I'screen CAP

Enzyme immunoassay for the detection of chloramphenicol (Cat.nr. HU0050001)

I'screen CAP **(Cat.nr. HU0050001)** is a kit prepared for an immunoenzymatic assay for the quantitative analysis of CAP and its glucuronide (major metabolite in urine). The kit contains the procedures and materials sufficient for 96 determinations (including standards).

Analysable samples
Honey, eggs, urine, water, milk, plasma, muscle (tissues), yoghurt and seafood.

This ELISA kit is not intended to be used for testing any kind of human samples.

Sample preparation
- Water, urine (method I): pH adjustment, dilution (optional)
- Urine (method II): pH adjustment, enzymatic hydrolysis, extraction with ethylacetate, evaporation, reconstitution.
- Serum and plasma: extraction with ethylacetate, centrifugation, evaporation, reconstitution.
- Eggs, tissues, seafood and yoghurt: homogenization, extraction with solvents, centrifugation, evaporation, reconstitution.
- Honey: dilution, extraction with ethylacetate, centrifugation, evaporation, reconstitution.
- Milk: centrifugation, extraction, centrifugation, evaporation, reconstitution.

Assay time: 45 minutes (sample preparation not included).

Detection limit
Urine, water, serum or plasma: 0.1 ppb
Milk: 0.01 ppb
Eggs, honey, yoghurt: 0.05 ppb
Muscle, seafood: 0.025 ppb

Specificity	
Compound	**Cross-reactivity (%)**
Chloramphenicol (CAP)	100
CAP glucuronide	70
Florfenicol	< 0.1
Tiamphenicol	< 0.1

1. TEST PRINCIPLE

The assay is performed in plastic microwells which have been coated with anti-sheep antibodies. Chloramphenicol standard solutions or samples, the enzyme conjugate chloramphenicol -HRP and a specific antibody anti-chloramphenicol are added to the microwells.
During the incubation, free chloramphenicol molecules and chloramphenicol-HRP compete for the anti-chloramphenicol antibodies binding sites. The anti-chloramphenicol antibodies are simultaneously bound to the solid phase. Any unbound substance is then removed in a washing step.
The bound enzyme activity is determined by adding a fixed amount of a chromogenic substrate. The enzyme converts the colorless chromogen into a blue product during the second incubation.
The addition of the stop reagent leads to a color change from blue to yellow. The absorbance is measured by a microplate reader at 450_{nm}. The color development is inversely proportional to chloramphenicol concentration in the sample.

2. REAGENTS PROVIDED

Microtiter plate: 96 wells (12 strips x 8 wells), coated with anti-sheep IgG antibodies.
As the strips are breakable, the wells can be used individually. For this purpose, it is sufficient to get out the wells from the frame and to break the joint.
Chloramphenicol Std: 6 amber plastic vials, each containing 1.5 ml of standard solutions, in the following concentrations: 0 ng/ml; 0.1 ng/ml; 0.2 ng/ml; 0.5 ng/ml; 1 ng/ml; 2 ng/ml.
CAP spiking solution: 1 plastic vial containing 1 ml of 100 ng/ml of chloramphenicol.
Enzyme conjugate:1 plastic vial containing 200 µl of enzyme conjugate.
Enzyme conjugate diluent: 1 plastic bottle containing 12 ml, red solution.
Anti-chloramphenicol antibody: 1 plastic bottle containing 12 ml of antibody, blue solution.
Dilution buffer 5x: 1 plastic bottle containing 50 ml, orange solution.
Washing buffer 10X:1 plastic bottle containing 50 ml.
Developing solution:1 amber plastic bottle containing 24 ml.
Stop solution:1 glass bottle containing 8 ml. White cap.

3. MATERIALS REQUIRED BUT NOT PROVIDED

- Distilled water
- Ethylacetate (for all matrices except urine, method I)
- Hexane (for muscle, seafood, yoghurt and in alternative for eggs)
- Acetic acid (for urine, method II)
- Helix pomatia juice (urine method II)
- Isooctane/chloroform (for eggs)

Equipment
- Vortex
- Homogeneizer
- Balance
- Centrifuge
- Waterbath
- Evaporator
- 20-200 µl micropipette with tips.
- 100-1000 µl micropipette with tips.
- 50 - 200 µl multichannel micropipette with tips.
- Microplate reader equipped with a 450nm filter.

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4. WARNING AND PRECAUTIONS FOR THE

- For *in vitro* diagnostic use only.
- The test is intended for screening samples of animal origin (no human origin) and is not IVD test for human use.
- Some reagents contain solutions that may be identified as dangerous substance by the Regulation (EC) No 1272/2008.
  Please refer to the Material Safety Data Sheet available *on both the Eurofins Tecna* (tecna.eurofins-technologies.com) *and Eurofins Technologies (*eurofins-technologies.com) *web site.*
- Handle the reagents with caution, avoiding contact with skin, eyes and mucous membranes.

