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ABRAXIS

## Importance of Ionophores 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, and Salinomycin 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.

The Ionophores Multi-Screen ELISA allows for the screening of 6 samples in duplicate determination for Monensin, Lasalocid, Maduramicin, Narasin, and Salinomycin. Less than 1 mL of sample extract is required. The test can be performed in less than 2 hours.

## Performance Data

Screening Level: For screening samples at 36 ppb.

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

Specificity: Cross-reactivity of the Abraxis Total Ionophores Multi-Screen ELISA Kit for the following Ionophores:

| | |
|---|---|
| Salinomycin | 150% |
| Monensin | 100% |
| Lasalocid | 100% |
| Maduramicin | 100% |
| Narasin | 100% |

Recoveries: Feed samples with no detectable Ionophores were analyzed unspiked and spiked with Monensin, Lasalocid, Narasin, and Salinomycin:

| Sample Type | Spike Level | Assay Result | Interpretation | Correlation |
|---|---|---|---|---|
| Dog Food | 0 ppb | < 30 ppb for each Ionophore | Negative | Yes |
| Dog Food | 36 ppb | > 30 ppb for each Ionophore | Positive | Yes |
| Vitamin Mix | 0 ppb | < 30 ppb for each Ionophore | Negative | Yes |
| Vitamin Mix | 36 ppb | > 30 ppb for each Ionophore | Positive | Yes |
| Rice Hulls | 0 ppb | < 30 ppb for each Ionophore | Negative | Yes |
| Rice Hulls | 36 ppb | > 30 ppb for each Ionophore | Positive | Yes |


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The Abraxis Ionophores Multi-Screen ELISA is an immunoassay for the screening of Monensin, Lasalocid, Maduramicin, Narasin, and Salinomycin in animal feed (wet and dry dog and cat food) and animal feed ingredients (beef and bone meal; chicken meal; fish meal; meat and bone meal; pork and bone meal; poultry meal; salmon meal; low micro yeast; vitamin mix; mineral premix; chelated mineral mix; egg powder; rice hulls; and beef tallow). Please refer to the appropriate technical bulletins for additional sample matrices. 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 Monensin, Lasalocid, Maduramicin, and Narasin. The substrate solution contains tetramethylbenzidine (TMB) and the stop solution contains diluted sulfuric acid. Avoid contact of the TMB and stopping solution with skin and mucous membranes. If these reagents come in contact with skin, wash with water.

### 3. Storage and Stability

The Ionophores Multi-Screen ELISA kit 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.

### 4. Test Principle

In order to facilitate the testing of multiple Ionophores on a single microtiter plate, the individual Ionophores are color-coded: blue coded reagents correspond to those specific for Monensin, yellow for Lasalocid, purple for Maduramicin, and green for Salinomycin/Narasin.

The test is a direct competitive ELISA based on the recognition of each Ionophore by specific antibodies. Ionophores, when present in a sample, and the appropriate Ionophore-HRP analogue compete for the binding sites of the corresponding antibodies in solution. The Ionophores antibodies are then bound by a second antibody (anti-rabbit or anti-sheep) 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 the Ionophore present in the sample. The color reaction is stopped after a specified time and the color is evaluated using an ELISA reader. The Ionophores content of the samples are determined by comparison to the standards analyzed with each run.

### 5. Limitations of the Ionophores Multi-Screen 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 cannot 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 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 (60 ± 5 minutes) and/or substrate (20 ± 2 minutes) reaction, and extreme temperatures (lower than 10°C or higher than 30°C) during the test performance.

The Abraxis Ionophores Multi-Screen 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. Color-coded microtiter plate coated with secondary antibodies, in a resealable aluminum pouch
2. Ionophores Calibrators/Standards (2): 0 and 30 ng/mL (ppb) extract equivalent, 1 mL each
3. Antibody Solutions (4) (anti-Monensin, anti-Lasalocid, anti-Maduramicin, and anti-Salinomycin/Narasin), 1.5 mL each
4. Conjugate Solutions (3) (Monensin-, Lasalocid-, and Maduramicin-HRP Conjugate Solutions), 1.5 mL each
5. Lyophilized Salinomycin/Narasin-HRP Conjugate, 3 vials, 1.5 mL/vial after reconstitution, see Test Preparation (Section C)
6. Salinomycin/Narasin Conjugate Diluent, 6 mL
7. Wash Solution (5X) Concentrate, 100 mL, must be diluted before use, see Test Preparation (Section C)
8. Sample Diluent, 30 mL
9. Substrate (Color) Solution (TMB), 16 mL
10. Stop Solution, 12 mL (handle with care)

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

1. Micro-pipettes with disposable plastic tips (20-200 µL)
2. Multi-channel pipette (50-300 µL) or stepper pipette with disposable plastic tips (50-300 µL)
3. Microtiter plate reader (wave length 450 nm)
4. Analytical balance
5. Food processor, blender, or coffee grinder
6. Reclosable plastic bags
7. Overhead tube rotator
8. Vortex mixer
9. Deionized or distilled water
10. Acetone, reagent grade
11. Methanol, reagent grade
12. Paper towels or equivalent absorbent material
13. Timer
14. Centrifuge capable of spinning at 3,000 x g
15. 15 mL conical tubes with caps
16. 4 mL glass vials with Teflon-lined caps

## C. Test Preparation

Micro-pipetting equipment and pipette tips for pipetting the standards and the samples are necessary. Please only use the reagents and standards from one package lot in one test, as they have been adjusted in combination. 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.

