.

Transfer 5.5 ml of the elute obtained after the primary solid phase extraction (paragraph 5), or 5.5 ml of the extract from the feed samples (paragraph 3), or 5 ml of the extract from the beverage samples (paragraph 4) 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 min. Evaporate the elute to dryness at a temperature of 50ºC under a mild stream of nitrogen. Dissolve the residue in 500 µl dilution buffer at a temperature of 37ºC during regular mixing using a vortex or using an ultrasonic bath. An aliquot of 2 times 50 µl is used in the ELISA test.

Reagent Preparation

The reagents included in the test-kit are sufficient to carry out at least 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 reagents not used should be put back into storage immediately at 2°C - 8°C.

Prepare reagents freshly before use

Microtitre plate

Return unused strips into plastic the resealable bag with desiccant and store at 2°C - 8°C for use in subsequent assays. Retain also the stripholder.

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 solution

Prepare a dilution range of the Medroxyprogesterone acetate standard. Reconstitute the lyophilised standard with 2 ml of dilution buffer. The concentration of the reconstituted standard is 40 ng MPA per ml.

Prepare a dilution range of the MPA standard containing 40, 20, 4, 2, 0.4 and 0.2 ng/ml.

Conjugate solution

Reconstitute the vial of lyophilised conjugate (MPA-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.

Assay Steps

Rinsing protocol

In EIA's, un-bound components have to be removed efficiently between each immunological incubation step. This is achieved by appropriate rinsing. It should be clear that each rinsing procedure must be carried out with care to guarantee good inter- and

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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 microtitre 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 microtitre 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.

1. Prepare samples according to Specimen Collection And Handling and prepare reagents according to Reagent Preparation. Microtitre plate is ready to use, do not wash.
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 (well C1,2 to H1,2).
3. Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtitre plate (40 samples; 80 wells).
4. Add 25 µl conjugate (MPA-HRPO) to all wells, except wells A1 and A2.
5. Add 25 µl antibody solution to all wells, except wells A1 and A2.
6. Seal the microtitre plate and shake the plate for a few seconds.
7. Incubate for 1 hour in the dark at 37°C.
8. Discard the solution from the microtitre plate and wash 3 times with rinsing buffer.
9. Pipette 100 µl substrate solution into each well. Incubate 30 min. at room temperature (20°C - 25°C).
10. Add 100 µl stop solution to each well.
11. Read the absorbance values immediately at 450 nm.

Calculation

The results obtained of the different samples can directly be expressed as ng MPA- equivalents per g or per ml of sample.

Typical Standard Curve

The values (% maximal absorbance) calculated for the standards are plotted (on the Y-axis) versus the Medroxyprogesterone acetate equivalent concentration (ng/ml) on a logarithmic X-axis. The calibration curve should be virtually linear in the 0.3 - 20 ng/ml range.

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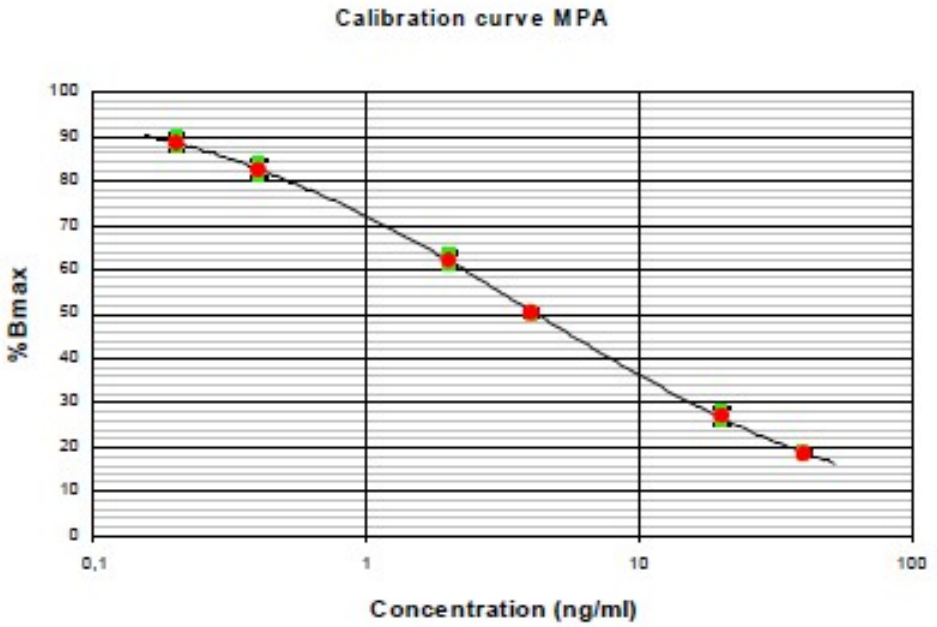

Figure 1: Example of a calibration curve

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

Interpretation of Results

Subtract the mean optical density (O.D.) of the 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 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 = % maximal absorbance
O.D. zero standard

Sensitivity

The sensitivity of the MPA ELISA-kit was found to be 0.5 ng/g for screening for MPA in kidney fat.

Specificity

The MPA-EIA utilises antibodies raised in rabbits against protein conjugated MPA. The reactivity pattern of the antibody is:
Cross- reactions:
Medroxyprogesterone acetate 100 %
Megestrol acetate 50 %
Melengestrol acetate 25 %
Chlormadinone acetate 50 %

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Precautions

1. The stop reagent contains 0.5 M sulphuric acid. Do not allow the reagent to get into contact with the skin.
2. Avoid contact of all biological materials with skin and mucous membranes.
3. Do not pipette by mouth.
4. Do not eat, drink, smoke, store or prepare foods, or apply cosmetics within the designated work area.
5. TMB is toxic by inhalation, in contact with skin and if swallowed; observe care when handling the substrate.
6. Do not use components past their expiration date and do not intermix components from different serial lots.
7. Each well is ultimately used as an optical cuvette. Therefore, do not touch the under surface of the wells, prevent damage and dirt.
8. All components should be completely dissolved before use. Take special attention to the substrate, which crystallises at 4 C.
9. 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.

REFERENCES

1. 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.
2. Hädrich J., Jarvers J., and Podestàt U. Immunoenzymatischer Nachweis von Acetylgestagenen in Fettgewebe. Validierung des Verfahrens gema DIN EN ISO/IEC 17025 und nach den Vorgaben der Entscheidung (EWG) Nr. 93/256 am Beispiel von 6-Methyl-17-hydroxyprogesteronacetat. Deutsche Lebensmittel-Rundschau: 97, 11, 409-414, 2001.

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