5. HANDLING AND STORAGE INSTRUCTIONS

- Store the kit at +2/+8 °C and never freeze.
- **Bring all reagents to room temperature before use (at least 1 hour). ATTENTION:** do not unseal the microplate until it reaches the room temperature**.**
- Reseal the unused strips of the microtiter plate in the bag together with the desiccant bag provided.
- Return all reagents to +2/+8 °C immediately after use.
- Do not use components after the expiration date.
- Do not intermix components from different kit lots.
- Do not use photocopies of the instruction booklet. Follow the original instruction booklet that is included with the kit.
- Do not change the assay procedure, in particular:
  - do not prolong the incubation times,
  - do not incubate the plate at temperatures higher than 25°C,
  - do not shake the plate during the incubations.
- Use accurate and precise micropipettes with suitable tips for dispensing.
- Once started, complete all the steps without interruption.
- The reproducibility of ELISA results depends largely upon the efficiency and uniformity of microwells washing; always keep to the described procedure.
- **Use a single disposable tip for each standard solution and sample to avoid cross-contamination.**
- Do not allow tips to contact the liquid already in the microwells.
- Avoid exposure to direct light during all incubations. It is recommended to cover the microtiter plate without using plate sealers.

6. SAMPLE PREPARATION

6.1 Serum and plasma samples

1) Add 2 ml of ethylacetate to 1 ml of serum/plasma.
2) Vortex for 1 minute.
3) Centrifuge 10 minutes at 2000 g.
4) Transfer 1 ml of the upper layer (ethylacetate) in a glass tube and evaporated at 50°C under a slow air or nitrogen stream.
5) Dissolve the residue in 500 µl of dilution buffer 1X.
6) The dilution factor is 1.

6.2 Water

1) Adjust pH value to 7±0.5. Use directly for 0.1-2 ppb dosage range.
2) Dilute 5x (1+4) with dilution buffer 1X for 0.5-10 ppb dosage range.
3) Dilute 10x (1+9) with dilution buffer 1X for 1-20 ppb dosage range.

6.3 Egg

1) Weigh 4 g of the homogenized sample in a tube.
2) Add 6 ml of ethylacetate and shake thoroughly for 10 minutes.
3) Centrifuge 10 minutes at 2000 g.
4) Transfer 3 ml of the ethylacetate in a glass tube and evaporate at 50°C under a slow air or nitrogen stream.
5) Dissolve the residue in 1.5 ml of isooctane/chloroform (2:3; v:v).
6) Add 1 ml of dilution buffer 1x. Vortex for 1 minute or mix on a rotatory wheel for 30 minutes.
7) Centrifuge 10 minutes at 2000 g.
8) Leave the tube in a water bath at 80°C for 5 minutes in order to eliminate the emulsion in the upper layer.
9) Centrifuge 10 minutes at 2000 g.
10) The upper layer is ready to use.
11) The dilution factor is 0.5.

Alternatively (from point 4):

5) Dissolve the residue in 1.5 ml of hexane
6) Add 1 ml of dilution buffer 1x. Vortex for 1 minute or mix on a rotatory wheel for 30 minutes.
7) Centrifuge 10 minutes at 2000 g.
8) Leave the tube in a water bath at 80°C for 5 minutes in order to eliminate the emulsion in the upper layer.
9) Centrifuge 10 minutes at 2000 g.
10) The lower layer is ready to use.
11) The dilution factor is 0.5.

6.4 Honey

1) Weigh 2 g of sample in a test tube
2) Add 4 ml of distilled water.
3) Warm the diluted sample to 60 °C for few minutes.
4) Add 4 ml of ethyl acetate and shake thoroughly for 10 minutes.
5) Centrifuge 10 minutes at 2000 g.
6) Transfer 2 ml of ethyl acetate (upper phase) in a glass tube. **ATTENTION**: the solution must be clear; if necessary, centrifuge again.
7) Evaporate at 50°C under a slow air or nitrogen stream.
8) Dissolve the residue in 500 µl of dilution buffer 1x.
9) The dilution factor is 0.5.

6.5 Muscle and seafood

1) Finely grind the sample.
2) Weigh 4 g of the homogenized sample.
3) Add 6 ml of ethylacetate and shake thoroughly for 10 minutes.
4) Centrifuge 10 minutes at 2000 g.
5) Transfer 3 ml of the ethylacetate in a glass tube and evaporate at 50°C under a slow air or nitrogen stream.
6) Dissolve the residue in 1 ml of hexane.
7) Add 500 µl of dilution buffer 1x. Vortex for 1 minute.
8) Centrifuge 10 minutes at 2000 g.
9) Leave the tube in a water bath at 80°C for 5 minutes in order to eliminate the emulsion in the interface.
10) Centrifuge 10 minutes at 2000 g.
11) Remove upper layer and interface.
12) The lower layer is ready to use.
13) The dilution factor is 0.25.

