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

CLG-CAM1.03		Page 1 of 9
Title: Screening for Chloramphenicol by ELISA		
Revision: 03	Replaces: CLG-CAM1.02	Effective: 04/15/2013

Contents

A. INTRODUCTION

1. Summary of Procedure

This method is based on a competitive-type ELISA (Enzyme Linked ImmunoSorbent Assay) for *in vitro* screening of chloramphenicol. The solid support of the reaction is a microtiter plate with divisible strips coated with sheep anti-rabbit IgG antibodies.

2. Applicability

This method is suitable for the screening of chloramphenicol in poultry, beef, swine, equine, and catfish muscle at levels ≥ 0.25 ppb.

Note: Refer to 21CFR for tolerance values set by FDA and 40CFR for tolerance values set by EPA.

B. EQUIPMENT

Note: Equivalent equipment may be substituted.

1. Apparatus

a. Analytical balance - Cat. No. BL15005, Sartorius.

b. Centrifuge - Model No. 5810, Eppendorf.

c. Mechanical shaker - Eberbach.

d. Polypropylene centrifuge tubes - 50 mL, Falcon.

e. Glass centrifuge tubes - 15 mL, HS No. 45600-15, Kimble.

f. Nitrogen Evaporator - Turbovap.

g. Vortex mixer - Genie 2, Scientific Industries.

h. Micropipettors - 2-250 µL, Rainin EDP.

i. Repeater pipet (Distriman) with 1250 µL mini syringes, Gilson.

j. Transfer pipettes - 10 mL in 1/10 serological, Kimble.

k. Transia Plate Chloramphenicol Test Kit - BioControl AB0299.

Note: Store kit components in the dark at 2 - 8 °C when not in use. Reagents with different batch numbers should not be interchanged between kits. The reagents should come to room temperature before use. The microtiter plate should be kept in the sealed pouch to avoid condensation. Each vial should be shaken well before use.

The Transia Plate Chloramphenicol Test Kit contains:

i. Microtiter plate with divisible strips, 96 wells (8 wells x 12 strips).

ii. Zip-lock bag for microtiter plate.

iii. Sample dilution buffer - concentration 4X, 1 x 20 mL.

iv. Standard - 0.025, 0.05, 0.1, 0.2, 0.5, and 2.0 ng/mL, chloramphenicol, ready to use, 1 x 1.0 mL.

Note: The reconstitution buffer serves as the zero standard.

v. Standard - 100 ng/mL, chloramphenicol, ready to use, 1 x 1.0 mL.

vi. Reconstitution buffer - ready to use, 1 x 10 mL.

vii. Conjugate - chloramphenicol conjugated to peroxidase, lyophilized.

viii. Anti-chloramphenicol antibody - lyophilized.

ix. Washing buffer - concentration 20X, 1 x 30 mL.

x. Substrate - TMB, ready to use, 1 x 12 mL.

Note: Avoid direct exposure of the substrate to light. A blue color of the substrate may indicate a deterioration of the reagent.

xi. Stop solution - 0.5M H_2SO_4, ready to use, 1 x 15 mL.

l. Absorbent paper - Wypall L40.

m. Robot-Coupe® processor - Robot Coupe U.S.A., Inc.

2. Instrumentation

a. Plate Reader - Elx808 Ultra Microplate Reader interfaced with a desktop computer, BioTek Instruments.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents / solutions may be substituted. The stability time frame of the solution is dependant on the expiration date of the components used or the listed expiration date, whichever occurs first.

1. Reagents

a. Ethyl acetate - HPLC Grade, Cat. No. 4601-7, Caledon.

b. n-hexane - HPLC Grade, Cat. No. 5601-7, Caledon.

c. Distilled water

2. Solutions

a. Washing buffer 1X:

Dilute the washing buffer to 1:20 in distilled water (Ex. Pipet 2 mL of washing buffer and 38 mL of distilled water into bottle). Mix well. The washing buffer can be stored at 2 - 8 °C until the expiration date stated on the kit label.

b. Sample dilution buffer 1X:

Dilute the sample dilution buffer to 1:4 in distilled water by pipetting 20 mL of sample dilution buffer and 60 mL of distilled water into a bottle. Mix well. The sample dilution buffer must be stored at 2 - 8 °C until the expiration date stated on the kit label.

c. Reconstitution of conjugate solution:

