INSTRUCTION FOR USE

Immunoport SEM

Enzyme immunoassay for the detection of nitrofurazone metabolite (SEM)

(Cat. No. IP601230)

Immunoport SEM is a kit prepared for a competitive immunoenzymatic assay for the quantitative analysis of semicarbazide (SEM), a metabolite of the nitrofurazone. The kit contains the procedure and the materials sufficient for 96 determinations, including standards.
For result evaluation a microtiter plate or strip photometer is required (manual or automatic ELISA reader).

Type of samples that can be analyzed (matrices)
Seafood, bovine and swine muscle, honey.

Sample preparation
Homogenization, derivatization/hydrolysis, organic solvent extraction, centrifugation, evaporation, reconstitution.

Assay time: 90 minutes (sample preparation not included).

Detection limit
0.2 ppb.

Specificity	
Coumpond	**Cross-reactivity (%)**
SEM	100
NITROFURAZONE	121±8
AHD	< 0.1
AOZ	< 0.1
AMOZ	< 0.1

1. TEST PRINCIPLE

The assay is performed in plastic microwells which have been coated with anti-SEM antibodies.
SEM standard solutions or samples and the enzyme labelled SEM-HRP are added to the microplate.
During the first incubation, free SEM molecules and SEM-HRP compete for the anti-SEM antibody binding site.Any unbound substance is then removed in a washing step.
The bound enzyme activity is determined adding a fixed amount of a chromogenic substrate. The enzyme converts the colourless chromogen into a blue product.
The addition of the stop reagent leads to a colour change from blue to yellow. The absorbance is measured with a microplate reader at 450 nm. The colour development is inversely proportional to the SEM concentration in the standard solution /sample.

2. PROVIDED REAGENTS

Microtiter plate: 96 wells (12 strips x 8 wells), coated with anti-SEM antibodies.
As the strips are breakable, the wells can be used individually. For this purpose, it is sufficient to take out the wells from the frame and to break the joint.
SEM Std: 6 plastic vials, each containing 1.5 ml of the following concentrations of SEM equivalents: 0 ng/ml; 0.1 ng/ml; 0.25 ng/ml; 0.5 ng/ml; 1 ng/ml; 2.5 ng/ml.
SEM Spiking solution 50 ppb: 1 plastic vial containing 1 ml of 50 ppb of SEM.
Enzyme conjugate: 1 plastic bottle containing 8 ml.
2-nitrobenzaldehyde 50mM: 1 glass vial containing 3.5 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
- Hydrochloric acid (HCl) 1M
- Potassium phosphate dibasic (K_2HPO_4) 0.1M
- Sodium hydroxide (NaOH) 1M
- Ethyl acetate
- Hexane

Equipment:

- Tissue homogenizer (for seafood and muscle analysis)
- Balance
- Incubator or water-bath (37°C or 55°C)
- Centrifuge (3000 x g)
- Vortex
- Evaporator
- 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.

  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 Muscle (bovine, swine, seafood): long procedure

1) For muscle: remove the fatty parts then grind the sample. For egg: homogenize the sample.
2) Weigh 1 g of homogenized sample and add 4 ml of distilled water, 0.5 ml of 1 M HCl, 60 µl of 50 mM 2-nitrobenzaldehyde solution.
3) Mix and incubate over night at 37°C.
4) Add 5 ml of 0.1 M K_2HPO_4, 0.4 ml of 1M NaOH and 5ml of ethyl acetate; shake vigorously for 30 seconds.
5) Centrifuge at 3000 g for 10 minutes at room temperature (RT).
6) Transfer 2.5 ml of the organic upper layer (ethyl acetate) and evaporate at 55°C, under a slow air or nitrogen stream.
7) Dissolve the residue in 1 ml of hexane and add 1 ml of washing buffer 1x; mix properly.
8) Centrifuge at 3000 g for 10 minutes at RT.
9) Transfer the aqueous lower phase in a new tube.
10) The dilution factor is 2.