6.6 Urine

Method I:

1) Adjust pH value to 7±0.5. Use directly for 0.1-2 ppb dosage range.
2) Dilute 5x (1+4) with dilution buffer 1X for 0.5-10 ppb dosage range.
3) Dilute 10x (1+9) with dilution buffer 1X for 1-20 ppb dosage range.

Method II:

1) Collect 1 ml of urine sample
2) Adjusted pH value to 4.8 by adding a few drops of 1M acetic acid.
3) Add 25 µl of *Helix Pomatia* juice.
4) Incubate for 2 hours at 55°C or overnight at 37°C.
5) Adjust pH value to 7±0.5.
6) Add 2 ml of ethylacetate and vortex for 1 minute.
7) Centrifuge the sample 10 minutes at 2000g.
8) Transfer 1 ml of the upper phase (ethylacetate) in a glass tube and evaporate at 50°C under a slow air or nitrogen stream.
9) Dissolve the residue in 500 µl of dilution buffer 1x.
10) The dilution factor is 1.

6.7 Milk

After milking, the milk has to be tested within 24 hours. Otherwise milk has to be stabilized (with Bronopol or similar substances) ***ATTENTION:*** *if stabilization of milk samples is required, do not use preservatives containing sodium azide or chloramphenicol (ex. Azidiol).*

1) Centrifuge at+2/+8°C 10 minutes at 2000g.
2) Add 5 ml of ethylacetate to 2.5 ml of defatted milk
3) Vortex for 1 minute
4) Centrifuge 5 minutes at 2000g.
5) Transfer 4 ml of the upper phase (ethylacetate) in a glass tube and evaporate at 50°C under a slow air or nitrogen stream
6) Dissolve the residue in 200 µl of dilution buffer 1x.
7) The dilution factor is 0.1.

6.8 Yogurt

1) Weigh 4 g of the homogenized sample in a tube.
2) Add 6 ml of ethylacetate and shake thoroughly for 10 minutes.
3) Centrifuge 10 minutes at 2000 g.
4) Transfer 3 ml of the ethylacetate in a glass tube and evaporate at 50°C under a slow air or nitrogen stream.
5) Dissolve the residue in 1.5 ml of hexane
6) Add 1 ml of dilution buffer 1x.
7) Vortex for 1 minute
8) Centrifuge 10 minutes at 2000 g.
9) The lower layer is ready to be tested.
10) The dilution factor is 0.5.

7. WORKING SOLUTIONS PREPARATION

Chloramphenicol Std: ready to use.
Enzyme conjugate diluent: ready to use.
Enzyme conjugate: **ATTENTION***: in order to recover the total amount of the conjugate, before use, centrifuge the vial for some seconds at low speed (spin-down).*
Calculate and prepare the necessary quantity for the experiment. Dilute the conjugate **1/200** in the enzyme diluent.

ATTENTION: In order not to take less than 20 µl of concentrate enzyme conjugate make two consecutive dilutions.
For example: prepare a semi concentrate **1/20** (take 20 µl of concentrate enzyme conjugate + 380 µl of enzyme diluent) and prepare the ready-to-use conjugate by diluting the semi concentrate **1/10** (take 200 µl of semi concentrate conjugate + 1800 µl of enzyme diluent). Shake gently. DO NOT VORTEX
Anti-chloramphenicol antibody: ready to use.
Dilution buffer 5x: dilute the concentrate 1:5 (1 ml+4 ml) with distilled water.
Washing buffer: dilute the concentrate 1:10 (1+9) with distilled water. **ATTENTION**: in presence of crystals, bring the solution at room temperature and stir in order to solve them completely.
The diluted washing buffer is stable at room temperature for 24 hours and at +2/+8°C for two weeks.
Developing solution: ready to use. **ATTENTION**: the solution is light sensitive and must be stored away from direct light.
Stop solution: ready to use. **ATTENTION**: it contains 1M sulphuric acid. Handle with care; in case of contact flush immediately with plenty of water.

8. ASSAY PROCEDURE

1) Predispose an assay layout, recording the standard and samples positions, taking into account that all have to be run in duplicate.
2) First incubation
   - Add 50 µl of each standard/ sample into the corresponding wells.
   - Using the multichannel pipet, add 50 µl of enzyme conjugate in each well.
   - Using the multichannel pipet, add 100 µl of antibody in each well.
   - Shake the plate gently with rotatory motion for few seconds.
   - Incubate 30 minutes at room temperature.
   - Do not prolong the first incubation time and do not use automatic shakers.
3) Washing
   - Pour the liquid out from the wells.
   - Fill completely all the wells with working wash solution using a squeeze bottle. Pour the liquid out from the wells.
   - Repeat the washing sequence four (4) times.
   - Remove the remaining droplets by tapping the microplate upside down vigorously against absorbent paper.