Reconstitute the lyophilized conjugate with 4 mL of reconstitution buffer. Mix thoroughly and keep in the dark until use. This solution can be stored in the dark at 2 - 8 °C for a maximum of 2 months. Alternatively, the reconstituted conjugate solution may be stored in a freezer at -20 °C until the expiration date on the kit. Freeze aliquots immediately after reconstitution.

d. Reconstitution of antibody solution:

Reconstitute the lyophilized antibodies with 4 mL reconstitution buffer. Mix thoroughly and keep in dark until use. This solution can be stored in the dark at 2 - 8 °C for a maximum of 6 months. Alternatively, the reconstituted antibody solution may be stored in a freezer at -20 °C until the expiration date on the kit. Freeze aliquots immediately after reconstitution.

D. STANDARD(S)

Note: Equivalent standards / solutions may be substituted. Purity and counterions are to be taken into account when calculating standard concentrations. The stability time frame of the solution is dependant on the expiration date of the components used or the listed expiration date, whichever occurs first.

1. Standard Information

a. Standards are provided with the ELISA kit and come ready to use. All standards must be stored in the dark at 2 - 8 °C until the expiration date stated on the kit.

2. Preparation of Standard Solution(s)

a. Fortification solution (10 ppb):

Add 100 µL of the 100 ng/mL standard and 900 µL of sample dilution buffer in a small bottle. Mix well.

E. SAMPLE PREPARATION

Homogenize samples prior to proceeding with extraction.

F. ANALYTICAL PROCEDURE

Note: The following steps are based on kit manufacturer instructions and may be subject to change. If any discrepancies exist, follow the current manufacturer instructions.

1. Preparation of Controls
   a. Weigh out 3.0 ± 0.1 g portions of a known blank tissue into a polypropylene centrifuge tube for each of the following Quality Control samples as needed:
      i. A tissue blank (negative control) - One needed for each analytical batch.
      ii. Positive control - Fortify the control sample with 75 µL of the 10 ppb fortification solution for a concentration of 0.25 ppb.
      iii. A recovery sample - Fortify the recovery sample with 75 µL of the 10 ppb fortification solution for a concentration of 0.25 ppb.
      iv. An internal check sample (as needed).

2. Extraction Procedure
   a. Weigh 3.0 ± 0.1 g of the homogenized tissue sample into a polypropylene centrifuge tube
   b. Add 6 mL of ethyl acetate to the sample and controls and mix on mechanical shaker on high for 10 minutes.
   c. Centrifuge for 10 minutes at 4000 rpm.
   d. Transfer 4 mL of the ethyl acetate to a glass centrifuge tube and evaporate at 55 ± 5 °C under a mild stream of nitrogen.
   e. Dissolve the residue in 1 mL of n-hexane.
   f. Add 1 mL of sample dilution buffer 1X and vortex for approximately 1 minute.
   g. Centrifuge for 10 minutes at 4000 rpm.

      Note: If an emulsion is present, put the tube in a water bath (80 °C) for approximately 5 minutes, or until the emulsion dissolves, and centrifuge again.
   h. Collect the lower, aqueous phase for the ELISA.

3. ELISA

   Note: The following steps are based on kit manufacturer instructions and may be subject to change. If any discrepancies exist, follow the current manufacturer instructions.

   Allow reagents and samples to come to room temperature. Vortex or shake the reagent vials before use. The microtiter plate must not be washed prior to use.

a. Analyze all controls, standards, and samples in duplicate. Attach the required number of strips to the plate: two wells each for plate blanks, zero standards, standards and samples. Any of the unused strips should be returned to the zip-lock bag containing dehydrating agent and sealed.

b. Place 100 µL of the reconstitution buffer in the first two wells to act as the plate blanks.

c. Add 50 µL of reconstitution buffer in the next two wells to act as the zero standard.

d. Transfer 50 µL of the 0.2 ppb and 0.5 ppb standards, in duplicate, to the next available vacant wells.

e. Transfer 50 µL aliquots of the samples, in duplicate, while randomly adding six positive controls throughout the sample set.

f. Add 25 µL of reconstituted conjugate to every well except the wells containing the plate blanks.

g. Add 25 µL of reconstituted antibody solution to every well except the wells containing the plate blanks.

h. Carefully shake the plate manually from side to side on a flat surface for one minute at room temperature (18 - 25 °C).

i. Incubate the plate in the dark at 2 - 8 °C for at least one hour.

