Melamine ELISA Kit
(OKAO00120)
Lot# KH0733

Instructions for Use

For the quantitative detection of Melamine (SDM) concentration in milk powder, milk, tissue, feed, egg and serum.

Variation between lots can occur. Refer to the manual provided with the kit.

This product is intended for research use only.

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1. Background

Principle

Aviva Systems Biology Melamine ELISA Kit (OKAO00120) is based on standard competitive inhibition enzyme-linked immuno-sorbent assay technology. Melamine has been pre-coated onto a 96-wellplate (12 x 8 Well Strips). Standards or test samples are added to the wells along with a rabbit anti-Melamine antibody and an anti-rabbit / HRP Conjugated antibody, then incubated. Wells are washed and detection substrates are added and incubated. An enzymatic reaction is produced through the addition of TMB which is catalyzed by HRP generating a blue color product that changes yellow after adding acidic stop solution. The density of yellow coloration read by absorbance at 450 nm which is quantitatively proportional to the amount of detector antibody bound in the well and inversely proportional to the amount of Melamine in the sample.

Background

Melamine is an organic base and a trimer of cyanamide, with a 1,3,5-triazine skeleton. Like cyanamide, it contains 67% nitrogen by mass and, if mixed with resins, has fire retardant properties due to its release of nitrogen gas when burned or charred, and has several other industrial uses. Melamine is also a metabolite of cyromazine, a pesticide. It is formed in the body of mammals that have ingested cyromazine. It has been reported that cyromazine can also be converted to melamine in plants. Melamine combines with cyanuric acid and related compounds to form melamine cyanurate and related crystal structures, which have been implicated as contaminants or biomarkers in Chinese protein adulterations.

General Specifications

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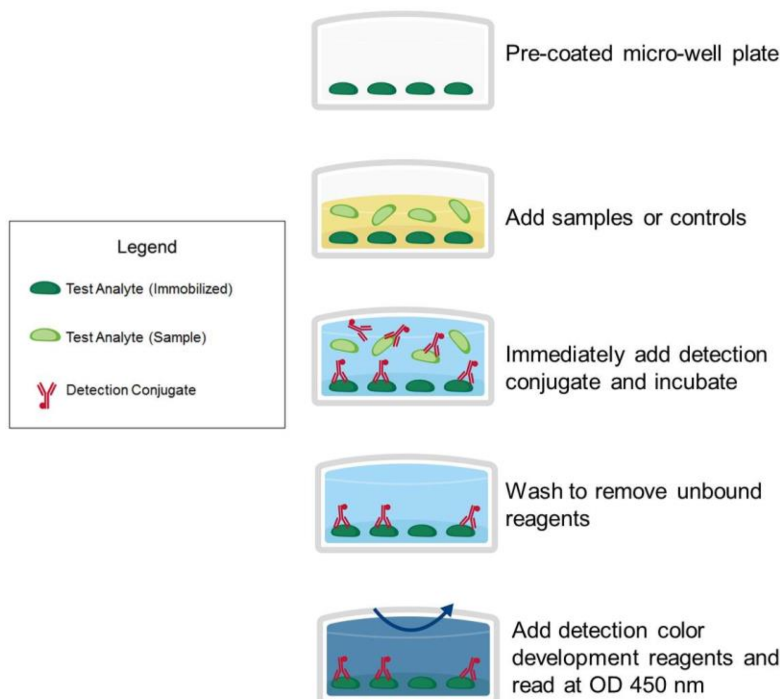
Range	2 – 162 ppb
LOD	2 ppb (Derived by linear regression of OD_{450} of the Mean Blank + 2xSD)
Specificity	Melamine CAS#: 108-78-1 PubChem#: 7955 Alias: Melamine; MELAMINE; 1,3,5-Triazine-2,4,6-triamine; Cyanurotriamide; Cyanuramide; Cyanurotriamine; 108-78-1
Cross-Reactivity	No detectable cross-reactivity with other relevant proteins

2. Assay Summary



3. Storage and Stability

- Upon receipt store kit at 4°C. Do not use past expiration date.

4. Kit Components

•The following reagents are the provided contents of the kit.

