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Title: Flunixin Residues in Bovine Liver by ELISA		
Revision **00**	Replaces: NA	Effective: 1/6/2003

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A. INTRODUCTION

1. Theory

   This procedure is used to screen bovine liver samples for Flunixin residues at concentrations of 50 ppb or higher. Blended liver sample is extracted with a dilute acidic phosphate buffer and analyzed using a commercial Flunixin ELISA kit (Flunixin Enhanced ELISA kit, Neogen Corporation.

2. Applicability

   This procedure is applicable to bovine liver.

B. EQUIPMENT

1. Apparatus

   Note: Equivalent apparatus may be substituted for those listed below.

   a. Balance, analytical. – Mettler model MT5, sensitive to 0.0001g.

   b. Balance, top loader. – Mettler model PM2000, sensitive to 0.01 g.

   c. Refrigerator, explosion-proof. – Lab-Line Frigid-Cab model 3566.

   d. Freezer, explosion-proof. – Lab-Line Frigid-Cab model 3552.

   e. Vortex mixer. – Lab-Line Super Mixer model 1290.

   f. Shaker (Two Speed), platform. – Eberhard Ultrasonic bath. –VWR Aquasonic model 150T.

   g. Centrifuge (high speed). – Jouan model KR22.

   h. pH meter. – Orion 611, readable to 0.01 pH.

   i. Volumetric dispenser, bottle-top. – Capable of delivering from 1 to 10 mL.

   j. Stirrer/hot plate. – Corning PC-4420.

   k. Micropipettor. – 20µL fixed.

   l. Micropipettors, Variable. – 10-100 µL, 20-200 µL, and 100-1000 µL.

   m. Centrifuge tubes, with caps, for use with high-speed centrifuge. – Nalgene. #3119-0050, 50-mL capacity: Description: Oakridge. Material: polyallomer.

   n. Glass storage bottles with caps. – 60-mL capacity, Daigger # LX2223C and 125-mL capacity, Daigger #LX2223C.

   o. Scintillation vials. – Disposable glass, with tops, Kimble #74505-20.

   p. Racks. – To hold Nalgene centrifuge tubes and working standards stored in

scintillation vials.

q. Volumetric flasks, Class A – 50, 100, 200, 500 and 1000 mL.

r. Pipettes, Class A. –1, 2, 5, 10 and 25 mL.

s. Graduated cylinders, Class A. – 100 and 250 mL.

t. HPLC-grade water system. – Ion pure plus 150.

2. Instrumentation

Note: Equivalent instrumentation may be substituted.

a. Plate Reader. – Biotec Autoreader, model ELx 808,equipped with 630 nm or 650 nm filter (ELISA Technologies). Reader should be calibrated to assure overall accuracy at the wavelength used.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents may be substituted.

1. Reagents

a. Test kits. – Flunixin Enhanced ELISA kit, Cat # 101910 (NEOGEN Corporation, ELISA Technologies Division, 628 E. 3rd Street, Lexington, Kentucky, 40505). Contents of bulk kit include the following:

i. EIA buffer (200 mL).

ii. 10x Wash buffer concentrate (100 mL). Dilute before use according to manufacturer instructions.

iii. K-Blue Substrate (100 mL). Stabilized 3,3' 5, 5' Tetramethylbenzidine (TMB) plus hydrogen peroxide in a single bottle. Light sensitive.

iv. Drug-Enzyme Conjugate (1 mL). Drug-horseradish peroxidase concentrate. Dilute before use according to manufacturer instructions.

v. Pre-coated Plates (5). 96 well Costar plates in strips of 8 breakaway wells coated with anti-drug antisera. Do not wash until the sample/drug-conjugate incubation is complete.

