COLISTIN ELISA

(5151COL[5]09.21)

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

EUROPROXIMA COLISTIN ELISA

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

TABLE OF CONTENTS

PAGE:

Brief Information……………………………………………… 2
1. Introduction…………………………………………………… 2
2. Principle of the colistin ELISA……………………………… 3
3. Specificity and Sensitivity…………………………………… 3
4. Handling and Storage……………………………………….. 4
5. Kit contents…………………………………………………… 5
6. Equipment and materials required but not provided……… 6
7. Precautions…………………………………………………… 6
8. Sample preparations………………………………………… 7
9. Preparation of reagents……………………………………… 8
10. Assay Procedure…………………………………………….. 10
11. Interpretation of results……………………………………… 11
12. Literature……………………………………………………… 13
13. Ordering information………………………………………… 13
14. Revision history……………………………………………… 13

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

5151COL[5]09.21

BRIEF INFORMATION

The EuroProxima Colistin ELISA is a competitive enzyme immunoassay for the screening and quantitative detection of colistin in various matrices. 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 with one kit.
The ELISA kit contains all the reagents, including standards, required to perform the test. However, no reagents for sample preparation are included.

1. INTRODUCTION

Colistin A and B are polypeptide antibiotics that have been used in medicine and veterinary for over 50 years. Due to the development of bacterial resistance to colistin European Medicines Agency has recenlty proposed to reduce colistin use in animals and restrict its application only in cases of infections for which no other effective treatments are available.

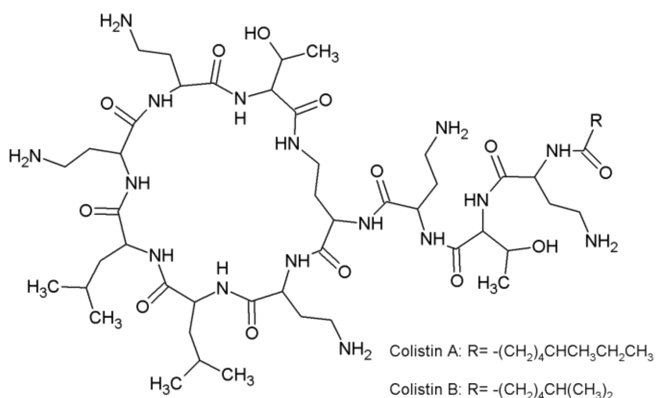


Figure 1: Colistin A and B structures

The Colistin ELISA can be used to screen for the presence of colistin residues in milk, meat, eggs, liver, fish and feed at the levels below the maximum residue limits (MRLs) set in the European Union. MRLs established by Commission Regulation 37/2010 for colistin in tissues of all food producing animals are as follows:

Target tissue	Muscle	Fat	Liver	Kidney	Milk	Eggs
MRL (µg/kg)	150	150	150	200	50	300

2. PRINCIPLE OF THE COLISTIN ELISA

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

3. SPECIFICITY AND SENSITIVITY

The cross-reactivity pattern of the antibody is as follows:

Colistin A and B	100%
Polymyxin B1 and B2	100%
Bacitracin	<0.1%

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 (ppb)	CCβ (ppb)
milk	8.1	4	10
eggs	8.2	22	30
chicken	8.2	12	30
pork	8.2	8	30
beef	8.2	15	30
liver	8.2	21	30
fish	8.2	12	30
feed	8.2	24	30

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.

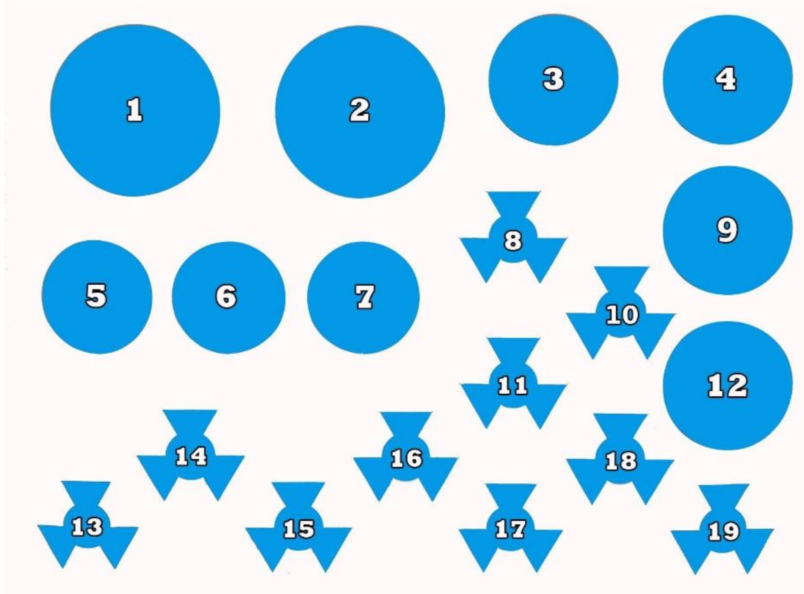
Degeneration of the reagents may have occurred when the following phenomena are observed:

- 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 with antibodies specific to colistin. 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. **Colistin standard** (lyophilized 4 ng/ml, black cap)
6. **Colistin standard** (lyophilized 4 ng/ml, black cap)
7. **Colistin standard** (lyophilized 4 ng/ml, black cap)
8. **Conjugate solution** (100x concentrated)
9. Not in use
10. Not in use
11. Not in use
12. Not in use
13. **Colistin spike standard** (lyophilized 1000 ng/ml, black cap)
14. Not in use
15. Not in use
16. Not in use
17. Not in use
18. Not in use
19. Not in use

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- 4 ml glass tubes
- 15 ml tubes with screw cap (polypropylene)
- Scales and weighing vessels
- Gloves
- Fume hood
- Homogeniser
- Vortex mixer
- Centrifuge (4000 x g)
- Automated microtiter plate washer or 8-channel micropipette 100 – 300 µl
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Micropipette 5 – 20 µl
- Micropipettes, 100 – 1000 µl
- Multipipette with 2.5 ml combitips
- Methanol, anhydrous
- Sulfuric acid (H_2SO_4) 1 M
- Sodium hydroxide (NaOH) 0.1 M
- n-Hexane
- 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.

R-Biopharm Nederland makes no warranty of any kind, either expressed or implied, except that the materials from which its products are made are of standard quality. There is no warranty of merchantability of this product, or of the fitness of the product for any purpose. R-Biopharm Nederland shall not be liable for any damages, including special or consequential damage, or expense arising directly or indirectly from the use of this product.

8. SAMPLE PREPARATION

8.1 Milk

- Pipette 1 ml of milk into a 15 ml polypropylene tube.
- Add 4 ml of distilled water and 1 ml of n-hexane.
- Mix head-over-head for 10 minutes (20ºC - 25ºC).
- Centrifuge the samples at 4000 x g for 10 minutes (20ºC - 25ºC).
- Remove the upper layer using a pipette.
- Add 100 µl of the bottom layer to 400 µl of sample dilution buffer (see chapter 9), vortex.
- Use 50 µl of this solution in the ELISA test.

8.2 Eggs, meat (chicken, pork, beef), liver, fish and feed

- Weigh 1 g of homogenized sample into a 15 ml polypropylene tube.
- Add 3.6 ml of distilled water, 0.4 ml of 1M H_2SO_4 and 1 ml of n-hexane.
- Mix head-over-head for 15 minutes (20ºC - 25ºC).
- Centrifuge at 4000 x g for 10 minutes (20ºC - 25ºC).
- Remove the upper layer using a pipette.
- Pipette 200 µl of the bottom layer into a tube, add 200 µl of 0.1 M NaOH, vortex.
- Dilute 25 µl of this solution further with 475 µl of sample dilution buffer (see chapter 9), vortex.
- Use 50 µl of this solution in the ELISA test.

9. PREPARATION OF REAGENTS

Before starting the assay, the reagents should be brought up to ambient temperature (20°C-25°C). Any unused reagents should be put back into storage immediately at 2ºC to 8ºC.

Microtiter plate
Bring the plate to ambient temperature before opening, to avoid condensation in the wells. Return unused strips into the zip 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 4x concentrated. Dilute the buffer 1:4 (1 ml buffer + 3 ml distilled water) before use. This buffer is for diluting conjugate and to prepare the sample dilution buffer.

Sample dilution buffer
Sample dilution buffer is not provided in the kit. Prepare this buffer as follows: take 18 ml of dilution buffer, add 2 ml of methanol, mix and store this buffer at 4ºC until use.