Description	Quantity	Storage Conditions
Melamine Microplate	96 Wells	Store at 4°C Do not use past expiration date
Melamine Standards	6 x 1 mL	
Melamine Detector Antibody	1 x 6 mL	
HRP Conjugate	1 x 6 mL	
Detection Reagent A	1 x 6 mL	
Detection Reagent B	1 x 6 mL	
2X Sample Prep Buffer	1 x 50 mL	
20X Wash Buffer	1 x 20 mL	
Stop Solution	1 x 6 mL	

5. Required Materials Not Supplied

- Microplate reader capable of reading absorbance at 450 nm.
- Automated plate washer (optional).
- Pipettes capable of precisely dispensing 0.5 µL through 1 mL volumes of aqueous solutions.
- Pipettes or volumetric glassware capable of precisely measuring 1 mL through 100 mL of aqueous solutions.
- New, clean tubes and/or micro-centrifuge tubes for the preparation of standards or samples.
- Absorbent paper or paper toweling.
- Distilled or deionized ultrapure water.
- Heater water bath
- Centrifuge
- Reagents: Methanol, n-Hexane.

6. Precautions

- Read instructions fully prior to beginning use of the assay kit.
- Any deviations or modifications from the described method or use of other reagents could result in a reduction of performance.
- Reduce exposure to potentially harmful substances by wearing personal protective lab equipment including lab coats, gloves and glasses.
- For information on hazardous substances included in the kit please refer to the Material Safety Data Sheet (MSDS).
- Kit cannot be used beyond the expiration date on the label.

7. Technical Application Tips

- Do not mix or substitute components from other kits.
- To ensure the validity of experimental operation, it is recommended that pilot experiments using standards and a small selection of sample dilutions to ensure optimal dilution range for quantitation.
- Samples exhibiting OD measurements higher than the highest standard should be diluted further in the appropriate sample dilution buffers.
- Prior to using the kit, briefly spin component tubes to collect all reagents at the bottom.
- Replicate wells are recommended for standards and samples.
- Cover microplate while incubating to prevent evaporation.
- Do not allow the microplate wells dry at any point during the assay procedure.
- Do not reuse tips or tube to prevent cross contamination.
- Avoid causing bubbles or foaming when pipetting, mixing or reconstituting.
- Completely remove of all liquids when washing to prevent cross contamination.
- Prepare reagents immediately prior to use and do not store, with the exception of the top standard.
- Equilibrate all materials to ambient room temperature prior to use (standards exception).
- For optimal results for inter- and intra-assay consistency, equilibrate all materials to 37°C prior to performing assay (standards exception) and perform all incubations at 37°C.
- Pipetting less than 1 μL is not recommended for optimal assay accuracy.
- Once the procedure has been started, all steps should be completed without interruption. Ensure that all reagents, materials and devices are ready at the appropriate time.
- Incubation times will affect results. All wells should be handled in the same sequential order and time intervals for optimal results.
- Samples containing precipitates, fibrin strands or bilirubin, or are hemolytic or lipemic might cause inaccurate results due to interfering factors.
- TMB Substrate is easily contaminated and should be colorless or light blue until added to plate. Handle carefully and protect from light.
- To minimize influences on the assay performance, operation procedures and lab conditions, especially room temperature, air humidity, incubator temperature should be strictly controlled. It is also strongly suggested that the whole assay is performed by the same operator from the beginning to the end.

8. Reagent Preparation

• Equilibrate all materials to room temperature prior to use and use prepare immediately prior to use.

8.1 1 M NaOH

8.1.1 Prepare 100 mL of fresh **0.1 M NaOH** by adding 4 g of NaOH to 100 mL of ultrapure water immediately prior to use.

8.1.2 Mix gently and thoroughly.

8.1.3 Do not store for future use.

8.2 1 M HCl

8.2.1 Prepare 100 mL of fresh **1 M HCl** immediately prior to use.

8.2.2 Add 8.6 mL HCl (36%) to 91.4 mL ultrapure water.

8.2.3 Mix gently and thoroughly.

8.2.4 Do not store for future use.

8.3 Acetonitrile- 0.1M NaOH

8.3.1 Combine **Acetonitrile** and **0.1M NaOH** at a ratio of 21:4 (V:V), with 21 parts Acetonitrile and 4 parts 0.1M NaOH.