Note: The washing step is very important. When washing, direct a strong jet of wash solution at the bottom of the well.

j. Carefully shake out the contents of the plate by holding the plate firmly and flicking your wrist. Wash each well using at least 300 µL of washing buffer per well. Tap the plate over a container and shake out liquid. Turn the plate upside down onto a paper towel and tap the plate firmly several times until all remaining liquid is removed. Repeat the washing at least three times.

Note: Gently blowing nitrogen gas into wells may help eliminate bubbles from wells.

k. Add 100 µL of the substrate to each well.

l. Incubate the plate at room temperature (18 - 25 °C) for 30 minutes.

m. Following the same sequence used when the substrate was added, place 100 µL of the stop solution to each well. Mix, for approximately 60 seconds, by sliding the plate back and forth on a flat surface thoroughly to ensure complete color conversion.

n. Read the plate as soon as possible following addition of stop solution on a plate reader set at 450 nm.

Note: The instrument parameters may be optimized to ensure system suitability.

4. Sample Set

Note: Each sample set must contain one QA sample/20 samples

a. Plate blank
b. Standards at 0, 0.2, and 0.5 ppb
c. Positive controls at 0.25 ppb (minimum of 6 values)
d. Blank control
e. Recovery at 0.25 ppb
f. Check sample (as needed)
g. Samples

G. CALCULATIONS / IDENTIFICATION

1. Calculate the average and standard deviation (SD) for the absorbance readings of the six positive control wells.

2. Use these values to calculate the decision level (DL) from the formula:

DL = average + (3*SD).

3. Average the duplicate wells for each sample. A sample will be identified as positive if its absorbance is less than or equal to the DL.

4. A plate must meet all of the following criteria:

a. The optical density of the plate blanks must be lower than 0.2.
b. The optical density of the zero standard must be higher than 0.8.
c. The standard curve absorbances continuously decrease in value from the 0 standard through each higher concentration standard.
d. A variability of less than ± 25% between duplicate sample wells is obtained.

Determined as follows: larger absorbance value divided by the smaller absorbance value is less than or equal to 1.25.

e. The CV for the six positive control replicates must be less than or equal to 20%.

H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Protective Equipment - Safety glasses, appropriate gloves, lab coat

2. Hazards

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Ethyl acetate	Flammable. Vapors are corrosive to the skin, eyes, and respiratory system.	Avoid contact or prolonged exposure to vapors. Work in a fume hood. Keep away from flame or heat.
Chloramphenicol	Toxic. Probable carcinogen. May cause heritable genetic damage. A possible risk to an unborn child. May cause sensitivity by inhalation and skin contact.	Work in a fume hood. Wear protective clothing, gloves and safety glasses.
TMB Substrate solution (tetramethylbenzidine)	Toxic in case of ingestion, inhalation and contact with skin.	Avoid contact with skin. Wear protective clothing, eyewear, and gloves.
Stop solution (sulfuric acid)	Skin and eye irritation.	Wear protective clothing, eyewear, and gloves.

3. Disposal Procedures

Follow local, state and federal guidelines for disposal.

I. QUALITY ASSURANCE PLAN

1. Performance Standard

See Section G.4. for criteria.

2. Critical Control Points and Specifications

Record	*Acceptable Control*
a. Shake time	1 minute.
b. Incubation time	30 minutes at room temperature (18 - 25 °C).
c. Substrate	Discard if it has turned blue in color.
d. Zero standard	Weak or absent color indicates degradation of reagents.
e. Liquid transfers into the ELISA wells.	Extreme care should be used to prevent the formation of bubbles in the ELISA wells.
f. Washing antibody wells	Wells should be tapped dry until all remaining wash solution is removed.

3. Intralaboratory Check Samples

   a. System, minimum contents.

      i. Frequency: One per week per analyst when samples analyzed.

      ii. Records are to be maintained.

   b. Acceptability criteria.

      Refer to I. 1.

      If unacceptable values are obtained, then:

      i. Investigate following established procedures.

      ii. Take corrective action as warranted.

4. Condition upon receipt: Cold.

   Note: Sample storage: 6 months frozen.

J. APPENDIX

1. References

   Test Kit Instructions, Transia Plate: *Chloramphenicol*, Test Kit AB0299, BioControl.

K. APPROVALS AND AUTHORITIES

1. Approvals on file.

2. Issuing Authority: Director, Laboratory Quality Assurance Division.