DNSH ELISA

5091DNSH[1]08.22

A competitive enzyme immunoassay
for the quantitative analysis of nifursol
metabolite DNSH

EUROPROXIMA DNSH ELISA

A competitive enzyme immunoassay for the quantitative analysis of DNSH

TABLE OF CONTENTS

The quality management system of R-Biopharm Nederland B.V. is ISO 9001:2015 certified

BRIEF INFORMATION

DNSH ELISA is a competitive enzyme immunoassay for the quantitative detection of nifursol metabolite 3,5-dinitrosalicylic acid hydrazide (DNSH) in meat and seafood. With this ELISA-kit 96 analyses can be performed. Samples and standards are measured in duplicate which means that a total of 42 samples can be analysed. Apart from acetonitrile, 1 M hydrochloric acid and deionised water, all reagents needed to perform the assay are included in the test kit.

1. INTRODUCTION

Nifursol is a nitrofuran antibiotic banned as a feed additive in the European Union and other countries. Nifursol is metabolised to 3,5-dinitrosalicyclic acid hydrazide (DNSH) in living organisms. DNSH is a marker for the detection of illegal use of nifursol in animal husbandry. The detection of DNSH by LC-MS/MS requires derivatisation of this metabolite with 2-nitrobenzaldehyde to NPDNSH, like other nitrofuran metabolites such as SEM, AHD, AMOZ and AOZ. In accordance with Commission Regulation (EU) 2019/1871 a reference point of action (RPA) of 0.5 µg/kg for DNSH and other nitrofuran metabolites applies from 28 November 2022.
DNSH ELISA is a specific method for the detection of DNSH in meat (poultry, pork, beef) and seafood (fish, shrimp, and prawn). The antibody used in this test detects DNSH without derivatisation with 2-nitrobenzaldehyde.

2. PRINCIPLE OF THE DNSH ELISA

The microtiter plate based DNSH ELISA consists of one plate (12 strips, 8 wells each) pre-coated with anti-DNSH polyclonal antibody. In the first step of the assay standards and samples are added to the pre-coated wells. DNSH present in the standard solutions or in the samples bind to the immobilised antibody. After incubation, the content of the wells is emptied. Then horseradish peroxidase (HRP) labeled with DNSH is added to each well and it binds to any available antibody (sequential incubation assay). After a washing step, the amount of bound complex is visualized by addition of enzyme/chromogen (hydrogen peroxide/tetramethylbenzidine, TMB). During the incubation the colourless chromogen is converted by the enzyme into a blue reaction product. The colour intensity is inversely proportional to the amount of DNSH in the sample/standard, i.e., the more DNSH present in the standard solution or sample, the less colour is developed. The colour development is stopped by addition of sulfuric acid. The intensity of yellow colour formed is measured photometrically at 450 nm.

3. SPECIFICITY AND SENSITIVITY

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

DNSH	100%
Nifursol	119%
AHD	<0.1%
AMOZ	<0.1%
AOZ	<0.1%
SEM	<0.1%

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	Procedure	LOD (µg/kg)	CCβ (µg/kg)
Meat	8.1	0.13	0.25
Fish and shellfish	8.1	0.15	0.25

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

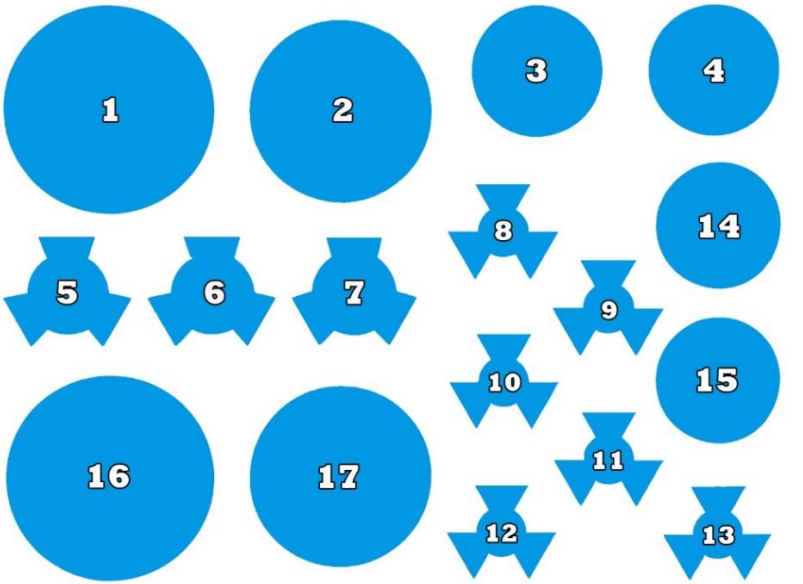
- Kit and kit components should be stored at 2°C to 8°C in a dark place. For repeated use store kit components as specified under chapter 9.
- After the expiry date of the kit and/or components has passed, no further quality guarantee is valid.
- Bring all kit components including the microtiter plate 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 with anti-DNSH antibody. Ready-to-use.
Position of the reagents in the kit. For preparation of the reagents see Chapter 9.