Note: The test kits must be stored in a refrigerator at 2-8ºC. Do not use past expiration date.

b. Sodium phosphate dibasic anhydrous – J.T. Baker # 3828-01, formula Na_2HPO_4, molecular weight 141.96.

c. Potassium phosphate monobasic crystal – J.T. Baker #-3246-01, formula KH_2PO_4, molecular weight 136.09.

d. Methanol, HPLC-grade –J.T. Baker # 9093-03.

e. Water, HPLC-grade – produced from Ion Pure Plus 150 system.

f. HCl, Concentrated (36.5-38.0%).

g. Sodium hydroxide pellets – Sigma ACS reagent S-0899. Formula NaOH, Molecular weight 40.00.

h. Buffer solution, pH 4.0 – Fisher Certified SB98-500.

i. Buffer solution, pH 7.0 – Fisher Certified SB108-500.

2. Solutions

a. Extraction Buffer: - Weigh 6.8 g of potassium phosphate monobasic, and 7.1 g of sodium phosphate dibasic into a 1 L class A volumetric flask. Dilute to volume with HPLC grade water. Adjust pH to 6.8 with 1M HCl or 1M NaOH solutions

b. 1 N HCl solution – Dilute 8.3 mL of concentrated HCl to 100 mL in HPLC-grade water in a 100-mL volumetric flask.

c. 1 N NaOH solution – Transfer 4 g NaOH into a 100-mL volumetric flask and dilute to mark with HPLC-grade water. Mix thoroughly.

d. Horseradish peroxidase (HRP) enzyme conjugate - EIA buffer dilution (1+180). (Both reagents are supplied with the bulk kit). Prepare in ratio of 1 µL HRP conjugate to 180 µL of EIA buffer on day of use.

Note: Allow HRP solution to warm up to room temperature before making dilutions.

Note: The Flunixin ELISA bulk kit supplies the analyst with approximately 1 mL of HRP enzyme conjugate and 200 mL of the EIA buffer. These volumes are about twice the theoretical volume needed to assay the 480 wells contained on the five plates in the bulk kit. However, the analyst is cautioned to conserve the volumes of the reagents in the kit, to allow for losses due to pipetting or mistakes. Calculate the number of wells to be used on a particular day to determine the amount of HRP conjugate to dilute. Instruction supplied with the bulk kit suggest that the analyst prepare sufficient HRP conjugate: EIA buffer solution to assay four extra wells (if using a single channel micropipettor) beyond the number of wells needed for sample extracts for the day, to account for loss by pipetting. If using a multi-channel micropipettor, allow for extra wells).

For example, if using all the wells on a 96-well plate, prepare sufficient volume of HRP conjugate: EIA buffer solution to assay 100 wells.

e. Wash buffer, dilution – Transfer 50 mL of the concentrated wash buffer (supplied with ELISA kit at 10x concentration) into a 500-mL volumetric flask, and dilute to mark with HPLC-grade water. This solution is assigned the same expiration date as the concentrated wash buffer and shall be stored at 2-8ºC.

D. STANDARDS

1. Flunixin Analytical Standard

   a. Standard Name: Flunixin N-methyl glucamine salt (Flunixin-NMG).

   b. Sources: Schering-Plough Corporation, P.O. Box 3182, Union, NJ. 07083-1982, Cat. No. SCH 14714, or U.S Pharmacopeia, 12601 Twinbrook Parkway, Rockville, MD 20852, Cat. No, 27460-7.

   c. CAS Registry No: 42461-84-7 (NMG salt) and 38677-85-9 (Acid).

   d. Chemical formula: $C_{14}H_{11}F_3N_2O_2$ (Acid), $C_{21}H_{28}F_3N_3O_7$ (NMG Salt).

   e. MW: 296 Acid and 491 (NMG Salt).

   f. Storage: Per manufacturer's instructions.

2. Preparation of Standard Solutions

   a. Stock Solution A (500 µg/mL free acid): - Weigh an equivalent of 50.0 mg of Flunixin-free acid (approximately 83.0 mg of Flunixin-NMG analytical standard; see formula below) into a 100-mL volumetric flask, dissolve the material, and dilute to the mark with methanol. This standard is stable for 6 months when stored at < -10ºC.

      Weight of Flunixin NMG (mg) = [(50.0 x 491)/ purity)] / 296.

