

FLORFENICOL-AMINE ELISA
5091FLOA[3]04.20

A competitive enzyme immunoassay for
screening and quantitative analysis of
Florfenicol-amine in various matrices

EUROPROXIMA FLORFENICOL-AMINE ELISA

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BRIEF INFORMATION

The Florfenicol-amine ELISA is a competitive enzyme immunoassay for the screening and quantitative detection of Florfenicol-amine in different commodities. With this ELISA-kit 96 analyses can be performed. Samples and standards are measured in duplicate which means that a total of 40 samples can be analysed.

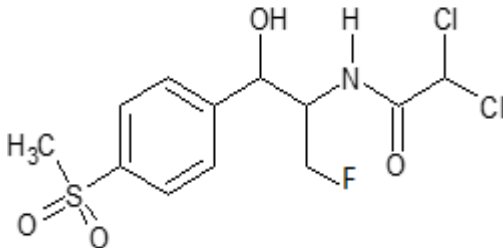
The ELISA kit contains all reagents to perform the test. Reagents for sample preparation are not included in the kit.

1. INTRODUCTION

Florfenicol is an effective broad-spectrum antibiotic and an analogue of chloramphenicol (CAP). Due to the ban of the use of CAP, in food-producing animals, Florfenicol is used to control infections in humans and in food-producing animals such as pigs, poultry, non-ruminating cattle and aquaculture. Florfenicol is used in cattle by the intramuscular route of administration, in porcine by intramuscular or oral routes and in chickens by oral route. In aquaculture, it is mainly administrated through medicated feeds.

Within the EU, Maximum Residue Limits (MRLs) for Florfenicol have been established, varying from 100 µg/kg to 3000 µg/kg.

These MRLs are described as: "Sum of florfenicol and its metabolites measured as florfenicol-amine". For this reason, EuroProxima has developed an ELISA that is specific for the measurement of florfenicol-amine.



Chemical structure of Florfenicol

2. PRINCIPLE OF THE FLORFENICOL-AMINE (FLOA) ELISA

The microtiter plate based FLOA ELISA consists of one precoated plate with antibody against FLOA (12 strips, 8 wells each). Horseradish peroxidase (-HRP) labeled FLOA and standard solution or sample are added to the wells. Free FLOA from the samples or standards and FLOA-HRP conjugate compete for the specific antibody binding sites (competitive enzyme immunoassay).

After an incubation step of 1 hour at room temperature, the non-bound reagents are removed in a washing step. The amount of bound FLOA-HRP conjugate is visualized by the addition of a substrate/chromogen solution (tetramethylbenzidine, TMB). Bound FLOA-HRP conjugate transforms the colourless chromogen into a coloured product. The substrate reaction is stopped by the addition of sulfuric acid. The colour intensity is measured photometrically at 450 nm. The optical density is inversely proportional to the FLOA concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The FLOA ELISA utilizes antibodies raised in rabbit against protein conjugated FLOA..

The cross-reactivity pattern of the antibody (as tested in buffer) is:

Florfenicol-amine	100%
Florfenicol	<10%
Thiamphenicol	<10%
Chloramphenicol	<10%

The cross-reactivities are determined in a buffer system. The reported values may be different in samples due to matrix effects.

The test cannot discriminate between analytes and cross-reactive substances.

The Limit of detection (LOD) and the detection capability (CC β) are determined under optimal conditions. Cut-off criteria need critical consideration.

Matrix	Procedure	LOD ppb	CC β ppb
Tissue	8.1	5	15
Fish	8.1	8	15
Kidney	8.1	12	15
Liver	8.1	7	15
Milk	8.3	2	8
Egg	8.2	5	8

If the sample is found to be non-compliant, the results shall be verified by re-analysis of the sample using a confirmatory method.

4. HANDLING AND STORAGE

- Kit and kit components should be stored at 2°C to 8°C in a dark place. For repeated use store kit components as specified under chapter 9.
- After the expiry date of the kit and/or components has passed, no further quality guarantee is valid.
- Bring all kit components including the microtiter plate to ambient (room) temperature before use.
- Dilute the kit components immediately before use, but after the components are brought to ambient temperature.
- Avoid condensation in the wells of the plate. Bring the sealed plate to ambient temperature before opening the plate sealing.
- The substrate chromogen solution can be stored in a refrigerator (2°C to 8°C) until the expiry date stated on the label.
- Exposure of the chromogen solution to light should be avoided.

