Importance of Salinomycin and Narasin Determination

The group of naturally occurring compounds known as "Ionophores" (ion bearer) are carboxylic polyether antibiotics which were initially developed for use in the prevention and treatment of coccidiosis in poultry. Ionophores form complexes with alkaline cations, creating lipophilic channels through hydrophobic lipid membranes. This facilitates the movement of metal ions across the membrane, interfering with the osmotic pressure of the cell. Ionophores such as Monensin, Lasalocid, Maduramicin, Narasin, Salinomycin and Semduramicin are active against Gram-positive bacteria, mycobacteria, some fungi and certain parasites and coccidia.

In addition to their use in the treatment and prevention of infection, Ionophores are also used at sub-therapeutic levels to improve feed efficiency in livestock. They are generally administered as feed additives. The withdrawal period for ionophores varies between 3-5 days. Although generally considered safe and effective at therapeutic doses in target animal species, accidental overdose, misuse, mixing errors, and accidental ingestion in non-target species can result in toxicity in a number of animals. Horses, certain avian species, dogs, and cats are especially sensitive to Ionophore toxicity. Effects of Ionophore toxicity, which includes muscle degeneration, neuropathy, and cardiac toxicity, are often fatal.

To protect humans, regulatory agencies around the world have imposed regulatory limits regarding the amount of each Ionophore allowable in products for human consumption, such as poultry and other edible animal tissues. In the US, the Acceptable Daily Intake (ADI) is 5 μg/kg of body weight of Salinomycin per day and 5 μg/kg of body weight of Narasin per day.

The Salinomycin/Narasin ELISA allows for the analysis of 43 samples in duplicate determination. Less than 1 mL of sample extract is required. The test can be performed in less than 2 hours.

Performance Data

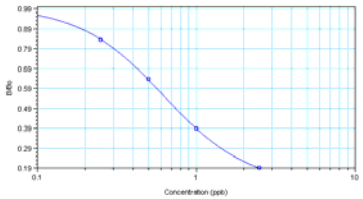
Test sensitivity: The limit of quantitation for Salinomycin/Narasin (90% B/B_0 calculated from the average of 30 calibration curves) is approximately 0.18 ng/mL. The middle of the test (50% B/B_0 calculated from the average of 30 calibration curves) is approximately 0.72 ng/mL (average of 30 calibration curves). Determinations closer to the middle of the calibration curve give the most accurate results.

Test reproducibility: Coefficients of variation (CVs) for standards: <10%; CVs for samples: <15%.

Specificity: Cross-reactivity of the Abraxis Salinomycin/Narasin Kit for related Ionophores:

Salinomycin	100%
Narasin	71%
Lasalocid, Maduramicin, Semduramicin	< 0.1 %.

Standard Curve:



For demonstration purposes only. Not for use in sample interpretation.

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Salinomycin/Narasin ELISA (Microtiter Plate)

Enzyme-Linked Immunosorbent Assay for the Determination of Salinomycin/Narasin in Feed and Contaminated Samples

Product No. 515795

1. General Description

The Abraxis Salinomycin/Narasin ELISA is an immunoassay for the quantitative and sensitive screening of Salinomycin and Narasin. This test is suitable for the quantitative and/or qualitative screening of Salinomycin and Narasin in animal feed and contaminated products (please refer to the appropriate technical bulletins for additional extraction/dilution procedures). Samples requiring regulatory action should be confirmed by HPLC, GC/MS, or other conventional methods.

2. Safety Instructions

The standard solutions in the test kit contain small amounts of Salinomycin. In addition, the substrate solution contains tetramethylbenzidine and the stop solution contains diluted sulfuric acid. Avoid contact of stopping solution with skin and mucous membranes. If these reagents come in contact with skin, wash with water.

3. Storage and Stability

The Salinomycin/Narasin ELISA should be stored in the refrigerator (4–8°C). The solutions must be allowed to reach room temperature (20-25°C) before use. Reagents may be used until the expiration date on the box. The conjugate is supplied in lyophilized form (3 vials). Before each assay, the required volume of lyophilized conjugate must be reconstituted with conjugate diluent (see Test Preparation section). Reconstitute only the amount needed for the samples to be run, as the reconstituted solution will only remain viable for one week (store refrigerated).

