

**United States Department of Agriculture  
Food Safety and Inspection Service, Office of Public Health Science**

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| Title: Screen for Melengestrol Acetate in Fat using ELISA |              |                       |
| Revision: 01  | Replaces: 00 | Effective: 02/01/2006 |

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**A. INTRODUCTION**

Melengestrol Acetate (MGA) is a licensed growth promoting feed additive that suppresses the estrus cycle in heifers. Residues accumulate preferentially in fatty tissues.

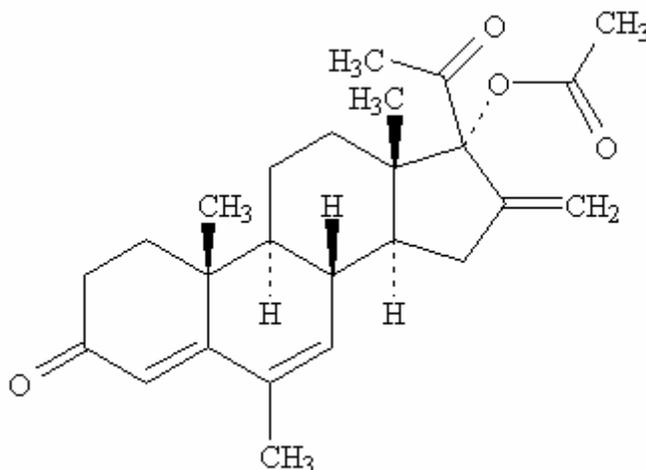
1. Theory

Rendered sample fat is dissolved in petroleum ether. This solution is chilled and centrifuged to separate congealed lipid materials. The supernate is decanted, evaporated to dryness, and the residue redissolved in MeOH. This solution is also chilled and centrifuged to precipitate residual lipids. The supernate is decanted, diluted with water, and further cleaned up on a RIDA<sup>®</sup> C18 column. The methanolic eluate is diluted with water and analyzed using a RIDASCREEN<sup>®</sup> MGA ELISA. See appendix (K.2) for an explanation of ELISA operating principles.

2. Applicability

This method will detect Melengestrol Acetate in rendered bovine fat at levels  $\geq 10$  ppb.

3. Structure



**B. EQUIPMENT**

Note: Equivalent equipment may be substituted for all but MGA ELISA kit (B.1.a).

1. Apparatus

- a. RIDASCREEN<sup>®</sup> MGA ELISA Kit - r-Biopharm No. R6502.
- b. Freezer (-60 °C) - Model No. SLT-25V-85A37, Kendro Lab Products.

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- c. Water bath - Techne Tecam Model No. UBI.
  - d. Refrigerated centrifuge - IEC, Model No. PR-7000, rotor #269, test tube holder Cat. No. 320A, test tube adaptor Cat. No. 1106.
  - e. Variable Volume Micropipettes - 2-20  $\mu$ L, 10-100  $\mu$ L, and 100-1000  $\mu$ L, 30-300  $\mu$ L multi-channel, Brinkman Instruments, Inc.
  - f. RIDA<sup>®</sup> C18 columns - r-Biopharm No. R2002. Optional: high volume adaptor kit, Varian, Cat. No. 2131001 (any appropriate reservoir may be used with adaptor kit i.e. a 5 mL disposable syringe with plunger removed).
  - g. SPE manifold - Waters Cat. No. WAT200677.
  - h. 50 mL glass centrifuge tubes - Pyrex No. 8422, or 50 mL polypropylene conical (Falcon<sup>™</sup>) tubes – Fisher Cat. No. 06-443-19.
  - i. 15 mL disposable glass centrifuge tubes - Kimble No. 73785-15.
  - j. Screw on caps for 15 mL glass centrifuge tubes – Fisher Cat. No. 05-569-5.
  - k. 15 mL disposable Falcon Tubes – Fisher Cat. No. 14-959-49B.
  - l. Test tubes - 16 x 100 mm, Kimble No. 73500 16100.
  - m. Graduated cylinders - Class A:
    - 50 mL - Fisherbrand Cat. No. 08-557C.
    - 500 mL - Fisherbrand Cat. No. 08-557F.
    - 1000 mL - Pyrex Cat. No. 3062.
  - n. Volumetric flasks - Amber, Class A, 10 mL, Pyrex Cat. No. 55640.
  - o. Balance - Mettler Model No. PJ3600 DeltaRange.
  - p. Balance - Sartorius Model No. AC 121S.
  - q. Solvent Evaporator - Meyer N-EVAP, Organomation.
  - r. Parafilm<sup>®</sup> M - use to cover/seal tubes, ELISA wells.
2. Instrumentation
- Microtiter plate spectrophotometer - BioTek Model No. ELx 808, 450 nm filter.

