INSTRUCTION FOR USE

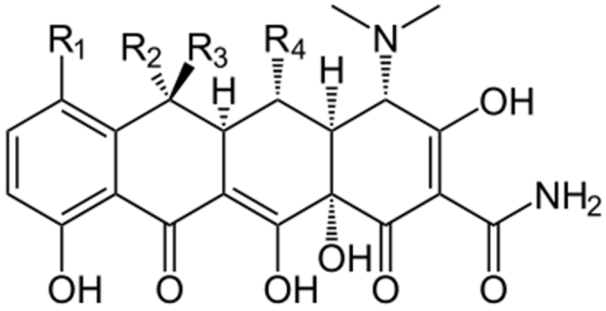
SENSI*Spec* ELISA Tetracycline 96 Tests

(Cat. nr. HU0050014)

Enzyme Immunoassay for the Quantitative Determination of Tetracycline in Food

Sensitivity	0.024 ng/mL
Recovery (spiked samples)	82 - 103%
Incubation Time	80 min

1. GENERAL INFORMATION



Tetracyclines belong to the group of antibiotics. The first identified tetracycline, *Chlortetracycline,* was isolated from Streptomyces aureofaciens by Benjamin Minge Duggar in 1948. In the following years many other tetracyclines were detected and characterized. Tetracyclines bind specifically to prokaryotic ribosomes, thereby inhibiting bacterial protein biosynthesis. In most countries tetracyclines are accepted in food production. Since the ingestion of tetracyclines presents a potential risk to the consumer the maximum amount of tetracycline residues in food is regulated in most countries. For example, in the EU Regulation No 37/2010 the maximum residue limits for the sum of the parent drug and its 4-epimer are defined as 100 µg/kg (ppb) for muscle and milk. Thus, a monitoring of food with respect to the concentration of tetracyclines is obligatory.

The **SENSI*Spec* ELISA Tetracycline** represents a highly sensitive detection system and is particularly capable of the rapid quantification of tetracycline contaminations in meat, milk, milk powder, cheese, shrimps and honey.

2. PRINCIPLE OF THE TEST

The **SENSI*Spec* Tetracycline** quantitative test is based on the principle of the enzyme-linked immunosorbent assay. An antibody directed against tetracyclines is coated on the surface of a microtiter plate. Tetracyclines containing samples or standards and a tetracycline-peroxidase conjugate are given into the wells of the microtiter plate. The conjugate competes with the tetracyclines of the samples/standards for the limited number of antibody sites. After 60 minutes of incubation at room temperature the wells are washed with diluted washing solution to remove unbound material. A substrate solution is added and incubated for 20 minutes, resulting in the development of a blue colour. The colour development is inhibited by the addition of a stop solution, and the colour turns yellow. The yellow colour is measured photometrically at 450 nm. The concentration of tetracyclines is indirectly proportional to the colour intensity of the test sample.

3. PRECAUTIONS

Full compliance of the following good laboratory practices (GLP) will determine the reliability of the results:

1) Prior to beginning the assay procedure, bring all reagents to room temperature (20-25°C).
2) All reagents should be mixed by gentle inversion or swirling prior to use. Do not induce foaming.
3) Once the assay has been started, all subsequent steps should be completed without interruption and within the recommended time limits.
4) Replace caps in all the reagents immediately after use. Do not interchange vial stoppers.
5) Use a separate disposable tip for each specimen to prevent cross-contamination.
6) All specimens and standards should be run at the same time, so that all conditions of testing are the same.
7) Do not mix components from different batches.
8) Do not use reagents after the expiration date.
9) Check both precision and accuracy of the laboratory equipment used during the procedure (micropipettes, ELISA reader etc.).

4. HEALTH AND SAFETY INSTRUCTIONS

1) Do not smoke or eat or drink or pipet by mouth in the laboratory.
2) Avoid contact of substrate and stop solution with skin and mucosa (possible irritation, burn or toxicity hazard). In case of contact, rinse the affected zone with plenty of water.
3) Handling and disposal of chemical products must be done according to good laboratory practices (GLP).

5. REAGENTS

The kit contains reagents for 96 determinations. They have to be stored at 2-8°C. Expiry dates are found on the labels of the bottles and the outer package.