1. **Extraction buffer** (55 ml, ready-to-use)
2. **Rinsing buffer** (30 ml, 20x concentrated)
3. **Substrate solution** (12 ml, ready-to-use)
4. **Stop solution** (12 ml, ready-to-use)
5. **Standard for meat** (4.4 µg/kg, lyophilised)
6. **Standard for meat** (4.4 µg/kg, lyophilised)
7. **Standard for seafood** (3.6 µg/kg lyophilised)
8. **Conjugate** (200 µl, 100x concentrated)
9. **Protease enzyme** (1.3 ml, 40x concentrated)
10. **Standard for seafood** (3.6 µg/kg lyophilised)
11. Not in use
12. Not in use
13. Not in use
14. Not in use
15. Not in use
16. Not in use
17. **Dilution buffer** (30 ml, 10x concentrated)

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Scales and weighing vessels
- Gloves
- Fume hood
- Homogeniser
- Vortex mixer
- Centrifuge (4000 x g)
- Automated microplate washer or 8-channel micropipette 100 – 300 µl
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Micropipettes 20 – 200 µl, 100 – 1000 µl
- Multipipette with 2.5 ml combitips
- Aluminium foil or parafilm
- Deionised or distilled water
- Acetonitrile
- 1 M hydrochloric acid (HCl)

7. PRECAUTIONS

- This kit may contain hazardous substances. For hazard notes please refer to the appropriate safety data sheets (SDS).
- **Please take extra care when working with protease enzyme as it can cause damage to skin and eyes. Always wear protective gloves and glasses**. Refer to the safety data sheet (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 and seafood
- Add 1 ml of diluted protease enzyme (Chapter 9) to 1 g of homogenised sample and vortex for 10 sec
- Incubate the sample for 1 h at 50°C
- Add 100 µl of 1 M hydrochloric acid (HCl) to the sample, vortex for 10 sec
- Incubate the sample for 3 h at 50°C
- Add 1 ml of acetonitrile to the samples and vortex for 10 sec
- Add 1 ml of the extraction buffer to the sample and vortex/shake for 30 sec
- Centrifuge the sample for 5 min at 4000 × g (20-25°C)
- Dilute the upper acetonitrile layer 8 times in the dilution buffer (Chapter 9), for example take 50 µl of the upper layer and add to 350 µl of the dilution buffer
- Mix well and use 100 µl directly in the ELISA.

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. For each strip 20 ml of diluted rinsing buffer is used (1 ml concentrated rinsing buffer + 19 ml distilled water).

Dilution buffer
The dilution buffer is 10 times concentrated. Mix well and dilute 10 times in deionised water, for example add 5 ml of concentrated buffer to 45 ml of water and mix well. Prepare fresh before use.

Standard dilution buffer
Standard dilution buffer is needed to dilute the standards. It is the dilution buffer containing 12.5% of acetonitrile. Prepare standard dilution buffer fresh before use, for example by adding 1.25 ml of acetonitrile to 8.75 ml of the diluted dilution buffer. Mix well.

Conjugate solution
The conjugate is delivered 100x concentrated. Spin down the conjugate in the vial by a short centrifugation step (1 min., 1000 x g). Add 20 µl of the concentrated conjugate

solution to 1980 µl of the dilution buffer (100 times diluted). Per 2 x 8 wells 1.6 ml of diluted conjugate is required. Store unused concentrated conjugate at +2ºC to +8ºC.

Protease enzyme
Protease enzyme is 40x concentrated. Mix well and dilute 40 times in deionised water, for example add 0.5 ml of protease enzyme to 19.5 ml of water. One ml of the diluted protease enzyme is needed per sample to be analysed. **Please refer to Chapter 7 for the safety advice concerning this reagent.**

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.

Standards
Prepare a dilution range of DNSH standards. For optimum calibration select the standard corresponding to the samples used. The standard is adjusted to provide good recovery.

Meat: add 2 ml of the **standard dilution buffer** (dilution buffer containing 12.5% acetonitrile) to DNSH “meat” standard and mix. This solution contains DNSH concentration corresponding to 4.4 µg/kg of DNSH in a sample. Pipette 0.4 ml of this solution into a clean tube and add 0.6 ml of the standard dilution buffer. Continue to make a dilution range of 1.76, 0.70, 0.28 and 0.11 µg/kg.