**C. REAGENTS AND SOLUTIONS**

Note: An equivalent reagent or solution may be substituted.

- 1. Reagents -
  - a. Tween<sup>™</sup> 20 - Fisher Cat. No BP337-100.
  - b. Methanol (MeOH) – 100%, HPLC Grade, Fisher Cat. No. A452-4.
  - c. Petroleum Ether - J.T. Baker Cat. No. 9268-22.

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- d. Tris-(hydroxymethyl)aminomethane (Tris) - Kodak Cat. No. 1174945.
- e. Provided with RIDASCREEN® ELISA:
  - i. MGA enzyme conjugate (bottle with red cap).
  - ii. anti-MGA antibody (bottle with black cap).

2. Solutions

Note: Distilled water or Milli-Q water may be used for the following solutions.

- a. 20/80 (v/v) MeOH/20 mM Tris-HCl, pH 8.5:  
Dissolve 2.42 g Tris-(hydroxymethyl)aminomethane in approx. 700 mL distilled water and 200 mL MeOH (100 %). Adjust to pH 8.5 with approx. 5 M HCl; then fill up to 1000 mL with distilled water. Solution is stable for 3 months when stored at 2 - 8 °C.
- b. 40/60 (v/v) MeOH/Water:  
Add 400 mL MeOH to 600 mL distilled water. Solution is stable for 3 months when stored at 2 - 8 °C.
- c. 80/20 (v/v) MeOH/Water:  
Add 800 mL MeOH to 200 mL distilled water. Solution is stable for 3 months when stored at 2 - 8 °C.
- d. MGA Enzyme Conjugate Working Solution:  
Dilute MGA enzyme conjugate 1+10 with kit buffer (e. g. 200 µL conjugate concentrate + 2 mL buffer, sufficient for 4 microtiter strips).  
Note: Before pipetting, the conjugate should be shaken carefully. Prepare only the amount needed fresh each day.
- e. Anti-MGA antibody Working Solution:  
Dilute Anti-MGA antibody 1+10 with kit buffer (e. g. 200 µL conjugate concentrate + 2 mL buffer, sufficient for 4 microtiter strips).  
Note: Before pipetting, the antibody should be shaken carefully. Prepare only the amount needed fresh each day.
- f. Wash Solution - 0.05% Tween 20 (e.g. 0.25 g Tween 20 to 500 mL distilled water). Prepare fresh each day.

**D. STANDARDS**

Note: MGA can degrade under UV light. All MGA solutions should be prepared in amber volumetric flasks and stored in amber vials at 2 - 8 °C.

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1. Source

Reference Standard: Melengestrol Acetate 250 mg, Cat. No. 0215895283, MP Biomedicals. Store at room temperature.

2. MGA Standard Solutions

Note: The analyst may prepare different volumes or concentrations, provided the fortification amounts and standard curve concentrations remain the same. The stock solution must be adjusted for purity during preparation.

a. Stock Standard 1 (1.0 mg/mL):

Add 10.0 ± 0.1 mg of MGA reference standard (adjusted for purity) to a 10 mL volumetric flask and dilute to volume with MeOH.

b. Stock Standard 2 (10 µg/mL):

Add 100 µL of Stock Standard 1 to a 10 mL volumetric flask and dilute to volume with MeOH.

c. Fortification Solution (250 ng/mL) MGA:

