BZERO QUINO

Enzyme immunoassay for the detection of quinolones (Cat.nr. HU0050005)

BZERO **QUINO** is a kit prepared for a competitive immunoenzymatic assay for the quantitative analysis of quinolones.
The kit contains the procedure and the materials sufficient for 96 determinations.
For result evaluation a microtiter plate or strip photometer is required (manual or automatic ELISA reader).

Type of samples that can be analyzed (matrices)
Muscle, fish, shrimp, egg, honey.

Sample preparation
- Muscle, fish, shrimp, egg, honey (procedure I): homogenization, extraction, centrifugation, dilution.
- Honey (procedure II): homogenization, solvent extraction, centrifugation, evaporation, reconstitution.

Assay time: 75 minutes (sample preparation not included).

Detection limit
- Muscle, fish, shrimp, egg: 0.60 ppb
- Honey (procedure I): 3 ppb
- Honey (procedure II): 0.72 ppb

Specificity	
Analyte	**Cross-reactivity (%)**
Oxolinic acid	>100
Norfloxacin	>100
Danofloxacin	>100
Enrofloxacin	100
Ciprofloxacin	100
Flumequine	81
Difloxacin	49
Sarafloxacin	43
Marbofloxacin	20

1. TEST PRINCIPLE

The assay is performed in plastic microwells which have been coated with the antigen.
Quinolones standard solutions, samples and a specific antibody anti-quinolones are added to the microwells
During the first incubation, free -quinolones molecules and the antigen coated on the solid phase, compete for the anti-quinolones antibody binding site. Any unbound substance is then removed in a washing step.
The enzyme conjugate is then added; during the second incubation it binds to the anti-quinolones antibodies that are bound on the solid phase.
After a washing step, the enzyme conjugate-antibody bound on the solid phase is detected by the addition of a colourless substrate/chromogen solution, which during the third incubation is converted by the enzyme into a blue coloured reaction product.
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 quinolones concentration in the sample.

2. PROVIDED REAGENTS

Microtiter plate: 96 wells (12 strips x 8 wells), coated with the antigen.
As the strips are breakable, the wells can be used individually. For this purpose, it is sufficient to break the joint.
Std 0: 1 plastic vial containing 3 ml of enrofloxacin standards of 0 ng/ml. **ATTENTION: Only the standard zero is provided. B/B0 values of calibration curve (0.04 - 0.8 ng/ml) are reported in the kit certificate of analysis.**
Enrofloxacin spiking solution 100 ng/ml: 1 plastic vial containing 1 ml of 100 ng/ml of Enrofloxacin.
Anti-quinolones Antibody: 1 plastic vial containing 8 ml. Blue solution.
Enzyme Conjugate: 1 plastic vial containing 14 ml. Red solution.
Extraction buffer 5x: 1 plastic bottle containing 50 ml.
Washing-buffer 20x: 1 plastic bottle containing 50 ml.
Developing solution: 1 plastic bottle containing 14 ml.
Stop solution: 1 glass bottle containing 8 ml. White cap.

3. MATERIALS REQUIRED BUT NOT PROVIDED

- Distilled water.
- Methanol
- Honey dilution buffer 10X: Potassium Phosphate buffer 0.1M, 9%, sodium chloride pH 7.4 (Eurofins Tecna Cat.nr.AB688)
- Acetonitrile and dichloromethane (honey, procedure II)
- HCl
- Tween 20

Equipment:
- Tissue homogenizer (for shrimp, fish and muscle analysis)
- Vortex
- Shaker

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- Evaporator
- Lab centrifuge (4000 g) and centrifuge tubes
- Balance
- Spectrophotometer and plastic cuvettes, or 96 wells inert microtiter plate (for honey analysis)
- 20-200 µl, micropipettes, tips.
- 100-1000 µl, micropipettes, tips.
- 50-250 µl, multichannel micropipette, tips.
- Microplate reader, filter 450nm

4. WARNING AND PRECAUTIONS FOR THE USERS

- The test is for *in vitro* diagnostic use only.
- Some reagents contain solutions that may be identified as dangerous substance by the Regulation (EC) *N° 1272/2008.* Please refer to *Material Safety Data Sheet* available on both the Eurofins Technologies and Eurofins Tecna (*tecna.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.
- **ATTENTION: It is strongly recommended to be careful to avoid cross-contamination events, especially when performing spiking experiments.**
- **ATTENTION: Avoid exposure to direct light during all incubations. It is recommended to cover the microtiter plate without using plate sealers.**