1) Microtiter plate consisting of 12 strips with 8 breakable wells each, coated with anti-tetracycline antibodies.
2) Tetracycline Standards (0; 0.04; 0.1; 0.4; 1; 4 ng/mL): 6 vials with 1.0 mL each as 100x concentrate, dyed red. Dilute 20 µL of standard with 1980 µL pre-diluted extraction and sample dilution buffer to achieve the concentrations mentioned above.
   For milk/milk powder containing samples dilute 20 µL of standard with 1980 µL Tetracycline Standard Diluent 2 (TCY-SV2) instead of pre-diluted extraction and sample dilution buffer to achieve the concentrations mentioned above.
   Stored at 4°C the diluted standards are stable for at least 12 hours.
   Note: The concentrations above refer to the 100x diluted standards.
3) Conjugate (Tetracycline-Peroxidase): 3 vials with 2.5 mL each, dyed red, lyophilized. The provided conjugate is freeze dried and has to be reconstituted before the test. For dissolution add 2.5 mL of distilled water per vial and shake well for 5 minutes. The redissolved conjugate can be stored frozen at -20 °C for at least 1 month. Repeated freezing and thawing should be avoided.
4) Substrate Solution (TMB): 15 mL, ready-to-use.
5) Stop Solution (0.5 M H_2SO_4): 15 mL, ready-to-use.
6) Extraction and sample dilution buffer (Tris): 2 x 60 mL as 10x concentrate, dyed red. Dilute 1+9 with distilled water. Stored at 4°C the diluted buffer is stable for at least one week. If during the cold storage crystals precipitate, the concentrate should be warmed up to 37°C for 15 minutes.
7) Sample Dilution Buffer A (Tris): 60 mL, dyed red, ready-to-use.
8) Sample Dilution Buffer B (Tris): 60 mL, dyed red, ready-to-use.
9) Washing Solution (PBS + Tween 20): 60 mL as 10x concentrate. Dilute 1+9 with distilled water. Stored at 4°C the diluted buffer is stable for at least 4 weeks. If during the cold storage crystals precipitate, the concentrate should be warmed up to 37°C for 15 minutes.
10) Plastic bag to store unused microtiter strips.
11) Instruction Manual.

6. ADDITIONAL INSTRUMENTATION AND REAGENTS (NOT PROVIDED)

6.1 Instrumentation

- 50 - 1000 µL micropipette
- Volumetric flask
- Analytical balance
- Mortar, mixer
- Water bath
- Centrifuge
- ELISA reader (450 nm)

6.2 Reagents

- Double distilled water
- Hexan

7. SAMPLE PREPARATION

Due to the high risk of cross-contamination, all applied instruments like applicator, mortar, glass vials etc. have to be **cleaned thoroughly** before and after each sample. The following sample preparation should be applied for milk and other liquid samples:

1) Defat milk if applicable. Therefore, the milk has to be centrifuged for 15 min at 4°C and at least 2000 g. Afterwards the upper fat layer should be removed.
2) Dilute 1 mL of previously defatted milk in 9 mL of **pre-diluted** extraction and sample dilution buffer. Afterwards the solution is shaken for 5 min at room temperature. The process is continued at point 3 of the solid sample extraction process.

The following sample preparation should be applied for solid samples

1) To maximize homogeneity and representativeness of the sample drawing, a minimum of 5 g sample should be pulverized finely in a mortar, impact mill etc.
2) 1 g of the homogenized mixture is suspended in 10 mL of **pre-diluted** extraction and sample dilution buffer. Afterwards the suspension is shaken for 10 min at 40°C (Cheese, Honey, Meat) or at 60°C (Nonfat dry milk).
3) The samples are centrifuged for 15 minutes with at least 2000 g. For a better separation of fat, the centrifuge should be cooled to 4°C if applicable. If it is not possible to separate the supernatant from the precipitate completely, the suspension should be filtered if necessary.
4) Further treatment:
   a. <u>Milk- and shrimps samples:</u>

Dilute 100 µL of particle-free solution with 100 µL of pre-diluted sample extraction buffer. 100 µL of the diluted extract are applied per well.
Dilution factor = 20

b. Meat samples:
Extract 1 mL of particle-free solution with 2 mL Hexane and discard the Hexane layer. Dilute 100 µL of extract with 100 µL of pre-diluted sample extraction buffer. 100 µL of the diluted extract are applied per well.
Dilution factor = 20

c. Cheese samples:
Extract 1 mL of particle-free solution with 2 mL Hexane and discard the Hexane layer. Dilute 100 µL of extract with 400 µL of pre-diluted sample extraction buffer. 100 µL of the diluted extract are applied per well.
Dilution factor = 50

d. Honey samples:
Dilute 100 µL of particle-free solution with 400 µL of pre-diluted sample extraction buffer. 100 µL of the diluted extract are applied per well.
Dilution factor = 50

e. Nonfat dry milk:
Dilute 50 µL of particle-free solution with 450 µL of pre-diluted sample extraction buffer. 100 µL of diluted extract are applied per well.
Dilution factor = 100

In case of too high concentrated samples, the sample extracts have to be further diluted with **pre-diluted** extraction and sample dilution buffer. The additional dilution factor has to be accounted for when calculating the results.

