

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

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Title: ELISA Screening for Clenbuterol Residue in Animal Retinal Tissue		
Revision: .00	Replaces: NA	Effective: 10/25/02

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**A. INTRODUCTION**

1. Theory

This qualitative method for the rapid screening of clenbuterol in animal retinal tissue uses a simple one-step extraction procedure. The choroid/pigmented retinal epithelium (choroid/PRE) is mixed with a phosphate buffer and mashed using a glass rod. The tissue extract is centrifuged at high speed to produce a clear supernatant. The supernatant, accompanied by a clenbuterol standard series as well as fortified tissue extracts, is then analyzed using the enzyme linked immuno-assay kit, which defines analyte concentration by color intensity. The autoreader quantifies the color intensity at 650 nm.

2. Applicability

This ELISA method is applicable to bovine, ovine, porcine, and caprine eyeballs. The Generic Bronchodilator kit is sensitive to at least the following four beta-agonists: terbutaline, clenbuterol, salbutamol/albuterol, and metaproterenol at levels  $\geq 3$  ppb.

3. Reference

Susan B. Clark, W. Douglas Rowe, and Jeffrey A. Hurlbut, FDA Laboratory Information Bulletin #43, July 1996.

**B. EQUIPMENT**

1. Apparatus

- a. Test Kit. ELISA Generic Bronchodilator Kit Neogen Corporation, ELISA Technologies Division, 628 E. 3rd Street, Lexington, Kentucky 40505 (CAT #100310, ELISA Technologies Div.)
- b. Plate Reader. Biotek Autoreader EL 311, with printer (650 nm filter used) ELISA Technologies.
- c. Centrifuge. International Equipment Company B-22M Superspeed Refrigerated Centrifuge with Rotor #876, (Refrigeration is not required) International Equipment Company, Clinical Centrifuge (CAT #20671-007, VWR Scientific, San Francisco, CA.)
- d. pH meter. Orion 601A, Calibrated at pH 4 and 7.
- e. Eppendorf pipettors. Variable volume, 5 $\mu$ L to 1000  $\mu$ L, Brinkmann Instruments, Inc., Westbury, NY 11590.

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- f. Transferpette, Multichannel Pipettes, Brinkmann, 50-200 µL #50-08-030-7 (CAT #53512-376 VWR Box 39396 Denver, CO 80239).
- g. Volumetric Glassware: 100 mL and 10 mL amber flasks Class A; 1.0 mL and 0.5 mL glass pipettes Class A.
- h. Glassware. 50 mL polyallomer tube with polypropylene screw closure (CAT #3139-0050, Nalge Company, Rochester NY 14602-0365.) Glass rods, 10 mm in diameter by 25 cm, fired and rounded at both ends. Disposable culture tubes, Borex, 12 X 75 mm.
- i. Razor blades. Single edge safety razor. American Safety Razor Co., Verona, VA 24482. Alternative choice: scalpel.
- j. Petri dish, disposable polystyrene. Optilux 100 X 20 mm style (CAT #1005, Becton Dickinson Labware, 2 Bridgewater Lane, Lincoln Park, NJ 07035).
- k. N-Dex Nitrile Gloves. LabSource Inc. (CAT # G59-200).

NOTE: Equivalent apparatus may be substituted for the above items.

**C. REAGENTS AND SOLUTIONS**

- a. Sodium Phosphate, dibasic, anhydrous.  $\text{Na}_2\text{HPO}_4$ , Aldrich Chemical Company, Inc., Milwaukee WI 53233
- b. Potassium Phosphate, Monobasic crystals.  $\text{KH}_2\text{PO}_4$ . Mallinckrodt
- c. Extraction Buffer. Weigh 13.6 g of potassium phosphate - monobasic, and 14.2 g of sodium phosphate, dibasic into a 1 L class A volumetric flask or graduated cylinder. Dilute to volume with deionized water. Adjust pH to 6.8 with 1M HCl or 1M NaOH solutions. Prepare extraction buffer fresh when it becomes cloudy. The pH should be checked periodically to verify that it is 6.8.
- d. Deionized water.
- e. Methanol. HPLC grade, Fisher Scientific
- f. 1M HCL. Fisher Scientific
- g. 1M NaOH. Fisher Scientific

NOTE: Equivalent reagents and solutions may be substituted for the above items.

**D. STANDARDS**

- 1. Source

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- a. Clenbuterol HCl (CLEN), Sigma Chemical Co., P.O. BOX 14508 St. Louis MO 63178 (CAT#C-5423) approximately 95% pure or equivalent.

