ERYTHROMYCIN ELISA

(5151ERY[4]04.20)

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EUROPROXIMA ERYTHROMYCIN ELISA

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BRIEF INFORMATION

The erythromycin ELISA is a competitive enzyme immunoassay. The test is based on antibodies directed against erythromycin. The ELISA kit contains a 96 well microtiter plate as well as all essential reagents including ready-to-use standards to perform the test. Methods for a fast and efficient extraction of erythromycin from different matrices are included in the kit manual.

1. INTRODUCTION

Erythromycin is an antibiotic that belongs to the group of Macrolides. It has a broad spectrum activity and can be used for people allergic to Penicillin's.
MRLs for Erythromycin for all food producing species are set for tissue at 200 ppb, milk at 40 ppb and eggs at 150 ppb.

2. PRINCIPLE OF THE ERYTHROMYCIN ELISA

The kit is based on a microtiter plate (12 strips, each 8 wells), precoated with a monoclonal antibody directed against erythromycin. Enzyme labelled erythromycin (HRP) and erythromycin standards or samples are added to the precoated wells. Free erythromycin (in the standard solution or in the sample) and enzyme labelled erythromycin compete for the specific antibody binding sites (competitive enzyme immunoassay).
After an incubation of one hour, the non-bound (enzyme labelled) reagents are removed in a washing step. The amount of bound erythromycin-HRP conjugate is visualized by the addition of a substrate/chromogen solution (erythromycin/TMB). Bound erythromycin–HRP conjugate transforms the 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 and is inversely proportional to the erythromycin concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The erythromycin ELISA utilizes antibodies raised in mouse against protein conjugated erythromycin. The reactivity pattern of the antibody is:

Cross-reactivity:	Erythromycin	100%
	Erythromycin ethylsuccinate	12%
	Tylosin	0%
	Spiramycin	0%

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) is determined under optimal conditions. Cut-off criteria need critical consideration.

Matrix	Procedure	LOD (ppb)
Milk	8.1	4
Urine	8.1	4
Liver	8.2	10
Shrimps	8.2	10
Egg	8.2	10
Fish	8.2	10

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:

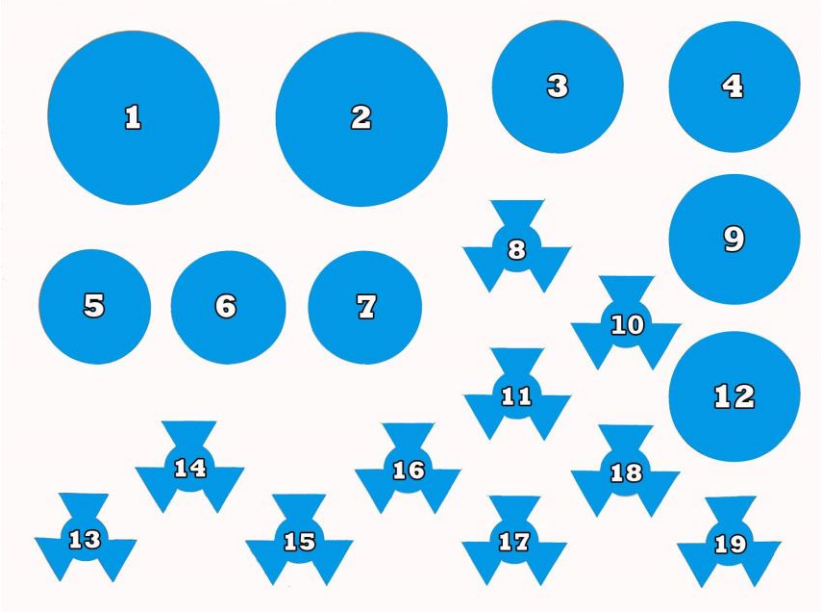
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

5. KIT CONTENTS

Manual

One sealed microtiter plate (12 strips, 8 wells each), coated with erythromycin antibody. Plate is ready-to-use.

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



1. **Dilution buffer** (20 ml, 4x 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** (100 µl, 100x concentrated)
9. Not in use
10. Not in use
11. Not in use
12. Not in use
13. **Zero standard solution** (2 ml, Ready-to-use)
14. **Standard 1 solution** (1 ml, Ready-to-use) **0.375 ng/ml**
15. **Standard 2 solution** (1 ml, Ready-to-use) **0.75 ng/ml**
16. **Standard 3 solution** (1 ml, Ready-to-use) **1.5 ng/ml**
17. **Standard 4 solution** (1 ml, Ready-to-use) **3 ng/ml**
18. **Standard 5 solution** (1 ml, Ready-to-use) **6 ng/ml**
19. Not in use

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Scales and weighing vessels
- Gloves
- Homogeniser (vortex, mixer)
- Centrifuge (2000 x g)
- Automated microtiter plate washer or 8-channel micropipette 100 – 300 µl
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Micropipettes, 100 – 1000 µl
- Multipipette with 2.5 ml combitips
- 15 ml tubes with screw cap (polypropylene)
- 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 Milk/Urine samples

- Homogenize the samples
- Pipette 125 µl milk or urine into a tube
- Add 875 µl dilution buffer (see chapter 9) , vortex
- Use 50 µl of this solution in the ELISA test.