Add 250 µL of Stock Standard 2 to a 10 mL volumetric flask and dilute to volume with MeOH.

d. Reference Stock Solution 1 (10 µg/mL):

Add 100 µL of Stock Standard 1 to a 10 mL volumetric flask and dilute to volume with 40/60 MeOH/water.

e. Reference Stock Solution 2 (100 ng/mL):

Add 100 µL of Reference Stock Solution 1 to a 10 mL volumetric flask and dilute to volume with 40/60 MeOH/water.

f. Reference Standard 3 (4 ng/mL):

Add 0.40 mL of Reference Stock Solution 2 to a 10 mL volumetric flask and dilute to volume with 40/60 MeOH/water.

g. Reference Standard 2 (2 ng/mL):

Add 0.20 mL of Reference Stock Solution 2 to a 10 mL volumetric flask and dilute to volume with 40/60 MeOH/water.

h. Reference Standard 1 (1 ng/mL):

Add 0.10 mL of Reference Stock Solution 2 to a 10 mL volumetric flask and dilute to volume with 40/60 MeOH/water.

i. Reference Standard 0 (0 ng/mL).

This is 40/60 MeOH/water.

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3. Storage and Stability

All stock and reference and fortification solutions are stable for 1 month when stored at 2-8 °C.

**E. SAMPLE PREPARATION**

Render fat in a heat safe container in an oven at 95 – 110 °C.

**F. ANALYTICAL PROCEDURE**

Note: MGA can degrade under UV light. Carry out extraction and analysis steps away from direct sunlight or other sources of strong UV light.

1. Extraction and Cleanup

- a. Weigh  $1.0 \pm 0.1$  of liquefied rendered fat into a 50 mL centrifuge tube. While fat is still warm, add 15 mL petroleum ether, vortex, cap and shake gently until fat is completely dissolved.

Note: Prepare positive controls at this time. Fortify six known blank fat samples at 10 ppb using 40  $\mu$ L of 250 ng/mL Fortification solution..

- b. Place tube in refrigerated centrifuge (-15 °C) and spin 15 minutes at 2000 - 3000 g. to congeal and separate lipids. Adjust centrifuge speed as needed to obtain a clear supernate.
- c. Decant the petroleum ether supernate into a new 15 mL disposable glass or polypropylene (Falcon™) centrifuge tube and evaporate it in a  $\leq 60$  °C water bath under a gentle stream of dry air/nitrogen. Note: Do not exceed 60 °C temperature. Solvent may begin to boil at higher temperatures which may result in loss of analyte.
- d. Redissolve the residue in 2 mL MeOH and vortex, on high, for approximately 1 minute. Cap tube tightly.
- e. Freeze vial contents for 45 minutes at a  $\leq -60$  °C to precipitate residual lipids.
- f. Place tube in refrigerated centrifuge (-15 °C) and spin 5 minutes at. 2000 - 3000g (Adjust centrifuge speed as needed to obtain a clear supernate).
- g. Decant the supernate into a new 15 mL disposable Falcon™ tube and dilute with 5 mL distilled water.

Note: Flow rate for all steps below should be approximately 1 drop per sec.

- h. Place RIDA® C18 column on SPE manifold.
- i. Rinse column twice with 1 mL MeOH.
- j. Equilibrate column twice with 1 successive 1 mL portions of 20/80 MeOH/20 mM Tris-HCl, pH 8.5.

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- k. Apply diluted sample (approx. 7 mL).
- l. Rinse the column twice with 1 mL 20/80 MeOH/20 mM Tris-HCl, pH 8.5.
- m. Rinse the column twice with 1 mL 40/60 MeOH/ distilled water.
- n. Remove fluid residues by applying positive pressure or vacuum. Continue application for 2 minutes after last drop of liquid has eluted to dry the column. Discard all drained liquids.
- o. Elute sample with 1 mL 80/20 MeOH/distilled water into a new 16 x 100 mm collection tube, applying pressure or vacuum to ensure a flow rate of 1 drop/sec. Continue until no liquid drains from the column.

