COLISTIN ELISA

(5151COL[1]01.17)

A competitive enzyme immunoassay for screening and quantitative analysis of colistin

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A competitive enzyme immunoassay for screening and quantitative analysis of COLISTIN

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BRIEF INFORMATION

The colistin ELISA is a competitive enzyme immunoassay for measurement of the concentration 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 [1].

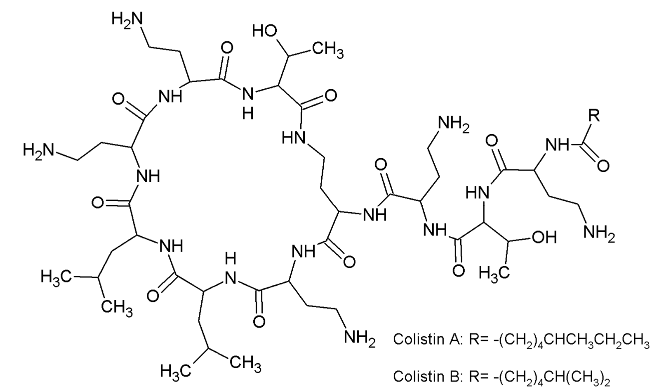


Figure 1: Colistin A and B structures

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 [2]:

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

Cross- reactions:		
	Colistin A and B	100%
	Polymyxin B1 and B2	100%
	Bacitracin	<0.1%

The limit of detection (LOD) is determined under optimal conditions. Cut-off values need critical consideration.

Matrix	Procedure	LOD [ppb]
milk	8.1	4
eggs	8.2	22
chicken	8.2	12
pork	8.2	8
beef	8.2	15
liver	8.2	21
fish	8.2	12
feed	8.2	24

12. LITERATURE

[1] European Medicines Agency. Countries should reduce use of colistin in animals to decrease the risk of antimicrobial resistance. 27 July 2016 EMA/480583/2016.
[2] 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 colistin ELISA kit, please use cat. code 5151COL.

EuroProxima B.V. TEL: + 31 26 3630364
Beijerinckweg 18 FAX: + 31 26 3645111
NL 6827 BN Arnhem Web-site:http://www.europroxima.com
The Netherlands E-mail: info@europroxima.com

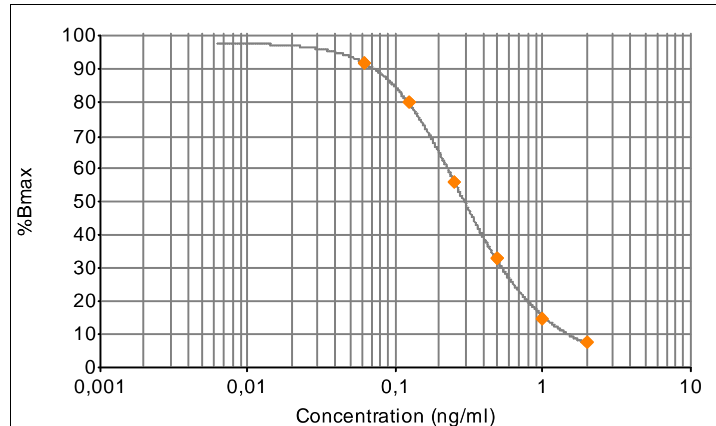


Figure 1 : Example of a calibration curve

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

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.

Remark: "positive" samples have to be confirmed by alternative (e.g. chromatographic) methods.

4. HANDLING AND STORAGE

- Kit and kit components should be stored at 2°C to 8°C in a dark place.
- 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.

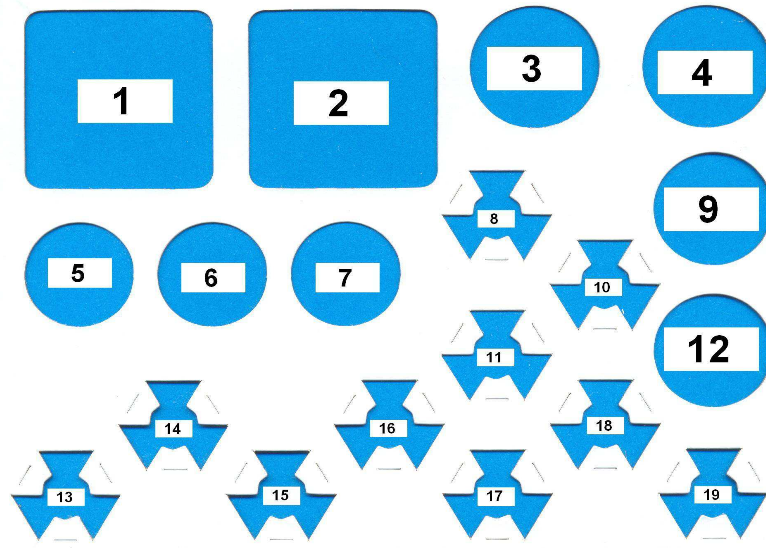
Degeneration of the reagents may have occurred when the following phenomena are observed:
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

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. Not in use
9. **Conjugate solution** (100x concentrated)
10. Not in use
11. Not in use
12. Not in use
13. Not in use
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. 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.