8.3.2 Mix gently and thoroughly.

8.3.3 Do not store for future use.

8.4 1X Sample Prep Buffer

8.4.1 Add 1 mL of ultra-pure water to 1 mL of **2X Sample Prep Buffer**

8.5 1X Wash Buffer

8.5.1 If crystals have formed in the **20X Wash Buffer** concentrate, equilibrate to room temperature and mix gently until crystals have completely dissolved.

8.5.2 Add the entire 20 mL contents of the **20X Wash Buffer** bottle to 380 mL of ultra-pure water to a clean > 1,000 mL bottle or other vessel.

8.5.3 Seal and mix gently by inversion. Avoid foaming or bubbles.

8.5.4 Store the **1X Wash Buffer** at room temperature until ready to use in the procedure. Store the prepared **1X Wash Buffer** at 4°C for no longer than 1 week. Do not freeze.

8.6 Microplate Preparation

•Micro-plates are provided ready to use and do not require rinsing or blocking.

•Unused well strips should be returned to the original packaging, sealed and stored at 4°C.

•Equilibrate microplates to ambient temperatures prior to opening to reduce potential condensation.

9. Sample Preparation

9.1 Sample Preparation and Storage

- Mix diluted samples gently and thoroughly.
- Pipetting less than 2 µL is not recommended for optimal assay accuracy.
- Untreated samples should be stored at -20°C.
- Prepared samples can be stable for 1 week at 2-8°C.

9.1.1 Tissue (Diluted 1:2, meat, chicken, duck, liver, shrimp, fish, egg, etc.)

9.1.1.1 Homogenize the sample at 10,000 rpm for 1 minute.

9.1.1.2 Weigh 2 ± 0.05 g of the homogenized sample into a 50 mL centrifugal tube.

9.1.1.3 Add 8 mL of Acetonitrile-0.1 M NaOH solution.

9.1.1.4 Mix for 2 minutes. Centrifuge at 4,000 rpm at room temperature (20-25 °C) for 10 minutes.

9.1.1.5 Transfer 2 mL of supernatant (approx. 1 g sample) into a new centrifugal tube, evaporate to dryness by nitrogen or air at 50-60 °C.

9.1.1.6 Add 1 mL **N-hexane**, vortex for 30 seconds, then add 1 mL of the diluted 1X Sample Prep.

9.1.1.7 Centrifuge at 4,000 rpm at room temperature for 5 min.

9.1.1.8 Discard the upper layer. Use 50 µL of lower layer for analysis.

9.1.2 Serum (Diluted 1:4)

9.1.2.1 Take 500 µL samples into 50 mL centrifugal tube.

9.1.2.2 Add 2 mL of **acetonitrile-0.1 M NaOH** solution

9.1.2.3 Mix for 2 minutes. Centrifuge at 4,000 rpm for 10 minutes.00

9.1.2.4 Transfer 1 mL of the supernatant into a new centrifugal tube, and evaporate to dryness by nitrogen or air at 50-60°C