4. Test Principle

The test is a direct competitive ELISA based on the recognition of Salinomycin and Narasin by specific antibodies. Salinomycin and Narasin, when present in a sample, and a Salinomycin-HRP analogue compete for the binding sites of rabbit anti-Salinomycin antibodies in solution. The Salinomycin antibodies are then bound by a second antibody (donkey anti-rabbit) immobilized on the wells of the microtiter plate. After a washing step and addition of the substrate solution, a color signal is generated. The intensity of the blue color is inversely proportional to the concentration of Salinomycin and/or Narasin present in the sample. The color reaction is stopped after a specified time and the color is evaluated using an ELISA reader. The concentrations of the samples are determined by interpolation using the standard curve constructed with each run.

5. Limitations of the Salinomycin/Narasin ELISA, Possible Test Interference

Although many organic and inorganic compounds commonly found in samples have been tested and found not to interfere with this test, due to the high variability of compounds that might be found in samples, test interferences caused by matrix effects can not be completely excluded.

Samples must be extracted and diluted as instructed in the sample preparation section (Section D) or appropriate technical bulletin before testing in the ELISA.

Mistakes in handling the test also can cause errors. Possible sources for such errors include: inadequate storage conditions of the test kit, incorrect pipetting sequence or inaccurate volumes of the reagents, too long or too short incubation times during the immune and/or substrate reaction, extreme temperatures (lower than 10°C or higher than 30°C) during the test performance.

The Abraxis Salinomycin/Narasin ELISA kit provides screening results. As with any analytical technique (GC, HPLC, etc.), samples requiring some regulatory action should be confirmed by an alternative method.

A. Reagents and Materials Provided

1. Microtiter plate (12 X 8 strips) coated with a secondary antibody, in a resealable aluminum pouch
2. Salinomycin/Narasin Calibrators/Standards (5): 0, 0.25, 0.50, 1.0, 2.5 ng/mL (ppb), 1 mL each
3. Antibody Solution (rabbit anti-Salinomycin), 6 mL
4. Salinomycin-HRP Conjugate, 3 vials (lyophilized), 3 mL/vial after reconstitution
5. Conjugate Diluent, 12 mL
6. Wash Solution (5X) Concentrate, 100 mL, must be diluted before use, see Test Preparation (Section C)
7. Sample Diluent, 2 bottles, 25 mL each
8. Substrate (Color) Solution (TMB), 16 mL
9. Stop Solution, 12 mL (handle with care)

B. Additional Materials (not delivered with the test kit)

1. Micro-pipettes with disposable plastic tips (50-200 µL)
2. Multi-channel pipette (50-250 µL) or stepper pipette with disposable plastic tips (50-250 µL)
3. Microtiter plate reader (wave length 450 nm)
4. Overhead tube rotator
5. Vortex mixer
6. Deionized or distilled water
7. Acetone, reagent grade
8. Paper towels or equivalent absorbent material
9. Timer
10. Centrifuge capable of spinning at 3,000 x g
11. 15 mL conical tubes with caps
12. 4 mL glass vials with Teflon-lined caps
13. Analytical 3 place balance

C. Test Preparation

Micro-pipetting equipment and pipette tips for pipetting the standards and the samples are necessary. In order to equalize the incubation periods on the entire microtiter plate, a multi-channel pipette or a stepping pipette is recommended for adding the enzyme conjugate, antibody, substrate, and stop solutions. Please only use the reagents and standards from one package lot in one test, as they have been adjusted in combination.

1. Adjust the microtiter plate and the reagents to room temperature before use.
2. Remove the number of microtiter plate strips required from the aluminum pouch. The remaining strips are stored in the aluminum pouch and zip-locked closed. Store the remaining kit in the refrigerator (4-8°C).
3. The standard solutions, antibody, substrate and stop solutions are ready to use and do not require any further dilutions.
4. The conjugate provided is lyophilized (3 vials). Before each assay, calculate the volume of conjugate needed (when reconstituted, each vial will provide enough conjugate for approximately 55 wells). Reconstitute only the amount necessary for the samples to be analyzed. Once reconstituted, the conjugate solution will only remain viable for 1 week (store refrigerated). If additional samples are to be analyzed greater than one week from reconstitution, a new vial of conjugate will need to be prepared. To reconstitute, add 3 mL of Conjugate Diluent to each vial of conjugate required and vortex thoroughly. If multiple vials are necessary at one time, reconstitute each vial, then combine the reconstituted solutions and vortex thoroughly.
5. Dilute the Wash Solution (5X) Concentrate at a ratio of 1:5. If using the entire bottle (100 mL), add to 400 mL of deionized or distilled water and mix thoroughly.
6. The stop solution must be handled with care as it contains diluted H_2SO_4.

D. Sample Preparation

Dog/Cat Food, Vitamin, and Rice Hull Extraction

Note: Dog or Cat Food which is in pressed pellet form must be ground into powder before extraction. Samples should be analyzed immediately after extraction.

