Version 23-04/24

User's Manual

Melengestrolacetat ELISA Kit

REF **DEIASL117**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

The Melengestrolacetat ELISA is a competitive enzyme immunoassay for the quantitative analysis of melengestrol acetate in bovine perirenal fat and muscle meat.

General Description

Synthetic gestagens can be used for estrus inhibition or synchronisation after break off and improvement of fertility. In addition one of these substances, melengestrol acetate (MLGA), is a licensed growth promoting feed additive for heifers in the USA and Canada. The admitted dose is 0.5 mg per day and head, given as a feed premix. Its mode of anabolic action is unclear, but it stimulates the ovarian synthesis of the endogenous anabolic steroid estradiol and that may have androgenic side effects. MLGA belongs to the most active synthetic gestagens. Its oral bioactivity is about 10 or 100 times higher than the activities of the gestagens chlormadinone acetate (CMA) or medroxyprogesterone acetate (MPA). If given parenterally the hormonal activity of MLGA is still 125 times higher than the one of progesterone. Due to its strongly lipophilic properties, MLGA is accumulated in fat 200-fold higher than in blood plasma. In the European Union the use of sexual hormones for growth promoting purposes is generally forbidden since 1988, and also the import of meat from hormone treated cattle into the EU is prohibited.

Principles of Testing

The basis of the test is the antigen-antibody reaction. The microtiter wells are coated with capture antibodies directed against anti-MLGA antibodies. Standards or sample solution, MLGA conjugate and anti-MLGA antibodies are added. Free and enzyme conjugated MLGA compete for the antibody binding sites (competitive enzyme immunoassay). At the same time, the anti-MLGA antibodies are also bound by the immobilized capture antibodies. Any unbound conjugate is then removed in a washing step. Substrate and chromogen are added to the wells and incubated. Bound conjugate converts the colorless chromogen into a blue product. The addition of the stop solution leads to a color change from blue to yellow. The measurement is made photometrically at 450 nm. The absorption is inversely proportional to the melengestrol acetate concentration in the sample.

Reagents And Materials Provided

1. Microtiter plate: 96 wells
2. Standard 1-6: 0 µg/l, 0.15 µg/l, 0.45 µg/l, 1.35 µg/l, 4.05 µg/l, 12.15 µg/l, 1.3mL for each vial.
3. Conjugate Concentrate (11x): 10ml
4. Antibody Concentrate (11x): 0.7ml
5. Conjugate/antibody buffer: 25ml
6. Substrate: 7ml
7. Chromogen: 7ml
8. Stop solution: 14ml

Materials Required But Not Supplied

Equipment:

1. Microtiter plate spectrophotometer (450 nm)
2. Graduated pipettes
3. Variable 20 µl - 200 µl and 200 - 1000 µl micropipettes
4. Refrigerated centrifuge
5. Shaker
6. Freezer (-60°C)
7. Mixer
8. Evaporator
9. Vortex
10. Waterbath

Reagents:

1. petroleum ether
2. C18 column
3. Methanol 100 % (v/v)
4. Methanol 80 % (v/v)
5. Methanol 40 % (v/v)
6. Methanol / 20 mM Tris-HCl (20/80, v/v)
7. dest. Wasser
8. dest. Wasser containing 0.05 % Tween20
9. 67 mM phosphate buffer pH 7.2

methanol/20 mM tris-HCl (20/80, v/v):

dissolve 2.42 g tris-(hydroxymethyl) aminomethane in 700 ml distilled water + 200 ml 100 % methanol, adjust pH 8.5 with 5 M HCl and fill up to 1000 ml with distilled water

67m M phosphate buffer, pH 7.2:

1. 79 g NaH_2PO_4 x H_2O, 9.61 g Na_2HPO4 x 2 H_2O + 8.7 g NaCl, fill up to 1000 ml with distilled water

Storage

Store the kit at 2 - 8 °C. Do not freeze any test kit components.

Return any unused microwells to their original foil bag, reseal them together with the desiccant provided and further store at 2 - 8 °C.

The colorless chromogen is light sensitive, therefore, avoid exposure to direct light.

No quality guarantee is accepted after the expiration date on the kit label.

Do not interchange individual reagents between kits of different lot numbers.

Specimen Collection And Preparation

Bovine perirenal fat

(1) grind the frozen kidney fat by scraping with a scalpel;

(2) weigh 1 g of sample into a 20 ml vial, add 15 ml petroleum ether and shake softly in a waterbath at 40 °C for extraction overnight;

(3) continue with "Sample Purification".

Muscle meat

(1) cut 50 - 300 g of muscle meat into cubes and add the corresponding volume of 67 mM of phosphate buffer (pH 7.2) and homogenize with an ultra turrax (e.g. 100 g muscle meat + 100 ml 67 mM of phosphate buffer);

(2) weigh 8 g of homogenized sample into a 20 ml vial, add 8 ml petroleum ether for extraction and shake softly in a waterbath at 40 °C for extraction overnight;

(3) continue with "Sample Purification".

Sample Purification

(1) continued sample preparation of perirenal fat and muscle meat;

(2) centrifuge: 15 min / 2000 g / - 15 °C (if a refrigerated centrifuge is not available, freeze the sample before centrifugation); (3) decant the petroleum ether supernatant into a new vial and evaporate to dryness under a mild nitrogen or air flow at 60 °C; (4) dissolve the residue in 2 ml methanol and vortex for 20 s (close vial tightly);

(5) for degreasing: freeze 45 min at - 60 °C;

(6) centrifuge: 5 min / 2000 g / - 15 °C;

(7) decant the supernatant into a new vial;

(8) dilute the methanolic supernatant with 5 ml distilled water.