9.1.2.5 Dissolve the dry residues in 1 mL **N-hexane**, vortex for 30 seconds.

9.1.2.6 Add 1mL of **1X Sample Prep Buffer and** mix properly for 30 seconds

9.1.2.7 Centrifuge at above 4,000 rpm at room temperature (20-25°C) for 5 minutes.

9.1.2.8 Discard the top layer. Use 50 µL of the lower layer for analysis.

9.1.3 Feed (Diluted 1:100)

9.1.3.1 Grind sample and weigh 2.0±0.05g sample into 50 mL conical tube.

9.1.3.2 Add 2 mL of **1 M HCl** and 16mL of **deionized water**.

9.1.3.3 Homogenize samples.

9.1.3.4 Vortex for one minute. Centrifuge greater than 4,000 rpm at room temperature (20-25°C) for 15 minutes.

9.1.3.5 Transfer 10 mL of clear supernatant into a new conical tube.

9.1.3.6 Add 0.5-1 mL pf **1 M NaOH**, adjust pH to 6-8.

9.1.3.7 Centrifuge greater than 4,000 rpm at room temperature (20-25°C) for 15 minutes.

9.1.3.8 Take 100 µL of the clear supernatant and add 900 µL of **1X Sample Prep Buffer**.

9.1.3.9 Use 50 µL of solution for analysis.

9.1.4 Milk (Diluted 1:27)

9.1.4.1 Transfer 600 µL of samples into 2 mL centrifugal tube and pipette 1 mL **acetonitrile**.

9.1.4.2 Mix for 5 minutes and centrifuge at 4,000 rpm for 5 minutes.

9.1.4.3 Take 100 µL of the supernatant, and add 900 µL of **1X Sample Prep Buffer**. Mix.

9.1.4.4 Take 50 µL of the lower layer for analysis

9.1.5 Milk powder (Diluted 1:20)

9.1.5.1 Transfer 2.0 ± 0.05 g samples into 50 mL centrifugal tube and pipette 4 mL of **methanol**.

9.1.5.2 Mix for 5 minutes and centrifuge at 4,000 rpm 10 minutes.

9.1.5.3 Take 100 µL of the supernatant and add 900 µL of **1X Sample Prep Buffer.** Mix.

9.1.5.4 Take 50 µL of the lower layer for analysis.

9.1.6 Egg (Diluted 1:20)

9.1.6.1 Weigh 2.0 ± 0.05 g of the homogenized sample (Egg white, egg yolk or whole egg) into 50 mL centrifugal tube.

9.1.6.2 Add 8 mL of **acetonitrile-0.1 M NaOH** solution.

9.1.6.3 Mix for 2 minutes and centrifuge at 4,000 rpm for 10 minutes.

9.1.6.4 Transfer 1 mL of the supernatant into a new centrifugal tube, and evaporate to dryness by nitrogen or air at 56°C.

9.1.6.5 Dissolve the dry residues in 1 mL **N-hexane** and vortex for 30 seconds

9.1.6.6 Add 1 mL of **1X Sample Prep Buffer** and mix for 30 seconds.

9.1.6.7 Centrifuge greater than 4,000 rpm at room temperature (20-25°C) for 5 minutes.

9.1.6.8 Discard the top layer.

9.1.6.9 Take 50 µL of the lower layer, and add 150 µL of **1X Sample Prep Buffer.**

9.1.6.10 Mix for 30 seconds. Use 50 µL of solution for analysis.

10. Assay Procedure

- Equilibrate all reagents and materials to ambient room temperature prior to use in the procedure.
- Temperature regulation for all procedures and incubations to 25°C is recommended for optimal reproducibility.
- Gently vortex each reagent prior to use.

10.1 Determine the required number of wells and return any remaining unused wells and desiccant to the pouch.

10.2 Add 50 µL of standards or samples into wells of the **Melamine Microplate**. At least two replicates of each standard, sample or blank is recommended.

10.3 Immediately add 50 µL of the **Melamine Rabbit Detector Antibody** and 50 µL of the **Anti-Rabbit / HRP Conjugate** to each well.

10.4 Cover the plate with the well plate lid, gently mix and incubate for 30 minutes.

10.5 Remove the plate lid and discard the liquid in the wells by rigorously flicking into an acceptable waste receptacle or aspiration.

10.6 Gently blot any remaining liquid from the wells by tapping inverted on the benchtop onto paper toweling. Do not allow the wells to completely dry at any time.

10.7 Wash plate 5 times with **1X Wash Buffer** as follows:

10.7.1 Add 250 µL of **1X Wash Buffer** to each assay well.

10.7.2 Incubate for 30 seconds.

10.7.3 Discard the liquid in the wells by rigorously flicking into an acceptable waste receptacle.

10.7.4 Gently blot any remaining liquid from the wells by tapping inverted on the benchtop onto paper toweling. Do not allow the wells to completely dry at any time.

10.7.5 Repeat steps 10.7.1 through 10.7.4 **four** more times.

10.8 Add 50 µL of prepared **Detection Reagent A** and 50 µL of **Detection Reagent B** to each well.

10.9 Gently mix the plate and incubate for 15 minutes **in the dark**.

10.10 Add 50 µL of **Stop Solution** to each well and mix gently. Well color should change to yellow immediately. Add the **Stop Solution** in the same well order as done for the **Detection Reagents**.

