TRIMETHOPRIM ELISA

5101TMP[2]01.24

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

EUROPROXIMA TRIMETHOPRIM ELISA

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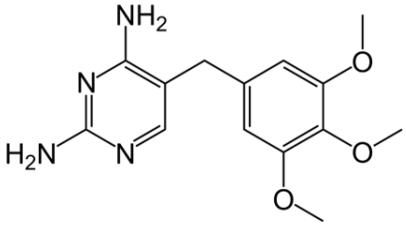
BRIEF INFORMATION

The Trimethoprim ELISA is a competitive enzyme immunoassay for the screening and quantitative detection of Trimethoprim in different commodities. With this ELISA-kit 96 analyses can be performed. Samples and standards are measured in duplicate which means that a total of 41 samples can be analyzed.
The ELISA kit contains all reagents to perform the test. Reagents required for sample preparation are included in the kit.

1. INTRODUCTION



chemical structure of trimethoprim

Trimethoprim (TMP) is a member of the dihyrofolate reductase inhibitors and is widely administered in combination with sulfonamides (among other antibiotics) for the treatment of animal infectious diseases. Maximum residue limits for TMP are established world-wide. In the European Union a maximum residue limit of 50 µg/kg in pork, chicken and fish is in force.

2. PRINCIPLE OF THE TRIMETHOPRIM (TMP) ELISA

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

3. SPECIFICITY AND SENSITIVITY

The TMP ELISA utilizes antibodies raised in rabbit against protein conjugated TMP.

The cross-reactivity pattern of the antibody (as tested in buffer) is:

Trimethoprim 100%

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	**LOD (µg/kg)**	**CCβ (µg/kg)**
Meat	1.42	5
Fish and shrimp	1.79	5
Milk and milk powder	1.92	5
Egg	0.66	4
Urine	1.32	5

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:

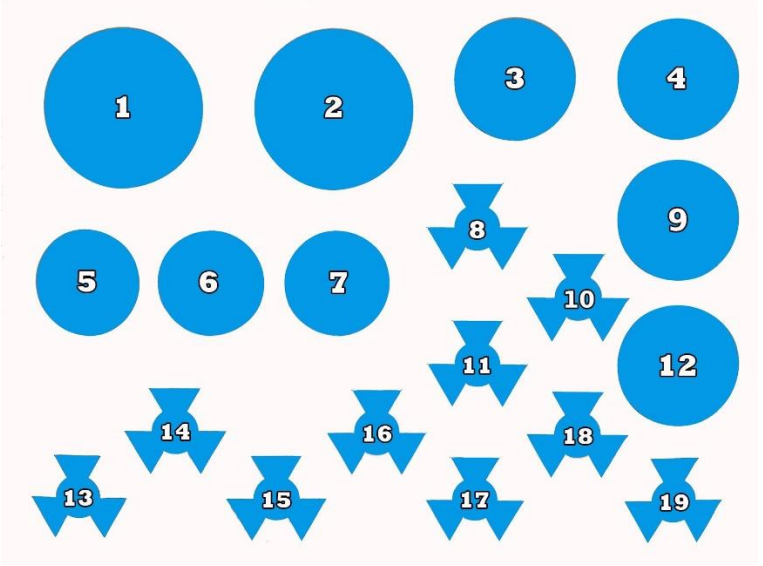
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

5. KIT CONTENTS

Manual

One sealed (96-wells) microtiter plate (12 strips, 8 wells each), coated antibody. 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. **Conjugate** (lyophilised, blue cap)
6. Not in use
7. Not in use
8. Not in use
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 solution 1** (1 ml, ready-to-use) 0.01875 ng/ml
15. **Standard solution 2** (1 ml, ready-to-use) 0.0375 ng/ml
16. **Standard solution 3** (1 ml, ready-to-use) 0.075 ng/ml
17. **Standard solution 4** (1 ml, ready-to-use) 0.15 ng/ml
18. **Standard solution 5** (1 ml, ready-to-use) 0.3 ng/ml
19. Not in use

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- 15 ml tubes with screw caps
- Scales and weighing vessels
- Gloves
- Fume hood
- Homogenizer (vortex, mixer)
- Centrifuge
- Automated microtiter plate washer or 8-channel micropipette 100 – 300 µl
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Glass test tubes (10 – 15 ml)
- Micropipettes, 100 – 1000 µl
- Multipipette with 2.5 ml combi tips
- Aluminum foil or parafilm
- Distilled water (bidest)
- Methanol
- N-hexane

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. Meat, fish, shrimp, egg, milk, milk powder* and urine.