2. Preparation of Standards

- a. Stock CLEN Standard Solution. 25 µg/mL in methanol. Accurately weigh  $2.5 \pm 0.1$  mg clenbuterol standard into a 100 mL amber volumetric flask. Dissolve and bring to volume with methanol. Stable for two months.
- b. Intermediate CLEN Standard Solution. 250 ng/mL in water. Pipet 1.00 mL  $\pm 0.01$  mL CLEN stock standard into a 100 mL amber volumetric flask and bring to volume with water. Stable for two months.
- c. Working CLEN Standard Solution 12.5 ng/mL in Extraction buffer. Prepare daily. Pipet 0.50 mL  $\pm 0.01$  mL CLEN Intermediate Standard Solution into a 10 mL volumetric flask and bring to volume with Extraction Buffer .

Plate standard series: Into individual 12 X 75mm test tubes containing 0.4mL , pipet 100µL, 50µL, 25µL, 10µL, and 5µL of Working CLEN Standard Solution. Vortex to mix.

The standard series – 2.5ng/mL, 1.4 ng/mL, 0.7 ng/ml, 0.3 ng/mL, 0.2 ng/mL CLEN—EIA buffer (found in the test kit) – are applied (0.02 mL) to the ELISA plate.

3. Storage conditions

Store Stock and Intermediate standard solutions in the refrigerator. Working CLEN standard solutions should be kept at room temperature until use.

4. Shelf Life Stability

- a. Stock and intermediate standards are stable for 2 months
- b. Working CLEN solution must be prepared fresh daily.

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**E. SAMPLE PREPARATION**

- a. Eyeballs should be thawed at room temperature just enough so that the outside tissues can be manipulated but the aqueous and vitreous humors are still frozen. This takes about 45 to 60 minutes. The eyeball will not deform any noticeable amount when squeezed.
- b. With a razor blade, incise the eyeball by slowly cutting across the cornea horizontally and vertically. The length of the incisions should include the full width and length of the cornea, and extend into the sclera to allow the eyeball to be everted. Force the semi-frozen contents (aqueous and vitreous humors and lens) out of the eyeball and retain in a separate whirl-pak bag. (Eye contents may be discarded if retinal results are negative for clenbuterol.)
- c. Evert the eyeball, and scrape the choroid/PRE layer (distinctive in bovine, ovine, and caprine due to bluish-green coloration on black) and neural retina (black filmy tissue emanating from the optic nerve area) into a petri dish. With a new razor blade, mince the tissue into fine pieces prior to weighing.

NOTE: The retina is composed of ten layers. The innermost nine layers are called the neural retina, which is unpigmented. The tenth layer of the retina is the highly pigmented epithelium and is intimately connected to the choroid, which is a pigmented, vascular coat of the eye. "Detached retina" refers to a condition in which the neural-retina (inner nine layers) is separated from the pigmented epithelium and, upon dissecting the eye, is found as a dull, gray, fold of tissue, approximately 30-50 mm in length, attached only at the optic disc (depressed area where optic nerve attaches to the back of the eye.) The everted eyeball is smooth and darkly pigmented with an iridescent metallic sheen (tapetum lucidum). This layer is the PRE with the adjacent choroid.

Everted porcine eyes do not display the greenish-blue metallic sheen. Typical choroid/PRE yields for bovine, ovine, caprine, and porcine are: 1.5 g, 0.8 g, 0.8 g, and 0.2 g, respectively.

**F. ANALYTICAL PROCEDURE**

- a. Weigh 0.1 g minced bovine, ovine, caprine, or porcine retinal tissue into a 50 mL polyallomer centrifuge tube. (When retinal tissue from a porcine eye is limited, weigh 0.05 g retinal tissue into a 50 mL centrifuge and add 0.2 mL Extraction buffer.)
- b. To fortify the 0.1 g daily retinal control tissue add:
  - i. Fortification Standards (1.5 ng/mL, and 0.75 ng/mL)

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Pipet 0.15 mL, and 0.075 mL of the intermediate CLEN standard solution into individual 25.0 mL volumetric flasks. Dilute to volume with Extraction Buffer.