6. SAMPLES PREPARATION

6.1 Shrimp

1) Remove the shell and the gut from shrimp sample.
2) Homogenize the sample.
3) Weigh 1 g of homogenized sample into a 15 ml centrifuge tube.
4) Add 2 ml of 55% methanol in extraction buffer 1X (i.e.: to prepare 50 ml solution: 27.5 ml of 100% methanol + 10 ml extraction buffer 5x + 12.5 ml distilled water)
5) Vortex for 30 seconds.
6) Shake thoroughly for 10 minutes.
7) Centrifuge at 4000 g for 10 minutes.
8) Collect the upper phase and dilute 1:5 in extraction buffer 1x (i.e.: 100 µl sample + 100 µl of extraction buffer 5x + 300 µl of distilled water).
9) The dilution factor is 15.

6.2 Fish, muscle (bovine, swine and chicken), egg

1) Homogenize the sample.
2) Weigh 1 g of homogenized sample into a 15 ml centrifuge tube.
3) Add 2 ml of 55% methanol in distilled water. (i.e.: to prepare 50 ml solution: 27.5 ml of 100% methanol + 22.5 ml distilled water)
4) Vortex for 30 seconds.
5) Shake thoroughly for 10 minutes.
6) Centrifuge at 4000 g for 10 minutes.
7) Collect the upper phase and dilute 1:5 in extraction buffer 1x (i.e.: 100 µl sample + 100 µl of extraction buffer 5X + 300 µl of distilled water).
8) The dilution factor is 15.

6.3 Honey

ATTENTION: Choose the procedure according to the honey type.

Procedure I

The procedure cannot be used for chestnut honey, for pure chestnut honey and for honey samples with an OD_{450nm} ≥ 0.15 (measured according to the method described below), because of the risk of false positive results.

If the USDA classification is used, honey samples lighter than or equal to Extra White are always analysable. Darker samples have to be measured as follows.

1) Homogenise the sample.
2) Weigh 1 g of honey and add 3 ml of distilled water.
3) Mix until the sample is completely dissolved.
4) Add 6 ml of 80% Methanol in distilled water.
5) Shake thoroughly for 10 minutes.
6) Centrifuge at 7000 g for 5 minutes.
7) Read the supernatant absorbance at 450nm, by subtracting the blank value prepared in the same way but without the honey.

Two different procedures can be followed:

a) Transfer 300 µl of sample extract into a 96 wells inert microtiter plate and read absorbance using an ELISA microtiter plate reader.

b) As an alternative, transfer 1 ml of sample extract into a plastic cuvette (1 cm optical path) and read the absorbance using a spectrophotometer.

If the OD is < 0.15, proceed as follows. Otherwise move to procedure II.

8) Prepare the 3.4% methanol in honey dilution buffer 1X. To prepare 50 ml buffer: 1.7 ml 100% methanol + 5 ml honey dilution buffer 10X (prepared as described in par. 3) + 43.3 ml of distilled water.
9) Dilute the supernatant 7.5 fold (i.e. 100 µl sample + 650 µl buffer).
10) The dilution factor is 75.

Procedure II

This procedure has **to be used only** for (**a**) pure chestnut honey or (**b**) honey containing chestnut and honey with $OD_{450} \geq 0.15$.

1) Homogenise the sample and weight 0.5 g
2) Add 3 ml of 90% acetonitrile solution, 15 mM HCl. For example, to prepare 50 ml solution: 45 ml acetonitrile + 5 ml of HCl 150 mM.
3) Mix gently on a rotary shaker for 10 minutes.
4) Add 1.5 ml Potassium Phosphate buffer 10 mM pH 7.4 containing 0.05% tween 20. i.e.: to prepare a 50 ml solution: 5 ml Potassium Phosphate buffer 0.1M pH 7.4 + 0.1 ml of 25% tween 20 solution + 44.9 ml distilled water.
5) Vortex at least 1 minute, until the complete melting of the sample.
6) Centrifuge at 4000 g for 10 minutes.
7) Collect 2 ml of the upper organic phase in a glass vial with 4 ml of dichloromethane.
8) Shake thoroughly for 5 minutes.
9) Centrifuge at 2000 g for 10 minutes.
10) Transfer 0,2 ml of the upper organic phase in a glass vial and evaporate at 50 – 60°C under a slow air or nitrogen stream. (**ATTENTION**: Collect the upper phase carefully, do not mix the two different phases; in case of mixing centrifuge again).