- Homogenize the sample to a fine mass
- Transfer 1 g or 1 ml of the homogenized sample into a screw cap vial, add 3 ml of Methanol, mix well (vortex for a few sec.)
- Add 1 ml of Hexane, mix well (vortex for a few sec.)
- Mix head over head for 10 minutes
- Centrifuge for 10 minutes at 4000x g at 20°C – 25°C
- Remove the upper Hexane layer
- Dilute the sample 30x in dilution buffer
- For example : 20µl of extracted sample to 580µl dilution buffer
- Use 50µl per well in the ELISA test (dilution factor 120)

*Milk powder samples
Reconstitute the milk powder according to manufacturers instruction.

9. PREPARATION OF REAGENTS

Before beginning the test, the reagents should be brought up to room 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 +2°C to +8°C for use in subsequent assays. Retain also the strip holder.

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

Dilution buffer
The dilution buffer is 4 times concentrated. Before dilution (20 ml buffer + 60 ml distilled water) the concentrated buffer should be at room temperature and thoroughly mixed. Concentrated buffer can show precipitates of the contents. Mix well before dilution with distilled water. The diluted buffer can be stored at +2ºC to +8ºC.

Conjugate
Reconstitute the vial of lyophilized conjugate (Trimethoprim-HRP) with 6 ml of dilution buffer, mix thoroughly and keep in the dark until use.

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

Unbound components have to be removed efficiently between incubation steps in ELISA tests. 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.
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 washing solution in 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 in duplicate (wells A1, A2, maximal signal).
   Pipette 50 µl of each of the standard solutions in duplicate (wells B1,2 to F1,2 i.e. 0.01875, 0.0375, 0.075, 0.15 and 0.3 ng/ml).

3. Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate (41 samples; 82 wells).

4. Pipette 50 µl of diluted conjugate (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 30 minutes in the dark 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 15 minutes 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 THE 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 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.

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

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 value of absorption (logit) calculation of the standards are plotted on Y-axis versus the analyte equivalent concentration on a logarithmic X-axis

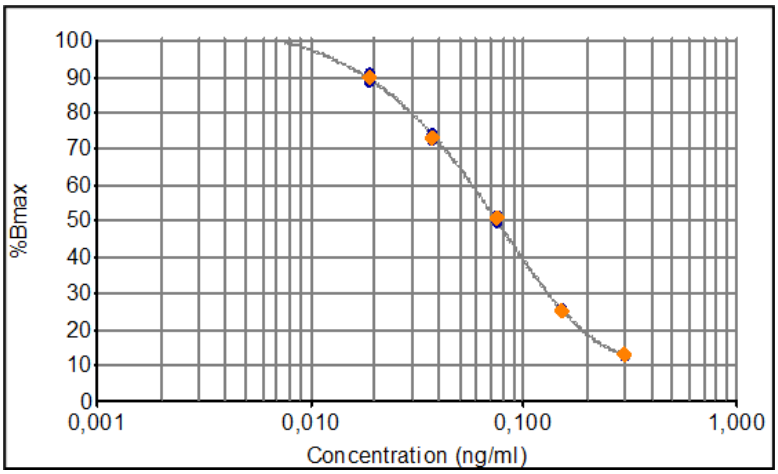


Figure 1: Example of a calibration curve

The amount of Trimethoprim (TMP) in the samples is expressed as TMP equivalents. The TMP equivalents (concentration) corresponding to the % maximal absorbance recorded for each sample can be read from the calibration curve.

8.1. Meat, fish, shrimp, egg, milk, milk powder, urine

The TMP equivalents, as read from the standard curve, should be multiplied by a factor 120 to obtain the TMP content in meat, fish, shrimp, egg, milk, milk powder and urine samples expressed in µg/kg.

12. LITERATURE

- Chair, M., Nelis, H., Leger, P., Sorgeloos, P., & De Leenheer, H. (1996). Accumulation of Trimethoprim, Sulfamethoxazole, and N-Acetylsulfamethoxazole in Fish and Shrimp Fed Medicated Artemia franciscana. *Antimicrobial Agents and Chemotherapy* , 1649–1652.
- EMEA. (2002). Trimethoprim. *European Medicines Evaluation Agency*, 1-2.
- Huovinen, P. (1987). Trimethoprim Resistance. *Antimicrobial Agents And Chemotherapy*, 1451-1456.

13. ORDERING INFORMATION

For ordering the Trimethoprim ELISA kit, please use cat. code 5101TMP.

14. REVISION HISTORY

Not applicable

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