PENICILLIN ELISA

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

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EUROPROXIMA PENICILLIN ELISA

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

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BRIEF INFORMATION

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

1. INTRODUCTION

Penicillin's are widely used in veterinary medicine and form the most important group of antibiotics. Penicillin's are strong inhibitors of bacterial growth, have a low toxicity, minimal side effects and are excreted rapidly after absorption.
In order to protect the consumer and secure dairy production the Committee for Veterinary Medical products has recommended maximum residue levels for six penicillin's. Commission Regulation (EU) No 37/2010.

2. PRINCIPLE OF THE PENICILLIN ELISA

The microtiter plate based penicillin ELISA consists of one precoated plate (12 strips, 8 wells each). Antibody, horseradish peroxidase (-HRP) labeled ampicillin and standard solution or sample are added to wells. Free Penicillin from the samples or standards and ampicillin-HRP conjugate compete for the specific antibody binding sites (competitive enzyme immunoassay).
After an incubation step of 1 hour the non-bound reagents are removed in a washing step. The amount of bound ampicillin-HRP conjugate is visualized by the addition of a substrate/chromogen solution (H_2O_2/TMB). Bound ampicillin-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 beta-lactam concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

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

	Cross-reactivity %
Ampicillin	100
Benzylpenicillin (Penicillin G)	100
Azlocillin	99
Piperacillin	88
Amoxicillin	85
Penicillin V	58
Oxacillin	40
Cloxacillin	30
Dicloxacillin	15
Nafcillin	3

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	0.08
Milk powder	8.2	1.52
Butter	8.3	0.43
Salmon	8.4	2.03
Shrimp	8.5	5.00
Chicken meat	8.6	5.00
Turkey meat	8.7	0.89

The LOD value is determined in many other matrices. Validation report PEN[val02]06.18 is available upon request.

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 are stored in a refrigerator (2°C to 8°C) before and immediately after use.
- 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 at ambient (room) temperature before use.
- Avoid condensation in the wells of the plate. Bring the sealed plate at ambient temperature before opening the plate sealing.
- Any direct action of light on the chromogen solution should be avoided.

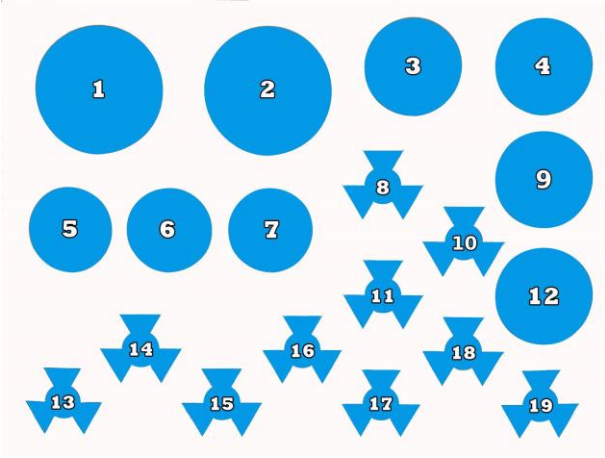
Degeneration of the reagents may have occurred when the following phenomena are observed:

- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or absent colour reaction of the maximum binding (zero standard) (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** (25 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. **Standard** (4 ng/ml ampicillin lyophilized)
6. **Standard** (4 ng/ml ampicillin lyophilized)
7. **Standard** (4 ng/ml ampicillin lyophilized)
8. **Conjugate solution** (100 µl; 100x concentrated)
9. not in use
10. **Antibody solution** (100 µl; 100x concentrated)
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. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Scales and weighing vessels
- Gloves
- Homogeniser (vortex, mixer)
- 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
- Methanol 100%
- Distilled water
- Extraction buffer see chapter 8.2

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
- Vortex the milk sample for 3 seconds
- Dilute 250 µl milk with 250 µl dilution buffer
- Vortex for 3 seconds
- Use 50 µl of the diluted milk sample in the ELISA.

8.2 Milk powder
- weigh 1 g of sample and fill up to 10 ml with deionized water
- Vortex until the sample is dissolved completely
- dilute reconstituted sample 1:2 with sample buffer (e.g. 1 ml sample + 1 ml sample buffer)
- Vortex for 3 seconds
- use 50 µl in the ELISA

8.3 Butter
- weigh 5 g of filtered butter into a 50 ml tube
- add 20 ml of deionized water and incubate at 40°C in a water bath until the butter is melted completely
- homogenise by vortexing
- centrifuge for 10 min at 4000 g at 4°C
- dilute lower aqueous phase 1:4 with sample buffer (e.g. 0.5 ml of lower phare + 1.5 ml of sample buffer)
- use 50 µl per well in the test

8.4 Salmon
- homogenise sample completely
- add 4 ml of deionized water to 1 g of the homogenized sample
- vortex and mix for 15 min (end-to-end shaker)
- centrifuge 10 min at 2000 g at room temperature (20-25 °C)
- dilute supernatant 1:8 with sample buffer (e.g. 50 µl of the supernatant + 350 µl of sample buffer)
- use 50 µl per well in the test

8.5 Shrimp
- homogenise sample completely
- add 4 ml of deionized water to 1 g of the homogenized sample
- vortex and mix for 15 min (end-to-end shaker)
- centrifuge 10 min at 2000 g at room temperature (20-25 °C)
- dilute supernatant 1:8 with sample buffer (e.g. 50 µl of the supernatant + 350 µl of sample buffer)
- use 50 µl per well in the test

