FLORFENICOL ELISA

5091FLORF[1]01.21

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

EUROPROXIMA FLORFENICOL ELISA

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The quality management system of R-Biopharm Nederland B.V. is ISO 9001:2015 certified

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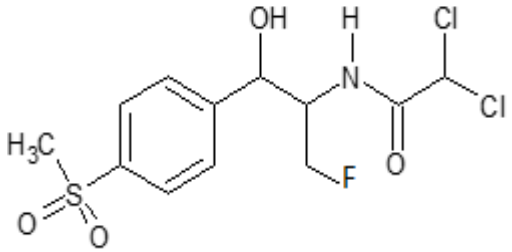
BRIEF INFORMATION

The Florfenicol ELISA is a competitive enzyme immunoassay for the screening and quantitative detection of Florfenicol 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 analyzed.
The ELISA kit contains all reagents to perform the test. Reagents required for sample preparation are included in the kit as well.

1. INTRODUCTION

Florfenicol is a fluorinated broed-spectrum antibiotic derivative of thiamphenicol (TAP) and an analogue of chloramphincol (CAP) which is used to control infections in humans and food-producing animals. In contrast to CAP and TAP, florfenicol is less toxic due to the lack of the nitro group which are present in CAP and TAP. Nevertheless, the adverse effects related to the consumption of animal-derived food containing florfenciol residues can not be excluded and testing of food for the presence of florfenicol residues is required in many countries worldwide. R-Biopharm Nederland has developed an ELISA that is specific for detection of florfenicol residues in a broad range of animal-derived foodstuffs.



Chemical structure of florfenicol

2. PRINCIPLE OF THE FLORFENICOL ELISA

The microtiter plate based Florfenicol ELISA consists of one precoated plate with antibody against florfenicol (12 strips, 8 wells each). Horseradish peroxidase (-HRP) labeled florfenicol and standard solution or sample are added to the wells. Free florfenicol from the samples or standards and florfenicol-HRP conjugate compete for the specific antibody binding sites (competitive enzyme immunoassay).
After an incubation step of 30 minutes at room temperature, the non-bound reagents are removed in a washing step. The amount of bound florfenicol-HRP conjugate is visualized by the addition of a substrate/chromogen solution (tetramethylbenzidine, TMB). Bound florfenicol-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 florefenicol concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The Florfenicol ELISA utilizes antibodies raised in rabbit against protein conjugated Florfenicol.
The cross-reactivity pattern of the antibody (as tested in buffer) is:

Florfenicol	100%
Florfenicol-amine	8%

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 (CCβ) are determined under optimal conditions. Cut-off criteria need critical consideration.

Matrix	Procedure	LOD (µg/kg)	CCβ (µg/kg)
Chicken and pork	8.1	0.2	0.4
Fish and shrimp	8.1	0.2	0.4
Egg	8.1	0.1	0.4

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 (2ºC to 8ºC) 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:

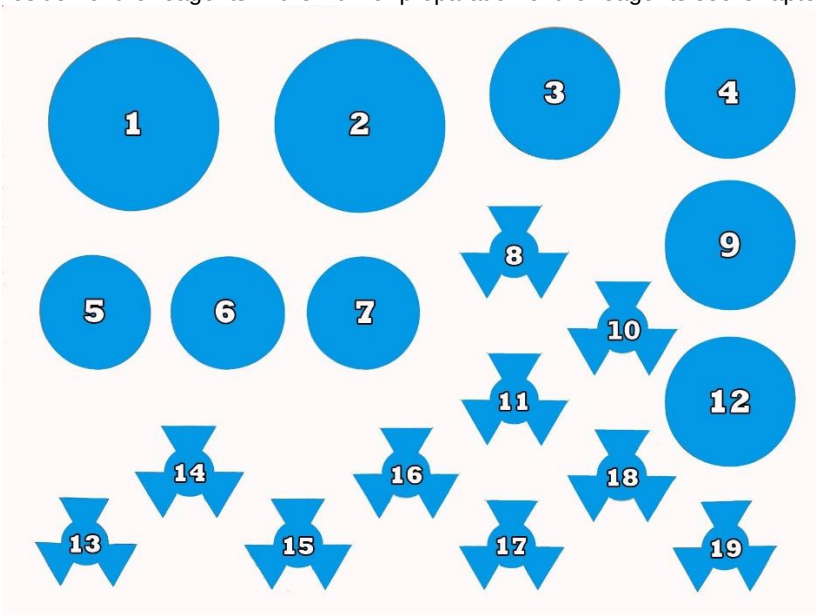
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

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** (30 ml, 10x concentrated)
2. **Rinsing buffer** (30 ml, 20x concentrated)
3. **Substrate solution** (12 ml, ready-to-use)
4. **Stop solution** (15 ml, ready-to-use)
5. Not in use
6. Not in use
7. Not in use
8. **Conjugate solution** (150 µl, 100 times concentrated)
9. **Extraction solution I** (5 ml, ready to use)
10. Not in use
11. Not in use
12. **Extraction solution II** (5 ml, ready to use)
13. **Zero Standard solution** (2 ml, ready-to-use)
14. **Standard solution 1** (1 ml, ready-to-use) **0.025 ng/ml**
15. **Standard solution 2** (1 ml, ready-to-use) **0.05 ng/ml**
16. **Standard solution 3** (1 ml, ready-to-use) **0.1 ng/ml**
17. **Standard solution 4** (1 ml, ready-to-use) **0.2 ng/ml**
18. **Standard solution 5** (1 ml, ready-to-use) **0.4 ng/ml**
19. **Standard solution 6** (1 ml, ready-to-use) **0.8 ng/ml**

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 µl
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Glass test tubes (10 – 15 ml)
- Micropipettes, 100 – 1000 µl
- Multipipette with 2.5 ml combi tips
- Aluminum foil or parafilm
- Distilled water

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 +4ºC.
- 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.