Degeneration of the reagents may have occurred when the following phenomena are observed:

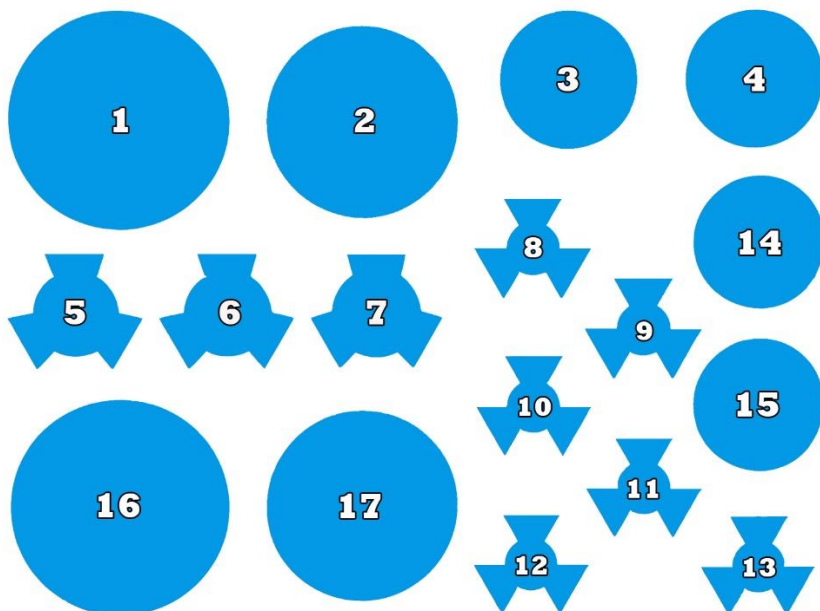
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells ($E_{450nm} < 0.8$).

5. KIT CONTENTS

Manual

One sealed (96-wells) microtiter plate (12 strips, 8 wells each), coated antibody. Ready-to-use.

Position of the reagents in the kit. For preparation of the reagents see Chapter 9.



1. **Dilution buffer** (40 ml, ready-to-use)
2. **Rinsing buffer** (30 ml, 20x concentrated)
3. **Substrate solution** (12 ml, ready-to-use)
4. **Stop solution** (15 ml, ready-to-use)
5. **Conjugate solution** (100 μ l, 100 times concentrated)
6. Not in use
7. **Zero Standard solution** (2 ml, ready-to-use)
8. **Standard solution 1** (1 ml, ready-to-use) **0.313 ng/ml**
9. **Standard solution 2** (1 ml, ready-to-use) **0.625 ng/ml**
10. **Standard solution 3** (1 ml, ready-to-use) **1.25 ng/ml**
11. **Standard solution 4** (1 ml, ready-to-use) **2.5 ng/ml**
12. **Standard solution 5** (1 ml, ready-to-use) **5.0 ng/ml**
13. **Standard solution 6** (1 ml, ready-to-use) **10.0 ng/ml**
14. Not in use
15. Not in use
16. Not in use
17. Not in use

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- 15 ml tubes with screw caps
- Scales and weighing vessels
- Gloves
- Fume hood
- Homogenizer (vortex, mixer)
- Centrifuge
- Automated microtiter plate washer or 8-channel micropipette 100 – 300 μ l
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Glass test tubes (10 – 15 ml)
- Micropipettes, 100 – 1000 μ l
- Multipipette with 2.5 ml combi tips
- Aluminum foil or parafilm
- Distilled water (bidest)

7. PRECAUTIONS

- This kit may contain hazardous substances. For hazard notes please refer to the appropriate safety data sheets (SDS).
- Avoid contact of all biological materials with skin and mucous membranes.
- Do not pipette by mouth.
- Do not eat, drink, smoke, store or prepare foods, or apply cosmetics within the designated work area.
- Do not use components past expiration date and do not use components from different lots.
- Each well is ultimately used as an optical cuvette. Therefore, do not touch the under surface of the wells, prevent damage and dirt.
- All components should be completely dissolved before use. Take special attention to the substrate and rinsing buffer, which crystallize at +4°C.
- Optimal results will be obtained by strict adherence to this protocol. Careful pipetting and washing throughout this procedure are necessary to maintain good precision and accuracy.