1. Weigh 1.0 g of feed, vitamin mix, or rice hulls into a 15 mL conical tube.
2. Add 5 mL of acetone.
3. Vortex for 30 seconds.
4. Mix using an overhead tube rotator for 15 minutes.
5. Centrifuge for 5 minutes at 3000 x g.
6. Dilute 40 µL of the supernatant solution into 920 µL of Sample Diluent (1:24 dilution). Vortex. This will then be analyzed as sample (Assay Procedure, step 1).

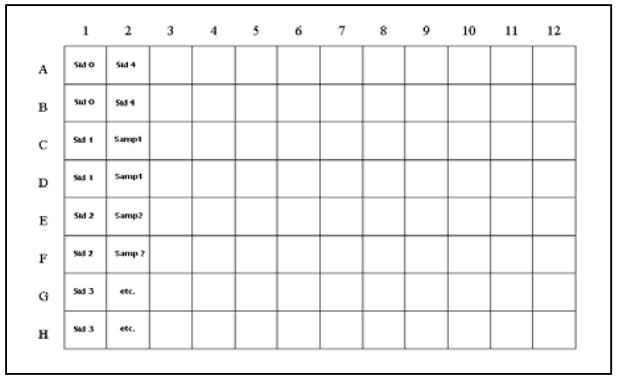
The Salinomycin/Narasin concentration in the sample is determined by multiplying the ELISA result by a factor of 120. Highly contaminated samples, those outside of the calibration range of the assay, must be diluted further and re-analyzed.

E. Working Scheme

The microtiter plate consists of 12 strips of 8 wells, which can be used individually for the test. The standards must be run with each test. Never use the values of standards which have been determined in a test performed previously.

Std 0-Std 4: Standards
(0; 0.25; 0.5; 1.0; 2.5 ppb)

Samp1, Samp2, etc.: Samples



F. Assay Procedure

1. Add 50 µL of the calibrator/standard solutions or sample extracts (Section D) into the wells of the test strips according to the working scheme given. Analysis in duplicate or triplicate is recommended.
2. Add 50 µL of reconstituted enzyme conjugate solution to the individual wells successively using a multi-channel pipette or a stepping pipette.
3. Add 50 µL of antibody solution to the individual wells successively using a multi-channel pipette or a stepping pipette. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a circular motion on the benchtop for 60 seconds. Be careful not to spill the contents.
4. Incubate the strips for 60 minutes at room temperature.
5. Remove the covering and decant the contents of the wells into a sink. Wash the strips four times using the diluted washing buffer solution. Please use at least a volume of 250 µL of washing buffer for each well in each washing step. Remaining buffer in the wells should be removed by patting the inverted plate dry on a stack of paper towels.
6. Add 150 µL of substrate (color) solution to the wells. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a circular motion on the benchtop for 60 seconds. Be careful not to spill the contents. Incubate the strips for 20 minutes at room temperature. Protect the strips from direct sunlight.
7. Add 100 µL of stop solution to the wells in the same sequence as for the substrate solution.
8. Read the absorbance at 450 nm using a microplate ELISA photometer within 15 minutes after the addition of stopping solution.

G. Evaluation

The evaluation of the ELISA can be performed using commercial ELISA evaluation programs such as 4-Parameter (preferred) or Logit/Log. For a manual evaluation, calculate the mean absorbance value for each of the standards. Calculate the $\%B/B_0$ for each standard by dividing the mean absorbance value for each standard by the Zero Standard (Standard 0) mean absorbance. Construct a standard curve by plotting the $\%B/B_0$ for each standard on the vertical linear (y) axis versus the corresponding Salinomycin/Narasin concentration on the horizontal logarithmic (x) axis on graph paper. $\%B/B_0$ for samples will then yield levels in ppb (or ng/g) of Salinomycin/Narasin by interpolation using the standard curve. Results can also be obtained by using a spreadsheet macro available from Abraxis upon request.

The concentrations of the samples are determined using the standard curve run with each test. Sample extracts showing a lower concentration of Salinomycin/Narasin than standard 1 (0.25 ppb) should be reported as containing < 30 ppb of Salinomycin/Narasin. Samples showing a higher concentration than standard 4 (2.5 ppb) must be diluted further with the provided sample diluent and re-analyzed.

Semi-quantitative results can be derived by simple comparison of the sample absorbances to the absorbances of the calibrators. Samples with lower absorbances than a calibrator will have concentrations of Salinomycin/Narasin greater than the concentration of that calibrator. Samples which have higher absorbances than a calibrator will have concentrations of Salinomycin/Narasin less than that calibrator.