Purify by C18 column as follows (flow rate: 1 drop/s):

(1) rinse column twice with 1 ml 100 % methanol;

(2) equilibrate column twice with 1 ml methanol/20 mM tris-HCl, pH 8.5 (20/80, v/v);

(3) apply diluted sample from 9.3. (approx. 7 ml);

(4) rinse the column twice with 1 ml methanol/20 mM tris-HCl, pH 8.5 (20/80, v/v);

(5) rinse the column twice with 1 ml 40 % methanol;

(6) remove residual fluid by positive pressure or vacuum and dry column for 2 min by floating it with air or nitrogen;

(7) elute sample with 1 ml 80 % methanol into a new vial;

(8) collect residual fluid by applying positive pressure or vacuum (flow rate: 1 drop/s);

(9) dilute the eluate 1:1 (1+1) with distilled water;

(10) employ 20 µl per well in the assay.

Note: Depending on the MLGA concentration in the sample further dilutions may be necessary. In this case use 40 % methanol for all further dilutions of the eluate, because the eluate has to be used in the test in 40 % methanol.

Reagent Preparation

Bring all reagents to room temperature (20 - 25 °C / 68 - 77 °F) before use.

The MLGA conjugate is provided as a concentrate. Since the diluted conjugate has a limited stability, only the amount which actually is needed should be reconstituted. Before pipetting, the conjugate concentrate should be shaken carefully. For reconstitution, the conjugate concentrate is diluted 1:11 (1+10) in buffer (e. g. 200 µl conjugate concentrate + 2 ml conjugate buffer, ready to use sufficient for 4 microtiter strips).

The anti-MLGA antibody solution is provided as a concentrate. Since the diluted antibody has a limited stability, only the amount which actually is needed should be reconstituted. Before pipetting, the antibody concentrate should be shaken thoroughly. For reconstitution, the antibody concentrate is diluted 1:11 (1+10) in buffer (e. g. 200 µl antibody concentrate + 2 ml antibody buffer, ready to use sufficient for 4 microtiter strips).

For the wash solution use distilled water with 0.05 % tween 20 (e. g. 0.5 g tween 20 in 1000 ml of distilled water). Prepare solution freshly for each test series.

Assay Procedure

Carefully follow the recommended washing procedure. Do not allow microwells to dry between working steps.

1. Insert a sufficient number of wells into the microwell holder for all standards and samples to be run in duplicate. Record standard and sample positions.
2. Add 20 µl of each standard or prepared sample to separate duplicate wells.
3. Add 50 µl of diluted conjugate to each well.
4. Add 50 µl of the diluted anti-MLGA antibody to each well. Mix gently by shaking the plate manually and incubate 2 h at room temperature 20 - 25 °C.
5. Pour the liquid out of the wells and tap the microwell holder upside down vigorously (three times in a row) against absorbent paper to ensure complete removal of liquid from the wells. Fill all wells with 250 µl of wash solution (see 10.1.) and pour out the liquid again. Repeat two more times.
6. Add 50 µl of substrate and 50 µl of chromogen to each well. Mix gently by shaking the plate manually and incubate for 30 min at room temperature (20 - 25 °C) in the dark.
7. Add 100 µl of the stop solution to each well. Mix gently by shaking the plate manually and measure the absorbance at 450 nm. Read within 30 minutes after addition of stop solution.

Calculation

The course of the standard curve is shown in the Quality Assurance Certificate, enclosed in the test kit. Remark for the calculation without software:

$$\frac{\text{absorbance standard (or sample)}}{\text{absorbance zero standard}} \times 100 = B/B_0\ (\%)$$

The zero standard is thus made equal to 100 % and the absorbance values are quoted in percentages. The values calculated for the standards are entered in a system of coordinates on semilogarithmic graph paper against the MLGA concentration [µg/kg].

In order to obtain the MLGA concentration in µg/kg (ppb) actually contained in a sample, the concentration read from the standard curve must be further multiplied by the corresponding dilution factor. When working in accordance with the regulation stated, the dilution factors are as follows:

bovine perirenal fat.....2

muscle meat 0.5

Specificity

The specificity of the Melengestrolacetat-test was determined by analyzing the cross-reactivities to corresponding substances in buffer system. In samples, the specificity may deviate from those determined in the buffer system due to matrix effects. Prior to the analysis of cross-reactive substances, the user has to determine the Limit of Detection and the Recovery for the substance in the respective sample matrix. The test cannot discriminate between analytes and cross-reactive substances.

Melengestrol acetate (MLGA)..... 100 %

Megestrol acetate (MGA) ..... 10 %

Medroxyprogesterone acetate (MPA) ..... 6.6 %

6- Methyl-17-OH-Progesterone acetate..... 5.2 %

Chlormadinone acetate (CMA)..... 4.8 %

17- Acetoxyprogesterone ..... 3.9 %

16- Methylprogesterone ..... 0.0035 %

all other tested substances..... 0.003 %

Precautions

This test should only be carried out by trained laboratory employees. The instruction for use must be strictly followed.