* Sample dilution buffer, see chapter 9.

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.

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

<u>Calibration curve:</u>
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.

<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.

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. Do not allow the wells dry out before the next reagent is dispensed.

Rinsing with automatic microtiter plate wash equipment

When using automatic plate wash equipment, make sure 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 (Sample preparation) and prepare reagents according to Chapter 9 (Preparation of reagents).

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. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- 4 ml glass tubes
- 15 ml tubes with screw cap (Greiner, 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
- H_2SO_4 1 M
- NaOH 0.1 M
- n-Hexane

7. PRECAUTIONS

- Colistin is a toxic compound. Avoid contact with mouth and skin. Avoid breathing vapors or mist.
- The stop reagent contains 0.5 M sulphuric acid. Do not allow the reagent to get into contact with the skin.
- 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.
- TMB is toxic by inhalation, in contact with skin and if swallowed; observe care when handling the substrate.
- Do not use components past expiration date and do not mix components from different serial lots.
- Each well is ultimately used as an optical cuvette. Therefore, do not touch the under surface of the wells, protect from damage and dirt.
- All components should be completely dissolved before use. Pay special attention to the substrate, which crystallises 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
- Pipette 1 ml of milk into a 15 ml polypropylene tube (Greiner).
- 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*, vortex.
- Use 50 µl of the diluted sample 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*, vortex.
- Use 50 µl of this solution in the ELISA test.

* Sample dilution buffer see chapter 9.

9. PREPARATION OF REAGENTS

Before beginning the test, the reagents should be brought up to ambient 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 2ºC to 8ºC for use in subsequent assays. Retain also the strip holder.

Dilution buffer
The dilution buffer (chapter 5 no.1) 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 3x 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.
For prolonged storage freeze aliquots at -20ºC.
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 40 ml of diluted rinsing buffer is used (2 ml concentrated rinsing buffer + 38 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.

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BRIEF INFORMATION

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The ELISA kit contains all the reagents, including standards, required to perform the test. However, no reagents for sample preparation are included.

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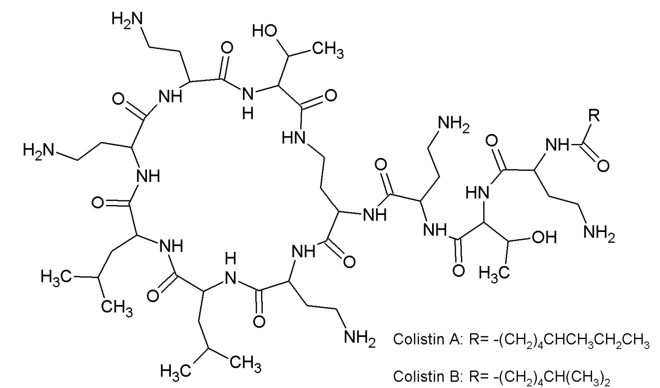

Figure 1: Colistin A and B structures

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 [2]:

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

Cross- reactions:	Colistin A and B	100%
	Polymyxin B1 and B2	100%
	Bacitracin	<0.1%

The limit of detection (LOD) is determined under optimal conditions. Cut-off values need critical consideration.

Matrix	Procedure	LOD [ppb]
milk	8.1	4
eggs	8.2	22
chicken	8.2	12
pork	8.2	8
beef	8.2	15
liver	8.2	21
fish	8.2	12
feed	8.2	24

12. LITERATURE

[1] European Medicines Agency. Countries should reduce use of colistin in animals to decrease the risk of antimicrobial resistance. 27 July 2016 EMA/480583/2016.
[2] 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 colistin ELISA kit, please use cat. code 5151COL.