Do not allow the wells to dry out

4) Developing
   - Using the multichannel pipet, add 200 µl of developing solution to each well.
   - Mix thoroughly with rotatory motion for few seconds.
   - Incubate for 15 minutes at room temperature.
5) Using a multichannel pipet, add 50 µl of stop solution to each well and mix thoroughly with rotatory motion for few seconds.
6) Measure the absorbance at 450 nm.
7) Read within 30 minutes.

9. CALCULATION OF RESULTS

- Calculate the mean absorbance of each standards and samples.

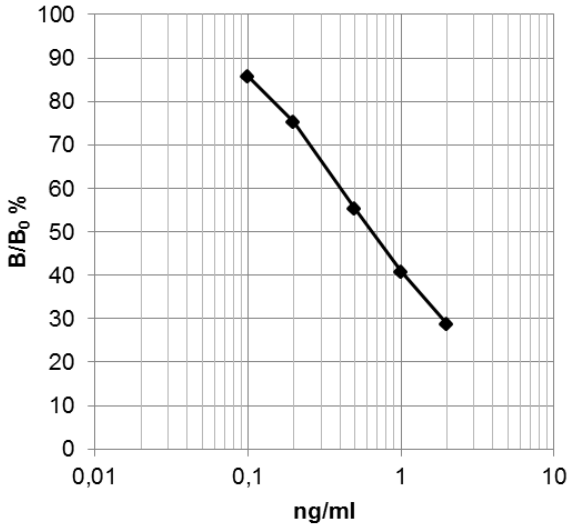
- Divide the mean absorbance value of each standard and sample by the mean absorbance of the Standard 0 (B_0) and multiply by 100; the Maximum Binding is thus made equal to 100% and the absorbance values are quoted in percentage:

$$\frac{\text{standard (or sample) absorbance}}{\text{standard 0 } (B_0) \text{ absorbance}} \times 100 = \frac{B}{B_0} (\%)$$

- Enter the B/B_0 values calculated for each standard in a semi-logarithmic system of coordinates and draw the standard curve.
- Interpolate B/B_0 value for each sample to the corresponding concentration from the calibration curve. Multiply this concentration for the dilution factor, as reported in chapter 6.

Please note: For results calculation, Excel spreadsheets are available on the Eurofins Tecna website (tecna.eurofins-technologies.com) and can be downloaded directly from the bottom of the product page.

10. STANDARD CURVE EXAMPLE



11. EVALUATION OF RESULTS

After processing the results, it is necessary to verify the assay performance. The verification is performed by comparison of obtained data with those given in the kit specifications (chapter 12).
If the values are out of specifications, then the results of the test are not assured, therefore the chloramphenicol concentration levels in the samples may not be valid.
In these cases, it is advised to check the expiry date of the kit, the wavelength at which the reading was performed, as well as the procedure followed. If operation errors do not emerge, contact our technical assistance.

WARNING: Replacements are only possible if the original component is returned. If any kind of substitution is requested, please STORE THE KIT in its integral version at +2/+8°C.

12. KIT SPECIFICATIONS

12.1 Assay specifications

Mean B_0 absorbance	$\geq$ 0.7 OD_{450nm}
B/B_0 50%	0.3 – 1.2 ng/ml
Std duplicates mean C.V.	$\leq$ 6 %

12.2 Assay performance

The kit performances hereby presented are from an in-house validation; Detection Capability (CCβ) was calculated as requested by of EU Decision 657/2002.

Detection Capability (CCβ)		
Analyte	**Muscle and seafood (ppb)**	**Honey, yoghurt (ppb)**
Chloramphenicol	0.1	0.15

Recovery	
Matrix	**%**
Muscle and seafood	75 ± 7
Yoghurt	115 ± 21

13. LIABILITY

Samples evaluated as positive using the kit have to be re-tested with a confirmation method.
Eurofins Technologies Hungary shall not be liable for any damages to the customer caused by the improper use of the kit and for any action undertaken as a consequence of results.
Eurofins Technologies Hungary shall not be liable for the unsafe use of the kit out of the current European safety regulations.

14. LITERATURE

Diana F., Puppini B., Bacer V., Postogna E., Persic L. and Paleologo M. Rapid and sensitive detection of antimicrobial residues in honey by binding assays. Poster presentation at the International Conference on Beekeeping Development and Honey Marketing. 30 October - 1 November, 2010 - Hanoi, Vietnam.

Zeleny R., Emteborg H. and Schimmel H. (2010). Assessment of commutability for candidate certified reference material ERM-BB130 "chloramphenicol in pork". Analytical and bioanalytical chemistry, 398(3), 1457-1465