Importance of Melamine Determination

Melamine is an organic base with the chemical formula $C_3H_6N_6$, and the IUPAC name **1,3,5-triazine-2,4,6-triamine**. Melamine is a trimer of cyanamide. Like cyanamide, it is 66% nitrogen (by mass) and provides flame retardant properties to resin formulas by releasing nitrogen when burned or charred. Dicyandiamide (or cyanoguanidine), the dimer of cyanamide, is also used as a flame retardant. Melamine is a metabolite of cyromazine, a pesticide. It is formed in the bodies of mammals who have ingested cyromazine. Cyromazine is also converted to melamine in plants.

Melamine is used in combination with formaldehyde to produce melamine resin, a very durable thermosetting plastic, and melamine foam, a polymeric cleaning product. The end products containing melamine include countertops, fabrics, glues and flame retardants. Melamine is one of the major components in Pigment Yellow 150, a colorant in inks and plastics. Melamine is also used to make fertilizers.

Ingestion of melamine may lead to reproductive damage, bladder or kidney stones, which can lead to bladder cancer. A study in 1953 reported that dogs fed 3% melamine for a year had the following changes in their urine: (1) reduced specific gravity, (2) increased output, (3) melamine crystalluria, and (4) protein and occult blood.

The practice of adding "melamine scrap" to animal feed in order to give the appearance of increased protein content is reported to be widespread in various countries. Melamine has also been intentionally added as a binding agent in fish and livestock feed. This practice can potentially contaminate animal products intended for human consumption such as meat and dairy products. Melamine has also been directly added to foods intended for human consumption. Recently, several companies and individuals were implicated in a scandal involving milk and infant formula which had been adulterated with melamine, leading to kidney stones and renal failure, causing four known infant deaths, and sickening nearly 53,000 infants.

The ABRAXIS® Melamine ELISA allows the determination of 42 samples in duplicate determination. Less than a mL of sample extract is required. The test can be performed in less than 1 hour.

Performance Data

Test sensitivity: The detection limit for Melamine is 10 µg/L (mean of 6 blank determinations minus 3 standard deviations). The middle of the test (50% B/B_0) is at approximately 150 µg/L. 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: The cross-reactivity of the ABRAXIS® Melamine Kit for various Traizines can be expressed as the least detectable dose (LDD), which is estimated at 90% B/Bo, or as the dose required for a 50% absorbance inhibition (50% B/Bo).

Compound	LDD (ppb)	50% B/Bo (ppb)	X-Reactivity (%)
Melamine	10	150	100
Ammeline	7	140	93
Ammelide	400	7,000	2.1
Cyanuric Acid	400	>10,000	< 1
Atrazine	>10,000	>10,000	< 1
Diamino Atrazine	0.1	2	> 500

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ABRAXIS® Melamine ELISA Microtiter Plate

Enzyme-Linked Immunosorbent Assay for the Determination of Melamine in Contaminated Samples

Product No. 50005B

1. General Description

The ABRAXIS® Melamine ELISA is an immunoassay for the quantitative and sensitive screening of Melamine. This test is suitable for the quantitative and/or qualitative screening of Melamine in various sample matrices (please contact Gold Standard Diagnostics for the appropriate extraction/dilution procedure technical bulletins). If necessary, samples requiring regulatory action can be confirmed by HPLC, GC/MS, or other conventional methods.

2. Safety Instructions

The standard solutions of the test kit contain small amounts of Melamine. 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 the skin, wash with water.

3. Storage and Stability

The ABRAXIS® Melamine ELISA should be stored in the refrigerator (2-8ºC). The solutions must be allowed to reach room temperature (20-25ºC) before use. Reagents may be used until the last day of the month as indicated by the expiration date on the box.

4. Test Principle

The test is based on the recognition of Melamine by antibodies. The standards, sample extracts, and Melamine HRP conjugate are pipetted into test wells coated with Melamine antibody to initiate the reaction. During the 30 minute incubation period, Melamine from the sample and Melamine HRP conjugate compete for binding to Melamine antibody. Following this 30 minute incubation, the contents of the well are removed and the wells are washed to remove any unbound Melamine and Melamine HRP conjugate. After washing with the diluted wash solution, a clear substrate is then added to the wells and any bound enzyme conjugate causes the conversion to a blue color. Following a 20 minute incubation, the reaction is stopped and the amount of color in each well is read using an ELISA reader. The color of the unknown samples is compared to the color of the standards and the Melamine concentration of the samples is derived. The concentrations of the samples are determined by interpolation using the standard curve constructed with each run.

5. Limitations of the ABRAXIS® Melamine ELISA, Possible Test Interference

Many organic and inorganic compounds commonly found in samples have been tested and found not to interfere with this test. However, due to the high variability of compounds that might be found in samples, test interferences caused by matrix effects cannot be completely excluded. Some matrices such as fatty foods, require a simple sample dilution before analysis to eliminate interferences (please contact Gold Standard Diagnostics for the appropriate extraction/dilution procedure technical bulletins).