1. Allow the reagents and sample extracts to reach room temperature before use.
2. Remove number of wells of the appropriate microtiter plate strips from the resealable pouch. The remaining wells are stored in the pouch with the desiccant (tightly sealed) in the refrigerator (4-8°C).
3. The standards, Monensin-, Lasalocid-, Maduramicin-HPR conjugates, all antibody solutions, substrate, and stop solutions are ready to use and do not require any further dilutions. Store in the refrigerator (4-8°C).
4. The Salinomycin/Narasin-HRP conjugate provided is lyophilized (3 vials). Store in the refrigerator (4-8°C). To reconstitute, add 1.5 mL of the Salinomycin/Narasin Conjugate Diluent to 1 vial of lyophilized conjugate and vortex thoroughly. Once reconstituted, the conjugate solution will only remain viable for 1 week and should be stored frozen. If additional samples are to be analyzed greater than one week from reconstitution, a new vial of conjugate will need to be prepared.
5. Dilute the Wash Solution (5X) Concentrate at a ratio of 1:5 with deionized or distilled water. 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; Beef and Bone Meal; Chicken Meal; Fish Meal; Meat and Bone Meal; Pork and Bone Meal; Salmon Meal; Low Micro Yeast; Vitamin Mix; Mineral Premix; Chelated Mineral Mix; Egg Powder; and Rice Hull Extraction

Note: 50 g of sample should be thoroughly homogenized prior to extraction to ensure a truly representative sample. Dog or Cat Food which is in pressed pellet form must be crushed or ground into a coarse powder before extraction. Moist samples should be homogenized into a paste-like consistency using a food processor or blender. Samples should be analyzed immediately after extraction. Procedure not for use with Super Premium Mineral Mix.

1. Weigh 1.0 g of homogenized sample 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 ± 1 minutes.
5. Centrifuge for 5 minutes at 3000 x g at room temperature.
6. Transfer supernatant to an appropriately labled 4 mL glass vial.
7. To a second appropriately labeled glass vial, add 920 µL of Sample Diluent. Add 40 µL of the supernatant solution (from step 6) to the diluent in the vial. Vortex. This will then be analyzed as sample (Assay Procedure, step 1).

Beef Tallow Extraction

Note: 50 g of sample should be placed in a reclosable plastic bag and thoroughly mashed until uniform in color and consistency to ensure a truly representative sample. Samples should be analyzed immediately after extraction.

1. Weigh 1.0 g of sample into a 15 mL conical tube.
2. Add 5 mL of methanol.
3. Vortex for 30 seconds.
4. Mix using an overhead tube rotator for 15 ± 1 minutes.
5. Centrifuge for 5 minutes at 3000 x g at room temperature.
6. Transfer supernatant to an appropriately labled 4 mL glass vial.
7. To a second appropriately labeled glass vial, add 920 µL of Sample Diluent. Add 40 µL of the supernatant solution (from step 6) to the diluent in the vial. Vortex. This will then be analyzed as sample (Assay Procedure, step 1).

## E. Working Scheme

The microtiter plate consists of 2 strips of 8 wells, color-coded for the appropriate Ionophore(s):

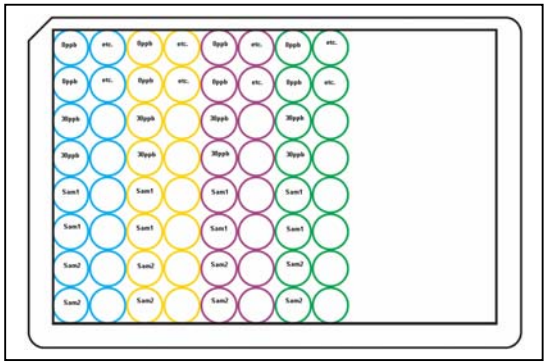
Blue = Monensin
Yellow = Lasalocid
Purple = Maduramicin
Green = Salinomycin/Narasin

Use only the color-coded antibody and conjugate solutions which correspond to the appropriate color-coded wells. Use of non-corresponding antibody or conjugate solutions (for example, using the purple-coded Maduramicin antibody or conjugate solution with the blue-coded Monensin microtiter plate wells) will not allow the necessary, highly specific, antibody/Ionophore/conjugate interaction to occur and no color signal will be generated for any of the incorrectly paired wells.

The standard solutions provided are composite standards. They contain Monensin, Lasalocid, Maduramicin, and Narasin at 0 ng/mL (ppb) and 30 ng/mL (ppb) extract equivalents. As they contain all of the Ionophores to be tested for, these two standards are used for wells of all colors. The standards must be run with each test. Never use the values of standards which have been determined in a test performed previously.

0 ppb and 30 ppb: Standards

Sam1, Sam2, etc.: Sample extracts



## 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 the appropriate color-coded **enzyme conjugate solution** to the corresponding color-coded wells successively using a multi-channel pipette or a stepping pipette.
3. Add 50 µL of the appropriate color-coded **antibody solution** to the corresponding color-coded 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 ± 5 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 ± 2 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. Results can also be obtained by using a spreadsheet macro available from Abraxis upon request.

The screening results for the samples are derived by simple comparison of the sample absorbances to the absorbances of the standards. The intensity of the color which develops (absorbance) is inversely proportional to the concentration of the Ionophores present in the sample. Based on a 70-130% extraction efficiency, sample extracts with higher absorbances than the 30 ppb equivalent standard should be reported as containing < 40 ppb of the corresponding Ionophore. Sample extracts with lower absorbances than the 30 ppb equivalent standard should be reporting as containing > 40 ppb of the corresponding Ionophore.