*Stopping point!* Eluate may be stored overnight at 2 - 8 °C in sealed or parafilm covered tube.

## 2. ELISA

Note: Bring all ELISA reagents to room temperature (20 - 25 °C) before use. Refrigerate all reagents immediately after use. Do not allow microwells to dry between working steps.

- a. Dilute the eluate 1:1 with distilled water to get a 40/60 (v/v) MeOH/distilled water solution.

Note: Depending on the MGA concentration in the sample further dilutions may be necessary. In this case use MeOH/distilled water [40/60 (v/v)] for all further dilutions (i.e. always maintain 40% MeOH).

- b. Remove microtiter plate from kit bag with appropriate number of strips to be used.

Note: Keep those wells not required together with the drying agent, well sealed in the foil bag and stored at 2 - 8 °C.

- c. Add 20 µL aliquots of each kit standard solution (0, 1.35, 4.05, 12.15 ng/mL) to duplicate wells. Note: Reference standards (0, 1, 2, 4 ng/mL) may be used in place of kit standards. Controls and samples may be placed in single or duplicate wells as appropriate.
- d. Add 50 µL of diluted enzyme conjugate to each well.
- e. Add 50 µL of the diluted anti-MGA antibody solution to each well. Mix gently by rocking the plate manually and incubate, covered, for 2 hours at room temperature out of direct sunlight.
- f. 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, pour out the liquid and tap the plate again. Repeat wash two more times. Important!: Reproducibility in any ELISA is largely dependent upon the consistency with

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which the microwells are washed. Follow wash instructions carefully.

- g. Add 50  $\mu$ L of substrate and 50  $\mu$ L of chromogen to each well. Mix gently by rocking the plate manually and incubate, covered, for 30 minutes at room temperature in the dark.
- h. Add 100  $\mu$ L of the stop solution to each well. Mix gently by rocking the plate manually and measure the absorbance at 450 nm. (If reference wavelength is used to correct readings, set reference to > 600 nm). Read wells within 60 minutes after addition of stop solution.

**G. CALCULATIONS**

1. Evaluate ELISA and method controls. Results may be reported only if:
  - a. The ELISA appears to be working properly based on analysis of the standard curve. (When concentrations of reference standards are plotted on a log scale x-axis against absorbance, a linear relationship is evident.)
  - b. The positive controls are screen positive.
2. Calculate the mean and standard deviation (SD) for the absorbance readings of the 10-ppb fortified controls included in the sample set.
3. Use the calculated mean for the 10-ppb fortified samples and the SD to determine a 95% prediction interval for an absorbance corresponding to 10 ppb. Set the cutoff limit as follows:

Cutoff = Mean + K x SD, where

K = 2.78 (applicable multiplier when 6 10-ppb fortified samples are used.)

4. Compare readings taken for all samples against the calculated cutoff value.
5. A sample run using a single aliquot is screen positive if the absorbance reading < calculated cut-off value. A sample run using duplicate aliquots is screen positive if it meets one of the following conditions:
  - a. Both absorbance readings are < calculated cutoff value.
  - b. One reading is < calculated cutoff value and the average of both readings is < cutoff and the relative difference between readings is less than 10%.

Note: If one well has an absorbance greater than the cutoff value and the relative difference between duplicate wells is greater than 10%, re-analyze the sample.

**H. SAFETY INFORMATION AND PRECAUTIONS**

1. Required Protective Equipment - Safety glasses, laboratory coat.

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2. Hazards

| <i>Procedure Step</i>                   | <i>Hazard</i>  | <i>Recommended Safe Procedures</i>   |
|---|--|--|
| Acid – HCl, Nitric Acid (stop solution) | Can cause burns on skin and eye injury. Corrosive.                                       | Wear gloves and protective clothing when handling.   |
| Organic Solvents: Petroleum Ether, MeOH | Flammable liquid and vapor, can cause eye, nose, and upper respiratory tract irritation. | Use in a fume hood, away from all open flames and electrical devices. Wear gloves when handling. |
| MGA                                     | May cause skin allergies or dermatitis   | Wear gloves and protective clothing when handling.   |