8. SAMPLE PREPARATION

8.1. Chicken, pork, fish, shrimp, egg (*measuring range 0.25-8 µg/kg)*

- Mix 1 g of the homogenised sample with 4 ml of the dilution buffer (see Chapter 9 for preparation)
- Vortex shortly (30 sec)
- Add 50 µl of Extraction Solution I and 50 µl of Extraction Solution II
- Vortex shortly (30 sec) and mix head over head for 10 min
- Centrifuge 5 minutes at 4000 x g at 20-25°C
- Dilute supernatant 2 times with the dilution buffer (150 µl of sample extract + 150 µl of the dilution buffer)
- Use 50 µl of this solution in the ELISA test

8.2. Chicken, pork, fish, shrimp, egg extended method

With this procedure the measuring range is extended to 5-160 µg/kg

- Samples from method 8.1. are diluted further
- Take 950 µl of the dilution buffer and add 50 µl of the diluted sample extracts from 8.1.
- Vortex shortly (30 sec)
- Use 50 µl of this solution 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 +2ºC to +8ºC. 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 is delivered 10 times concentrated. Prepare dilutions freshly before use. For 40 samples at least 180 ml is needed (18 ml concentrated dilution buffer + 162 ml distilled water). The dilution buffer is used to dilute the samples and the conjugate.

Conjugate solution (150 µl)
The conjugate (FLOR-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 the dilution buffer. Per 2 x 8 wells 800 µl of the diluted conjugate is required. Store unused concentrated conjugate at +2ºC to +8ºC.

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

Extraction solutions I and II
Extraction solutions I and II are supplied in the kit ready to use. These solutions are used during sample preparations to eliminate components that can interfere during the assay (e.g., proteins and fats) and to reduce matrix effect.

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 µl) 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 µl of the zero standard in duplicate (wells H1, H2, blank).
   Pipette 50 µl of the zero standard in duplicate (wells A1, A2, maximal signal).
   Pipette 50 µl of each of the standard solutions in duplicate (wells B1,2 to G1,2 i.e.0.025, 0.05, 0.1, 0.2, 0.4 and 0.8 ng/ml).

3. Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate.

4. Pipette 50 µl of diluted 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 30 minutes in the dark at room temperature (20ºC to 25ºC).

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 15 minutes at room temperature (20ºC to 25ºC).

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.

$$\frac{\text{O.D. standard (or sample)}}{\text{O.D. zero standard/Bmax}} \times 100 = \text{percentage maximal absorbance}$$

Calibration curve:
The values (percentage 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

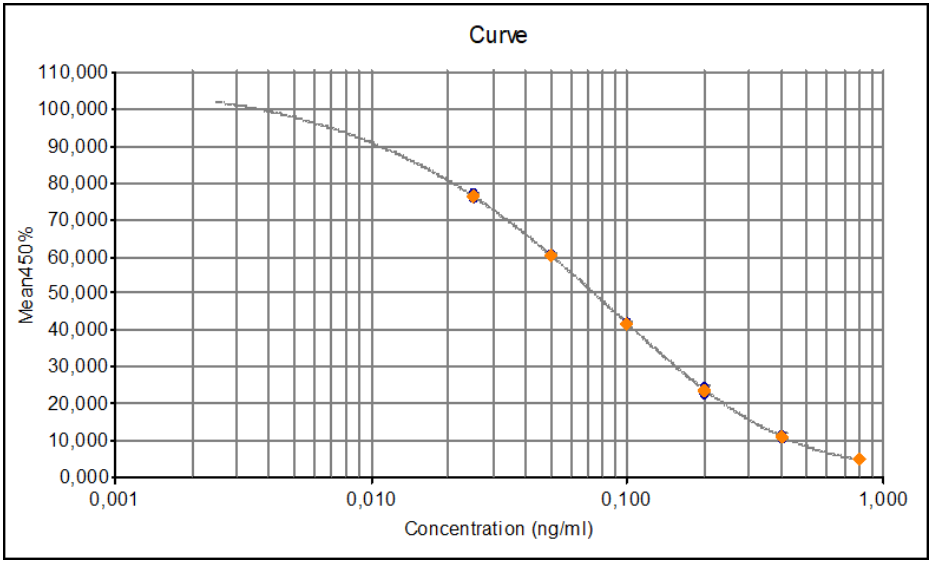


Figure 1: Example of a calibration curve

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

8.1. Chicken, pork, fish, shrimp, egg

The FLOR equivalents, as read from the standard curve, should be multiplied by a factor 10 to obtain the florfenicol content in chicken, pork, fish, shrimp and egg samples expressed in µg/kg.

8.2. Chicken, pork, fish, shrimp, egg extended method

The FLOR equivalents, as read from the standard curve, should be multiplied by a factor 200 to obtain the florfenicol content in chicken, pork, fish, shrimp and egg samples expressed in µg/kg.

12. LITERATURE

Tao, X., Jiang, H., Yu, X., Zhu, J., Wang, X., Wang, Z., ... & Shen, J. (2012). Development and validation of a chemiluminescent ELISA for simultaneous determination of florfenicol and its metabolite florfenicol amine in chicken muscle. Analytical Methods, 4(12), 4083-4090.

13. ORDERING INFORMATION

For ordering the Florfenicol ELISA kit, please use cat. code 5091FLORF.

14. REVISION HISTORY

Not applicable

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