Standard Operating Procedure

for

Detection of Penicillin in Bovine Milk and Beef Muscles by Enzyme-Linked Immunosorbent Assay (ELISA)

(ELISA Kit 5091PEN, EuroProxima)

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Issued to	**Lab Manager (LM)**

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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 (MRL) for six penicillin's. Commission Regulation (EU) No 37/2010 (1).

2. SCOPE

This Standard Operating Procedure (SOP) describes the procedure for screening of Penicillin residues in Bovine milk and Beef muscles, using competitive enzyme immunoassay.

3. OBJECTIVE / PURPOSE

To ensure the accuracy, quality and reproducibility of the procedure / protocol.

4. ABBREVIATIONS

4.1. PEN: Penicillin

4.2. ELISA: Enzyme-Linked Immunosorbent Assay

4.3. HRP: Horseradish Peroxidase

4.4. TMB: Tetra Methyl Benzidine (chromogen)

4.5. O.D.: Optical Density

4.6. LOD: Limit of Detection

4.7. WS: Working Standard

4.8. CC: Calibration Curve

4.9. D.F.: Dilution Factor

5. PRINCIPLE OF THE METHOD

The microtiter plate based penicillin ELISA consists of one precoated plate (12 strips, 8 wells each). Antibody, 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.

6. SPECIFICITY AND SENSITIVITY

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

Metabolites	MRL Edible Tissue (ppb)	MRL Milk (ppb)	Cross-reactivity (%)
Ampicillin	50	4	100
Benzylpenicillin (PenicillinG)	50	4	100
Azlocillin	-	-	99
Piperacillin	-	-	88
Amoxicillin	50	4	85
Penicillin V	25	-	58

Oxacillin	300	30	40
Cloxacillin	300	30	30
Dicloxacillin	300	30	15
Nafcillin	300	30	3

No cross reactivity with Cephalosporins is observed.

The LOD is calculated as: Xn+3SD and is determined under optimal conditions.

Matrix	Procedure	LOD
Milk	8.1	0.08
Tissue	8.2	2.5

7. 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).

8. EQUIPMENT AND MATERIALS REQUIRED

- Scalpel and blades
- Analytical Balance (5 decimal points)
- Polythene Gloves
- Homogenizer (HG-15D, DIAHAN Scientific, USA)
- Vortex mixer (VM-10, DIAHAN Scientific, USA)
- Refrigerated Centrifuge 5430R, Eppendorf
- Romer's Evap system of dri-block and sample concentrator
- Microtiter plate washer (ELx50™, BioTek)
- Microtiter plate reader (ELx808™, BioTek) with 450 nm filter
- Micropipettes, 100 – 1000 µL
- Commercial ELISA Kit (5091PEN. EuroProxima)
- Double Distilled water

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.

9.1 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.

9.2 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.

9.3 Working Standard

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 WS 3 vials concentrated ampicillin are supplied in the kit.

9.4 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.

9.5 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.

9.6 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).

9.7 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. SAMPLE PREPARATION

10.1 Bovine 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.

10.2 Beef Tissue

- Weigh 1 gram homogenized sample into a tube
- Add 4 mL distilled water
- Vortex, mix for 15 minutes head over head (rotor)
- Centrifuge (10 minutes, 2000 x g, 20°C to 25°C)
- Dilute 50 µL of the upper layer with 350 µL dilution buffer
- Use 50 µL in the ELISA

11. ASSAY PROCEDURE

11.1 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:

11.2 Manual rinsing

- 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.
- Fill all the wells to the rims (300 µL) with rinsing solution.
- This rinsing cycle (1 and 2) should be carried out 3 times.
- Turn the plate upside down and empty the wells by a firm short vertical
- movement.
- Place the inverted plate on absorbent paper towels and tap the plate firmly to
- remove residual rinsing solution from the wells.
- Take care that none of the wells dry out before the next reagent is dispensed.

11.3 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.

11.4 Assay Protocol

- Prepare samples according and prepare reagents.
- Pipette 100 µL of dilution buffer in duplicate.
- Pipette 50 µL of dilution buffer (zero standard, Bmax) in duplicate.
- Pipette 50 µL of each of the standard in duplicate (0.125, 0.25, 0.5, 1.0, 2.0 and 4.0 ng/mL).

- Pipette 50 µL of each sample solution in duplicate into the remaining wells of the microtiter plate.
- Pipette 25 µL of conjugate (ampicillin-HRP) to all wells.
- Pipette 25 µL of antibody solution to all wells.
- Seal the microtiter plate and shake the plate for a few seconds on a microtiter plate shaker.
- Incubate for 1 hour in the dark at 4°C.
- Discard the solution from the microtiter plate and wash 3 times with rinsing buffer. Pipette 100 µL of substrate solution into each well.
- Incubate 30 minutes in the dark at 20ºC to 25ºC.
- Add 100 µL of stop solution to each well.
- Read the absorbance values immediately at 450 nm.

12. INTERPRETATION OF RESULTS

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.

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

12.1 Calibration curve (CC):

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

12.2 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. The amount of penicillin in the samples is expressed as penicillin equivalents. The beta-lactam equivalents in the samples (ng/mL) corresponding to the % maximal absorbance of each extract can be read from the CC.

12.3 Milk samples

To obtain the penicillin content in milk samples, the calculated penicillin concentration has to be multiplied by a D.F 2.

12.4 Tissue samples

To obtain the penicillin content in tissue samples, the calculated penicillin concentration has to be multiplied by a D.F 40.

13. PRECAUTIONS

- The stop reagent contains 0.5 M sulfuric 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; take care when handling the substrate.
- 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, which crystallizes 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.

14. REFERENCES

1. 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.
2. Insert brochure for commercial ELISA Kit 5091PEN, EuroProxima.