3. Disposal Procedures

| <i>Procedure Step</i>                   | <i>Hazard</i> | <i>Recommended Safe Procedures</i>   |
|---|---------------|--|
| Acids                                   | See Above     | Neutralize the acid for disposal down the drain in accordance with local, state and federal regulations.   |
| Organic Solvents: Petroleum Ether, MeOH | See Above     | Collect waste in tightly sealed container and store away from non-compatibles in a cool, well ventilated, flammable liquid storage area/cabinet for disposal in accordance with local, state, and federal regulations. |

**I. QUALITY ASSURANCE PLAN**

1. ELISA Kit Suitability

Using the kit standards 0.0 ppb, 1.35 ppb, 4.05 ppb, 12.15 ppb, plot concentration vs. absorbance and compare with in-house standards 0.0 ppb, 1, 2 and 4 ppb. If results show linearity the entire lot of ELISA kits is acceptable for use.

2. Performance Standard

| <i>Analyte</i> | <i>Analytical Range</i> | <i>Acceptable Condition</i>                  |
|----------------|-------------------------|--|
| MGA            | ≥ 10 ppb                | 10 ppb fortified control is screen positive. |

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3. Readiness To Perform (FSIS Training Plan)
  - a. Familiarization
    - i. Phase I: Standards- Analyze duplicate standard curve using kit standards 0.0 ppb, 1.35 ppb, 4.05 ppb, 12.15 ppb on 3 days.
    - ii. Phase II: Fortified samples- Analyze a minimum of 3 replicates at 0 (tissue blank) and 10 ppb over a period of 3 different days.  
NOTE: Phase I and Phase II may be performed concurrently.
    - iii. Phase III: Check samples for analyst accreditation.
      - (a) Using the full method, analyze a minimum of 6 samples that are blind to the analyst. At least one, but no more than two of the samples may be blanks. The rest are to be fortified at 10 ppb. Only known blank tissue may be used for this trial.
      - (b) Report analytical findings to QAM.
      - (c) Letter from QAM is required to commence official analysis.
  - b. Acceptability criteria.  
Refer to I.2.
4. Intralaboratory Check Samples
  - a. System, minimum contents.
    - i. Frequency: One per week when analyses are being conducted.
    - ii. Records are to be maintained
  - b. Acceptability criteria.  
Refer to I.2  
If unacceptable values are obtained, then:
    - i. Stop all official analyses by that analyst.
    - ii. Take corrective action.
5. Sample Acceptability and Stability
  - a. Keep samples frozen before use.
  - b. Sample storage:
    - i. Time: 1 month.
    - ii. Condition: < -20 °C.

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6. Sample Set

- a. At least 6 10-ppb fortified tissue controls
- b. At least 1 unfortified blank tissue control
- c. Samples

7. Sensitivity

- a. Minimum proficiency level (MPL): 10 ppb in sample tissues.

**J. WORKSHEET**

[RESERVED]

**K. APPENDIX**

1. Reference

With a few minor exceptions, the method as written has been taken directly from the R-Biopharm kit instructions for Melengestrol Acetate. RIDASCREEN® Melengestrol acetate 01-11-26, R-Biopharm, USA.

2. ELISA Operating Principles

The following explanation is based on Kit documentation (see Reference):

The basis of this test is the antigen-antibody reaction. The microtiter wells are coated with capture antibodies directed against anti-MLGA antibodies. Standards or sample solution, MLGA enzyme 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 enzyme conjugate is then removed in a washing step. Enzyme substrate (urea peroxide) and chromogen (tetramethylbenzidine) are added to the wells and incubated. Bound enzyme 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 (optional reference wavelength > 600 nm). Absorbance is inversely proportional to the melengestrol acetate concentration in the sample.

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**L. APPROVALS AND AUTHORITIES**

1. Approved by:

In Suk Kim

David Martin

Jess Rajan

Phyllis Sparling

\*Charles Pixley

*Approval signatures on file.*

2. \*Issuing Authority: Laboratory Quality Assurance Division (LQAD)