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8. SAMPLE PREPARATION

8.1 Tissue, Fish, Kidney and Liver

- Mix 1 gram of the homogenised sample with 4ml of distilled water
- Vortex shortly (30 sec) and mix head over head for 15 min
- Centrifuge 5 minutes at 4000 x g at 20-25°C
- Dilute supernatant 8 times with dilution buffer (50 µl + 350 µl dilution buffer)
- Use 50 µl per well in the ELISA test

8.2 Egg

- Mix 1 gr of homogenised sample with 7 ml of distilled water
- Vortex shortly (30 sec) and mix head over head for 15 min
- Centrifuge 5 min at 4000 x g at 20-25°C
- Dilute supernatant 2 times with dilution buffer (100 µl + 100 µl dilution buffer)
- Use 50 µl per well in the ELISA test

8.3 Milk

- Mix 1 ml of homogenised sample with 7 ml of distilled water
- Vortex shortly (30 sec) and mix head over head for 15 min
- Dilute supernatant 2 times in dilution buffer (100 µl + 100 µl of dilution buffer)
- Use 50 µl per well in the ELISA test

9. PREPARATION OF REAGENTS

Before beginning the test, the reagents should be brought up to room temperature. Any reagents not used should be put back into storage immediately at +2°C to +8°C. Prepare reagents freshly before use

Microtiter plate

Return unused strips into the resealable bag with desiccant and store at +2°C to +8°C for use in subsequent assays. Retain also the strip holder.

Rinsing buffer

The rinsing buffer is delivered 20 times concentrated. Prepare dilutions freshly before use. Per strip 20 ml of diluted rinsing buffer is used (1 ml concentrated rinsing buffer + 19 ml distilled water).

Dilution buffer

The dilution buffer (ready to use) is used to dilute the samples and the conjugate.

Conjugate solution (100 µl)

The conjugate (FLOA-HRP) is delivered 100x concentrated. Spin down the conjugate in the vial by a short centrifugation step (1 minute, 1000 x g). Add 10 µl of the concentrated conjugate solution to 990 µl of dilution buffer. Per 2 x 8 wells 800 µl of diluted conjugate is required. Store unused concentrated conjugate at +2°C to +8°C.

Substrate/chromogen solution

The substrate/chromogen solution (ready-to-use) tends to precipitate at +4°C. Take care that this vial is at room temperature when used (keep in the dark) and mix the content before pipetting into the wells.

10. ASSAY PROCEDURE

Rinsing protocol

Unbound components have to be removed efficiently between incubation steps in ELISA tests. This is reached by appropriate rinsing. It should be clear that each rinsing procedure must be carried out with care to guarantee good inter- and intra-assay results.

Manual rinsing or rinsing with automatic plate wash equipment can be performed as follows:

Manual rinsing

1. Empty the contents of each well by turning the microtiter plate upside down and remove residual liquid by striking the plate against a paper towel.
2. Fill all the wells to the rims (300 μ l) with rinsing solution.
3. This rinsing cycle (1 and 2) should be carried out 3 times.
4. Turn the plate upside down and empty the wells by a firm short vertical movement.
5. Place the inverted plate on absorbent paper towels and tap the plate firmly to remove residual washing solution in the wells.
6. Take care that none of the wells dry out before the next reagent is dispensed.

Rinsing with automatic microtiter plate wash equipment

When using automatic plate wash equipment, check that all wells can be aspirated completely, that the rinsing solution is nicely dispensed reaching the rim of each well during each rinsing cycle. The washer should be programmed to execute three rinsing cycles.

Assay Protocol

1. Prepare samples according to Chapter 8 and prepare reagents according to Chapter 9.
2. Pipette 100 μ l of the zero standard in duplicate (wells H1, H2, blank).
Pipette 50 μ l of the zero standard in duplicate (wells A1, A2, maximal signal).
Pipette 50 μ l of each of the standard solutions in duplicate (wells B1,2 to G1,2 i.e. 0.313, 0.625, 1.25, 2.5, 5.0 and 10 ng/ml).
3. Pipette 50 μ l of each sample solution in duplicate into the remaining wells of the microtiter plate (40 samples; 80 wells).
4. Pipette 50 μ l of conjugate (HRP) to all wells, except H1 and H2.
5. Seal the microtiter plate and shake the plate for a few seconds on a microtiter plate shaker.