Seafood: add 2 ml of the **standard dilution buffer** (dilution buffer containing 12.5% acetonitrile) to the DNSH “seafood” standard and mix. This solution contains DNSH concentration corresponding to 3.6 µg/kg of DNSH in a sample. Pipette 0.4 ml of this solution into a clean tube and add 0.6 ml of the dilution buffer. Continue to make a dilution range of 1.44, 0.58, 0.23 and 0.09 µg/kg.

The reconstituted lyophilised standard can be stored at -20°C for at least 1 month and used again.

10. ASSAY PROCEDURE

Rinsing protocol

In ELISAs, 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.
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 followed by a firm short vertical movement.
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 standard dilution buffer (diluted dilution buffer containing 12.5% acetonitrile) (wells G1, G2, blank). Pipette 100 µl of the standard dilution buffer in duplicate (wells A1, A2, maximal signal).
   Pipette 100 µl of each of the standard solutions in duplicate (wells B1,2 to F1,2) i.e., 0.11, 0.28, 0.70, 1.76 and 4.4 µg/kg for meat;
   0.09, 0.23, 0.58, 1.44 and 3.6 µg/kg for seafood)

3. Pipette 100 µl of each sample solution in duplicate into the remaining wells of the microtiter plate.

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 (20-25°C).

7. Discard the solution from the microtiter plate and tap dry onto a paper towel. Do not wash!

8. Pipette 100 µl of conjugate into each well, except G1 and G2.

9. Seal the microtiter plate and shake the plate for a few seconds on a microtiter plate shaker.

10. Incubate for 15 minutes in the dark at room temperature (20-25°C).

11. Discard the solution from the microtiter plate and wash 3 times with rinsing buffer.

12. Pipette 100 µl of substrate solution into each well.

13. Incubate 15 minutes at room temperature (20ºC - 25ºC) in the dark.

14. Add 100 µl of stop solution into each well.

15. Read the absorbance values immediately at 450 nm.

11. INTERPRETATION OF RESULTS

Subtract the mean optical density (O.D.) of the wells G1 and G2 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 (wells A1 and A2) and multiplied by 100. The zero standard is thus made equal to 100% (maximal absorbance) and the other O.D. values are quoted in percentages of the maximal absorbance.

$$\frac{O.D.\,standard\ (or\ sample)}{O.D.\ zero\ standard} x\ 100 = \ percentage\ maximal\ absorbance$$

Calibration curve:
The values (percentage maximal absorbance) calculated for the standards are plotted (on the Y-axis) versus the DNSH concentration (ng/ml) on a logarithmic X-axis.

Alternative for calibration curve:
The values of absorption (logit) calculation of the standards are plotted on Y-axis versus the analyte equivalent concentration on a logarithmic X-axis

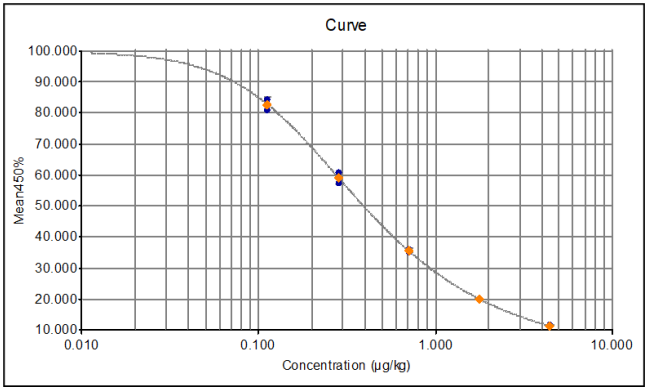


Figure 1: Example of a calibration curve

Calculation factors

The DNSH concentration in meat and seafood expressed in µg/kg can be read directly from the calibration curve.

12. LITERATURE

The European Commission, 2019. Commission Regulation (EU) 2019/1871 of 7 November 2019 on reference points for action for non-allowed pharmacologically active substances present in food of animal origin and repealing Decision 2005/34/EC. *Official Journal of the European Union,* L 289, 41-46.

13. ORDERING INFORMATION

For ordering the DNSH ELISA, please use catalogue code 5091DNSH.

14. REVISION HISTORY

Not applicable

R-Biopharm Nederland B.V.
Beijerinckweg 18
6827 BN Arnhem
The Netherlands

Tel: + 31 26 3630364
www.europroxima.com
info@r-biopharm.nl