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

The ABRAXIS® Melamine ELISA kit provides screening results. As with any analytical technique (GC, HPLC, etc.) samples requiring regulatory action should be confirmed by alternative methods.

A. Reagent and Materials Provided

1. Microtiter plate (12 X 8 strips) coated with polyclonal anti-melamine antibody, in an aluminum pouch
2. Standards (6): 0, 20, 50, 100, 200, 500 ng/mL (ppb), 2 mL each
3. Melamine-HRP Conjugate, 6 mL
4. ABRAXIS® Wash Buffer (5X) Concentrate, 100 mL, must be diluted prior to use, see Test Preparation (Section C)
5. Substrate (Color) Solution (TMB), 12 mL
6. 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), stepper pipette (50-250 µL), or electronic repeating pipette with disposable plastic tips
3. Microtiter plate washer (optional)
4. Microtiter plate reader (wave length 450 nm)
5. Shaker for microtiter plates (optional)
6. Deionized or distilled water
7. Container with 500 mL capacity (for diluted 1X Wash Buffer, see Test Preparation, Section C)
8. Paper towels or equivalent absorbent material
9. Timer

C. Test Preparation

Micro-pipetting equipment and pipette tips for pipetting the standards and the samples are necessary. We recommend using a multi-channel pipette, stepping pipette, or electronic repeating pipette for adding the enzyme conjugate, the substrate (color) solution and the stop solution in order to equalize the incubations periods of the standard solutions and the samples on the entire microtiter plate. 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 (2-8ºC).
3. The standards, enzyme conjugate, substrate, and stop solution are ready to use and do not require any further dilutions.
4. Dilute the ABRAXIS® Wash Buffer (5X) Concentrate at a ratio of 1:5. If using the entire bottle (100 mL), add to 400 mL of deionized or distilled water.
5. The stop solution must be handled with care as it contains diluted H_2SO_4.

D. 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 5: Standards
(0; 20; 50; 100; 200; 500 ppb)

Sam1, Sam2, etc.: Samples

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 0	Std 4	etc.									
B	Std 0	Std 4	etc.									
C	Std 1	Std 5										
D	Std 1	Std 5										
E	Std 2	Sam 1										
F	Std 2	Sam 1										
G	Std 3	Sam 2										
H	Std 3	Sam 2										

E. Assay Procedure

1. **Add 100 µL of the standards or sample extracts** 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 enzyme conjugate solution** to the individual wells successively using a multi-channel, stepping, or electronic repeating pipette. Cover the wells with parafilm or tape and mix the contents by moving the plate on the bench top in a circular motion for about 30 seconds (be careful not to spill the contents).
3. **Incubate the strips for 30 minutes at room temperature**.
4. **Wash the strips four times using the diluted wash buffer**. Please use at least a volume of 250 µL of 1X wash buffer for each well and each washing step. Remaining buffer in the wells should be removed by patting the inverted plate dry on a stack of paper towels.
5. **Add 100 µL of substrate (color) solution** to the wells successively using a multi- channel, stepping, or electronic repeating pipette. Cover the wells with parafilm or tape and mix the contents by moving the plate on the bench top in a circular motion for about 30 seconds (be careful not to spill the contents). **Incubated the strips for 20 minutes at room temperature**. Protect the strips from sunlight.
6. **Add 100 µL of stop solution** to the wells in the same sequence as for the substrate solution using a multi- channel, stepping, or electronic repeating pipette.
7. Read the absorbance at 450 nm using a microplate ELISA photometer within 15 minutes after the addition of stopping solution.

F. Evaluation

The evaluation of the ELISA can be performed using commercial ELISA evaluation programs such as Logit/Log or 4-Parameter (preferred). 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 Melamine concentration on the horizontal logarithmic (x) axis on graph paper. $\%B/B_0$ for samples will then yield levels in ppb of Melamine by interpolation using the standard curve. Results can also be obtained by using a spreadsheet macro available from Gold Standard Diagnostics upon request.

The concentrations of the samples are determined using the standard curve run with each test. Samples showing a lower concentration of Melamine compared to standard 1 (20 µg/L or ppb) must be reported as containing < 20 ppb Melamine. Samples showing a higher concentration than standard 5 (500 µg/L) must be diluted further to obtain accurate results.

Semi-quantitative results can be derived by simple comparison of the sample absorbance to the absorbance of the standards. Sample containing less color than a standard will have a concentration of Melamine greater than the concentration of the standard. Samples containing more color than a standard will have a concentration less than the concentration of the standard.

G. Standard Curve (For demonstration purposes; not for use in sample interpretation)