- ii. One blank sample spiked by the analyst at 3 ppb must be distributed randomly into at least 6 wells.
  - c. Add 0.40 mL  $\pm$  0.01 mL Extraction Buffer to centrifuge tube.
  - d. Vigorously mash retinal tissue with glass rod for one minute.
  - e. Cap and centrifuge tube at 15,000 RPM for 15 minutes.
  - f. Pour supernatant into a 12 X 75 mm test tube.
  - g. Aliquot 0.020 mL supernatant into duplicate wells of the Generic Bronchodilator ELISA plate.
  - h. Following ELISA kit directions, add 180  $\mu$ L diluted Terbutalene-HRP Conjugate Solution (HRP) to each well. The HRP solution and EIA buffers are supplied in the test kit and are diluted 1:180 HRP:EIA. After addition of the HRP:EIA solution to each sample, mix the solutions by gently vibrating the plate on a flat surface.
  - i. Cover plate to avoid possible dust/dirt contamination. Incubate for one hour at room temperature. Shake gently at least twice during the incubation period.
  - j. Invert the plate after the incubation period, to remove matrix solutions.
  - k. Wash the wells four times with 300  $\mu$ L each of diluted washing buffer (diluted according to kit instructions supplied in the test kit). Tamp the inverted plate on a paper towel.
  - l. Add 150  $\mu$ L of the K-Blue substrate supplied in the test kit to each well. Allow the reaction to proceed for 15 minutes with intermittent gentle shaking of plate, especially before taking an absorbance reading.
  - m. Read results at 650nm. Continue to take readings every five minutes until an optimum absorbance reading (although the absorbance values continue to increase, "optimum" is considered to be between 1.0 and 2.0) is obtained for the control tissue (read against air.) Color development may take 20 to 30 minutes.
5. Instrumental Settings —
- Read plate at 650 nm.

**G. CALCULATIONS**

Note: Since the ELISA antibody integrates its response over the entire spectrum of

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compounds that it recognizes, it is not possible to determine the identity or actual concentrations of the beta-agonists present in a positive sample. All positives are identified as ppb Clenbuterol.

Evaluate sample results based on absorbance values for fortified control tissues. For this ELISA plate, clenbuterol concentration is related to intensities of the color blue. (The highest concentration of clenbuterol after 30 minutes is a very pale light blue-whereas the reagent blank is a deep blue color).

Calculate the mean and standard deviation (SD) for the absorbance readings of the six 3 ppb spiked replicates. Use these to calculate a decision level using the formula  $DL = \text{Mean} + 3 \cdot SD$ . A sample will be identified as positive if its absorbance is less than the decision level.

The CV calculated for the six 3 ppb spiked replicates is  $\leq 20\%$ .

**H. HAZARD ANALYSIS**

1. Method Title — ELISA Screening for Clenbuterol Residue in Animal Retinal Tissue.
2. Required Protective Equipment — Lab coat, safety glasses. Gloves should be worn when working with the eyeballs.
3. Hazards

<i>Procedure Step</i>	<i>Hazard</i>	<i>Recommended Safe Procedures</i>
Methanol	This solvent may be flammable and may produce toxic effects to skin, eyes and the respiratory system.	Use reagents in an efficient fume hood away from all electrical devices and open flames.

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4. Disposal Procedures

<i>Procedure Step</i>	<i>Hazard</i>	<i>Recommended Safe Procedures</i>
Methanol	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. Performance Standard

a. A plate should meet all the following criteria:

- i. The control tissue absorbance should be between 1.0 and 2.0 absorbance units vs. air,
- ii. The standard curve has absorbencies continuously increasing from the 2.5ng/mL through each lower concentration standard through the EIA buffer,
- iii. The absorbencies continuously increase from the 3 ppb to the 0 ppb fortified control tissue wells, and,
- iv. A variability of less than  $\pm 25\%$  between duplicate sample wells is obtained. Determine acceptable variability by using the following:

$$\frac{\text{Larger Absorbance Value}}{\text{Smaller Absorbance Value}} \leq 1.25$$

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2. Critical Control Points and Specifications

<i><b>Record</b></i>	<i><b>Acceptable Control</b></i>
a. Variability between duplicate wells.	± 25%
b. Time of retinal tissue contact with extraction solution.	At least 10 minutes.
c. After applying aliquots to the ELISA plate the plate is devoid of bubble and liquid prior to the addition of the K-blue solution.	Evacuate plate of all liquid or bubbles prior to the addition of the K-blue solution.

3. Readiness To Perform

- a. Analyst Training
- i. Phase I: Standards- Duplicate external standard curves on each of 3 consecutive days, which will include the following:
    - (a) Blank
    - (b) 0.2 ng/mL
    - (c) 0.3 ng/mL
    - (d) 0.7 ng/mL
    - (e) 1.4 ng/mL
    - (f) 2.5 ng/mL
  - ii. Phase II: Fortified samples- 3 replicates at 0, 3.0, and 6.0 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) 30 unknown beef eyeball samples. The sample fortifications

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including the number of blanks are to be blind to the analyst. 10-15 of the 30 should be blank and the rest spiked at 3 ppb. All samples should be put in duplicate wells. The samples must be randomized throughout the set. An external curve must be run to help monitor plate acceptability.