EuroProxima B.V. TEL: + 31 26 3630364
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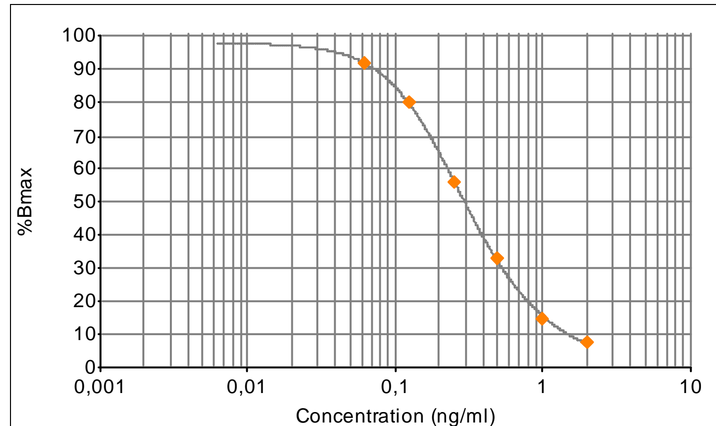


Figure 1 : Example of a calibration curve

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

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.

Remark: "positive" samples have to be confirmed by alternative (e.g. chromatographic) methods.

4. HANDLING AND STORAGE

- Kit and kit components should be stored at 2°C to 8°C in a dark place.
- 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:

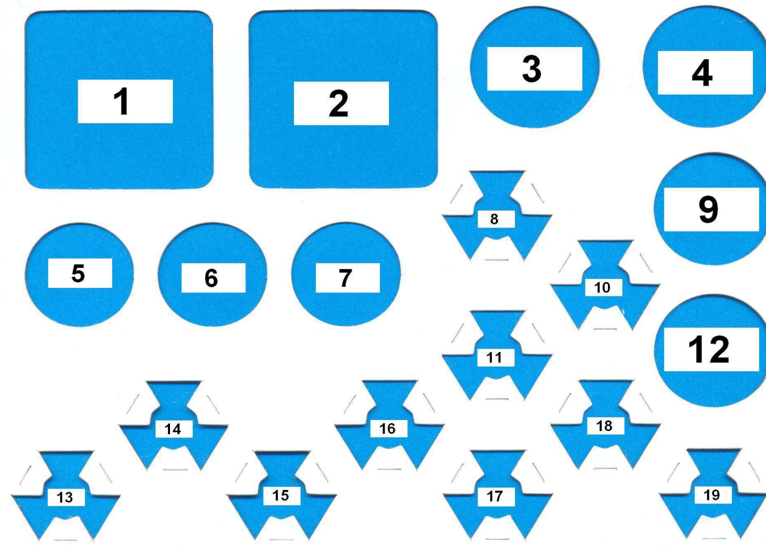
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

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. Not in use
9. **Conjugate solution** (100x concentrated)
10. Not in use
11. Not in use
12. Not in use
13. Not in use
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. 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.

* Sample dilution buffer, see chapter 9.

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.

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

<u>Calibration curve:</u>
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.

<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.

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. Do not allow the wells dry out before the next reagent is dispensed.

Rinsing with automatic microtiter plate wash equipment

When using automatic plate wash equipment, make sure 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 (Sample preparation) and prepare reagents according to Chapter 9 (Preparation of reagents).

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. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- 4 ml glass tubes
- 15 ml tubes with screw cap (Greiner, 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
- H_2SO_4 1 M
- NaOH 0.1 M
- n-Hexane

7. PRECAUTIONS

- Colistin is a toxic compound. Avoid contact with mouth and skin. Avoid breathing vapors or mist.
- The stop reagent contains 0.5 M sulphuric acid. Do not allow the reagent to get into contact with the skin.
- 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.
- TMB is toxic by inhalation, in contact with skin and if swallowed; observe care when handling the substrate.
- Do not use components past expiration date and do not mix components from different serial lots.
- Each well is ultimately used as an optical cuvette. Therefore, do not touch the under surface of the wells, protect from damage and dirt.
- All components should be completely dissolved before use. Pay special attention to the substrate, which crystallises 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

- Pipette 1 ml of milk into a 15 ml polypropylene tube (Greiner).
- 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*, vortex.
- Use 50 µl of the diluted sample 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*, vortex.
- Use 50 µl of this solution in the ELISA test.

* Sample dilution buffer see chapter 9.

9. PREPARATION OF REAGENTS

Before beginning the test, the reagents should be brought up to ambient 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 2ºC to 8ºC for use in subsequent assays. Retain also the strip holder.

Dilution buffer
The dilution buffer (chapter 5 no.1) 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 3x 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.
For prolonged storage freeze aliquots at -20ºC.
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 40 ml of diluted rinsing buffer is used (2 ml concentrated rinsing buffer + 38 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.