8.2 Tissue (fish, shrimps, liver) and egg samples

- Weigh 1 gram of homogenized sample into a 15 ml tube
- Add 4 ml distilled water
- Vortex and mix the samples head-over-head for 15 minutes
- Centrifuge 10 minutes, 2000 x g at 20°C - 25°C
- Pipette 250 µl of the upper layer into a clean tube
- Add 750 µl dilution buffer (see chapter 9), vortex
- Use 50 µl of this solution in the ELISA test.

9. PREPARATION OF REAGENTS

Before starting the test, allow the reagents to come to ambient temperature. Any reagents not used should be put back into storage immediately at 2°C to 8°C.
Prepare reagents fresh 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.

Dilution buffer
The dilution buffer is 4 times concentrated. Before dilution (20 ml buffer + 60 ml distilled water) the concentrated buffer should be at 20°C to 25°C and thoroughly mixed. Concentrated buffer can show precipitates of the contents. Mix well before dilution with distilled water.

Conjugate
The conjugate 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 dilution buffer. Per 2 x 8 wells 800 µl is required. Store unused concentrated conjugate at 2°C to 8°C.

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

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.

10. ASSAY PROCEDURE

Rinsing protocol

In ELISA's, between each immunological incubation step, unbound components have to be removed efficiently. 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.
Basically, 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 rinsing solution from 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 G1, G2, blank).
   Pipette 50 µl of the zero standard (Bmax) in duplicate (wells A1, A2).
   Pipette 50 µl of each of the standard solutions in duplicate (wells B1,2 to F1,2 i.e. 0.375, 0.75, 1.5, 3 and 6.0 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 conjugate (erythromycin-HRP) to all wells, except G1 and G2.

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 4°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 in the dark at 20°C to 25°C.

10. Pipette 100 µl of stop solution to each well.

11. Read the absorbance values immediately at 450 nm.

11. INTERPRETATION OF RESULTS

Subtract the mean optical density (O.D.) of the wells G1 and G2 (Blank) from the individual O.D. of the wells containing the standards and the samples.

The O.D. values of the five 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 (% 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 absorption value of the standards is plotted on the Y-axis versus the concentration on the X-axis. The Y-axis is in logit the Y-axis is logarithmic.

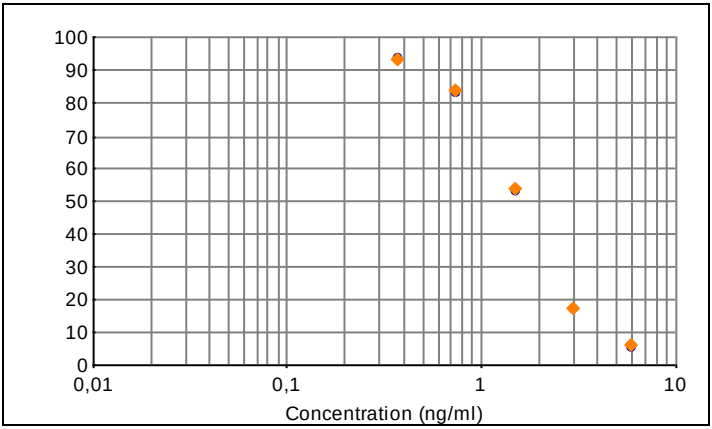


Figure 1 : Example of a calibration curve

The amount of erythromycin in the samples is expressed as erythromycin equivalents. The erythromycin equivalents in the samples (ng/ml) corresponding to the % maximal absorbance of each extract can be read from the calibration curve.

8.1 Milk and urine

The erythromycin equivalents, as read from the standard curve, have to be multiplied by a factor 8 to obtain the erythromycin content in milk and urine samples.

8.2 Tissue (fish, shrimps, liver) and egg samples

The erythromycin equivalents, as read from the standard curve, have to be multiplied by a factor 20 to obtain the erythromycin content in tissue (fish, shrimps, liver) and egg samples.

12. LITERATURE

Commission Regulation (EU) No 37/2010. Off. J. European Union, **L15**, 1.

Anonymous. 2007. CRL Guidance paper of 7th December 2007. CRLs view on state of the art analytical methods for national residue control plans; [cited 2007 December 7]. Available from: http://www.rivm.nl/bibliotheek/digitaaldepot/crlguidance2007.pdf

13. ORDERING INFORMATION

For ordering the erythromycin ELISA kit, please use cat. code 5151ERY.

14. REVISION HISTORY

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

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