10.11 Read the O.D. absorbance at 450 nm with a standard microplate reader within 5 minutes of stopping the reaction in step 10.10. If wavelength correction is available, set to 630 nm.

11. Calculation of Results

For analysis of the assay results, first derive the **Percent Relative Absorbance** for each sample or standard by calculating the mean absorbance between replicate wells. This value is then divided by the mean replicate Blank (0 ppb) absorbance, then multiplying by 100%:

$$\text{Percent Relative Absorbance (Sample or Standard)} = \frac{\text{Mean Absorbance (Sample or Standard)}}{\text{Mean Blank Absorbance}} \times 100\%$$

A standard curve is generated by plotting the **Percent Relative Absorbance** of each standard serial dilution point vs. the respective standard concentration. The Melamine concentration contained in the samples can be interpolated by using linear regression of each sample **Percent Relative Absorbance** against the standard curve. This is best achieved using curve fitting software.

Standard #	**0**	**1**	**2**	**3**	**4**	**5**
ppb	**0**	**2.0**	**6.0**	**18**	**54**	**162**

Note: If wavelength correction readings were available, subtract the readings at 630 nm from the readings at 450 nm. This may provide greater reading accuracy.

Note: If the samples measured were diluted, multiply the derived mean sample concentration by the dilution factor for a final sample concentration.

12. Typical Expected Data

12.1 Limit of Detection

Derived by linear regression of OD450 of the Mean Blank + 2xSD

Sample	Mean Recovery
Milk powder	40 ppb
Milk	54 ppb
Milk powder/Milk (Methods 2)	2 ppb
Tissue	4 ppb
Feed	200 ppb
Egg	40 ppb
Serum	8 ppb

12.2 Reproducibility

Three samples of known concentrations were measured in 20 replicates within an assay plate to assess intra-assay reproducibility. Three samples of known concentrations were measured across 40 replicate assays to assess inter-assay reproducibility:

Sample	Intra-Assay			Inter-Assay		
	1	2	3	1	2	3
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (ppb)	2.411	7.382	18.394	2.837	8.175	17.228
SD	0.171	0.546	1.435	0.199	0.646	1.516
CV (%)	7.1	7.4	7.8	7.0	7.9	8.8

12.3 Cross-Reactivity Rate

Substance	Cross Reactivity Rate
Melamine	100%
Cyanuric acid	60%
Triazine	<1%
Diamino atrazine	<1%

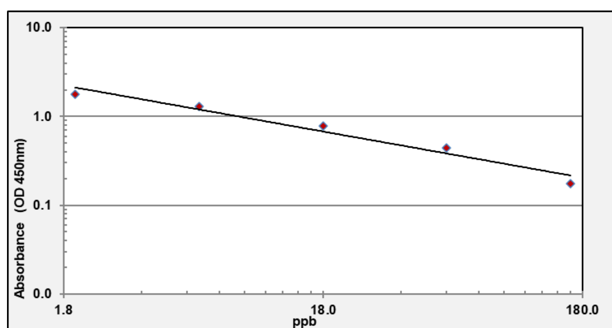
12.4 Recovery

The recovery of Melamine spiked at levels throughout the range of the assay in various matrices was evaluated. Samples were diluted prior to assay as directed in the Sample Preparation section.

Sample	Mean Recovery
Milk powder/Milk	90% +/- 20%
Tissue	85% +/- 20%
Feed	85% +/- 20%
Egg	80% +/- 20%
Serum	90% +/- 15%

12.5 Typical Data

For convenience in result calculation, absorbance as abscissa and standard concentrations can be used as ordinate. The standard curve data provided in the manual is only for reference; experimenters should draw the standard curve based on their own data.



ppb	Absorbance		Mean Absorbance	B/B0(%)
	Rep 1	Rep 2		
162.0	0.176	0.178	0.177	8.5
54.0	0.445	0.415	0.430	20.5
18.0	0.779	0.900	0.840	40.1
6.0	1.303	1.304	1.304	62.2
2.0	1.788	1.755	1.772	84.6
0.0	2.160	2.028	2.094	100.0

13. Technical Resources

Technical Support:

For optimal service please be prepared to supply the lot number of the kit used.

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