      For a purity of 99.0%, substitute 0.990 into the above equation.

   b. Stock Solution B (50 µg/mL free acid): - Pipet 10 mL of stock solution A into a 100-mL volumetric flask and dilute to the mark with methanol. This standard is stable for 6 months when stored at < -10ºC.

   c. Working standard solutions:

      i. 50 ng/mL (50 ppb): - Pipet 100 µL of stock solution B into a 100-mL volumetric flask and dilute to mark with HPLC-grade water. Mix thoroughly. This standard is stable for one month when stored at 2-8ºC.

      ii. 120 ng/mL - Pipet 240 µL of stock solution B into a 100 mL volumetric flask and dilute to mark with HPLC-grade water. Mix thoroughly. This standard is stable for one month when stored at 2-8ºC.

d. External Standards. Prepare fresh daily

i. 15 ng/mL (Equivalent to 100 ppb in sample). Add 100 µL 120 ng/mL working standard (D.2.c.ii) to 700 µL extraction buffer in an appropriate vial and mix.

ii. 7.5 ng/mL (Equivalent to 50 ppb in sample). Mix equal volumes of 15 ng/mL (D.2.d.i) standard and extraction buffer in an appropriate vial.

E. SAMPLE PREPARATION

After slaughter, bovine liver samples are frozen, packaged with cold-frozen gel packs, and shipped overnight to the laboratory. The samples are partially thawed and blended, then stored in the freezer at <-10ºC.

F. ANALYTICAL PROCEDURE

1. Sample Set Requirements

a. Each sample set will contain a negative control and a 50 ppb positive control plus any number of samples that can be accommodated in a set.

2. Control sample preparation

a. Weigh 1.0 ± 0.10 g portion of blank liver into two 50-mL centrifuge tubes. (A blank is previously analyzed tissue found to contain no Flunixin residue and exhibit minimal background activity). Use one tube as negative control.

b. For fortification, add 1-mL of 50 ng/mL working standard (D.2.c.i) to the remaining tube.

3. Extraction Procedure

a. Weigh a 1.0-± 0.10 g portion of each frozen liver tissue sample into a 50 mL centrifuge tube. Allow liver to thaw.

b. Add 6 mL of phosphate extraction buffer (C.2.a) to the negative tube and sample tubes. Add 5 mL buffer to positive tissue control tube, which already contains 1 mL working standard.

c. Mix contents of tube on vortex mixer for 5 seconds.

d. Shake tube vigorously for 5 min.

e. Sonicate tube for 1 min.

f. Centrifuge at 15,000 RPM at 4ºC for 15 min.

g. Keep tube refrigerated until ready for ELISA.

4. ELISA

a. Apply 20 µL portions of control tissue and sample extracts in duplicate to the ELISA plate.

Note*:* An estimation of well to well repeatability is necessary to properly set a cutoff based on the response of the 50 ppb control. This may be estimated by calculating the standard deviation of the normalized absolute differences between all duplicate wells of 50 ppb replicate controls.

Distribute randomly the 50 ppb fortified control extracts in 20 µL portions into 3 pairs of wells over the area of the ELISA plate utilized.

b. Following ELISA kit directions, add 180 µL diluted drug-HRP Conjugate Solution (HRP) to each well. (The HRP solution and EIA buffer are supplied in the test kit and are diluted 1:l80 HRP: EIA or per Kit Instruction). After addition of the HRP: EIA solution to each sample, mix the solutions in plate wells by gently vibrating the plate on a flat surface.

c. Cover plate to avoid possible dust/dirt contamination.

d. Incubate for one hour at room temperature. Shake gently at least twice during the incubation period.

e. Invert the plate after the incubation period, to remove matrix solutions.

f. Wash the wells 3 times with (300 µL/time) with diluted washing buffer (diluted 1:10 with water and supplied with the test kit). Tamp the inverted plate on a paper towel between washings.

g. Add 150 µL K-Blue substrate (another reagent supplied in the test kit) to each well. Allow the reaction to proceed for 30 minutes with intermittent gentle shaking of plate, especially before taking an optical density (absorbance) reading.

h. Read results at 630nm or 650nm on a Biotec Autoreader ELx 808. The optimum absorbance value is considered to be between 1.0 to 2.0 for the negative control read against air.