6. Incubate for 1 hour in the dark at room temperature (20°C to 25°C).
7. Discard the solution from the microtiter plate and wash 3 times with rinsing buffer.
8. Pipette 100 µl of substrate solution into each well.
9. Incubate 30 minutes at room temperature (20°C to 25°C).
10. Add 100 µl of stop solution to each well.
11. Read the absorbance values immediately at 450 nm.

11. INTERPRETATION OF THE RESULTS

Subtract the mean optical density (O.D.) of the wells H1 and H2 (Blank) from the individual O.D. of the wells containing the standards and the samples.

The O.D. values of the six standards and the samples (mean values of the duplicates) are divided by the mean O.D. value of the zero standard/Bmax (wells A1 and A2) and multiplied by 100. The zero standard/BMax is thus made equal to 100% (maximal absorbance) and the other O.D. values are quoted in percentages of the maximal absorbance.

O.D. standard (or sample)

-----x 100 = percentage maximal absorbance

O.D. zero standard/Bmax

Calibration curve:

The values (% maximal absorbance) calculated for the standards are plotted (on the Y-axis) versus the analyte equivalent concentration (ng/ml) on a logarithmic X-axis.

Alternative for calibration curve:

The value of absorption (logit) calculation of the standards are plotted on Y-axis versus the analyte equivalent concentration on a logarithmic X-axis

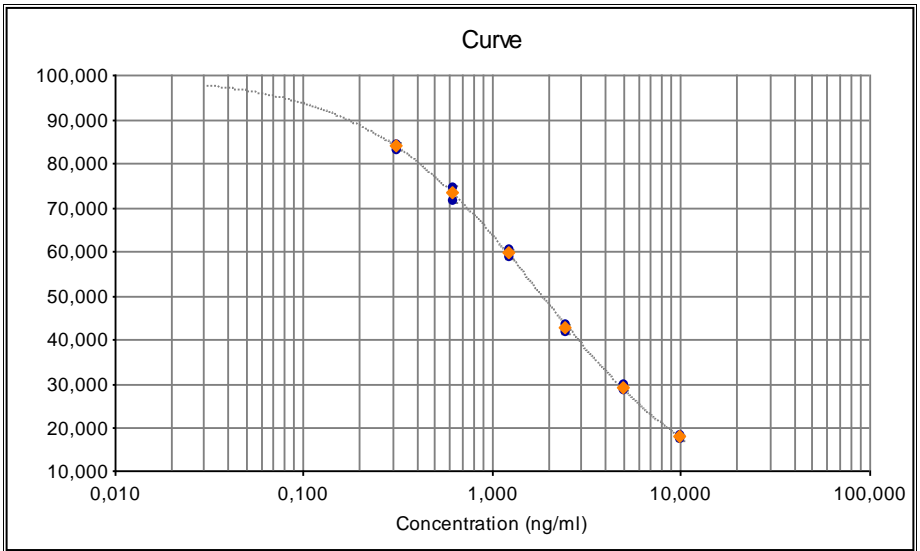


Figure 1: Example of a calibration curve

The amount of florfenicol (FLOA) in the samples is expressed as FLOA equivalents. The FLOA equivalents (concentration) corresponding to the % maximal absorbance recorded for each sample can be read from the calibration curve.

8.1 Tissue, Fish, Kidney and Liver samples

The FLOA equivalents, as read from the standard curve, should be multiplied by a factor 32 to obtain the FLOA content in tissue, fish, kidney and liver samples expressed in ppb (ng/g).

8.2 Egg

The FLOA equivalents, as read from the standard curve, should be multiplied by a factor 14 to obtain the FLOA content in egg samples expressed in ppb (ng/g).

8.3 Milk

The FLOA equivalents, as read from the standard curve, should be multiplied by a factor 16 to obtain the FLOA content in milk samples expressed in ppb (ng/g).

12. LITERATURE

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- EC 37/2010. Commission Regulation (EU) No 37/2010 of 22 December on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. Official Journal of the European Union, L15/1-72.

13. ORDERING INFORMATION

For ordering the Florfenicol-amine ELISA kit, please use cat. code 5091FLOA.

14. REVISION HISTORY

The manual is adapted to a new layout of the test kit. Several textual changes are added.