8. PROCEDURE

In order to compensate for matrix effects for milk or milk powder containing samples, the dilution of the standards should be done with separately available Tetracycline Standard Diluent 2 (TCY-SV2). For any other samples the standards should be diluted with pre-diluted extraction and sample dilution buffer

1) Prepare samples as described above.
2) Pipet 100 µL **diluted** standards or prepared samples in duplicate into the appropriate wells of the microtiter plate.
3) Add 50 µL of redissolved tetracycline-peroxidase conjugate into each well.
4) Incubate for 60 minutes at room temperature
5) Wash the plate three times as follows: Discard the contents of the wells (dump or aspirate). Pipet 300 µL of diluted washing solution into each well. After the third repetition empty the wells again and remove residual liquid by striking the plate against a paper towel. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbances.
6) Pipet 100 µL of substrate solution into each well.
7) Allow the reaction to develop in the dark (e.g. cupboard or drawer; the chromogen is light-sensitive) for 20 minutes at room temperature.
8) Stop enzyme reaction by adding 100 µL of stop solution (0.5 M H_2SO_4) into each well. The blue colour will turn yellow upon addition.
9) After thorough mixing, measure absorbance at 450 nm (reference wavelength 620 nm), using an ELISA reader. The colour is stable for 30 minutes.

9. CALCULATION OF RESULTS

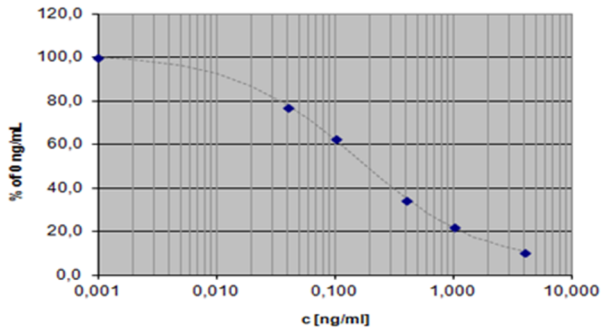
1) Calculate the average optical density (OD 450 nm) for each set of reference standards or samples.
2) Construct a standard curve by plotting the mean optical density obtained for each reference standard against its concentration in ppm on semi-log graph paper with the optical density on the vertical (y) axis and the concentration on the horizontal (x) axis. Alternatively, the evaluation can be carried out by software. In this case the 4-parameter method should be preferred.
3) The diluted samples must be further converted by the appropriate **sample dilution factor** for calculating the sample concentration in ppb. The factors for each sample matrix are listed in the *sample preparation* section.

Example:
A honey sample prepared as described above results in 0.6 ng/mL. The concentration of the sample is calculated as follows:
C_{sample} = **0.6** (ng/mL) * **50** (ppb*ml/ng) = **30 ppb**

10. TYPICAL STANDARD VALUES

The following table contains an example for a typical standard curve. The binding is calculated as percent of the absorption of the 0 ng/mL standard. These values are only an example and should not be used instead of the standard curve which has to be measured in each new test.

Tetracycline (ng/mL)	(% binding 0 ng/mL)
0	100
0.04	77
0.1	63
0.4	35
1	22
4	11



11. PERFORMANCE

11.1 Sensitivity

The limit of detection (LOD) of the **Tetracycline test** is 0.024 ng/mL.

The limit of quantification (LOQ) of the **Tetracycline test** is 0.072 ng/mL.

Validation experiments with common matrices resulted in the following LODs and LOQs [ppb].

Matrix	LOD	LOQ
Meat	0.5	1.4
Milk	1.1	2.0
Nonfat Dry Milk	6.7	14.0
Cheese	1.4	3.2
Shrimps	0.5	1.1
Honey	2.2	3.3

11.2 Recovery

Meat	87%
Milk	94%
Nonfat Dry Milk	90%
Cheese	82%
Shrimps	89%
Honey	103%

11.3 Linearity

The serial dilution of spiked samples (meat, milk, nonfat dry milk, cheese, shrimps and honey) resulted in a dilution linearity of 83-110%.

11.4 Precision

Intra-Assay Precision	8.0%
Inter-Assay Precision	6.6%

11.5 Reactivity

Tetracycline	100%
4-Epitetracycline	111%
Rolitetracycline	82%
Chlortetracycline	42%
Doxycycline	41%
Demeclocycline	37%
Oxytetracycline	34%
4-Epioxytetracycline	34%
4-Epi Chlortetracycline	11%
Methacycline	9%
Minocycline	1%

12. LIABILITY

Gold Standard Diagnostics Budapest 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 the results.

Gold Standard Diagnostics Budapest shall not be liable for the unsafe use of the kit out of the current European safety regulations.

13. REFERENCES

1) Lee H-J, et al. (2001) – Enzyme-linked immunosorbent assay for screening the plasma residues of tetracycline antibiotics in pigs. J Vet Med Sci, 63(5):553-556