G. CALCULATIONS

1. Evaluate sample results based on absorbance values for fortified control tissue. Flunixin concentration is inversely related to intensities of blue color.

2. Calculate the mean and standard deviation (SD) for the absorbance readings of the six 50-ppb control replicates (see F.4.a). Use these to determine a decision level (DL) using the formula DL = Mean + 3*SD. Identify sample as positive if its average absorbance is less than the decision level.

H. HAZARD ANALYSIS

1. Method Title — Flunixin Residues in Bovine Liver by ELISA.

2. Required Protective Equipment — Lab coat, safety glasses, and gloves.

3. Hazards

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Primary standard	Causes skin irritation and possible pulmonary edema after prolonged inhalation of this compound	Handle primary standard in a fume hood. Use safety glasses and gloves when handling the primary standard.
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.
Acid and base	Corrosive	Wear gloves and safety glasses.

4. Disposal Procedures

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Methanol	Can cause burns on skin and eye injury	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.
Acid and base	See Above	Neutralize the acid or base for disposal down the drain in accordance with local, State and Federal regulations.
Used plate(s)	None	Dispose in accordance with local, State and Federal regulations.

I. QUALITY ASSURANCE PLAN

1. Each sample set must meet the following criteria before data can be reported:
   a. The control tissue absorbance should be between 1.0 to 2.0 absorbance units vs. air.
   b. Absorbance must decrease with increasing Flunixin concentration for all positive controls.
   c. Inspection of the plate reveals no obvious areas of non-uniform response or other anomalies that would seriously affect qualitative accuracy.

2. Performance Standard

Analyte	*Analytical Range*	*Acceptable False Negatives*	*Acceptable False Positives*
Flunixin	≥ 50 ppb	0%	< 15%

3. Critical Control Points and Specifications

	Record	*Acceptable Control*
a.	F.3.a: Sample Weight	Weigh 1.00 ± 0.10 g.
b.	F.3.g: Extract storage condition	Store in refrigerator until ready for ELISA kit.
c.	C.2.d. and F.4.b. Horseradish peroxidase dilution	Prepare on the day of use. Dilute properly.

4. Readiness To Perform
   a. Familiarization:

      Note: Phase I and Phase II may be performed concurrently.

i. Phase I: When possible, the analyst being trained may observe the method being performed by an analyst who has previously qualified in the method.

Run duplicate external standard curves on 3 working days, which will include the following:

(a) 0 ppb (1 mL buffer solution).

(b) 50 ppb - (Working Standard (D.2.d.ii) diluted 1:7 with buffer).

(c) 100 ppb - (Working Standard (D.2.d.i) diluted 1:7 with buffer).

ii. Phase II: Fortified samples- 3 sets of 10 blank and 10 fortified (50 ppb) samples over a period of 3 working days.

iii. Phase III. Check samples for analyst accreditation.

(a). The analyst is to receive 30 beef liver samples. The sample fortifications, including the number of blanks, are to be blind to the analyst. At least 10 of the 30 samples should be blank and the rest spiked at 50 ppb level. These samples will be prepared and provided by Supervisor or Quality Assurance Manager (QAM). All samples should be analyzed in duplicate wells. The samples must be randomized throughout the set.

(b). An external curve must be generated using 0, 50 and 100 ppb, respectively to help monitor plate acceptability.

(c). Report analytical findings to the QAM.

(d). A letter from the QAM is required to commence analyzing official samples.

b. Acceptability criteria:

Refer to sections I.1-2 above.

5. Intralaboratory Check Samples

a. System, minimum contents:

i. Frequency: One Check sample should be analyzed per twenty samples.

ii. Records are to be maintained for:

(a) False positives/negatives.

b. Acceptability criteria:

i. If unacceptable values are obtained,

ii. Then take corrective action.

6. Sample Acceptability and Stability:
   a. Matrix: Bovine liver.
   b. Sample receipt size: Minimum 20 – 25 g bovine liver.
   c. Sample receipt condition: Frozen shipped with frozen gel-pak.
7. Sample storage:
   a. Blended bovine liver sample stored at < –10ºC is stable for two months.
8. Sample Set:
   a. Each sample set contains
      i. One control tissue blank.
      ii. 1 tissue blank fortified at 50-ppb level.
      iii. Samples.
9. Sensitivity

   Minimum proficiency level (MPL): 50 ppb.