8.6 Chicken meat
- homogenise sample completely
- add 4 ml of deionized water to 1 g of the homogenized sample
- vortex and mix for 15 min (end-to-end shaker)
- dilute supernatant 1:8 with sample buffer (e.g. 50 µl of the supernatant + 350 µl of sample buffer)
- use 50 µl per well in the test

8.7 Turkey meat
- homogenise sample completely
- add 4 ml of deionized water to 1 g of the homogenized sample
- vortex and mix for 15 min (end-to-end shaker)
- dilute supernatant 1:8 with sample buffer (e.g. 50 µl of the supernatant + 350 µl of sample buffer)
- use 50 µl per well in the test

9. PREPARATION OF REAGENTS

Before starting 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 fresh 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
This buffer is used for the dilution of conjugate, antibody and samples. The dilution buffer is 4x concentrated. Dilute the buffer 1:4 (10 ml buffer + 30 ml distilled water) before use. The concentrated buffer should be at room temperature (20ºC to 25ºC) and thoroughly mixed. Concentrated buffer can show precipitates, mix well before dilution. The diluted buffer can be stored at 2ºC to 8ºC.

Standard (3 x 4 ng/ml)
Prepare a dilution range of ampicillin standards. Add 2 ml of dilution buffer to the concentrated standard and mix. This solution contains 4 ng ampicillin per ml. Pipette 0.25 ml of this solution into a clean tube and add 0.25 ml of dilution buffer. Continue to make a dilution range of 4.0, 2.0, 1.0, 0.5, 0.25 and 0.125 ng/ml.
For prolonged storage: freeze aliquots at -20ºC.
For preparation of fresh standards 3 vials concentrated ampicillin are supplied in the kit.

Conjugate
The conjugate is 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 dilution buffer. Per 2 x 8 wells 400 µl is required. Store unused concentrated conjugate at 2°C to 8°C.

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

Rinsing buffer
The rinsing buffer is delivered 20x concentrated. Prepare dilutions freshly 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. 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 (Sample preparation) and prepare reagents according to Chapter 9 (Preparation of reagents).

2. Pipette 100 µl of dilution buffer in duplicate (wells H1, H2, blank).
   Pipette 50 µl of dilution buffer (zero standard, Bmax) in duplicate (wells A1, A2).
   Pipette 50 µl of each of the standard 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 25 µl of conjugate (ampicillin-HRP) to all wells, except H1 and H2.

5. Pipette 25 µl of antibody solution to all wells except H1 and H2.

6. Seal the microtiter plate and shake the plate for a few seconds on a microtiter plate shaker.

7. Incubate for 1 hour in the dark at 4°C.

8. Discard the solution from the microtiter plate and wash 3 times with rinsing buffer.

9. Pipette 100 µl of substrate solution into each well.

10. Incubate 30 minutes in the dark at 20ºC to 25ºC.

11. Add 100 µl of stop solution to each well.

12. 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 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.

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

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

Alternative for calibration curve:
The absorbtion 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.

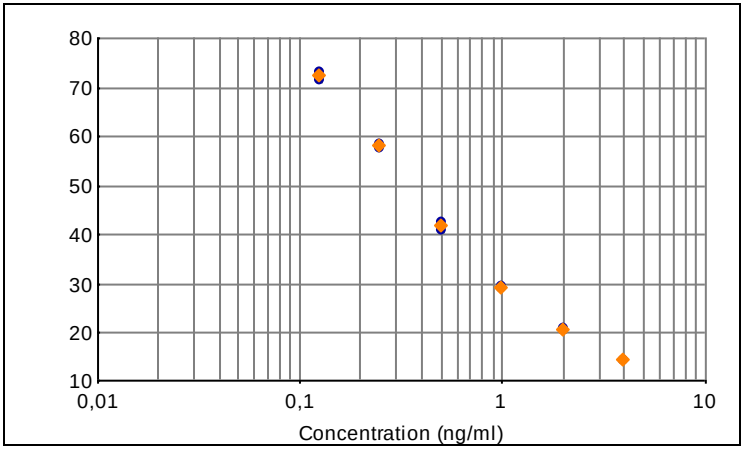


Figure 1: Example of a calibration curve

The amount of penicillins in the samples is expressed as penicillins equivalents. The penicillins 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 penicillins content in milk samples, the calculated penicillin concentration has to be multiplied by a factor 2.

8.2 Milk powder
To obtain the penicillins content in milk powder samples, the calculated penicillin concentration has to be multiplied by a factor 20.

8.3 Butter
To obtain the penicillins content in butter samples, the calculated penicillin concentration has to be multiplied by a factor 20.

8.4 Salmon
To obtain the penicillins content in salmon samples, the calculated penicillin concentration has to be multiplied by a factor 40.

8.5 Shrimp
To obtain the penicillins content in shrimp samples, the calculated penicillin concentration has to be multiplied by a factor 40.

8.6 Chicken meat
To obtain the penicillins content in chicken samples, the calculated penicillin concentration has to be multiplied by a factor 40.

8.7 Turkey meat
To obtain the penicillins content in turkey samples, the calculated penicillin concentration has to be multiplied by a factor 40.

12. LITERATURE

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.

13. ORDERING INFORMATION

For ordering the penicillin ELISA kit, please use cat. code 5091PEN

14. REVISION HISTORY

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

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