a) Pure chestnut honey:

11) Reconstitute with 1.2 ml of washing buffer containing 5% methanol (for example, to prepare a 50 ml solution: 2.5 ml of washing buffer 20X + 2.5 ml of 100% methanol + 45 ml distilled water)
12) Vortex for 1 minute.
13) The dilution factor is 18.

b) Other honey:

11) Reconstitute with 1.2 ml of honey dilution buffer containing 10% methanol (for example, to prepare 50 ml solution: 5 ml of honey dilution buffer 10x + 5 ml of 100% methanol + 40 ml of distilled water).
12) Vortex for 1 minute.
13) The dilution factor is 18.

7. WORKING SOLUTIONS PREPARATION

Std.0: ready to use.
Antibody: ready to use.
Enzyme conjugate: ready to use.
Washing buffer: dilute the concentrated buffer **1/20** (1+19) 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.
Extraction buffer 5x: dilute the concentrated buffer **1/5** (1+4) with distilled water. **ATTENTION**: In presence of crystals, bring the solution at room temperature and stir in order to solve them completely.
Developing solution: ready to use; this solution is light sensitive: keep 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 antibody in each well.
   - Shake the plate gently with rotatory motion for few seconds.
   - Incubate 30 minutes at room temperature, away from direct light.
   - 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) Second incubation
   - Using the multichannel micropipette, add 100 µl of enzyme conjugate in each well
   - Shake the plate gently with rotatory motion for few seconds.
   - Incubate 30 minutes at room temperature, away from direct light.
   - Do not prolong the first incubation time and do not use automatic shakers.
5) Repeat step 3
6) Developing
   - Using the multichannel pipet, add 100 µl of developing solution to each well.
   - Mix thoroughly with rotatory motion for few seconds.
   - Incubate for 15 minutes at room temperature, away from direct light
7) Using a multichannel pipet, add 50 µl of stop solution to each well and mix thoroughly with rotatory motion for few seconds.
8) Measure the absorbance at 450 nm.
9) Read within 15 minutes.

9. CALCULATION OF RESULTS

- Calculate the mean absorbance of each sample and standard 0
- Divide the mean absorbance value of each sample by the mean absorbance of the standard 0 (B_0) and multiply by 100; the standard 0 is thus made equal to 100% and all the other absorbance values are expressed as percentages:

$$\frac{\text{Sample absorbance}}{\text{Standard 0 } (B_0) \text{ absorbance}} \times 100 = \frac{B}{B_0}(\%)$$

- Enter the B/B_0 values provided in the certificate of analysis for each standard (0.04; 0.08; 0.16; 0.32; 0.80 ng/ml) in a semi-logarithmic system of coordinates against the quinolones concentration and draw the standard curve
- Interpolate the B/B_0 value of each sample to the corresponding concentration from the calibration curve. For dilution applications multiply this concentration for the dilution factor, as reported in chapter 6.

Please note: to build the calibration curve use the "point to point" curve. Excel spreadsheet are available on the website tecna.eurofins-technologies.com and can be downloaded directly from the bottom of the product page.

10.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 kit specifications (see chapter 11).
If the values are outside the specifications given, then the results of the test are not assured, therefore the quinolones 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 are not identified as cause, contact our technical assistance.

In order to avoid false positive results, for honey and fish analysis, it is necessary to adopt a decision limit (CCα). It is suggested to adopt an internal CCα. For further information contact the technical assistance

WARNING: Kit replacement will only be possible in case of return. The kit must be stored in its integral version at +2/+8°C.

11. KIT SPECIFICATIONS

11.1 Assay specification

Description	Specifications
Mean B_0 absorbance	$\geq$ 0.7 OD_{450nm}

11.2 Assay performances

The kit performances were assessed within an *in-house* validation. The Detection Capability (CCβ) was calculated in compliancy to EU Decision 657/2002. A performance data sheet (PDS) is available upon request.

Detection capability (CCβ)	
Shrimp	2 ppb sarafloxacin
Fish (iridescent shark)	3 ppb sarafloxacin
Muscle	5 ppb sarafloxacin
Egg	2 ppb sarafloxacin
Honey procedure I	5 ppb enrofloxacin
Honey procedure II	5 ppb enrofloxacin 5 ppb ciprofloxacin

12. 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.