Standard 4 ng/ml
Prepare a dilution range of colistin standards. Add 2 ml of sample dilution buffer to the colistin standard and mix. This solution contains 4 ng colistin per ml. Pipette 0.25 ml of this solution into a clean tube and add 0.25 ml of sample dilution buffer. Continue to make a dilution range of 2.0, 1.0, 0.5, 0.25 and 0.125 ng/ml.
Three vials of lyophilized colistin are supplied in the kit for preparation of fresh standards.

Conjugate solution (100 µl)
The conjugate is delivered 100x concentrated. Spin down the conjugate in the vial by a short centrifugation step (1 minute, 1000 x g). Add 5 µl of the concentrated conjugate solution to 495 µl of dilution buffer. Per 2 x 8 wells 400 µl is required. Store unused concentrated conjugate at 2°C to 8°C.

Rinsing buffer
The rinsing buffer is delivered 20 times concentrated. Prepare fresh dilution 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. Bring the vial to room temperature (20°C to 25°C, kept in the dark) and mix it well before pipetting into the wells.

Spike standard
Add 2 ml of distilled or deionized water to the lyophilized colistin spike standard vial and mix well to obtain 1000 ng/ml solution. Spike the samples with appropriate volume of the spiking solution to obtain the required spiking level. The recommended spiking

volume is 20-100 µl. If needed, dilute the spiking solution further in water to 100 ng/ml or 50 ng/ml to avoid using very small volumes for spiking.

10. ASSAY PROCEDURE

Rinsing protocol

Unbound components have to be removed efficiently between each incubation step in ELISAs. This is achieved by appropriate rinsing. 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 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 and 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 sample dilution buffer in duplicate (wells H1, H2, blank).
   Pipette 50 µl of the sample dilution buffer (zero standard, Bmax) in duplicate (wells A1, A2).
   Pipette 50 µl of each of the standard solutions in duplicate (wells B1,2 to G1,2 i.e. 0.125, 0.25, 0.5, 1.0, 2.0 and 4.0 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 (colistin-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 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 30 minutes in the dark 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 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 sample dilution buffer/Bmax (wells A1 and A2) and multiplied by 100. The sample dilution buffer/Bmax is thus made equal to 100% (maximal absorbance) and the other O.D. values are quoted in percentages of the maximal absorbance.

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

<u>Calibration curve:</u>
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.

<u>Alternative for calibration curve:</u>
The O.D. values of the standards are plotted on the Y-axis versus the concentration on the X-axis. The scale of the Y-axis is logit and the X-axis is logarithmic.

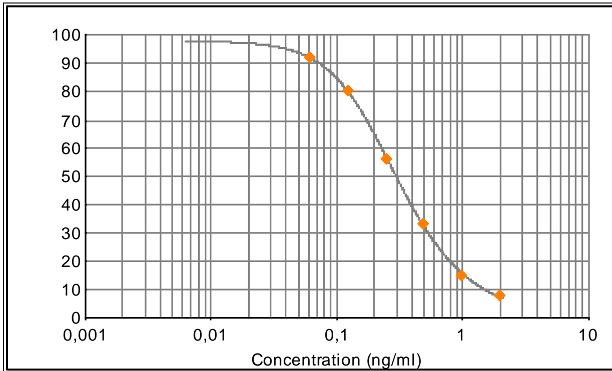


Figure 1 : Example of a calibration curve

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

Calculation factor

8.1 Milk

To obtain the colistin content in milk samples, the equivalents, as read from the standard curve, have to be multiplied by a factor 25.

8.2 Eggs, meat, liver, fish and feed

To obtain the colistin content in eggs, meat, fish and feed, the equivalents, as read from the standard curve, have to be multiplied by a factor 200.

12. LITERATURE

European Medicines Agency. Countries should reduce use of colistin in animals to decrease the risk of antimicrobial resistance. 27 July 2016 EMA/480583/2016.

Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. Official Journal of the European Union L 15/1.

13. ORDERING INFORMATION

For ordering the EuroProxima Colistin ELISA kit, please use the catalogue code 5151COL.

14. REVISION HISTORY

A spike standard is added to the test kit, see chapter 5 and 9.

R-Biopharm Nederland B.V.
Beijerinckweg 18
6827 BN Arnhem
The Netherlands

Tel: + 31 26 3630364
www.europroxima.com
info@r-biopharm.nl