6.2 Muscle (bovine, swine, seafood): short procedure (alternative)

1) For muscle: remove the fatty parts then grind the sample. For egg: homogenize the sample.
2) Weigh 1 g of homogenized sample and add 4 ml of distilled water, 0.5 ml of 1 M HCl, 60 µl of 50 mM 2-nitrobenzaldehyde solution.
3) Mix and incubate 2 hours at 55°C.
4) Add 5 ml of 0.1 M K_2HPO_4, 0.4 ml of 1M NaOH and 5ml of ethyl acetate; shake vigorously for 30 seconds.
5) Centrifuge at 3000 g for 10 minutes at room temperature (RT).
6) Transfer 2.5 ml of the organic upper layer (ethyl acetate) and evaporate at 55°C, under a slow air or nitrogen stream.
7) Dissolve the residue in 1 ml of hexane and add 1 ml of washing buffer 1x; mix properly.
8) Centrifuge at 3000 *g* for 10 minutes at RT.
9) Transfer the aqueous lower phase in a new tube.
10) The dilution factor is 2.

6.3. Honey

1) Weigh 1 g of honey sample.
2) Add 5 ml of hexane, 0.5 ml of 1M HCl and 4 ml of distilled water.
3) Vortex for 1 minute.
4) Centrifuge at 3000 g for 10 minutes at room temperature (RT).
5) Take all the lower aqueous phase and add 40 µl of 50 mM 2-nitrobenzaldehyde solution; vortex for few seconds.
6) Incubate over night at +37°C.
7) Add 5 ml of 0.1M K2HPO4, 0.4 ml of 1M NaOH and 5 ml of ethylacetate.
8) Vortex for 1 minute.
9) Centrifuge at 3000 g for 10 minutes at RT.
10) Transfer 2.5 ml of the upper organic phase in a glass vial and evaporate at 50 – 60°C under a slow air or nitrogen stream.
11) Dissolve the residue in 1 ml of washing buffer 1x by vortexing for 1 minute.
12) The dilution factor is 2.

7. WORKING SOLUTIONS PREPARATION

SEM Standards: ready to use, (shake gently prior to use).
SEM Spiking solution: ready to use (shake gently prior to use).
Enzyme Conjugate: ready to use .
2-nitrobenzaldehyde 50mM: ready to use (shake gently prior 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.
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 enzyme conjugate in each well.
   - Shake the plate gently with rotatory motion for few seconds.
   - Incubate 60 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 100 µl of developing solution to each well.
   - Mix thoroughly with rotatory motion for few seconds.
   - Incubate for 30 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 60 minutes.

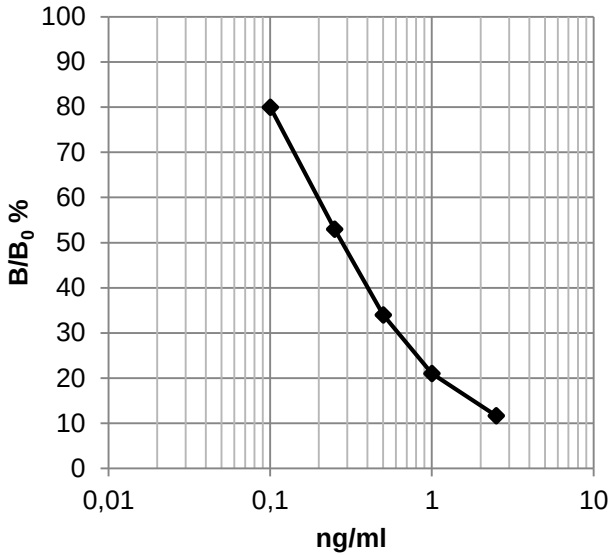
9. CALCULATION OF RESULTS

- Calculate the mean absorbance of each standard and sample.
- 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 standard 0 is thus made equal to 100% and all the other absorbance values are expressed as percentages:

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

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

10. CALIBRATION 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 kit specifications (see chapter 12).

If the values are outside the specifications given, then the results of the test are not assured, therefore the SEM 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 it is necessary to adopt a decision limit (CCα). The decision limit varies according to the type of sample. It is suggested to determine a decision limit for each matrix routinely analysed in your laboratory.

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

12.KIT SPECIFICATIONS

12.1 Assay specification

Description	Specifications
Mean B_0 absorbance	$\geq$ 0.7 OD_{450nm}
B/B_0 50 %	0.21 – 0.38 ng/ml
Std duplicates mean C.V.	$\leq$ 6 %

12.2 Assay performances

The kit performances were assessed within an *in-house* validation; data can be requested to the technical assistance.

Detection capability (CCβ)	
Shrimp	0.5 ppb

13.LIABILITY

Samples evaluated as positive using the kit have to be re-tested with a confirmation method.

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