J. WORKSHEET

Examples of worksheets, on the following two pages, may be copied for use.

CLG-FLX3.00		Page 13 of 16
Title: Flunixin Residues in Bovine Liver by ELISA		
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FLUNIXIN ELISA WORKSHEET
(Page 1 of 2)

Analyst: _____
Date Started: _____
Date Completed: _____
Set Number: _____
Reviewed By Initials/Date: _____

Standards/Reagents Used	
Fortification standards (6)	
Sodium phosphate, dibasic buffer	
Potassium phosphate , monobasic buffer	
0.2M mixed phosphate buffer	
Diluted Flunixin-HRP conjugate	
Diluted washing buffer	
K-Blue substrate	
FX test kit lot #	
Equipment	
Refrigerator (REF)	
Freezer (FRZ)	
Balance (BAL)	
Micropipettors (MCP)	
pH meter (PHM)	
Shaker (TIM)	
Centrifuge (CNT)	
Optical density reader (SPC)	

Reader Wavelength (nm) _____

ELISA Plate Layout

Location	1	2	3	4	5	6	7	8	9	10	11	12
A	1	5	9	13	17	21	25	29	33	37	41	45
B	Dup-1	Dup-5	Dup-9	Dup-13	Dup-17	Dup-21	Dup-25	Dup-29	Dup-33	Dup-37	Dup-41	Dup-45
C	2	6	10	14	18	22	26	30	34	38	42	46
D	Dup-2	Dup-6	Dup-10	Dup-14	Dup-18	Dup-22	Dup-26	Dup-30	Dup-34	Dup-38	Dup-42	Dup-46
E	3	7	11	15	19	23	27	31	35	39	43	47
F	Dup-3	Dup-7	Dup-11	Dup-15	Dup-19	Dup-23	Dup-27	Dup-31	Dup-35	Dup-39	Dup-43	Dup-47
G	4	8	12	16	20	24	28	32	36	40	44	48
H	Dup-4	Dup-8	Dup-12	Dup-16	Dup-20	Dup-24	Dup-28	Dup-32	Dup-36	Dup-40	Dup-44	Dup-48

Estimation of Decision Level (DL)

(Based on 6 replicates of one 50 ppb fortification sample)

Replicate Numbers	Amount/20 µL/Well	Absorbance	Normalized value
Tissue Blank	0.00 ng		–
1	0.17 ng (calculated)		
2	0.17 ng (calculated)		
3	0.17 ng (calculated)		
4	0.17 ng (calculated)		
5	0.17 ng (calculated)		
6	0.17 ng (calculated)		
		Avg:	

SD: _____

DL = SD x 3 = _____

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FLUNIXIN ELISA WORKSHEET
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Analyst: _____
Date Started: _____
Date Completed: _____
Set Number: _____
Reviewed By Initials/Date: _____

DL =Decision Level Absorbance Calculated from ELISA Worksheet Page 1 =
Note: A Sample is FLX positive (+) if its absorbance is < DL.

Position Number	Lab Number	Form Number	Sample Wt. 1.00 ± 0.10 g	Results (+) or (-)
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				

Position Number	Lab Number	Form Number	Sample Wt. 1.00 ± 0.10 g	Results (+) or (-)
25				
26				
27				
28				
29				
30				
31				
32				
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K. REFERENCES

Susan B. Clark, Sherri B. Turnipseed, Gene J, Nandrea, Mark R. Madson, and Emma R. Singleton, Identification *and Confirmation of Flunixin Meglumine and Phenylbutazone Residues in Animal Kidney by ELISA Screening and Liquid Chromatography Mass Spectrometry,* US FDA Laboratory Information Bulletin, #4246, May 2001.

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Approved by:	Date
Sydney Griffith	12/05/2002
Thomas Mallinson	12/09/2002
Terry Dutko	12/05/2002
Jess Rajan	12/09/2002