- (b) Report analytical findings to the Laboratory Quality Assurance Manager (QAM).
    - (c) Letter from QAM is required to commence official analysis.
  - b. Acceptability criteria.
    - i. No false negatives at the 3 ppb level.
    - ii. Refer to section I.1 above.
- 4. Intralaboratory Check Samples
  - a. System, minimum contents.
    - i. Frequency: At least 1 weekly per analyst if samples analyzed.
    - ii. Records are to be maintained by the analyst and reviewed by the supervisor and Laboratory QAM for:
      - (a) Positive or negative results for QA samples.
  - b. Acceptability criteria.

If unacceptable values are obtained, then:

    - i. Stop all official analyses by that analyst.
    - ii. Take corrective action.
- 5. Sample Acceptability and Stability
  - a. Matrix: Eyeball
  - b. Sample receipt size:
  - c. Sample receipt condition: Frozen
  - d. Sample storage:
    - i. Time: Eyeballs should be stored at - 20 °C or lower. They can be kept

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for one year. Extracted retinal tissue may be stored for one week at  
- 40°C or lower (longer tends to dry the tissue).

6. Sample Set
  - a. Each sample set must contain:
    - i. Blank
    - ii. 3 Fortified controls. (Two at 3 ppb and 1 at 6 ppb.)
    - iii. Samples
  
7. Sensitivity
  - a. Lowest detectable level (LDL): 3 ppb.
  - b. Minimum proficiency level (MPL): 3 ppb.

**J. WORKSHEET**

An example of a worksheet on the following page can be removed from this book for photocopying.

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**BETA-AGONIST WORKSHEET  
(Clenbuterol – Bovine Eyes)**

DATE: \_\_\_\_\_  
SUPERVISOR REVIEWED BY: \_\_\_\_\_

ANALYST: \_\_\_\_\_  
REVIEWED BY: \_\_\_\_\_  
INSTRUMENTS AND

SETTING

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

PLATE READER: \_\_\_\_\_  
 PLATE READER FILTER: \_\_\_\_\_  
 PLATE READER MODE: ABS1 \_\_\_\_\_  
 CENTRIFUGE: \_\_\_\_\_  
 CENTRIFUGE SPEED: \_\_\_\_\_  
 CENTRIFUGE TIME: \_\_\_\_\_  
 CENTRIFUGE TEMP: \_\_\_\_\_  
 CENTRIFUGE ROTOR: \_\_\_\_\_  
 PIPETTE: \_\_\_\_\_  
 FREEZER: \_\_\_\_\_  
 BALANCE: \_\_\_\_\_  
 pH METER: \_\_\_\_\_  
 REFRIGERATOR: \_\_\_\_\_

STANDARDS AND REAGENTS

BETA-AGONIST STOCK: \_\_\_\_\_  
 BETA-AGONIST INTERMEDIATE: \_\_\_\_\_  
 BETA-AGONIST WORKING SOL: \_\_\_\_\_  
 BETA-AGONIST SPIKING SOL: 0.75 ng/ml  
 BETA-AGONIST SIKING SOL: 1.5 ng/ml  
 BETA-AGONIST SPIKING SOL: 3 ng/ml

	CLEN	SAL	CIM

STANDARDS

	CLEN	SAL	CIM
0.2 ng/ml			
0.3 ng/ml			
0.7 ng/ml			
1.4 ng/ml			
2.5 ng/ml			

PHOSPHATE BUFFER: \_\_\_\_\_  
 WASH BUFFER: \_\_\_\_\_  
 DILUTED DRUG ENZYME CONJUGATE: \_\_\_\_\_  
 KIT #: \_\_\_\_\_  
 COMMENTS: \_\_\_\_\_

Calculations:  
 Sample size = 0.10 gram  
 Weighing Date: \_\_\_\_\_  
 Weighing Analyst: \_\_\_\_\_  
 Amount of drug enzyme conjugate needed. Calculation A  
 A = No. of wells used \_\_\_\_ + 8 = \_\_\_\_ (round to the nearest 5's) \_\_\_\_  
 Milliliters of EIA buffer needed: Calculation B  
 B = ((Calculation A) \_\_\_\_ X 180)/1000 = \_\_\_\_  
 C = (((Calculation A) \_\_\_\_ ) x 300 x 4)/ 1000 = \_\_\_\_ (round up to the nearest 10's)  
 Amount of deionized water needed: C – 10% C \_\_\_\_ - (0.1 x \_\_\_\_ ) = \_\_\_\_  
 Amount of wash buffer needed: 10% of C 0.1 x \_\_\_\_ = \_\_\_\_

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Approved by:	Date
Stephen Powell	10-15-02
Leon Ilnicki	10-15-02
Jess Rajan	10-9-02